Title

PLASMA TISSUE FACTOR, TISSUE FACTOR PATHWAY INHIBITOR AND RESTENOSIS FOLLOWING FEMOROPOPLITEAL PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY IN CLAUDICANTS

By

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The candidate confirms that the work submitted is his and the appropriate credit has been given where reference has been made to the work of others.

Dedication

I have been very fortunate to have an extremely supportive family. I dedicate this thesis to my Grandmother, and parents, who built the foundations upon which I based my values and judgment. I also dedicate this thesis to my wife Komal and my wonderful daughter Annanya and son Aarav, while I thought I was taking care of them, they were taking care of me. I also dedicate this thesis to my supervisors Prof. P T McCollum, Dr. Camille Ettelaie and Mr. I C Chetter who provided intellectual and personal support through out the course of my study and without whose help this thesis would not have been possible.

Abstract

Introduction: The primary goal in the management of intermittent claudication is to decrease cardiovascular morbidity and mortality by disease modifying therapy. However percutaneous transluminal angioplasty (PTA) is often performed to improve limb related symptoms. Claudicants may have variation in symptomatology and disease progression while awaiting PTA Moreover restenosis following PTA is a major therapeutic problem despite high initial success rate, limiting the long-term efficacy of the procedure. There is growing evidence that the tissue factor-tissue factor pathway inhibitor (TF-TFPI) interaction, a major regulator of haemostasis, plays an important role in the healing response of arteries following PTA. In this study, 1) we reassessed claudicants awaiting femoropopliteal PTA for more than 3 months to determine changes in patient symptomatology and disease distribution during the wait period and 2) we investigated any association between plasma TF and TFPI concentration and the development of restenosis following PTA.

Patients and methods: In the first part of study, 47 claudicants with a median waiting period of 9.6 (interquartile range 4-21) months for femoro-popliteal PTA were assessed. Assessment included symptom and co-morbidity appraisal. Arterial duplex was performed on the symptomatic limb and compared with previous duplex study for assessment of disease progression.

In the second part, 52 unilateral claudicants undergoing femoropopliteal PTA and 10 healthy controls were studied for association of plasma TF and TFPI level with peripheral arterial disease and restenosis. Baseline (Pre PTA) plasma samples were collected from healthy controls and claudicants before PTA. Claudicants underwent arterial Duplex prior to and

at 24 hours, one month, three month and six months following PTA to identify restenosis. Plasma TF and TFPI levels were measured using standard enzyme linked immunosorbent assay (ELISA).

Results: On reassessment of claudicants awaiting PTA, 30(64%) patients were stable whilst 10 (21%) and 7 (15%) had symptomatically improved or deteriorated respectively. 9 (19%) patients demonstrated disease progression on arterial duplex. As a result of reassessment, 21 patients (45%) were removed from the PTA waiting list.

Plasma TF and TFPI concentrations were significantly higher in claudicants compared to healthy controls. The baseline plasma TF and TFPI concentrations were significantly higher in claudicants who developed restenosis following PTA than those who did not. Following PTA, claudicants who developed restenosis showed higher concentrations of plasma TF at 24 hour, 1 month, 3 month and 6 month follow-up intervals. Conversely, the TFPI levels were significantly lower in the restenosis group at month 1 and 6 following PTA. The group differences did not reach statistical significance at day 1 and month 3 following PTA. The ratio of baseline plasma TF and TFPI was found to be raised significantly in claudicants who developed restenosis than those whose angioplasty site remained patent.

Conclusions: 1.Claudicants awaiting PTA may experience a change in symptomatology with time which might alter management decisions. In units with a long waiting list, patients should be reassessed in the clinic to review symptom and evidence of disease progression or regression.

2. Increased baseline plasma TF and TFPI levels are associated with restenosis following PTA; however it is more likely that an altered TF/TFPI balance could be involved in the occurrence of restenosis.

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Table of contents

List of figures

List of Tables

Chapter 1 Introduction

1.1. PERIPHERAL ARTERIAL DISEASE (PAD) AND INTERMITTENT CLAUDICATION (IC)

PAD is an important cause of morbidity and indicator of mortality for people in many Western countries. About 20% of the UK population aged 55–75 years have evidence of lower extremity peripheral arterial disease, of whom 5% presents with symptoms (Norgren, Hiatt et al. 2007). In USA, the findings from a national cross-sectional survey of PARTNERS (PAD Awareness, Risk, and Treatment: New Resources for Survival) found that PAD affects 29% of patients aged ≥70 years or aged 50–69 years with a risk factor for vascular disease (smoking, diabetes)(Hirsch and Hiatt 2001). The National Health and Nutritional Examination Survey reported the prevalence of PAD 2.5% in the age group 50–59 years to 14.5% in subjects >70 years on an unselected population of 2174 subjects aged ≥40 years (Selvin and Erlinger 2004). The clinical presentation of PAD may vary from no symptoms to intermittent claudication, atypical leg pain, rest pain, ischemic ulcers, or gangrene. Intermittent claudication (IC) is the commonest manifestation of symptomatic PAD. It is a cramping pain in leg that develops when walking and is relieved with rest. The prevalence of IC varies in different population studies due to reporting bias. The Rotterdam study reported IC in 6.3% of patients with PAD (Meijer, Hoes et al. 1998) In the Edinburgh artery study the prevalence of IC was 4.5% in 1592 patients with age group 55-74 years. The prevalence of IC increases with age and in the relatively younger age group, claudication is more common in men but at older ages there is little difference between men and women (Fowkes, Housley et al. 1991).

IC has 2 major consequences: The first is a decrease in overall well-being and quality of life due to pain on walking and the second is a markedly increased cardiovascular

morbidity (myocardial infarction and stroke) and mortality.

Population follow-up studies suggest that symptoms in up to 50 % of patients with IC remain stable or may even improve. Only approximately one quarter of patients with intermittent claudication deteriorate progressively with an intervention rate of approximately 5% (Jelnes, Gaardsting et al. 1986; Dormandy, Heeck et al. 1999).

Patients with IC have have a 2-4 % risk of undergoing non fatal cardiovascular event within 1st year of diagnosis and a 1-3% yearly incidence thereafter (Norgren, Hiatt et al. 2007). In the Framingham Study, mortality in patients with IC was 2–3 times higher than in age- and sex-matched control patients, with 75% of PAD patients dying from cardiovascular events. The Edinburgh artery study reported increased cardiovascular events and death in both asymptomatic PAD and IC (Fowkes, Housley et al. 1991).

1.2. MANAGEMENT OF INTERMITTENT CLAUDICATION

The most important issue in the management of patients presenting with intermittent claudication is the significant risk of developing cardiovascular complications; their most serious problem is not the limitation of walking, although claudication may be the only symptom. The clinical course of claudication is benign in most of the cases i.e. symptoms usually remain stable or improve. Only approximately one quarter of patients with intermittent claudication deteriorate progressively with an intervention rate of approximately 5% (Jelnes, Gaardsting et al. 1986; Dormandy, Heeck et al. 1999)

The two primary goals in managing patients with IC are

1) Decrease cardiovascular morbidity and mortality by disease modifying therapy for secondary prevention.

2) Improve limb related symptom and quality of life.

1.2.1. MODIFICATION OF RISK FACTORS

SMOKING CESSATION

Successful cessation of smoking in patients with IC is associated with decreased disease progression, critical limb ischaemia, amputation rate, myocardial infarction, stroke and increased long term survival (Quick and Cotton 1982; Jonason and Bergstrom 1987).

In smokers, advised to stop smoking along with a formal cessation program and nicotine replacement therapy (NRT), a cessation rate of 22% have been observed at 5 years (Norgren, Hiatt et al. 2007). Randomized studies have supported use of bupropion in patients with cardiovascular disease (Tonstad, Farsang et al. 2003). Combining bupropion and NRT has been shown to be more effective than either therapy alone (Jorenby, Leischow et al. 1999). Varenicline a partial acetylcholine nicotinic receptor agonist has been shown to be more effective than bupropion in achieving higher quit rate (Cahill,

Stead et al. 2007).

LIPID LOWERING THERAPY

Low HDL (high density lipoprotein) or High LDL (low density lipoprotein): HDL ratio are independent risk factor for PAD (Fowkes, Housley et al. 1991). Evidence supporting the use of statins to lower LDL cholesterol levels in PAD comes from the Heart Protection Study (Heart Protection Study Collaborative Group 2002). 20,536 subjects at high risk for cardiovascular events including 6748 patients with PAD were randomized to simvastatin, antioxidant vitamins, a combination of treatments, or placebo with a 5-year follow up. Use of simvastatin was associated with a 12% reduction in total mortality, 17% reduction in vascular mortality, 24% reduction in coronary heart disease events, 27% reduction in all strokes and a 16% reduction in non-coronary revascularizations. A Cochrane review of studies in patients with PAD concluded that lipid lowering therapy may be useful in preventing deterioration of underlying disease and symptom alleviation (Leng, Price et al. 2000).

Trials have shown that use of statin was associated with increase in pain free walking distance and quality of life (Mohler, Hiatt et al. 2003; Erez and Leitersdorf 2007).

A recent meta-analysis also showed that patients with PAD benefits from statin therapy irrespective of baseline cholesterol concentration (Lewington, Whitlock et al. 2007).

DIABETES

Diabetes increases the risk of PAD approximately three to four-fold, and the risk of claudication two-fold (Norgren, Hiatt et al. 2007). Edinburgh artery study and Health Professionals Follow-up study showed 1.5-2.5 fold increase risk of symptomatic and asymptomatic PAD in diabetics (Fowkes, Housley et al. 1991; Al-Delaimy, Merchant et al. 2004). The UK prospective diabetes study (UKPDS) reported strong association of

haemoglobin A1c (HbA1c) with risk of PDA (Adler, Stevens et al. 2002). The TASC (TransAtlantic Inter-Society Consensus) guideline suggests that patients with diabetes and PAD should have aggressive control of blood glucose levels with the aim of keeping HbA1c of <7.0% or as close to 6% as possible (Norgren, Hiatt et al. 2007).

HYPERTENSION

Hypertension is a major cardiovascular risk factor present in up to 55% patients with PAD (Lip and Makin 2003). Effective antihypertensive therapy can reduce progression of PAD and mortality from myocardial infarction and stroke (Ostergren, Sleight et al. 2004; Feringa, van Waning et al. 2006).

ANTIPLATELET THERAPY

Long-term use of antiplatelet agents reduces the rate of MI and ischaemic stroke in patients with symptomatic PAD (Peripheral Arterial Disease Antiplatelet Consensus Group 2003). The CAPRIE trial evaluated efficacy and safety of clopidrogel compared with aspirin for secondary prevention in patients with cardiovascular disease; It showed a reduction in the risk of MI, stroke and vascular death in favour of clopidrogel (CAPRIE Steering Committee). The Peripheral Arterial Disease Antiplatelet Consensus Group recommends long term use of either Aspirin 75-325 mg daily or Clopidogrel 75 mg per day in all patients with intermittent claudication or who have had previous vascular intervention (Peripheral Arterial Disease Antiplatelet Consensus Group 2003).

1.2.2. IMPROVEMENT OF LIMB RELATED SYMPTOM AND QUALITY OF LIFE

EXERCISE PROGRAMME

Exercise training significantly improves walking capability in patients with intermittent claudication. A meta-analysis performed by Gardner et al suggestesd the efficacy of

exercise in improving the claudication related pain (Gardner and Poehlman 1995). A systematic review of randomized controlled clinical trials by the Cochrane collaboration group compared the effect of exercise programmes on IC with usual care or placebo (Watson, Ellis et al. 2008). Exercise produced significant improvement in walking time and walking distance. A systematic review of randomised controlled trials comparing supervised vs unsupervised exercise programs for IC showed statistically significant improvement in treadmill walking distance associated with Supervised exercise programme (SEP) compared with non-supervised exercise therapy regimens (Bendermacher, Willigendael et al. 2006). Supervised exercise programme (SEP) have been recommended as first line treatment for treatment of claudication (Stewart, Hiatt et al. 2002; Norgren, Hiatt et al. 2007).

PHARMACOLOGICAL THERAPY TO IMPROVE SYMPTOM OF IC.

Cilostazol is the drug with best evidence in support of its indication in treatment of IC. It is a phosphodiesterase III inhibitor and is reported to have anteplatelet and vasodilator effect. Two large systematic reviews have shown improvement in walking distance along with improvement of quality of life (Regensteiner, Ware et al. 2002; Thompson, Zimet et al. 2002). In these trials, the best benefits were observed in patients with short distance claudication. Cilostazol is licensed in UK for symptomatic relief of IC.

Naftidrofuryl is reported to have vasodilator effect and is thought to improve tissue oxygenation. Scottish Intercollegiate Guidelines Network suggests consideration to use naftidrofuryl in patients with IC and a poor quality of life (Hainsworth 2006).

ENDOVASCULAR TREATMENT FOR CLAUDICATION

Interventional therapy is considered in a minority of patients with intermittent claudication after balancing patient disability against procedural complication and

likelihood of long-term success of the procedure (Dormandy 2000).

In general an intervention or revascularisation procedure is indicated in 1) significant deterioration of symptom so as to severely handicap the patient in terms of preferred daily activities and job. 2) Severe handicap of patient at presentation and 3) development of critical ischaemia (Dormandy 2000).

The morphological categorization of femoropopliteal lesions may help to identify the preferred therapeutic option. According to the Transatlantic Inter-Society Consensus recommendation, PTA is the treatment of choice for TASC type A femoropopliteal lesions (Table 1), and in selected patients with TASC type B and C lesions but more evidence is required to make a firm recommendation about the preferred therapeutic option (Dormandy 2000).

Table 1. TASC grading for femoropopliteal disease*

TASC grade	Lesion Characteristics
TASC A	Single stenosis up to 3 cm; Not at superficial femoral artery origin or distal popliteal artery.
TASC B	▪ Single stenosis/occlusion 3-5 cm; not at distal popliteal artery. ▪ Heavy calcified stenosis up to 3 cm. ▪ Multiple lesions, each < 3 cm ▪ Single/ Multiple lesions in absence of continuous tibial runoff.
TASC C	▪ Single stenosis/occlusion > 5 cm ▪ Multiple Single stenosis/occlusions each 3-5 cm with or without heavy calcification.
TASC D	Complete common femoral or superficial femoral artery occlusions or complete popliteal and proximal trifurcation occlusions

***** TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). J Vasc Surg, 2000.

SUPERVISED EXERCISE VS ENDOVASCULAR TREATMENT FOR IC

Evidence is limited for exercise compared with angioplasty in treatment of intermittent claudication due to small number of trials. Angioplasty is thought to produce greater improvement in symptom in the short term but the effect may not be sustained (Watson, Ellis et al. 2008). The randomized trial comparing exercise training vs. angioplasty at Oxford showed greater rise in ABPI following angioplasty compared to exercise. However exercise training confers greater improvement in claudication distance and maximum walking distance (Perkins, Collin et al. 1996).

A randomized control trial of SEP, angioplasty and combined therapy in IC shows greater benefit in clinical outcome in patients treated with both SEP and angioplasty compared to patients treated by angioplasty or SEP (Mazari, Gulati et al.).

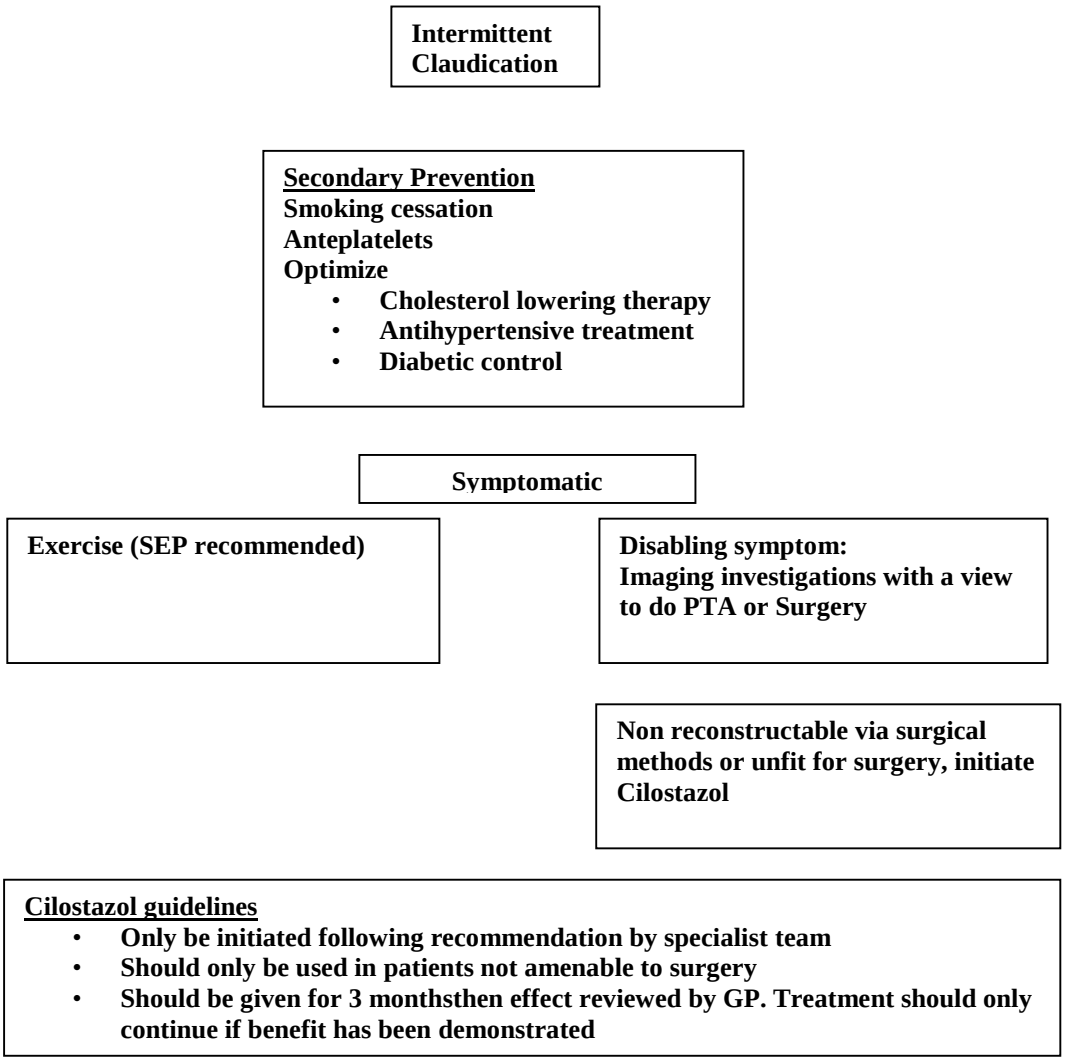
A prospective multicentre randomized controlled trial the CLEVER study is under way to compare the clinical and cost effectiveness between SEP vs. endoluminal revascularization in claudicants (Murphy, Hirsch et al. 2009).

1.3. ENDOVASCULAR TREATMENT OF LOWER LIMB PAD IN HULL

As per mid year report 2009 from Office of National Statistics, Hull has a population of 262,400 with a population density of 3,673. Vascular disease is not uncommon in Hull, partly attributable to prevalence of diabetes (4.5% in year 2006/2007 as per General Practioners registers) and high prevalence of smoking. Hull's Health and Lifestyle Survey, 2007 by the Primary Care Trust reports an overall smoking prevalence of 32%, which is way above the National prevalence of 22% in (2006).

In the year 2009 more than 300 cases of lower limb PTA was performed at Hull Royal Infirmary. The therapeutic algorithm followed for treating patients with diagnosed intermittent claudication is shown in figure 1. Table 2 and 3 shows the demographic profile and the lower limb PTAs performed in 2009.

Figure 1. Alogrithm for the Treatment of Intermittent Claudication



Author: P Kendrewand, Mr P Renwick, Hull and East Yorkshire Hospitals NHS Trust. Approved March 2008.

Table 2. Demographic profile of patient who underwent PTA of lower limb vessels at Hull Royal infirmary (Jan 2009- Dec 2009)

N= 316 Age= Median 70 (Iqr 62-77) years M:F= 3.6:2	
	Percentage
Hypertension	45.1
Diabetes	24.6
Hypercholesterolemia	35
Renal failure	6
Current smoker Ex smoker	20.2 38.8
Angina MI	14.2 14.5
Symptomatic carotid disease	5.6

Table 3. Endovascular treatment of lower limb vessels at Hull Royal infirmary (Jan 2009- Dec 2009)

PTA site	Number (percentage)	Bilateral	Stent use
Iliac	105 (33.2)	45 (66.2)	48 (77.4)
Ileo femoral	41 (13)	14 (20.6)	7 (11.3)
Femoro popliteal	149 (47.2)	6 (8.8)	7 (11.3)
Ileo femoral with distal vessels	15 (4.7)	3 (4.4)	0
Distal vessels only	6 (1.9)	0	0
Total	316	68 (100)	62 (100)

1.4. Factors affecting outcome of femoropopliteal PTA

Despite increasing numbers of femoropopliteal PTA performed through out the world, controversy still exists concerning outcomes, because often the reporting of indications and results are not standardised. Varying reporting criteria may account for up to twofold difference in reported five-year patency following femoropopliteal PTA (Matsi PJ, Manninen HI 1995). Many studies group PTA for claudication and critical ischaemia together and others fail to stratify the results (Harris RW et al 1991).

The factors that influence the outcome of PTA for lower extremity PAD include;

- Lesion characteristics
- Pattern of arterial disease
- Patient demographics
- Clinical presentation
- Intra procedural factors

1.4.1. Lesion Characteristics

1. *Location*

Long term patency following PTA is dependent on anatomical location of the lesion. Initial and long-term results are best in proximal arteries with decreasing long-term patency for more distal lesions. The early and late outcomes of femoropopliteal angioplasty are inferior to that of iliac angioplasty. Twice the initial and early failure rate at one year has been reported for PTA of femoropopliteal arteries than that of iliac arteries (Rutherford RB 1992). Similarly PTA of above knee popliteal lesions is associated with better results than PTA of below-knee popliteal lesions (Johnston 1992).

2. S*tenosis versus occlusion*

In femoral and popliteal arteries higher rates of technical failures have been reported in

occlusive lesions compared to stenosis (18% versus 7%)(Capek, McLean et al. 1991). However the long-term patency rate in these two types of lesions may be similar. Some series have shown better outcomes in stenosis rather than occlusion, whereas others have found no difference (Rutherford 2000).

3. *Length of the lesion*

Length of the lesion directly affects the patency rates following PTA. Longer lesions have a significant negative effect on the results of femoropopliteal PTA (Rutherford 2000). Capek et al reported that lesion length correlates with outcome following PTA, with short stenoses faring better with longer long term patency than long lesions (Capek, McLean et al. 1991). The overall vessel patency rates after femoropopliteal PTA in this study were 81%, 61%, and 58% at one, three, and five years.

4. Multiple stenoses in same segment

Lesions with multiple stenoses are associated with more frequent residual stenosis following PTA and higher restenosis rates. The outcome following femoropopliteal PTA involving multiple stenosis are inferior to those following PTA of focal stenosis (Johnston 1992).

5. *Other Morphologic features*

Calcification of stenotic lesions may affect outcome following PTA. Heavily calcified lesions are more prone to rupture, dissection and residual stenosis following PTA (Rutherford 2000). Eccentricity of the lesion has been considered to correlate with poor outcome following PTA in some studies, however this may not be an important factor for long term patency following PTA provided good haemodynamic correction

has been achieved. In femoropopliteal PTA lesion eccentricity is associated with higher rates of early failure due to dissection (Capek, McLean et al. 1991).

1.4.2. Pattern of arterial disease

The pattern of disease indicates the extent of atherosclerotic involvement and the runoff status, which might influence the success rate of PTA.

1. *Multilevel Occlusive disease*

PTA may be indicated to treat stenosis or occlusion at more than one arterial segment. Each site has its own failure rate and failure at one site may negatively affect the outcome at other sites.

2. *Runoff status*

Runoff status has been found to be a significant prognostic factor in the outcome following endovascular intervention (Capek, McLean et al. 1991; Johnston 1992). Studies on femoropopliteal PTA have shown that outcome is influenced by the runoff status. Krepel et al (1985) reported a five-year success rate of 77% and 59% for patients with good runoff and poor runoff, respectively. Gallino and associates have reported better early and late success rates following femoropopliteal PTA in patients with two or three patent tibial arteries than in patients with one or no tibial arteries (Gallino, Mahler et al. 1984). Stanley et al reported a 55% 2-year patency rate in limbs with good runoff and 23% in limbs with poor runoff following femoropopliteal PTA (Stanley, Teague et al. 1996).

1.4.3. Patient demographics

1. *Gender*

Women tend to have a lower patency rate following PTA than men (Mehilli, Kastrati et al. 2003).

2. *Diabetes*

Presence of diabetes correlates poorly with outcome (Gallino, Mahler et al. 1984; Jeans, Cole et al. 1994). However this may be due to the pattern / distribution of atherosclerotic

lesions in the diabetic population, which have a predominance in the

infrapopliteal vessels. For the same combination of occlusive lesions, no significant difference between diabetes and non-diabetics was observed (Davies, Cole et al. 1992). Diabetic patients with good runoff fare better than those with poor runoff after femoropopliteal PTA (Stokes, Strunk et al. 1990)

3. *End stage renal disease*

Increased incidence of restenosis has been reported in patients with end stage renal disease following coronary angioplasty (Schoebel, Gradaus et al. 1997). This is considered to be due increased prothrombotic risk associated with higher fibrinogen concentrations in these patients (Rabelink, Zwaginga et al. 1994; Schoebel, Gradaus et al. 1997).

1.4.4. Clinical presentation

Indication for intervention is one of the strongest predictors of outcome following femoropopliteal PTA. Successful long-term outcome following femoropopliteal PTA is less likely with more advanced lower limb ischaemia. Patients with claudication tend to have a more benign overall disease than those with critical ischaemia. Claudicants have better long-term success rates than patients with critical limb ischaemia (Johnston 1992). Hunick and associates reported better five-year patency rates in claudicants than those who had critical ischaemia (55% vs. 29%) following PTA for stenotic lesions (Hunink, Donaldson et al. 1993).

1.4.5. Intra-procedural factors

Events occurring during the angioplasty might influence early and long term outcome. Intra-procedural complications like dissection, significant residual stenosis and poor haemodynamic result may influence long term outcome following PTA (Rutherford 2000).

1.5. MECHANISM OF PTA

Dr. C. Dotter performed the first endovascular procedure by dilating an iliac artery stenosis with the sequential passage of progressively larger catheters across the lesion (Dotter CT, Judkins MP 1964). Andreas Gruntzig introduced the concept of the double lumen catheter and increased the application of percutaneous catheter based vascular dilatation (Gruntzig AR 1979). Balloon catheters allowed vascular dilatation through sites remote from the target lesion. In the past decades, the development of flexible guide wires, has facilitated access for the dilatation of lesions in distant smaller tortuous arteries. Percutaneous transluminal angioplasty is an increasingly common endovascular procedure performed in the aorta, iliac, femoral, tibial and coronary arteries with varying degrees of success.

Balloon angioplasty expands a narrowed arterial segment in a manner that retains the expansion thereby improving blood flow through the dilated arterial segment. Balloon angioplasty functions by exerting a circumferential force on the arterial wall as the balloon expands. The pressure generated by fluid forced in to the contained space of the balloon during dilatation results in the generation of a circumferential stretching force on the arterial wall. This circumferential stretching force acting on the balloon surface is called 'Hoop stress' and may be calculated using the equation,

Hoop stress = hydrostatic pressure X balloon diameter (Abele 1980).

Hoop stress generates both longitudinal and radial force vectors with the radial component being the dilating force. The radial force is directly proportional to the diameter of the fully inflated balloon and the pressure used for balloon inflation (Castaneda-Zuniga, Formanek et al. 1980). The radial force is thus greater than the hydrostatic force of fluid injected in the balloon. During angioplasty the increase in intraluminal pressure and radius results in an increased tangential force, which keeps the arterial wall expanded. The force

generated by the balloon in a stenotic area varies with radial force vectors being greatest at the area of greatest stenosis (Abele 1980; Castaneda-Zuniga, Formanek et al. 1980). The hoop stress also acts on the adjacent normal compliant artery thus causing elastic stretching of the compliant adjacent artery. Angioplasty balloons are made of non-compliant material thus retain their diameter with increasing pressure. This prevents excessive overstretching of the adjacent normal arterial segment during balloon dilatation.

Current knowledge regarding the mechanism of luminal gain following PTA is based on animal models, autopsy and atherectomy specimens, and intravascular ultrasound studies. Balloon dilatation of the inelastic stenotic lesions causes significant intimal trauma (Castaneda-Zuniga, Formanek et al. 1980; Lyon, Zarins et al. 1987). When a fibrocalcific plaque is dilated during PTA, the radial force vectors separate the inelastic plaque from adjacent compliant artery. The shearing force at the interface separates the plaque and the compliant arterial wall (usually tunica media). This separation creates a dissection that is rapidly extended radially by the pressure exerted by the balloon. This dissection is necessary for the irreversible expansion of the arterial lumen (Neville RF 1997). The resultant healing process and arterial remodelling obliterates the dissection planes, rendering it angiographically invisible within 3 to 30 days post PTA (Lyon, Zarins et al. 1987).

1.6. RESTENOSIS FOLLOWING ANGIOPLASTY

Restenosis may be defined as the recurrence of impaired lumen area after successful angioplasty. This has been characterized conventionally by quantitative angiography and most commonly confirmed by a diameter reduction of 50% or more at follow-up following a successful angioplasty.

1.7. METHODS OF DETERMINATION OF RESTENOSIS

Restenosis following endovascular intervention can be identified by conventional angiography, duplex sonography, intravascular ultrasound, angio-computed tomography and magnetic resonance imaging. Despite all these techniques, restenosis is often diagnosed by symptom recurrence and resulting end organ dysfunction.

1.7.1. Contrast angiography

Angiography is considered the gold standard for defining both normal vascular anatomy and pathology. It remains the only universally accepted imaging modality to guide percutaneous intervention. Over the decades although many definitions of angiographic restenosis have been used, the most widely used and accepted is recurrent stenosis greater than 50% (Miller J M 1999). Arterial remodelling offers particular challenges to angiographic interpretations because angiography only visualizes the contrast filled vessel lumen (Topol and Nissen 1995). Up to 40 % of the internal elastic lamina may be occupied before the lumen begins to narrow (Glagov, Weisenberg et al. 1987). Angiography is considered to be an end point in defining clinical restenosis however discrepancies between angiographic and clinical outcomes are not unusual (Miller J M 1999).

1.7.2. Duplex ultrasound

Duplex ultrasound is useful to detect stenosis and determine its anatomical location in lower extremity PAD. The most commonly used quantitative criteria to detect stenosis is

the ratio of the peak systolic velocity within the stenosis and the adjacent normal arterial segment. A ratio of greater than 2 is commonly used to diagnose a stenosis which reduces the luminal area of the artery by greater than 50% (Fletcher, Kershaw et al. 1990; Moneta, Yeager et al. 1993; Pinto, Lencioni et al. 1996). There is conflicting evidence regarding the value of duplex ultrasound in surveillance following angioplasty. Studies suggest that immediately after angioplasty, velocities in the treated segment are abnormally elevated and do not predict subsequent patency rates (Sacks, Robinson et al. 1990; Sacks, Robinson et al. 1994). Ultrasound however is valuable in the evaluation of chronic recurrent stenoses (Spijkerboer, Nass et al. 1996; Vroegindeweij, Tielbeek et al. 1997).

1.7.3. Magnetic resonance imaging (MRA)

Similar to duplex imaging, MRA can be used to delineate anatomical location and degree of stenosis in patients with PAD. MRA has also been used in assessment of post endovascular intervention of the lower extremities. MRA in post procedure evaluation of angioplasty sites demonstrates concordance with catheter angiography in 80-95% cases (Davis, Schopke et al. 1997).

1.7.4. Intravascular Ultrasonography

The use of intravascular ultrasound has broadened our understanding of plaque morphology and arterial remodelling following percutaneous intervention. As it can measure both external and internal vessel diameter, it has been used to confirm Glagov phenomenon (Glagov, Weisenberg et al. 1987) which describes that arteries remodel to maintain constant blood flow despite increase in atherosclerotic lesion mass. Mintz et al demonstrated negative arterial remodelling by performing serial intravascular ultrasound studies of angioplastied vessels in human coronary arteries (Mintz, Popma et al. 1996).

1.8. CURRENT CONCEPT IN MECHANISM OF RESTENOSIS

The development of a restenotic lesion differs from the development of a primary atherosclerotic lesion in many ways. Primary atherosclerotic lesion develops over years, whilst restenosis most commonly occurs within the first 6 months following intervention (Nobuyoshi, Kimura et al. 1988; Serruys, Luijten et al. 1988). The pathophysiology of restenosis is complex and incompletely understood. Current evidence suggests that restenosis is an abnormal response of the artery to angioplasty trauma and consists of thrombosis, inflammation, cellular proliferation, and extra cellular matrix production (Nikol, Huehns et al. 1996). The lumen loss after angioplasty may be divided in to 3 distinct stages: early luminal loss associated with elastic recoil, late loss secondary to negative vascular remodelling and neointimal hyperplasia (Rajagopal and Rockson 2003).

1.8.1. Elastic recoil

Elastic recoil is a progressive phenomenon that occurs immediately following angioplasty resulting in immediate luminal loss. By using quantitative coronary angiography in 154 angina patients undergoing PTCA, Caixeta and colleague observed a 34% loss in lumen diameter within 15 minutes of balloon deflation (Caixeta, Arie et al. 1996). Studies indicate that elastic recoil may account for up to 50% of the loss in lumen gain during angioplasty (Rozenman, Gilon et al. 1993; Rodriguez, Palacios et al. 1995). The degree of elastic recoil following angioplasty varies in different arteries due to variation in elastin content. Most studies of arterial wall recoil are focussed on coronary arteries (Rensing, Hermans et al. 1990; Kimball, Bui et al. 1992; Caixeta, Arie et al. 1996; Bermejo, Botas et al. 1998). Pasterkamp et al reported 11% elastic recoil in femoral arteries following angioplasty, using intravascular ultrasound (Pasterkamp, Borst et al. 1995).

Gardiner et al reported 32% elastic recoil in a study involving balloon angioplasty for iliac artery stenosis and showed the influence of balloon artery ratio on the degree of elastic recoil (Gardiner, Bonn et al. 2001). Elastic recoil following angioplasty may be reduced by stent implantation. However even after stenting, luminal diameter is usually <60% of the maximum achievable diameter due to suboptimal stent deployment and elastic recoil (Bermejo, Botas et al. 1998).

1.8.2. Vascular Remodelling

Vascular remodelling occurs naturally in atherosclerosis. Glagov et al noted compensatory enlargement of human coronary arteries in response to plaque formation to reduce the effect of arterial lumen narrowing (Glagov, Weisenberg et al. 1987). In vivo confirmation of this positive remodelling was reported by Losordo et al (Losordo, Rosenfield et al.1994). Studies involving animal and human subjects have established an alternate form of arterial remodelling, the constrictive or negative remodelling. Following angioplasty constrictive remodelling results in narrowing of vessel diameter causing restenosis of the angioplastied segment. Mintz et al, using serial intravascular ultrasound, documented negative remodelling resulting in restenosis following human percutaneous transluminal coronary angioplasties (PTCA) (Mintz, Popma et al. 1996). They observed that the change in lumen area between post PTCA and late angiographic follow-up correlated strongly to a change in vessel size (area circumscribed by the external elastic lamina) rather than the change in area of atherosclerotic plaque plus medial wall of the artery. The study showed that the change in vessel size is bi-directional. Some lesions showed an increase in vessel size where as others showed a decrease in vessel size. Lesions that exhibited an increase in vessel size had no change in lumen area in contrast to the lesions that exhibited a decrease in vessel size associated with reduction of lumen area. Figure 2 shows a simplied diagram of

positive and vascular remodeling in response to atherosclerotic plaque formation. The precise mechanism of arterial remodelling after angioplasty is uncertain but is likely to be multifactorial and include a combination of elastic recoil, disordered flow mediated remodelling, adventitial scar contraction and thinning of tunica media (Isner 1994). Current data suggest vascular remodelling may be due to adventitial thickening and scar contraction as a result of replacement of hyaluronic acid with collagen in the extra cellular matrix (Riessen, Wight et al. 1996). The extent, negative remodelling plays in restenosis is debated, but is thought to be more predominant following simple angioplasty whereas neointimal hyperplasia is thought to predominate in restenosis following angioplasty and stenting (Bauters and Isner 1997).

Figure 2. Positive and negative arterial remodelling in response to atherosclerotic plaque formation

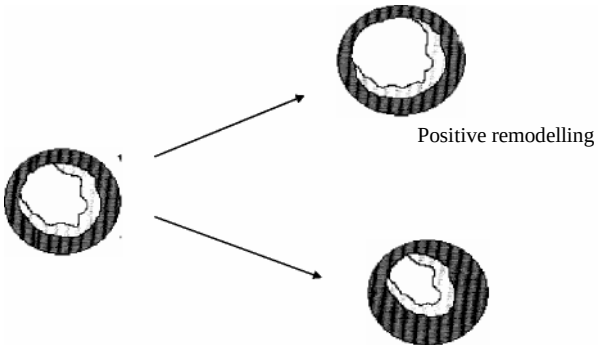


Figure showing two types of remodeling in a blood vessel in response to atherosclerotic plaque formation. Positive remodeling is an adaptative expansion of the arterial wall to maintain the blood flow through blood vessels in response to atherosclerotic plaque growth. In negative remodeling there is a failure of the arterial wall to expand or an inward modeling resulting in reduction in luminal area (Glagov, Weisenberg et al. 1987).

1.8.3. Neointimal Hyperplasia

Intimal hyperplasia is an exuberant response to healing in blood vessels following an open or endovascular procedure that can compromise vessel lumen, restrict blood flow and may ultimately result in failure of the interventional procedure. Intimal hyperplasia occurs within months of intervention and is a complex cellular process predominantly involving the intimal layer of the artery. It may be triggered by a combination of trauma causing endothelial injury coupled with turbulent blood flow. Immediately following angioplasty, fracture of atherosclerotic plaque exposes its thrombogenic contents triggering platelet adhesion, activation and thrombosis (Fuster et al. 1990; Casscells, Engler et al. 1994). Endothelial denudation results in the loss of antithrombotic factors (nitric oxide, prostacyclin, tissue plasminogen activators), further contributing to platelet adhesion and aggregation. Activated platelets release mitogens including thromboxane A2, serotonin, platelet derived growth factor which promote smooth muscle cell proliferation and migration(Bauters and Isner 1997; Dorn 1997; Pakala, Willerson et al. 1997). Concurrently the levels of mitogenic protooncogenes (c-fos, c-jun, fosB, junB and junD) increase in the smooth muscle cells, altering their phenotype from contractile to synthetic type (Miano, Vlasic et al. 1993). In contrast to the vasoactive, contractile (differentiated) smooth muscle cells, the synthetic type (undifferentiated) are highly proliferative (Chamley, Campbell et al. 1977). Additionally smooth muscle cells secrete promigratory proteins including CD44v6, urokinase plasminogen activator receptor, integrin alpha(v)ss, transforming growth factor ss, MDC9 and ss-inducible gene h3 (Bauters and Isner 1997; Rajagopal and Rockson 2003). Consequently, many activated medial smooth muscle cells migrate to the intima (Miano, Vlasic et al. 1993). An injured, dysfunctional endothelium may also contribute to smooth muscle proliferation and migration as an intact endothelium

may exert antiproliferative influence on the underlying smooth muscle cells possibly mediated by prostacyclin and nitric oxide, heparin sulphate and nitric oxide (Clowes, Clowes et al. 1986; Bauters and Isner 1997). The proliferative response of the smooth muscle cells peaks 4 weeks after initial injury before slowing down until the number of intimal smooth muscle cells stabilizes 6 months post injury. At this point much of the hyperplastic response is contributed by increase in extra cellular matrix from secretory activity of synthetic smooth muscle cells and fibroblasts. The mature intimal lesion is 60%-80% extra cellular matrix by volume (Neville and Sidawy 1998). The extra cellular matrix continues to increase despite reestablishment of endothelial layer. One year post injury lesions exhibit decreased smooth muscle cellularity with a persistent increase in extra cellular matrix (Fuster et al. 1990; Clowes and Reidy 1991).

1.9. MOLECULES INVOLVED IN INTIMAL HYPERPLASIA

1.9.1. Mitogen activated protein kinases (MAPKs)

MAPKs are a family of serine threonine protein kinases activated by a range of stimuli including growth factors, cytokines, shear stress, vasoactive agents, and oxidative stress (Khan, Bianchi et al. 2004). The MAPK family members are involved in intracellular signalling cascades that transduce extra cellular stimuli into a cellular response by activating cytoplasmic and nuclear effector proteins, ultimately modifying gene transcription. The three major members of MAPK family, implicated in vascular response to injury are: a) extra cellular signal regulated kinases (ERKs); b) p38 MAPK , and c) c-Jun N-terminal kinases (JNKs) (Koyama, Olson et al. 1998; Tanaka, Oda et al. 1998; Yu, Ferrari et al. 2007). Activation of certain members of MAPKs regulates inflammatory response to vascular injury. p38 MAPK activation is involved in production of proinflammatory cytokines (IL-1β, TNF- α) and activation of monocytes, macrophages and T- lymphocytes (Lee, Laydon et al. 1994). JNK up regulates expression of proinflammatory genes encoding for cell surface receptors, cell adhesion molecules, growth factors and cytokines (Manning and Davis 2003). MAPKs also mediate endothelial and vascular smooth muscle cells (VSMC) proliferation and migration induced by growth factors involved in development of intimal hyperplasia (Yu, Ferrari et al. 2007).

1.9.2. Thrombin

Thrombin is a serine protease which, in addition to its well recognised enzymatic role in the coagulation cascade, serves as an effector molecule to elicit cellular responses. Thrombin affects different cell types through activation of protease activated receptors (PARS) which translates extra cellular proteolytic events in to intracellular signalling (Coughlin 2005). Thrombin is a strong mediator of inflammation, a process intimately linked to

development of intimal hyperplasia (Cirino, Cicala et al. 1996; Erlich, Boyle et al. 2000; Fukuda, Shimada et al. 2004). Thrombin promotes leukocyte migration, stimulates secretion of growth factors and platelet activating factor (Strukova 2001; Minami, Sugiyama et al. 2004). Migration and proliferation of vascular smooth muscle cells in response to injury are also stimulated by thrombin (Li, Garnette et al. 2000; Cao, Dronadula et al. 2006).

1.9.3. Platelet derived growth factors (PDGFs)

PDGFs are peptide growth factors that are released predominantly by activated platelets at the site of vascular injury and by stress stimulated endothelial cells (Dardik, Yamashita et al. 2005). PDGFs play important role in neointimal hyperplasia. One isoform of PDGF (PDGF-BB) is a very potent stimulator of VSMC migration. Another isoform, PDGF-AA stimulates VSMC proliferation but not migration (Koyama, Hart et al. 1994; Uchida, Sasahara et al. 1996). In vitro studies have shown that the effect of PDGFs on VSMC are mediated by MAPKs (Kingsley, Huff et al. 2002; Zhan, Kim et al. 2003).

1.9.4. Fibroblast growth factor-2 (FGF-2)

FGF-2 is a pleiotropic growth factor and controls a variety of physiological and pathological functions including angiogenesis and neointimal hyperplasia (Bikfalvi, Klein et al. 1997; Ornitz and Itoh 2001). Tissue damage including vascular injury induces both the release and expression of FGF-2 (Pintucci, Steinberg et al. 1999; Rhoads, Eskin et al. 2000) and FGF-2 then induces VSMC proliferation and migration (Rauch, Millette et al. 2005). FGF-2 mediated activation of MAPKs is implicated in reendothelialization and VSMC proliferation and migration following vascular injury (Pintucci, Steinberg et al. 1999).

1.9.5.Transforming growth factor- f3 (TGF-f3)

TGF-f3 is a pleiotropic cytokine produced by platelets, vascular endothelial cells, VSMCs,

and inflammatory cells. It is a key regulator of cell migration, proliferation, differentiation and apoptosis (Roberts 1998; Schulick, Taylor et al. 1998). Induction of TGF-f3 has been implicated in the regulation of the cellular response to vascular injury. Haemodynamic shear forces secondary to vascular injury induce release of TGF-f3 from endothelial cells (Song, Kocharyan et al. 2000). It is uncertain whether TGF-f3 is a positive or negative regulator of intimal hyperplasia. TGF-f3 increases ECM deposition and some studies suggest that it promotes intimal hyperplasia (Schulick, Taylor et al. 1998). More recently it has been suggested that TGF-f3 could inhibit VSMC proliferation and migration (Seay, Sedding et al. 2005).

1.9.6. Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are extra cellular matrix (ECM)-modifying enzymes that play a significant role in pathogenesis of restenosis by regulating ECM degradation and release of matrix degrading MMPs. Two types MMPs have been particularly implicated in restenosis namely MMP-2, and MMP-9 (Dollery, McEwan et al. 1995; Loftus and Thompson 2002). Remodelling of ECM after vascular injury facilitates migration and proliferation of VSMCs, and MMPs play an essential role in this (Galis and Khatri 2002). Induction of MMP is considered important in the development of intimal hyperplasia. Inflammatory mediators that play a role in the development of intimal hyperplasia are involved in the control of MMPs activity (Yu, Ferrari et al. 2007). MMPs play important role in constrictive arterial remodelling following vascular injury. Constrictive remodelling in response to angioplasty is believed to be the result of changes in collagen and elastin metabolism mediated by alterations in MMP activity after angioplasty (Post, Borst et al. 1995). Several studies demonstrate a reduction in post angioplasty constrictive remodelling and neointimal hyperplasia after administration of

MMP inhibitors (de Smet, de Kleijn et al. 2000; Peterson, Porter et al. 2000; Sierevogel, Pasterkamp et al. 2001).

1.10. TISSUE FACTOR

Tissue factor, formerly known as thromboplastin is a 47-kDa cell bound transmembrane glycoprotein expressed in both vascular and nonvascular cells. TF has been considered an important initiator of coagulation in *vivo* since its discovery in the 19th century (Rapaport and Rao 1995). Traditionally TF is believed to be the initiator of the extrinsic pathway of coagulation. However with the increase in understanding of the role of TF and its regulator, the tissue factor pathway inhibitor (TFPI), TF has also been shown to be a cellular signalling receptor (Petersen, Freskgard et al. 2000) and has several non-haemostatic actions. The pathways by which TF initiates cellular mechanisms are not clear. It has been suggested that TF may influence coagulation dependent mechanisms involving coagulation proteases (Abe, Shoji et al. 1999; Camerer, Rottingen et al. 1999; Ollivier, Chabbat et al. 2000; Petersen, Freskgard et al. 2000) and coagulation independent mechanisms involving its cytoplasmic domain (Abe, Shoji et al. 1999; Ruf and Mueller 1999).

1.11. TISSUE FACTOR AND COAGULATION

Tissue factor, a class 2 cytokine receptor, is a transmembrane glycoprotein that consists of three sections: a large extra cellular domain, a transmembrane domain and a cytoplasmic tail (Edgington, Mackman et al. 1991; Martin, Boys et al. 1995). The extra cellular domain is important for haemostatic activity (McVey 1999). The transmembrane portion is necessary for stabilization of the molecule and its complex. The function of the cytoplasmic domain, although not yet fully determined, is considered to be involved in the coagulation independent functions of TF (Abe, Shoji et al. 1999; Ruf and Mueller 1999). Traditionally TF was thought to initiate the extrinsic coagulation pathway, with collagen playing the same role in the intrinsic pathway. The biochemical events of coagulation are organised into the extrinsic, intrinsic and common pathways in the cascade mechanism of coagulation (Fig. 3) (Davie and Ratnoff 1964; Macfarlane 1964). The extrinsic pathway is initiated by TF interacting with factor VII to form activated factor VII, which in turn activates factor X to form activated factor X (Xa). The intrinsic pathway is initiated when factor XII comes in contact with negative charges underlying endothelium and also generates factor Xa. Factor Xa catalyses conversion of prothrombin to thrombin which combines with factor XIII to generate a fibrin plug. Several clinical and experiential observations suggest that the cascade/ waterfall hypothesis does not accurately reflect the events of *in vivo* haemostasis (Biggs and Mac 1951; Macfarlane, Biggs et al. 1964; Ragni, Lewis et al. 1981). Ostend and Rapaport provided experimental evidence that factor VIIa-TF complex activates both factor X and IX indicating a central role of TF in initiation of coagulation (Osterud and Rapaport 1977). The discovery of circulatory inhibitor of factor VIIa-TF complex suggested an alternative ‘network’ model of coagulation, which involves linkage of two pathways and is regulated by positive and

negative feedback loops (Broze 1992; Rapaport and Rao 1992; McVey 1999). The modern concept of coagulation incorporates cell surfaces in the coagulation process and TF has a central role in this new concept of coagulation (Fig 4). The process of clot formation is considered to occur in two stages: a) initiation of coagulation and b) propagation of the resultant thrombus. The initiation phase begins when disruption of the vessel wall exposes TF to circulating factor VII, forming a proteolytic active TF/ factor VIIa complex (TF-FVIIa) (Higashi and Iwanaga 1998). TF-FVIIa complex activates factor IX as well as factor X on the sub endothelial surface (Bauer, Kass et al. 1990; Butenas, van 't Veer et al. 1997). The amount of FXa generated during this phase is extremely low for formation of a fibrin plug. Trace amount of thrombin are generated which initiates back-activation of factors V, VIII and possibly XI. Factor VIIIa then complexes with IXa to generate a sufficient amount of Factor Xa that will sustain clot formation (propagation phase). Traditionally it was believed that TF was only expressed in extra vascular tissues by macrophages, monocytes, and fibroblasts (Wilcox, Smith et al. 1989; Fleck, Rao et al. 1990). However TF is also found in the adventitia of blood vessels, organ capsules, and epithelium of skin and mucous membranes. TF is unable to interact with coagulation factors, and thereby initiates thrombosis at these sites when vessel wall damage occurs. Circulating TF is present in both the whole blood and serum of healthy individuals (Giesen, Rauch et al. 1999; Giesen and Nemerson 2000). Normally, circulating TF is present at extremely low levels, and is present in an inactive encrypted form, and therefore does not initiate coagulation. TF inactivity may be caused by asymmetrical distribution of negatively charged phospholipids across the cell membrane (Bevers, Comfurius et al. 1998). The phospholipids, required for binding of coagulation factors to cell membrane and TF-VIIa complex, may be presented

by cell membrane disruption. In the revised hypothesis of coagulation, TF rather than the contact factors is responsible for initiation of coagulation. Factor IX and factor VII are necessary for enhanced factor Xa generation and sustained coagulation.

Figure 3. Cascade/ waterfall theories of coagulation

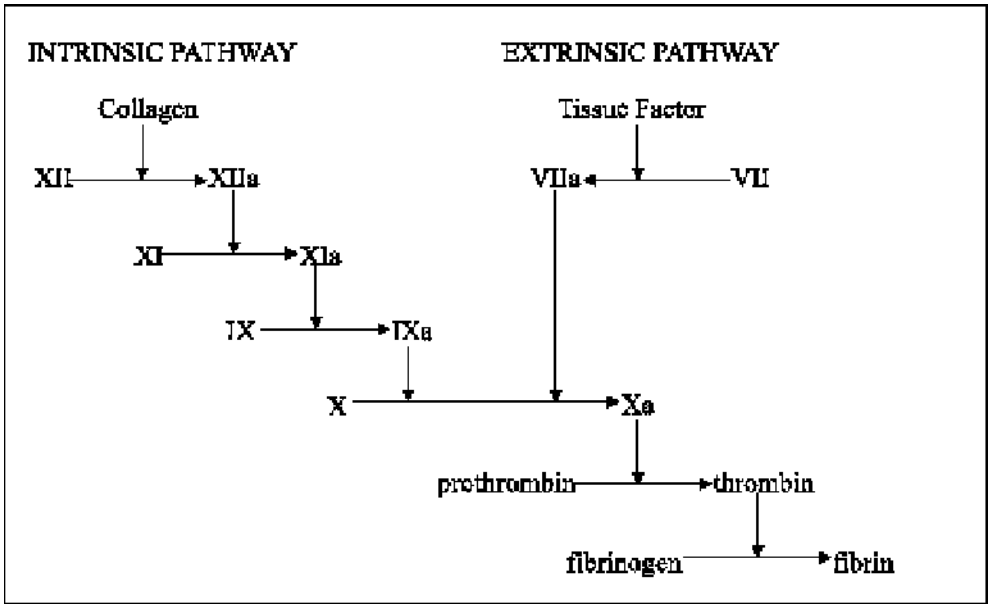
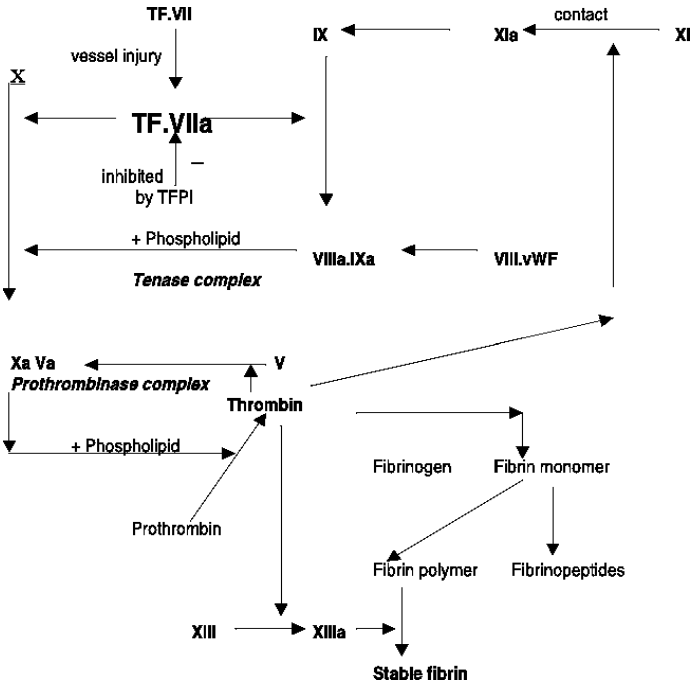


Figure showing cascade model of coagulation in which the biochemical events of coagulation are organised into the extrinsic, intrinsic and common pathways. The extrinsic pathway is initiated by TF interacting with factor VII to form activated factor VII, which in turn activates factor X to form activated factor X (Xa). The intrinsic pathway is initiated when factor XII comes in contact with negative charges underlying endothelium and also generates factor Xa. Factor Xa catalyses conversion of prothrombin to thrombin which combines with factor XIII to generate a fibrin plug.

Figure 4. Revised theory of coagulation



In the revised model, the clot formation occurs in two stages: a) initiation of coagulation and b) propagation of the resultant thrombus. The initiation phase begins when injury to the vessel wall exposes TF to circulating factor VII, forming TF-VIIa complex. TF-FVIIa complex activates factor IX as well as factor X on the sub endothelial surface. The thrombin generated in the process initiates back-activation of coagulation factors V, VIII and possibly XI. Factor VIIIa then complexes with IXa to generate a sufficient amount of Factor Xa that will sustain clot formation (propagation phase).

1.12. TISSUE FACTOR IN INTRACELLULAR SIGNALING

The structural similarities between TF and the super family of cytokine receptors, especially interferon α, β and γ receptors were identified in 1990 (Bazan 1990). However it took sometime before TF induced intracellular signalling was identified. Intracellular signalling by TF-FVIIa complex mediates the non-haemostatic functions of tissue factor including induction of proinflammatory response, migration and proliferation of VSMC, tumour angiogenesis and metastasis. Binding of FVIIa to membrane bound TF causes several intracellular effects (Petersen, Freskgard et al. 2000; Monroe and Key 2007) including: a) mobilization of intracellular calcium stores (Rottingen, Enden et al. 1995) b) transient phosphorylation of intracellular proteins like MAPKs (Poulsen, Jacobsen et al. 1998; Versteeg, Bresser et al. 2003). Phophorylated MAPKs enter the cell nucleus and activate several transcription factors. MAPKs activity are implicated in tumour metastasis, VSMC proliferation and migration. Although the precise pathway of intracellular signalling by TF-FVIIa and target cell effect is not clear, it is likely that TF-FVIIa signals through activation of members of the family of protease activated receptors (PARS) (Camerer, Huang et al. 2000; Riewald and Ruf 2002).

1.13. TISSUE FACTOR PATHWAY INHIBITOR (TFPI) AND REGULATION OF COAGULATION

TFPI is a multivalent, kunitz type, protease inhibitor which produces FXa dependent feedback inhibition of TF- FVIIa complex (Broze 2003). The TFPI has a N-terminal acidic region, three kunitz type domains (stable peptide domains with ability to recognize specific protein structures and also act as competitive protease inhibitors) separated by two linker regions, and a C-terminal basic region. K1 inhibits factor VIIa complexed to TF. K2 inhibits factor Xa (FXa). No direct protease inhibiting action has been demonstrated for K3. The C terminal basic region is demonstrated to be required for rapid inhibition of coagulation and for inhibition of smooth muscle proliferation (Bajaj, Birktoft et al. 2001). The heparin binding sites of TFPI are located in the K3 domain and the C terminal basic region of the molecule (Kato 2002).

The anticoagulant action of TFPI is a two stage process (Fig. 4). At first the TFPI binds to FXa to form TFPI-FXa complex. The TFPI-FXa complex then binds to TF-FVIIa forming a quaternary complex which completely blocks TF-VIIa activity and prevents further activation of factor X (Price GC 2004). This process does not occur in the absence of FXa indicating coagulation must be initiated before TFPI can function. There are three pools of TFPI distribution in vivo. The first pool of TFPI is the largest (80-85%) and is bound to vascular endothelium. The second pool (10%) circulates in plasma, primarily in association with lipoproteins but a small amount is in a free form. The third TFPI pool is the smallest (approximately 3%) and is found within platelets (Broze, Lange et al. 1994; Bajaj, Birktoft et al. 2001). In the second pool, the lipoprotein associated TFPI is C-terminally truncated and thus exhibits less anticoagulant activity. The free plasma TFPI is full length but its concentration is insufficient to influence coagulation (Broze,

Lange et al. 1994; Broze 1995). In the first pool TFPI is constitutively expressed on the endothelial cell surface and is stored intracellularly (Kato 2002). Thrombin induces release of TFPI from the intracellular stores and alters the cell surface distribution (Lupu, Lupu et al. 1995). The synthesis and secretion of TFPI is also increased by heparin thereby decreasing the procoagulant activity of the cells. Serum growth factors and shear stress also up regulate TF expression (Hansen, Svensson et al. 2000; Kato 2002).

1.14. TF AND TFPI IN CARDIOVASCULAR DISEASE

Hypertension

Tissue factor antigen in plasma is elevated in hypertensive subjects compared to normotensive controls and can be lowered by antihypertensive drugs (Felmeden, Spencer et al. 2003). Angiotensin II has been shown to induce TF expression in monocytes, endothelial cells and VSMCs through angiotensin type I receptor (AT-I) (Taubman, Marmur et al. 1993; He, He et al. 2006). Both ACE inhibitors and AT-I receptor blockers reduce plasma TF activity in hypertensive patients (Koh, Chung et al. 2004).

Hyperglycaemia

In vitro studies suggest that hyperglycaemia induces thrombin induced TF expression of endothelial cells (Boeri, Almus et al. 1989). In healthy humans, glucose intake also up regulates monocyte TF expression. Increased plasma levels of TF are observed in patients with diabetes mellitus and TF levels are reduced by improving glycaemic control (Sambola, Osende et al. 2003). It has also been suggested that increased TF level is associated with micro vascular and neurogenic complications in patients with Type 2 diabetes and is possibly a marker for micro vascular disease progression (Sommeijer, Hansen et al. 2006). Alterations in TFPI activity in diabetics have been considered to reflect early endothelial dysfunction (Leurs, van Oerle et al. 1997). Increased TFPI levels have been reported in patients with diabetes mellitus, particularly in those with nephropathy associated with insulin dependent diabetes (Yokoyama, Myrup et al. 1996; Leurs, van Oerle et al. 1997).

Hyperlipidaemia

Oxidised LDL increases TF expression in endothelial cells, monocytes and macrophages (Drake, Hannani et al. 1991; Wada, Kaneko et al. 1994) where as HDL

has been shown to inhibit endothelial TF expression (Viswambharan, Ming et al. 2004). Patients with elevated LDL levels show increased plasma TF activity (Sambola, Osende et al. 2003). Statins reduce TF expression in monocytes, endothelial cells, and VSMCs (Eto, Kozai et al. 2002). Based on animal studies, it has been suggested that the reduction in TF expression is, at least in part, related to pleiotropic anti inflammatory effect of these drugs (Bea, Blessing et al. 2003). The relationship between TFPI synthesis and lipid metabolism is controversial (Ettelaie, Wilbourn et al. 1999; Kawaguchi, Miyao et al. 2000). Kato et al (2002) found an inverse relationship between free TFPI level and HDL cholesterol level.

Smoking

Nicotine induces TF expression in endothelial cells and VSMCs in cell culture studies (Cirillo, S et al. 2006). Smoking in vivo is associated with increased plasma TF activity and a strong correlation has been observed between the number of cigarettes smoked and plasma TF expression (Sambola, Osende et al. 2003).

1.15. TF IN CORONARY ARTERY DISEASE (CAD)

Coronary artery disease (CAD) is the result of chronic inflammation within the arterial wall, the pathogenesis of which is still not completely understood (Ross 1999). Several inflammatory markers have been associated with increased risk of CAD. Recently TF, which activates coagulation and affects inflammation has been implicated in the pathogenesis of CAD (Moons, Levi et al. 2002; Steffel, Luscher et al. 2006). TF is extensively expressed in all stages of atherosclerotic lesions (Landers, Gupta et al. 1994; Hatakeyama, Asada et al. 1997). TF antigen, and activity have been detected in various cell types within plaque including endothelial cells, VSMCs, monocytes and foam cells (Wilcox, Smith et al. 1989; Marmur, Thiruvikraman et al. 1996; Hatakeyama, Asada et al. 1997). Tissue factor, probably of macrophage origin, has been found in abundance in the extra cellular matrix of the necrotic core of atherosclerotic plaques (Thiruvikraman, Guha et al. 1996; Toschi, Gallo et al. 1997). TF in atherosclerotic plaques may play a major role in the initiation of thrombus formation. Fibrin deposition in the atherosclerotic intima is occasionally located around macrophages and VSMCs, which express TF in abundance (Ichikawa, Nakagawa et al. 1996). In human coronary atherectomy specimens, TF antigen concentration and activity were found to be higher in patients with myocardial infarction or unstable angina (associated with increased plaque thrombogenicity) compared to those with stable angina (Annex, Denning et al. 1995; Marmur, Thiruvikraman et al. 1996; Ardissino, Merlini et al. 1997).

Significantly higher levels of circulating soluble TF are found in patients with acute MI or unstable angina compared to patients with stable angina or healthy controls (Suefuji, Ogawa et al. 1997; Misumi, Ogawa et al. 1998; Cunningham, Romas et al. 1999; Mallat, Benamer et al. 2000). Vascular smooth muscle cells, circulating leukocytes and

aggregating platelets may be a source of elevated circulating TF level (Suefuji, Ogawa et al. 1997). Plaque rupture may also contribute to elevated plasma TF level by exposing highly procoagulant plaque content to the circulation (Mallat, Benamer et al. 2000). In patients with acute myocardial infarction elevated TF level may result from release of TF containing microparticles from the lipid core following endothelial erosion of athesclerotic lesions (Virmani, Kolodgie et al. 2000).

1.16. TFPI IN CORONARY ARTERY DISEASE (CAD)

Atherosclerotic plaque disruption, complicated by local thrombosis, is considered to be the key factor in the development of acute coronary syndrome including unstable angina and myocardial infarction. Several reports have demonstrated co- localization ofTFPI and TF in atherosclerotic plaques, suggesting a role for TFPI in the modulation of plaque thrombogenicity by attenuating TF activity (Caplice, Mueske et al. 1998; Kaikita, Takeya et al. 1999; Crawley, Lupu et al. 2000). Over expression of TFPI reduces thrombus formation after vascular injury and reduced endogenous TFPI activity may enhance thrombus formation. Studies have demonstrated beneficial effects of recombinant TFPI on TF induced arterial thrombosis (Haskel, Torr et al. 1991; Badimon, Lettino et al. 1999; Roque, Reis et al. 2000). Although the physiological aspects of TFPI suggest that its potential deficiency should lead to thrombosis (Bajaj, Birktoft et al. 2001), the relationship of TFPI and atherothrombosis is less clear. The Prospective Epidemiological study of Myocardial Infarction (PRIME) demonstrated the association of low free TFPI levels with an increased risk of coronary events (Morange, Simon et al. 2004). However elevated plasma TFPI levels have been reported in patients with ischaemic heart disease (Moor, Hamsten et al. 1994; Falciani, Gori et al. 1998; Soejima, Ogawa et al. 1999). Soejima et al reported increased plasma free TFPI levels in patients with unstable angina, and its association with an adverse outcome. The Athero Gene study also demonstrated a raised plasma free TFPI level in acute coronary syndrome when compared to those with stable angina or healthy controls (Morange, Blankenberg et al. 2007). Free plasma TFPI has been suggested to be a marker of myocardial damage in coronary disease. TFPI concentrations are markedly increased in patients with myocardial infarction (especially those with ST elevation) and there is a strong correlation of TFPI levels with troponin

levels in patients with acute coronary syndrome (Morange, Simon et al. 2004; Morange, Blankenberg et al. 2007).

1.17. TF AND TFPI IN PERIPHERAL ARTERIAL DISEASE (PAD)

In contrast to TF pathway in CAD, limited data exists on the relationship of TF and TFPI in patients with peripheral arterial disease (PAD). PAD is known to confer a prothrombotic state, with an intimate relationship between thrombogenesis and atherogenesis (Makin, Silverman et al. 2002). Recent evidence suggests TF to be considered as one possible index of a prothrombotic state (Makin, Chung et al. 2003). Increased level of TF have been reported in patients with PAD compared to healthy controls (Blann, Amiral et al. 2000; Makin, Chung et al. 2003). In contrast to reports of increased levels of TFPI activity in coronary artery disease, Blann et al. found lower level of total TFPI in patients with PAD compared to healthy controls (Sandset, Sirnes et al. 1989; Falciani, Gori et al. 1998; Blann, Amiral et al. 2000). Blann et al suggest low TFPI level to be at least in part responsible for a increased tendency of thrombosis in patients with PAD.

1.18. TF AND TFPI IN RESTENOSIS FOLLOWING ANGIOPLASTY (PTA)

The mechanical injury sustained during percutaneous transluminal angioplasty provokes a distinct biological event, which is different from spontaneous atherosclerosis (Orford, Selwyn et al. 2000). Angioplasty induced vascular injury may lead to neointimal hyperplasia which involves platelet deposition, VSMC proliferation and migration, and synthesis and deposition of extra cellular matrix. The mechanical injury may also lead to negative vascular remodelling with vessel constriction and reduced vessel lumen due to adventitial scarring. Thrombus formation and the inflammatory process play a crucial role in this vascular response to arterial balloon dilatation (Davis, Fischer et al. 2003). Inflammation can cause local thrombosis, which may again amplify inflammation (Libby and Simon 2001).

Experimental evidence suggests that TF may play an important role in the pathogenesis of restenosis following percutaneous transluminal angioplasty or bypass operations. In vitro studies have demonstrated up regulation of TF in the arterial wall and raised TF levels in the developing neointima following balloon angioplasty (Hatakeyama, Asada et al. 1998; Giesen, Fyfe et al. 2000; D'Andrea, Ravera et al. 2003). TF has been demonstrated to contribute to the inflammatory response, thrombus propagation, vascular smooth muscle cell migration and proliferation. Each of these processes are considered to be important steps in genesis of restenosis (Sato, Asada et al. 1996; Giesen, Rauch et al. 1999; Chu 2005). In animal studies, TF inhibition reduces intimal hyperplasia following angioplasty (St Pierre, Yang et al. 1999; Roque, Reis et al. 2000). Human studies have predominantly focussed on the role of TF in restenosis following coronary percutaneous transluminal angioplasty (PTA). Marcucci et al observed a positive correlation between plasma TF level and angiographically documented clinical recurrences after coronary

PTA in ischaemic heart disease patients (Marcucci, Prisco et al. 2000). Mizuno et al demonstrated that elevated levels of TF antigen in the coronary sinus blood after coronary PTA in IHD patients are associated with late restenosis (Mizuno, Ikeda et al. 2001). Extending this observation further, Tutar et al demonstrated that whole blood TF procoagulant activity was a marker for restenosis following coronary PTA and stent implantation (Tutar, Ozcan et al. 2003).

TFPI represents the only physiological inhibitor of the TF: Factor VII complex and has been implicated in the inhibition of neointima formation following balloon angioplasty in animal models (Jang, Guzman et al. 1995; Brown, Kania et al. 1996). TFPI has been suggested to inhibit neointimal growth following angioplasty by inhibiting TF induced thrombus propagation and by inhibiting VSMC proliferation and migration (Sato, Asada et al. 1997; Sato, Kataoka et al. 1999; Yutani, Imakita et al. 1999).

Chapter 2. Materials and Methods

2.1. Aim of the study

1) To determine variation in symptomatology and disease progression in unilateral claudicants awaiting femoropopliteal PTA in the waiting list for more than 3 months.

2) To determine the association between plasma TF, TFPI concentration and the development of restenosis in unilateral claudicants following femoro popliteal percutaneous transluminal angioplasty (PTA).

2.2. Design of the study

2.2.1. *Ethical approval*

A full study protocol was submitted to the Local Regional Ethics Committee- Hull and East Riding and to the NHS Research and Development Department of the Hull and East Yorkshire NHS Trust. Full ethical approval was obtained from both organisations before the start of the study.

2.2.2. *Research staff*

The staff involved in the study comprised of one Professor of Vascular Surgery, one consultant Vascular Surgeon (Senior lecturer), one Biomedical Scientist, one Vascular technician and the author of this thesis.

2.2.3. *Patient selection*

1) For assessing variation in symptomatology and disease progression 47 patients were identified that had been waiting for elective femoropopliteal PTA more than 3 months.

2) For study to determine association between plasma TF, TFPI and restenosis following femoropopliteal PTA, unilateral claudicants scheduled to undergo femoropopliteal angioplasty were included in the study after obtaining informed written consent.

2.2.4. *Control population*

Ten age and sex matched control subjects with normal ankle: brachial pressure indices

(ABPI) were recruited from patients attending for hernia repair, or other minor operations, or healthy hospital staff for measurement of plasma TF and TFPI and written informed consent was obtained.

2.2.5. *Exclusion criteria's*

1. Patients with recent M.I. (<3 months)
2. Disseminated malignancy
3. Systemic sepsis
4. Acuteor chronic renal impairment.

2.2.6. *Sample size calculation*

From previously published work it is known that the incidence of restenosis following femoropopliteal angioplasty ranges from 30-40 %. The sample size was calculated using base line mean TF concentration and a 6 month restenosis rate of 30% in previously published study (Tutar, Ozcan et al. 2003). The minimum number of patients needed for 80% power was 46. Allowing for the risk of dropouts and interventional complications the total target was set at 52 patients.

2.2.7. *Patient recruitment*

1) For assessing variation in symptomatology and disease progression, 47 patients were identified that had been waiting for elective femoropopliteal PTA more than 3 months.
2) For study to determine association between plasma TF, TFPI and restenosis following femoropopliteal PTA, all patients with unilateral claudication scheduled to undergo femoropopliteal PTA were considered for the study. Patients who met the criteria for the study were approached by the author. The purpose of the study and the follow up schedule were explained to the patient. Patients' questions were answered and they were provided with a patient information leaflet. If the patient then elected to participate in the study,

written consent was obtained.

2.2.8. *End-point*

The end point for this study was restenosis of the angioplastied segment at 6 months.

2.3. Intervention

a) For study to determine variation in symptomatology and disease progression in unilateral claudicants awaiting femoropopliteal PTA in the waiting list:

1) Case note review: A comprehensive case note review was performed for all patients. Data collected included age, gender, vascular symptoms, patient reported walking distance (PRWD), ankle brachial pressure index at rest (ABPI), initial duplex assessment, and date of acceptance to the PTA waiting list.

2) Reassessment: A clinical and imaging assessment was performed in a consultant led clinic assisted by an experienced vascular technologist. and a research fellow following informed consent. A proforma was completed for each patient. Clinical and imaging data was recorded in the proforma including claudication symptomatology (stable, improved or deteriorated), co morbidities, and patient reported walking distance (PRWD). Ankle Brachial pressure indices were measured at rest (ABPI) and an arterial duplex was performed on the symptomatic limb by an experienced vascular scientist and compared with the initial duplex assessment to assess disease progression. All arterial lesions were graded according to TransAtlantic Inter-Society Consensus (TASC) guideline.

b) For study to determine the association between plasma TF and TFPI concentration and the development of restenosis:

2.3.1 *Pre angioplasty protocol for the study group*

Patients enrolled for the study underwent assessment in the vascular research laboratory at Hull Royal infirmary. A proforma was completed for patients' symptom and risk factors

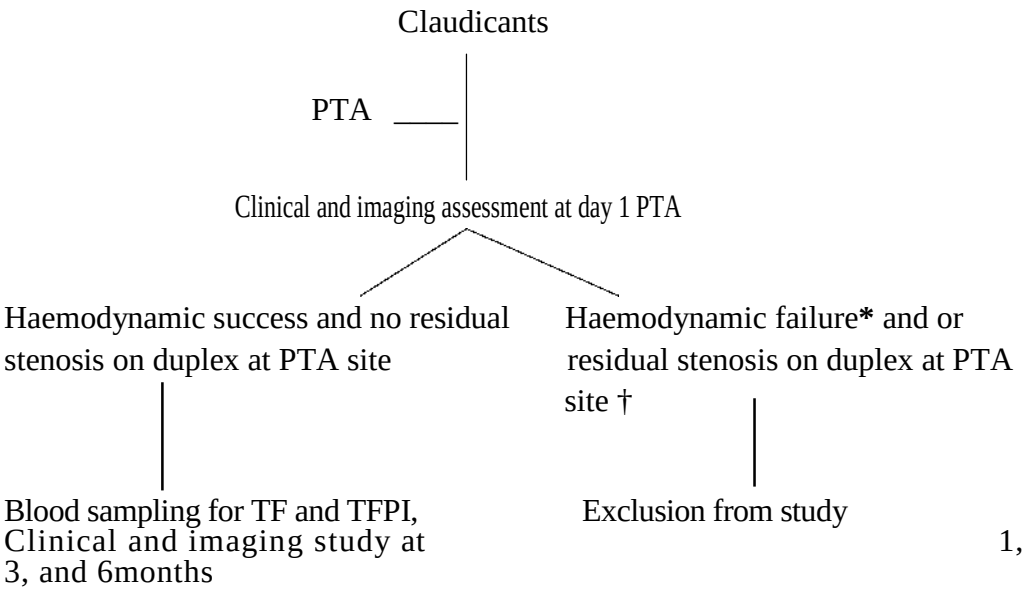
for peripheral vascular disease (hypertension, diabetes, smoking, hyperlipidemia, cardiac disease). Resting ankle brachial pressure index (or toe brachial pressure index in diabetics) and duplex ultrasound assessment of the symptomatic limb were performed and recorded in the proforma. Venous blood samples were obtained from the study group and centrifuged to obtain plasma which was stored in aliquots at -80^0 C for future measurement of TF and TFPI.

2.3.2. *Percutaneous transluminal angioplasty*: PTA were performed by experienced consultant interventional radiologists with no deviation from a standard protocol. At the end of the procedure, the radiologist marked the site of the angioplastied arterial segment on the patients skin. This angioplastied segment was then accurately recorded in the proforma by measuring the distance from a fixed bony point.

2.3.3. *Post angioplasty follow-up*

As shown in Figure 5, all patients were assessed 24 hours following femoropoliteal angioplasty. Only patients with a haemodynamically successful PTA with no significant residual stenosis were followed up at 1, 3, and 6 months. Haemodynamic success was defined by an increase in ankle brachial pressure index by at least 0.1 following angioplasty (Rutherford 1997). A significant residual stenosis was defined as as peak systolic velocity at the angioplastied segment greater than 2.5 times that of the adjacent normal arterial segment at 24hours post PTA. Assessment at 1, 3, and 6 months entailed clinical appraisal, blood sampling for TF and TFPI measurement and colour Duplex ultrasound imaging for detection of restenosis. Restenosis was defined as peak systolic velocity at the angioplastied segment greater than 2.5 times that of the adjacent normal arterial segment.

Figure 5. Post angioplasty follow-up protocol



***** No change In ABPI or an increase in ABPI of < 0.1

† Peak systolic velocity of angioplastied segment >2.5 times that of adjacent normal segment.

2.3.4. *Blood Sampling and storage*

Venous blood samples were collected from basilic vein in vacutainer test tubes containing sodium citrate. The blood samples were immediately centrifuged for 15 minutes at 3000rpm at 4^0C. The plasma thus obtained was stored in aliquots at -80^0C for subsequent analysis.

2.3.5. *Measurement of plasma TF*

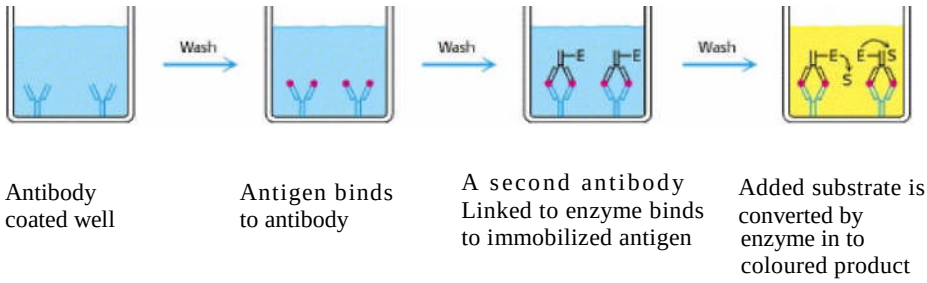
The plasma TF was measured by a highly sensitive enzyme linked immunosorbent assay (ELISA).The sandwich variant of the ELISA was used for measurement of plasma TF (Affinity Biological Inc).

Principle of Sandwich-style ELISA for TF (Fig.6)

Monoclonal antibody to Tissue Factor (TF) was coated onto the wells of a micro titre plate. Any remaining binding sites on the plastic wells were blocked with an excess of bovine serum albumin. The plates were washed and biological fluids containing TF were applied. The coated antibody captured the TF in the sample. After washing the plate to remove unbound material, a peroxidase conjugated polyclonal antibody to TF was added to the plate to bind to the captured TF. After washing the plate to remove unbound conjugated antibody, the peroxidase activity was expressed by incubation with o-phenylenediamine (OPD). After a fixed development time the reaction was quenched with the addition of H_2SO_4 and the colour produced was quantified using a micro plate reader. The colour generated is proportional to the concentration of TF present in the sample.

Figure 6. Principle of Sandwich ELISA

R. A. Goldsby, T. J. Kindt, B. A. Osborne, *Kuby Immunology,* 4th ed. (W. H. Freeman and Company, 2000), p. 162.



In the Sandwich ELISA technique, Plate is coated with known amount of capture antibody and any nonspecific binding site is blocked with blocking buffer. An antigen containg sample is added to the plate to allow the antigen to bind to the capture antibody. Plate is then washed to remove unbound antigens. A detecting antibody is then added, which binds to the antigen. An Enzyme-linked secondary antibody is then added, that binds to the detecting antibody. A substrate is added, and is converted by the enzyme to a detectable coloured form.

Materials and reagents for assay

1. Capture Antibody (Affinity Biologicals^{TM})
2. Detecting Antibody (Affinity Biologicals^{TM})
3. Purified recombinant apo-tissue factor (American Dignostica)
4. Coating Buffer: 50 mM Carbonate(pH 9.6)
5. PBS (pH7.4)
6. Wash Buffer: PBS-Tween (pH 7.4)
7. Blocking Buffer: PBS-BSA (pH 7.4)
8. Sample Diluent: HBS-TX100 (pH 7.2)
9. Conjugate Diluent: HBS-BSA-T20
10. Substrate Buffer (pH 5.0)
11. OPD Substrate (Sigma # P-69 12)
12. Stopping solution: 2.5M H_2SO_4
13. 96 well microplates, microplate washer, microplate reader

Assay Procedure:

1. Coating of plates:

Capture antibody was diluted to 1/100 in coating buffer and immediately 100 µl added to every well in the plate. The plate was then incubated overnight at 4^{o}C.

2. Blocking:

Contents of plate were emptied and 150 µl of blocking buffer added to every well and incubated for 90 minutes at 22^{o}C. Plate was then washed 3 times with wash buffer.

3. Preparation of Reference Standards and Test Samples:

Reconstituted the vial of recombinant apo-TF to 10 µg/ml in water (unused material frozen in aliquots and stored at -70^{o}C). The stock TF 1/100 was further diluted in

sample diluent to achieve a final concentration of 100 ng/ml, then serial 1/2's down to 1/3200 (3.13 ng/ml). Test samples were diluted 1/4 and 1/8. All dilutions were made in sample diluent. 100 µl/well was applied and the plate was incubated at 22^{o}C for 60 minutes. The plate was then washed three times with wash buffer.

4. Detecting Antibody:

Detecting antibody was diluted 1/100 in conjugate diluent. 100 µl of this was then applied to each well and incubated the plate at 22^{0}C for 60 minutes. The plate was then washed three times with wash buffer.

5. OPD Substrate:

100 µl of freshly prepared OPD substrate was applied to every well. Colour was allowed to develop for 5-10 minutes and then the colour reaction was stopped with addition of 50 µl/well of 2.5 M H2SO4. The plate was then read at wavelength of 490 nm. 6. Calculation of Results:

The standard curve was constructed by plotting the mean absorbance value for each TF standard versus the corresponding concentration of TF in ng/ml. The TF concentrations for diluted samples were interpolated directly from the standard curve. A standard curve was generated each time the assay was performed.

2.3.6. *Measurement of plasma TFPI*

The plasma TFPI was measured by a highly sensitive enzyme linked immunosorbent assay (American Diagnostica Inc.) This ELISA detects both intact and truncated forms of TFPI as well as complexes with tissue Factor (TF) and factor VIIa (TF/VIIa/TFPI).

Principle of Sandwich-style ELISA for TFPI

The IMUBIND Total TFPI ELISA is a "sandwich" ELISA employing a rabbit antihuman TFPI polyclonal antibody as the capture antibody. Specificity of the capture antibody for native, complexed and truncated TFPI was confirmed by Western blot analysis, visualizing a single band at 34 kDa, corresponding to the mobility of intact native TFPI and visualizing a single band at 21 kDa, corresponding to the mobility of a truncated form of TFPI. Diluted plasma samples incubated in micro-test wells precoated with this capture antibody. TFPI is detected using a biotinylated monoclonal antibody specific for the specific domain of TFPI. The subsequent binding of the streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody enzyme detection complex. The addition of TMB substrate and its subsequent reaction with HRP provided a blue colour. Sensitivity was increased by addition of a 0.5M sulphuric acid stop solution, yielding a yellow colour. TFPI levels were determined by measuring sample solution absorbance at 450 nm and comparison against those of a standard curve developed using native TFPI.

Materials and reagents for assay

1. TFPI Standard, 5 ng/mL (lyophilized)
2. TFPI Depleted Plasma (lyophilized)
3. TFPI Reference Plasma (lyophilized)

4. Detection Antibody, biotinylated anti-human TFPI F (ab') 2 (lyophilized)

5. Enzyme Conjugate, Streptavidin-horseradish peroxidase

6. Enzyme Conjugate Diluent (lyophilized)

7. TMB substrate

8. Wash Buffer (pH 7.4)

9. 0.5M H_2SO_4

10. Bovine Serum Albumin (BSA, e.g. Sigma A-7030)

11. Micro-test plate reader, 6 x 16 well precoated micro-test strips with holder and lid

Sample preparation

1. Frozen plasma samples collected from the study group were thawed at 37°C for 15 minutes.

2. These plasma samples and the TFPI reference Plasma were diluted 1:40 in Sample Buffer.

Assay procedure

Day one

The precoated micro-test strips were placed on a plate holder and 100 µL of TFPI Standard, TFPI Reference Plasma or diluted sample were added to micro-test wells, covered with lid and incubated overnight at +4°C. Measurements were performed in duplicate.

Day 2

1. The wells were washed 4 times with Wash Buffer.

2. 100 µl of Detection Antibody was added to each well, covered with lid and incubated for 1 hour at room temperature.

3. Wells were washed 4 times with Wash Buffer.

4. 12 µL of Enzyme Conjugate was added to 12 mL of Enzyme Conjugate Diluent. 100 microlitre of diluted enzyme conjugate was added to each well, covered with lid and then incubated for one hour at room temperature.

5. Wells were washed 4 times with Wash Buffer.

6. 100 µL of Substrate solution was added to each well, and covered with lid and incubated for 20 minutes at room temperature. A blue colour developed.

7. The enzymatic reaction was stopped by adding 50 µL of 0.5M H_2SO_4. The solution colour turned yellow. The absorbances were read on a micro-test plate reader at a wavelength of 450 nm within 30 minutes. The background average of the blanks was deducted from the standards and sample readings.

Calculation of results

The standard curve was constructed by plotting the mean absorbance value for each TFPI standard versus the corresponding concentration of TFPI in ng/mL(Figure 7). The TFPI concentrations for diluted samples were interpolated directly from the standard curve. A standard curve was generated each time the assay was performed.

TFPI concentrations obtained for each test sample, as interpolated from the standard curve were averaged and multiplied by the dilution factor of the sample(40 in this study) to calculate the TFPI concentration of the original sample.

Figure 7. Standard curve for TFPI measurement

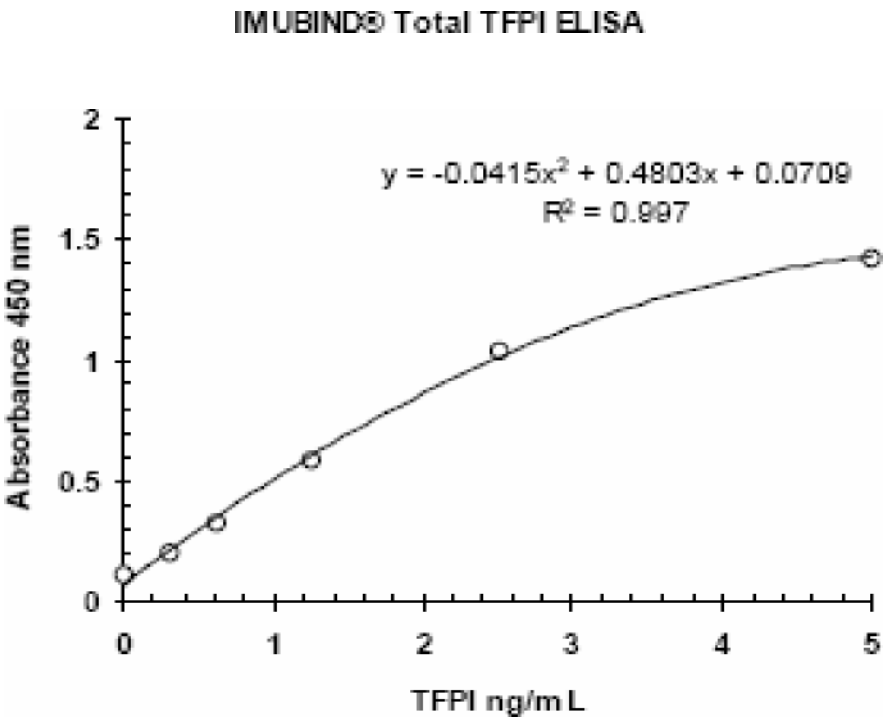


Figure shows a standard curve used in quantitave ELISA used for measurement of plasma TFPI. The standard curve was constructed by plotting the mean absorbance value for each TFPI standard versus the corresponding concentration of TFPI in ng/mL followed by plotting a curve through the points. Microsoft excel 2003 was used for plotting the standard curve in our study.

2.3.7. *Internal validation of effect of storage on plasma TF and TFPI level*

Plasma samples were stored at -80^{0}C and analysed in batches at a later date. Internal validation was performed to observe the effect of storage on plasma TF and TFPI levels. Separate batches of stored plasma samples of ten individuals were analysed for TF and TFPI on fresh samples and at one month and two months after storage.

2.4. Statistical analysis

2.4.1. *For determination of variation in symptomatology and disease progression in claudicants awaiting PTA:* for more than 3 months: All continuous variables were reported as median values with interquartile range (IQR). Discrete variables are presented as counts and percentages. Mann-Whitney U tests were used to compare unpaired continuous variables and Wilcoxon paired tests were used to analyze paired continuous variables. A two sided p-value <0.05 was considered statistically significant. All statistical analysis was performed using SPSS for Windows (version 11.5, SPSS Inc., IL, USA).

2.4.2. *For determination of effect of storage on plasma TF and TFPI levels:* The averages of assay values for TF and TFPI for each ten individuals were analysed using Friedman's test. The tests were considered significant if p value was less than 0.05.

2.4.3. *For determination of the association between plasma TF and TFPI concentration and the development of restenosis:* Plasma TF and TFPI concentrations were reported as median values with interquartile range (IQR). Nonparametric ANOVA test was used to compare plasma TF or TFPI concentration with occurrence of restenosis. The association of plasma TF and TFPI with occurrence of restenosis was analysed by Mann Whitney U test. Analysis for the difference of TF and TFPI levels from the pre PTA baseline levels at different intervals following PTA were done separately for the patent and restenotic groups using Wilcoxon's sign rank test for two paired samples. The tests were considered significant if p value was less than 0.05. All statistical analysis was performed using SPSS for Windows (version 11.5, SPSS Inc., IL, USA).

Chapter 3. Results

3.1 VARIATION IN PATIENT SYMPTOMATOLOGY AND DISEASE DISTRIBUTION IN CLAUDICANTS AWAITING FEMOROPOPLITEAL PTA

3.1.1 Epidemiology

Forty seven claudicants (M: F; 34:13), median age 69 (interquartile range 46-89) years with a median waiting period of 9.6 (interquartile range 4-21) months for underwent the reassessment. Patients' epidemiological characteristics, smoking habit and associated co morbidities based on case note review and initial assessment are summarised in Table 4.

Table 4. Study population characteristics and co morbidities

Median Age (IQR), years		69(46-89)
	No. of patients	Percentage
Gender		
Male	34	72.3
Female	13	27.7
Hypertension	30	63.8
Diabetes	9	19.1
Cardiac disease	13	27.7
Renal disease	0	0
Carotid disease	9	19.1
COPD*	10	21.2
Arthritis	11	21.2
Tobacco use	29	61.7

*Chronic obstructive pulmonary disease

3.1.2. Comparison of symptom and disease progression at reassessment of claudicants awaiting PTA.

At reassessment, 30 (64%) patients were found to be symptomatically stable whilst 10(21%) and 7(15%) had improved or deteriorated respectively from baseline.

Duplex USS performed at reassessment demonstrated 9 (19%) TASC A, 15 (32%) TASC B, 19 (40%) TASC C and 4 (9%) TASC D lesions of the index limb (planned for PTA). Comparison with baseline duplex ultrasound demonstrated a disease progression in 9(19%) patients. The majority remained stable (Table 5). There was a significant association between waiting time and change in symptomatology ($p=0.008$, Mann Whitney U test); however no significant association was found between the waiting time and change in disease pattern at baseline and reassessment ($p=0.88$, Mann Whitney U test).

Table 5. Waiting period, symptom and disease progression at reassessment of claudicants awaiting PTA.

Median waiting period (IQR), months	9.6 (4- 21)	
Symptom at assessment	No. of patients	Percentage
Stable	30	64.0
Improved	10	21.3
Deteriorated	7	14.8
Disease assessment on duplex USS		
Stable	38	80.9
Deteriorated	9	19.1

* Data reported as median and interquartile range (IQR).

Table showing comparison of claudication symptom and duplex USS assessment in patients awaiting femoropopliteal PTA at baseline and at reassessment.

At reassessment, 64% patients were found to be symptomatically stable whilst 21% and 15% had improved or deteriorated respectively from baseline.

Comparison of duplex USS finding at baseline and at reassessment showed disease progression in 19% patients while majority remained stable. There was a significant association between waiting time and change in symptomatology (p=0.008, Mann Whitney U test); however no significant association was found between the waiting time and change in disease pattern at baseline and reassessment (p=0.88, Mann Whitney U test).

3.1.3. Comparison of Walking distance and ABPI in claudicants awaiting PTA at baseline and reassessment (Table 6)

The median baseline patient reported walking distance (PRWD) was 100m (interquartile range 0-400) while at the time of reassessment, the median PRWD was 100m (interquartile range 10-1500) ($p=0.19$, Mann Whitney U test).

The median resting ABPI was 0.65 (interquartile range 0.41-0.98) at baseline and 0.71 (interquartile range 0.48-1.09) at reassessment ($p=0.06$, Mann Whitney U test).

Claudicants who remained stable, demonstrated stable PRWD and ABPI at reassessment. Claudicants who improved symptomatically, demonstrated significant improvement in PRWD and ABPI at reassessment ($p<0.05$, Mann Whitney U test) . Claudicants who symptomatically deteriorated, demonstrated a significant deterioration in PRWD but no significant deterioration in ABPI at reassessment.

Table 6. Comparison of Walking distance and ABPI in patients with stable claudication, clinical improvement, and clinical deterioration at baseline and reassessment.

	Baseline	At assessment	P-value (Mann Whitney U)
Patients with stable claudication (n= 30)			
Walking distance	100(20-400)	100(50-800)	0.37
ABPI	0.70(0.42-0.98)	0.66(0.48-0.99)	0.89
Patients with clinical improvement (n=10)			
Walking distance	100(20-400)	350(50-1500)	0.018
ABPI	0.61(0.41-0.72)	0.84(0.65-1.09)	0.009
Patients with clinical deterioration (n=7)			
Walking distance	200(100-400)	50(10-200)	0.04
ABPI	0.60(0.53-0.75)	0.68(0.58-0.82)	0.59

*Data reported as median and interquartile range (IQR).

Table showing comparison of patient reported walking(PRWD) distance and ABPI in claudicants who remained symptomatically stable, improved or deteriorated at reassessment. Claudicants who remained stable, demonstrated stable PRWD and ABPI at reassessment. Claudicants who improved symptomatically, demonstrated significant improvement in PRWD and ABPI at reassessment ($p<0.05$, Mann Whitney U test) . Claudicants who symptomatically deteriorated, demonstrated a significant deterioration in PRWD but no significant deterioration in ABPI at reassessment.

3.2 PLASMA TF, TFPI AND RESTENOSIS FOLLOWING FEMOROPOPLITEAL PTA

3.2.1. Epidemiology

52 claudicants (M: F; 3:1), median age 69 (interquartile range 46-86) years underwent initial assessment prior to angioplasty. Patients' epidemiological characteristics, smoking habit and associated co morbidities are summarised in Table 7.

3.2.2. Patient symptomatology and lesion characteristics

The median patient reported walking distance (PRWD) of the study group was 100 (interquartile range 50-180) meters and the median resting ABPI was 0.65 (interquartile range 0.50-0.83). Duplex performed at initial assessment demonstrated 16 (30.8%) TASC A lesions, 19(36.5%) TASC B lesions, and 17 (32.7%) TASC C lesions of the index limb planned for PTA (Table 8).

Table 7. Demographic profile of the study population

Median Age (IQR), years		69(46-82)
Male: Female		3:1
	No. of patients	Percentage
Hypertension	26	53.1
Diabetes	7	14.3
Cardiac disease	11	22.4
Carotid disease	2	4.1
COPD*	8	16.3
Tobacco use	28	57.1
Hyperlipidaemia	17	34.7

*Chronic obstructive pulmonary disease

Table 8. TASC grading for femoropopliteal disease of the index limb*

TASC grade	Lesion Characteristics	Percentage study population
TASC A	▪ Single stenosis up to 3 cm; Not at SFA origin or distal popliteal artery.	30.8%
TASCB	▪ Single stenosis/occlusion 3-5 cm; not at distal popliteal artery. ▪ Heavy calcified stenosis up to 3 cm. ▪ Multiple lesions, each < 3 cm ▪ Single/ Multiple lesions in absence of continuous tibial runoff.	36.5%
TASC C	▪ Single stenosis/occlusion > 5 cm ▪ Multiple Single stenosis/occlusions each 3-5 cm with or without heavy calcification.	32.7%
TASC D	Complete CFA or SFA occlusions or complete popliteal and proximal trifurcation occlusions	0

***** TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). J Vasc Surg, 2000.

3.2.3. Femoropopliteal PTA and outcome in the follow up assessments:

Of the 52 patients enrolled in the study, 49 claudicants underwent successful femoropopliteal PTA.Technical failure occurred in three (5.7%) patients, due to inability to cross the lesion with a guide wire. Majority of the angioplatied segments were were located in the SFA. ABPI and duplex assessment of the angioplastied limb at 24 hours following PTA, identified thrombotic occlusion in two patients. Overall 47 patients hence underwent further follow up for detection of restenosis. 16 claudicants (32.6%) developed restenosis within 6 months following angioplasty. Based on ankle brachial index, haemodynamic failure was noted in 19 patients. The relationship of the PTA type, restenosis and haemodynamic failure are summarised in Tables 9-13.

Table 9. Femoropopliteal Percutaneous Ttransluminal Angioplasty in the study group

Procedural success Haemodynamic success following PTA*		49 47
PTA type	No. of patients	Percentage
Superficial femoral artery (SFA)	22	44.9
SFA- Popliteal	17	34.7
Popliteal(POP)	5	10.2
SFA/POP with TPT **	5	10.2

* Defined as an increase in the ABPI by at least 0.1 at 24 hours following PTA

** Tibio peroneal trunk

Table 10. Duplex USS assessed outcome of angioplastied limb following femoropopliteal PTA

PTA	Follow up			Total
	Patent	Restenosis**	Thrombotic occlusion*	
SFA	15	7	0	22
SFAPOP	11	5	1	17
POP	2	3	0	5
SFA/ POP-TPT	3	1	1	5
Total	31	16	2	49

The restenosis rate in patients who underwent SFA PTA only and those undergoing SFA and popliteal artery PTA were comparable (31% vs 29%). Patients undergoing popliteal artery PTA had the highest incidence of restenosis (60%). Although 20% of patients undergoing SFA/ POP-TPT developed restenosis, 20% had post procedural thrombotic occlusion.

* Thrombotic occlusion within 24 hour following PTA; Not followed up further.

** Restenosis as identified on duplex imaging.

Table 11. Haemodynamic outcome following femoropopliteal PTA

	Haemodynamic outcome			Total
	Haemodynamic success	Haemodynamic failure*	Early haemodynamic failure**	
SFA	15	7	0	22
SFAPOP	8	8	1	17
POP	2	3	0	5
SFA/POP-TPT	3	1	1	5
Total	28	19	2	49

Overall haemodynamic failure following PTA was 40%. Majority of the haemodynamic failures occurred in patients undergoing popliteal or popliteal and tibioperoneal trunk (TPT) PTA. 2 patients had thrombotic occlusion following PTA and were not followed up for restenosis.

* > 0.1 reduction of ABPI from the post PTA ABPI (Rutherford et al. 1997).

** Thrombotic occlusion within 24 hour following PTA; not followed up further.

Table 12. TASC grading and restenosis following femoropopliteal PTA

	TASC A	TASC B	TASC C	Total
Patent	11	13	7	31
Restenosis	5	5	6	16
Total	16	18	13	47

Highest incidence of restenosis was seen in claudicants undergoing PTA of TASC C lesions. Outcome following PTA of TASC A and TASC B lesions were comparable.

Table 13. Correlation between restenosis and haemodynamic outcome* following femoropopliteal PTA

		Haemodynamic outcome		
		Success	Failure	Total
Duplex USS follow up	Patent	20	11	31
	Restenosis	8	8	16
Total		28	19	47

Haemodynamic outcome at follow-up intervals, after PTA did not correlate with the occurrence of restenosis as identified by duplex ultrasound. 35% (11/31) of the claudicants whose PTA site remained patent at 6 month had haemodynamic failure. 50 % (8/16) of the claudicants who developed restenosis did not have haemodynamic failure.

3.2.4. Effect of plasma sample storage on TF and TFPI level

As samples were analysed in batch, the results for separate bath of stored samples were analysed on fresh samples and after storage for one and two months to observe the effect of storage on plasma TF and TFPI levels.

The three averages of the two assay values for TF for each 10 individuals, analysed using Friedman's test showed no statistically significant change of plasma TF levels over time during storage ($p= 0.721$; Friedman's test; Fig. 8). Similar analysis for plasma TFPI also did not show significant change over time ($p= 0.368$; Friedman's test ; Fig. 9).

Fig. 8. Plasma TF levels at different time points during storage at -80^{0}C

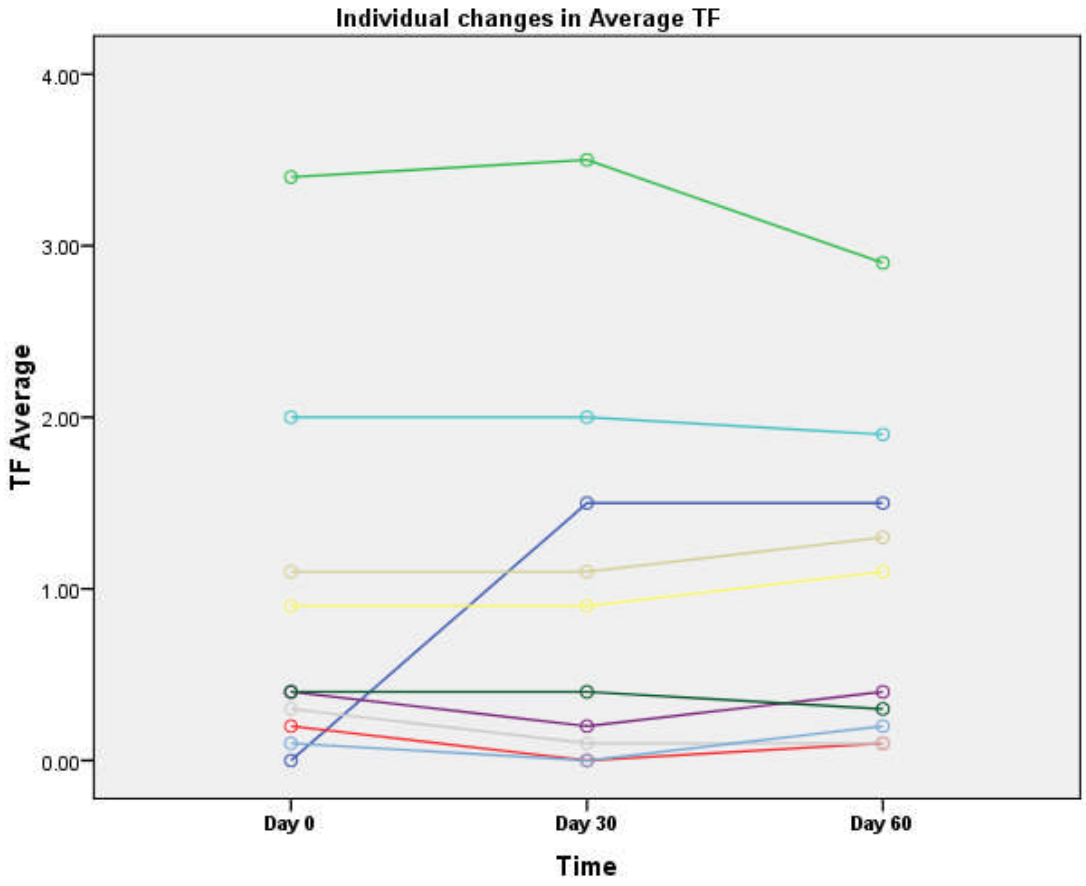


Fig 8 shows a line chart showing plasma TF levels at different interval during storage of plasma samples at -80^{0}C. No statistically significant change of TF levels was observed over time (p= 0.721, Friedman's test).

Fig. 9. Plasma TFPI levels at different time points during storage at -80^{0}C

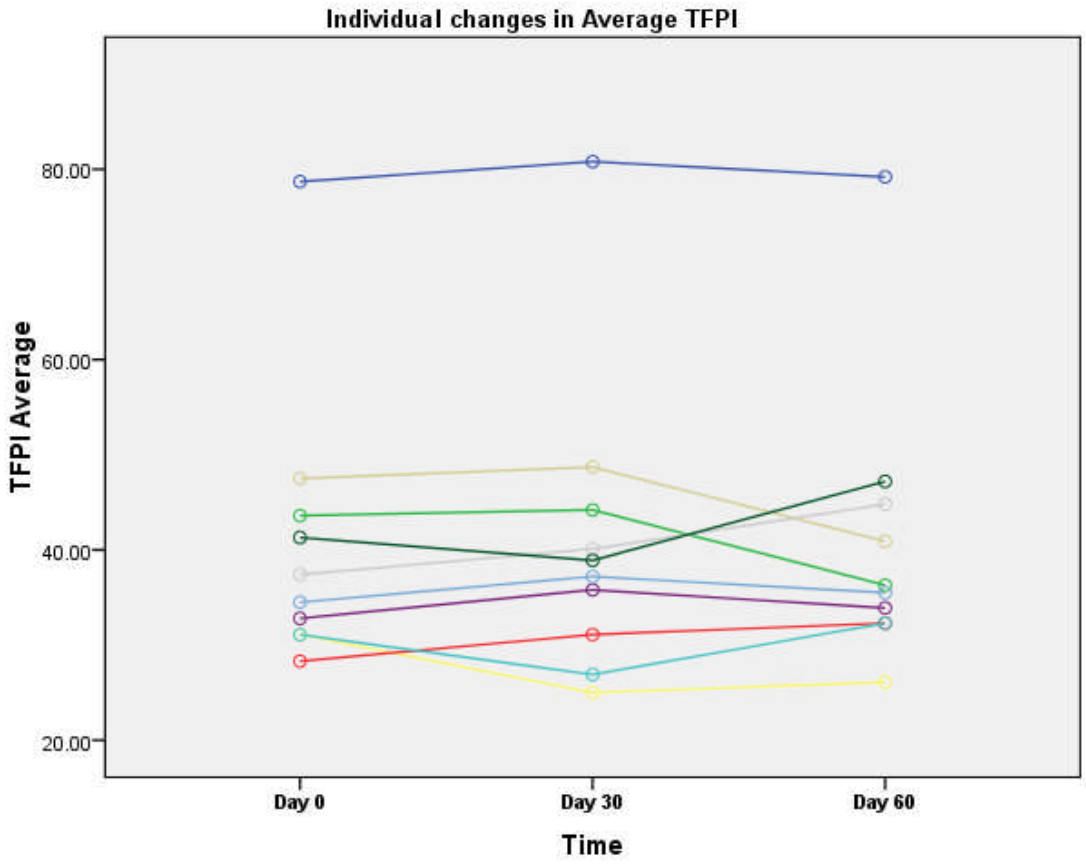
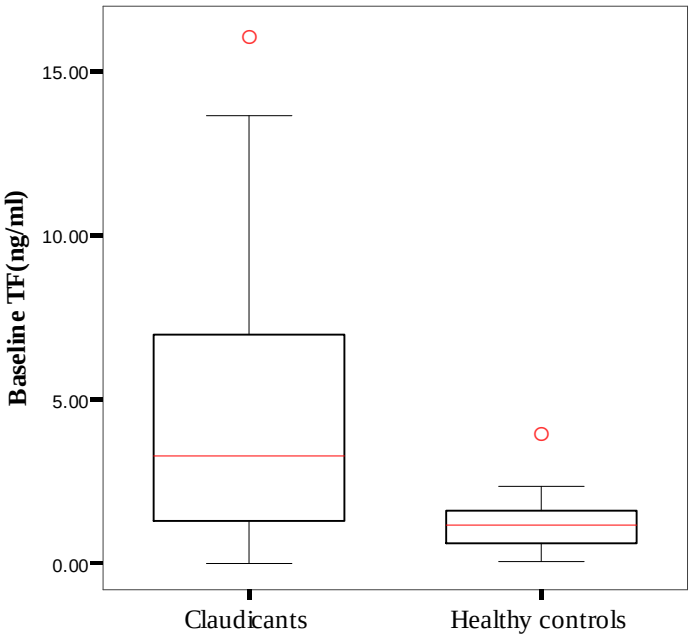


Fig 9 shows a line chart showing plasma TF levels at different interval during storage of plasma samples at -80^{0}C. No statistically significant change of TF levels was observed over time (p= 0.368, Friedman's test).

3.2.5. Plasma TF levels in healthy control and claudicants at baseline.

Plasma TF levels in healthy controls and in the claudicants before PTA are shown in Figure 10. Claudicants demonstrated significantly higher baseline plasma TF than control subjects ($p<0.05$, Mann-Whitney U). The median plasma TF concentration in claudicants was 3.3ng/ml (interquartile range 1.3-7.1). The plasma TF concentration in healthy controls was median 1.2 ng/ml (interquartile range 0.4-1.8).

Figure 10. Plasma TF in healthy controls and claudicants

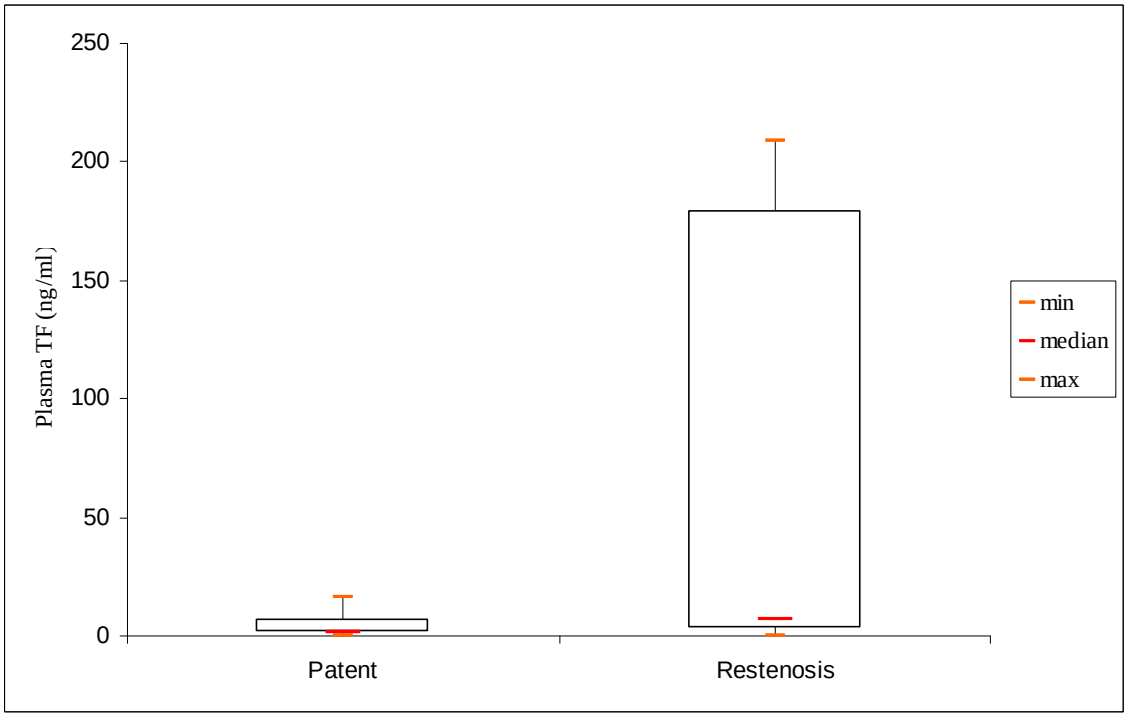


Box whisker plot showing difference between plasma TF concentration in healthy controls and claudicants. The box represents the interquartile range (IQR) and the horizontal line in the box being the median value. The whiskers represent the minimum and maximum range of the data. Claudicants demonstrated significantly higher baseline plasma TF than control subjects ($p<0.05$, Mann-Whitney U).

3.2.6. Baseline (pre PTA) Plasma TF levels in claudicants undergoing femoro-popliteal PTA and its relationship with restenosis.

In claudicants, baseline TF levels were significantly higher in those who developed restenosis following PTA than those who did not (p=0.03, Mann-Whitney U) – Figure 11. The median baseline value of plasma TF concentration in claudicants who remained patent following PTA was 1 .64ng/ml (1.3-7.2), whilst in those developing restenosis the median baseline plasma TF concentration was 6.96ng/ml (3.36-179).

Fig. 11. Baseline plasma TF levels in claudicants underoing femoropopliteal PTA and its relationship with restenosis.



Box whisker plot showing difference between plasma TF concentration in claudicants undergoing femoropopliteal PTA. The box represents the interquartile range (IQR) and the horizontal line in the box being the median value. The whiskers represent the minimum and maximum range of the data. The baseline TF levels were significantly higher in those who developed restenosis following PTA than those who did not (p=0.03, Mann-Whitney U).

3.2.7. Changes in plasma TF concentration following femoropopliteal PTA

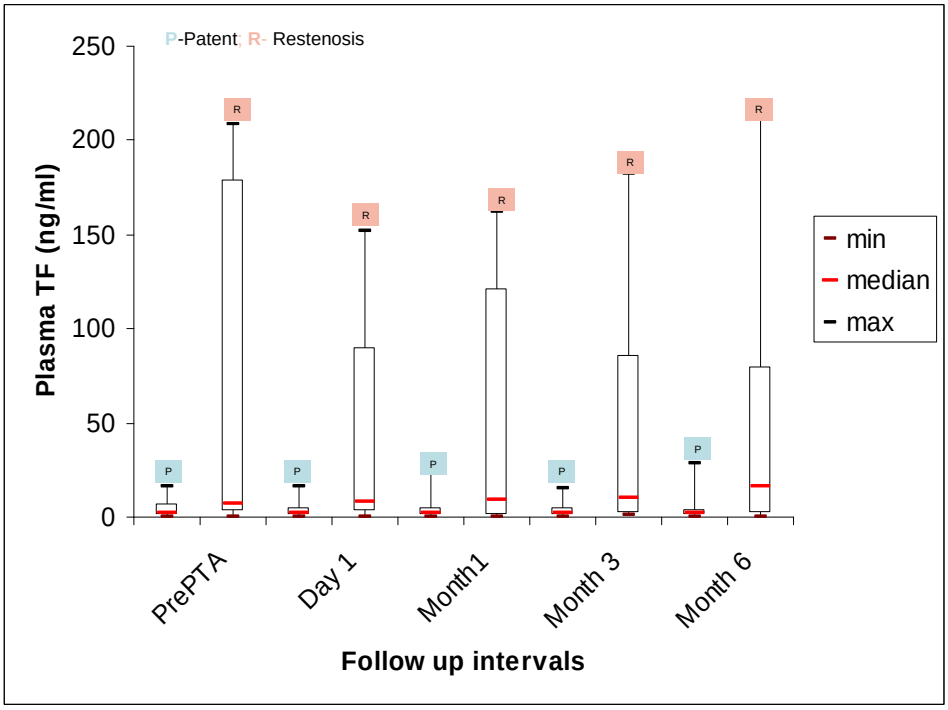
Plasma TF levels at baseline prior to angioplasty, and at 24 hours, 1 month, 3 months and 6 months following angioplasty are displayed in Table 14 and Figure 12. Plasma TF concentrations at all follow up visits were raised in patients who restenosed, compared to those who remained patent. The differences between those who restenosed and those who remained patent were statistically significant except at month 1 following PTA. The difference of TF concentrations at followup intervals from the baseline levels in either of the groups were not found to be statistically significant ($p>0.05$, Wilcoxon's sign rank test)-Table15.

Table 14. Plasma TF levels of the Claudicants at baseline and post PTA follow up intervals

	Median TF conc. in ng/ml (interquartile range)	Mean rank	p (Mann Whitney U)
PRE PTA			
Patent	1.64 (1.3-7.2)	16.33	0.03
Restenosis	6.96 (3.3-179)	25.00	
DAY1			
Patent	2.40 (1.2-4.8)	16.52	0.05
Restenosis	8.44 (2.5-89.9)	24.44	
MONTH 1			
Patent	1.98 (0.9-4.6)	15.92	0.12
Restenosis	9.06 (0.6-121.6))	21.89	
MONTH 3			
Patent	2.43 (1.2-4.7)	16.44	0.04
Restenosis	10.07 (2.5-86.3)	24.67	
MONTH 6			
Patent	1.99 (1.1-4.1)	16.33	0.03
Restenosis	16.16 (1.7-79.6)	25.00	

Table showing plasma TF levels of patients who remained patent and those who developed restenosis following PTA at baseline and at post PTA follow up intervals. The TF levels were significantly higher in those who developed restenosis following PTA at all the follow up intervals except at month 1, than those who remained patent ($p<0.05$, mann whitney U test).

Figure 12. Plasma TF concentration at baseline and at intervals following PTA



Box and whisker plot showing baseline plasma TF concentrations in claudicants undergoing femoropopliteal PTA and relationship with restenosis. The box represents the interquartile range and the horizontal line in the box being the median value. The whiskers represent the range of the data. The TF levels were significantly higher in those who developed restenosis following PTA at all the follow up intervals except at month 1, than those who remained patent ($p<0.05$, mann whitney U test).

Table 15. The difference of TF concentrations from pre angioplasty TF levels at follow up intervals follwing PTA.

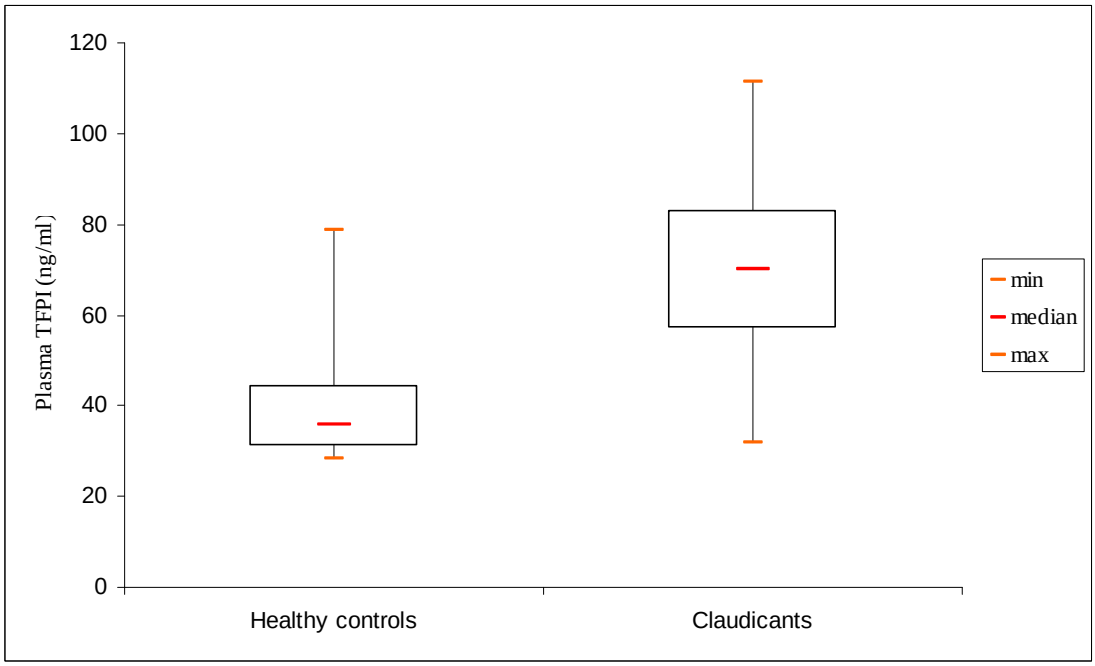
		Negative mean ranks	Positve mean ranks	P (Wilcoxon sign rank test)
Day1	Patent	11.29	7.4	0.90
	Restenosis	10.14	7.22	0.88
Month 1	Patent	9.33	8.62	0.72
	Restenosis	9.50	7.50	0.68
Month 3	Patent	12.89	7.40	0.39
	Restenosis	9.71	7.56	1.00
Month 6	Patent	11.36	8.12	0.23
	Restenosis	7.12	9.88	0.57

Wilcoxon sign rank test based on ranking the difference between baseline (pre PTA) plasma TF and TF levels at different time points following PTA. Calculations performed separately for the groups who remained patent and the ones who developed restenosis. The difference of TF concentrations at all followup intervals from the baseline levels in either of the groups were not found to be statistically significant ($p>0.05$, Wilcoxon's sign rank test).

3.2.8. Plasma TFPI in healthy controls and claudicants at baseline

Plasma TFPI levels in healthy controls and in the claudicants before PTA are shown in Figure 13. Claudicants demonstrated significantly higher baseline plasma TFPI than control subjects (p=0.001, Mann-Whitney U). The median plasma TFPI concentration in claudicants was 69.97ng/ml (interquartile range 57.13- 83.11). The plasma TFPI concentration in healthy controls was median 35.95 ng/ml (interquartile range 31.10-44.57).

Figure 13. Plasma TFPI in healthy controls and claudicants at baseline

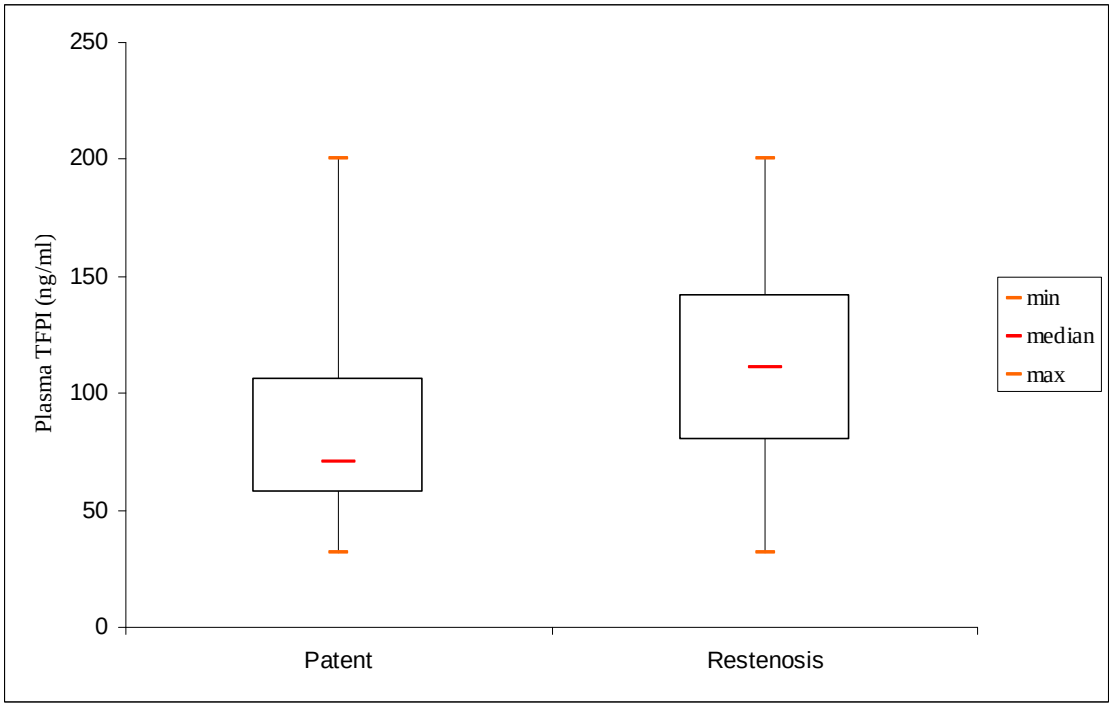


Box whisker plot showing difference between plasma TFPI concentration in healthy controls and claudicants. The box represents the interquartile range (IQR) and the horizontal line in the box being the median value. The whiskers represent the minimum and maximum range of the data. Claudicants demonstrated significantly higher baseline plasma TFPI than control subjects (p=0.001, Mann-Whitney U).

3.2.9. Baseline (pre PTA) Plasma TFPI levels in claudicants undergoing femoropopliteal PTA and its relationship with restenosis

In claudicants, baseline TFPI levels were significantly higher in those who developed restenosis following PTA than those who did not (p=0.05, Mann-Whitney U test) – Figure 14. The median value of plasma TFPI concentration in claudicants who remained patent following PTA was 70.64ng/ml (57.3-106.25); while in those developing restenosis the median plasma TFPI concentration was 111 .36ng/ml (80-142).

Figure 14. Baseline plasma TFPI concentration (ng/ml) in claudiants undergoing femoropopliteal PTA



Box and whisker plot showing baseline plasma TFPI concentration in claudicants undergoing femoropopliteal PTA and relationship with restenosis. The box represents the interquartile range (IQR) and the horizontal line in the box being the median value. The whiskers represent the range of the data. The baseline TFPI levels were significantly higher in those who developed restenosis following PTA than those who did not (p=0.05, Mann-Whitney U test).

3.2.10. Change in plasma TFPI concentration following PTA

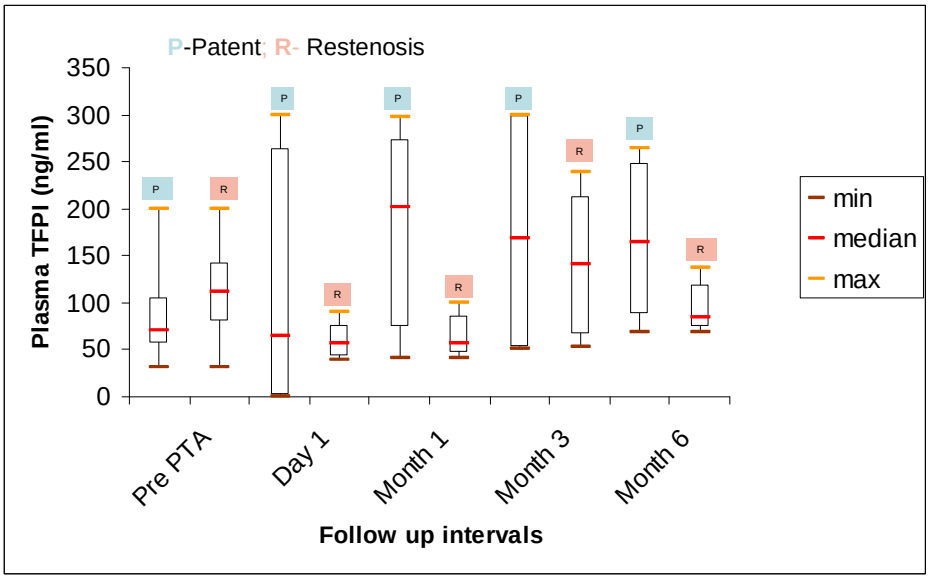
The baseline (Pre-PTA) plasma TFPI concentration was found to be significantly higher in claudicants who developed restenosis following PTA compared to those whose angioplasty site remained patent. Conversely, the TFPI levels were significantly lower in the restenosis group at month 1 and 6 following PTA. The group differences did not reach statistical significance at day 1 and month 3 following PTA (Table 16 and Fig.15). The difference of TFPI concentrations at followup intervals from the baseline levels in either of the groups were not found to be statistically significant ($p>0.05$, Wilcoxon's sign rank test) except the borderline significance at month 1 following PTA for the group who did not develop restenosis (Table 17).

Table 16. Plasma TFPI level of the Claudicants at baseline and post PTA follow up intervals

	Median TFPI conc. in ng/ml (inter quartile range)	Mean rank	p (Mann Whitney U)
PRE PTA Patent Restenosis	 70.64 (57.3-106.2) 111.36 (80.1-142.2)	 13.31 19.73	0.05
DAY1 Patent Restenosis	 64.14 (2.73-264.72) 56.46 (42.3-77.1)	 9.0 23.38	0.33
MONTH 1 Patent Restenosis	 201 (73.5-272.9) 57.46 (46.6-86.1)	 15.8 20.75	0.02
MONTH 3 Patent Restenosis	 169.06 (52.45-300) 140 (65.67-212.42)	 16.11 24.38	0.6
MONTH 6 Patent Restenosis	 164.2 (87.6-249.1) 84.30 (74.6-118.6)	 16.19 24.13	0.03

Table showing plasma TFPI levels of patients who remained patent and those who developed restenosis following PTA at baseline and at post PTA follow up intervals. The TFPI levels were significantly higher in those who developed restenosis following PTA at baseline, but significantly lower at month 1 and month 6 following PTA (Mann whitney U test).

Figure 15. Change in plasma TFPI concentration following PTA



Box and whisker plot showing baseline plasma TFPI concentration in claudicants undergoing femoropopliteal PTA and relationship with restenosis. The box represents the interquartile range and the horizontal line in the box being the median value. The whiskers represent the range of the data. The baseline TFPI levels were significantly higher in those who developed restenosis following PTA than those who did not (p=0.05, Mann-Whitney U test).

Table 17. The difference of TFPI concentrations from pre angioplasty TFPI levels at follow up intervals follwing PTA.

		Negative mean rank	Positve mean rank	P (Wilcoxon sign rank test)
Day1	Patent	3.00	3.00	0.6
	Restenosis	2.00	2.59	0.59
Month 1	Patent	2.00	5.00	0.04
	Restenosis	3.50	2.00	0.28
Month 3	Patent	1.00	3.50	0.08
	Restenosis	1.00	2.50	0.28
Month 6	Patent	2.00	3.40	0.14
	Restenosis	2.00	2.23	0.10

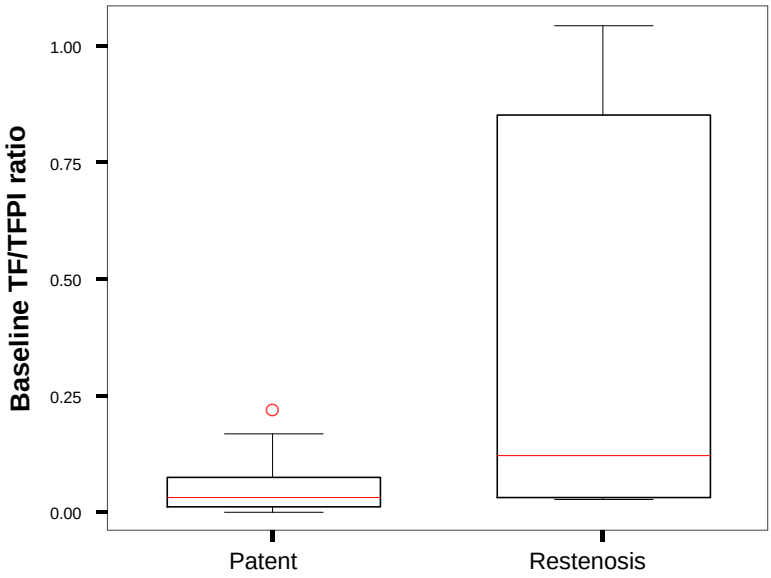
Wilcoxon sign rank test based on ranking the difference between baseline (pre PTA) plasma TFPI and TFPI levels at different time points following PTA. Calculations performed separately for the groups who remained patent and the ones who developed restenosis. The difference of TFPI concentrations at followup intervals from the baseline levels in either of the groups were not found to be statistically significant ($p>0.05$, Wilcoxon's sign rank test) except the borderline significance at month1 following PTA for the group who did not develop restenosis.

3.2.11. Plasma TF/ TFPI ratio and restenosis following femoropopliteal PTA.

The ratio of baseline plasma TF and TFPI was found to be raised significantly ($p=0.04$; Mann Whitney U) in claudicants who developed restenosis [median 0.12 (0.02- 0.89)] than those whose angioplasty site remained patent [median 0.02 (0.01-0.07)]-Figure 16.

Following PTA, although the sample median TF/TFPI ratio was higher for the restenosis group at day 1, month 3 and month 6, and was lower for the restenosis group at month 1, the group differences did not reach statistical significance at all time points ($p>0.05$; Mann Whitney U)-Table 18.

Figure 16. Baseline (Pre PTA) plasma TF/TFPI ratio and restenosis following femoropopliteal PTA



Box and whisker plot showing baseline plasma TF/TFPI ratio in claudicants undergoing femoropopliteal PTA and relationship with restenosis. The box represents the interquartile range (IQR) and the horizontal line in the box being the median value. The whiskers represent the range of the data. The baseline TF/TFPI ratio was significantly higher in those who developed restenosis following PTA (p=0.04; Mann Whitney U test).

Table18. Plasma TF/ TFPI ratio and restenosis following femoropopliteal PTA

	Median TFTFPI ratio (interquartile range)	Mean rank	p (Mann Whitney U)
PRE PTA			
Patent	0.02 (0.01-0.07)	14.40	0.04
Restenosis	0.12 (0.02-0.89)	22.67	
DAY1			
Patent	0.018 (0.004-0.07)	11.50	0.4
Restenosis	0.110 (0.005-0.320)	13.90	
MONTH 1			
Patent	0.015 (0.003-0.043)	10.79	0.8
Restenosis	0.003 (0.002-0.63)	11.43	
MONTH 3			
Patent	0.010 (0.004-0.02)	9.36	0.08
Restenosis	0.046 (0.006-0.623)	14.29	
MONTH 6			
Patent	0.009 (0.002-0.01)	9.21	0.06
Restenosis	0.047 (0.005-0.64)	14.57	

The table shows significant association of baseline TF/TFPI ratio with restenosis following PTA ($p<0.05$, Mann Whitney U). Although the post PTA plasma TF/TFPI ratio were found to be higher at day 1, month 3 and month 6 and lower at month 1 for the restenosis group, the group differences did not reach statistical significance ($p> 0.05$, Mann Whitney U).

Chapter 4. Discussion and Conclusions

DISCUSSION

This study assessed two groups of patients: the first group were evaluated for changes in symptoms whilst on a National Health Service waiting list for angioplasty (Clinical study) and in the second group the role of TF and its natural inhibitor TFPI was assessed in relationship to the development of restenosis after a technically successful percutaneous balloon angioplasty of the index lesion (Laboratory study).

Clinical study

The first part of the study reassessed claudicants who had been awaiting angioplasty for more than three months. Fifteen percent of the patients deteriorated clinically, but none required urgent intervention to prevent limb loss.

Published literature on natural history studies and trials of conservative management of claudicants suggests a relatively benign lower extremity outcome (Cox, Hertzer et al. 1993; Dormandy, Heeck et al. 1999; Amighi, Sabeti et al. 2004; Norgren, Hiatt et al. 2007). The finding of our study correlates well with these studies despite the fact that the patients in our study were symptomatically severe at baseline and warranted PTA. This symptomatic stabilization may be due to development of collaterals, metabolic adaptation of ischaemic muscle, or the preferential alteration of gait by the claudicants to favour non-ischemic muscle groups (Norgren, Hiatt et al. 2007). Only 19% of the patients showed disease progression on duplex ultrasound at reassessment. We found no association between disease progression and symptomatic deterioration in our patient cohort. This data is consistent with previously published literature suggesting lack of association between atherosclerotic disease progression and symptomatic deterioration in claudicants (Da Silva, Widmer et al. 1979). The ABPI and reported maximum walking distance showed an expected increase in the patients who improved symptomatically. The

ABPI in clinically stable patients and those with deterioration of symptoms did not show substantial change despite significant reduction in reported maximum walking distance. In the group which showed deterioration, this finding does not support previous reports suggesting significant correlation between ABPI and walking distance (McDermott, Mehta et al. 1999; McDermott, Greenland et al. 2002). The lack of change in ABPI in this group suggests that disease progression may not be the most important factor in the observed decline in walking distance. This may be explained by the influence of atypical exertional leg pain, produced by other co morbid conditions that may have contributed additional effect on intermittent claudication. A wide range of leg symptoms other than that of intermittent claudication in peripheral arterial disease has been reported in published studies (McDermott, Mehta et al. 1999; McDermott, Greenland et al. 2001). McDermott and SS Mehta reported a prevalence of 28.5% intermittent claudication, 56.2% atypical leg symptoms and absence of exertional pain in 15.3% of patients with PAD identified by ankle-brachial index screening. Similarly, McDermott and Greenland et al reported wide range of leg symptoms in patients with PAD. They have suggested Comorbid diseases particularly diabetes and the presence of associated spinal stenosis may contribute to atypical symptoms in patients with PAD.

As a result of the study, we identified claudicants with symptom change, and have been able to prioritize patients with progression of symptom for early endovascular intervention. Similarly, patients with improved symptom were managed conservatively instead of PTA resulting in reduction in the non-urgent waiting time for femoropopliteal PTA.

Nevertheless this study had certain weaknesses. Firstly, the lack of data on objective measure of walking distance at baseline restricted us to compare patient reported walking

distance at baseline and reassessment. Although symptoms reported by patients and impairment of lifestyle are considered most important factor for considering revascularisation, there is lack of correlation between symptoms and objective measure of walking distance (McDermott, Mehta et al. 1999). Secondly, lack of objective data on regular physical training by these patients during the waiting period may allow a bias in comparing the outcomes (Menard, Smith et al. 2004). Thirdly, the true interval to reassessment varied. We therefore can not make a definite recommendation as to the best time for reassessment of patients on a waiting list. Finally, a significant proportion of our patients could not tolerate treadmill test at reassessment due to shortness of breath, arthritis or inability to walk at 2 miles per hour. Other tests including long distance corridor walk and 6 minute walk work (Simonsick, Montgomery et al. 2001; Carter, Holiday et al. 2003) may be useful for such cases.

In summary, findings in this part of study suggests

- Majority of patients with intermittent claudication remains symptomatically stable over a period of time while waiting for angioplasty. Some of the claudicants however symptomatically improve with time and their symptoms correlate well with the ankle brachial pressure index.
- Symptomatic deterioration occurs in some claudicants without clinical correlation with ABPI, suggesting possible presence of atypical leg symptoms.

Laboratory study

The second part of the study involved restenosis following femoropopliteal PTA and association with plasma TF and TFPI.

Restenosis following femoropopliteal PTA is a major therapeutic problem, limiting the long term efficacy of the procedure despite a high initial success rate. The technical success

rate of the femoropopliteal PTA in this study was 94.3% which is similar to other published figures (Karch, Mattos et al. 2000; Lofberg, Karacagil et al. 2001). Inability to cross the lesions was the cause of technical failure in all cases in this study. The overall restenosis rate at 6 months following femoropopliteal PTA was 32% which correlates well with the previously published reports (Johnston 1992; Koppensteiner, Spring et al. 2006). In our study, majority of the claudicants had either a TASC A or B lesion with 27% having TASC C lesions. The incidence of restenosis was higher in TASC C lesion although no significant difference in restenosis rate was found between TASC A and B lesions. Previously published literature suggests TASC classification of femoropopliteal lesion may be reliable predictor of patency following PTA. TASC C and D lesions are reported to be associated with increased incidence of failure and re occlusion following PTA (Surowiec, Davies et al. 2005; Conrad, Cambria et al. 2006).

Ninety six percent of the claudicants who had successful PTA had early haemodynamic success at 24 hour following PTA as assessed by ankle-brachial pressure index. However 40% amongst them were found to have haemodynamic failure at follow-up assessments. The haemodynamic failure following PTA did not correlate with the occurrence of restenosis as identified by duplex ultrasound scan. Similar observations have been reported in other studies. Nyamekye et al compared duplex assessment and ABPI with angiography for surveillance of femoropopliteal lesions following PTA. The resting and post exercise ABPI did not correlate with colour duplex assessment or formal angiogram in detecting restenosis (Nyamekye, Sommerville et al. 1996). Golledge et al assessed patient symptom, duplex assessment and ABPI measurement following femoropopliteal PTA; They found no correlation between anatomical assessment using duplex USS and ABPI in detection of restenosis (Golledge, Ferguson et al. 1999).

Our study prospectively identifies an association between plasma TF and TFPI concentrations with development of restenosis in claudicants undergoing femoropopliteal PTA. TF is a transmembrane glycoprotein that is constitutively expressed in adventitial and medial cells of blood vessel, and acts as a cofactor for clotting factor VIIa following disruption of endothelial layer such as during angioplasty. Neither peripheral blood cells nor endothelial cells express TF under normal conditions (Moons, Levi et al. 2002). TF synthesis however can be induced in peripheral blood cells under certain pathologic circumstances including sepsis, renal impairment, myocardial or cerebro vascular events and malignancy (Donati MB 1984; G.C. Price 2004). In this study we were able to rule out changes of TF due to these causes by our exclusion criteria. Increased level of circulating TF activity has also been reported to be associated with diabetes mellitus, hyperlipidemia and smoking (Sambola, Osende et al. 2003). Smoking, hyperlipidaemia and diabetes may have an implication in our study group, as 57% of our study cohort were smokers while 35% were hyperlipidaemic and 14% being diabetic. However our small study was not powered to explore these associations.

Our study detected tissue factor in the plasma of both healthy controls and the claudicants. Presence of TF antigen in the peripheral blood of healthy individuals are also documented in studies by Koyama et al and Abrecht et al. (Koyama 1994; Abrecht 1996). The TF in the peripheral blood may be derived from circulating leucocytes, platelets or may be associated with micro particles (Mackman 2006). Micro particles are exocytotic products of plasma membrane and are supposed to originate preferentially from platelets. In response to vascular injury in vivo, micro particles are rapidly recruited to the injury site, triggering a TF dependent coagulation pathway (Falati 2003). No published

literature has clarified the relative proportions of TF in monocytes, platelets and micro particles in health and diseased states. Since our assay measures the TF concentration in the plasma, one possibility is that we may have detected the micro particle associated TF in the patient population.

We found plasma TF and TFPI levels to be significantly higher in claudicants than in the healthy control group. Blann et al reported a similar result of increased plasma TF levels in patients with peripheral arterial disease as compared to healthy subjects (Andrew D. Blann 2000). Gorsk-Bierska et al also reported higher level of tissue factor in 62 patients with intermittent claudication compared with healthy controls (Gosk-Bierska, Wysokin Ski et al. 2008).

We found significant association of high plasma level of TF in claudicants who developed restenosis following femoropopliteal PTA. The study shows that the association of restenosis with increased plasma TF concentrations predates the angioplasty and therefore cannot be procedure related. Previous studies have demonstrated an association between circulating TF and restenosis after coronary revascularization (Marcucci, Prisco et al. 2000; Mizuno, Ikeda et al. 2001; Tutar, Ozcan et al. 2003). Mizuno et al demonstrated correlation between elevated levels of TF antigen in the coronary sinus blood after coronary angioplasty and late restenosis (Mizuno, Ikeda et al. 2001). Tutar et al similarly demonstrated similar association of raised whole blood TF activity with development of restenosis in 61 patients with stable angina undergoing coronary angioplasty (Tutar, Ozcan et al. 2003). A study by Wahlgren et al failed to show any association between TF and restenosis after PTA, although 29 of the 34 patients underwent iliac angioplasty and the restenosis rate was extremely low (Wahlgren, Sten-Linder et al. 2006). Our present study not only extends these observations in peripheral

angioplasty but also examines the change in plasma TF levels following PTA over a period of time. We found significantly raised plasma TF levels at 24 hour, 3 month and 6 months following PTA in claudicants who developed restenosis compared to those who remained patent. At 1 month following PTA, the median plasma TF level was higher in the restenosis group, but this was not statistically significant. This finding at present remains unexplained but may involve complex interaction at cellular level following angioplasty related trauma. However the study findings show a prolonged augmentation of plasma TF level following the angioplasty in patients who developed restenosis. This may suggest that prolonged up regulation of TF following the arterial injury may be associated with the development of restenosis. Although the exact mechanism of influence of TF in restenosis is unknown, it could be possible that higher levels of TF are deposited at the injured or angioplasty site, promoting release of growth factors and inducing vascular smooth muscle cell proliferation and migration. These are both important in formation of neointimal hyperplasia.

The study also identified an increased concentration of baseline (Pre PTA) TFPI in claudicants who developed restenosis following femoropopliteal PTA.

TFPI is a multivalent kunitz type protease inhibitor and produces FXa dependent feedback inhibition of TF-FVIIa complex. (Broze 2003). TFPI is thought to be distributed in three pools in vivo; 80-85% in endothelial cell surface, 10% in plasma and 3% in platelets (Bajaj, Birktoft et al. 2001). The endothelium bound TFPI is thought to contribute towards maintaining the vessel lumen in antithrombotic state. Increased plasma levels of TFPI thus may reflect endothelial injury. Increased plasma concentration of TFPI has been reported in patients with ischemic heart disease (Moor, Hamsten et al. 1994; Falciani, Gori et al. 1998; Soejima, Ogawa et al. 1999).

The finding of raised baseline TFPI concentration in patients developing restenosis following PTA does not correlate with experimental studies that have shown the role TFPI in reducing restenosis following PTA (Brown, Kania et al. 1996; Sato, Asada et al. 1996). On analyzing the TF/TFPI ratio, the claudicants who developed restenosis showed significantly higher baseline TF/TFPI ratio as compared to those whose angioplasty site remained patent following femoropopliteal PTA. A raised TF/TFPI ratio indicates a relative over activity of TF which may play important role in the development of restenosis. This finding suggests that it may be the relative imbalance of TF and TFPI which is more important in predicting restenosis than the absolute values of plasma TF or TFPI levels. Following femoropopliteal the TFPI concentrations were found to be lower at all the follow-up intervals in patients who developed restenosis as compared to those who did not. This may indicate a relative augmented TF activity compared to that TFPI following PTA in claudicants who developed restenosis. Our present study does not contribute to understanding this assumption and further research is needed in this area.

Although this study demonstrates association between increased plasma TF with subsequent development of restenosis, they do not establish causality. Indeed it is possible that a significant fraction of TF may have been released from the atherosclerotic plaque, which is known to be rich in micro particle associated TF (Moons, Levi et al. 2002). In that case, raised level of plasma TF may represent a marker of more extensive atherosclerotic disease which may be the true risk factor for the development of restenosis. Only unilateral claudicants were taken in this study who may have disease in the contra lateral limb without symptom. The diseased asymptomatic limb may also contribute towards higher values of TF concentration.

Despite the above-mentioned limitation it is seems clear that an augmented TF

concentration may relate to the development of restenosis. The study also suggests this augmentation of TF may be to an extent related to a TF/ TFPI imbalance. Further studies are needed to understand the TF/TFPI interaction in the development of restenosis.

In summary, findings in this part of study suggests

- There is a significant incidence of restenosis in claudicants following femoropopliteal PTA.
- Plasma TF and TFPI levels in claudicants are higher compared to healthy controls indicating their association with peripheral arterial disease.
- High plasma levels of TF or an imbalance of TF-TFPI may be associated with occurrence of restenosis following angioplasty.

CONCLUSIONS

1. Claudicants awaiting PTA may experience a change in symptomatology with time which might alter management decisions. In units with a long waiting list, patients should be reassessed in the clinic to review symptom and evidence of disease progression or regression.

2. Increased plasma TF concentration is associated with restenosis following PTA; however it is more likely that an altered TF/TFPI balance could be involved in the occurrence of restenosis.

Further studies are necessary to evaluate relationship between specific mechanisms in the development of restenosis and complex TF- TFPI interactions in peripheral arterial disease.

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Chapter 6. Appendices

6.1. Protocol for preparation of reagents required in TF measurement

1. Coating Buffer: 50 mM Carbonate

1.59g of Na_2CO3 and 2.93g of NaHCO3 made up to 1 litre. pH adjusted to 9.6 and stored at 2-8 ^{0}C maximum up to 1 month.

2. PBS: (base for wash buffer and Blocking buffer) - 8.0g NaCl, 1.1 5g Na_2HPO4, 0.2g KH_2PO4 and 0.2g KCl, made up to 1 litre. pH adjusted to 7.4 and stored up to 1 month at2- 8^{0}C.

3. Wash Buffer: PBS-Tween (0.1%, v/v)

1.0 mlof Tween-20 was added to 1 litre of PBS. pH is adjusted to 7.4 and stored at 2- 8^{0}C up to 1 week.

4. Blocking Buffer: PBS-BSA (2%, w/v)

5.0 g of Bovine Serum Albumin (Sigma-RIA grade) was dissolved in 200 ml of PBS. The pH adjusted to 7.4, if required. Volume was made up to 250 ml with PBS and and stored frozen in aliquots at -20^{0}C.

5. Sample Diluent: HBS-TX100

5.95g HEPES (free acid) and 1.46 g NaCl dissolved in 200 ml H_2O. 0.25 ml of Triton X-100 was then added and pH adjusted to 7.2 with NaOH. The final volume of 250 ml was made by adding H_2O. The diluent then stored frozen in aliquots at -20^{0}C.

6. Conjugate Diluent: HBS-BSA-T20

5.95g HEPES (free acid), 1.46 g NaCl, 2.5 g Bovine Serum Albumin (Sigma, RIA grade) dissolved in 200 ml H_2O. 0.25 ml of Tween-20 was then added and pH adjusted to 7.2 with NaOH. The final volume of 250 ml was made by adding H_2O. The diluent then stored frozen in aliquots at -20^{0}C.

7. **Substrate Buffer:** Citrate-Phosphate buffer pH 5.0

2.6g Citric acid and 6.9g Na_2HPO_4 made up to a final volume of 500 ml with purified H_2O and stored at 2-8^{0}C up to 1 month.

8. *OPD Substrate:* (o- Phenylenediamine.2HCl (5mg tablets: Sigma # P-6912). Made up immediately before use. 5 mg OPD was dissolved in 12 ml substrate buffer and then 12 µl 30% H_2O_2 was added.

6.2. Protocol for preparation of reagents required in TFPI measurement

A. **Standards**

1. 0.5 mL of cold (2-8°C) distilled H_2O was added to the TFPI Depleted Plasma vial. The vial was allowed to stand on ice for 2-3 minutes.

2. A solution of 5% TFPI depleted plasma was then prepared by diluting the TFPI depleted plasma 1:20 with Sample Buffer. The solution was let stand for 5 minutes after gentle mixing.

3. 1 mL of the 5% TFPI depleted plasma was added to the 5 ng/mL TFPI standard vial.

4. Standards of 2.5, 1.25, 0.625 and 0.3 12 ng/mL concentration were generated by serially diluting the 5 ng/mL TFPI plasma standard. Tubes were labelled and 0.5 mL of 5% TFPI depleted plasma was pipetted into each tube. 0.5 mL of the 5 ng/mL TFPI plasma standard was then added into the 2.5 ng/mL labelled tube and mixed. 0.5 mL was then transferred from the 2.5 ng/mL tube into the 1.25 ng/mL labelled tubes and mixed. Same process was continued for the 0.625 ng/mL and 0.3 12 ng/mL labelled tubes.

5. 5% TFPI depleted plasma was used as the "0" standard.

B. **TFPI Reference Plasma**

0.5 mL of cold filtered deionised H_2O was added to the vial and gently mixed for 2 minutes.

C. Detection Antibody

5.5 mL filtered deionised H_2O was added per vial and mixed gently for 3 minutes.

D. Enzyme Conjugate Diluent

20 mL filtered deionised H_2O was added to the vial and mixed well.

E. **Wash Buffer**

The content of PBS packet was dissolved in 900 mL of filtered deionised H_2O, Mixed well and diluted to a final volume of 1 litre with filtered deionised H_2O

F. **Sample Buffer**

Appropriate amount of Sample Buffer was prepared by adding BSA to Wash Buffer to a final concentration of 1% w/v (1 gmBSA/100 mL Wash Buffer).

6.3. Patient information leaflet

Thank you for reading this

Study Title:

The influence of plasma Tissue factor and Tissue factor pathway inhibitor concentration in restenosis following femoro popliteal percutaneous transluminal angioplasty.

Invitation:

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

What is the purpose of the study and why have I been chosen?

You have been chosen to be a part of the study because you do not suffer from poor leg circulation caused by narrowing in the arteries supplying blood to your leg. Certain people suffer from poor leg circulation due to narrowing in the arteries supplying blood to their leg. This condition may be treated by balloon "angioplasty" to widen narrowed part of the artery. The initial result of this procedure is usually very good and will gives prompt relief from symptoms. A significant number of people may develop repeat narrowing of their arteries over a period of time (20%-40% at 1 year, 50% at 3yr and approximately 60% at 5 year according to published literature) and may require repeat angioplasty. We know there are certain protein substances in the blood, which are involved in repeat narrowing of the arteries. Early detection of these substances may help in identifying the problem early. In future it may also be possible to modify the action of this substances to prevent re-narrowing of the arteries.

The main aim of this study is to define the link between these substances and repeat narrowing of the leg arteries following angioplasty. It is also important to compare the blood level of these substances in healthy person and people with poor leg circulation to know if these are present only in patients with poor leg circulation. As you do not suffer from this condition you are suitable being a healthy control for this study.

Do I have to take part?

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

What will happen to me if I take part?

If you agree to take part in the study, you will be asked to sign a consent form after discussion regarding the nature of the study. You will then have a blood sample collected by needle puncture.

What do I have to do?

No extra demands will be made of you apart from taking blood samples from you.

Do I have to take part?

Only if you want to. Participation is voluntary, you may refuse to participate or withdraw from the study at any time. You do not need to tell us why you do not want to take part.

Are there any risks involved?

Essentially there are no side effects or disadvantage of participating in this study. The potential benefit of the study is that we may be able find factors, which are in someway responsible for repeat narrowing of arteries following angioplasty. The information we

get from this study may help us to treat future patients with narrowing of leg arteries better.

Are there any costs involved?

There is no cost involved on your part for being a part of the study.

Confidentiality

In order to meet legal obligations, a member of the of the department of vascular surgery, academic vascular surgery unit, Hull Royal Infirmary may inspect your hospital records. Details of your treatment and your past relevant medical history as required for the study, will be recorded on a Case Record Form (CRF) the information from which will be entered onto computer in the Department of Vascular Surgery, Hull Royal Infirmary. A CRF includes all information collected in the course of the research study. This information will be retained by the Department of Vascular Surgery and may be passed on to the authorized regulatory authorities. The records will identify you only by a number (not your hospital number) and your initials. All information in your notes and CRF will be treated in strict confidence. A copy of this Informed Consent Form will be kept with the CRF and you will be given a copy. The information from this study will be retained until the data are analyzed for two years in the Department of Vascular Surgery, Hull Royal Infirmary.

By signing the attached consent form you give permission for the above to occur.

If you agree to participate in this study, your General Practitioner will be informed, unless you state otherwise.

Your rights

Your participation in this study is entirely voluntary and refusal will not affect any other medical treatment. You may, without giving reason, refuse to take part in the trial, and this will not in any way affect your continuing treatment by your doctor. Your doctor will

give you any relevant updated information about any relevant updated information about further plan of instituting any treatment or investigation that may occur during the study.

Who is organising and funding the research?

This study is organised by the Academic Vascular Unit at Hull Royal Infirmary and funded by grants that it receives.

Trial-related injury

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Thank you for taking the time to read this information sheet and consenting to participate in the study. You will be given a copy of this information sheet and a copy of your consent to participate form. If you have any further queries or questions please don’t hesitate to contact:

Mr Biswajit Ray FRCS.

Clinical Research Fellow

Vascular Laboratory, Hull Royal Infirmary, Hull HU3 2JZ.

Tel:01482674178.

6.4. Consent Form

March 2003
Study number:

Patient ID number:

Title of project:
Plasma tissue factor, tissue factor pathway inhibitor and restenosis following femoropopliteal percutaneous transluminal angioplasty in claudicants

Invitation:

Names of researchers:
Mr. Biswajit Ray FRCS. Research Fellow in Vascular Surgery, the University of Hull
Mr IC Chetter MBChB FRCS. Lecturer in Vascular Surgery, the University of Hull.
Dr C Ettelaie, Lecturer, Department of Biological Sciences, the University of Hull
Prof. PT McCollum MCh, FRCS. Professor of Vascular Surgery, the University of Hull

Contact Address:
Academic Vascular Unit
Anlaby Alderson House, Hull Royal Infirmary
Road, Hull HU3 2JZ Please initial

Telephone No: 01482 674178

Fax No: 01482 675665

1. I confirm that I have read and understood the information sheet dated February 2003 for the above study and have had the opportunity to ask questions -------

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected --------

3. I understand that sections of any of my medical notes may be looked at by responsible individuals where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. --------

4. I agree to take part in the above study --------

----------	----------	----------
Name of patient	Date	Signature

Name of person taking consent	Date	Signature

Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes
February 2003

6.5. Letter to patient`s GP

Date

Dear Dr.

Re: <Patient Name and Address>.

.....

.....

"Plasma tissue factor, tissue factor pathway inhibitor and restenosis following femoropopliteal percutaneous transluminal angioplasty in claudicants"

I am writing to inform you that your patient has been enrolled into the above research study.

The purpose of the study is to evaluate the role of above named substances in the blood and restenosis following angioplasty of femoropopliteal arteries.

The subject consented for blood samples being taken for the above study before and after undergoing angioplasty. Follow-up will be arranged at 3 months and 6 months at the Academic vascular surgical unit, during which time, subject will undergo blood sampling, ABPI measurement and Doppler USG assessment of angioplastied artery. The clinical implications of this study will be to possibly identify restenosis following angioplasty at an early stage by detecting these substances early. In future it may also be possible to modify the action of these substances to prevent re-narrowing of the arteries. If you have any questions regarding any of the above, please feel free to contact me on 01482 674178.

Yours sincerely

Mr B.Ray FRCS

Research Fellow, Vascular Surgery

Vascular Laboratory, Alderson House

Hull Royal Infirmary

Hull, HU3 2 JZ.

6.6. List of abbreviations

ABPI	Ankle: brachial pressure indexe
ACE	Acetyl coenzyme
ANOVA	Analysis of variance
AT-I	Angiotensin type I receptor
BSA	Bovine Serum Albumin
0_C	Degree Celcius
CAD	Coronary artery disease
COPD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FGF-2	Fibroblast growth factor-2
FXa	Activated factor Xa
g	Gram
H_2O	Water
H_2 SO4	Sulphuric acid
HEPES	N-(2-Hydroxyethyl)piperazine-N' -(2-ethanesulfonic acid)
HRP	horseradish peroxidase
IHD	Ischemic heart disease
IQR	interquartile range
K1,2,3	Kunitz domain1,2,3
kDa	killodalton
KH2PO4	Potassium biphosphate
LDL	Low density lipoprotein
M:F	Male to female ratio

MAPKs	Mitogen activated protein kinases
MI	Myocardial infarction
ml	Millilitre
MMP	Matrix metalloproteinase
MRA	Magnetic resonance angiogram
Na2CO3	Sodium carbonate
Na2HPO4	Sodium biphosphate
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NaOH	Sodium hydroxide
ng	Nanogram
NHS	National health services
nm	Nanometre
OPD	o-phenylenediamine
PAD	Peripheral arterial disease
PARS	Protease activated receptors
PBS	Phosphate buffer solution
PDGF	Platelet derived growth factors
POP	Popliteal artery
PRIME	Prospective Epidemiological study of Myocardial Infarction
PTA	Percutaneous transluminal angioplasty
PTCA	Percutaneous transluminal coronary angioplasty
RPM	Revolution per minute

SFA	Superficial femoral artery
T-20	Tween 20
TASC	Transatlantic Inter-Society Consensus
TF	Tissue factor
TF-EIA-C	Capture antibody for tissue factor
TF-EIA-D	Detecting antibody for tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor- β
TMB	3,3’ ,5,5 ’-Tetramethylbenzidine
TPT	Tibio peroneal trunk
VSMC	Vascular smooth muscle cell
µl	Microlitre

6.7. Publications and Presentations arising from the thesis

Plasma tissue factor is a predictor for restenosis after femoropopliteal angioplasty *Br J Surg.* 2007 Sep;94(9):1092-5.

- *Original article*

Presentations to learned societies and published abstracts

1. Plasma tissue factor, a predictor for restenosis following percutaneous transluminal angioplasty (PTA).

Published in abstract form in BJS Volume 92, Number 4, April 2005, p 502

2. "Prognostic implication of plasma tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in restenosis following percutaneous transluminal angioplasty (PTA)"- *Society ofAcademic and Research Surgery,* Edinburgh, January 2006.

Published in abstract form in BJS Volume 93 Issue S3, Pages 1 - 57 (July 2006)

3. "Restenosis following femoropopliteal percutaneous transluminal angioplasty is predicted by plasma tissue factor "– *15th Annual Winter Meeting of the Peripheral Vascular Surgery Society,* Steamboat Springs, Colorado, January, 2005.

4. "Impact of reassessment on a waiting list for percutaneous transluminal angioplasty (PTA). *12th International Conference ofEuropean Association ofEndoscopic Surgeons,* Barcelona, June 2004.