Smoking and Plasma Fibrinogen, Lipoprotein (a) and Serotinin are Markers for Postoperative Infrainguinal Graft Stenosis

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Objectives: A number of systemic variables are associated with infrainguinal graft failure and also with experimental smooth muscle hyperplasia. Stenosis is the most common cause of infrainguinal graft thrombosis but it is not known if systemic variables are associated with stenosis.

Design, materials and methods: In this study, clinical and serological factors were measured and correlated with stenosis development in 81 infrainguinal bypass grafts (52 vein, 29 PTFE; 28 with stenosis) in prospective (n = 46) and retrospective (n = 35) groups. Pre-existing stenosis was excluded by perioperative graft assessment.

Results: There was a significantly greater proportion of smokers in the patients who developed stenosis (11/18; 61%) compared with those who did not (6/28; 21%, p = 0.006; χ^2). Patients who developed stenosis also had significantly (Mann Whitney U-tests), higher circulating levels of [median (interquartile range)] fibrinogen (412.5 (356–484.5) vs. 339 (300–397.7) mg/100ml, p = 0.003), Lipoprotein (a) (0.20 (0.05–0.45) vs. 0.085 (0.05–0.23) g/l, p = 0.03) and 5-hydroxytryptamine (14.1 (6.6–45) vs. 4.41 (3–8.39) nmol/l, p = 0.005), than those without stenosis.

By logistic regression, these associations were independent of graft material and whether grafts were studied prospectively or retrospectively.

Conclusions: Smoking and plasma fibrinogen, Lp(a) and 5-hydroxytryptamine are markers for postoperative infrainguinal graft stenosis.

Key Words: Intimal hyperplasia; Smoking; Lipoprotein (a); Fibrinogen; Serotonin; Graft Stenosis.

Introduction

A number of patient related variables, notably smoking, hyperfibrinogenaemia and hyperlipidaemia have been shown to be associated with reduced patency after arterial reconstruction.[1–4]

In vitro and animal studies of intimal hyperplasia and atherogenesis have demonstrated the potential of tobacco smoke derivatives,[5–8] coagulation cascade proteins,[9–12] cholesterol and apolipoproteins[13–15] and platelet releasates[16–18] to stimulate proliferation of smooth muscle cells. Migration and proliferation of medially derived smooth muscle cells produces vessel wall thickening after arterial reconstruction[19,20] and results in localised stenosis in approximately 25% of infrainguinal bypass grafts.[21,22] The adverse haemodynamic consequences of stenosis are believed to cause up to 80% of infrainguinal graft thromboses.[21,23,24] Thus, clinical and serological variables may influence graft stenosis development.

In this study we measured a number of factors, all of which are associated either with increased risk of failure after arterial reconstruction or experimental proliferation of smooth muscle cells (both in most cases) and compared these in patients who did or did not develop hyperplastic stenosis in infrainguinal bypass grafts.

Methods

Ethical committee approval was granted for this study.

Patients

Clinical and serological variables were measured in 81 patients undergoing infrainguinal bypass grafting under the care of a single surgeon. Autologous vein

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conduits were used in 52 bypasses and the remaining 29 were 6mm expanded polytetrafluoroethylene (PTFE) grafts (Impra U.K.). There were 53 femoropopliteal grafts and 28 femorocrural grafts. A distal vein collar[25] was used with prosthetic grafts to crural vessels.

Patients were recruited in two groups. The prospectively followed group consists of 46 consecutive patients (30 autologous vein and 16 PTFE grafts) who underwent arterial reconstruction during the study period. Variables were measured immediately prior to arterial reconstruction in all cases. Median follow-up is 17.5 months with a minimum follow up of 12 months.

The retrospective study group consists of 35 patients (22 autologous vein and 13 PTFE grafts) in whom risk factors were measured a mean of 10 months (standard deviation + /− 5) after reconstruction. These patients were recruited randomly from those attending for graft follow up during the study period. All had undergone infrainguinal reconstruction by the same surgeon and had standardised Duplex surveillance data recorded. Entry criteria included perioperative assessment, a patent graft which had not undergone thrombectomy or thrombolysis and completion of at least 1 year of surveillance (described below).

Exclusion of non-hyperplastic graft stenoses

All patients included in this study underwent intraoperative Doppler studies[26] and predischarge Duplex scanning to exclude fixed, non hyperplastic causes of graft stenosis such as retained valve cusps, intramural venous disease or anastomotic technical error. Stenoses identified intraoperatively were corrected during primary reconstruction and results confirmed with arteriography.

Graft surveillance and stenosis detection

Following discharge, patients underwent Duplex scanning at 6 weeks, and 3, 6, 9 and 12 months post-reconstruction using the V2:V1 technique.[27] Severe stenoses (V2:V1 > 2 on Duplex), confirmed by angiography were corrected by percutaneous balloon dilatation, patch angioplasty or jump grafting.

For the purposes of this study graft stenosis is defined as a new stenosis detected within the first postoperative year, requiring correction. Non-sten-

Table 1. Risk factors studied

Clinical factors	Smoking (breath CO analysis), hypertension, diabetes mellitus (types I and II)
Lipids/lipoproteins	Total triglycerides and cholesterol
Serum	High/low density lipoproteins cholesterol Apolipoproteins A, B and lipoprotein (a)
Platelet activation marker Plasma	Serotonin (5-HT)
Coagulation/fibrinolysis Plasma	Fibrinogen Factor VII and VIII activity Plasminogen Tissue plasminogen activator (tPA) Plasminogen activator inhibitor (PAI-1)

osed grafts are those which remained patent and have undergone regular surveillance for at least 1 post-operative year, during which no stenosis was detected.

Risk factor measurement

The clinical and serological variables measured in this study have all been shown to be associated either with increased risk of failure after arterial reconstruction or experimental proliferation of smooth muscle cells and are shown in Table 1.

It was not possible to measure all risk factors in all patients; assessment of smoking and hypertension relevant to the time of stenosis development was only possible in the prospective group. In addition to blood samples invalidated for technical reasons, patients were required to travel between two hospitals (St Mary's and The Royal Free) for laboratory analysis. Although this policy minimised error caused by *in vitro* changes due to delay in analysis, inevitably some patients were unable to attend both hospitals. It is appreciated that these losses may increase the likelihood of statistical error, but the selection of patients was not subject to influence by the authors. The numbers of patients studied in each group is shown in the relevant results section.

Clinical risk factors Smoking was assessed by direct questioning and validated by measurement of breath Carbon Monoxide (CO) concentration using the Microsmokalyser (Bedfont Scientific, Upchurch, Kent, U.K.). Readings above 10 ppm expired CO were considered indicative of current smoking as recommended by the manufacturer.

Hypertension was defined as current use of one or

more anti-hypertensive agents to maintain normal blood pressure and diabetes mellitus as current diet, oral or insulin therapy for control of serum glucose.

Lipids and lipoproteins Whole blood was collected from an antecubital vein after a minimum 12 h fast and stored in plain tubes. Serum was prepared by centrifugation at $3000 \times g$.

Serum cholesterol and triglycerides were determined enzymatically using a Centrifichem centrifugal analyser (Baker Instruments, Windsor, U.K.), Low density lipoprotein (LDL) cholesterol was calculated by the Friedwald equation.[28] High density lipoprotein (HDL) was isolated by the dextran sulphate-Mg Cl_2 method[29] and the cholesterol measurement performed as above. Apolipoproteins were measured using immunonephelometry (Beckman Auto Immuno-Chemical Systems, Beckman Instruments, High Wycombe, U.K.)

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

Plasma was prepared by centrifuging blood for 20 min at $1500 \times g$ at 4°C. The supernatant was collected and spun again as above and kept frozen at -40°C.

Platelet rich plasma (PRP) was prepared using the technique described by Hardisty *et al.*[30] Nine volumes of venous blood were collected in a polystyrene syringe and mixed with one part 3.8% trisodium citrate in a polystyrene tube. PRP was prepared by centrifuging blood at $160 \times g$ for 15 min at room temperature.

Platelet counts were performed using a Coulter T-890 blood counter (Coulter Electronics, Luton, U.K.). Following counting, the PRP was centrifuged at $1000 \times g$ for 10 min to prepare platelet pellets. The pellets were washed with Isoton II (Coulter Electronics, Luton, U.K.) and stored at -40°C until analysis. Whole blood aggregation was evaluated by a free platelet count method using a blood cell counter (Coulter T-890; see above).

Plasms Platelet Serotonin and β-thromboglobulin assay Platelet pellets were resuspended in physiological saline (0.9% w/v) and platelets lysed using the MSE-Soniprep sonicator (MSE, Sussex, U.K.). Plasma serotonin (5-hydroxytrptamine; 5-HT) concentrations

were estimated using a radioimmunoassay (Biogenesis Ltd., Bournemouth, U.K.).

Coagulation and fibrinolytic activity Blood was collected from an antecubital vein with minimal stasis and anticoagulated with 3.8% trisodium citrate in proportions of 9:1, respectively, in precooled tubes. Samples were centrifuged at $3000 \times g$ for 15 min at 4°C and plasma was stored at -70°C until analysis.

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

For the quantitative determination of tissue plasminogen activator (t-PA) citrated blood was collected in concentrations of nine parts blood to one part 3.8% trisodium citrate as described above. Samples were processed as recommended by Chandler *et al.*[31] For the quantitative determination of plasminogen activator inhibitor (PAI-1) concentration, blood was collected into citrate as already described, supplemented with the following anti-platelet agents: indomethacin (20 mg/l final concentration F.C.), theophylline (F.C. 3mmol/l) and adenosine (F.C. 1 mmol/l). t-PA and PAI-1 antigen were quantitated using Enzymoimmunoassay Kits (Kabi Diagnostica, Sweden).

Statistical analysis

The data was analysed with help from Mrs. Jane Wadsworth in the Academic Department of Public Health at St.Mary's Hospital Medical School.

This study compares a number of variables between patients who developed graft stenosis and those who did not; it was considered important to include the influence of study groups (retrospective or prospective) and graft type (vein or PTFE) on the comparison. A two part analysis was therefore used; primary examination of all data by univariate analysis, followed by logistic regression of those factors associated with stenosis also including the effects of prospective and retrospective groups and vein and PTFE grafts.

The Chi-square and Mann-Whitney U tests were used for univariate analysis. Results are presented as median and interquartile range with individual data points shown on figures.

Table 2. Clinical risk factor profiles in prospectively studied patients (*n*=46)

	Stenosed grafts	Non-stenosed grafts	*p*= (χ^2 test) + Yates' Correction
Diabetes mellitus	3/18	5/28	0.91
Hypertension	8/18	13/28	0.89
Smoking	11/18	6/28	0.016
Female sex	6/18	7/28	0.54

Results

Stenosis rates and histological assessment

During follow-up in the prospective group of patients, graft stenoses requiring intervention were detected in 12/30 vein grafts (40%) and 6/16 PTFE grafts (37.5%). Twenty-eight grafts remained stenosis free during a minimum of 1 year surveillance.

In the retrospective group, 10 patients (five each vein grafts and PTFE grafts) had developed graft stenosis requiring correction during the first postoperative year and 25 patients were stenosis free.

Tissue from stenoses was obtained from 12 patients treated by excision or patch angioplasty (other cases were corrected by percutaneous angioplasty or jump grafting). Histology confirmed intimal thickening due to smooth muscle hyperplasia in all cases.

Clinical variables

Comparison of clinical variables between patients who did and did not develop stenosis was only undertaken on the prospective group to reflect circumstances at the time of stenosis development. Table 2 compares the clinical risk factor profiles of prospectively studied patients who did and did not develop stenosis.

By univariate analysis, a significantly greater proportion of patients who developed stenosis continued to smoke after reconstruction; stenosis group 11/18(61%) compared with 6/28 (21%) in the non stenosis, $p = 0.006$, $\chi2$ test ($p = 0.016$ after Yates Correction) see Fig. 1.

Regression analysis showed that smoking differences between the two groups were independent of the graft material (maximum likelihood estimates; smokers *vs.* non-smokers, —2.507 $p = 0.0007$: vein *vs.* PTFE, 0.286 $p = 0.707$).

Clotting and fibrinolysis studies
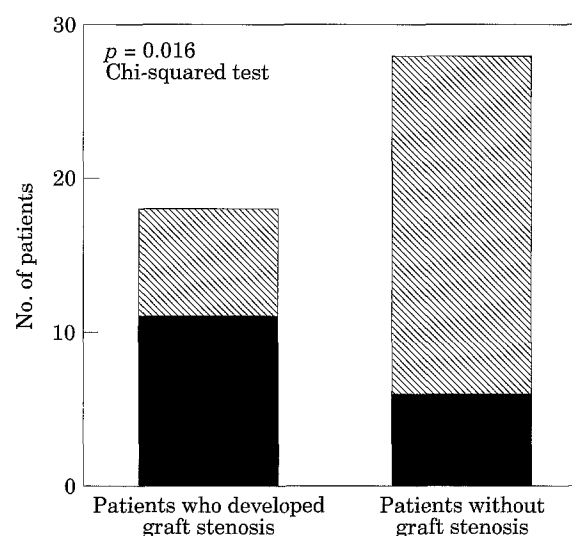
Results of coagulation //fibrinolysis analysis were

Fig. 1. Bar chart showing patients who continued to smoke after reconstructioon as a proportion of patients who did and did not develop graft stenosis. (■) smokers; (▨) non-smokers.

available for 53 patients (21 stenosed and 32 non stenosed grafts). The plasma concentration of fibrinogen was significantly higher in patients with grafts which developed stenosis (413 (356–4845) *vs.* 339 (300–398) mg/100ml, $p = 0.003$, Mann Whitney U test, Fig. 2). By regression analysis, these differences were independent of graft material or study group (maximum likelihood estimates: fibrinogen, –0.0163 $p = 0.0013$: vein *vs.* PTFE, 0.2053 $p = 0.7$: prospective *vs.* retrospective, –1.239 $p = 0.08$).

There were no significant differences in activity of factors VII or VIII, tPA or PAI-1 or plasminogen concentration between stenosed and non stenosed grafts (results not shown).
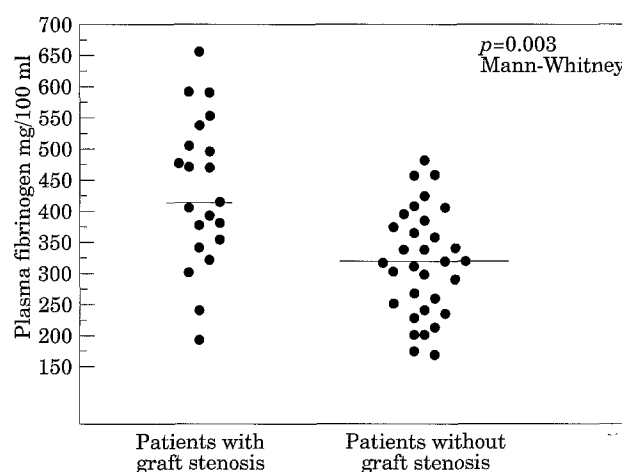

Fig. 2. Scattergram showing plasma fibrinogen concentration in vein and PTFE grafts which developed stenosis and those that did not. The bar shows median fibrinogen concentration.
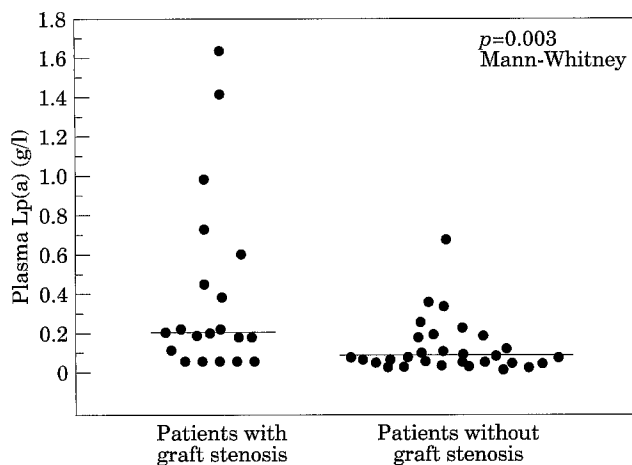
Fig. 3. Scattergram showing serum lipoprotein (a) concentration in vein and PTFE grafts which developed stenosis and those that did not. The bar shows median Lp(a) concentration.
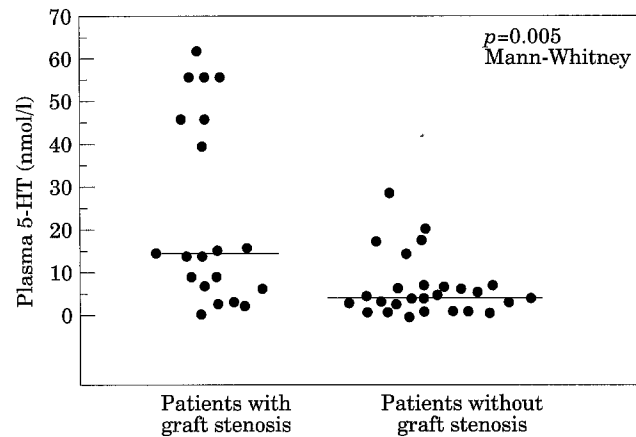

Fig. 4. Scattergram showing plasma 5-HT concentration in vein and PTFE grafts which developed stenosis and those that did not. The bar shows median 5-HT concentration.

Lipids and lipoproteins

Results of lipid/lipoprotein analysis were available for fifty patients (20 stenosed and 30 non stenosed grafts). Median serum concentration of lipoprotein (a) was higher in patients whose grafts developed stenosis than those who did not (0.20 (0.05–0.45) vs. 0.085 (0.05–0.23) g/l, p = 0.03 Mann Whitney test, Fig. 3). By regression analysis, these differences were independent of graft material or study group (maximum likelihood estimates: Lp(a) −1.518 p = 0.04, vein vs. PTFE 0.128 p = 0.8, prospective vs. retrospective 0.335 p = 0.6).

There were no significant differences in levels of triglycerides, cholesterol, HDL, LDL or apolipoproteins A or B between the two groups (results not shown).

Plasma serotonin and platelet function studies

Results of platelet function analysis were available for 47 patients (20 stenosed and 27 non-stenosed grafts).

Plasma 5-HT concentration was higher in patients who developed stenosis compared with those who did not (14.1 (6.6–45) vs. 4.4 (3–8.39) nmol/l p = 0.005, Mann Whitney test, Fig. 4). By regression analysis, these differences were independent of graft material or study group (maximum likelihood estimates: 5HT 1.822 p = 0.018, vein vs. PTFE 0.099 p = 0.8, prospective vs. retrospective 0.691 p = 0.3).

By univariate analysis, there were differences in platelet aggregation between stenosed and non-sten-

osed grafts when adrenalin was used as an agonist but these failed to remain significant when the effects of graft material and study group were included in the regression analysis. There were no other significant differences in platelet aggregation or plasma and intraplatelet levels of releasates between those patients who developed stenosis and those who did not.

Discussion

Haemodynamically significant stenoses develop in approximately 25% of infrainguinal bypass grafts, usually within the first 12 months after surgery[21] and may be associated with up to 80% of postoperative graft thromboses.[23] This has led to the establishment of stenosis surveillance and correction programmes in many vascular surgical centres. Reported stenosis rates are often higher from centres with an interest in graft surveillance and this is borne out by the prospective finding of stenoses in 37.5% of PTFE and 40% of autologous vein grafts in the current series. Histological examination of stenoses from both vein and prosthetic grafts reveals similar pathology; a localised, subintimal proliferation of smooth muscle cells with a surrounding matrix of proteoglycan and collagen.[32] Although the sequence of events leading to this intimal hyperplasia is now understood[18,33,34] the stimuli responsible for smooth muscle cell migration to the subintima and their subsequent proliferation, remain unclear.

A number of recent publications have identified clinical and serological variables associated with

increased failure rates after arterial reconstruction[2–4,35] and there is overlap between these factors and those known to stimulate smooth muscle cell proliferation in experimental models.[9,10,12,16,36–39] This suggests that the observed increase in graft failure rates associated with these risk factors may be a consequence of increased stenosis development.

In the present study we have demonstrated an association between stenosis of infrainguinal bypass grafts and plasma levels of lipoprotein (a), fibrinogen and serotonin as well as an increased incidence of smoking. These results have two potentially important implications. As preoperative markers of graft stenosis, these variables may be of value in identifying patients and grafts at increased risk of developing stenosis and thus thrombosis. This could be used to target a more aggressive and potentially more cost effective graft surveillance programme. Secondly, the data supports a large body of circumstantial evidence suggesting a causative relationship between circulating factors and hyperplasia of mural smooth muscle cells in both graft intimal hyperplasia and in atherosclerosis. This is important as it infers that correction of risk factors may reduce the incidence of stenosis. It is of value therefore to review the data implicating the above variables in experimental smooth muscle cell mitosis.

Tobacco smoking is one of the major risk factors for atheroma development in man since it accelerates atherosclerosis in both the peripheral and coronary circulation.[40,41] The mechanism of action is unknown, but hypothetical mechanisms include vessel injury through vasospasm, hypoxia or endothelial cell loss[7,42,43] and platelet activation.[44,45] Smoking-related endothelial injury in an infrainguinal graft and the perianastomotic vessels (perhaps exacerbated by operative trauma) could expose medial smooth muscle cells to a variety of mitogens both in the plasma and in platelets (perhaps activated by smoking), resulting in the development of a localised proliferative lesion in a similar manner to that proposed in the early genesis of atheroma.[46] This could explain the observations in the current study and suggests a possible causal relationship.

Fibrinogen is the soluble precursor of fibrin. Epidemiological studies have demonstrated that elevated levels of circulating fibrinogen are associated with accelerated development of atheroma and coronary events in man.[47,48] Cell culture studies have shown that fibrinogen and its derivatives are chemotactic and mitogenic to smooth muscle cells.[11,12,49] Despite this, it is still unclear whether the observed adverse cardiovascular effects of hyperfibrinogenaemia are mediated through a hypercoaguable state or at the vessel wall

level. The present findings show fibrinogen to be associated with infrainguinal graft disease in the absence of overt thrombosis. Additionally, there were no differences in plasma levels of other coagulation or fibrinolytic factors between patients with and without stenosis and this suggests that in infrainguinal bypass grafts, fibrinogen acts via a mechanism independent of the coagulation cascade.

Lipoprotein (a) is a protein-lipid complex, similar in composition to LDL but containing apolipoprotein (a). Attention has been focused on this molecule since it was shown to be an independent predictive factor for the development of atheroma in man.[50,51] Its mechanism of action is unclear; circulating Lp(a) may affect the vessel in similar ways to those proposed for other atherogenic lipoproteins (increasing endothelial permeability to lipids or by oxidisation and direct stimulation of smooth muscle mitosis). However, apo(a) also has structural similarity to plasminogen[52] and can compete with plasminogen and t-PA for fibrinogen binding *in vitro*[53,54] and may thus possess pro-coagulant properties. Our observation that there were no differences in plasminogen or t-PA activity between stenosed and non stenosed groups of patients suggests that the association between Lp(a) and infrainguinal graft stenosis is independent of coagulation effects but interpretation of these results is difficult as fibrinogen levels also varied between patient groups.

Platelets are excellent candidates as initiators of intimal hyperplasia as they are known to contain at least two mitogens for vascular smooth muscle cells— platelet derived growth factor (PGDF)[16] and epidermal growth factor (EGF)[55]— both of which are released during activation. As well as being mitogenic, PGDF is also a chemotactic agent for smooth muscle cells[56] and at least in theory, could induce both smooth muscle cell migration from the media and subsequent subintimal proliferation. Epidemiological evidence suggests an association between platelet activation and the development of atherosclerosis[57–61] and animal studies have demonstrated increased intimal thickening in bypass grafts with high levels of labelled platelet adherence[39] as well as the protective effects of anti-platelet therapy.[62,63] In excess of 99% of whole blood 5-HT is contained within the dense granules of platelets in humans and a number of studies have demonstrated that platelet degranulation is associated with elevation of the plasma 5-HT level.[64–66] 5-HT has also been shown to possess mitogenic properties and to significantly enhance the mitogenic properties of PDGF.[67] The data presented above may demonstrate an association between platelet activation and the development of graft stenosis. However, other sources

of 5HT (most importantly endothelial cells) may contribute to circulating levels making interpretation of plasma results difficult. Because we were unable to demonstrate other evidence of platelet activation in association with graft stenosis we believe that the current data is insufficient to show a conclusive association between platelet activation and infrainguinal graft stenosis.

The present study demonstrates that cigarette smoking, plasma fibrinogen, Lp(a) and 5-HT are markers for the postoperative development of stenosis in infrainguinal bypass grafts. These variables are known to be associated with the proliferation of smooth muscle cells in experimental models and have been indirectly associated with increased rates of failure after arterial reconstruction suggesting that they may be associated with increased smooth muscle cell mitosis in the graft wall. A randomised study of the effects of perioperative correction of these 'risk' factors on the incidence of graft stenosis may be justified.

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