Risk Factors in Infra-Inguinal Graft Stenosis

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Statement of Originality

The work described in this thesis is solely the work of the author. The studies were devised and undertaken by the author, with technical assistance where indicated.

This work contributes to original literature by (i) considering circulating risk factors in a human model of intimal hyperplasia and (ii) by considering the effects of a number of risk factors in a single model.

Preceding work has demonstrated associations between risk factors and intimal hyperplasia in animal models or smooth muscle cells in culture, but very little in-vivo data exists in man. Data associating risk factors and stenosis in animal studies are derived from observations at a large number of anatomical sites and include arteries, veins and prosthetic vessels.
'Well in our country' said Alice, still panting a little, 'you'd generally get to somewhere else - if you ran very fast for a long time, as we've been doing.'

'A slow sort of country!' said the Queen. 'Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!'

'If I wasn't real,' Alice said - half laughing through her tears, it all seemed so ridiculous - 'I shouldn't be able to cry.'

'I hope you don't suppose those are real tears?' Tweedledum interrupted in a tone of great contempt.

Through the Looking-Glass
Lewis Carroll
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Abstract

Intimal hyperplasia appears to be the universal vessel response to injury and is important in atherogenesis. In animal and in-vitro models, the initiation and promotion of this process has been associated with a number of risk factors found in the circulation. In man, approximately 25% of infrainguinal bypass grafts develop stenosis due to smooth muscle cell proliferation but it is not known if circulating risk factors influence this process.

In these studies, circulating risk factors have been correlated with the development of hyperplastic stenoses in infrainguinal bypass grafts. Non-hyperplastic causes of stenosis have been excluded by peri-operative graft assessment. Duplex scanning and angiography were used to detect new stenoses occurring in the first postoperative year.

Logistic regression shows association between graft stenoses and; continued cigarette smoking and elevated circulating levels of fibrinogen, Lp(a) and 5-HT. These associations are independent of graft material or whether grafts were studied prospectively or retrospectively. There were some differences in stenosis associated risk factors between vein and PTFE graft groups on univariate analysis.

These results suggest that circulating risk factors do play a role in intimal hyperplasia and stenosis of human infrainguinal bypass grafts and support other work demonstrating a reduction in graft patency in association with similar abnormalities.
Introduction

During the last three decades, great advances have been made in our understanding of the common diseases affecting the cardiovascular system. Studies in the field of endothelial injury and the resulting vessel wall response, has been particularly fruitful; migration and hyperplasia of smooth muscle cells of medial origin is now understood to be the universal response to vessel injury (Chervu & Moore 1975, Clowes et al 1983a, Ip et al 1990) and is one of the earliest changes seen in areas of developing atherosclerosis (Ross & Glomset 1976).


Most of these observations, however, have been made in animal models, at a number of different anatomical sites, and include observations in arteries, veins and prosthetic grafts. It remains unclear whether all risk factors act at all anatomical sites and which factors, if any, are relevant to intimal hyperplasia in man.

Between 10% and 75% of femoropopliteal or femorocrural bypass grafts thrombose in the first post-operative year (European Concensus Document on Critical Limb Ischaemia 1989) and up to 80% of these may be associated with graft stenosis (Brewster et al 1983). After peri-operative assessment to exclude pre-existing disease, or poor surgical technique, stenosis of infrainguinal bypass grafts is caused by intimal hyperplasia (Szilagy et al 1973, Fuchs et al 1978, DeWeese 1978). These stenoses
can be detected by regular postoperative graft surveillance (Grigg et al 1988, Chang et al 1990, Harris 1992).

This thesis examines the association between the development of hyperplastic stenoses in infrainguinal bypass grafts, and a number of the serological risk factors for intimal hyperplasia which have been identified in other models.

Identification of serological risk factors associated with infrainguinal graft stenosis may be of value in recognising grafts at higher risk of developing stenosis, and may indicate where pharmacological intervention could be of value in preventing stenosis development. Results may complement studies associating risk factors with infrainguinal graft patency.
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Chapter 1

Atherosclerosis.

1.1 Introduction

Despite a five to ten percent fall per decade in the death rate from atherosclerotic disease in Europe and the U.S.A. since the late 1960's (Gordon and Thom 1975, Walker 1977), cardiovascular disease remains the commonest cause of death in the western world. The majority of these deaths are the consequences of atherosclerosis and its complications (Report of the Working Group of Atherosclerosis of the National Heart Blood and Lung Institute 1981). At its peak atherosclerosis assumed almost epidemic proportions; in the early and mid sixties almost one-half of all deaths in the U.S. were attributed to the disease (Atherosclerosis. A report by the National Heart and Lung Institute Task Force on arteriosclerosis 1971). Although the current trends in prevalence of the disease are favourable there remains much that is not fully understood about the condition. In this chapter the nature and effects of atherosclerosis and a review of the current theories of its development are discussed.

Terminology

The term atherosclerosis was introduced in 1904 by Marchand to describe a frequently observed arterial lesion consisting of an amorphous, lipid containing centre with an overlying fibrous cap. The term has been extended by some to include other arteriopathies in which hardening of the vessel wall is prominent, such as Monckeberg's medial calcific sclerosis and hypertensive arteriolosclerosis. In addition, 'atherosclerosis' is commonly used in the generic sense to describe the functional and clinical effects of the disease on target organs. In the following account the term will be used in its widest sense with regard to degenerative arterial disease (but not including conditions such as Monckeberg's sclerosis) and ischaemic effects. When
required, the vascular lesions of the disease will be referred to as atheroma or atherosclerotic plaque.

1.2 The Morphology of Atheroma

The presence of atherosclerosis in the human circulation and the recognition of atheromata by pathologists is not new; Lesions have been identified in Egyptian and Grecian mummies dating from almost 3,500 years ago (Ruffer 1911, Cockburn et al 1975) and atheroma was initially recognised by physicians in the sixteenth century (Long 1933). The first detailed descriptions of the morphology of the disease were made by the great European pathologists of the nineteenth century such as Virchow (1856), however it was not until the early twentieth century that the causative role of atheromatous plaques in the genesis of coronary and other vascular diseases was recognised (Herrick 1912). Since that time the development of modern imaging techniques such as transmission and scanning electron microscopy, and pressure fixation facilities to correctly maintain the in vivo relationship between plaque and the vessel lumen, have permitted great strides in our understanding of the morphology of atheroma to be made (Geer et al 1961, Stary 1983). Despite these technological breakthroughs, the aetiology of the disease remains unclear.

Histologically, there are two pathologically distinct entities present in the vessel wall which are thought to represent differing stages of the development of atherosclerosis;

Fatty Streaks in the Tunica Intima

These are the simplest of atherosclerotic lesions but contain most of the elements seen in advanced plaques (Figure 1(1)). They are found commonly in the aorta and coronary arteries of young adults and even children (Klotz & Manning 1911).
Macroscopically there is a circumscribed flat or very slightly raised, yellow lesion in the vessel wall varying in form from a dot to a streak and several lesions may coalesce to cover a large intimal surface (Haust 1983). Microscopically there is a considerable range of changes but usually this includes an accumulation of intimal smooth muscle cells containing varying degrees of intracellular lipid (Stary 1983) (figure 1(2)), which may be surrounded by free lipids (Geer 1965). Macrophages are also present and when these cells ingest lipid they produce a characteristic 'foam cell' appearance, typical of atheroma (Haust 1983, Schwartz et al 1989).
Stary (1983) examined fatty streaks in children and his observations of the anatomical sites of their development in the coronary arteries provided confirmation of previous work (Geer et al 1961, Strong & McGill 1962) suggesting that fatty streaks occur at the sites of mature atherosclerotic plaque development in older age groups. The hypothesis that the fatty streak is the earliest detectable lesion of atherosclerosis has recently been supported by the work of Faggioto & Ross (1984), who used electron microscopic techniques to show that in some fatty streaks the overlying endothelium is attenuated and fragile and thus predisposed to injury - as described below, endothelial injury is currently believed to be a vital initiating event in atheroma formation (Ross & Glomset 1976).

Figure 1(2); Electron micrograph showing fat filled smooth muscle cell in a fatty streak. Surrounding ground substance containing collagen and chips of fibrin (arrowed) is evident. (X5000)
From Haust 1983
Despite this circumstantial evidence, there remains some doubt about the association between fatty streaks and mature atherosclerotic plaque, particularly as fatty streaks are more common in young women and people of Negro origin, two groups who are least likely to develop atheroma later in life (Haust 1983). To account for this observation, Pearson et al (1980) have postulated that there may be two types of fatty streak lesion, differentiated by the clones of cells they contain, and have experimental evidence to support this contention using the enzyme glucose-6-phosphate dehydrogenase as a cellular marker. These authors suggest that the different types of lesions may have separate roles, one important in the progression of early lesions to mature atherosclerosis and the other undergoing spontaneous regression. At the time of writing the validity of these observations remains to be established.

The most widely held current view is that the fatty streak represents the earliest macroscopic change detectable in the wall of a vessel developing atherosclerosis (Ross 1986) but the factors which influence the development, progression or regression of these lesions is not fully understood.

Advanced Atheromata

This lesion is the hallmark of atherosclerosis and its role in vascular disease seems clear. Like fatty streaks, the composition of the plaque is variable within given limits. Macroscopically the lesions lie in the intima (or intima and media if very advanced), may vary from a few millimetres to several centimetres in diameter and are usually elongated in the direction of blood flow in the vessel (Haust 1983). There is a dense white 'fibrous' cap on the luminal surface, with an underlying cellular region which may contain a deposit of lipid (commonly cholesterol crystals) and amorphous cellular debris (Ross et al 1984). The term atheroma is derived from the Greek athere (porridge) and refers to the observations of this viscous paste in the centre of atheromatous lesions by early pathologists.
Microscopically, the cap of 'fibrous' tissue consist of smooth muscle cells lying in lacunae between alternating layers of connective tissue containing collagen and proteoglycan (Haust 1983, Ross et al 1984), believed to be produced by the smooth muscle cell (figure 1(3)). The region between the cap and amorphous centre also consists of large numbers of proliferated smooth muscle cells but these lie in a less dense ground substance (Geer et al 1961, Ghidoni & O'Neal 1967, McGill 1968, Ip et al 1990, Wissler 1991). In an electron microscopy study of human plaques obtained at the time of reconstructive surgery, Ross et al (1984) showed that the ground substance in this region also consists of proteoglycan and collagen fibres.

Figure 1(3); Smooth muscle cell from atheromatous plaque surrounded by secreted collagen (black stained) and ground substance. (Pentachrome II stain, X 1,460) From Haust 1983
It is of note that these histological features are almost identical to those found in intimal hyperplastic lesions in human infrainguinal and coronary bypass grafts and in restenosis after angioplasty (Sottiurai et al 1983 and 1989, Johnson et al 1990, Garrat et al 1991).

**Plaque Complications**

Large atheromatous plaques can produce haemodynamic disturbance by impinging directly on the lumen of small or medium sized arteries. More often, and particularly in the large vessels such as the aorta, the clinical effects of atherosclerosis are produced by the development of plaque complications, the most common of which are calcification, fissuring / ulceration and intraplaque haemorrhage (Haust 1983) (figures 1(4) - 1(7)). Complications most commonly occur in more advanced lesions and can result in rapid alteration of plaque morphology with potentially catastrophic effects.

![Figure 1(4); Plates / shelves of calcification (black) in the superficial layers of the fibrous cap of a mature atheromatous plaque.](image)

(Alcian blue - PAS - Haematoxylin - Orange G stains, X 39) From Haust 1983
Figure 1(5): A fissure on human aortic endothelium; a linear area of endothelial loss can be seen in the middle of the diseased area. (X 3)
From Haust 1983

Figure 1(6): Section through atheromatous plaque showing loss of endothelium and ulceration (top right).
(Massons trichrome stain, X 5.5) From Haust 1983
Figure 1(7); Thrombus on areas of aortic ulceration (arrowed). The pale grey area between the thrombi is subendothelial hemorrhage. 
(Magnification X 3) From Haust 1983
Calcium deposition represents dystrophic calcification and occurs in the body of the atheroma itself, the fibrous cap or both. This results in a local reduction of vessel wall elasticity which may predispose to fissure splitting of the fibrous cap under the effects of arterial blood pressure (Haust 1983). Coalescence of fissures may produce more substantial endothelial loss and result in the formation of an endothelial ulcer. Both of these latter changes expose thrombogenic material from the media to the circulating blood and may promote the formation of a clot on the vessel wall - the so-called mural thrombus. Widening of a fissure by haemodynamic forces followed by intramural haemorrhage may produce acute enlargement of a plaque which can progress to luminal occlusion. As well as producing occlusion of the vessel lumen, mural thrombosis and intramural haemorrhage may contribute to atheroma progression (Ross & Glomset 1976 - see response to injury hypothesis below).

1.3 The Aetiology of Atherosclerosis

The Imbibation and Incrustation theories of Virchow and Rokitanski.

The classical theories of atherogenesis, taught to medical students for many years were based on the mutually exclusive imbibation and incrustation hypotheses proposed in the nineteenth century and supported by Virchow (1856) and Von Rokitanski (1852) respectively.

The imbibation theory proposed the accumulation of fats in the vessel wall as the initiating factor in atherogenesis. The fats were thought to be mostly cholesterol and were believed to be derived from the circulation. Support for this theory came from observations of the prolific nature of atheroma-like lesions in animals and humans with hypercholesterolaemia along with the observation that many patients with atheroma have abnormal lipid profiles (reviewed by Inkeles and Eisenberg 1981).

The opposing view, first expounded by Von Rokitanski in the 1840's, but revived by Duguid over 100 years later (Duguid 1946), regarded atheroma as incrusted mural thrombus with varying degrees of re-endothelialisation. In
Rokitanski's era this theory was based on observation alone but considerable support was added by the demonstration of fibrin (figures 1(2) & 1(8)) and platelet breakdown products in atheroma of all stages by modern imaging methods (Duguid 1946, Woolf 1961).

Figure 1(8); Thrombus on the surface of atheromatous plaque (top). The darker regions in the middle of the figure are fibrinous material within the plaque. (Massons trichrome stain, X 98) From Haust 1983

For many years these theories were considered mutually exclusive and debate raged over the relative merits of the two schools of thought. Supporters of the
imbibation hypothesis pointed to the lack of atheroma development in peripheral veins, where thrombosis is more common, to detract from the incrustation theory, whilst Duguid and his supporters pointed to the inability of macromolecules such as lipoproteins to cross an intact endothelium in vivo to cast doubt on the validity of the imbibation hypothesis. This feud was ended in the 1976 when Ross proposed a plausible theory encompassing elements of both imbibation and incrustation; the response to injury hypothesis. This has been refined in the light of further understanding of the nature of endothelial injury and the cellular and biochemical changes that occur in atheroma prone regions of arterial walls, and the current theory has now become established as the likely aetiology of atheroma.

**The Response-to-Injury theory of Ross.**

The major components of atherosclerotic plaque have been described elsewhere and consist mainly of subintimal deposits of lipids and proliferated arterial smooth muscle cells surrounded by a collagen/proteoglycan matrix (Geer et al 1961, Ghidoni & O'Neal 1967, McGill 1968, Ip et al 1990, Wissler 1991). In 1976 Ross and Glomset proposed a theory explaining the development of these morphologic abnormalities in the intima of arteries and also encompassing the observations and theories of imbibation and incrustation described above. In the response to injury theory, endothelial damage, possibly without cell loss (type I injury - a functional, non denuding alteration of endothelial cells {Ip et al 1990} ) is believed to be central to the initiation of atherosclerosis. Such an injury may be caused by abnormalities of blood flow such as hypertension or turbulence, as well as direct physical or chemical insult (Fry 1972, Stehbens 1974). The injury is believed to lead to the subintimal accumulation of lipoproteins or macrophages (or both) due to localised loss of endothelial barrier function. Alternatively, if the severity of the injury is great enough (i.e. Type II or III denuding endothelial injury {Ip et al 1990}), direct adherence of platelets and monocytes to the vessel wall may occur. Following injury, the combined chemotactic and stimulatory effects of lipids (possibly after oxidation-
see lipids and atheroma below) and mitogens released from monocytes and / or platelets on medial myocytes is believed to initiate subintimal smooth muscle cell hyperplasia. Subsequent growth of the plaque occurs by a continuing cycle of injury and stimulation or by the inclusion of overt mural thrombus. Mural thrombosis may occur as a consequence of overlying endothelial cell loss or retraction in the region of the lesion, itself possibly a result of the adverse effects of hypercholesterolaemia on endothelial cells (Faggioto & Ross 1984, Jackson & Gotto 1976 described in detail below).

Incorporation of platelet rich thrombus which develops in this way may also be a further (and particularly powerful) stimulant to hyperplasia of smooth muscle cells already present in an early lesion, as platelets are known to be rich sources of smooth muscle cell mitogens (Ross et al 1974, Oka & Orth 1983).

In their study of human superficial femoral artery lesions, Ross et al (1984) cultured smooth muscle cells obtained from plaques in vitro, and demonstrated a growth behaviour pattern similar to that of senescent smooth muscle cells in culture. This suggested that the cells from the lesion had undergone numerous doublings prior to removal and were themselves effectively senescent. This indirect data supports the hypothesis that smooth muscle cells undergo hyperplasia during the development of atheroma (see aetiology of atheroma below).

There have been many additional studies which have suggested that circulating agents may influence the development of intimal hyperplasia in atherosclerosis; in addition, population based studies have been able to correlate the subsequent development of atheroma with levels of some blood components. Current understanding of the circulating risk factors is reviewed in the relevant experimental chapters in this thesis.
1.4 The Anatomical Distribution of Atheroma

Despite the systemic nature of the known risk factors for atherosclerosis, the disease has a distinctly localised nature. This may represent focal regions where many risk factors act together to produce deleterious effects, but the mechanism of this remains unclear. Characteristically, the most significant plaques develop at the sites of bifurcation of major arteries, areas of acute angulation of arteries and regions where arteries are relatively fixed (Texon et al 1965). This suggests that haemodynamic factors may be important in the aetiology (this is discussed more fully in chapter 9).

It has been suggested that systemic risk factors exert differing effects in different locations; cigarette smoking appears to be the major risk factor for peripheral vascular disease whereas hypertension is the major risk factor for cerebrovascular disease (Greenhalgh 1978). However as coronary, cerebral and peripheral atherosclerosis commonly co-exist (Turnipseed et al 1980, Hertzer et al 1984) and single site disease development is rare, this differentiation may be of little clinical value.

1.5 The Effects of Atherosclerotic Stenosis

Haemodynamics

The effects of arterial narrowing on the blood flow through a vessel are described in most circumstances by the Poiseuille equation shown below (although it is important to apprecaite that this is derived from observation of Newtonian fluids).

\[ P_1 - P_2 = \frac{\nu \cdot 8L\eta}{r^2} = \frac{Q \cdot 8L\eta}{\pi r^4} \]
\[ P_1 - P_2 = \text{Pressure differential} \]

\[ V = \text{Mean flow velocity} \]

\[ Q = \text{Flow} \]

\[ \eta = \text{Viscosity} \]

\[ r = \text{Radius} \]

\[ L = \text{Length} \]

Where

From this equation it can be deduced that the effects of a stenosis on flow (for a given driving pressure) are directly proportional to the fourth power of the radius of the stenosis. Thus reduction of flow is exquisitely sensitive to reduction of the vessel lumen. Furthermore, the effect of stenoses in series, such as a common iliac stenosis followed by a common femoral stenosis, a condition common in human atherosclerosis, is shown in the formula;

\[ R_{\text{total}} = R_1 + R_2 + \ldots + R_n \]

The effects of multiple stenoses are additive. Thus the combined effects of two or three relatively minor stenoses on overall flow may be very significant.

These mathematical considerations allow us to explain some of the syndromes associated with vascular insufficiency and their diagnostic tests. For example, in conditions of flow limitation by a fixed stenosis or series of stenoses, exercise and distal vasodilatation increases flow through the narrowed segment. As maximal flow is reached, in the presence of fixed stenosis and blood viscosity, continuing exercise can only result in increased flow if the driving pressure is increased. Therefore at maximum flow rates, distal perfusion pressure \((P_2)\) falls and this fall is detected under controlled circumstances in the treadmill ankle/brachial pressure index test.

The clinical syndromes associated with vascular stenoses and occlusions in the leg are described below.
The Collateral Circulation

The progressive narrowing of extremity vessels by atheroma encourages the opening of collateral vessels to improve flow to the distal portion of the limb. This flow is thought to develop through pre-existing anastomoses between major arteries, usually around joints and involves reversal of flow through one set of vessels (the re-entry arteries) and enlargement of another group (the stem arteries). The stimulus to this occurrence is not clear but alteration of the pressure differential across the anastomotic bed by the atherosclerotic process is a likely candidate. Exercise is beneficial in stimulating the development of collaterals which can often achieve sufficient flow to maintain the vitality of a limb at rest. The effects of exercise may be mediated by the development of relative hypoxia, and thus vasodilatation, with a corresponding rise in the pressure gradient between the stem and re-entry arteries (Kempczinski & Bernhard 1989).

Fortunately, there are rich collateral beds in the hip and thigh which evolve according to logical anatomical rules. In human atherosclerosis most important of these develop between the internal iliac and the profunda femoris arteries in common femoral disease (via the gluteal and deep circumflex femoral branches respectively - the so called cruciate anastomosis), and between the perforating branches of the profunda femoris and the popliteal arteries in superficial femoral artery disease (via genicular branches of both arteries). In aortic stenosis, the supra-renal branches of the aorta (superior mesenteric artery and the intercostal arteries) and the profunda femoris artery may produce important collateral flow, via the iliolumbar, epigastric and hypogastric arteries and the circumflex femoral arteries. The relatively poor collateral network in the calf accounts for the severity of ischaemia seen when atherosclerosis affects the trifurcation of the popliteal artery or the crural vessels. Understanding of the collateral circulation is of importance when planning dissection or incisions during operations on ischaemic limbs as poorly planned surgery may disrupt important collateral vessels, exacerbating the degree of ischaemia. Occlusion of
collaterals by worsening atheroma or thrombosis may be a factor in the development of acute on chronic ischaemia in a limb with known vascular insufficiency but with reasonable compensation prior to the acute event.
Chapter 2
Infrainguinal Arterial Disease
and its Management

2.1 Introduction

Although death from atherosclerosis is most commonly due to disease in the coronary or cerebral circulation (Ross 1986), vascular disease in the leg is a source of significant morbidity which may substantially reduce the quality of life of a patient whose long term prognosis is limited (Jurgens et al 1960). In Western Europe, occlusive arterial disease in the leg affects approximately 5% of the male population aged fifty or over (European Concensus Document on Critical Limb Ischaemia 1989) and whilst the disease is thought to take a relatively benign course in most of these patients (see natural history), intervention is required in a sufficient proportion for leg ischaemia to be a major part of the vascular surgeon's workload; in the U.K. it is estimated that between 27 - 55,000 patients present annually with critical leg ischaemia and between 6 - 7,000 major amputations are performed each year (European Concensus Document on Critical Limb Ischaemia 1989).

There have been many advances in the management of leg ischaemia since the widespread introduction of arterial reconstruction in the 1950's. Improvements in angiographic visualisation of the arterial tree, along with non-invasive investigation allow the modern vascular surgeon to assess both the anatomy and physiology of the patients' disease. Techniques of vein grafting continue to be improved and there are now a number of artificial conduits with differing properties which can be used if suitable autogenous vein is not available. The introduction of percutaneous angioplasty has further added to management options and the expanding range of applications of this technique require constant reappraisal. Finally a better understanding of the natural history of leg ischaemia and the outcome of reconstruction and amputation has clarified the aims of treatment for leg ischaemia and enabled vascular surgeons to direct...
appropriate therapy to appropriate patients. This chapter discusses atheroma in the leg and its effects, along with the common forms of management.

2.2 The Anatomical Distribution of Atheroma in the leg

The distribution of atheroma in the leg follows the pathophysiological rules for general atherosclerosis described by Texon et al. (1965) above; the superficial femoral artery in Hunters Canal at the junction of the terminal superficial femoral artery and the origin of the popliteal artery is the commonest site of plaque development (Mavor 1956). This corresponds not only to the site of angulation of the artery over the adductor magnus muscle, but also to an area of relative fixation of the artery, and the site of origin of the large supra genicular branches. The posterior wall of the common femoral artery is affected with almost similar frequency and for similar reasons. Plaque in this region often extends into the origins of the superficial femoral and profunda femoris arteries. Additionally, atheroma is common in the lower aorta and common iliac arteries, particularly between the inferior mesenteric artery and the bifurcation of the origin of the internal iliac arteries (Kempczinski and Bernard 1989).

Plaques in the infrainguinal arteries commonly cause stenosis and occlusion and therefore present predominantly with symptoms of chronic ischaemia, although complicated plaque may thrombose a major vessel, or embolise loose thrombus into the leg and produce acute ischaemia. Disease in the more proximal vessels, particularly the lower aorta, often weakens the media and allows aneurysmal dilatation. The present chapter deals only with the effects stenosis and occlusion in the infrainguinal arteries.
2.3 The Clinical Syndromes of Vascular Insufficiency in the Leg

**Intermittent Claudication**

Intermittent claudication is the most common symptom of mild and moderate impairment of arterial flow into the leg. The term is derived from the Latin *claudio* - to limp, and describes the syndrome of tight pain in a muscle group on walking a fixed distance at a given speed. The ankle flexors in the calf are the most commonly affected group in keeping with the most common site of atheroma development in Hunter's canal. The patient classically describes a tight or burning pain in the back of the calf which occurs only whilst walking, and is immediately relieved by stopping. Characteristically he is able to walk a similar distance before the onset of pain again. The haemodynamic changes occurring during intermittent claudication have already been described. The pain experienced by the patient is a consequence of the local build up of metabolites in the muscle, as a result of the limitation of arterial inflow and the reduced perfusion pressure in the muscle bed (Taylor and Porter 1989).

**Ischaemic Rest Pain**

A pathophysiological stage on from intermittent claudication in the evolution of vascular disease in the leg is the occurrence of ischaemic rest pain. In its earliest phase, this can occur only at night and may be relieved by making the limb dependant (i.e. hanging it over the side of the bed). Later the pain is present at all the time, rendering the patient immobile and dependant on analgesics. Unlike intermittent claudication this pain is not felt in a muscle group but more commonly in the forefoot, and is the sign that resting flow through supplying vessels (usually collaterals around an major artery which is occluded by the time rest pain has developed) is insufficient to meet the basal demands of the tissues (Taylor and Porter 1989). Although this is an ominous sign, it may not herald impending limb loss, as collaterals may develop. Rest pain in the presence of ischaemic tissue necrosis is much more serious and usually indicates
impending limb loss if intervention is not undertaken. The term 'gangrene' applied to mummified, ischaemic tissues (particularly digits) is something of a misnomer. In pathological terms gangrene means the infection of dead tissue with putrefactive bacteria, and most of the lesions occurring in peripheral vascular disease are relatively sterile. However the term gangrene is widely used and understood by vascular surgeons and is unlikely to be replaced on the grounds of mere pedantry.

**Critical Leg Ischaemia**

The definition of critical ischaemia was developed by a working party for the International Vascular Symposium (Bell et al 1982) to improve standardisation of reports dealing with the management of severe leg ischaemia. The definition aims to identify the at risk limb needing intervention to salvage or remove ischaemic tissue. There have been minor modifications to the original recommendations of this group in the light of subsequent studies and the current criteria are: rest pain requiring regular analgesia of more than 2 weeks duration and/or ulceration or gangrene in the foot or toes, plus Doppler ankle systolic pressure <50mmHg. For published reports this information must be supported by additional evidence of ischaemia such as angiography (European Consensus Document on Critical Limb Ischaemia 1989).

The predictive value of this definition has been demonstrated by the Joint Vascular Research Group in the U.K. who showed that outcome in patients with critical leg ischaemia was similar to that for patients in stage IV of the previously widely applied Fontaine classification (defined as presence of ulceration and gangrene), whether ischaemic tissue loss was present or not (Wolfe 1986). The definition of CLI therefore seems to be of value in identifying the severely ischaemic leg before tissue necrosis occurs.

When considering management options, however, it is important to realise that not all patients presenting with CLI will require limb salvage - a term which intimates impending loss of the affected limb if arterial reconstruction is not undertaken. This is due at least in part to the inclusion of patients with small vessel disease in the foot or
distal embolic disease, who fulfil the definition of CLI but are at low risk of limb loss and may respond to local treatment.

2.4 The Natural History of Leg Ischaemia

Assessing the natural history of any disease is difficult, if not impossible once treatment regimes are established as moral issues and pressure from society prevent randomised trial (Dormandy et al 1989). Despite this, valuable data concerning progression of untreated peripheral vascular disease can be gained from observational studies of subclinical and non limb-threatening leg ischaemia in the community. These data can be used to advise patients presenting with mild or moderate ischaemia. It is generally held that the risk of disease progression from claudication to more severe ischaemia is low; in the Framingham Study, observation of a large number of claudicants in the community showed that under 2% progressed to require amputation over an eight year period (Kannel et al 1970), a figure similar to that found in another large population based epidemiological study by Widmer et al (1985). However, not all studies have shown such a low incidence of advancement; Cronenwett et al (1984) showed clinical disease progression in 60% of claudicants over a two and a half year observation period, and over 1/3 of these patients required operative intervention to prevent amputation. Boyd (1960) showed that 12% of claudicants required amputation during a 10 year follow up period. The latter two studies include only patients who have already developed intermittent claudication of sufficient severity to present to a doctor however, rather than those with symptomatic disease in the community and this makes interpretation and comparison of these results difficult, and serves to illustrate some of the difficulties encountered in measuring disease progression. Boyd's study was conducted before arterial reconstruction became widely available and thus may more closely represent the natural history of untreated intermittent claudication than recent studies.
Disease progression in more advanced stages of leg ischaemia seems to be much worse than in earlier stages; in a study of severe ischaemia undertaken by Bloor in 1961, also prior to the widespread application of vascular reconstruction, 47% of patients with rest pain and or ischaemic ulcers progressed to amputation. Recently, Szilagyi et al (1979) have suggested that almost one quarter of patients with severe leg ischaemia eventually require amputation over a 10 year period, even if arterial reconstruction is undertaken.

Perhaps as important as local disease progression in the study of infrainguinal atherosclerosis, is the effect of vascular disease on the heart and cerebrum on the survival of patients with leg ischaemia. Studies have shown that both coronary and carotid atheromatous disease is common in patients presenting for arterial reconstruction in the leg (Hertzer et al 1984, Turnipseed et al 1980); Hertzer showed that up to 90% of patients admitted for surgical intervention for leg ischaemia have angiographically demonstrable coronary disease. Furthermore, this was clinically detectable in only about half of these patients. The high incidence of concurrent disease is reflected in survival figures after surgical intervention for leg ischaemia of all types which range from 74-80% at five years to 50-64% at 10 years (Jurgens et al 1960, Crawford et al 1981, Hertzer 1981, Szilagyi et al 1986).

2.5 Non-Operative Management Of Leg Ischaemia

- DRUG TREATMENT

Bearing in mind that leg ischaemia may have a relatively benign natural history and a relatively poor long term outlook, attempts to manage peripheral vascular disease non operatively have been tried for many years. The principle options seem to be correction of cardiovascular risk factors (particularly smoking), graded exercise, drug therapy and percutaneous treatment.

As described in chapter 5, the pathophysiology of smoking and atheroma formation is not understood although an epidemiological association has been clearly
established (reviewed by Fielding 1985). It has also been demonstrated that stopping smoking after initial presentation with peripheral vascular disease is associated with a rapid increase in walking ability (Quick and Cotton 1982) and may produce a reduction in the likelihood of amputation (Jurgens et al 1960). Further, Kempczinski & Bernhard (1989) have suggested that up to 50% of patients with symptomatic leg ischaemia (including those with severe disease) will improve if smoking and other risk factors are corrected. Despite such strongly persuasive data and the presence of a vociferous anti-smoking lobby in the western world, Clyne et al (1982) showed that almost 2/3 of peripheral vascular disease patients attending British hospitals, did not understand the implications of smoking and their disease. It is therefore apparent that continued education of patients with peripheral vascular disease is a vital component of treatment by the vascular surgeon.

Graded exercise in the management of intermittent claudication has been tried by some groups and seems to produce clinically detectable improvement; Ekroth et al (1978) were able to demonstrate an improvement in claudication distance in excess of 200% in a large group of claudicants treated with supervised exercise classes. In a smaller study in 1980, Clifford et al used similar classes and published a more modest 80% improvement in walking distance.

The methods by which exercise reduces claudication symptoms are not clear; it is known that the improvements seen after exercise programmes are not associated with an increase in ankle-brachial pressure index, calf blood flow (Bylund et al 1976) or oxygen content in the popliteal vein (Sorlie and Myhre 1978). These observations along with muscle physiological studies suggest that a metabolic alteration in the ischaemic muscles is responsible for the improvement (Bylund et al 1976).

Raising the shoe heel to reduce the amount of work performed by the calf muscles during walking has been tried but with little success (Chavatzas and Jamieson 1974). The role of other physical methods such as transcutaneous nerve stimulation...
and pneumatic massage in improving muscle blood flow is dubious and probably limited (reviewed by Ernst 1989).

Various categories of drugs could be of theoretical benefit in peripheral vascular disease. Adrenergic vasodilatation by either α-receptor blockade or β-receptor stimulation theoretically should improve blood supply by reduction of vasomotor tone, however as maximal vasodilatation is probably already present in ischaemic muscle beds, the effects of these drugs may be limited. Trubestein (1981) suggested that adrenergic modifying drugs may influence limb blood flow by enhancing the collateral circulation, but this remains to be proven and most clinical studies to date have failed to demonstrate significant improvement using vasodilator drugs in peripheral vascular disease. Some of the newer agents, such as Cinnarizine and Buflomedil may have a role in mild peripheral vascular disease and a small number of studies have purported to show an improvement in symptoms using these drugs (Mashiah et al 1978, Trubestein et al 1984).

Haemorheologically active drugs are also attractive for the treatment of intermittent claudication, as most patients with peripheral vascular disease have increased whole blood viscosity (Dormandy et al 1973a). Oxpentifylline may increase red cell deformability and has been shown to be of benefit by some although measured improvements in blood flow were small and considered not to be of clinical significance (Reich et al 1984). In addition a recent clinical study was unable to show any improvement using the drug (Lloyd et al 1987). Other viscosity reducing agents such as the anabolic steroid stanozolol and the fibrinogen reducing agent clofibrate are associated with side effects which may limit their therapeutic use (Oliver et al 1978).

Naftidrofuryl is a different class of drug used in intermittent claudication and is claimed to increase oxygen extraction by ischaemic muscles. This is probably spurious, as basic physiological studies of ischaemic muscle suggest that O2 extraction in ischaemia is virtually maximal (Pentecost et al 1966). Regardless of this, a modest improvement in symptoms has been reported by Adhoute et al (1986) although other studies have contradicted this (Ruckley et al 1978).
Antiplatelet agents have been used as treatment for vascular disease but effects seem to be limited to the inhibition of progression of established atheroma (Hess et al 1985) rather than any clinically detectable improvement in symptoms or blood flow. These findings may be expected in the light of the current understanding of platelets and atherogenesis.

**Angioplasty**

Balloon angioplasty for peripheral vascular disease is useful alone or in conjunction with conventional operation. The advantages of angioplasty include reduction in cost, repeatability, and reduced complications in high risk patients.

The technique of percutaneous transluminal angioplasty was first described in 1964 by Dotter and Judkins. The widespread application of the technique did not occur until Gruntzig and Hopff developed the double lumen balloon in 1974. Further advances in balloon and guide wire technology since that time have greatly enhanced therapeutic options.

The technique of angioplasty includes an initial arteriogram to define the pathology, followed by catheter introduction under local anaesthesia. A guide wire is passed through the stenosis followed by the balloon catheter. Inflation of the balloon to dilate the stenosis to the diameter of healthy native vessel is then usually undertaken (O'Keefe et al 1991). Data from post mortem specimens of successful angioplasty, suggests that plaque fracture and medial dissection are characteristic findings (Castenada-Zuniga et al 1980). It is unclear whether plaque compression or vessel stretching are important effects in angioplasty (Waller 1989). Minimal restriction of activity is needed after the procedure and some institutions have performed angioplasty on an outpatient basis (LeMarbe et al 1987).

Success of angioplasty depends on the morphology of the lesion and the skill of the operator. Generally, short segment stenoses, under 3 cm. length, are associated with the best results and lesions over 10cm. are associated with very poor success rates.
In addition, the best results seem to be obtained with angioplasty in larger vessels.

Iliac stenosis is probably the most common indication for angioplasty in the peripheral vascular tree and reported success rates are as high as 90% at 5 years - but this is often based on symptomatic improvement rather than angiographic data (van Andel et al 1985). In the femoropopliteal segment, angioplasty has been associated with an 80% initial success rate; 3 year reported success is a more modest 70% for stenoses and 55% for occlusions (O'Keefe et al 1991). Angioplasty of infrapopliteal vessels is less successful than in larger vessels although techniques are improving constantly (London et al 1993). Angioplasty has also been used with good effect in both venous and prosthetic bypass grafts although the mechanism of angioplasty action on hyperplastic graft stenoses is not clear and the technique may be less successful for this condition than for native vessel atherosclerosis.

2.6 The Surgical Management of Leg Ischaemia

The History of Arterial Reconstruction

The use of a surgically constructed conduit to bypass diseased native arteries in the leg was first described by Goyanes in Madrid in 1906, who used autologous saphenous vein to exclude an aneurysmal popliteal artery in a human subject. Although the subject of this paper survived, the disastrous consequences of infection and poor anaesthetic technique on the outcome of surgery, meant that the method had little application around the turn of the century.

Kunlin in L'Hôpital Cantor Ville in Paris, revived interest in lower limb arterial reconstruction in 1948 when he recorded four autogenous vein bypass grafts, two of which survived until the death of their recipients 6 months after surgery. However it was not until the Korean war of 1954-6, which combined modern high velocity ammunition with the ability to evacuate the injured and prevent loss of life on the
battlefield, that significant experience of arterial reconstruction was gained. U.S. army surgeons performed in excess of 1,000 infrainguinal reconstructions during and immediately after the war in which 1000 men were killed and almost 10,000 injured.

The appearance of a body of surgeons with substantial experience of this kind of procedure rapidly led to its application to civilian non-traumatic arterial occlusive disease as originally described in Spain and France. Improvements in the diagnosis of vascular disease occurred throughout the 1950's and 60's, as a product of advancing technology, which allowed enhanced images of diseased vessels to be obtained, along with better understanding of the nature of the haemodynamic changes caused by arterial disease.

Aortoiliac Reconstruction

The aortoiliac tree is a common site for atheromatous stenosis and occlusion, and a degree of proximal disease may be present in many patients who require intervention for leg ischaemia (Mozersky et al 1978, Darling et al 1979). There have been many advances in the management of aortoiliac disease in the last 30 years and the high success rates following surgical reconstruction in this region make the management of aortoiliac disease one of the most rewarding in vascular surgery.

Aortoiliac disease co-existing with more distal disease most commonly occurs in the elderly hypertensive and / or diabetic male (Mozersky et al 1978). Darling et al (1979) have suggested that localised aortoiliac disease without significant distal occlusion or stenosis is more common in younger men with lipid abnormalities but has estimated that this pattern of disease represents only 10% of presentations with clinically significant aortic or iliac disease. Cronenwett et al (1980) identified women as another group in whom localised aortoiliac disease may occur. The importance of proximal disease in patients requiring more distal reconstruction is considered along with femoropopliteal and femorotibial reconstruction below.
When intervention is required, direct reconstruction using bypass grafting is often the most definitive and desirable means of correction (Brewster & Darling 1978, Crawford et al 1981). Percutaneous transluminal dilatation of arteries is successful in the aortoiliac tree but is probably best limited to presentations with short localized stenoses (Johnston et al 1982) as results are much poorer after percutaneous dilatation of occlusions or long stenoses (Morin et al 1986).

The technique of direct reconstruction most often employs a prosthetic graft placed in or near the aortoiliac bed, although extra-anatomic operations are used in high risk patients unable to withstand transabdominal surgery, and these operations will be considered separately. Successful arterial reconstruction in the aortoiliac tree requires the optimal anastomosis at the correct site in the arterial tree proximal and distal to the lesion to be bypassed.

The proximal anastomosis is usually constructed high in the aorta (as close to the renal vessels as can safely be achieved) to prevent progression of atheroma in the distal abdominal aorta compromising inflow. Controversy continues concerning the optimal type of proximal anastomosis. Brewster and Darling (1978) and Mulcare et al (1978) have shown that graft patency after end-to-end anastomosis is superior to that after end-to-side anastomosis but neither of these is based on a randomised, controlled trial. Other proponents of end-to-end anastomosis have shown that the incidence of the potentially lethal complication of aortoenteric fistula formation is reduced after end-to-end anastomosis compared with end-to-side techniques (Mulcare et al 1978, Dunn et al 1982). This is probably as a result of the anastomosis being wrapped in the aortic wall after placement. Furthermore, there are theoretical disadvantages in the end-to-side operation when using a side-biting aortic clamp which may dislodge tenuous intraluminal thrombus. End-to-side graft-aortic anastomosis should be used when there are large patent branches present below the renal vessels or if the common and internal iliac arteries are patent. Exclusion of these vessels by the end-to-end technique may
produce acute ischaemia with irreversible effects on the intestine (chiefly the colon) or spinal cord (Ernst 1983, Picone et al 1986).

At the present time the absolute indications for end-to-end or end-to-side anastomosis are not clearly established but the above outlines the principal points on which further studies will be based.

The sites of construction of the distal anastomoses are usually dictated by the pattern of disease as shown on arteriography and clinical examination. In some patients it may be technically possible to construct the distal anastomoses at the level of the external iliac arteries but this may not be the optimal site as progression of iliac disease can jeopardise graft function and has been shown to result in a higher incidence of late graft failure and subsequent distal operations (Baird et al 1977, Brewster & Darling 1978). It has been suggested on theoretical grounds that bypass to the femoral artery in the groin may be associated with an increased risk of graft infection but this has not been borne out by studies (Moore et al 1968, Crawford et al 1981).

**Femoropopliteal Reconstruction**

The femoro-popliteal segment is the most common site for atheroma formation in the leg (Mavor 1956) and the consequences of stenosis and occlusion in this region are regularly seen in vascular clinics and on vascular surgical wards. Clinically, femoropopliteal disease presents as intermittent claudication in the calf or critical leg ischaemia in the presence of weak or absent popliteal and pedal pulses. Of available surgical techniques, direct reconstruction using a bypass graft is the most durable and commonly applied operation. Successful bypass grafting in the femoropopliteal segment requires adequate arterial inflow to the conduit, patent distal vessels with sufficient run off to accommodate a good graft flow, and a reliable graft material.
Femoropopliteal grafting is indicated for limb threatening ischaemia of the leg due to occlusive arterial disease in the femoropopliteal segment but can also be applied with relative freedom to patients with claudication which limits the desired lifestyle (Linton & Darling 1962, Szilagyi et al 1964, Donaldson & Mannick 1980). This relatively wide range of indications compared with more distal reconstruction is a consequence of the relative success and safety of the procedure; reported operative mortality rates vary between 1% and 7% with most studies suggesting a rate around 3% (Kacoyanis et al 1981, Veith et al 1986). Most of these deaths are due to atheroma in the coronary or cerebral circulation (DeWeese & Robb 1977). Despite this good record, femoropopliteal reconstruction should not be performed in circumstances of mild claudication due to femoropopliteal occlusion of recent occurrence as in these circumstances the outlook is favourable and a collateral circulation is likely to develop which may render symptoms negligible (Boyd 1960). A second reason for avoiding unnecessary reconstruction is the fear that graft failure, by the propagating occlusive thrombus into previously healthy arteries, may result in a higher level of amputation than would have been possible had no preceding surgery been undertaken. Although this remains unproved, the phenomenon has been suggested by some authors (Dardik et al 1982, Evans et al 1990) and the fear remains great enough to restrict reconstruction to disease which significantly affects the patient's lifestyle. Szilagyi et al (1979) have added to this list of relative contraindications and suggested that coexisting disease which limits the life expectancy of the patient to 1 year or less should deter the surgeon from advising reconstruction for his patient.

Preoperative assessment of patients for femoropopliteal bypass should include arteriography and attempts should be made to demonstrate all vessels from the abdominal aorta to the pedal arch. Modern techniques of subtraction angiography are usually able to demonstrate patent segments of named vessels in most patients, but if this is not available or if vessels are not seen then intraoperative angiography should be undertaken directly through the popliteal artery. Good arteriography allows optimal planning of proximal and distal graft sites and may demonstrate any unsuspected inflow
or outflow tract disease which may be repaired, prior to, or at the time of, infrainguinal grafting. Non invasive flow studies should also be undertaken to give further clues to the state of the native vessels. This should include characterisation of the Doppler flow waveforms in the femoral, popliteal and pedal arteries, sequential pressure measurements and pulse generated runoff. The latter technique is able to demonstrate the presence of patent infrapopliteal arteries in conditions of low flow. The number of such vessels and their continuity with the pedal arch has been correlated with subsequent graft outcome (Scott et al 1990). If an autogenous vein graft is to be used, preoperative venous duplex scanning may be used to establish suitability and to mark the site of the vein.

Adequate aortoiliac inflow is vital to the success of femoropopliteal grafting and if stenosis or occlusion in this segment is seen on preoperative angiography (particularly in conjunction with low thigh systolic pressures or a damped femoral artery waveform on non-invasive testing) then this should be corrected prior to femoropopliteal reconstruction (Flanigan et al 1984). A popliteal segment not in continuity with the infra popliteal arteries on arteriography (blind popliteal segment) may be used as a distal anastomosis site to relieve claudication or mild rest pain associated with reasonable ankle pressure (Lye et al 1976) but if necrosis is present then either segmental or infrapopliteal reconstruction is required (Flinn et al 1980).

Operative techniques for femoropopliteal bypass involve exposure of the common femoral artery in the groin and the popliteal artery, either above or below knee, as indicated by preoperative investigations. The chosen conduit is then anastomosed to the exposed vessels. This requires exposure of the whole length of the long saphenous vein if it is to be used, or the development of a subcutaneous or submuscular tunnel to transmit a prosthetic or arm vein graft.

Vein grafts have traditionally been the conduit of choice for reconstruction below the inguinal ligament, but recently interest has been expressed in the use of expanded polytetrafluoroethylene grafts (ePTFE) for above knee femoropopliteal reconstruction; medium term patency of both vein and PTFE grafts appears to be
similar at this site (Veith et al 1986, The European Consensus Document on Critical Ischaemia 1989) and use of the prosthetic conduit is associated with a reduction in operating time which may be beneficial to frail or high risk patients (Veith et al 1986). Perhaps more importantly, the use of PTFE preserves a functionally useful long saphenous vein should more distal reconstruction in the leg, or coronary artery reconstruction, be required at a later date.

Outcome after femoropopliteal reconstruction is described below.

Femorotibial and Femoroperoneal Reconstruction

When occlusive arterial disease extends to the popliteal artery or its trifurcation, then revascularisation requires arterial bypass to the infrapopliteal tibial arteries (anterior and posterior tibial arteries) or to the peroneal artery. This is a much more serious undertaking than femoropopliteal bypass as graft failure rates are higher in the calf and the potential for thrombosed grafts to raise the level of a subsequent amputation (see above) cannot be ignored. Therefore, femoro-infrapopliteal reconstruction should not be undertaken unless there is critical ischaemia and thus a high likelihood of limb loss without successful revascularisation (Bernhard et al 1972, Kahn et al 1973, Bell 1985, Veith et al 1986). The clinical presentation of distal disease may be identical to femoropopliteal disease (although a popliteal pulse may sometimes be present). The pattern may only become recognisable when arteriography is performed.

Preoperative assessment is identical to femoropopliteal bypass. At operation exposure of the crural arteries may require incision along the medial border of the tibia (from which the posterior tibial and peroneal arteries can be exposed), an anteromedial incision over the anterior muscle compartment (to expose the anterior tibial artery) or a lateral incision excising a portion of fibula to expose the peroneal artery laterally. The choice of crural vessel to anastomose to will commonly be dictated by angiographic
findings. There is data to suggest that grafting to a tibial artery in direct continuity with the pedal arch may give favourable results (Imparato et al 1973, O'Mara et al 1981), whilst bypass to the peroneal artery (particularly without good angiographic collaterals between the peroneal artery and the pedal arch) may be associated with poorer results (Imparato et al 1973, Szilagyi et al 1979) although these findings are not clearly established.

The choice of conduit for grafting to the infrapopliteal arteries is much more critical than for femoropopliteal bypass. Graft patency in the medium term is significantly reduced when prosthetic grafts are used and this may therefore be associated with a higher rate of limb loss (Veith et al 1986, European Concensus Document on Critical limb Ischaemia 1989). The use of a vein cuff at the distal anastomosis of ePTFE and crural vessels (Miller 1989) may be associated with an improvement in prosthetic graft patency (Wolfe & Tyrell 1991) but this improvement does not bring prosthetic success rates up to the level of vein grafts in this location and thus cannot justify the use of these grafts if adequate vein is present.

**Extra-anatomic Reconstruction**

The term extra-anatomic arterial reconstruction is generally applied to bypass grafts which assume a path which is significantly different from the route taken by the normal blood flow. The term is rather inconsistent as most arterial reconstructions bypass diseased vessels using an 'extra-anatomic' route except perhaps aortic inlay grafts. However, the most commonly used 'extra-anatomic' grafts are the femoro-femoral cross over graft and the axillo-femoral or axillo-bifemoral grafts. These grafts are used when the local anatomical or pathological conditions are such that deliberate avoidance of the abdomen is preferable. Frequently encountered conditions and bypasses include; infection in the aortic bed or co-existent intra-abdominal pathology such as malignancy or sepsis; axillo-femoral grafting may be used in either of these conditions (Rutherford & Baue 1989). Unilateral iliac disease in a patient unfit for an
abdominal operation is usually amenable to femoro-femoral cross over grafting. Other extra-anatomic operations such as carotid-subclavian, axillo-axillary and carotid-carotid bypass are used from time to time but will not be described here.

Haemodynamics of Extra-anatomic bypass

Without an understanding of haemodynamic principles, arterial 'steal' phenomena might be expected from the donor artery into the revascularised limb after extra-anatomic reconstruction as the operation usually utilises a patent feeding vessel to another limb or organ. However unlike resistance in series, resistance circuits in parallel produce a fall in overall resistance (Weale 1964). The total resistance to flow produced by circuits in parallel is given by the formula;

\[
\frac{1}{R_{\text{total}}} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} \ldots \ldots \ldots \frac{1}{R_n}
\]

Therefore, theoretically, the addition of a further circuit by extra-anatomic reconstruction is unlikely to be associated with haemodynamic deterioration. Such a calculation is supported by flow measurements in vivo using flow meters (Parsonnet et al 1970). It has been calculated that the flow in a donor vessel can be increased 10 fold without any loss of driving pressure to the distal arteries (Ehrenfeld et al 1968).

Donor vessel steal may occur if there is significant outflow stenosis in the donor artery as in these circumstances, flow from the common channel may preferentially take the path of least resistance. This phenomenon accounts for the drop in the ankle perfusion pressure that can be detected in the donor limb of up to 80% of femoro-femoral cross over grafts (Flanigan et al 1978). Flanigan was also able to correlate the presence of inflow and (recipient) outflow tract occlusive disease with femoro-femoral cross over graft patency and showed success ranging from 90% when both of these vessels were healthy to nil if both vessels had significant occlusive disease.
Femoro-Femoral Bypass

This operation may be used in high risk patients in whom it is desirable to avoid a transperitoneal operation. The operation may be performed under regional or even local anaesthesia if necessary; reported operative mortality ranges from 0-15% (Rutherford et al 1987 and Eugene et al 1976 respectively) with most series showing mortality under 5%. It is also durable; 5 year primary patency ranges from 44% - 76% (same reported series).

Alternatives to femoro-femoral bypass are percutaneous transluminal angioplasty if the lesion is suitable (i.e. short occlusion or short to medium length (<5cm) stenoses), unilateral ilio-femoral bypass, or aortobifemoral grafting in a fit patient with bilateral aortoiliac stenosis.

Axillo-Femoral Bypass

This operation is commonly performed to exclude an infected retroperitoneum after failed aortic grafting or to restore lower limb flow in an unfit patient with aortic occlusion. Reported patency rates for unilateral bypass are poor at 5 years ranging from 19% (Rutherford et al 1987) to 67% (Ray et al 1979). Because of this the operation is often reserved for patients with limb threatening ischaemia who are poor anaesthetic risk - a fact that may contribute to the high reported operative mortality rate (Rutherford et al 1987). Axillobifemoral grafts have better patency rates at 5 years (up to 77% secondary patency -Ray et al 1979) but should be considered for use only if aorto-bifemoral grafting cannot be undertaken. Axillo-femoral or bifemoral grafting can be performed under local or regional anaesthesia when necessary but is not as amenable as femoro-femoral grafting. Usually the operation can be undertaken with light general anaesthesia with minimal risk to the patient (Rutherford & Baue 1989).
Chapter 3

Infrainguinal Graft failure, Stenosis and Intimal Hyperplasia

3.1 Introduction

From the preceding chapter it is evident that bypass grafting using either autologous vein grafts or a prosthetic conduit is the most common form of surgical treatment for severe arterial disease in the infrainguinal arterial tree. Failure of infrainguinal grafts is usually associated with intragraft thrombosis although leg ischaemia can recur in the presence of a functioning graft if distal native vessels are occluded leaving only retrograde flow in the recipient artery. The consequences of graft failure may be disastrous for the limb, particularly if grafting was initially performed for critical ischaemia, and may lead to limb loss, although there is some data to suggest that temporary patency may enable a critically ischaemic limb to recover and in some hands limb salvage rates after infrainguinal reconstruction are significantly higher than graft patency rates (Bernhard et al 1972, Veith et al 1990). It is important to realise, however, that even this data does not advocate reconstruction for all patients with critical leg ischaemia, as several studies have suggested that the level of subsequent amputation may be adversely affected by a failed revascularisation (Dardik et al 1982, Evans et al 1990) and the cost in human terms of failed attempts at reconstruction followed by amputation cannot be calculated.

Current evidence points to the post operative development of graft stenoses as the most common cause of graft failure. In this chapter infrainguinal graft failure and stenosis will be considered and the importance of intimal hyperplasia as cause of stenosis will be illustrated.
3.2 Graft Failure Rates

An appraisal of the incidence of graft failure is provided by the European Concensus Document on Critical Leg Ischaemia (1989) which estimates average 1 year graft patency rates for femoro-above knee-popliteal grafts of 85% when vein is used and 80% for PTFE or other prosthetic grafts. Femorocrural grafts in the lower 1/3 of the calf yield 1 year success rates of 55% and 25% for vein and prosthetic grafts respectively (Figure 3(1)). These results are the concensus view of many surgeons in different countries and individual units may achieve better results than this. These patency figures include those grafts which have thrombosed but have undergone secondary intervention which has restored patency. The document highlights the basic premise that increased graft length and the presence of prosthetic material both increase the likelihood of graft failure.

Figure 3(1) Graph showing 1 year patency of vein and PTFE (hatched) infrainguinal grafts with different levels of distal anastomosis.
(derived from the European Concensus Document on Critical Leg Ischaemia 1989)
3.3 The Aetiology and the Timing of Graft Failure

The timing of graft failure in relation to the primary reconstruction gives a clue to the aetiology (fig 3 (2)). Early failure, within one month of surgery, is commonly due to errors in patient selection or operative technique. Approximately 10% of grafts will fail in this time period (Whittemore et al 1981).

When grafts thrombose after 2 years, progression of native vessel atherosclerosis, either proximally or distally, is the usual cause (Whittemore et al 1981). This accounts for 2-3% of all graft failures each year.

The most common time for grafts to fail is between 1 month and 2 years, the same period in which graft stenoses are known to develop, and it is believed that 80% of all graft failures occur secondary to graft stenosis (Whittemore et al 1981, Taylor et al 1990a, Harris 1992).

Figure 3(2) Graph showing timing of infrainguinal graft failure and cause of failure (Derived from Brewster et al 1983)
3.4 Graft Stenoses

Incidence and Aetiology

The post-operative development of graft stenoses was first described by Szilagyi in 1973 and since that pioneering work many groups have studied the phenomenon. Szilagyi identified causes of graft stenosis in a study of postoperative patency using arteriography supported by histological examination whenever tissue became available. Based on aetiology/pathology Szilagyi described five types of graft stenosis (in descending order of frequency);

1. Intimal Thickening
2. Atherosclerosis of the graft
3. Fibrotic valve cusps
4. Fibrotic stenoses (diseased vein grafts or clamp injury)
5. Suture stenosis (branches tied too close to vessel)

Although this study attempted to identify the aetiology of stenoses from angiographic appearances without histology in most cases, intimal thickening and graft atherosclerosis accounted for almost 50% of stenoses. The overall rate of angiographic stenosis was 33% and the mean time of stenosis detection after grafting was 16 months for intimal thickening and 45 months for atherosclerotic plaques. More recent work has shown that stenoses occur in 20-35% of grafts (Table 3 (i)) and usually to develop within the first 1 or 2 years of graft implantation (Grigg 1988, Taylor 1990a, Bell & Brennan 1991, Harris 1992). Additionally, other recent studies, have refuted the development of stenosis at the site of clamping of vein grafts (category iv); Moody et al (1990) studied stenoses using angiography and were unable to show any correlation between sites of graft clamping marked with a radio-opaque clip and the subsequent development of a stenosis. Stenoses caused by side branch sutures or stenotic venous valves are by definition present at the time of graft implantation and can thus be detected and corrected intra-operatively (Tyrell et al 1990). For the purposes of
the current studies all grafts underwent perioerpative surveillance to exclude stenoses present at the time of implantation, thus stenoses detected within the first post operative year, were presumed to be of intimal hyperplasia unless this proved otherwise at time of operation (see stenosis detection methods below).

Table 3 (i).
Studies of Graft Stenosis Rates

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Number of grafts</th>
<th>% Stenosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bandyk et al</td>
<td>1988</td>
<td>197</td>
<td>28</td>
</tr>
<tr>
<td>Harris</td>
<td>1991</td>
<td>80</td>
<td>28</td>
</tr>
<tr>
<td>Taylor et al</td>
<td>1990</td>
<td>412</td>
<td>16</td>
</tr>
<tr>
<td>Chang et al</td>
<td>1990</td>
<td>350</td>
<td>20</td>
</tr>
<tr>
<td>Mills et al</td>
<td>1990</td>
<td>379</td>
<td>6</td>
</tr>
</tbody>
</table>

In Taylor’s study of 412 infra-inguinal grafts (1990) he demonstrated a graft stenosis incidence of 16% and there were no differences between stenosis rates in autologous vein or prosthetic grafts. Sixty-five percent of stenoses developed within 6 months of the primary operation, and no new graft related stenoses were detected after 12 months. Other groups have found late stenoses but these developed in series in which regular surveillance was not undertaken during the first year and it is possible that these lesions had been present for some time (Berkowitz 1981, Disselhoff 1989).

Although evidence implicating stenoses in medium-term graft failure remains circumstantial, Grigg et al (1988) were able to show a positive correlation between the presence of graft stenoses and graft thrombosis and Moody et al (1990) have calculated a 3 fold increase in graft occlusion rate in association with stenoses.
Stenosis Detection

It is evident that significant improvement in graft survival can be achieved by reduction of stenosis associated failure (potentially 80% of all failing grafts). This has led many groups to look for methods of early detection of these lesions, before significant haemodynamic compromise occurs. Subsequent correction of these stenoses should reduce the number of graft failures.

The ideal stenosis detection programme provides non-invasive, accurate information about the graft. It should also be safe, easy to perform and cheap. Numerous techniques have been used with varying degrees of success and whilst the ideal has not yet have been determined, much has been learned of the abnormalities produced in a graft when a stenosis develops; in most cases patients remain symptom free until a disastrous graft failure occurs. Flow abnormalities are mostly limited to the region of the stenosis until the critical point when flow ceases (Bandyk 1988).

Clinical Examination and Ankle/Brachial Pressure Ratios

The earliest studies of graft stenosis used clinical techniques to detect stenoses, chiefly graft pulse palpation supplemented with exercise ankle/brachial pressure ratios. The presence of a graft pulse means only that a pressure wave is passing along the graft and can occur in the absence of flow, it is therefore insensitive to the minor flow abnormalities associated with early stenoses. The addition of ankle pressure measurements improves sensitivity and can detect stenoses before graft failure. Although the method is cheap and simple, in most studies it detects only about 50% of lesions demonstrable by either duplex scanning or angiography (Wolfe et al 1987, Berkowitz et al 1981, Campbell & Wolfe 1987) although recently Brennan and Bell (1991) have shown promising results by increasing the sensitivity of the technique. Using a drop in ankle/brachial ratio between 0.1 and 0.2 and using an experienced...
observer they were able to detect over 70% of stenoses when compared with duplex scanning and angiography.

**Duplex Scanning**

The availability of duplex scanning in the early 1980's provoked great interest amongst vascular surgeons looking for the ideal graft surveillance tool. Almost 10 years later debate continues over the optimal application of this powerful device. Some centres have shown an ability to detect stenoses using a single flow parameter such as peak systolic flow velocity, taken at a single point within a graft (Bandyk et al 1988, Chang et al 1990, Mills et al 1990). Whilst this technique is simple to execute not all groups have been able to demonstrate a correlation between such a measurement and angiographic findings in normal and stenosed grafts (Grigg et al 1988). This has led to the use of more complex methods of duplex graft interrogation such as the V2:V1 ratio (Grigg et al 1988). This techniques seems to correlate well with the presence of a graft stenosis but requires more time and training to execute (see stenosis detection methods below).

**Impedance Analysis**

A significant stenosis in a major vessel increases the impedance to flow and various centres have tried to measure this. A computerised device has now been produced which purports to accurately identify stenoses through a single noninvasive measurement (Wyatt et al 1991). This technique is interesting but requires critical assessment in a number of centres before any recommendation can be given.
Angiography

This remains the gold standard against which all new methods of stenosis detection are measured (Wolfe et al 1987). It must be performed in two planes at 90 degrees to detect all stenoses and is invasive and expensive.

Outpatient intravenous digital subtraction angiography or day case intra-arterial studies performed through small cannulae, are improving the acceptability of angiography but cost remains prohibitive for routine screening use. Furthermore the warm flush experienced during contrast infusion can reduce patient compliance to serial monitoring.

3.5 Intimal Hyperplasia in Infrainguinal Grafts

Incidence

Thickening of the intimal layer of autologous vein grafts and at the anastomoses of prosthetic bypass grafts is currently believed to be the primary cause of stenosis following arterial reconstruction (Szilagyi et al 1973, Fuchs et al 1978, DeWeese 1978 Sottiurai 1990, Chervu and Moore 1975), particularly if mechanical causes of stenosis are excluded by the use of intraoperative doppler. A degree of intimal thickening appears to be the normal response of injured vessels (Clowes et al 1983a) and is probably important in the physiological regulation of arterial maturation (Hughes 1937, Rodbard 1970, Langille & O'Donnell 1986) and vessel responses to changes in blood flow (Holman 1949, Kamiya & Togawa 1980, Zarins et al 1987). In failed infrainguinal bypass grafts in humans, Sottiurai has suggested that intimal hyperplasia at the distal anastomosis may be a ubiquitous finding, and has been able to demonstrate the characteristic cytoarchitecture in 65 occluded femoro-distal bypass grafts removed at the time of reconstruction or amputation (Sottiurai et al 1983).

The development of intimal hyperplasia is not limited to infrainguinal grafts however, and current understanding is based on studies of the phenomenon in coronary arteries following balloon angioplasty, after carotid endarterectomy, in bypass graft
models and following balloon arterial de-endothelialisation in animal models (Chervu & Moore 1975).

Morphology

Macroscopically intimai hyperplasia consists of a glistening white raised area on the luminal surface of the vessel (DeWeese 1978). A similar in appearance was described in 1906 by Carrell & Guthrie during their observations on vascular anastomoses used in experimental kidney transplantation and this is perhaps the first report of intimai hyperplasia in the literature. Microscopically, in infrainguinal bypass grafts, the lesion is identical irrespective of the graft material; smooth muscle cells, believed to originate in the arterial media, are present in abundance and are surrounded by extracellular matrix consisting chiefly of collagen Figure 3 (3) (Sottiurai et al 1983). This cytoarchitecture is common to lesions examined from thrombosed human bypass grafts or from experimentally induced graft intimai hyperplasia in animal models (Sottiurai et al 1983 & 1989).

Percutaneous atherectomy techniques have proved a unique medium for obtaining primary and restenosing lesions from native vessels and vein grafts in both the coronary and peripheral vascular trees. As well as confirming the presence of smooth muscle cells and ground substance as described by Sottiurai and colleagues (1983), two comparative studies have shown that primary and secondary lesions resected in this manner have similar constituents but that the proportion of fibrous and necrotic elements is higher in primary lesions (i.e. atheroma), whilst restenoses (intimai hyperplasia) contain more viable hyperplastic cells (Johnson et al 1990, Garrat et al 1991).

It is worth pointing out the morphological similarity between the lesion of intimai hyperplasia and the description of maturing atheroma given in chapter 1. A comparison of atheroma and intimai hyperplasia (or 'accelerated atheroma') as described by Ip et al (1990) is shown in table 3(ii).
Figure 3(3) Composite electron micrograph of intimal hyperplasia; samples are taken from the lumen (A), middle (B) and graft (C) surfaces of the lesion. Myofibroblasts (MF) orientated in the direction of blood flow and surrounded by collagen (C) and proteoglycan can be seen. LC - luminal cells X3600

From Sottiurai 1990
### Table 3 (ii).
Comparison of Spontaneous and Accelerated Atherosclerosis
(from Ip et al 1990)

<table>
<thead>
<tr>
<th>Endothelial Injury Type</th>
<th>Spontaneous Atherosclerosis</th>
<th>Intimal Hyperplasia and Accelerated atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early platelet involvement</td>
<td>Type I</td>
<td>Types I and II</td>
</tr>
<tr>
<td>Early monocyte involvement</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sources of Growth Factors</td>
<td>Endothelium/Monocyte/SMC/Platelet</td>
<td>Endothelium/Platelet/SMC</td>
</tr>
<tr>
<td>Initial Pathology</td>
<td>Lipid deposition, monocyte proliferation and platelet adhesion</td>
<td>Thrombosis, intimal SMC proliferation, fibrosis</td>
</tr>
<tr>
<td>Late Pathology</td>
<td>Intimal SMC proliferation, Fibrosis</td>
<td>Lipid deposition</td>
</tr>
<tr>
<td>Complication</td>
<td>Plaque rupture, thrombosis</td>
<td>Plaque rupture, thrombosis</td>
</tr>
<tr>
<td>Duration of process</td>
<td>Decades</td>
<td>3 months-2 years</td>
</tr>
</tbody>
</table>


Aetiology

The cause of intimal hyperplasia in bypass grafts is not known, although its occurrence at the site of balloon de-endothelialisation in arteries (Chidi & DePalma 1978, Greenwood et al 1984, Clowes et al 1989) suggests that intraoperative and haemodynamic injury to the graft endothelium may be important, at least in the initiation of the process. This theory is supported by observations demonstrating correlation between the degree of intimal injury and the extent of subsequent intimal hyperplasia in balloon injury models (Liu et al 1989, Steele 1985, Ip et al 1990). Other mechanisms which may regulate intimal hyperplasia in arterial bypass grafts include wall shear stress (see rheology and intimal hyperplasia below) as well as interaction between the vessel wall and components of the circulating blood (see lipids, platelets, clotting and fibrinolysis and intimal hyperplasia below).

Although the aetiology of intimal hyperplasia remains obscure, much has been learned about the development of the lesion. Central to most current studies and hypotheses is the response to injury of the vascular smooth muscle cell, a feature also known to be important in the development of atherosclerosis. In balloon de-endothelialisation models of intimal hyperplasia, replication of smooth muscle cells has been shown to commence in the media (Clowes & Schwartz 1985) followed by subsequent migration into the subintimal space where further hyperplasia and the production of extracellular matrix occurs (Clowes et al 1986 & 1989). Clowes has demonstrated a 200-400 fold increase in the smooth muscle cell proliferation rate within 48 hours of a balloon catheter injury in the carotid artery of the rat using a Thymidine labelling index (Clowes et al 1983b) and was also able to show that this growing fraction does not increase beyond 72 hours after the injury (Clowes et al 1985).

How this injury/hyperplasia response is mediated is unclear, some available evidence points to direct smooth muscle cell stimulation (Ross et al 1974, Gasic et al 1992) whilst several investigators have shown that hyperplasia ceases if complete endothelial regeneration occurs (Fishman et al 1975, Haudenschild & Schwartz 1979,
Clowes et al 1989), suggesting that endothelial cells may mediate the effects of the products of injury.

Sites of Occurrence

Intimal thickening have been shown to develop at any or several points along the body of vein grafts transplanted end-to-end into the arterial tree (Zwolak et al 1987). Additionally, the process occurs at graft to artery anastomoses, particularly in end to side configurations, an arrangement not found in mammalian adult arteries (Sottiurai 1990). At the distal anastomosis, Sottiurai (1990) has demonstrated the consistent build up of localised intimal hyperplasia on the floor of the native artery and at the heel and toe of the graft (figure 3 (4)) and has suggested that, at least in part, this is due to the flow pattern at the anastomosis which is known to be abnormal in these regions (Scharfstein et al 1963, Guvstein et al 1968). This has led Sottiurai to propose a theory of the aetiology of intimal hyperplasia in which turbulent or varying flow patterns enable platelets, complement and monocytes to interact with subendothelial tissues by an as yet undetermined series of events but which may involve the 'shingle' effect (reverse flow on overlapping endothelial cells) (Sottiurai 1990).

Risk Factors and Intimal Hyperplasia

A large body of work now exists associating recognised cardiovascular risk factors with smooth muscle cell proliferation in animal models of intimal hyperplasia. Evidence of the role of these risk factors in the initiation or promotion of intimal hyperplasia and the data correlating risk factors with the degree of intimal thickening will be presented in the relevant experimental chapters.
3.6 The Management of Graft Stenosis and Failure

Stenoses

There is overwhelming evidence that correction of haemodynamically compromised grafts which are still patent is associated with a significant improvement in medium and long term patency compared with procedures undertaken after graft thrombosis. Whittemore in Boston has shown that graft patency after stenosis correction is 82% at 5 year follow-up compared with 28% 5 year patency subsequent...
to operations following graft thrombosis (Whittemore et al 1981). In addition some centres are now able to report the effects of their graft surveillance and stenosis repair programmes. Moody et al (1990) have demonstrated a 15% improvement in overall medium term patency with his policy of aggressive re-intervention.

Intragraft stenoses are amenable to either open surgery or percutaneous transluminal angioplasty (PTA). When stenosis correction was first undertaken, reports of the success of re-intervention varied widely between different centres, particularly when PTA was used (Table 3(iii)).

<table>
<thead>
<tr>
<th>Author</th>
<th>Number of Grafts</th>
<th>Balloon Dilatation (Patency)</th>
<th>Surgery (Patency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bandyk</td>
<td>197</td>
<td>18 (100%)</td>
<td>8 (90%)</td>
</tr>
<tr>
<td>Veith</td>
<td>191</td>
<td>30 (90%)</td>
<td></td>
</tr>
<tr>
<td>Berkowitz</td>
<td>134</td>
<td>27 (80%)</td>
<td></td>
</tr>
<tr>
<td>Shanik (1)</td>
<td>49</td>
<td>7 (43%)</td>
<td>5 (80%)</td>
</tr>
<tr>
<td>Mannick (7)</td>
<td>322</td>
<td>14 (50%)</td>
<td>23 (70%)</td>
</tr>
<tr>
<td>Wolfe</td>
<td>412</td>
<td>18 (100%)</td>
<td>8 (90%)</td>
</tr>
</tbody>
</table>

In a multicentre analysis of PTA the single most important factor predicting outcome was length of stenosis; lesions under 1 cm had a 73% success rate at 2 year follow up whereas stenoses longer than 3 cm had a significantly reduced success rate of 25% over the same period of follow up (Taylor et al 1990b).

The obvious advantages of percutaneous transluminal angioplasty are its simplicity, repeatability and relative inexpense. Whilst open surgery is more successful, in most cases it does require general anaesthesia and a longer stay in hospital for the patient.

2 Cohen JR Mannick JA Couch NP Whittemore AD 1986 Recognition and management of impending vein graft failure Arch Surg 121(7) 758-759
Currently, it seems that a programme of graft surveillance combined with the correct intervention following stenosis detection is vital to the survival of infra-inguinal bypass grafts (Harris 1992). The techniques of surveillance are relatively simple and should be available to all vascular surgeons undertaking regular infra-inguinal arterial reconstruction. Although the optimal methods of stenosis repair require clarification, work is underway and it is hoped that in the future the 80% of graft failures occurring between 1 month and 2 years can be reduced significantly.

Thrombosed Grafts

The treatment of patients presenting with a thrombosed infra-inguinal graft is dictated by the viability of the leg at presentation. In all cases arteriography should be performed initially to confirm the diagnosis and demonstrate any immediately correctable or unsuspected pathology.

The Acute Graft Thrombosis with Viable Limb

In these circumstances the aim of treatment is to restore function to the original graft and correct any lesion within the graft or native vessels which may have caused thrombosis; Circumstances ideally suited to the use of thrombolysis.

Various agents can now be used, including Streptokinase, Urokinase and Tissue Plasminogen Activator (TPA). All are administered through an intra-arterial cannula, preferably within a sheath with side holes through which low dose thrombolytic agent can also be infused. Such an arrangement facilitates easy change of cannula whilst minimising chances of bleeding around the puncture site and vessel trauma. The secondary infusion along the sheath prevent thrombus forming around the catheter.

The tip of the main cannula delivering thrombolytic agent should be impacted in the top of the thrombus to be lysed under angiographic control and the progress of
thrombolysis monitored with 6 or 8 hourly angiography until the graft is cleared and clinical improvement occurs.

The advantage of this form of treatment is that the cause of the occlusion can be identified by arteriography once the graft is cleared. When a graft stenosis is detected, it can be corrected without need for replacement of the whole graft and loss of valuable autogenous vein. Other advantages are its relatively non-invasive nature (compared with open embolectomy) and the ability to lyse thrombus in both the graft and the smaller distal vessels, which may not be amenable to standard balloon embolectomy.

The most obvious disadvantage of the technique is the danger of bleeding associated with systemic escape or localised build up of the thrombolytic agent. Groin haemorrhage is the most common complication and is reported in 10 - 30% of cases when thrombolysis is used for leg ischaemia. Recent work in the UK has suggested that TPA may be associated with fewer bleeding complications than other agents (.5% groin haemorrhage incidence using TPA and cannula with sheath, compared with 33% incidence using Streptokinase without the sheath), particularly when used as described above (Dawson et al 1991).

Disadvantages and potential complications of this form of treatment are; the inherent time delay between commencing treatment and re-establishment of flow, that it may not lyse all thrombus, especially if this has been present for some time, and rarely, anaphylaxis can occur with Streptokinase and Urokinase if they are used repeatedly.

Acute Thrombosis Non-Viable Limb

In these circumstances emergency surgery is required to salvage the limb. The patient should be prepared for operation immediately. After administration of antibiotic prophylaxis and systemic heparin further action depends on the type of graft.
Thrombosed Vein Grafts

If a thrombosed vein graft can be operated on within 6 hours of onset of acute symptoms then the aim of treatment should be the restoration of graft flow with detection and correction of any underlying abnormalities. At operation the hood of the distal anastomosis should be opened and the graft and distal vessels cleared of thrombus using standard techniques. Once flow is established, on table angiography is performed and any graft or native vessel abnormality corrected with a vein patch or jump graft as described previously. Infusion of 100,000 units of Streptokinase into the graft may lyse distal thrombi if these are visualised on the arteriogram.

In the more usual case when surgery is undertaken later than 6 hours after thrombosis, consideration should be given to excision of the graft and its replacement with vein from another site or a PTFE graft. In the latter case retention of the distal 1-2cm of the original graft and its use as a vein cuff may be advantageous.

We have adopted this policy since few of our vein grafts which have been surgically thrombectomised at greater than 6 hours after failure have remained patent.

Thrombosed PTFE Grafts

With PTFE grafts the timing of reoperation in relation to thrombosis is not as critical to further graft survival, and in all cases the surgeon should aim to restore function to the original graft with or without a stenosis correcting procedure. Since PTFE is more thrombogenic than vein, even at higher flow rates and the graft can thrombose without any obvious local pathology.

In all circumstances both proximal and distal graft hoods need to be opened. Thrombus can then be cleared using saline. If good back flow from distal vessels is
obtained, the graftotomies can be closed and an on table arteriogram performed. Once again any lesion in the graft or native vessels must then be corrected. In a study of 104 failed PTFE grafts Veith described corrective procedures such as patch angioplasty were required in 70% of cases (Veith et al 1989)
Chapter 4

Patient recruitment and Statistical Analysis

In this chapter recruitment of patients for risk factor / graft stenosis studies will be described, along with the statistical methods used for data analysis.

4.1 Patient Recruitment

All patients included in the following studies were recruited from the femoropopliteal vascular clinic at St. Mary's Hospital between May 1990 and June 1991, under the care of a single consultant surgeon (Mr. J.H. N. Wolfe).

The femoropopliteal clinic is manned by a research fellow (N. Cheshire during the study period) who co-ordinates follow up and graft surveillance for all patients who undergo infrainguinal arterial reconstruction. Following femoropopliteal or femorocrural bypass, patients are seen initially at 6 weeks post reconstruction and then at 3, 6, 9 and 12 months. Long-term follow up then continues on a 6 monthly basis.

Post operative stenosis surveillance is undertaken on all infrainguinal grafts and has been since 1986. Therefore, in addition to recently grafted patients entering into the surveillance programme (see chapter 5), the clinic also follows a cohort of patients with mature grafts who have previously undergone stenosis surveillance. Since 1988, duplex doppler surveillance (using techniques described below) has been in operation, and results have been systematically documented. This data provides a group of patients suitable for investigation in a retrospective manner.

All patients recruited into the studies gave verbal consent to inclusion; details of previous vascular history and clinical risk factors were recorded on a proforma; data concerning age, sex, smoking habits, diabetes mellitus and hypertension were obtained and substantiated where necessary (particularly smoking - see chapter 5).
That only 15-30% of infrainguinal grafts develop stenosis (Bandyk et al 1988, Harris et al 1989, Taylor et al 1990, Chang et al 1990) presents a problem to a study aiming to compare risk factors between stenosed and non stenosed grafts, as the number of patients likely to develop stenosis within a given study cohort is small unless the recruitment period is prohibitively long. Analysis using a limited number of patients with stenoses could produce statistical errors.

It was considered important to obtain data not just from a prospective group of patients, but also from patients who had completed a full surveillance programme recently, and had developed graft stenosis, or whose grafts remained stenosis free, and were still attending for follow up.

This policy resulted in the recruitment of two groups of patients and these are described below.

Prospective Patient Cohort

The prospective arm of this study included all sixty patients who underwent infrainguinal reconstruction during the period May 1990 to June 1991. Median follow up in this group of patients is 17.5 months and minimum follow up is 12 months. Patients who did not complete the scheduled stenosis surveillance programme (n=9, in all cases because the patient was referred from another hospital and lived too far from St Mary's to attend for regular follow up) or whose grafts occluded within the first month after surgery (n=5) were excluded from the study; early occlusion of grafts is usually associated with technical error either in patient selection or intra-operative technique (Brewster et al 1983), and thus inclusion of the latter group of patients would have unnecessarily complicated analysis of results.

Thus, forty-six patients were available for prospective follow up. In thirty of these patients (65%), in-situ autologous vein grafts were used. The remaining sixteen patients were reconstructed using 6mm diameter expanded Polytetrafluoroethylene grafts with external support (PTFE, Imppra UK). Distal vein collars (Miller 1989) were...
used with all PTFE grafts to crural arteries. For femoropopliteal bypass patients were entered into the Joint Vascular Research Group's femoropopliteal PTFE versus PTFE and collar trial, and were randomised to receive a vein collar or not. All grafts underwent peri-operative assessment to exclude non-hyperplastic causes of graft stenosis as described below. During follow up, graft stenoses requiring intervention were detected on duplex surveillance and confirmed by angiography in 12 vein grafts (40%) and 6 PTFE grafts (37.5%). The types and outcome of grafts in the prospective group are shown in table 4.1.

Table 4.1 Prospective grafts included in risk factor studies, and outcome

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. Patients in Study</strong></td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td><strong>Femoropopliteal Grafts</strong></td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td><strong>Femorocrural Grafts</strong></td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td><strong>Stenoses (%)</strong></td>
<td>12 (40%)</td>
<td>6 (37.5%)</td>
</tr>
</tbody>
</table>

Retrospective Patient Cohort

Patients in the retrospective study were recruited randomly from those attending for follow up. All had undergone infrainguinal reconstruction between 1988 (when duplex surveillance data collection became standardised) and 1990 (when the current study began). Entry criteria included complete peri-operative assessment to exclude pre-existing graft stenosis (see chapter 5), a patent graft which had not undergone thrombectomy or thrombolysis, a clearly documented surveillance history (recorded on successive duplex scan reports) and completion of at least 1 year surveillance as per the surveillance programme described below (see chapter 5).

Two groups of patients were identified and recruited: a retrospective stenosed group and a retrospective non stenosed group. Criteria for inclusion in the retrospective
stenosed group were angiographically proven graft stenosis requiring operative or percutaneous correction during the first post operative year. Criteria for inclusion in the retrospective non stenosed group were the demonstration of a graft free from stenosis (on regular duplex scanning or angiography) for a minimum of 1 year post reconstruction. Patients with occluded grafts or poorly documented surveillance studies were excluded from the study for the same reasons as described in the prospective group.

Of the thirty-five patients recruited retrospectively into the study, twenty-two had autologous vein grafts (17 in-situ and 5 reverse saphenous or arm vein / composite vein grafts) and thirteen had been reconstructed with PTFE grafts +/- a vein collar. In the latter group, all femorocrural operations included a distal vein collar. Collars were not used in femoropopliteal reconstructions prior to the JVRG trial (see above). Stenoses requiring intervention and correction had been previously detected in ten of these grafts (5 vein grafts and 5 PTFE grafts). The types and outcome of grafts in the retrospective group are shown in Table 4.2.

Table 4.2: Retrospective grafts included in risk factor studies, and outcome

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Patients in Study</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>Femoropopliteal Grafts</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Femorocrural Grafts</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>No. of Stenoses</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Results from both prospective and retrospective groups were used to compare risk factors between stenosed and non stenosed grafts. The statistical methods used to account for differences between these groups (and different graft materials), are described below.
4.2 Statistical Analysis

This thesis considers vascular risk factors in patients undergoing infrainguinal bypass and compares the prevalence or scale of these factors in patients whose grafts develop stenosis due to intimal hyperplasia with those whose grafts remain stenosis free. The methods used to exclude causes of graft stenosis other than intimal hyperplasia (and to detect hyperplastic stenoses) will be described in chapter 5. The statistical methods used to analyse results require explanation and justification. It is timely to describe these at this point.

Analysis of a dataset containing category and continuous variables in stenosed and non stenosed grafts of two different types (vein and PTFE), studied in prospective and retrospective groups presented a number of problems and required advice from medical statisticians. The analysis used has been developed with help from Mrs. Jane Wadsworth in department of medical statistics at St. Mary's Hospital Medical School.

Analysis Problems

A major potential danger in this analysis is the division of patients and grafts into a large number of groups, each containing small numbers of results (for example, prospectively studied vein grafts with stenosis, retrospectively studied PTFE grafts without stenosis etc.). Intra-group analysis after division in this manner requires a large number of univariate comparative tests to be undertaken. Although such an analysis may take into account the effects of some potentially confounding variables, two serious potential sources of error are created:

1. Statistically significant results may be randomly generated as a consequence of the large number of comparisons performed (with a risk of 1 in 20 if significance is taken at the 5% level).
ii Type II error may occur due to the reduced sample size in the large number of groups.

In this thesis, variables were gathered into larger subgroups (such as measures of platelet activation or abnormalities of lipids and lipoproteins). Although this may reduce the possibility of drawing erroneous conclusions from randomly generated differences (as isolated differences alone should not influence interpretation of the results for the subgroup as a whole), the risk of statistical errors of the types described above was considered too great to analyse the data by subdivision into multiple small groups.

It is clearly important to consider the influence of retrospective and prospective study groups, and autologous vein and PTFE grafts, on the comparison of variables between stenosed and non-stenosed grafts, as differences in risk factors between these groups may affect conclusions. However, this must not allow real differences to be masked by analysis of small groups. Similarly, over interpretation of comparisons between heterogeneous groups of stenosed and non-stenosed grafts containing grafts of different materials, studied in different ways must also be guarded against.

Therefore, in consultation with statisticians, the following analysis was devised:

i. A primary examination of data, using a limited number of univariate analyses, to identify factors which may be associated with stenosis development.

ii. Subsequent logistic regression analysis of factors associated with stenosis development by univariate analysis, including effects of prospective and retrospective groups, vein and PTFE grafts, as well as stenosed and non stenosed grafts.
Analysis in this manner allows the relatively large number of measured risk factors to be compared between stenosed and non stenosed grafts in suitably sized groups, after which more detailed analysis can be used to account for the influence of study groups (prospective and retrospective) and graft material (vein and PTFE) on results.

In this model, the univariate analyses serve to identify factors which may be associated with graft stenosis and therefore to reduce background 'noise' in the logistic regression analysis.

Univariate Analysis; the primary data examination

In the development of an initial analysis to identify risk factors associated with stenosis development, vein and PTFE grafts were analysed separately to maximise the chances of identifying differences in risk factors between stenosed and non stenosed grafts. Analysis of these two groups separately is valid for a number of reasons:

(i)  The origin of smooth muscle cells causing intimal hyperplasia and stenosis in the two types of graft is probably different (see chapter 3).

(ii) The effects of the graft itself on levels and activity of circulating agents in the retrospective group, may be different between the two types of material.

(iii) Local flow (and therefore rheological variables) may be influenced by graft compliance. This may differ between vein and PTFE grafts.

(iv) Systemic conditions may be different between groups of patients who are reconstructed with PTFE grafts compared with those grafted with autologous vein.
Choice of Comparative Tests

As a number of the variables under consideration (notably smoking and Lp(a)) are not drawn from a normal distribution in the population, parametric comparative tests were avoided for univariate analysis; Chi-squared testing was used for frequency analysis between stenosed and non-stenosed grafts and the Mann-Whitney U test was used for comparison of continuous variables. Results of the univariate analyses are presented as individual data points and medians.

Logistic Regression of Associated Risk Factors

After factors associated with graft stenosis were identified as described above, relevant results were entered into a logistic regression analysis, utilising all available data. For entered variables, results were compared between stenosed and non-stenosed grafts and the influence of vein and PTFE graft groups and the prospective / retrospective studies groups was also measured. Logistic regression analysis was also used to develop a mathematical model of outcome when variables were serially 'fitted' into the model in a defined manner.

This analysis allows:

(i) Confirmation (or not) of the results of the primary survey (when each variable is entered into the model first)

(ii) A comparison of the interaction between variables (mathematically) such that the predictive power of dependant variables can be calculated after the influence of the another variable has been accounted for.

It is important to stress that the relationships demonstrated in the latter analysis require interpretation with physiological and pathological knowledge if they are to be of value.
Logistic regression analysis allows the influence of prospective and retrospective study groups, and the effects of graft material, to be taken into account in the analysis, and thus avoidance of errors due to assumptions about these different groups.
Chapter 5

Perioperative Graft Assessment, Stenosis Surveillance
Methods and Clinical Risk Factor Profiles

In this chapter the methods of peri-operative assessment used to exclude causes of graft stenosis other than intimal hyperplasia will be described, along with the postoperative duplex surveillance techniques used to detect new stenoses. In addition, examples of histological analysis of resected stenoses will be shown. In the final section, a review of the literature associating clinical risk factors with the development of atherosclerosis and intimal hyperplasia will be presented and the clinical risk factor profiles of patients included in the study will be compared in stenosed and non-stenosed groups.

5.1 Peri-Operative Graft Assessment

Techniques and Timing

The causes of graft stenosis have already been described in chapter 2; Szilagyi et al. in their seminal paper in 1973 described the causes of infrainguinal graft stenosis (in descending order of frequency) as:

- Intimal thickening
- Graft atherosclerosis
- Fibrotic valve cusps
- Fibrotic stenoses
- Suture stenosis

As the current studies concern only in the effects of vascular risk factors on the development of graft stenosis due to intimal hyperplasia, it is important that other (less frequent) causes of stenosis are excluded.
The studies consider only stenoses which develop within the first postoperative year, therefore, graft atherosclerosis can be largely ignored, as these lesions are known to develop over a protracted period (mean of 48 months in Szilagyi's study) and calcified plaques are identifiable on X-rays (all grafts with stenosis in these studies had confirmatory angiography).

To exclude graft stenosis due to fixed anatomical lesions such as poorly stripped vein valves, inappropriately applied side branch ligatures or segments of diseased vein, intraoperative doppler surveillance of completed grafts was undertaken using a hand held 4 MHz doppler device inside a sterile glove as described by Tyrell et al (1990) and was supplemented by full duplex scanning of the graft prior to discharge using the technique described below. Intraoperative angiography was also used following femoro-crural reconstruction and in a proportion of femoro-popliteal grafts.

When stenoses were detected by intra-operative assessment, correction was undertaken by further valvotomy, ligature removal or patch angioplasty as appropriate.

Only patients with grafts free from stenosis on intraoperative doppler and postoperative duplex scanning were included in follow up studies.

**Evaluation of Intraoperative Studies**

To evaluate the technique of intraoperative doppler assessment used as described above, a study of intraoperative hand held doppler assessment versus duplex scanning prior to discharge was undertaken in 58 infrainguinal reconstructions. All doppler assessments were undertaken by a single research fellow (NC), early duplex scanning using the V2:V1 technique (see below) was undertaken by a research fellow prior to discharge. Results are shown in table 5.1
Overall there was a 29% incidence of intraoperative abnormality detected. Graft stenoses were detected intra-operatively in 17% of grafts. Correction of abnormalities in this group resulted in no further stenoses being detected by duplex scanning before discharge. Eighty-four percent of arteriovenous fistulae were detected during the operation. This latter figure is perhaps not surprising as patent branches of the long saphenous vein are understood to dilate in the post-operative period and thus may not be detectable as arteriovenous fistulae at the time of grafting.

These results suggest that intra-operative graft assessment using simple hand held doppler device is valuable in detecting graft stenoses present at the time of reconstruction and may be used to detect correctable abnormalities present at the time of operation.

5.2 Stenosis Detection

Duplex doppler stenosis surveillance was undertaken on all grafts at six and twelve weeks post reconstruction, and then every three months for the first post operative year. These intervals were chosen as recent studies have shown that stenoses can develop early, and that few develop after 12 months (Taylor et al 1990a).

Stenoses were detected using the V2:V1 technique of graft interrogation (Grigg et al 1988) by one of two research fellows (N. Labropoulus & M. Leone). This involves sampling of velocity at 2cm intervals along the graft. As flow at any point in a graft is constant (as an arterial conduit is a tube without branches), any reduction in the cross sectional area must produce a proportional increase in velocity (because flow is the product of cross sectional area and velocity) (fig 5.1). Intrgraft-stenoses
produce a localised increase in velocity which then returns to prestenosis levels downstream. Vein calibre alters at major branch sites and these are regions of potential error - however these changes produce a persistent velocity increase, maintained throughout the length of the smaller calibre vein. An increase in velocity greater than 100% (V2:V1 ratio >2, fig 5.1) is associated with a luminal reduction of 50%. V1:V2 ratio measured in this manner using duplex doppler has been shown to correlate well with angiographic stenosis (Grigg et al 1988). In addition to V2:V1 measurement, peak systolic flow velocity, taken at a single point within the graft was also used for surveillance. One of the weaknesses of the V2:V1 technique is the difficulty in obtaining accurate velocity information from small native vessels distal to the graft (particularly crural vessels), and these areas may be important. An average peak systolic flow velocity under 45 cm/s second has been proposed by Bandyk (1988) to detect of distal native vessel lesions and was used as an adjunct to V2:V1 measurements during the study period.

\[
\text{Flow}_1 = \text{Flow}_2
\]

\[
\text{Flow}_1 = \text{Velocity}_1 \times \text{Area}_1 = \text{Flow}_2 = \text{Velocity}_2 \times \text{Area}_2
\]

Figure 5.1 Line drawing showing unbranched graft in which flow at all points is equal. As flow is the product of fluid velocity and cross sectional area, a reduction in cross sectional area caused by a stenosis as in region 2, produces a corresponding increase in velocity. This can be detected by duplex scanning.

Duplex detected abnormalities (of V2:V1 or peak systolic flow velocity) were investigated by angiography (either intra-venous or intra-arterial digital subtraction.
angiography) to define the nature of the lesion. Stenoses which were demonstrable on biplanar angiography as greater than 50% of the graft lumen and which had a V2:V1 ratio of 2 or greater were corrected by open operation or percutaneous balloon angioplasty as appropriate. Lesser stenoses were monitored with six-weekly duplex and or angiographic assessment; fulfilment of the above criteria then led to correction. After detection and correction of a stenosis, surveillance began again at six and twelve weeks, 3 months, 6 months etc.

It is worth emphasising again at this point that the definition of stenosed graft for the purposes of this study includes the demonstration of a stenosis free graft at the time of reconstruction, followed by the detection and confirmation of a new stenosis on duplex scanning and angiography which must fulfil the criteria for correction outlined above. This definition excludes patients who may have had a minor duplex abnormality detected on a single scan but not confirmed on angiography or subsequent duplex examination. Non stenosed grafts were defined as those completing at least 12 months regular surveillance during which the graft remained patent and no duplex abnormality was detected.

5.3 Histological Analysis

Haemodynamically significant stenoses detected by post-operative surveillance were corrected using percutaneous balloon dilatation, patch angioplasty or jump grafting as described in chapter 3. It was therefore not possible to obtain tissue for histological analysis from all grafts with significant stenosis. Tissue was obtained in a proportion of grafts which developed stenosis (n=12, 43% of all stenoses included in the study). Histology confirmed intimal thickening due to smooth muscle hyperplasia as the cause in all cases.

In autologous vein grafts, hyperplastic stenoses were found in the body of the graft (n=5) as well as around the distal anastomosis (n=12).

In PTFE grafts stenoses occurred predominantly at the distal anastomosis (n=9), with a small number of stenoses developing at the proximal anastomosis (n=2). When distal vein collars were used, a characteristic sparing of the native artery was
noted, with the accumulation of intimal hyperplasia at the PTFE vein collar interface (fig 5.6) - a mechanism which has been proposed in the improvement of graft patency associated with the use of vein collars (Tyrell et al 1992).

Representative sections of stenoses are shown in figures 5.2 - 5.5.
Arteriograms demonstrating the effects of vein collars on stenosis development are shown in figures 5.6 and 5.7.
Figure 5.2. Histological section through a vein graft stenosis showing proliferated smooth muscle cells producing intimal thickening - marked A. B shows the region of the internal elastic lamina. H & E stain.
Figure 5.3 Histological section through native artery at the site of a perianastomotic PTFE graft stenosis. Section shows proliferated smooth muscle cells (A) producing thickening of the native vessel intima (H&E stain)
Figure 5.4 Section through perianatomotic native artery at the site of a PTFE graft stenosis. Stained with Elastic Van-Geeson.
Figure 5.5 Section through perianatomotic native artery at the site of a PTFE graft stenosis. Stained with Elastic Van-Geeson.
Figure 5.6. Intra-arterial subtraction angiogram after TPA infusion for graft occlusion showing anastomotic stricture at site of PTFE / collar anastomosis with sparing of the collar / artery anastomosis.
Figure 5.7. Intra-arterial subtraction angiogram showing stricture developing at the heel of PTFE/collar anastomosis with sparing of the anastomosis between the collar and artery.
5.4 Clinical Risk Factors and Atheroma

The association between smoking, hypertension and diabetes mellitus with the development of atheroma is well established from epidemiological studies however relatively little work has been undertaken to demonstrate the mechanisms of their action (Haust 1983). In reviewing the current literature, each of these factors will be considered separately.

**Smoking**

Epidemiological evidence associating smoking and atherosclerosis is strong; several prospective studies of the development of coronary or cerebral vascular disease have shown a significantly higher incidence amongst smokers (Doll & Peto 1976, Kannel 1981) and a reduction in risk if smoking is stopped (Doll & Hill 1964, Gordon et al 1974).

Carbon monoxide (Armitage et al 1976) and nicotine (Fisher et al 1973) from cigarette smoke have both been implicated in the genesis of atheroma although the mechanism of action of both remains unclear; carbon monoxide in the blood has been shown to increase the permeability of vessel walls to large molecules such as fibrinogen (Allen et al 1988) and to increase the rate of endothelial cell loss (Davis et al 1985) - two effects consistent with the modern hypotheses of atheroma initiation. The effects of nicotine are less clearly linked to atherogenesis but circulating nicotine is associated with alteration of blood pressure and heart rate and a reduction of peripheral blood flow (Couch 1986), probably mediated via the autonomic nervous system, as plasma adrenalin and noradrenalin levels are known to rise after smoking (Feyerabend &
Russell 1979). These effects may alter the local haemodynamic environment and influence the rheological variables thought to be important in atherogenesis, as described in chapter nine.

A recent study of vein grafts has shown that the nitrous oxide (NO - also known as endothelial derived relaxing factor) dependent endothelial effects are attenuated in smokers (Hageman et al 1993). This is a potential mechanism for atherosclerosis as reduced NO is associated with an increase in endothelial permeability and may have direct effects on smooth muscle cells.

In addition to direct effects of tobacco smoke on atherogenesis, chronic smoking is also associated with a detrimental trend in other risk factors for vascular disease such as increased plasma fibrinogen (Lowe et al 1980, Meade et al 1987) and enhanced platelet adhesiveness and aggregation (Glynn et al 1966, Hawkins 1972, Saba & Mason 1975). Whether smoking exerts an atherogenic effect via these risk factors is not clear.

**Hypertension**

The role of hypertension in the development of atheroma is not understood. Animal work has demonstrated acceleration of atheroma formation in hyperlipidaemic primates with associated hypertension (Robertson & Strong 1968), and a similar association has been suggested in human diabetes (Pick et al 1974). These observations led to the belief that hypertension could not influence the development of atheroma without the presence of additional vascular risk factors, but in another primate study, using an atherogenic diet followed by a return to normal feed, Xu et al (1991) have recently been able to show that the presence of hypertension (induced by aortic coarctation) is associated with continued plaque progression after the serum lipid profiles have returned to normal. This work suggests that hypertension may be important in disease progression after plaque initiation, particularly as other studies
have been unable to demonstrate the induction of atheroma in animal models by hypertension alone (Chobanian 1983).

Other schools of thought hold that hypertension and atherosclerosis may be different expressions of a single, possibly genetic, defect which renders the vessel wall hyper-responsive to a number of stimuli (Baudouin-Legros & Meyer 1990, Bondjers et al 1991) or that the consequences of hypertension may be mediated by adverse effects on platelet function or lipid profiles (Ding et al 1991).

Although these studies are by no means complete, it is clear that hypertension (or the underlying condition predisposing to hypertension) has wide-ranging effects on the vasculature, many of which are detrimental and may accelerate the development of atheroma.

**Diabetes Mellitus**

Like hypertension and smoking, the role of diabetes in the development of atheroma is not fully understood, but there is epidemiological evidence of a link between the two diseases - in the Framingham study (Garcia et al 1974) diabetics had an incidence of intermittent claudication in excess of three times that in the non-diabetic population. Further it has also been suggested that diabetics have a distribution of occlusive vascular disease which may be less favourable for arterial reconstruction than that in the normal population, with a higher proportion of occlusions in the crural vessels (Garcia et al 1974). It is important to emphasise here that the clinical syndrome of vascular insufficiency seen in diabetics may not solely reflect atheromatous disease in the large or intermediate vessels, as diabetics are affected by other vascular diseases such as medial calcification (Edmonds et al 1982) and alteration in capillary numbers and function (Ward 1982). These factors, together with an increase in the incidence of atheroma (combined with other pathology such as peripheral neuropathy) contribute to the high incidence of amputations seen in diabetic patients.
Many causes have been suggested for the increased incidence of vascular
disease seen in diabetics including a microangiopathy of the vasa vasorum of the large
and intermediate vessels (Moore and Frew 1965), abnormalities of the endothelial cell
(Aanderud et al 1985), abnormalities of lipid metabolism (Kannel et al 1971, Stanton
1978) and haemostatic dysfunction (McMillan 1975, Badawi et al 1970) but at the time
of writing non of these mechanisms has been directly shown to influence
atherosclerosis in diabetics.

5.5 Clinical Risk Factors and Intimal Hyperplasia

The most successfully studied models of intimal hyperplasia currently in use are
smooth muscle cells in culture and animal vessel-injury models; using these techniques,
very little experimental work on the role of clinical risk factors in intimal hyperplasia
has been undertaken. The spontaneously hypertensive rat model (SHR) is an exception,
and has provided some evidence of abnormal smooth muscle cell function in conditions
which may be similar to human essential hypertension; vascular smooth muscle cells
from SHR's appear to be more responsive to mitogenic stimulation in-vitro than cells of
non-hypertensive rats, and thus after stimulation in-vivo (perhaps by vessel injury), the
vessels of such animals may be more prone to develop intimal hyperplasia.

Restenosis within the first few months after carotid endarterectomy has been
shown histologically to consist of hyperplastic smooth muscle cells (Cossman et al
1978) and this has proved to be one of the few human models of intimal hyperplasia in-
vivo. In a small series of recurrent carotid disease, Cossman and colleagues (1978)
showed that the incidence of diabetes (5 of 7 patients with recurrent disease),
hypertension (5 of 7 patients) and hyperlipidaemia (6 of 7 patients) were higher in
recurrent disease patients than in the group of patients undergoing endarterectomy as a
whole. They also suggested that patients with recurrent disease tended to be younger
than others, and in a separate report showed that women were more likely to develop
restenosis (Cossman et al 1980). In a similar study of 172 endarterectomy patients, Cantelmo et al (1981) used non invasive methods to detect 24 recurrent stenoses and demonstrated an increased incidence of hypertension (75%), hyperlipidaemia (60%) and diabetes (33%) in the restenosed group compared with all endarterectomies. However, in a matched case control study of twenty-one patients with recurrent carotid disease, Clagett et al were unable to show any differences in the incidence of hypertension or diabetes. Interestingly however, this study showed that 95% of patients with recurrent disease, continued to smoke after their operation - a incidence much higher than that in the non stenosed group and a factor not reported by other studies. This study also showed that recurrent disease occurred in a disproportionate number of women.

In experimental models of intimal hyperplasia there is some evidence that clinical risk factors can influence smooth muscle cell activity. Prostaglandin production (a potential pathway for platelet inhibition) by smooth muscle cells in culture is reduced by the addition of nicotine (Bauch et al 1987) and by norepinephrine and platelets are rich sources of smooth muscle cell mitogens. Some groups have suggested that intrinsic smooth muscle cell response to mitogens is greater in hypertensive patients and that the platelets of these individuals contain higher concentrations of mitogenic agents (Hadrava et al 1991) - however these findings have not been widely reported. There is little data concerning smooth muscle cell function in diabetics.

5.6 Clinical profiles of study patients

In the following study, the prevalence of clinical risk factors during the first postoperative year (the time when the majority graft stenoses develop (Taylor et al 1990a)) have been compared in patients who developed graft stenosis and those who did not; to avoid error caused by poor recollection (particularly of smoking) in the retrospective group of patients, this study includes only the prospective study group. Patients were classified as hypertensive requiring treatment or non hypertensive,
Smoking was assessed by direct questioning and validated by measurement of breath CO concentration using the Microsmokalyser (Bedfont Scientific, Upchurch, Kent U.K.). Readings above 10 ppm expired CO were considered indicative of current smoking as recommended by the manufacturer. As has been demonstrated in other studies (Galvin et al 1991), direct questioning produced a degree of factual economy amongst smokers but the face to face nature of history taking in the clinic and the immediacy of results from the smokalyser (combined with some humour) allowed patients who were smoking to admit so. Additionally, CO analysis was repeated at subsequent visits.

Negative or minimal breath CO readings were obtained in a proportion of patients who admitted to occasional smoking on direct questioning; at the time of writing, it has not been reported that non smoking patients may give false negative information in this manner, and it was presumed that the early time of our femoropopliteal clinic, and the low reported number of cigarettes smoked by this particular group of patients contributed to these aberrant results. Patients who repeatedly admitted to smoking but registered non smoking results on the breath CO analysis were classified as current smokers.

Age and Sex

There were no age differences between patients who developed stenosis and those who did not (table 5.2). However there was a higher proportion of women amongst PTFE grafts which developed stenosis (table 5.3). This difference was marginal, reaching significance on chi-squared testing without continuity correction, but failing to reach significance after Yates correction.
<table>
<thead>
<tr>
<th>Mean Age (yrs)</th>
<th>Stenosed Grafts</th>
<th>Non-stenosed Grafts</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein Grafts</td>
<td>68</td>
<td>71.5</td>
<td>NS</td>
</tr>
<tr>
<td>PTFE Grafts</td>
<td>67</td>
<td>70</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 5.2: Mean age in patients with stenosed and non-stenosed grafts

<table>
<thead>
<tr>
<th></th>
<th>Stenosed Grafts</th>
<th>Non-Stenosed Grafts</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vein Grafts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Sex</td>
<td>5/17 (29%)</td>
<td>11/35 (31%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PTFE Grafts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Sex</td>
<td>4/11 (36%)</td>
<td>1/18 (5%)</td>
<td>0.03 (NS after Yates)</td>
</tr>
</tbody>
</table>

Table 5.3: Sex distribution amongst patients with vein and PTFE grafts which did and did not develop stenosis

**Smoking**

Univariate analysis demonstrated association between patients who continued to smoke after reconstruction (based on direct questioning and breath CO analysis) and the development of graft stenosis; vein grafts 8/14 smokers in stenosed groups compared with 1/18 in non-stenosed, p=0.01 \(\chi^2\) test (0.04 after Yates Correction) figure 5.6, PTFE grafts 7/9 smokers in stenosed group compared with 3/12 in non-stenosed group, p=0.003 \(\chi^2\) test (0.01 after Yates correction, figure 5.7).

Regression analysis confirmed the findings of the univariate tests; as smoking was only measured in prospective groups, only the effects of graft material and smoking on stenosis were accounted for in the model. Analysis of maximum likelihood estimates is shown in table 5.4.
Table 5.4 Analysis of maximum likelihood estimates.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein v PTFE</td>
<td>-0.2863</td>
<td>0.7629</td>
<td>0.14</td>
<td>0.707</td>
</tr>
<tr>
<td>Smokers v Non Smokers</td>
<td>-2.507</td>
<td>0.7422</td>
<td>11.41</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

This table shows that smoking is significantly associated with the development of graft stenosis and that this independent of the type of graft material used.

The strength of the association between smoking and graft stenosis was of such magnitude that numbers of smoking patients in the non stenosed group (n=4) were insufficient for analysis of the effects of smoking and non smoking on other variables associated with the development of stenosis (i.e. the effects of smoking could not be entered into subsequent logistic regression in which other variables were also fitted).

**Hypertension**

There were no significant differences in the incidence of hypertension requiring treatment between patients who developed stenosis and those who did not (vein grafts 4/14 v 7/18 p=NS figure 5.8, PTFE grafts 7/9 v 5/12 p=NS figure 5.9).

**Diabetes Mellitus**

There were no significant differences in the incidence of diabetes mellitus between grafts which developed stenosis during follow up and those that did not (vein grafts 3/14 v 1/18 grafts, p=NS, Figure 5.10, PTFE grafts 2/9 v 2/12 p=NS figure 5.11).
Figure 5.6: Bar chart showing incidence of current smokers and non-smokers amongst vein grafts which developed stenosis during follow up and those that did not.
Figure 5.7: Bar chart showing incidence of current smokers and non-smokers amongst PTFE grafts which developed stenosis during follow up and those that did not.

$p=0.003$
Chi-squared test
Figure 5.8: Bar chart showing incidence of hypertension requiring treatment amongst vein grafts which developed stenosis during follow up and those that did not.
Chi squared test

Figure 5.9: Bar chart showing incidence of hypertension requiring treatment amongst PTFE grafts which developed stenosis during follow up and those that did not.
Figure 5.10: Bar chart showing incidence of diabetes amongst vein grafts which developed stenosis during follow up and those that did not.

Chi squared test

p=NS

Diabetics

Non diabetics
Figure 5.11: Bar chart showing incidence of diabetes amongst PTFE grafts which developed stenosis during follow up and those that did not.
5.7 Discussion

The results of these studies demonstrate an association between cigarette smoking (as measured in a prospective cohort of patients undergoing infrainguinal reconstruction), and the postoperative development of graft stenosis. This association is demonstrable by univariate analysis of autologous vein and PTFE grafts, and also in a logistic regression model including all data from both groups.

In addition, a marginal association between female sex and stenosis development in PTFE grafts has been suggested by univariate testing. The study failed to show a relationship between diabetes mellitus or hypertension and graft stenosis.

As other common causes of graft stenosis were excluded by the use of perioperative graft assessment (see above), these results suggest an association between smoking and intimal hyperplasia in infrainguinal bypass grafts.

It was not considered suitable to use data from retrospectively studied patients as smoking history requires verification by other tests (Galvin et al. 1991) and retrospective collection of data in this manner (it would be necessary for patients to recall if they were smoking during the first post-operative year) is open to bias and impossible to verify.

These results support observations by Clagett et al. (1983) described above, showing that 95% of patients who developed hyperplastic recurrent carotid stenosis following endarterectomy in one study cohort, were smokers, compared with less than a third of those patients who did not develop stenosis, and that recurrent carotid disease was more common in women. Some groups have demonstrated an association between hypertension and diabetes and intimal hyperplasia after carotid endarterectomy (Cossman et al. 1978, Cantelmo et al. 1981) in contrast to the current findings, but these were retrospective analyses with unmatched controls and may not be reliable (see above).
As it is now generally accepted that most medium term failures occur as a consequence of the adverse haemodynamic effects of hyperplastic graft stenoses (Brewster et al 1983, Taylor et al 1990a), the current results support a growing body of data demonstrating reduced infrainguinal graft patency in patients who continue to smoke after reconstruction (Myers et al 1978, Wiseman et al 1989 and 1990).

The mechanisms by which smoking may promote intimal hyperplasia have been discussed at the beginning of this chapter. Details remain unclear, but may involve direct smooth muscle stimulation, perhaps via effects on endothelial nitrous oxide (Higman et al 1993). In addition, the association between smoking and increased plasma fibrinogen (Lowe et al 1980) and catechole-amine levels (Feyerabend et al 1986), may adversely influence graft wall shear which has been linked to intimal injury and subsequent smooth muscle hyperplasia (Caro et al 1971, Zarins et al 1983, Sprague et al 1987, Schwartz et al 1989) - see haemorheology and intimal hyperplasia, chapter 9.

Activation of platelets by tobacco smoking (Glynn et al 1966, Hawkins 1972, Saba & Mason 1975) may also stimulate intimal hyperplasia as platelet adherence to arterial grafts has been shown to be correlated with the degree intimal hyperplasia in experimental models (Fuster et al 1979, Yukizane et al 1991) and with graft patency in humans (Goldman et al 1983) - see chapter 8.

Furthermore, smoking has been associated with an increase in endothelial permeability to circulating macromolecules, and an increase in the rate of endothelial cell loss (Allen et al 1988, Davis et al 1985) - changes which may expose medial smooth muscle cells to mitogens in the circulation (Ross 1986).

The effects of smoking on other variables associated with graft stenosis in this study could not be measured in logistic regression analysis as the strong association between smoking and stenosis meant that very few smoking patients did not developed stenosis.

Little information exists to suggest why females may be more prone to develop intimal hyperplasia. In recurrent carotid disease, Cossman and colleagues (1978)
suggested that the smaller size of the carotid artery in women may predispose the vessel to restenosis. Such an effect may occur in smaller infrainguinal grafts and recipient vessels in females, although we have no data in the current study to support or refute this claim. Clagett (1983), suggested that measured carotid size was not related to the observed higher restenosis incidence in women in his study.

The theoretical support for an association between smoking and smooth muscle cell proliferation, along with data associating smoking with intimal hyperplasia in the carotid artery and observations of reduced infrainguinal graft patency in smokers, support the findings in the current study and serve to emphasise the importance of stopping smoking after arterial reconstruction. The value of stopping smoking to improve infrainguinal graft patency has been recognised for some time but it has previously been unclear whether progression of native vessel atheroma, intragraft disease or systemic effects were the cause of the observed patency reduction. As graft stenosis is now thought to be associated with a high proportion of the graft failures which occur within the first one or two years after reconstruction (Brewster et al 1983, Taylor et al 1990a, Harris 1992), it seems likely that at least some of the adverse effects of smoking on graft patency are mediated through the development of hyperplastic graft stenoses.

This data underlines the importance of strong anti smoking advice for patients who undergo infrainguinal reconstruction, particularly women, if prolonged graft patency, and thus limb salvage, is to be achieved.
Chapter 6

Studies of Serum Lipids, Lipoproteins and Apolipoproteins and Graft Stenosis

6.1 Introduction

The following studies correlate the development of graft stenosis with the serum concentration of lipids, lipoproteins and apolipoproteins. Elevation of these factors has been associated with atherogenesis and intimal hyperplasia, and the supporting evidence for this will also be reviewed, along with a description of lipid and lipoprotein structure and function. The close morphologic similarity between intimal hyperplasia and early atheromatous lesions has been described earlier.

6.2 Lipids in the circulation, Lipoproteins and Apolipoproteins

Water insoluble lipids and phospholipids are transported in the circulation - to their sites of utilisation - bound to protein. Macheboeuf and Viscontini in 1946 first recognised that the combinations of protein and lipid commonly found in the circulation have a constant composition. Although this suggested the presence of specific lipid binding proteins, the first attempts to classify the lipid-protein combinations were based on electrophoretic mobility, a non-specific physical property. As mobility of the lipoproteins was similar to that of the \( \alpha_1 \)- and \( \beta \)-globulins, these terms were also applied to the lipoproteins. In 1954 Gofman et al used ultracentrifuge techniques to demonstrate that the range of particle size and densities of human lipoproteins was unevenly distributed between densities of 0.92 and 1.25 g/ml. Based on these observations, five major density classes of lipoproteins were designated; chylomicrons (\( d < 0.94 \) g/ml), very low density lipoproteins (VLDL, \( d = 0.94 - 1.006 \) g/ml), low density lipoproteins (LDL, \( d = 1.006 - 1.063 \) g/ml), high density lipoproteins (HDL, \( d = 1.063 - 1.21 \) g/ml) and the lowest fraction with a density > 1.21 g/ml (sometimes
known as very high density lipoproteins). Subsequent distribution studies showed that the lipoproteins were highly heterogeneous within these density groups and that subclasses differed in the proportion of neutral lipids and phospholipids or the lipid/protein ratio contained within (Lossow et al 1969, Anderson et al 1977, Fisher 1983).

As techniques for investigation improved, electrophoretic and immunological studies indicated that the lipoproteins contained two distinct proteins; one associated with the higher density lipoproteins (or α-lipoproteins) and termed α-protein, and the other associated with the lower (or β) densities and thus termed β-protein (Cohn 1945). The importance of these proteins was not appreciated immediately however, as the concept of lipoprotein density (mostly a product of the lipid content) as the most important indicator of the pathological properties of the molecule took hold and appeared to be supported by emerging epidemiological data showing a positive correlation between the development of atherosclerosis and the serum concentrations of low and very low density lipoproteins, along with a negative correlation with high density lipoproteins (see lipids and atherosclerosis below).

Characterisation of the protein moiety of lipoproteins began in earnest in the late 1960's and it was rapidly recognised that the system was infinitely more complex than the α-protein, β-protein hypothesis suggested. The newly discovered proteins were termed apolipoproteins and assigned capital letters (apoA, apo E etc.). Roman numerals were used to identify constitutive polypeptide differences within the same class of protein (e.g. apoA-I, apoA-II - indicates two molecules with physical characteristics of apoA but containing at least one different polypeptide chain) and polymorphic forms of either apolipoproteins or polypeptides are designated by roman numerals (e.g. apoB-48, apoB-100 - indicates two molecules with physical characteristics of apoB, overlapping polypeptide chains but one chain larger than the other) (Alaupovic 1980, 1991). An apolipoprotein is defined as a lipid binding protein (single or multiple polypeptides) with the capacity to form a system of soluble, polydisperse lipoprotein particles.
The main protein molecule of HDL is apolipoprotein A (apoA), and has multiple subfractions (apoA-I to apoA-IV at least). ApoA-I and apoA-II account for 75-80% of total protein content of HDL. The main protein of LDL is apoB. Two subfractions exist, (apoB-48 and apoB-100) varying in size and accounting for 85-90% of total LDL protein content. VLDL's contains apoB (40-50% of total apolipoprotein content), and apoC (25-40% of total apolipoprotein content). Several other apolipoproteins (apo D-J) and apo (a) are also known to exist. The continuing interest in this field marks the shift in our pathophysiological understanding of lipoproteins away from the lipid component and onto the protein moiety. The special role of lipoprotein (a) and apolipoprotein (a) is described below.

The discovery of these apolipoproteins has prompted some to suggest that the concept of lipoproteins grouped by physical properties (such as density) is now outmoded and that we should now consider family groups of lipoproteins containing similar apolipoproteins (Alaupovic 1991). In this theory, lipoproteins containing a single apolipoprotein are termed simple lipoproteins whilst those containing two or more apoproteins are called complex lipoproteins. Unfortunately, such a system does not fit well with the widely accepted density bands of lipoproteins as the lipid 'saturation' of a given apolipoprotein (and thus its density) can vary under different metabolic conditions. The theory of lipoprotein families is based on the proposition that apolipoproteins are the main determinants of the structural, functional and pathological properties of lipoproteins. Data to support this hypothesis is emerging; apoA-I and apoE containing lipoproteins have been shown to play a role in the regulation of cholesterol flux between plasma and cultured fibroblasts (Fielding & Fielding 1980, 1981) and the hepatic catabolism of triglyceride rich lipoproteins containing apolipoprotein B, appears to be dependant on the content of apoE (Koren et al 1987). There is recent work to suggest that the apolipoprotein content of lipoproteins dictates the atherogenic potential of the molecule; apoB containing lipoproteins such as LDL and VLDL are associated with the accelerated development of atheroma in the coronary
circulation, whilst apoA containing molecules such as HDL are cardio-protective (see lipids and atheroma below).

The liver and intestine are the major sites of biosynthesis, assembly and secretion of plasma destined lipoproteins (Dashti 1991). The liver seems to predominantly produce triglyceride rich and cholesterol-ester poor VLDL and LDL's, whilst enterocytes also form VLDL's and LDL's, as well as postprandial chylomicrons (Bisgaier & Glickman 1983, Hamilton 1983, Johnson et al 1983). Little is known of the generation of HDL's. The plasma concentrations of lipoproteins are markedly influenced by the nutritional status of the animal. It is generally agreed in humans that increased dietary cholesterol and saturated fat results in elevated LDL-cholesterol levels, whilst substitution of polyunsaturated fats for saturates, reduces LDL-cholesterol (Dashti 1991). In the latter case ω-3 fatty acids (found in fish oils) seem to be very effective in lowering both plasma LDL and VLDL levels (Harris 1989). Although a high cholesterol diet has been shown to elevate plasma apoB levels in some species this has not been shown in humans, although polyunsaturated feeding may decrease the synthetic rate of apoB whilst simultaneously increasing the catabolic rate (Goodnight et al 1982).

6.3 Lipoprotein (a) and Apolipoprotein (a)

Lipoprotein (a) denotes a spectrum of cholesterol rich particles with a general composition similar to LDL, but characterised by a specific additional protein termed apo(a) - described initially by Berg in 1963. At present there is some doubt as to whether this molecule fulfils the definition of an apolipoprotein. There are at least 11 isoforms of apo(a), varying in size from 400 to 800 kilodaltons (Gaubatz et al 1983 & 1990) and there is a significant inverse correlation between the size of the apo(a) isoform and the serum level of lipoprotein (a). Amino acid sequencing studies have shown that apo(a) is very similar to human plasminogen (McLean et al 1987) and this
similarity is believed to account for at least some of the highly atherogenic properties associated with the molecule (see lipids and atheroma below).

It has been shown by several groups that Lp(a) competes with plasminogen and t-PA for fibrin binding in vitro (Hoff et al 1988, Hajjar et al 1989). Consequently, the presence of Lp(a) attenuates t-PA activity (and thus clot lysis) in the presence of fibrin. A similar competitive inhibition of endothelial plasminogen receptors by Lp(a) has also been demonstrated (Miles et al 1989) and could have similar pro-thrombotic effects. These effects on the coagulation cascade remain theoretical and at present there is little in-vitro evidence to show that Lp(a) inhibits plasmin mediated thrombolysis (Smith & Croxible 1991). Another mechanism by which Lp(a) may influence atherogenesis is by selective uptake or retention in developing atheroma; the ratio of apo(a) to apoB is higher in homogenates of atheromatous plaque than in the circulating plasma, suggesting that it is preferentially bound to atheroma prone regions of the arterial tree. Lp(a) is known to bind with high affinity to glycosaminoglycans which are present in the intimal lattice (Cushing et al 1989). The pathophysiology of Lp(a) is currently the subject of intense research which will hopefully unravel some of the mechanisms underlying the epidemiological association between the molecule and vascular disease.

6.4 Lipids and Atheroma

A large number of studies have demonstrated an association between chronic elevation of levels of serum cholesterol and the cholesterol containing low and very-low density lipoproteins in the plasma and atherosclerosis (reviewed by Inkeles and Eisenberg 1981). Whether circulating triglyceride levels are correlated with the development of atherosclerosis remains controversial - many studies have shown a positive correlation in univariate analysis but in most this has weakened or disappeared when HDL-cholesterol level is taken into account on multivariate analysis (Stein & Gotto 1992).
To explain the observed relationship between lipids and atherogenesis, recent data suggests that hypercholesterolaemia may precipitate the type of subtle, non-denuding endothelial injury now thought important in the initiation of atheroma, possibly by increasing the cholesterol/phospholipid ratio in the membrane of endothelial cells (Jackson & Gotto 1976) thereby producing an increase in endothelial cell membrane viscosity. This could lead to endothelial cell retraction at sites of altered shear stress. Such retraction has been demonstrated in early atheromatous lesions in animals (Faggiotto & Ross 1984).

A second mechanism of lipid dependant atherogenesis has been postulated as a consequence of the observation that the monocyte/macrophage cell system can oxidise low density lipoprotein and that this modified molecule is cytotoxic (Catheart at al 1985) and chemoattractant (Steinberg et al 1988) for monocytes. Oxidised LDL has been demonstrated in atheroma in humans (Yla-Herttuala et al 1989) and may therefore stimulate atherogenesis by chemotraction of macrophages, conversion of macrophages to foam cells (Steinberg 1988) or even by a direct cytotoxic effect on endothelial cells producing localised injury (Catheart at al 1985, Carew 1989). Additionally, a direct stimulatory action of oxidised LDL on platelets and macrophages causing release of smooth muscle cell mitogens may play a role in atherogenesis when the molecule is formed freely in the vessel wall (Carew 1989).

There are other mechanisms by which lipids and lipoproteins may enhance atherogenesis; lipoproteins have activating effects on platelets - platelet shape change (a precursor of contraction and degranulation) and aggregation responses are enhanced by altered plasma LDL:HDL ratio (De Clerck 1986) and LDL alone can enhance platelet aggregation in response to other agents such as serotonin (Fetkovska et al 1989, Amstein et al 1991).

Recently, data has emerged to suggest that apolipoprotein B-100 (apoB), the major constituent of lower density lipoproteins, plays an important role in atherogenesis and may be the earliest detectable lipid in atheroma prone areas of intima.
(Wissler 1991). Furthermore, the plasma level of another low density lipoprotein fraction, Lp(a), has been shown to be a powerful predictor of atherosclerosis in man in the coronary (Murai et al 1986) and cerebral (Zenker et al 1986) circulation’s. The molecule has also been demonstrated in the lesions of human atheroma (Walton et al 1974).

Taken together these data suggest that high plasma concentrations of lipid, particularly cholesterol and some of the fractions of the low density lipoproteins which carry cholesterol, may be important in the pathogenesis of atherosclerosis. Although the exact mechanism remains unclear, circulating lipids and lipoprotein seem to play a role in the endothelial injury thought to be important in the initiation of atheroma, whilst lipoprotein sequestered in the vessel wall may directly or indirectly stimulate smooth muscle hyperplasia.

6.5 Lipids and Intimal Hyperplasia

Evidence to implicate circulating lipids in the genesis of intimal hyperplasia can be divided into two categories. Direct evidence from experimentally induced intimal hyperplasia with and without lipid abnormalities, and indirect evidence, mostly observational studies of arterial grafts in the presence of lipid abnormalities. Whilst evidence from human studies is mostly indirect, both types of study provide useful information.

Lipids and Experimentally induced intimal hyperplasia.

A recent in vitro study of vein grafts subject to arterial conditions in organ culture has shown that developing lesions incorporate apolipoprotein B in the interstices between hyperplastic smooth muscle cells (Berceli et al 1991). Whilst this study did not suggest that the lipoprotein content may contribute to lesion progression there is strong circumstantial evidence if it is remembered that oxidised apoB products may be cytotoxic (Cathcart et al 1985), chemoattractant to macrophages (Steinberg et al 1988)
and platelets stimulants (Carew 1989). Data demonstrating a relationship between the level of circulating lipids and the degree of intimal hyperplasia has been provided by experiments on animal models. In the rabbit, Weidinger et al (1991) showed that balloon denudation of the iliac artery in the presence of hypercholesterolaemia was associated with a significantly increased proliferative response by smooth muscle cells than that caused by denudation or hypercholesterolaemia alone. Minick et al (1979) in an earlier study also using rabbits were likewise able to show that high levels of circulating lipids (particularly hypercholesterolaemia) were associated with enhanced intimal hyperplasia. This study used balloon de- endothelialisation in normo-lipidaemic rabbits, some of whom were subsequently given a high lipid diet (beginning 4-9 weeks after injury), and implied that the phenomenon only occurred in re-endothelialised areas- a finding slightly at odds with current thinking on smooth muscle mitogenesis.

Patient Studies

There is little data associating elevated lipid and lipoprotein levels with intimal hyperplasia in humans, and most that does exist is indirect due to the obvious difficulties in obtaining histological evidence in vivo.

In a prospective multicentre study of femoropopliteal grafts Wiseman et al (1989) showed that apolipoprotein A1 and Lipoprotein (a) were higher in those vein grafts which were occluded at 1 year than those that remained patent. If it is accepted that stenoses account for most graft failures, then this finding can be interpreted as supporting evidence of lipid factors in graft stenosis and intimal hyperplasia. It must be appreciated however that causes of grafts stenosis other than intimal hyperplasia were not excluded in these studies. No account was taken of progression of native vessel disease as a cause of graft failure although it might be added that progression of native atheroma to the extent of graft failure within one year in a patient who has (presumably) undergone full preoperative work up may be unlikely.
In a retrospective study of late graft failure and stenosis in coronary bypass grafts, Campnaeu et al (1984) showed that VLDL and LDL levels were higher, and HDL levels lower in patients who developed progressive stenoses in the graft or native vessels. This study considers native vessel and graft stenosis together and this makes interpretation of data difficult. In addition, the author concludes (based on angiographic findings) that the lesions present in the grafts at 10 years after surgery represent atheromata in the veins. This study is of interest therefore only if we accept that intimal hyperplasia and atherosclerosis have a common pathophysiological origin; whilst this has been suggested in the carotid artery following endarterectomy (Clagett et al 1986), the claim remains unsubstantiated in arterial bypass grafts.

Following carotid endarterectomy Colyvas et al (1992) were able to demonstrate significantly higher levels of apolipoprotein B, and lower levels of high density lipoprotein cholesterol in patients who developed restenosis of sufficient severity to warrant reoperation. Although the end point of this study (required reoperation) is rather subjective, the work supports a previous study by Salenius et al (1989), which showed that patients with favourable lipid profiles had significantly fewer high grade restenoses after carotid endarterectomy.

Restenosis following coronary angioplasty has been shown to be associated with low HDL-cholesterol levels by Shah and Amin (1992) and this supports work by others demonstrating some abnormalities in lipid profiles in patients who develop restenosis (Bergelson et al 1989, Harlan et al 1990, Reis et al 1990). This data must be carefully scrutinised however as the association between the two variables is often weak (i.e. only demonstrable between restenosis and lipid levels measured 3-6 months after angioplasty [Reis et al 1990]) and other studies have failed to show any association between lipid levels and restenosis (MacDonald et al 1987).

Indirect evidence of an association between lipids and intimal hyperplasia is provided by studies of dietary or pharmacological manipulation of circulating lipids and their effects on post angioplasty restenosis. The administration of fish oil supplements
containing ω-3 fatty acids (known to favourably alter the plasma lipoprotein profile - see above) after coronary angioplasty has produced varying results; Dehmer et al (1988) and Milner et al (1989) were able to show a significantly reduced restenosis rate when ω-3 fatty acids were given for 6 months commencing at the time of angioplasty compared with untreated controls. However both Grigg et al (1989) and Reis et al (1989) in double blind placebo controlled studies of similar treatment, failed to observe any difference in restenosis rates between the treated or untreated groups. It is difficult to explain the variations seen in these studies unless the effects of treatment are very small.

With regard to pharmacological reduction of circulating lipids, results to date are also inconclusive. In a recent study Sahni et al (1991) were able to show a reduction in restenosis after coronary angioplasty by the use of Lovastatin, a drug which inhibits the rate limiting enzyme in cholesterol synthesis. Similar findings have also been demonstrated in rabbit arteries after balloon de-endothelialisation when Lovastatin is administered (Gellman et al 1991). These findings must be considered in the light of work by Hollman and colleagues (1989) however who, in a preliminary report, observed no change in restenosis rates after angioplasty despite treatment with a more aggressive lipid reducing policy.
Studies of Lipids, lipoproteins and graft stenosis

6.6 Patients

All patients undergoing infrainguinal reconstruction under the care of Mr JHN Wolfe during the study period were recruited into the studies along with a retrospective cohort of patients as described in chapter 4.

Lipid profiles were measured in 59 patients undergoing femoropopliteal or femorocrural bypass grafting, in prospective (n=36) and retrospective (n=23) groups. Lipid measurements in the prospective group were measured preoperatively, at the time of clinic attendance or admission to hospital, and patients were followed for the development of graft stenosis. In the retrospective group, lipid measurements were made on patients known to have developed graft stenosis within the first post operative year (retrospective stenosed group) and on a similar group of patients who had previously undergone reconstruction and in whom graft surveillance has shown no abnormality for a minimum of 1 years post operation (retrospective non stenosed group).

Table L1 shows total numbers of vein and PTFE grafts studied along with the number of stenosed grafts in each group. Numbers of each type of graft followed prospectively and retrospectively are shown in table L2.

<table>
<thead>
<tr>
<th></th>
<th>Stenosis</th>
<th>No Stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein Grafts</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>PTFE Grafts</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Table L1: total numbers of vein and prosthetic grafts in lipid studies. Also shown are numbers with and without stenosis.
Inclusion of patients into this study deserves further mention; of fifty two vein grafts entered into the study as a whole, lipids and lipoprotein levels were measured in thirty-five patients (69%); results were available for twenty-four of twenty-nine enrolled patients with PTFE grafts (82%). Omissions were due to spoiling of collected samples in a minority of cases.

As has already been described (see chapter 4), the majority of missing results were a consequence of the requirement for patients to be transferred between St. Mary’s Hospital to the Royal Free Hospital where coagulation and platelet function studies were performed on freshly drawn blood. This inevitably led to a random sample of patients who were not available for blood sampling at both hospitals. Although this does increase the possibility of sample error and bias, it must be emphasised that patient availability was entirely random and not subject to control or selection by the author or anyone involved in sampling other than the patient himself.

Grafts which thrombosed (except one which occluded whilst awaiting correction of an angiographically proven stenosis) and those not available for stenosis surveillance were excluded from study to avoid difficulty in data interpretation (see chapter 4).
6.7 Methods

Causes of graft stenosis other than intimal hyperplasia were excluded by intraoperative graft assessment using doppler and angiography, histological proof was obtained when possible. Details of stenosis surveillance techniques and the intraoperative and post operative doppler studies used to exclude fixed graft stenosis have been described in chapter 5.

Blood Collection and lipid measurements

Whole blood was collected from a antecubital vein after a minimum 12 hour fast and stored in plain tubes. Serum was prepared by centrifugation at 3000 rpm. Cholesterol and triglycerides were determined enzymatically using a Centrifichem centrifugal analyser (Baker Instruments Windsor UK), LDL cholesterol was calculated by the Friedwald equation (Friedwald et al 1972). HDL was isolated by the dextran sulphate-Mg Cl2 method (Warnick et al 1982) and the cholesterol measurement performed as above. Apolipoproteins were measured using immuno-nephelometry (Beckman Auto Immuno-Chemical Systems, Beckman Instruments, High Wycombe UK).
6.8 Results

Lp(a) and graft stenosis

Univariate analysis showed significant differences in serum Lp(a) levels in PTFE grafts which developed stenosis and those that did not; median serum Lp(a) concentration in PTFE grafts with stenosis was 0.3 g/L compared with 0.05 g/L in non stenosed grafts (p< 0.01 Mann Whitney U test, figure L1).

![Image of Lp(a) concentrations in PTFE grafts which developed stenosis and those that did not. The bar shows median concentration.

95% confidence interval of difference - 0.656 to - 0.0202

Figure L1: Lp(a) concentrations in PTFE grafts which developed stenosis and those that did not. The bar shows median concentration.
In autologous vein grafts, there were no statistically significant differences in Lp(a) levels between grafts which developed stenosis and those that did not (median Lp(a) concentrations 0.15 g/L v 0.111 g/L respectively, figure L2).

Logistic regression was used to estimate the relationship between graft stenosis and Lp(a) after allowing for the confounding effects of graft material and study group. Results are shown as maximum-likelihood estimates in table L3.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp(a)</td>
<td>-1.5186</td>
<td>0.7696</td>
<td>3.89</td>
<td>0.048</td>
</tr>
<tr>
<td>Prospective v</td>
<td>0.3356</td>
<td>0.6795</td>
<td>0.24</td>
<td>0.621</td>
</tr>
<tr>
<td>Retrospective</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>0.128</td>
<td>0.6617</td>
<td>0.04</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table L3: Analysis of maximum likelihood estimates.

This data shows that serum Lp(a) concentration is associated with the development of graft stenosis and this effect is independent of graft material or whether patients were studied in prospective or retrospective groups.

The results of logistic regression fitting all variables associated with stenoses into the same model are shown on chapter 9.
Figure L2: Lp(a) concentrations in vein grafts which developed stenosis and those that did not. The bar shows median Lp(a) concentration.
Triglycerides, Total cholesterol and graft stenosis

In PTFE grafts which developed stenosis, median serum triglyceride concentration was significantly lower than that in non stenosed grafts (median serum triglyceride concentration, 1.59 mmol/L v 2.55 mmol/L p=0.03 Mann Whitney U test, Figure L3). Median serum total cholesterol was not significantly different the two groups of PTFE grafts (median total cholesterol concentration in stenosed grafts 6.03 mmol/L, median cholesterol concentration in non stenosed grafts 5.71 mmol/L, P/NS Mann Whitney U test, figure L4).

Serum triglyceride and total cholesterol levels were not significantly different in stenosed and non stenosed vein grafts (median serum triglycerides 1.63 mmol/L v 2.16 mmol/L respectively figure L5, p=NS, median serum total cholesterol 6.15 mmol/L v 5.51 mmol/L respectively figure L6, p=NS both groups Mann Whitney U test ).

Regression analysis estimates of the association between triglycerides and graft stenosis are shown in table L4

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Triglycerides</td>
<td>1.919</td>
<td>1.127</td>
<td>2.9</td>
<td>0.088</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>-0.6636</td>
<td>0.6136</td>
<td>1.17</td>
<td>0.279</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>0.8036</td>
<td>0.598</td>
<td>1.8</td>
<td>0.179</td>
</tr>
</tbody>
</table>

Table L4

These results show that the relationship between serum triglycerides and graft stenosis is not independent of the effects of graft material or study group and suggest that differences between these groups may account for the results of univariate analysis.
Figure L3: serum triglyceride concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median concentration.

95% confidence interval of difference 0.078 to 1.31
Figure L4: serum cholesterol concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median concentration.
p=NS (Mann Whitney U Test)

Figure L5: serum triglyceride concentration in vein grafts which developed stenosis and those that did not. The bar shows median triglyceride concentrations.
Figure L6: serum cholesterol concentration in vein grafts which developed stenosis and those that did not. The bar shows median cholesterol concentration.
HDL-Cholesterol and graft stenosis

Serum high density lipoprotein - cholesterol levels were not significantly different between stenosed or non stenosed grafts when either PTFE or vein conduits were considered;

PTFE grafts; median HDL1-cholesterol 0.0.85 mmol/L and 0.66 mmol/L respectively, p=NS Mann Whitney U test, figure L7, median HDL2 levels 0.23 mmol/L and 0.22 mmol/L respectively p=NS Mann Whitney U test, figure L7, median HDL3 levels 0.60 mmol/L and 0.47 mmol/L respectively p=NS Mann Whitney U test, figure L8.

Vein grafts; median HDL1 levels 0.77 mmol/L and 0.61 mmol/L respectively p=NS Mann Whitney U test, figure L9, median HDL2 levels 0.2 mmol/L and 0.219 mmol/L respectively p=NS Mann Whitney U test figure L9, median HDL3 levels 0.48 mmol/L and 0.41 mmol/L respectively p=NS, figure L10.
p=NS, Both groups, Mann Whitney U test

Figure L7: Serum HDL-1 cholesterol (open circles) and HDL-2 cholesterol (closed circles) in PTFE grafts which developed stenosis compared with those that did not. Median values for each group are shown by bars.
Figure L8: serum HDL-3 cholesterol in PTFE grafts which developed stenosis and those that did not. Median values are shown by the bar.
Figure L9: Serum HDL-1 cholesterol (open circles) and HDL-2 cholesterol (closed circles) in vein grafts which developed stenosis compared with those that did not. Median values for each group are shown by bars.

p=NS, Both groups, Mann Whitney U test
ApoB and graft stenosis

There were no significant differences in serum apolipoprotein B concentrations between stenosed or non stenosed PTFE grafts (median concentrations 141 g/L and 136.5 g/L respectively p=NS figure L11). ApoB concentration was non significantly elevated in
stenosed vein grafts compared to non stenosed (median apoB concentrations 131 g/L and 112 g/L respectively p=0.10 Mann Whitney U test, figure L12) and differences of a similar magnitude were present in prospectively studied grafts alone (median apoB concentrations 129 g/L and 112.0 g/L respectively p=0.14, figure L12).

Figure L11: serum apolipoprotein B concentration in PTFE grafts which developed stenosis and those which did not. Medians shown by the bar
Figure 1.12: Serum apolipoprotein B concentration in all vein grafts (closed circles) and prospective vein grafts (open circles). The bars shown median concentration.

p=NS Mann Whitney
Both Groups

n=9 n=15 n=8 n=13
ApoA1 and graft stenosis

Serum apolipoprotein A1 concentrations were not different in stenosed or non stenosed grafts in either PTFE or vein conduit (PTFE grafts median concentrations 169.5 mg/dL and 141.5 mg/dL, p=NS figure L13, vein grafts, median concentrations 166 mg/dL and 163 mg/dL, p=NS figure L14).

\[ p=NS \quad (\text{Mann Whitney U Test}) \]

![Graph showing serum apolipoprotein A1 concentration in stenosed and non-stenosed grafts.](image)

**Figure L13**: serum apolipoprotein A1 concentration in PTFE grafts which developed stenosis and those that did not. Median concentration is shown by the bar.
p = NS (Mann Whitney U Test)

Figure L14: serum apolipoprotein A1 concentration in vein grafts which developed stenosis and those that did not. Medians shown by the bar.
6.9 Discussion

These studies have compared serum lipid and lipoprotein levels in patients with infrainguinal bypass grafts which developed hyperplastic stenosis with patients whose grafts remained stenosis free. Non hyperplastic causes of graft stenosis have been excluded by intraoperative and perioperative graft assessment with doppler, duplex scanning and angiography and intimal hyperplasia has been proven on histology whenever possible (see chapter 5).

Results show that elevated levels of serum Lp(a) are associated with graft stenosis development, and that this relationship is statistically independent of the type of graft material used and whether grafts were studied in prospective or retrospective groups. PTFE graft stenosis was associated with reduced serum triglycerides by univariate analysis but logistic regression suggests that this observation may be a product of differences in triglyceride levels between prospective and retrospectively studied grafts.

There were no significant differences in concentrations of total-, HDL-, or LDL-related cholesterol, or in apolipoproteins B-100 or A-1, between stenosed and non stenosed grafts.

Lp(a) denotes a family of lipoproteins with structural homology to LDL but also containing an additional apolipoprotein, apo(a). Experimental data to support an association between elevated LDL and Lp(a) and the development of intimal hyperplasia and graft stenosis has been described earlier in this chapter; raised LDL has been associated with the retrospective development of stenoses in coronary artery bypass grafts (Campneau et al 1984, Hoff et al 1988), and in the British Multicentre Femoro-Popliteal Trial, elevation of plasma LDL and Lp(a) was associated with reduced 1 year patency in femoropopliteal and femorocrural grafts (Wiseman et al 1989 and 1990). As hyperplastic stenoses are believed to be the most common cause of intermediate graft failure (Szilagyi et al 1973, Brewster et al 1983), this
observation indirectly implies an association between LDL and Lp(a) and graft stenosis.

Relatively little human work has been undertaken to directly study any association between lipoprotein abnormalities and intimal hyperplasia, but there is a well established link between elevated lipoproteins and atherosclerosis; migration and proliferation of smooth muscle cells (in a manner very similar to intimal hyperplasia in bypass grafts) is now believed to be the first abnormality to develop in atherosclerotic plaques (Ross & Glomset 1976). The association between raised serum lipoproteins and atherosclerosis suggests that lipoproteins may be important in the development of human smooth muscle cell pathology. The actual mechanism of this association is unclear, but it has been suggested that oxidised lipoproteins may be chemotactic and mitogenic to smooth muscle cells - either directly or via effects on intermediate cells such as macrophages (Cathcart et al 1985, Carew 1989), see above. This may explain the observed relationship.

Lp(a) is associated with accelerated atherosclerosis in man (Murai et al 1986, Zenker et al 1986) and can be demonstrated in human atheromata (Walton et al 1974) and thrombosed coronary vein grafts (Cushing et al 1989), but whether this molecule acts as a smooth muscle mitogen as proposed for other apoproteins, or whether its unique structural homology to plasminogen (Hoff et al 1988, Hajjar et al 1989) affects its atherogenic properties is unknown.

That serum triglyceride levels were lower in stenosed PTFE grafts than in non stenosed grafts on univariate analysis, appears to be a product of triglyceride differences between prospective and retrospective groups of grafts; at the time of writing there is no data to support an association between reduced serum triglycerides and the development of intimal hyperplasia, and conversely, no data to show protection against intimal hyperplasia by elevated triglyceride levels. Additionally,
serum triglycerides are known to vary depending on diet (unlike Lp(a) levels, Krempler et al 1980, MBewu & Durrington 1990) and therefore may be subject to variation caused by poor compliance with the request to fast before bloods were taken.

These results suggest that elevated circulating levels of lipoprotein (a), a molecule associated with accelerated atherosclerosis development, are also related to the development of intimal hyperplasia and graft stenosis in infrainguinal bypass grafts.
Chapter 7

Studies of Coagulation/Fibrinolytic Activity & Graft Stenosis

7.1 Introduction

The following studies correlate the development of graft stenosis with activity of plasma coagulation and fibrinolytic factors.

A summary of the pro- and anti-coagulation cascades, and a review of the literature associating activation of these factors with atherogenesis and intimal hyperplasia and will also be presented. The close morphologic similarity between intimal hyperplasia and early atheromatous lesions has been described earlier.

7.2 Physiological Coagulation and Fibrinolysis.

The Clotting Cascade

When a break occurs in the continuity of a blood vessel the haemostatic response must be rapid, localised and controlled. In humans this requires a carefully orchestrated combination of responses by the coagulation cascade in conjunction with other circulating factors such as platelets. The changes occurring in the coagulation cascade will be described here, the response of platelets will be considered elsewhere.

The component parts of the clotting cascade were elucidated some years ago and are well understood; tissue deep to the site of vascular injury leaks tissue factor and ADP. The released tissue factor activates Factor VII to VIIa, thereby initiating the extrinsic pathway of coagulation (figure C1). Factor VIIa directly converts Factor X to Xa, and thus rapidly generates thrombin (factor IIa) and therefore fibrin from fibrinogen. The presence of thrombin further amplifies the coagulation cascade by activating factors V and VIII (Schrier 1986).

Exposure of damaged endothelium and underlying tissue also activates factor XII (Hageman factor), which in a complex series of enzymatic reactions (involving vital...
circulating, non-enzymatic co-factors), triggers the intrinsic coagulation pathway (figure C1). Although relatively slower, the intrinsic pathway is quantitatively important in normal clotting (Schrier 1986). Activation of the extrinsic and intrinsic coagulation pathways result in the conversion of soluble fibrinogen into insoluble fibrin monomers. Aggregation of these monomers then results in the formation of strands of fibrin polymer which interact with thrombin and platelets to form a plug, able to occlude the site of haemorrhage.

All of the plasma procoagulants except von Willebrand factor (a component of factor VIII) are synthesised in the liver. The production of factors II, VII, IX and X are dependant on vitamin K. Von Willebrand factor is synthesised in megakaryocytes and may be important in platelet activation after dissociation from the inactive factor VIII complex (George et al 1984).

The importance of this cascade in human disease is obvious, as thrombosis is known to be one of the final crucial event in serious disorders such as myocardial infarction (DeWood et al 1980, Davies and Thomas 1984). Furthermore, the presence of fibrin molecules in human atheroma (see below) and the incrustation theory of atherogenesis suggest that non occluding thrombus may be important in the development of atherosclerosis, the single most important cause of death in the western world. The demonstration that some of the recognised risk factors for atheroma affect haemostatic function (Packham & Mustard 1986) gives further impetus to the suggestion that the clotting cascade is important in vascular disease. The evidence linking coagulation with atheroma development and intimal hyperplasia is detailed below.
Fibrinolysis and Inhibition of Coagulation

If the autoamplification properties of the coagulation cascade were left unchecked, then any haemorrhage would result in generalised coagulation and widespread vessel occlusion. Such a disaster is opposed by the removal of procoagulant by flowing blood and/or the reticuloendothelial system. The active breakdown of polymerised fibrin (fibrinolysis) and antagonism of activated coagulation factors by circulating inhibitors are also important in the localisation of pro-coagulant activity.

Physiological fibrinolysis is effected through plasmin, an enzyme produced from its zymogen, plasminogen, by proteolytic cleavage (Aoki & Harpel 1984). As well as breaking down fibrin, unopposed plasmin has a broad action and will also cleave fibrinogen, Factors V and VIII and complement activity (Schrier 1986).

Plasminogen is activated by two physiological enzymes, tissue plasminogen activator (t-PA) and urokinase. Tissue plasminogen activator is a serine protease produced by endothelial cells (Aoki & Harpel 1984). Its ability to activate plasminogen is markedly enhanced by the presence of fibrin, on which it forms a complex with plasminogen - a mechanism ideally suited to the local production of thrombolytic agents in areas where they are needed. Tissue plasminogen activator is itself inhibited by a three types of agent; the most important of these is plasminogen activator inhibitor one (PAI-1), also produced by endothelial cells and also present in platelet granules (Aoki And Harpel 1984).

Circulating plasmin is rapidly inactivated by anti plasmins, the most important of which is α-2-antiplasmin (Aoki & Harpel 1984, Linjen & Collen 1989). This molecule binds plasmin at the functionally important lysine binding site on one of the five kringles (complex triple looped polypeptides) which are it's most important structures. This mechanism of action is similar to those of the clinically available anti-fibrinolytic agents tranexamic acid and ε-aminocaproic acid.

The most important inhibitors of coagulation are antithrombin III and protein C. Antithrombin III opposes the action of the serine protease procoagulants, Factors XIIa,
XIa, IXa, Xa and Ila. AT-III binds heparin, producing a conformational change which makes AT-III more active (estimated increase in AT-III activity by x750-1000) (Rosenberg & Bauer 1986) - the mechanism of action of heparin in clinical use. Heparin like proteoglycans on the surface of endothelial cells are thought to potentiate the action of AT-III in vivo. Protein C is a vitamin K dependant anticoagulant synthesised in the liver. On activation, it is converted into a serine protease which inhibits factors Va and VIIIa, the two major procoagulants not opposed by AT-III (Schrier 1986). Activated protein C also inhibit the inhibitors of tissue plasminogen activator (e.g. PAI-1 see below), allowing t-PA to activate plasminogen (Clouse & Comp 1986).

Data correlating the role of coagulation/fibrinolytic activity with atherogenesis and intimal hyperplasia is provided below.

The current understanding of the balance between pro and anticoagulant activity is summarised in figure C1.
Figure C1: Simplified plan of pro- and anti-coagulant activity in the circulation. Lines with arrow heads show activation of profactors or macromolecule breakdown. Enzymes stimulating these actions are shown by plain lines. The thick lines show inhibition.

PL=platelet factor 3
7.3 Coagulation, fibrinolysis and Atheroma

That abnormalities of the clotting and fibrinolytic cascades may play a role in atherogenesis came from early pathological observations of fibrin (figures 1(2) & 1(8)) and platelet degradation products in mature atheroma. More recently, supporting evidence for this theory has been provided by the demonstration of enhanced coagulation activity in patients who develop atherosclerosis.

Pathological studies demonstrating the presence of thrombus and its constituents in atheroma were described by Von Rokitanski in 1852 and were important in the formation of the incrustation theory of atherogenesis. The availability of techniques such as electron microscopy and immunocytochemistry, combined with the development of monoclonal antibody technology, has confirmed the presence fibrin within mature atheromatous plaque (Woolf & Carstairs 1967, Kao & Wissler 1965, Haust et al 1965). A recent study has been able to differentiate between fibrin and fibrinogen within plaque and demonstrated the presence of both molecules (Bini et al 1989). This led the authors to suggest that there may be two mechanisms of coagulation factor inclusion and/or promotion of developing atheroma; one involves the inclusion of non occluding mural thrombus into established plaque and explains the presence of fibrin polymers. The presence of soluble fibrinogen monomers is explained as a consequence of the increase in vessel wall permeability proposed in the response to injury hypothesis by Ross; injury is believed to render the vessel wall permeable to macromolecules, and although this is commonly cited to explain the presence of lipoproteins within developing plaques, it may also allow insudation of coagulation factors such as fibrinogen (Bini et al 1989). This hypothesis achieves further credibility when it is remembered that some coagulation factors (including fibrinogen) have been shown to be mitogenic and or chemoattractant to smooth muscle cells in vitro (see below) - and smooth cellular proliferation is understood to be an important element in early atheroma formation.
Other studies correlating atherosclerosis and clotting disorders have concentrated mostly on the coronary circulation. In the Northwick Park Heart Study, plasma fibrinogen concentration and clotting factor activity in a large cohort of men were measured and correlated prospectively with cardiac mortality and myocardial events. Results showed that plasma fibrinogen concentration and factor VII activity were closely correlated with the development of ischaemic heart disease over a mean 10 year follow up period and that all clotting factors measured (factors III, V, VII and VIII) had an independent association with the development of coronary disease (Meade et al 1986). Plasma fibrinogen concentration was correlated with both myocardial infarction and stroke in another prospective study in Sweden, with a mean follow up of 13 years (Wilhelmsen et al 1984).

Although data from clinical studies such as these could represent the effects of a predisposition to thrombosis rather than the progression of atherosclerosis, the histological demonstration of intra-plaque fibrin combined with the in vitro mitogenic activity of coagulation factors (see below), suggests at least that thrombus can be incorporated into developing plaque (and therefore contribute to size) and that some elements of the encrusted thrombus may directly stimulate smooth muscle cell proliferation.

7.4 Coagulation, Fibrinolysis and Intimal Hyperplasia

Evidence suggesting that coagulation factors may play a role in intimal hyperplasia has been provided by studies of the behaviour of vascular smooth muscle cells in culture. It was observed some years ago, that the addition of serum to an in-vitro preparation of smooth muscle cells stimulates mitosis in most species - investigation of this phenomenon led to the discovery of the platelet derived growth factor (Ross et al 1974). However it has also been shown that inactivation of the established serum mitogens such as PGDF by monoclonal antibodies, only reduces in
vitro cell stimulation by approximately 20% (Ferns et al 1991), and that addition of
serum containing PDGF concentrations which varying by a factor of ten, have almost
equal effects on smooth muscle cell growth (Bowen-Pope et al 1989). These data
suggest that serum factors other than PDGF may be important in smooth muscle
mitosis in culture, and therefore that these additional factors may influence smooth
muscle growth in vivo (as endothelial injury exposes medial myocytes to circulating
blood); Carney et al in 1984 and Huang & Ives in 1987, demonstrated that thrombin is
mitogenic to vascular smooth muscle cells in culture. In similar studies, Gasic et al
(1992) have demonstrated increased tritiated-thymidine uptake by cultured rat aortic
smooth muscle cells following stimulation by numerous purified coagulation cascade
proteins at physiological concentrations. Maximal increases were observed after
stimulation with activated factor (Xa) and protein S, and lesser responses with
unactivated factors X and IX. Only stimulation of the model with factor VII or protein
C did not produce any increase in thymidine uptake. These authors suggested that
repeated Epidermal Growth Factor-like domains, known to be present on some
coagulation protein molecules (Furie & Furie 1988), may be partly responsible for the
observed mitogenic effects. Using a time course experiment, they have also postulated
that the mitogenic effects of Xa and protein C are not mediated by the autocrine
induction of PDGF as has been suggested after smooth muscle cell stimulation by
interleukin 1 (Raines et al 1989). They suggest that coagulation cascade proteins act by
direct stimulation of the cells.

In more recent work, Naito and colleagues have shown that fibrinogen is
chemotactic for smooth muscle cells (Naito et al 1989 & 1990) and also that fibrin and
fibrinogen deposited in the vessel wall may bind smooth muscle cells and thus act as a
'scaffold' for migrating cells. Singh et al (1990), using a rabbit model, impregnated
silk sutures with fibrinopeptide B (a cleavage product of fibrinogen), to show that this
factor is associated with increased smooth muscle cell proliferation. These authors
suggested that this stimulation may occur secondary to the monocyte chemotactic
effects of FpB. Furthermore, interaction between fibrinogen and low density
lipoproteins is thought be a potent source of FpB. Increased availability of FpB in hyperfibrinogenaemic and hyperlipoproteinaemic states, may be a factor in the association of these conditions with the development of atherosclerosis.


**Studies of Coagulation / Fibrinolysis and Graft Stenosis**

**7.5 Patients and Methods**

Patients

All patients undergoing infrainguinal reconstruction under the care of Mr JHN Wolfe during the study period were recruited into the studies along with a retrospective cohort of patients as described in chapter 4.

Fibrinogen Studies

Regarding inclusion of patients, forty of fifty-two patients with vein grafts had plasma fibrinogen measured (77%) and twenty-five of twenty-nine patients with PTFE grafts had fibrinogen measurements (86%). Lost samples in the fibrinogen group were mostly spoiled after sampling and therefore entirely random. Fibrinogen level in the prospective group was measured preoperatively, at the time of clinic attendance or admission to hospital, and patients were followed for the development of graft stenosis. In the retrospective group, measurements were made on patients known to have developed graft stenosis within the first post operative year (retrospective stenosed group) and on a similar group of patients who had previously undergone reconstruction and graft surveillance, without abnormality for a minimum of 1 year post operation (retrospective non stenosed group).
Table C1 shows total numbers of vein and PTFE grafts studied along with the number of stenosed grafts in each group. Numbers of each type of graft followed prospectively and retrospectively are shown in table C2.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>No Stenosis</td>
<td>24</td>
<td>14</td>
</tr>
</tbody>
</table>

Table C1: Numbers of vein and prosthetic grafts in fibrinogen studies. Also shown are numbers with and without stenosis.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospective Study</td>
<td>21 (11)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Retrospective Study</td>
<td>19 (5)</td>
<td>9 (5)</td>
</tr>
</tbody>
</table>

Table C2: Numbers of patients included in prospective and retrospective fibrinogen studies. Bold figures are totals, figures in brackets indicate numbers with stenosis.

Clotting Factor and Fibrinolysis Analysis

Activity of factors VII and VIII, plasminogen, PAI and tPA were measured at the Royal Free Hospital academic department of clinical chemistry. Samples were taken at the Royal Free to avoid in-vitro activation, thereby requiring patients to travel. These factors were therefore measured in a smaller population of randomly available patients who were fit and/or able to travel across the city: thirty five patients with vein grafts (48%) and thirteen (45%) patients with PTFE grafts were so able. Although these omissions increase the possibility of sample error and bias, it must be emphasised that
patient availability was entirely random and not subject to control or selection by the author or anyone involved in sampling other than the patient himself.

The timing of clotting/fibrinolysis activity measurements in the prospective group and retrospective groups was the same as described for fibrinogen measurements above. Retrospective patients were studied in two similar groups to those described for fibrinogen studies (retrospective stenosed and retrospective non-stenosed groups).

Table C3 shows total numbers of vein and PTFE grafts in whom plasma factor VII, factor VIII and fibrinolytic activity were measured, along with the number of stenosed grafts in each group. Numbers of each type of graft followed prospectively and retrospectively in these studies are shown in table C4.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>No Stenosis</td>
<td>21</td>
<td>7</td>
</tr>
</tbody>
</table>

Table C3: Numbers of vein and prosthetic grafts in coagulation factor & fibrinolysis studies. Also shown are numbers with and without stenosis.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospective Study</td>
<td>19 (10)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Retrospective Study</td>
<td>16 (4)</td>
<td>7 (2)</td>
</tr>
</tbody>
</table>

Table C4: Numbers of patients included in prospective and retrospective coagulation factor & fibrinolysis studies. Bold figures are totals, figures in brackets indicate numbers with stenosis.
7.6 Methods

Causes of graft stenosis other than intimal hyperplasia were excluded by perioperative graft assessment using hand held doppler, angiography and duplex scanning. Histological proof was obtained when possible. Details of stenosis surveillance techniques and the intraoperative and post operative doppler studies used to exclude non hyperplastic causes of graft stenosis have been described in chapter 4.

Blood Collection and Plasma Preparation for Coagulation Studies

Blood was collected from an antecubital vein with minimal stasis and anticoagulated with 3.18% trisodium citrate in proportions of 9:1 respectively in precooled tubes. Samples were centrifuged at 3000g for 15 minutes at 4°C and plasma was stored at -70°C until analysis.

Fibrinogen, Plasminogen, Factor VII and Factor VIII Assay

Levels of plasma fibrinogen and plasminogen and activity of factors VII and VIII (percentage activity relative to standardised plasma) were assayed using an automated coagulation analyser (ACL 300 Research Instrumentation Laboratory Ltd, Warrington Cheshire UK - see figure C2).

Blood Collection and Plasma Preparation for Fibrinolytic Studies

For the quantitative determination of tissue plasminogen activator (t-PA) citrated blood was collected in concentrations of 9 part blood to 1 part 3.18% trisodium citrate as described above. Samples were processed as has been recommended by Chandler et al (1989); briefly, 0.5 ml of blood was added to 0.25 ml of Sodium acetate buffer (0.5 mol/ l at pH 4.2) and immediately centrifuged at 10,000g for 2 minutes. The resulting supernatant was collected and frozen at -70°C until assay.

For the quantitative determination of plasminogen activator inhibitor concentration, blood was collected into citrate as already described, supplemented with the following
anti-platelet agents; indomethacin (20 μg/ml final concentration [F.C.]), theophylline (F.C. 3mmol/L) and adenosine (F.C. 1 mmol/L). These antiplatelet agents were added since a large amount of PAI-1 is contained within platelets (Booth et al 1988) and this may be released from hyperaggregable platelets in patients with peripheral vascular disease during processing.

**7.7 Selection of Coagulation Factors for Analysis**

Fibrinogen and factors VII and VIII were chosen for analysis in this study because of the recognised association of each with the development or progression of atheroma in the native arterial tree (see above) and also as they are representative of the intrinsic, extrinsic and common pathways in the coagulation cascade as shown in figure C1.

![Image of ACL 300 automated coagulation analyser](Fig C2; The ACL 300 automated coagulation analyser)
7.8 Results

Fibrinogen and Graft Stenosis

In univariate analysis, plasma fibrinogen concentration was significantly higher in patients who developed stenosis than those who did not; in vein grafts which developed stenosis median fibrinogen concentration was 447.5 mg/100ml compared with 339.3 mg/100ml in patients who did not (p=0.01 Mann Whitney U test, figure C3).

Figure C3: plasma fibrinogen concentration in vein grafts which developed stenosis and those that did not. The bar shows median values.

In PTFE grafts median fibrinogen concentration in stenosed grafts was 391.0 mg/100ml compared with 300.0 mg/100ml in non stenosed grafts (p=0.003 Mann Whitney, figure C4).
Figure C4: Plasma fibrinogen concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median values.

95% confidence interval of difference: -202.5 to 43.1
Logistic regression was used to estimate the relationship between stenosis and fibrinogen after allowing for the confounding effects of study group (prospective and retrospective) and graft material; results are shown in table C5 as analysis of maximum-likelihood estimates.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Fibrinogen</td>
<td>-0.0163</td>
<td>0.00508</td>
<td>10.27</td>
<td>0.0013</td>
</tr>
<tr>
<td>Prospective v</td>
<td>-1.239</td>
<td>0.7147</td>
<td>3.01</td>
<td>0.083</td>
</tr>
<tr>
<td>Retrospective</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>0.2053</td>
<td>0.7615</td>
<td>0.07</td>
<td>0.787</td>
</tr>
</tbody>
</table>

Table C5: Analysis of likelihood estimates: Fibrinogen

This data shows that there are significant differences in plasma fibrinogen concentration between patients whose grafts develop stenosis and those who do not and that this relationship is independent of prospective and retrospective study groups and the type of graft material used.

The results of logistic regression fitting all variables associated with graft stenosis into the same model are described at the end of chapter nine.
Activity of factors VII / VIII and Graft Stenosis

Plasma factor VIII activity was similar in stenosed and non stenosed grafts in both vein and PTFE groups (Vein grafts; medians; 95% of control activity in stenosed v 72% in non stenosed grafts, p=NS Mann Whitney U test, figure C5. PTFE grafts, median activity 77% control v 92% respectively, p=NS Mann Whitney U test, figure C6).

Figure C5: plasma factor VIII activity in vein grafts which developed stenosis and those that did not. The bar shows median values.
Figure C6: Plasma factor VIII activity in PTFE grafts which developed stenosis and those that did not. The bar shows median activity.

p=NS
Mann Whitney U Test

<table>
<thead>
<tr>
<th>Stenosed Grafts</th>
<th>Non stenosed Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=5</td>
<td>n=6</td>
</tr>
</tbody>
</table>
Factor VII activity in both graft types was not different in stenosed or non-stenosed grafts (vein grafts; median activity 96% v 109%, p=NS, PTFE grafts; median activity 88% v 102%, p=NS, figure C7).

Figure C7: Plasma factor VII activity in PTFE grafts (open circles) and vein grafts (closed circles) which developed stenosis and those that did not. The bars show median values.
Thrombolytic Activity and Graft Stenosis

There were no significant differences in plasminogen activity between stenosed and non stenosed vein grafts (median plasminogen activity 110 % v 104 % p=NS Mann Whitney, figure C8).

There was a tendency toward higher plasma concentrations of plasminogen activator inhibitor (PAI) in non stenosed vein grafts (median PAI concentrations 30.1 mg/L and 46.7 mg/L, p=NS, figure C9).

Plasma tissue plasminogen activator (tPA) concentration was not different between stenosed and non stenosed vein grafts (median tPA concentration 4.8mg/L and 4.3 mg/L, p=NS, figure C10).

In PTFE grafts there were no differences in plasminogen activity between stenosed and non stenosed grafts (median activity 108 % v 139% respectively, p=NS, figure C8).

As with vein grafts, there was a non significant trend toward elevation of plasma PAI in non stenosed grafts (median PAI concentration 47.3 mg/L in stenosed grafts and 83.4 mg/L in non stenosed grafts, p=NS, figure C9).

Plasma tPA concentration was not significantly different between stenosed and non stenosed PTFE grafts (median tPA concentration, 4.36 mg/L and 4.71 mg/L p=NS, figure C10).
Figure C8: Plasminogen activity in PTFE grafts (open circles) and vein grafts (closed circles) which developed stenosis and those that did not. The bars show median values.

n=5  n=6  n=12  n=16
Figure C9: Plasma Plasminogen activator inhibitor concentration (PAI-1) in PTFE grafts (open circles) and vein grafts (closed circles) which developed stenosis and those that did not. The bars show median values.

p=NS Both Groups

n=5  n=4  n=9  n=15
Figure C10: Plasma tissue plasminogen activator (t-PA) concentration in PTFE grafts (open circles) and vein grafts (closed circles) which developed stenosis and those that did not. The bars show median values.
7.9 Discussion

The preceding studies have compared plasma activity of coagulation and fibrinolytic factors in patients with infrainguinal bypass grafts which developed stenosis with patients whose grafts remained stenosis free. Non hyperplastic causes of graft stenosis have been excluded by intraoperative and perioperative graft assessment and intimal hyperplasia proven on histology whenever possible (see chapter 5).

Results show that elevated levels of plasma fibrinogen are associated with the development of hyperplastic graft stenoses, independent of the type of graft material used or whether grafts were studied prospectively or retrospectively. There were no differences in plasma fibrinolytic activity or the activity of other coagulation factors between stenosed and non stenosed grafts, although a trend towards elevated plasminogen activator inhibitor (PAI) in non stenosed autologous vein and PTFE grafts was seen.

These findings support data from groups who have studied infrainguinal graft patency (as graft stenosis is believed to be the commonest cause of medium term graft failure; Brewster et al 1983); Wiseman et al, demonstrated reduced 1 year patency of both vein and PTFE infrainguinal grafts in association with hyperfibrinogenaemia in the British Multicentre Femoro-Popliteal Trial (Wiseman et al 1989 and 1990).

Epidemiological data in human atherosclerosis also suggests that raised fibrinogen may be associated with intimal hyperplasia; it is now understood that migration and proliferation of smooth muscle cells is the first abnormality to develop in atherosclerosis (Ross & Glomset 1976) and in long term prospective studies of coronary atherosclerosis, high levels of plasma fibrinogen at recruitment have correlated well with subsequent disease development (Meade et al 1986, Wilkinson et al 1984). Furthermore, fibrinogen monomers have been demonstrated in fatty streak lesions (Stastny & Fosslien 1992) and mature atheromatous plaques in man (Bini et al 1989).
The mechanisms by which fibrinogen and intimal hyperplasia may be linked remain unclear however. There are a number of theories; Bini (1989) has proposed that the presence of fibrinogen molecules in proliferative lesions in arteries may simply reflect the local increase in vessel wall permeability to macromolecules implied in Ross' response to injury hypothesis (Ross & Glomset 1976 – see chapter 1). In Bini's interpretation, following endothelial injury, additional large molecular weight molecules (such as fibrinogen) may enter the subintimal space in the same way as platelet release substances and other mitogenic agents (Ross & Glomset 1976). Bini's theory therefore implies that fibrinogen is not causally associated with localised development of intimal hyperplasia and does not explain the currently demonstrated association between elevated circulating levels of fibrinogen and the development of stenosis.

There are two mechanisms by which circulating fibrinogen may promote intimal hyperplasia; coagulation factors may act as direct smooth muscle mitogens or chemoattractants - activated factor X (Gasic et al 1992) and the fibrinogen cleavage product fibrinopeptide B (Singh et al 1990) have been shown to stimulate smooth muscle proliferation, and fibrinogen itself is chemotactic and haptotactic for smooth muscle cells in culture (Naito et al 1989, Naito et al 1990). At the time of writing the author is unaware of any data demonstrating mitogenic action by fibrinogen on smooth muscle cells, but this work is urgently required and of major vascular biology interest.

Fibrinogen may also stimulate intimal hyperplasia via effects on haemorheology; plasma fibrinogen (along with haematocrit) is a major determinant of whole blood viscosity and therefore of blood flow characteristics; localised abnormalities of wall shear rate (a product of flow velocity and fluid viscosity) have been implicated in the genesis of atherosclerosis (Caro et al 1971, Zarins et al 1983) and intimal hyperplasia at bypass graft anastomoses (White et al 1990). The mechanisms are not understood, but probably include endothelial injury and/or platelet activation, possibly combined in regions of complex flow (see haemorheology and atherosclerosis, chapter nine). This hypothesis is refuted in the current study, however,
as we have also measured whole blood and plasma viscosity in the same cohort of patients and failed to demonstrate any differences between stenosed and non-stenosed grafts (see chapter 9).

It is of interest that in this study and in studies of infrainguinal graft patency (Wiseman et al 1989 and 1990), an increased incidence of smoking was found in association with graft stenosis or thrombosis. Smoking is known to be associated with elevation of plasma fibrinogen (Lowe et al 1980) and thus these findings may be linked (this is not to say that circulating fibrinogen does not play a role in intimal hyperplasia development, as the molecule may be the effector through which smoking produces adverse effects). In the present work, the effect of smoking on fibrinogen and graft stenosis could not be taken into account in a logistic regression model, as the association between smoking and stenosis was so great that very few smokers did not develop graft stenosis (see chapter 9), and results of multivariate analysis using these small numbers would be unreliable.

Taken together the above results, along with the supporting data described in this chapter, suggest that elevation of plasma fibrinogen may be important in the genesis of smooth muscle proliferation within the vascular wall and is associated with the development of infrainguinal graft stenosis.

The tendency towards elevated plasminogen activator inhibitor (PAI) activity in grafts which did not develop stenosis deserves mention. Although these differences were not significant, they were evident in univariate analysis in both vein and PTFE graft groups. PAI is theoretically capable of promoting smooth muscle cell migration; following the earliest phases of proliferation within the media, smooth muscle cells are known to begin to secrete proteases such as collagenases and tissue metallo-proteases, about 4-5 days after endothelial injury. These enzymes digest surrounding matrix and facilitate cell migration (Kenagy et al 1993, Zempo et al 1993). A number of these proteases are known to be activated by plasmin (Kenagy et al 1993) and thus reduction of PAI may allow local plasmin concentrations to rise and thus promote cell migration.
Emerging details of the process of intimal hyperplasia underline the potential importance of interactions between circulating factors and the vessel wall.
Chapter 8

Studies of Platelet Function and
Graft Stenosis

8.1 Introduction

The following studies correlate plasma and intracellular levels of the platelet granule substances 5-hydroxytryptamine (5-HT), β-thromboglobulin (β–TG) and platelet derived growth factor (PDGF) with the development of graft stenosis in infrainguinal bypass grafts. Spontaneous and stimulated in-vitro platelet aggregation has also been measured and correlated with stenosis development.

A review of platelet function, and the current evidence associating platelets with atherogenesis and intimal hyperplasia will be also presented. The close morphologic similarity between intimal hyperplasia and early atheromatous lesions has been described earlier.

8.2 Platelets and Platelet Function

The platelet is a highly evolved, specialised structure, derived from megakaryocytes in the haemopoetic bone marrow. Megakaryocytes are derived from multipotential myeloid stem cells, and the hormone megakaryocyte colony stimulating factor (Meg-CSA) appears to control the cell proliferation of pluripotential cells into megakaryocytes (Gewirtz and Hoffman 1986). Platelet production appears to be controlled by a second hormone, thrombopoietin, which enhances the final maturation of platelets from the megakaryocyte. Mature megakaryocytes are large and polyploidal with abundant cytoplasm, platelets are produced by rearrangement of the cytoplasm into thin strands which subsequently become beaded and fragment, producing the characteristic, non-nucleated platelet (Haller and Radley 1983). Large platelets called megathrombocytes are sometimes seen in the peripheral circulation, particularly during recovery from
immune thrombocytopenia. Whether or not these represent young platelets is controversial (Schrier 1986).

Platelets entering the circulation survive between 8.5 and 10 days (Schrier 1986) and have a half life of about 4 days. Approximately 30-40% of platelets are present in a splenic pool that can freely exchange with the circulation (Hill-Zoebel et al 1986).

The function of platelets is to act as a temporary plug at sites of vessel damage, and to achieve this the cell membrane contains specialised glycoproteins to enable it to bind to de-endothelialised surfaces. Glycoprotein Iβ (GPIb) is one such molecule that binds activated platelets to von Willebrand factor (see coagulation - chapter 7)(George et al 1984). It is believed that von Willebrand factor binds to exposed subendothelium and can act as a bridge to anchor platelets to areas of vessel damage. Such an action is vital if platelets are to resist the tendency of flowing blood to sweep them onward in the circulation. Glycoproteins IIb and IIIa, form a dimer when platelets are activated and the newly formed molecule is a receptor for fibrinogen (Schrier 1986) - another mechanism acting to localise platelets to areas of injury. Thrombospondin is also a glycoprotein, contained in platelet α - granules (Leung and Nachman 1982). Like GP IIb and IIIa, this also binds to fibrinogen and thus acts an endogenous platelet ligand.

After adherence of platelets to an injured area, the cells undergo morphological changes, from their normal disc shape, into a sphere. They also begin to release contained granular substances. There are two important granules; the α - granules, contain β-thromboglobulin, platelet derived growth factor (PDGF), thrombospondin, von Willebrand factor, fibrinogen, fibronectin, platelet factor 4, transforming growth factor - β, albumin and high molecular weight kininogen. The dense granules contain 5-hydroxytryptamine, ADP, pyrophosphate, calcium and ATP (Schrier 1986). Platelets also contain lysosomes in which acid hydrolases are stored (George et al 1984). Both reversible morphological changes and granule release, and irreversible aggregation, with degranulation of α and dense granules are common in patients with
cardiovascular disease and this is believed to be due to vessel wall damage as found in atherosclerosis and diabetes (Heistad et al. 1984, Nevelsteen et al. 1984). These reactions release 5-HT, PF₄, and β-thromboglobulin into the circulation and may raise plasma levels.

The intracellular mechanisms underlying platelet release and aggregation following platelet adherence, appear to be dependant on the formation of cyclic endoperoxides, which can be initiated by phospholipase A₂, an enzyme which converts platelet phospholipids into arachidonic acid (Schrier 1986). Cyclic AMP blocks the production of arachidonic acid (Minkes et al. 1977) and thus inhibits platelet aggregation and release. Elevation of cAMP is the mechanism of anti-platelet action of prostaglandin E₁.

Substances contained in platelet granules and released on activation have numerous potentially important functions in human disease as well as in the promotion of haemostasis and healing. They are also pro-aggregants; stimulated in vitro platelet aggregation occurs in a rapid wave, followed momentarily by a second wave, believed to be due to stimulation by substances released from the platelets themselves (Weiss 1975a & 1975b). PDGF appears to be a chemoattractant and mitogen of smooth muscle cells and fibroblasts and thus may be important in wound maturation and healing. It has also been implicated in a number of vascular diseases (see below). Transforming growth factor-β causes rapid angiogenic and fibrogenic responses important in wound healing and may also control the mitogenic responses of some of the cells involved in healing, to other growth factors. β - Thromboglobulin is a platelet specific marker protein of degranulation but its physiological role is unclear (Zahavi and Kakkar 1980). The role of 5-Hydroxytryptamine (5-HT or serotonin) in human health and disease remains controversial and warrants further discussion.
5-hydroxytryptamine is found in significant quantities in the central nervous system (which contains special 5-HT neurones in various regions), the enterochromaffin cells in the intestine, the gastro-intestinal wall, and in platelets. The concentration of free 5-HT circulating in the blood is very low and probably physiologically irrelevant (Erspamer 1958, Engbaek & Voldby 1982). Receptors for 5-HT have been detected in various tissues, including the central nervous system, gastrointestinal tract, blood vessels (in particular, endothelial cells), platelets, autonomic nerve endings, and the myocardium. The functional relevance in each of these sites is not clear.

Owing to the development of selective agonists and antagonists, the distinction of several subtypes of 5-HT receptors is generally accepted. It is thought that 5-HT itself is a non-selective agonist that can stimulate most subtypes of 5-HT receptors. (Reviewed by Gothert & Schikler 1987). Because of the large number, and opposing nature, of 5-HT receptors the pharmacological effects of the agent are variable but include both vasoconstriction and vasodilatation; these effects have been studied in some detail and are reviewed by Leijsen (1985). Circulating 5-HT which escapes hepatic and endothelial inactivation is avidly taken up by platelets and stored in the dense granules - thus there is a dynamic balance between intraplatelet and plasma 5-HT concentrations. In the platelet, 5-HT achieves a high concentration, and this compartment is believed to be an important storage site of 5-HT in humans. Conversely, platelets can release 5-HT when stimulated by a variety of agents, including 5-HT itself.

8.3 Platelets and Atheroma

Platelets are excellent candidates as initiators of the smooth muscle cell hyperplasia seen in early atheromatous lesions as they are known to contain at least two mitogens for vascular smooth muscle cells - platelet derived growth factor (PDGF) (Ross et al 1974, Ross et al 1986) and epidermal growth factor (EGF) (Oka & Orth
As well as being mitogenic, PGDF is also a chemotactic agent for smooth muscle cells (Grotendorst et al. 1982) and at least in theory, could induce both the smooth muscle cell migration from the media and subsequent subintimal proliferation believed to occur in evolving atheroma. Epidemiological evidence suggests an association between platelet activation and the development of atherosclerosis; Haerem (1972) first reported the presence of platelet aggregates post mortem in patients dying from coronary thrombosis. This was followed by studies showing raised in-vitro platelet aggregation rates (Gormsen et al. 1977, Schwartz et al. 1980) and elevated plasma levels of the platelet release substances β-Thromboglobulin and platelet factor 4 (Zahavi & Kakkar 1980, Levine et al. 1981) in patients with known ischaemic heart disease. Two recent population based prospective studies have also correlated platelet aggregation with myocardial infarction (Elwood et al. 1991) and fatal myocardial events (Thaulow et al. 1991) in large groups of patients. Although these data may demonstrate an association between platelet dependant thrombosis and disease (particularly in the case of Thaulow's study) the cellular biology work on role of PDGF in smooth muscle hyperplasia means that the possibility that platelet activation may be stimulating atheroma formation cannot be ignored. This hypothesis is supported by the beneficial effects of anti-platelet therapy in both the primary prevention of atherosclerosis and reduction of disease progression (ISIS-2 1988, Antiplatelet trialists collaboration 1988).

Experimental data supports a proposed association between platelets and smooth muscle cell proliferation in some models of atherogenesis such as balloon denudationalisation (Stemerman 1972, Clowes et al. 1983a, Walker & Bowyer 1984, Clowes et al. 1986). Furthermore, exclusion or inhibition of platelets in these models can reduce or abolish smooth muscle hyperplasia (Moore et al. 1976, Friedman et al. 1977, Harker et al. 1983). The role of platelets in promoting cellular proliferation after the less severe, non denuding injuries now believed to be important in the genesis of spontaneous atheroma (Ip et al. 1990) is less certain. Fuster et al. (1978) and Fuster & Griggs (1986) have demonstrated a reduction in intimal thickening and atheroma.
formation in hypercholesterolaemic atherogenic pigs with a defect in platelet - vessel wall interaction (Von Willebrand's disease), and rabbits on a high cholesterol diet with drug induced thrombocytopenia have been shown to develop atheroma more slowly than control animals on the same diet (Cohen & McCombs 1968).

Conversely, several hypotheses of spontaneous atherogenesis exist in which platelets do not seem to take an obvious part (Faggiotto & Ross 1984, Aquel et al 1985, Gown et al 1986, Seddon et al 1987). These findings combined with the discovery that many of the cell types other than platelets involved in the formation of atheroma can produce PDGF-like mitogens (particularly endothelial cells {Gajdusek et al 1980} and smooth muscle cells themselves {Manderson et al 1989, Nilson et al 1986}) has led some groups to postulate platelets mainly in the role of atheroma promotion following complications such as plaque splitting or fissuring. This important event would commonly be followed by thrombosis, which if not occlusive and catastrophic, could result in thrombus incorporation into the plaque with release of contained platelet mitogens (Ip et al 1990, Ross 1986) which would then act as a potent stimulant to atheroma progression. This idea is a modern interpretation of the original 'incrustation' hypothesis supported by von Rokitansky over 100 years ago (1852).

8.4 Platelets and Intimal Hyperplasia

It is a measure of the overlap between intimal hyperplasia and atherogenesis that some of the work associating platelets with intimal hyperplasia (such as balloon de-endothelialisation models) have already been described above. Additional studies will be described here.

Animal Studies

Direct evidence implicating platelets in infrainguinal graft stenosis development in animals has recently been provided by Yukizane et al (1991), who demonstrated
adherence of radiolabelled platelets to femoral artery vein grafts during the first week after implantation (the period of endothelial loss) in a canine model and showed a direct correlation between counts of platelet adherence and the degree of intimal hyperplasia on histological section of the grafts at sacrifice. This study excluded radioactivity counts from surrounding tissues- a criticism of similar earlier work, by use of a lead shield around the vein graft. In prosthetic grafts, Hagen et al (1982) demonstrated a reduction in the degree of distal anastomotic narrowing in small calibre PTFE aortic grafts in monkeys when aspirin and dipyridamole were used. Electron microscopy was used in this study to confirm that hyperplastic smooth muscle cells were the cause of narrowing in both groups. Fuster et al (1979) demonstrated similar platelet adherence to graft endothelium and subsequent intimal thickening in coronary vein grafts in an animal model very similar to that described above in lower limb grafts. However, this study also considered the effects of antiplatelet drug therapy and showed that although numbers of adherent platelets were reduced by treatment, platelet-vessel wall interaction was not abolished. This data may be of value when considering studies associating antiplatelet agents and graft patency, however altered graft patency in antiplatelet treatment trials may not mean that platelets are associated with the development of graft stenosis.

Human Work

There are obvious difficulties investigating intimal hyperplasia in humans; to this end some available data is indirect and depends on an assumption that bypass graft patency is reduced by graft stenosis development, and that most graft stenoses are secondary to intimal hyperplasia. Whilst there is data to support these assumptions, data relying on these variable steps is not without flaws and must be interpreted with this in mind;

Gavaghan et al (1990) has demonstrated significantly elevated preoperative serum levels of the platelet specific a granule component β-thromboglobulin in patients whose aortocoronary grafts occluded in the first postoperative year. Although
no attempt to identify graft stenosis was undertaken in this study. Preoperative antplatelet therapy (low dose aspirin) was shown to reduce β-thromboglobulin levels and was associated with improved 1 year graft patency. Taking β-thromboglobulin levels as a marker of platelet activation (Zahavi & Kakkar 1980) these preoperative findings do little to clarify the role of platelets in graft stenosis development and can be used to support the contention of Fuster and colleagues or the hypothesis that platelet/graft interactions are responsible for graft stenosis.

The results of trials of antiplatelet drugs after autologous vein aortocoronary bypass seem easier to understand. Several large, prospective studies have demonstrated the beneficial effects of antiplatelet drugs (commenced prior to grafting and continued afterwards usually for at least 1 year) on coronary graft patency (Mayer et al 1981, Chesebro et al 1982, Fuster and Chesebro 1986, Verstraete et al 1986).

Interpretation of these results as supporting evidence of the association between platelet function and intimal hyperplasia demands that the post operative changes occurring in coronary grafts are similar to those known to occur in infrainguinal vein grafts. The case for this is not proven due to the obvious difficulties encountered in visualising grafts placed in the thorax. In addition, studies which have attempted limited stenosis detection programmes and have correlated stenosis development and antiplatelet agents in aorto-coronary bypass grafts have failed to show any benefit; in a prospective, double blind, randomised trial of antiplatelet therapy versus placebo in over 400 patients undergoing aortocoronary vein grafting in whom graft stenosis was assessed by biplanar angiography at 1 year, Fuster & Chesebro (1986) concluded that antiplatelet agents do not prevent intimal hyperplasia but improve patency probably by exerting an anti thrombotic effect.

It has also recently been demonstrated that the beneficial effects of antiplatelet agents on aortocoronary graft patency is almost identical to the effects of anticoagulation with coumarins (Pfisterer et al 1989). Although this statement may appear to support Fuster's claims that platelets cause thrombosis rather than graft
stenosis (above) it must be remembered that coumarin agents reduce fibrinogen levels and this factor may be important in graft stenosis development (see chapter 8).

Controlled trials of graft patency using antiplatelet agents may be expected to provide circumstantial evidence to associate platelet activity and graft stenosis if it is accepted that the majority of graft failures occur secondary to stenosis. There are surprisingly few such studies in infrainguinal grafts but in the British Multicentre Femoropopliteal Trial (McCollum et al 1991) prospective follow up of a large number of vein grafts randomised to antiplatelet drugs (aspirin and dipyridamole) or placebo failed to show any benefit from treatment at 3 years despite initially promising results. The authors suggested that poor compliance in the treatment group and the inadvertent use of aspirin preparations by the placebo group may have been responsible for the lack of demonstrable benefit from treatment, however somewhat confusingly they then go on to show that coronary mortality was significantly reduced in the treated group. This makes interpretation of their suggestions difficult.

As with vein grafts, indirect evidence of an association between platelets and stenosis of prosthetic infrainguinal grafts can be inferred from studies of graft patency in the presence of antiplatelet agents. The British Multicentre Femoropopliteal Trial studied the effects of antiplatelet agents on prosthetic graft patency. Although all patients with prosthetic grafts were prescribed antiplatelet drugs measurement of serum salicylate levels and pill counts allowed division of participants into good or poor compliers. Analysis showed significantly improved graft patency in good compliers compared with poor compliers (Edwards and McCollum 1991). This data supports the results of several studies showing similar benefits (Green et al 1982, Clyne et al 1987). Furthermore, Goldman et al (1983) demonstrated that the rate of accumulation of radiolabelled platelets onto prosthetic human grafts in the first week after grafting was a sensitive indicator of subsequent graft thrombosis. The potential mechanisms of platelet mediated thrombosis of prosthetic grafts may be more complex than those involved in vein graft stenosis and appear to be two fold; (i) by
immunocytochemistry, platelets can be demonstrated in layered non occlusive (stenosing), organised thrombus occurring in the non endothelialised central portion of PTFE and Dacron grafts of varying age in the femoropopliteal segment (Walton et al 1986); (ii) platelets may play a central role in the initiation and progression of the smooth muscle cell hyperplasia seen at the distal arterial anastomosis of prosthetic grafts (Hagen et al 1982, Sottiurai 1990). The potential of both such lesions and thus of platelets for precipitation of graft thrombosis is obvious. The cytological and extracellular matrix characteristics of intimal hyperplasia at the distal anastomosis of PTFE grafts is identical to intimal hyperplasia of autologous vein grafts (Sottiurai 1990).

Studies of Platelet Function and Graft Stenosis

8.5 Patients

Patient recruitment methods have been described in chapter 4.

Plasma and intraplatelet levels of 5-HT, b-TG and PDGF, and in-vitro aggregation studies were measured in 48 patients undergoing femoropopliteal or femorocrural bypass grafting, in prospective (n=25) and retrospective (n=23) groups. Plasma samples for platelet function measurements in the prospective group were taken preoperatively, at the time of clinic attendance or admission to hospital, and patients were followed for the development of graft stenosis. In the retrospective group, samples were taken from patients known to have developed graft stenosis within the first post operative year (retrospective stenosed group) and on a similar group of patients who had previously undergone reconstruction and in whom graft surveillance has shown no abnormality for a minimum of 1 years post operation (retrospective non stenosed group) - see chapter 4.
Platelet function studies were undertaken at the Royal Free Hospital and patients (rather than blood samples) were taken to the laboratory for immediate processing, to ensure accuracy of results. As not all patients were available for transfer prior to operation or because they lived a prohibitive distance from either hospital, it was only possible to measure platelet function on a limited number of patients; thirty-four of fifty-two enrolled patients with vein grafts had platelet function measured (60%) and twelve of twenty-nine patients with PTFE grafts had platelet measurements (41%). These omissions increase the possibility of sample error and bias, however it must be emphasised that patient availability was entirely random and not subject to control or selection by the author or anyone involved in sampling other than the patient himself.

Table P1 shows total numbers of vein and PTFE grafts studied along with the number of stenosed grafts in each group. Numbers of each type of graft followed prospectively and retrospectively are shown in table P2.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>No Stenosis</td>
<td>20</td>
<td>8</td>
</tr>
</tbody>
</table>

Table P1: Total numbers of vein and prosthetic grafts in platelet function studies. Also shown are numbers with and without stenosis.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospective Study</td>
<td>18 (10)</td>
<td>7 (4)</td>
</tr>
<tr>
<td>Retrospective Study</td>
<td>16 (4)</td>
<td>7 (2)</td>
</tr>
</tbody>
</table>

Table P2: Numbers of patients included in prospective and retrospective platelet function studies. Bold figures are totals, figures in brackets indicate numbers with stenosis.
Grafts which thrombosed and those not available for stenosis surveillance were excluded from the study to avoid difficulty in data interpretation.

Aspirin Usage

It is policy on this vascular unit to prescribe low dose aspirin (25-50mg daily) to patients with femoro-crural grafts or prosthetic femoro - below knee popliteal grafts, unless there are major contraindications. Proximal vein grafts and femoro - above knee popliteal PTFE grafts are routinely prescribed antiplatelet agents.

Aspirin use was similar amongst patients with vein grafts who developed stenosis (9/14, 64%) and those who did not (21/35, 60%). Amongst patients with PTFE grafts, aspirin use was universal amongst patients whose grafts developed stenosis (6/6), whilst non of the patients with non stenosed grafts were taking aspirin (0/8).

8.6 Methods

Details of stenosis surveillance techniques and the intraoperative and postoperative doppler studies used to detect graft stenosis have been described in chapter 5.

Platelet Function Tests

Sample Collection and processing

Blood was taken from an antecubital vein with minimal stasis. For aggregation studies, nine parts of blood were added to one part of 3.8% w/v trisodium citrate (BDH Poole Dorset U.K.). For platelet release substances, citrated blood containing indomethacin (20 mg/ml), theophylline (3mmol/L) and adenosine (1mmol/L) was prepared.

Plasma was prepared by centrifuging blood at for 20 mins at 1500xg at 4° C. The supernatant was collected and spun again as above and kept frozen at -40° C.

Platelet rich plasma (PRP) was prepared using the technique described by Hardisty et al (1970). Nine volumes of venous blood were collected in a polystyrene syringe and mixed with one part 3.8% trisodium citrate in a siliconed glass tube. The packed cell
volume of the citrated sample was determined and PRP was prepared by centrifuging blood at 250 g for 10 minutes. Plasma was handled and kept in silicone glassware at room temperature (18-22° C) until tested.

Platelet counts were performed using a Coulter T-890 blood counter (Coulter Electronics, Luton U.K.) . Following counting, the PRP was centrifuged at 1000xg for 10 minutes to prepare platelet pellets. The pellets were washed with isoton II (Coulter) and stored at -40° C until analysis.

Plasma and platelet 5-HT and β-thromboglobulin assay

Platelet pellets were lysed as follows; platelet pellets were resuspended in physiological saline (0.9% w/v) and a platelets lysed using the MSE-Soniprep sonicator (MSE Sussex UK). In order to check that the procedure fully disrupted cells, platelet size and population numbers were counted before and after sonification in a healthy group of volunteers. The above parameters were assessed using a Coulter ZM counter with a channelizer C-100 and X-Y recorder (Coulter electronics Limited). Following sonification platelet count was reduced to >5% of original, mean platelet volume was immeasurable and a graphical plot of population was indistinguishable from that of a platelet diluent (Isoton II).

The 5-HT content in platelet lysates was assayed using a modification of the spectrofluorimetric method of Drummond and Gordon (Drummond & Gordon 1974, Dangelmmeier & Holmeson 1983). Briefly, 6M trichloroacetic acid (BDH, Poole Dorset UK) was added to the platelet lysates to precipitate the proteins. The samples were then centrifuged at 10,000 g for 4 minutes. A portion of each supernatant was removed and transferred to glass test tubes and ophthaldialdehyde (Sigma Chemicals Co. Poole Dorset UK) in HCl was added and the mixture heated to in boiling water for 10 minutes. The tubes were then cooled in ice and washed twice with chloroform 'Analar' (BDH) to remove any traces of trichloroacetic acid. The aqueous phase was removed and the fluorescence read in a Perkin-Elmer MPF-3 fluorescence spectrophotometer (Hitachi Ltd. Tokyo Japan), with excitation and emission
wavelengths of 360 nm and 475 nm respectively. Standards and blanks were processed in the same way as the platelet lysates. The intra-assay coefficient of variation for the whole procedure was 4.0% (n=20) and the interassay coefficient of variance for the whole procedure was 12% (n=8).

Plasma serotonin concentrations were estimated using a radioimmunoassay. Antisera, standards and reagents were purchased from Immunodiagnostics Ltd (Washington Tyne & Wear UK). The intra assay coefficient of variance for this method was 3.1% (n=10) and the interassay variance was 5.1% (n=10).

Whole Blood Platelet Aggregation
Whole blood platelet aggregation in citrated blood was assessed by counting free platelets using a Coulter T-890 whole blood counter. Prior to the addition of agonists to induce aggregation, blood was pre-incubated for 1 minute with a Teflon coated magnet spinning at 1000 rpm at 37°C, in a plastic cuvette placed in a chronolog aggregometer (Chronolog model 540, Labmedics Stockport U.K.). After addition of agonist, samples (150 µl) were withdrawn from the cuvettes using a pipette with a plastic tip. The timing of the samples and the agonists used are shown in table P3. Platelet aggregation was calculated on the basis of the number of free platelets and expressed as a percentage of the basal platelet count.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Time after commencing to first sampling</th>
<th>Time after commencing to second sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous platelet</td>
<td>6 mins</td>
<td>15 mins</td>
</tr>
<tr>
<td>aggregation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>1 min</td>
<td>3 mins</td>
</tr>
<tr>
<td>Adrenalin</td>
<td>1 min</td>
<td>3 mins</td>
</tr>
<tr>
<td>Serotonin</td>
<td>30 secs</td>
<td>1 min</td>
</tr>
<tr>
<td>Bovine lung Heparin</td>
<td>1 min</td>
<td>3 mins</td>
</tr>
</tbody>
</table>

Table P3: The timing of platelet aggregation measurements and agonists used.
8.7 Results

**Plasma 5-HT and Graft Stenosis**

The concentration of plasma 5-HT was significantly higher in vein grafts which developed stenosis compared with those that did not (median concentrations, 14.1 nmol/L and 4.11 nmol/L respectively, \( p < 0.01 \) Mann Whitney U Test Figure P1).

\[ p < 0.01 \text{ (Mann Whitney U Test)} \]

![Graph showing plasma 5-HT concentration in vein grafts](image.png)

Figure P1: plasma 5-HT concentration in vein grafts which developed stenosis and those that did not. The bar shows median values.

95% confidence interval of difference - 27.19 to - 6.44
Plasma levels of 5-HT were non significantly elevated in PTFE grafts which developed stenosis (Median concentrations 11.76 nmol/L v 5.5 nmol/L, p=NS, figure P2).

Figure P2: plasma 5-HT concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median values.
Logistic regression was used to estimate the relationship between stenosis and 5-HT after allowing for the confounding effects of study group (prospective and retrospective) and graft material; results are shown in table P4 as analysis of maximum-likelihood estimates.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>χ²</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 5-HT</td>
<td>-1.8229</td>
<td>0.7708</td>
<td>5.59</td>
<td>0.018</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>-0.6911</td>
<td>0.700</td>
<td>0.97</td>
<td>0.32</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>0.099</td>
<td>0.731</td>
<td>0.02</td>
<td>0.89</td>
</tr>
</tbody>
</table>

This data shows that there are significant differences in plasma 5-HT concentration between patients whose grafts develop stenosis and those who do not and that this relationship is independent of prospective and retrospective study groups and the type of graft material used.

The results of logistic regression fitting all variables associated with graft stenosis into the same model are described at the end of chapter nine.
Plasma β-TG, Intraplatelet Releasates and Graft Stenosis

Plasma levels of β-thromboglobulin did not vary between vein grafts which developed stenosis and those that did not (median concentrations 50.5 nmol/L vs 51.5 nmol/L, p=NS Mann Whitney U test, figure P3), and there were no significant differences between stenosed and non-stenosed vein grafts in intraplatelet 5-HT (median intraplatelet concentrations 2.0 and 2.06 nmol/10^9 platelets respectively, p=NS, figure P4), intraplatelet β-TG (median intraplatelet concentrations 52.1 and 46.9 μg/10^9 platelets respectively, p=NS, figure P5) or intraplatelet PDGF (median intraplatelet concentrations, 30.6 and 28.9 ng/10^9 platelets respectively, p=NS, figure P6).
p=NS (Mann Whitney U Test)

Figure P3: plasma $\beta$-thromboglobulin concentration in vein grafts which developed stenosis and those that did not. The bar shows median value.

Stenosed Grafts  Non-stenosed Grafts

n=14  n=18
Figure P4: intraplatelet 5-HT concentration in vein grafts which developed stenosis and those that did not. The bar shows median value.
p=NS (Mann Whitney U Test)

Figure F5: intraplatelet β-thromboglobulin concentration in vein grafts which developed stenosis and those that did not. The bar shows median values.
Figure P6: intraplatelet PDGF concentration in vein grafts which developed stenosis and those that did not. The bar shows median values.

p=NS (Mann Whitney U Test)
In patients with PTFE grafts which developed stenosis, significantly increased levels of plasma β-thromboglobulin (median plasma concentrations 65.6 nmol/L and 44.2 nmol/L, \( p=0.008 \) Mann Whitney U test, figure P7) and significantly reduced levels of intraplatelet PDGF (median intraplatelet concentrations, 26.2 ng/10^9 platelets v 36.2 ng/10^9 platelets, \( p=0.02 \) Mann Whitney U test, figure P8) were found, compared with patients who did not develop stenosis. Intraplatelet concentrations of 5-HT and β-TG were not significantly different between stenosed and non stenosed PTFE grafts (intraplatelet 5-HT; 1.85 v 1.85 mmol/10^9 platelets, \( p=NS \), intraplatelet β-TG 42.9 v 53.1 μg/10^9 platelets, \( p=NS \)).

There were no detectable plasma levels of PDGF in stenosed or non stenosed vein or PTFE grafts.

\[ p=0.008 \text{ (Mann Whitney U Test)} \]

![Figure P7: plasma β-thromboglobulin concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median value.](image)

95% confidence interval of difference -45.1 to -9.9
Figure P8: intraplatelet PDGF concentration in PTFE grafts which developed stenosis and those that did not. The bar shows median values.

95% confidence interval of difference: 2.44 to 32.02
Logistic regression was used to estimate the relationship between stenosis and β-thromboglobulin, and stenosis and intraplatelet PGDF, after allowing for the confounding effects of study group (prospective and retrospective) and graft material; results for β-TG are shown in table P5, and for intraplatelet PGDF in table P6, as analysis of maximum-likelihood estimates.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>χ²</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma β-TG</td>
<td>-0.2062</td>
<td>0.236</td>
<td>0.76</td>
<td>0.382</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>-1.306</td>
<td>0.652</td>
<td>4.01</td>
<td>0.045</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>-0.066</td>
<td>0.720</td>
<td>0.01</td>
<td>0.926</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>χ²</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraplatelet PDGF</td>
<td>0.0309</td>
<td>0.038</td>
<td>0.89</td>
<td>0.346</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>-1.02</td>
<td>0.658</td>
<td>2.81</td>
<td>0.094</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>-0.597</td>
<td>0.736</td>
<td>0.66</td>
<td>0.417</td>
</tr>
</tbody>
</table>

These results show that differences in plasma β-TG and intraplatelet PGDF between stenosed and non-stenosed graft groups are not maintained when the effects of graft material and study group are taken into account. These two variables are not independently associated with stenosis.
In-vitro Platelet Aggregation and Graft Stenosis

There were some differences in in-vitro platelet aggregation between stenosed and non-stenosed vein grafts, after 3 minutes incubation with adrenalin, median aggregation in combined groups was higher in platelets of patients whose grafts developed stenosis (19% free platelets remaining compared with 38% of platelets from non stenosed patients, p=0.01 Mann Whitney U test, figure P9).

There were no demonstrable differences in in-vitro platelet aggregation when ADP (figure P10), bovine lung heparin (BLH - figure P11) or 5-HT (figure P12) were used as agonists or in spontaneous platelet aggregation (SPA) (figure P13).
p=0.01 at 3 minutes (Mann Whitney U Test)

Figure P9: adrenalin stimulated platelet aggregation (% unagreggated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed vein grafts. The bar shows median values.

95% confidence interval of difference 4.2 to 33.7
Figure P10: ADP Stimulated Platelet Aggregation (% unaggregated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed vein grafts. The bar shows median values.

p=NS both groups (Mann Whitney U Test)
Figure P11: Bovine lung heparin stimulated platelet aggregation (% unaggregated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed vein grafts. The bar shows median values.
p=NS Both Groups (Mann Whitney U Test)

Figure P12: 5-HT stimulated platelet aggregation (% unaggregated platelets) at 0.5 (open circles) & 1 (closed circles) minutes in stenosed and non-stenosed vein grafts. The bar shows median values.
Figure P13: spontaneous platelet aggregation (% unaggregated platelets) at 6 (open circles) & 15 (closed circles) minutes in stenosed and non-stenosed vein grafts. The bar shows median values.
There were aggregation differences in platelets of patients with PTFE grafts which developed stenosis compared to those who did not. Significantly higher aggregation in response to adrenalin stimulation was observed at 3 minutes, compared to those patients who did not develop stenosis (3 minutes stimulation with adrenalin, median free platelets. 18% in stenosed grafts v 59% in non stenosed, p=0.05 Mann Whitney, figure P14).

Figure P14: adrenalin stimulated platelet aggregation (% unaggregated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed PTFE grafts. The bar shows median values.

95% confidence interval of difference 11.8 to 67.7
Platelet aggregation in response to 5-HT, BLH and ADP stimulation, as well as spontaneous aggregation are compared in figures P15-18. No significant differences in in-vitro aggregation were demonstrable between stenosed and non stenosed grafts.

**Figure P15:** 5-HT stimulated platelet aggregation (% unaggregated platelets) at 0.5 (open circles) & 1 (closed circles) minutes in stenosed and non-stenosed PTFE grafts. The bar shows median values.
Figure P16: Bovine lung heparin stimulated platelet aggregation (% unaggregated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed PTFE grafts. The bar shows median values.

p=NS Both Groups (Mann Whitney U Test)
Figure P17: spontaneous platelet aggregation (% unaggregated platelets) at 6 (open circles) & 15 (closed circles) minutes in stenosed and non-stenosed PTFE grafts. The bar shows median values.
Figure P18: ADP Stimulated Platelet Aggregation (% unaggregated platelets) at 1 (open circles) & 3 (closed circles) minutes in stenosed and non-stenosed PTFE grafts. The bar shows median values.

\( p = \text{NS Both Groups (Mann Whitney U Test)} \)
Logistic regression was used to estimate the relationship between stenosis and adrenalin stimulated in-vitro aggregation after allowing for the confounding effects of study group (prospective and retrospective) and graft material; results are shown in table P7 as analysis of maximum-likelihood estimates.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenalin aggregation</td>
<td>-0.2062</td>
<td>0.236</td>
<td>0.76</td>
<td>0.382</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>1.306</td>
<td>0.652</td>
<td>4.01</td>
<td>0.045</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>-0.066</td>
<td>0.720</td>
<td>0.01</td>
<td>0.926</td>
</tr>
</tbody>
</table>

These results show that differences in adrenalin stimulated in-vitro platelet aggregation between stenosed and non stenosed grafts seen on univariate analysis groups are not maintained when the effects of graft material and study group are taken into account. This variable is not independently associated with stenosis.
8.8 Discussion

In this chapter a number of indices associated with platelet activation have been measured in patients undergoing infrainguinal bypass grafting and results have been compared between the group who developed stenosis and those whose grafts remained stenosis free. Logistic regression shows that elevation of plasma 5-HT is associated with the development of graft stenoses and this relationship is independent of confounding variables such as graft material or study group.

On univariate testing, elevated plasma β-thromboglobulin and reduced intraplatelet PDGF were associated with stenosis in PTFE grafts. Increased in-vitro aggregation to adrenalin stimulation was associated with stenosis in both autologous vein and PTFE grafts. The significance of these relationships, however, was lost when the effects of prospective / retrospective study groups and graft material were taken into account in logistic regression. It is therefore possible that observed differences in these variables on univariate analysis may be produced by differences between prospective and retrospective graft groups.

There were no significant differences between stenosed and non stenosed grafts in intraplatelet levels of 5-HT or β-thromboglobulin, or in-vitro platelet aggregation in response to other agonists.

As non hyperplastic causes of graft stenosis have been excluded in these studies by perioperative graft assessment, these results suggest an association between raised plasma 5-HT and intimal hyperplasia in arterial bypass grafts.

Five-hydroxytryptamine is mitogenic to vascular smooth muscle cells in culture (Nemecek et al 1986) and therefore may promote intimal thickening in-vivo if medial smooth muscle cells are exposed to elevated circulating levels. Platelets are the principle source of free plasma 5-HT. Over 95% of whole blood 5-HT content is stored in the dense granules (see platelet function above) and this may be liberated, along with β-thromboglobulin and PDGF, when platelets undergo aggregation or pre-aggregation.
release reactions (see platelet function above). Recent studies with results which may be relevant to the current findings, have shown that 5-HT may be liberated very early in platelet activation, in a selective reaction which may not involve release of other granule substances. This could produce isolated elevation of plasma 5-HT, without raised levels of other releasates, in association with platelet activation.

Therefore, this data may also provide evidence of platelet activation in association with stenosis development. However, platelets act only as storage organs, and 5-HT is synthesised by a number of different cells such as argentaffin cells in the gut and neurones. Plasma levels are therefore subject to a number of influences. In addition, there are conflicting results within the study; if measured plasma 5-HT was platelet derived, it might reasonably be expected that elevated plasma levels would be complemented by a reduction in intraplatelet 5-HT. This was not seen. The univariate association between PTFE graft stenosis and other granule substances appears to be a consequence of confounding variables (see above), and finally, there were no significant differences in the majority of in-vitro indices of platelet activation between stenosed and non stenosed grafts. The inability to demonstrate circulating levels of PGDF in either stenosed or non stenosed grafts may be expected as the molecule has a short half life in plasma \([t_{1/2} >2\text{mins}]\) (Ross 1986).

However, there is reason to believe that platelet activation and intimal hyperplasia/graf stenosis may be associated; adherence of radiolabelled platelets to arterial bypass grafts has been correlated with the subsequent degree of intimal thickening in animals (Fuster et al 1979, Yukizane et al 1991) and with graft failure in man (Goldman et al 1983). Antiplatelet drug therapy has been shown to reduce intimal hyperplasia at arterial/prosthetic graft anastomoses (Hagen et al 1982).

Despite this theoretical association, it cannot be concluded from the above results that graft stenosis is associated with platelet activation, and further studies are necessary, perhaps utilising additional platelet specific plasma measures, such as
platelet factor 4, if this question is to resolved (see conclusions and suggestions for further study).
Chapter 9

Studies of Haemorheology and Graft Stenosis

9.1 Introduction

The following studies correlate the development of graft stenosis with haematocrit, whole blood viscosity and plasma viscosity.

A review of haemorheology and the determinants of viscosity will also be presented, along with the evidence associating abnormalities of blood viscosity and haemorheology with atherogenesis and intimal hyperplasia. The close morphologic similarity between intimal hyperplasia and early atheromatous lesions has been described earlier.

9.2 Principles

Haemorheology is the study of the flow properties of blood and the deformation of its components. Fluid flowing in a straight, cylindrical tube at a steady rate exhibits laminar flow if the Reynolds number is not exceeded. Shearing is the action of theoretical cylinders of such a fluid sliding over each and the vessel wall, other to produce velocity differentials. Laminar flow produces a parabolic velocity profile; the lowest velocities (and highest shear stress) lies in the outer layers near to the vessel wall (Koenig & Ernst 1992). The axial (central) layer flows at the maximum velocity with the lowest shear stresses. Resistance to shearing and flow comes from friction between adjacent fluid layers - a product of the viscosity of the fluid. Shear stress (τ) is the force per unit area required to produce movement in one layer of the fluid relative to adjacent layers - also a product of the fluid velocity. Shear rate (γ) is the velocity gradient between two adjacent fluid layers. Viscosity can be calculated from the ratio of shear stress to shear rate. Blood is a non-Newtonian fluid and its viscosity varies
inversely with the shear forces applied, therefore to describe whole blood viscosity, measurements at several shear rates are required. Whole blood viscosity is usually quoted at the extremes of physiological shear (Koenig and Ernst 1992).

9.3 The determinants of blood rheology

The viscosity of blood depends in a large part on the cells contained within, i.e., the concentration of red blood cells, leukocytes and platelets. The effect of platelets or leukocytes on viscosity can be ignored, since they are either relatively small in relation to red cells. A linear increase in haematocrit leads to an exponential rise in blood viscosity.

The viscosity of plasma is mainly determined by the concentration of non-spherical, high molecular weight proteins such as fibrinogen, alpha-2 macroglobulins and immunoglobulins of the M class.

Red cell aggregation is a reversible process caused by plasma macromolecules like fibrinogen and globulins, linking one red blood cell to another. The physiological relevance of this process is complex and not fully understood. In large vessels aggregation favours the migration of red cells to the vessel axis, thereby increasing blood fluidity and oxygen transport. In low flow conditions, however, aggregation most certainly increase blood viscosity resulting in hypoperfusion.

Finally, red blood cell deformability is determined by extrinsic and intrinsic factors; extrinsic factors include a normal excess of surface-area to volume ratio (normally about 40%, due to incomplete filling) and the cell morphology. Intrinsic factors are the mechanical properties of the membrane and the viscosity of the cell content (largely a Newtonian haemoglobin solution). The lipid composition of the double-layer membrane is a major determinant of membrane deformability and hence of blood cell deformability.
Flow of blood therefore is determined by (1) the flow conditions, i.e., geometry of blood vessels, perfusion pressure, endothelial functions; (2) the flow properties of blood, i.e., blood viscosity, haematocrit, plasma viscosity, red cell aggregation and deformability and axial migration.

The prognostic significance of measured abnormalities in haemorheological function is described in the section below

9.4 Haemorheology and Atheroma

The local haemodynamic environment is a logical candidate in the search for the final factor which may bring together the known systemic risk factors for atheroma to produce the sharply localised nature of plaque formation seen clinically. However a suitable mode of study of complex pulsatile arterial flow has proved difficult to develop.

Caro observed the selective development of aortic atheroma at sites of low shear stress and based on this proposed a theory of increased uptake of lipids and lipoproteins in regions of low shear, possibly by retardation of the transport of atherogenic substances such as lipoproteins and platelets away from the vessel wall (Caro et al 1971) or by interference with endothelial metabolic function by reduction of surface turnover of metabolically active molecules (Robertson 1968). This is supported by the work of Zarins et al (1983) who used a glass-dye model to calculate shear stress at the carotid bifurcation and showed that the commonest site for plaque deposition (on the lateral wall opposite the flow divider) is associated with low or near zero shear stress.

However, Caro’s low shear theory is opposed by other work demonstrating significantly increased low density lipoprotein endocytosis by cultured endothelial cells subject to high shear stresses, a device apparently mediated by an increase in the number of LDL binding sites (Sprague et al 1987, Schwartz et al 1989). The recently
demonstrated reduction of endothelial cell membrane fluidity and cellular compliance by high shear- a mechanism possibly responsible for the cell retraction seen on the surface of some early atheromatous lesions (Faggioto & Ross 1984) also detracts from the appeal of the low shear hypothesis.

The contradictory findings in studies associating rheological factors and atheroma, in either human or animal models, has been included in a hypothesis of human carotid bifurcation atherosclerosis which involves activation of platelets by high shear forces, and the subsequent stimulation of smooth muscle cells by the transport of these activated cells to areas of low shear and prolonged vessel wall contact time (Brown et al 1975, Chervu and Moore 1975). This theory is supported by glass-hydrogen bubble flow models of the human carotid bifurcation show complex streaming of high velocity fluid from the centre of the common carotid artery into the flow divider (high shear region) and then around the vessel circumference into the area of flow separation in the carotid sinus (low shear region) - the commonest site of carotid atherosclerosis development (LoGerfo et al 1981, Logerfo et al 1985).

Furthermore, Ip and colleagues (1990) have postulated a mechanism of atheroma progression involving haemodynamic induction of fissures in developing plaques, resulting in the incorporation of non-occluding thrombus into the lesion with subsequent stimulation of myocytes by mitogens contained in the thrombus or in platelets.

If either low or high shear (or both) at the vessel wall is important in the initiation or progression of atherosclerosis, then rheological factors such as blood viscosity may also be important, as shear under given flow circumstances is proportional to viscosity. Dormandy provided indirect evidence of a role for rheological factors in atherogenesis by showing that whole blood viscosity was significantly higher in patients with peripheral vascular disease than aged matched controls at both low and high shear rates and that within the patient group viscosity was higher in those patients with more severe vascular disease (evidenced by the
presence of both intermittent claudication and ischaemic heart disease) than those with intermittent claudication alone (Dormandy et al 1973a). In a prospective study of the effects of hyperviscosity he was also able to demonstrate a correlation between increasing whole blood viscosity and the subsequent deterioration of peripheral vascular disease (Dormandy et al 1973b). This study counteracted critics who suggested that the observed rheological findings in his first study were secondary to the degree of ischaemia. Increases in viscosity during exercise in patients with peripheral vascular disease have also been demonstrated (Ciufetti et al 1989) and these may contribute to exacerbation of ischaemic symptoms during exercise.

9.5 Haemorheology and Intimal Hyperplasia

Almost the whole range of haemodynamic factors have been implicated in the aetiology of intimal hyperplasia, including high and low flow rates, separation of flow, high and low wall shear rates and compliance mismatch (reviewed by Chervu and Moore 1975).

Human Studies

Due to the obvious difficulties of accurate flow measurement in vivo, there are relatively few direct studies of haemorheology and intimal hyperplasia. Some studies have used bench models of human arteries to demonstrate flow; in simulated end to side vascular anastomoses, White et al (1990) have demonstrated areas of low shear stress and prolonged particle residency time in regions which coincide with the sites of maximal intimal hyperplasia (as described by Sottiurai 1990 see figure 3(4). Intimal hyperplasia after coronary angioplasty has been associated with haemorheological factors; poor dilatation of the initial stenosis is known to predispose to accelerated intimal hyperplasia and restenosis (Leimgruber et al 1986) and it has been postulated that this is due to shear abnormalities in the region of the poorly dilated stenosis, resulting in platelet deposition (Badimon et al 1986, Chesebro et al 1987).
Animal Studies

Reduced wall shear forces have been shown to be associated with the development of intimal hyperplasia in several animal models of bypass grafts but results are conflicting: Berger et al (1980) used a carotid interposition vein graft in the dog to show that regions of low flow developed a significantly thickened intimal layer, and postulated that this was due to reduction in shear rate. Similarly, Rittgers et al (1978), also in a dog model, used iliac cross over grafts at different angles to blood flow and were able to demonstrate an inverse association between calculated shear rate and intimal thickness at various points within the grafts. In the baboon, Kohler et al (1991) used endothelialised prosthetic aorto-iliac grafts to show that increased graft flow and shear rate was associated with a reduction in the degree of neointimal hyperplasia.

In contrast to these animal findings however, high wall shear has been shown to be associated with endothelial injury (Fry 1968) and enhanced platelet adherence in a pig model (Badimon et al 1986) - two mechanisms postulated as stimuli for intimal hyperplasia development.

Bypass grafting in 'normal' and 'poor run off' animal models provides indirect evidence relating haemodynamic factors and intimal hyperplasia - differences in the two models are mostly flow and haemodynamics. Several of these studies have shown that reduced flow conditions (mostly produced by the ligation of run off vessels distal to the anastomosis) are associated with an increase in both the rate and extent of intimal hyperplasia development (Morinaga et al 1987, Hehrlein et al 1991, Yukizane et al 1991); Morinaga suggested that intimal hyperplasia was associated with a reduction in wall shear variation during the cardiac cycle, calculated from electromagnetic flow meters in autologous vein grafts in dogs. Yukizane and colleagues were able to demonstrate enhanced platelet adherence to grafts under reduced flow conditions - these may be a source of smooth muscle cell mitogens, see platelets and intimal hyperplasia, chapter 6.
In studies of the factors important in intimal hyperplasia progression, Clowes et al. (1987) have shown that medial hyperplasia of smooth muscle cells following endothelial cell loss in the rat, can be reproduced by hydrostatic distension alone. However, in this model, subsequent migration of smooth muscle cells and extracellular matrix synthesis in the intima does not seem to occur. This observation has led Clowes to conclude that vessel distension may be an important initiating event in intimal hyperplasia, but that its role in the progression of the disease process is unclear.

Studies of Haemorheology and Graft Stenosis

9.6 Patients

Patient recruitment methods have been described in chapter 4. Viscosity measurements were made in 60 patients undergoing femoropopliteal or femorocrural bypass grafting in prospective (n=40) and retrospective (n=20) groups. Viscosity measurements in the prospective group were made preoperatively, at the time of clinic attendance or admission to hospital, and patients were followed for the development of graft stenosis. In the retrospective group, measurements were made on patients known to have developed graft stenosis within the first post operative year (stenosed group) and on a similar group of patients who had previously undergone reconstruction and graft surveillance, without abnormality for a minimum of 1 year post operation (control group).

Forty of fifty two patients with vein grafts enrolled into the study as a whole (77%) and twenty of twenty-nine patients with PTFE grafts (69%), had haemorheology measured. As in preceding chapters, omissions were random; a small proportion of samples were lost after sampling whilst in other cases, patients failed to attend for follow up after having been studied in the platelet function lab or for smoking breath testing etc. These factors were not under the control of the author.
Table R1 shows total numbers of vein and PTFE grafts studied along with the number of stenosed grafts in each group. Numbers of each type of graft followed prospectively and retrospectively are shown in table R2.

<table>
<thead>
<tr>
<th></th>
<th>Vein Grafts</th>
<th>PTFE Grafts</th>
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</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>No Stenosis</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>Total No studied</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

Table R1: Total numbers of vein and prosthetic grafts in rheological studies. Also shown are numbers with and without stenosis.

<table>
<thead>
<tr>
<th></th>
<th>Prospective Study</th>
<th>Retrospective Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein Grafts</td>
<td>24 (11)</td>
<td>16 (3)</td>
</tr>
<tr>
<td>PTFE Grafts</td>
<td>11 (4)</td>
<td>9 (5)</td>
</tr>
</tbody>
</table>

Table R2: Numbers of patients included in prospective and retrospective rheology studies. Bold figures are totals, figures in brackets indicate numbers with stenosis.

Grafts which thrombosed (except one which occluded whilst awaiting correction of an angiographically proven stenosis) and those not available for stenosis surveillance were excluded from the study to avoid difficulty in data interpretation.

9.7 Methods

Causes of graft stenosis other than intimal hyperplasia were excluded by intraoperative graft assessment using doppler and angiography. Histological proof was
obtained when possible. Details of stenosis surveillance techniques and the intraoperative and post operative doppler studies used to exclude fixed graft stenosis have been described in chapter 4.

**Viscosity Studies**

Five millilitres of blood was taken from an antecubital vein using minimal tourniquet time, and stored in lithium heparin tubes. All measurements were performed within 12 hours of the sample being taken. Samples which were not processed immediately were stored at 5°C. All blood was equilibrated in a water bath at 37°C prior to measurement of haematocrit or viscosity.

**Haematocrit Measurement**

After thorough mixing, 2 samples of whole blood were taken into capillary tubes without suction. The ends of each tube were sealed using flame or wax. Samples were spun at 15,000 rpm for 5 mins using a microcentrifuge (Hawksley U.K.). Haematocrit readings were then estimated using a linear scale haematocrit reader. The patient's haematocrit was taken as the mean of the two samples. If wide discrepancy between the two samples was present, further samples were taken and spun.

**Viscosity Measurements**

During viscosity measurements of whole blood and plasma, samples were stored at 37°C in the water bath. Measurements of viscosity were made on a Contraves Low Shear 30 device (figure R.1) connected to the water bath to maintain all surfaces which contact blood at temperature 37°C. Viscosity measurements were made on all specimens at native haematocrit. In those samples with haematocrit outside the range 0.43 - 0.47, whole blood viscosity was also measured after correction to haematocrit of 0.45. Whole blood viscosity at haematocrit 0.45 in those samples with haematocrits of 0.43-0.44 and 0.46-0.47 were calculated using standard tables.
Haematocrit Correction

Correction of haematocrit was undertaken for samples with Hct. >0.47 or <0.43. Heparinised blood was spun at 5000 rpm for 5 minutes using a centrifuge. The amount of plasma to remove or add was calculated as follows:

\[
\text{Total required volume} = \frac{\text{Measured Haematocrit} \times \text{Sample Volume}}{\text{Required Haematocrit}}
\]

The required plasma volume was removed or added using micropipettes. After correction and mixing, haematocrit measurements were again made using the technique described above.

Figure R.1: The Contraves Low Shear 30 device: The bob is suspended in the blood to be measured, which is contained in the cup. This can be rotated at varying speeds to produce different shear rates.
Viscosity Measurement

The Contraves low shear 30 machine measures viscosity using a variable shear system in which a centrally suspended stainless steel bob is suspended in a cup containing the blood to be tested (figures R.1 and R.2). The cup and blood are spun at different speeds thus applying shear forces to the bob whose tangential deflection is measured. The viscosity of the sample in the cup is directly proportional to the tangential deflection of the suspended bob and is calculated at different spin speeds/shear rates using standard tables (see appendix R(i)). Deflection on the contraves device was displayed in numerically (figure R.3) and graphically (figure R.4). Peak deflection was used for all viscosity calculations.

The graphic display allows the operator to undertake a simple from of audit: whole blood viscosity curves follow a characteristic trace as red cells begin to settle.
during measurement (see appendix R (ii)). Readings from curves which did not fit this 'normal' pattern were repeated.

As blood is non-Newtonian, viscosity measurements were carried out at a range of viscosities. We measured viscosity at 8 shear intervals in the range 0.277 in s\(^{-1}\) to 128.5 in s\(^{-1}\). Plotting measured viscosity against a log scale of shear rate within this range produces a characteristic 2 part curve (see appendix R(iii)) as whole blood viscosity at low shear is primarily dependent on plasma proteins whilst at higher rates red cell deformability becomes more important. All measured viscosity samples were plotted against shear in this manner as a second form of audit; any samples in which the viscosity curve was not 'normal' were repeated.

Results are expressed as whole blood viscosity in mPa.S\(^{-1}\) at the minimum and maximum shear rates for both native and corrected (0.45) haematocrit.

**Plasma Viscosity**

After measurement of whole blood viscosity, blood samples were spun again at 2000 rpm for 5 minutes. Plasma was removed from the top of the specimen obtained using a micropipette. For accuracy measurements were made at 4 shear rates between 20.4 in. s\(^{-1}\) and 128.5 in.s\(^{-1}\) (plasma is a Newtonian fluid and thus could be measured at a single shear rate). Results shown are the mean of the four measurements in mPa.S\(^{-1}\).
Figure R.3: Numerical read out of viscosity measurements from Contraves

Figure R.4: Analogue Contraves read out
9.8 Results

Haematocrit

There were no significant differences in haematocrit between patients who developed graft stenosis and those who did not in either vein grafts (median haematocrit 45% both groups, p=NS Mann Whitney U test, figure R.5) or PTFE grafts (medians 42% v 45% respectively, p=NS Mann Whitney U test, figure R.5).

Figure R.5: Haematocrit in stenosed and non stenosed vein (closed circles) and PTFE (open circles) grafts. The bar shows median haematocrit.
Whole Blood Viscosity

Comparisons of whole blood viscosity at native haematocrit (high and low shear rates) in vein grafts which developed stenosis and those that did not are shown in figure R.6. Median viscosity at low shear rate in stenosed vein grafts was 58.2 mPa.S$^{-1}$ compared with 60.3 mPa.S$^{-1}$ in non stenosed grafts (p=NS Mann Whitney U test). At high shear rate, median viscosities were 4.67 mPa.S$^{-1}$ and 5.09 mPa.S$^{-1}$ respectively p=NS Mann Whitney U test.

![Figure R.6: Whole blood viscosity at native haematocrit in vein grafts which developed stenosis and those which did not. Results are shown at low (closed circles) and high (open circles) shear rates.](image)

respectively p=NS Mann Whitney U test.
Whole blood viscosity at native haematocrit in stenosed and nonstenosed PTFE grafts is shown in figure R.7. Median viscosity at low shear was higher in stenosed grafts compared with non-stenosed grafts in combined prospective and retrospective groups but this failed to reach statistical significance (median results, combined group 58.6 mPa.S$^{-1}$ v 47.6 mPa.S$^{-1}$, p=NS Mann Whitney U test). At high shear, median viscosity was 4.60 mPa.S$^{-1}$ in both stenosed and non-stenosed grafts.

![Figure R.7: Whole blood viscosity at native haematocrit in PTFE grafts which developed stenosis and those which did not. Results are shown at low (closed circles) and high (open circles) shear rates.](image-url)
Whole blood viscosity measurements at after correction of haematocrit to 45% are shown in figures R.8 & R.9. In patients with vein grafts, median viscosity at low shear in stenosed grafts was 53.5 mPa.S⁻¹ compared with 60.6 mPa.S⁻¹ in non stenosed grafts (p=NS Mann Whitney U test, figure R.8). At high shear, vein graft median viscosities were 4.53 mPa.S⁻¹ and 5.15 mPa.S⁻¹ respectively, p=NS Mann Whitney U test, figure R.8.

In patients with PTFE grafts, median whole blood viscosity after correction of haematocrit was higher in grafts which developed stenosis than those who did not (medians 57.6 and 45.5 mPa.S⁻¹ respectively at low shear rate, p=NS, figure R.9, and 4.8 v. 4.62 mPa.S⁻¹ at high shear, p=NS, figure R.9.)
Figure R.8: Whole blood viscosity at hematocrit of 45% in vein grafts which developed stenosis and those which did not. Results are shown at low (closed circles) and high (open circles) shear rates.

<table>
<thead>
<tr>
<th></th>
<th>Stenosed Grafts</th>
<th>Non stenosed Grafts</th>
<th>Stenosed Grafts</th>
<th>Non stenosed Grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>13</td>
<td>14</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

p=NS Both Groups
As smoking may have an important influence on viscosity (Lowe et al 1980, Meade et al 1987) we also compared whole blood viscosities in smokers and non smokers. There were no demonstrable differences in whole blood viscosity between smokers and non smokers at native haematocrit (at low shear rate, median viscosities were 58.2 mPa.S\(^{-1}\) and 53.8 mPa.S\(^{-1}\) respectively, at high shear, median viscosities were 5.54 mPa.S\(^{-1}\) and 4.65 mPa.S\(^{-1}\) respectively, p=NS both groups, Mann Whitney U test) or after haematocrit correction to 45% (low shear rate, median viscosities 56.2 mPa.S\(^{-1}\) and 55.1 mPa.S\(^{-1}\) respectively, high shear, median viscosities 4.98 mPa.S\(^{-1}\) and 4.07 mPa.S\(^{-1}\) respectively, p=NS both groups, Mann Whitney U test)
Plasma Viscosity

Plasma viscosity in stenosed and non stenosed vein and PTFE grafts is compared in figures R.10 and R.11. There were no demonstrable differences in plasma viscosity between stenosed and non stenosed grafts. Plasma viscosity in stenosed vein grafts was 1.48 mPa.S⁻¹ compared with 1.56 mPa.S⁻¹ in non stenosed grafts (p=NS Mann Whitney U test, figure R.10). In PTFE grafts plasma viscosity was 1.56 mPa.S⁻¹ in patients whose grafts developed stenosis, and 1.58 mPa.S⁻¹ in patients who did not develop stenosis (p=NS Mann Whitney U test, figure R.11).
Figure R.10: plasma viscosity in vein grafts which developed stenosis and those that did not. The bar shows median values.

p=NS (Mann Whitney U Test)
p=NS (Mann Whitney U Test)

Figure R.11: Plasma viscosity in PTFE grafts which developed stenosis and those that did not. The bar shows median values.
9.9 Discussion

These studies have measured haematocrit, whole blood viscosity and plasma viscosity in patients undergoing infrainguinal bypass grafts and have compared results between those who developed stenosis and patients whose grafts remained stenosis free. Non hyperplastic causes of graft stenosis have been excluded by peri-operative graft assessment and intimal hyperplasia has been proven with histology whenever possible (see chapter 5).

Results show no demonstrable differences in haemorheological parameters between the two groups although a trend toward elevation of whole blood viscosity in association stenosis was seen in PTFE grafts.

These results are perhaps somewhat surprising as association between raised fibrinogen and stenoses has been shown in the same cohort of patients (see chapter 7), and fibrinogen is an important determinant of plasma viscosity. It is conceivable that the tendency toward elevated whole blood viscosity seen in stenosed PTFE grafts reflects fibrinogen differences but such an explanation defies the lack of rheological differences seen in vein grafts. Furthermore, it must be emphasised that no rheological differences reached statistical significance, and any assumptions are speculative.

The theoretical reasons why abnormalities of viscosity may influence the development of intimal hyperplasia and graft stenosis have been described earlier in this chapter; many of the risk factors for vascular disease, identified from epidemiological studies, are associated with abnormal viscosity states (Lowe et al 1982, Lowe 1987, Yarnell et al 1991), however establishing the link between altered haemorheology and smooth muscle cell pathology has proved elusive, and theories remain controversial. Association between abnormalities of flow and intimal hyperplasia are attractive however, as the recognised vascular risk factors act systemically, whereas most vascular disease is localised. Regional shear rate abnormality is an obvious candidate as the missing factor. There is conflict in our understanding of how flow may adversely
affect the vessel wall; if the response to injury theory of atherogenesis is correct, it
would be reasonable to expect high shear (related to high flow and viscosity) to cause
most injury (Ip et al 1990), whereas in-vivo evidence implicates a reduction of the wall
shear rate in the stimulation of smooth muscle cell mitosis (Caro et al 1971, Rittgers et
White et al 1991), and it has been postulated that his may be mediated by an endothelial
'shear detection' mechanism, and effected by endothelial derived growth factors
(Davies et al 1992). For a given driving pressure and vessel diameter, wall shear is
directly proportional to the viscosity of the blood (the Poiseuille equation), thus
abnormalities of shear rate may in part be determined by viscosity.

Although these data suggest that small bore, high flow grafts may be
advantageous in clinical practice, Binns' study (1989) not only showed that intimal
hyperplasia was reduced in small prosthetic grafts, but also that the spontaneous
thrombosis rate was highest in this group!

A more precise mechanism by which low shear may be associated with smooth
muscle cell hyperplasia has been proposed by Caro et al (1971), who suggested that a
prolonged contact time between the vessel wall and mitogenic factors, such as
lipoproteins and activated platelets, may occur secondary to the reduction of physical
transportation in low shear regions, and this hypothesis is included in the modern
theory of atherosclerosis development in the carotid bulb (Zarins 1983, Logerfo et al
1981 & 1985, Brown et al 1975). This theory is able to account for the contrasting
observations of both high shear and low shear dependent stimulation of intimal
hyperplasia, but may not be applicable to all circumstances where intimal hyperplasia is
known to develop.

In a separate prospective study we have recently been able to show increased
whole blood and plasma viscosity in patients' who developed early graft failure after
infrainguinal bypass compared to those with primary graft patency (Cheshire et al
1991), however it is likely that the mechanisms of early failure involve factors other
than intimal hyperplasia (Brewster et al 1983).
These results suggest that if rheological factors do play a role in graft failure after infrainguinal reconstruction, this is not secondary to graft stenosis development or stimulation of intimal hyperplasia. Studies of haemorheology or viscosity modulating drugs in graft patency may be more appropriate in early graft failure rather than the medium term or long term failure associated with intimal hyperplasia.
9.10 Relationships between Variables Associated with Stenosis

Logistic regression analysis has also been used to estimate the relationship between those variables shown to be independently associated with stenosis development. Results are shown as analysis of maximum-likelihood estimates in table R3.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>χ²</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 5-HT</td>
<td>-2.208</td>
<td>0.9812</td>
<td>5.06</td>
<td>0.0244</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.0157</td>
<td>0.0061</td>
<td>6.55</td>
<td>0.0105</td>
</tr>
<tr>
<td>Prospective v Retrospective</td>
<td>1.165</td>
<td>0.8663</td>
<td>1.81</td>
<td>0.179</td>
</tr>
<tr>
<td>Vein v PTFE</td>
<td>-0.8413</td>
<td>0.9753</td>
<td>0.74</td>
<td>0.388</td>
</tr>
</tbody>
</table>

Serum Lp(a) was not significantly associated with stenosis after the effects of other variables were taken into account.

As there is no data to suggest that elevated plasma 5-HT or fibrinogen may cause serum Lp(a) levels to rise, this finding may demonstrate that the predictive power of elevated plasma 5-HT and fibrinogen is greater than that of lipoprotein (a). Inclusion of smoking effects in this model may be particularly interesting because of the recognised associations between smoking and platelet activation (one of the causes of elevated plasma 5-HT) and smoking and plasma fibrinogen. The small proportion of smoking patients who developed stenosis means that this data cannot be included in this final analysis and this is a serious weakness.
10.1 Conclusions

The following conclusions can be drawn from the results described above.

Concerning the post-operative development of graft stenoses in infrainguinal bypass grafts:

- Cigarette smoking
- Elevated plasma fibrinogen
- Elevated serum Lp(a), and
- Elevated plasma 5-HT

are associated with stenosis development independent of graft material or the manner in which grafts are studied.

As pre-existing, non hyperplastic causes of stenosis were excluded (see chapter 5), these results demonstrate an association between systemic risk factors and intimal hyperplasia in human bypass grafts.

There may be some differences in associated risk factors between vein and PTFE grafts.

There is evidence to support an association between circulating risk factors and the development of intimal hyperplasia, this has been described in the relevant results chapters. Most data is derived from animal or in-vitro studies considering isolated risk factors. Few studies have considered risk factors at a single anatomical site as in this study. Very few studies have directly examined risk factors in human
intimal hyperplasia (due to obvious problems), but hyperplastic stenoses are believed to cause most medium term graft failures (Brewster et al 1983) and there is concordance between the present results and risk factors known to be associated with reduced infrainguinal graft patency at 1 year (Wiseman et al 1989 & 1990).

These results suggest that the post-operative development of stenoses in infrainguinal arterial grafts may be influenced by systemic, patient determined variables and are therefore not solely a consequence of intraoperative injury or insult to the graft or its immediate surroundings. This conflicts with the often localised nature of stenosis development but attention is drawn to the localised nature of atherosclerosis, a process also involving proliferation of smooth muscle cells and which appears to be subject to similar risk factors. Systemic, patient related risk factors, perhaps combined with local endothelial compromise (as a consequence of operative injury and/or shear forces determined by local flow characteristics) may result in the pattern of graft stenosis seen in up to 1/3 of arterial grafts in the leg. These results are of interest as this association has not been shown before at this anatomical site despite an explosion of interest in graft stenosis and the role of stenoses in graft failure. The results stress the systemic nature of atherosclerosis and suggest that management of vascular disease in a solely localised manner (eg bypass grafting) may not produce optimal outcome.

10.2 Criticism

A number of flaws in the study design and execution limit interpretation of results and these will be discussed here:

Although grafts were analysed by type (vein or PTFE) when univariate tests were used to establish associated factors, subsequent logistic regression shows factors associated with stenosis in combined vein and PTFE groups. Although use of all available data in this way strengthens the value of the logistic regression results, some differences were observed between vein and PTFE grafts by univariate analysis. The
statistical methods used in these studies, and the numbers of patients included meant that it was not possible to examine these variations further.

It was not possible to fit smoking into the regression model of all variables associated with stenosis. This is a major limitation on interpretation of the final results as differences in variables, such as fibrinogen, between stenosed and non stenosed grafts could be a consequence of differences in smoking between the two groups. The small number of smokers who did not develop stenosis means that the results of multivariate analysis including the effects of smoking would have been worthless.

The division of patients into stenosed and non stenosed groups may be artificial, as it is believed that a degree of intimal hyperplasia occurs in most bypass grafts. It is therefore likely that there is a continuum of graft changes ranging from very minor thickening, through increasing degrees of smooth muscle hyperplasia, to grafts in which the process is most pronounced and impinges on the graft lumen. If this is so, then samples have been compared between groups of grafts at either end of this spectrum of change. This may account for some of the overlap in risk factors seen between stenosed and non stenosed grafts. Interpretation of results should not be affected.

In many of the cases, it has been assumed that intimal hyperplasia is the cause of stenoses detected by surveillance without histological proof. Measures were taken to exclude other common causes of stenosis (described in chapter 5) but it was only possible to obtain histological confirmation in a proportion of cases - when open correction was undertaken. Although non hyperplastic causes of stenosis are unlikely after peri-operative assessment, the nature of the non resected lesions remains presumptive.

Inclusions and exclusions of patients into individual studies has been described in relevant chapters. All patients undergoing femoro-popliteal or femoro-crural
reconstruction during the study period were enrolled, along with a number of adequately documented retrospective patients (see chapter 4). Not all enrolled patients had all investigations performed and reasons for this have been described. Inclusion was determined by individual patients availability and was therefore random.

The inclusion of a retrospective cohort of patients has been described in chapter 4; as only 25-30% of grafts develop stenosis, a prospective study alone would have required a prohibitively long recruitment time to generate a sufficient number of stenosed grafts for analysis. The statistical methods used to account for confounding effects caused by the inclusion of prospective and retrospective groups have also been described in chapter 4.

The number of risk factors measured is comparatively large for the number of patients included and this may lead to accusations of 'fishing' and the potential for random generation of statistically significant differences. Statistical methods have been described in chapter 4; this possibility was minimised by the use of univariate tests only to identify risk factors which may be associated with stenosis and the subsequent inclusion of these into logistic regression analysis using all available data and accounting for the effects of confounding variables.

10.3 Suggestions for Further Study

Identification of risk factors predisposing to the development of intimal hyperplasia is important in infrainguinal graft stenosis and also in the study of atherosclerosis - the commonest cause of death in the western world. Initiating and controlling influences are not fully understood and we are ignorant of why the disease has such a sharply demarcated anatomical distribution and why some individuals appear more prone to its development than others. Identification of components of the flowing blood which
may interact with the vessel wall to stimulate smooth muscle cells, may suggest how
dietary or pharmacological manipulations may reduce intimal hyperplasia. The
current results therefore suggest a number of further studies;

Bypass grafts and platelet function;
The results described above demonstrate differences between some measures of
platelet activation in association with stenosis development, but this data is difficult
to interpret. Furthermore, differences appear to be greatest in PTFE graft but may be
influenced by the inclusion of prospective and retrospective grafts. Continued
activation of platelets by the graft could explain these observations. There is evidence
to show that turnover of radiolabelled platelets on Dacron grafts continues for some
years after implantation (Goldman et al 1982a & 1982b) but it is not clear whether
this is associated with activation of platelets. Measurement of platelet function pre-
operatively and at intervals post-operation (for example 3, 6 and 12 months) would
show whether grafts do continue to activate platelets. Results may aid interpretation
of platelet results seen in this study and may provide a more rational basis for the use
of antiplatelet agents after arterial reconstruction.

Rheology in patients with peripheral vascular disease
The findings of elevated fibrinogen but not whole blood and plasma viscosity in
association with stenosis were surprising. The variation in viscosity measurements in
both stenosed and non-stenosed groups of patients suggests that other variables may
be important in determining rheology in patients who require arterial reconstruction.
Immunoglobulins, acute phase proteins and white cell rheology are minor
determinants of rheology in healthy individuals but may be important in patients with
critical leg ischaemia. Measurement of whole blood viscosity, fibrinogen and these other rheological variables may determine the role of these agents in arteriopathes.

**Effects of manipulation of risk factors:**

It is possible to selectively reduce plasma fibrinogen using pharmacological agents (such as the enzymatic agents ancrod and batroxobin, or the lipid modulating agent fenafibrate). Administration to patients or animal models with arterial grafts may demonstrate the effects of fibrinogen on stenosis development. In patients, fenafibrate may be used to reduce fibrinogen (as ancrod is associated with side effects and has never been administered for longer than seven weeks in humans -Bell 1987). A randomised, blinded trial of this agent in patients undergoing bypass grafting and stenosis surveillance, could compare stenosis incidence between treated and placebo groups. There are some drawbacks as fenafibrate also affects plasma LDL levels and may have intrinsic anti-smooth muscle proliferative effects (Munro unpublished data).

Use of ancrod or batroxobin in an animal model may yield more specific results as these agents reduce fibrinogen in isolation, are effective in smaller mammals such as rabbits and rats (Bell 1987), and the degree of graft intimal thickening can be directly measured at sacrifice (rather than relying on the development of stenoses). Autologous vein and prosthetic bypass grafts, as well as the well established endothelial injury models may be studied in this way.

Plasma Lp(a) is not easily amenable to pharmacological manipulation. Attempts to reduce 5-HT may produce a number confounding effects on platelets. Anti-smoking advice is routinely given to patients after arterial reconstruction but effects are variable. For these reasons, attempts to study the effects on stenosis development of manipulation of these factors (along the lines described for fibrinogen above) are not currently viable.
Smoking effects

It is possible that the observed association between stenosis and fibrinogen is a consequence of the differences in smoking between stenosed and non stenosed grafts. If plasma 5-HT is accepted as a measure of platelet activation, this may also be secondary to smoking.

The effects of smoking in the current study could not be calculated because the strength of the association between continued smoking and stenosis development was so great that too few negative associations were found in a study of this size for further analysis to be possible. A prolonged prospective study similar to that used in this thesis (perhaps also including urinary or plasma cotinine as an additional, objective measure of smoking), including a larger number of patients, may be of value in resolving this question.

Sex and Graft Stenosis

Analysis of clinical risk factors suggested an association between female sex and PTFE graft stenosis. An increased incidence of intimal hyperplasia in the carotid artery following endarterectomy has also been suggested (see chapter 5). A study of stenosis incidence in women with infringuinal grafts, including larger numbers than have been recruited in this study, is potentially of great significance. Graft surveillance as described in this study could be used to detect stenoses.
The in-vitro effects of risk factors on smooth muscle cells in culture

Although epidemiological data associating some risk factors with intimal hyperplasia development is strong, little is known of how (or if) these factors stimulate smooth muscle cells to reproduce and migrate. Smooth muscle cells in-vitro, may be cultured in mixed media known to stimulate mitosis (eg foetal calf serum). The effects of blocking or removing fibrinogen, 5-HT or LDL's Lp(a) (eg by antibody neutralisation) on smooth muscle proliferation could be of value, particularly if combined with proliferation time course studies (such as tritiated thymidine uptake).
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Key to data spreadsheet

| Column A | Group (Gp) | P= Prospective  
| R= Retrospective |
| Column B | Type | v= vein graft  
| p= PTFE graft |
| Column C | Asprin Stat. | A= taking asprin  
| NA= no asprin |
| Column D | Category | s= Stenosis  
| n= No stenosis |
| Column E | Name | pts surname prefix v= vein graft  
| p=PTFE graft |
| Column F | Plasma 5-HT |
| Column G | Plasma beta-TG |
| Column H | SPA 6 | Spontaneous platelet aggregation  
| at 6 minutes |
| Column I | SPA 15 | Spontaneous platelet aggregation  
| at 15 minutes |
| Column J | ADP1 | ADP stimulated aggregation,  
| 1 minutes |
| Column K | ADP2 | ADP stimulated aggregation  
| 2 minutes |
| Column L | BLH1 | Bovine lung heparin stimulated  
| aggregation, 1 minute |
| Column M | BLH3 | Bovine lung heparin stimulated  
| aggregation, 3 minutes |
| Column N | ADR1 | Adrenalin stimulated  
| aggregation, 1 minute |
| Column O | ADR3 | Adrenalin stimulated  
| aggregation, 3 minutes |
| Column Q | GSHT 5 | 5-HT stimulated aggregation, 30  
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