UNIVERSITY OF LONDON

OBJECTIVE ASSESSMENT OF MICROCIRCULATORY RESPONSE IN VENOUS DISEASE TO THERAPY

A Thesis Submitted To University College London
In Fulfilment of the Requirements of the Master of Surgery
(M.S.)
by

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ABSTRACT

Venous stasis is associated with leucocyte & endothelial activation as well as local growth factor response in chronic venous distasease (CVD). There is no good data on the response of this increased activation to treatment. The theme of this thesis was to show changes in leucocyte/endothelial activation as well as microcirculatory stasis in response to medical, surgical and compression therapy. Thus these may act as objective measures of response to treatment.

Vascular endothelial growth factor (VEGF) is being investigated extensively in various arterial scenarios I demonstrated high plasma levels of among patients with CVD for the first time. Levels in patients were about 60% higher (82pg Vs 52 pg in controls). This may represent an (reparative!) angiogenic response existing along with the leucocyte inflammatory response. I used a model of medical treatment (60 days oral flavonoid therapy) and demonstrated significant change in plasma VEGF (50% reduction i.e. 98 pg to 57 pg/dl), ICAM (32%), VCAM (29%) & lactoferrin (36%) levels in patients. Thus I showed that endothelial cell activation (ECA) as well as VEGF might be used as an objective surrogate marker in CVD. I propose that amelioration of endothelial activation may be a mechanism of action for these compounds.

I studied the response of these parameters to surgical treatment of varicose veins in 20 patients. I showed that there is an increased plasma lactoferrin at 4 weeks that goes below starting base line levels at 6 months (865 Vs 870 Vs 519). VEGF levels continued to increase (65 Vs 83 Vs 134 pg/dl) in these patients and this may represent vascular remodelling. Although not all of them are easily explainable, the microcirculatory parameters were shown to have a definable response to therapy.

I used a new apparatus (Laser capillary anemometer) to assess the response of the velocity of blood in the microvasculature of patients with venous, arterial or mixed disease. I demonstrated that compression increases velocity of blood in the sub-papillary plexus & lower levels of compression (20 mm Hg) are more effective in increasing velocity in patients with mixed disease. This may explain the basis for using compression therapy in these patients.

Thus I have shown that various parameters of microcirculation may be used to assess the response to therapy in CVD. Future uses of these findings may include design of new and novel therapeutic approaches and to prognosticate for the development of skin changes and ulceration of the leg in CVD.

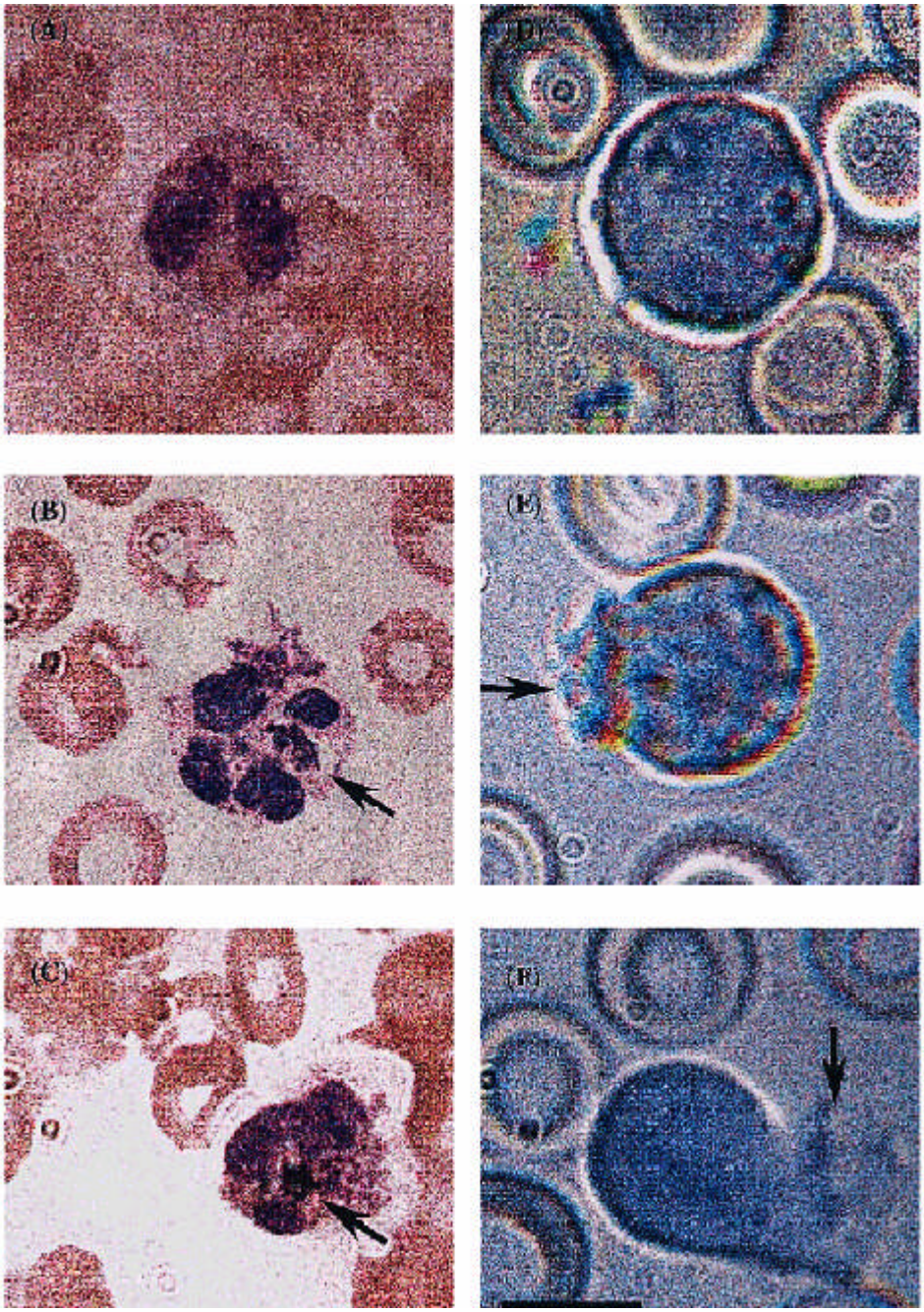

Figure 1 Illustrates both the leucocyte priming as well as presence of factors in the serum that mediate it in CVD. Micrographs of polymorphonuclear granulocytes showing tetrazolium crystal formation after nitroblue tetrazolium reduction (A,B,C) and projection of pseudopods (D,E,F). Control blood of individuals without symptoms (A,D), patient whole blood (B,E), and native granulocytes incubated in patient plasma (C,F). Bar represents 10 μm. Magnification is same for all patients. From Takase S et al with permission.[1]

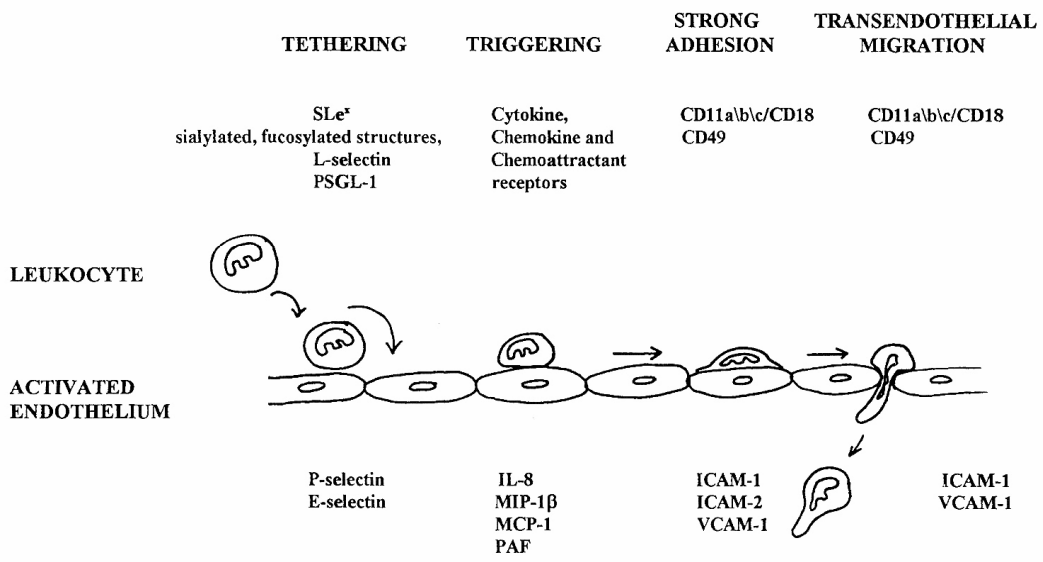

Figure 2 depicts the interaction between the leucocytes and the endothelium that has become so important in the understanding of tissue damage in CVD. The reduced arterio-venous pressure gradient (P_A-P_V) due to lower flow rates leads to leucocyte margination (decreased shear/ physical characteristics of the leucocyte). This leads to increased cell adhesion molecule expression. There is initial weak adhesion followed by stronger adhesion. Different adhesion molecules are involved in these processes. These leucocytes eventually diapedese. These activated leucocytes may cause tissue damage locally either directly or by cytokine release triggering a chemotactic reaction for other white cells. The adhesion molecule expression has been used in many of the studies that are part of this thesis (From Hunt & Jurd 1998, by permission).[60]

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Statement of Originality

All the experimental work described in the thesis is original and was undertaken by me, as a member of the research team at the vascular laboratory at the department of surgery UCL- Medical School between 1996 and 1998. The thesis is based entirely on original observations carried out by me. The work descried in this thesis ‘Objective assessment of the microcirculatory response in venous disease to therapy’’ stems from ideas developed jointly by Mr. PD Coleridge Smith and myself. Neither this thesis nor any part of it has been submitted before to this or any other university in consideration for a degree.

ABBREVIATIONS	
APG	Air Plethysmography
CAM	Cell adhesion molecule
CEAP	Clinical, Etiological, Anatomical, Pathological classification
CVD	Chronic Venous Disease
DVI	Deep Venous Insufficiency
ECA	Endothelial Cell Activation
GSV	Long Saphenous Vein
PAF	Platelet activating Factor
PPG	Photoplethysmography
PV	Perforating Veins
SFJ	Saphenofemoral Junction
SPJ	Saphenopopliteal Junction
SSV	Small Saphenous Vein
SVI	Superficial Venous Insufficiency
VEGF	Vascular Endothelial Growth Factor
VWF	Von Willebrand's Factor

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During submission of this thesis Professor P Boulos took over as my supervisor and I am grateful to him for giving me the chance to complete my work. I thank Professor Charles McCollum for his painstaking reading of the thesis and extremely helpful suggestions.

To Mr. J Farah, Mr. S A Aly, Mr. M Saharay Ms. C Butler and all the staff at the Vascular Laboratory for their help and encouragement. I am greatly indebted to all those colleagues in my Unit and others that I met during various conferences that no doubt helped me shape my thoughts.

I also thank Mr. Philip Coleridge Smith for his supervision and allowing me to refer back to some of his work during the completion of this thesis. Thanks to Dr. J Porter for his help in elucidating many points during the projects regarding the immunological investigations. I thank him also for letting me use his laboratory.

Last, but not the least I am indebted to my both my wife as well as my daughter, Maryam Shoab for their patience with the time I needed for this thesis.

PART I

INTRODUCTION

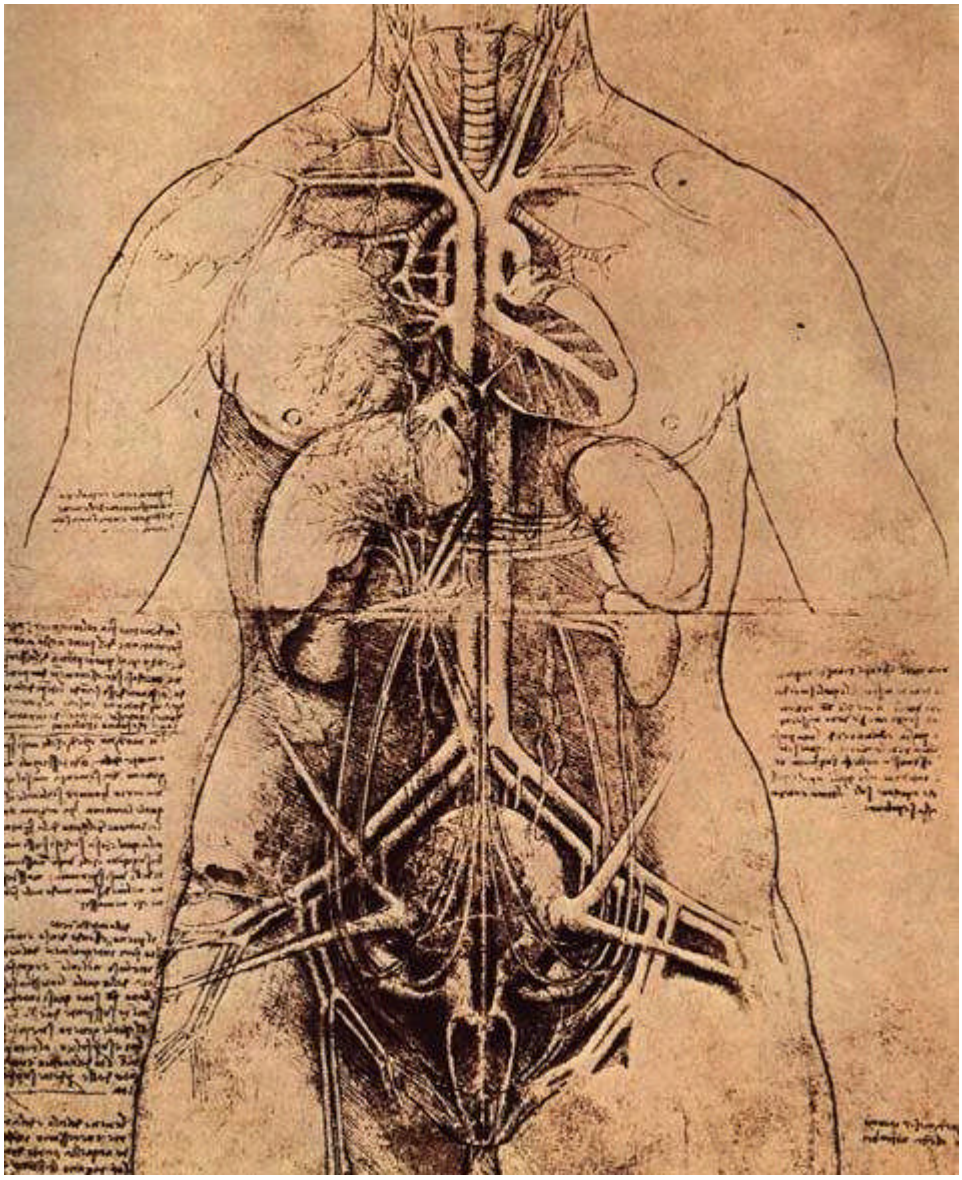

Figure 3 Leonardo Da Vinci provided drawings of the vascular system around 1452. This figure is a reproduction of one of them.

1.1 Development of the Understanding of CVD

The clinical presentations of CVD have been recognised since antiquity. Relics suggest that the ancients had noted the presence of venous ‘swellings’ on the lower limbs and that they regarded them as abnormal. The Ebers papyrus of 1550 BC is the first recorded evidence of varicose veins regarding them as ‘serpentine windings’. A Greek illustration from Athens dated fourth century BC shows the medial side of a massive leg with what appears to be a varicose vein.[2] Presently the changes in the microcirculation of the skin in patients with chronic venous disease (CVD) can now be studied with the help of recent advances in technology.

Figure 4. Leonardo Da Vinci’s detailed drawing of the tributaries at the sapheno femoral junction.

Following this, from the 10th to the 18th century, various physicians, including Haly Abbas, Avicenna and Fallopio, attributed ulceration of the legs to the accumulation of black bile, bad humors, menstrual blood and faeculant humors, and that ulceration in the legs served a useful purpose in getting rid of these vile substances,[3] such that if an ulcer healed it was deliberately broken down. However, Maitre Henri de Mondeville realized that compression bandages helped ulcer healing (drove out evil humors), Ambroise Paré also realised this in 1533.[4]

Figure 5. An illustration of a 'venous ulcer', perforator veins and associated varicosities of below knee veins by John Gay (1866)

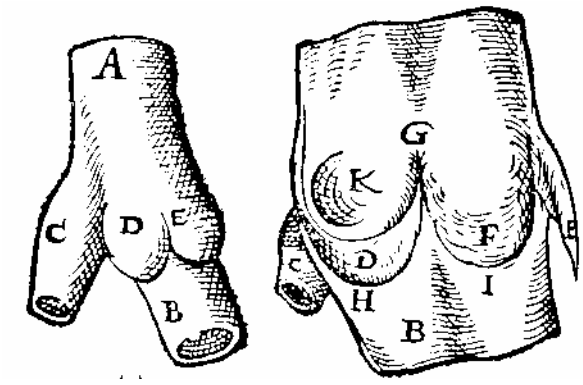

Figure 6.The first recorded drawings of venous valves by Saloman Alberti circa 1585

Trendelenberg (1891) described ligation of the long saphenous vein in the upper third of the thigh. The operation of flush ligation of the sapheno-femoral junction has since been wrongly ascribed to him. Keller and Mayo wrote about stripping of the long saphenous vein in 1905. The work on vascular anastomoses that earned Alexis Carrel his Nobel Prize included attempts at vein transplantation for deep venous reconstruction. Homans in 1916 clearly established the relationship between previous deep vein thrombosis, recanalisation, valve destruction and ulceration of the Leg. Homans described two types of ulcers; varicose ulcers associated with superficial varicose veins and generally cured by removal of these veins, and 'post-phlebitic' venous ulcers, which were intractable to palliative treatment and generally incurable by removal of varicose vein alone. Linton[5] and Cockett have since drawn attention to incompetence of the communicating veins of the calf as a potential cause of venous ulceration. Linton in 1938 described the anatomy and pathological significance of the perforating veins of the leg and performed sub-fascial ligation of incompetent perforators to control venous ulcers. Cockett in 1955 hypothesized that transmission of high pressure to the skin through incompetent perforating veins led to skin changes characteristic of venous insufficiency. He described extra-fascial ligation of perforating veins believing that preservation of the deep fascia was important in preserving effective calf pump function.[6] Palma used the femoro-femoral bypass operation for treating iliac vein obstruction and published it three years later.

PATHOPHYSIOLOGY-DEVELOPMENT OF CONCEPTS

Interest in micro-circulatory aspects of CVD mostly stems from the skin complications caused by it. The prevailing theory about venous ulceration has been that of some sort of 'impaired circulation' leading to diminished nutrition. John Homans of Harvard first

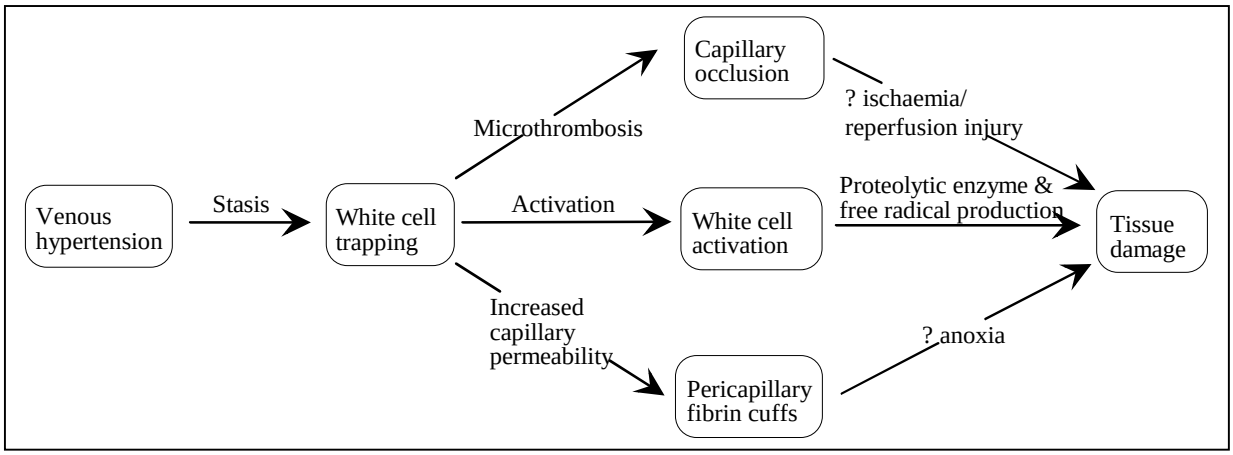

Figure 7 The white cell trapping hypothesis according to Coleridge Smith et al. This theory draws on earlier studies that showed increased leucocyte-endothelial interaction, capillary micro-thromboses, leucocyte activation and sequestration in CVD.

proposed the theory of venous stasis in 1916.[7] He coined the term postphlebitic syndrome to describe the skin changes in CVD. He stated ''over-stretching of the vein walls and destruction of the valves……interferes with the nutrition of the skin……therefore, skin, which is bathed under pressure with stagnant venous blood will form permanent open sores or ulcers''.

Blalock showed that the o2 content of blood from the femoral vein in CVD was higher than that from the contra lateral side. This led to the (not conclusively proven) hypothesis regarding arterio-venous shunting of blood in CVD.[8]

Vein Valve Damage

There are suggestions that venous hypertension causes a shear stress dependent leucocyte- endothelial interaction, which has the manifestation of chronic inflammation. There is subsequent migration of inflammatory cells through the endothelium into the

vein wall and the parenchyma of the valves. There may be elastin and collagen damage as a result of macrophage activity. This may be a mechanism of damage to these valves.[9]

Skin Damage

Leucocytes are also implicated in the skin changes seen in CVD. Thomas, working with Dormandy reported that 25 % fewer white cells left the dependent foot of the patients with venous hypertension.They concluded that this was due to trapping of leucocytes in the microcirculation secondary to venous hypertension.They sepeculated that these had become activated and caused damage to the microcirculation and the overlying skin.[10]

The white-cell trapping theory has established itself as the most plausible theory amongst contemporary phlebologists. Schmid-Schonbein showed that the likelihood of the white cell to adhere to the endothelium increased with decreasing shear stress (as would be seen in venous hypertension due to the decreased arterio-venous gradient i.e. P_A-P_V across the capillary)[11]. They also showed that the leucocyte being larger and less deformable than the erythrocytes are pushed to the periphery in the capillaries as the red cells overtake them. It has also been shown that cellular adhesion molecule (CAM) expression is encouraged by leucocyte-endothelium interaction. Thus the contact between the leucocyte and the endothelium is increased in CVD. This fact led investigators to think, and prove, that some leucocytes were sequestered in the leg during venous hypertension[12].

The types of leucocytes directly involved in the skin changes have remained controversial. T-lymphocytes, macrophages and mast cells have been identified. Skin biopsies have shown that macrophages and lymphocytes predominate in the liposclerotic skin.[13] It is possible that ‘massive’ activation of these mononuclear cells may precipitate skin ulceration.Cytokines have since been shown to be involved actively in this process of leucocyte activation-adhesion-migration-tissue damage sequence. Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are two of the more widely studied[14]. Both cytokines reduce fibrinolytic activity of the monocytes that may be contributory to the fibrin cuffs seen in CVD.

Flow may be non-uniform in CVD. There may be micro-thromboses; this may lead to organized localized heterogeneous perfusion. There is peri-capillary oedema and erythrocytes extravasation. The exact steps in the mechanism of these changes are unknown. Continuing leucocyte mediated injury along with the reparative response suggested by my work may be important contributory factors.[15]

1.2 Epidemiology of CVD

Venous disease of the legs is common, hence the justification for the extensive research devoted to CVD. CVD may affect up to 20% of the total population,[16], [17] a risk that increases with age. The prevalence of healed and/or active leg ulceratiuon in the population is approximately 1%. [18]

In one study from London age and female sex were independent risk factors for varicose veins and phlebitis. Thrombosis and pulmonary embolism were both significantly associated with the presence of diabetes, female sex, and increased age. A quarter of the sample had varicose veins, and that nearly a third had had venous disease of some kind at some time. This suggested that venous disease may place a heavier burden on health resources than had been realised[19].

The Edinburgh Study

The Edinburgh study was a cross sectional study of a random sample of 1566 subjects 18 to 64 years of age. It reported telangiectasis in 80 % of men and 85% of women. Varicose veins were present in 40% men and 16% women. Ankle oedema was seen in 7% men and 16% women. Active or healed venous leg ulcers occurred in approximately1% of the general population.

Analysis of varicose veins in Edinburgh population (Lee et al 2003) provided the following results of inter-relationships between a range of lifestyle factors and risk of varicose veins. This was to identify which factors may be implicated in the aetiology. An age-stratified random sample of 1566 subjects (699 men and 867 women) aged 18 to 64 years from 12 general practices throughout Edinburgh was used. A detailed self-administered questionnaire was completed, and a comprehensive physical examination

determined the presence and severity of varicose veins. Self-reported evidence suggested a familial susceptibility. Future genetic studies may be warranted.[20] The slightly higher age-adjusted prevalence of varicose veins in men than in women (39.7% versus 32.2%) was not explained by adjustment for an extensive range of lifestyle risk factors (male odds ratio [OR] 2.11, 95% confidence interval [CI] 1.51–2.96). In both sexes, increasing height showed a significant relationship with varicose veins (male OR 1.50, 95% CI 1.18–1.93 and female OR 1.26, 95% CI 1.01–1.58). Among women, body mass index was associated with an increased risk of varicose veins (OR 1.26, 95% CI 1.02–1.54). No consistent relationship with any lifestyle factor was shown. It was also concluded that Using RD > 0.5 as a cut-off point decreases the specificity and risks defining more normal veins as incompetent, whereas using RD > 1.0 as a cut-off point decreases the sensitivity and risks defining more incompetent veins as normal. There are obvious limitations in using presence of reflux in individual vein segments as a test for the presence or absence of "venous disease." Accepting these limitations, calculations of sensitivity and specificity tended to support the use of 0.5 seconds as the cut-off point for RD in the below knee popliteal vein segment as a test for venous disease. In the upper LSV, however, there was little difference in the sensitivity and specificity between RD >0.5 and RD >1.0.

European Studies

The French study reported in 2004[21] documented the prevalence of varicose veins, skin trophic changes, and venous symptoms in a sample of the general population of France. It also looked at their main risk factors and assessed relationships between them. This cross-sectional epidemiologic study was carried out in the general population of 4 locations in France: Tarentaise, Grenoble, Nyons, and Toulon. Random samples of 2000 subjects per location were interviewed by telephone, and a sub-sample of subjects completed medical interviews and underwent physical examination, and the presence of varicose veins, trophic changes, and venous symptoms was recorded.

Prevalence of varicose veins, skin trophic changes, and venous symptoms was not statistically different in the 4 locations. In contrast, sex-related differences were observed: varicose veins were found in 50.5% of women versus 30.1% of men (P < .001); trophic skin changes were found in 2.8% of women versus 5.4% of men (P _

NS), and venous symptoms were found in 51.3% of women 51.3% versus 20.4% of men (P < .001). Main risk factors for varicose veins were age and family history in both sexes, and pregnancy in women. Female sex was a significant factor only for non-saphenous varicose veins. Varicose veins, age, and pitting edema were the most significant risk factors for trophic skin changes. The risk factors for venous symptoms were female sex, varicose veins, and prolonged sitting or standing. A negative relationship with age was found in women.

Cost Considerations

In the UK the total cost to the National Health Service is substantial. Data from the Riverside study in London suggested that between £230 and £600 million is spent annually to care for patients suffering with venous ulcers.[22] Much of this is spent on care in the community, with 30% of community nursing time spent on treating leg ulcers. Even a small improvement in healing and recurrence rates may produce substantial savings. Furthermore, the indirect costs arising from leg ulcer disease, for example, time lost from work, permanent disability and early retirement, are even higher. It has been estimated that, in the UK, five hundred thousand working days are lost per year due to this condition.[23]

Prevalence Studies outside UK

The prevalence of CVD is much lower in African and Asian or Australasian aborigine populations although immigrant subjects from these regions have the same risk as the population of their host country. Sedentary habits, obesity, tight clothing may provide part of the explanation.

The first major study into the epidemiology of varicose veins was the US National Health Survey of 1935-6, which, in a questionnaire of 2.8 million people, estimated that varicose veins were present in 1.75 million of the total population of the United States. This survey was repeated in 1959-61, which suggested a point prevalence of 2.25% for severe varicose veins (0.8% men, 3.5% women). A similar study in the UK based on interviews gave a prevalence of 2.25% for varicose veins (1.41% men, 3.74% women), and in Denmark a survey of patients attending hospital with varicose veins found an incidence of 1.7% of males and 2% of females in one year. However, Borschberg (Basel survey) has criticized these surveys, which concluded that statistically acceptable

evidence on the prevalence of varicose veins could not be drawn from them. There have since been several major regional surveys into both varicose veins and venous ulceration: in a study from Sweden[24] the point prevalence of leg ulceration was estimated at 6.4 per 1000 in subjects. In a further study from Sweden,[25] the period prevalence per year from a retrospective analysis of medical records was found to be two to four per thousand, and a more recent Swedish study has found the point prevalence to be 3.05 per 1000.[26] The Tecumseh Community Health Study in Michigan suggested that 24 million Americans had "significant" varicose veins (7.4% men and 16.6% women), with 5% having skin changes and 0.5% having active or healed ulcers. In Czechoslovakia, 6.6% of men and 14% of women had varicose veins, and 1% of the population over 15 was found to have present or past leg ulceration. The Basel III study found a prevalence of 4.2% for severe varicose veins and 1% for active and healed ulcers in a highly selected population of industrial workers, and in New Zealand Beaglehole *et al.*[27] found varicose veins in 36.3% of Maori men and 47.4% of Maori women, compared to 21.5% of men and 40.4% of women in the white population. Overall, it would appear that there is a prevalence of varicose veins of approximately 2%, although local surveys have shown higher levels. Data from the Framingham study supports this.[28]

Prevalence of Venous Leg Ulceration

There have been several studies on the prevalence of active ulceration. In Ireland, the prevalence of current ulcers was found to be approximately 1.5% of the population. In Australia the point prevalence of active leg ulcers of greater than four weeks duration was found to be 1.05-1.1 per 1000 of the population.[29,30] Sixty-seven percent of these ulcers had venous pathology, 27% had arterial disease, and the rest suffered from diabetes, rheumatoid arthritis and other miscellaneous conditions. In Scotland the point prevalence of active leg ulceration was found to be 1.48 per 1000 of the population, and in England the point prevalence of current ulcers was found to be 1.8 per 1000.It has also been estimated that for every patient with an ulcer there are 20-30 patients with the characteristic skin changes of lipodermatosclerosis, which precede ulceration.[31] Overall, Callam, reviewing the literature, suggests a point prevalence of 0.1-0.2% for

active ulceration in Western Europe, with 1% of the population suffering from ulceration at some time in their lives.[32]

Sex Distribution

It is generally believed that varicose veins and ulcers are more common in women. All the above mentioned studies with the exception of the Basel III study (carried out in factory workers with relatively few women) have shown a female preponderance, ranging from 1.5:1 in the Swedish and Czech studies, to 2:1 in the studies from Australia, Ireland and England. Females may also be more prone to varicose veins as pregnancy is often thought to be a risk factor for their development. In 1942 Lake had observed that in a group of 563 women that 67% of childless women and 80% of women with children had varicose veins. It is estimated that between 8 and 20% of women develop varicose veins during pregnancy.[33] It has been shown that multiple pregnancies increase the prevalence of varicose veins.[34] The role for progestogen receptors is discussed in the chapter on pathology.

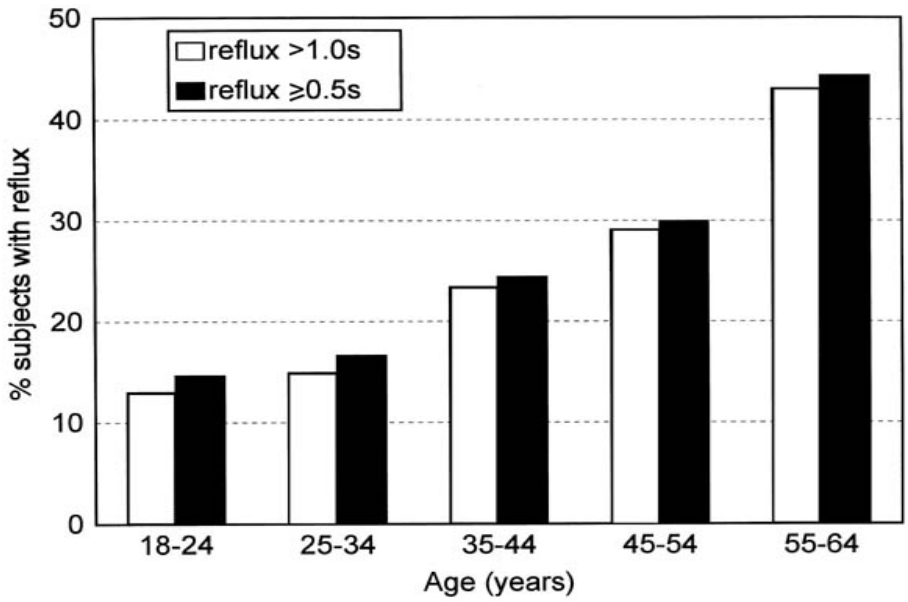

Figure 8 Results of DUPLEX confirmed reflux in the lower limbs of a cross sectional study of 1566 subjects. Proportion of participants either leg, by age. Data is grouped reflux of 0.5 seconds or more duration and reflux of more than 1.0 seconds. This graph is from the Edinburgh Vein study and shows the increasing prevalence of reflux with age

However, in patients with symptomatic veins GSV reflux combined either with SSV reflux and/ or perforator reflux has been shown to correlate with severity of symptoms and skin changes (The Edinburgh Vein Study)[17].

Age Distribution

The Basel III study has shown an age-related increase in the prevalence of ulceration from nil under the age of 35 to 4.8% in women over the age of 50. Data from the other studies show similar trends. The cross sectional Edinburgh study of DUPLEX examination of lower limbs also found increasing venous insufficiency with age.[17]

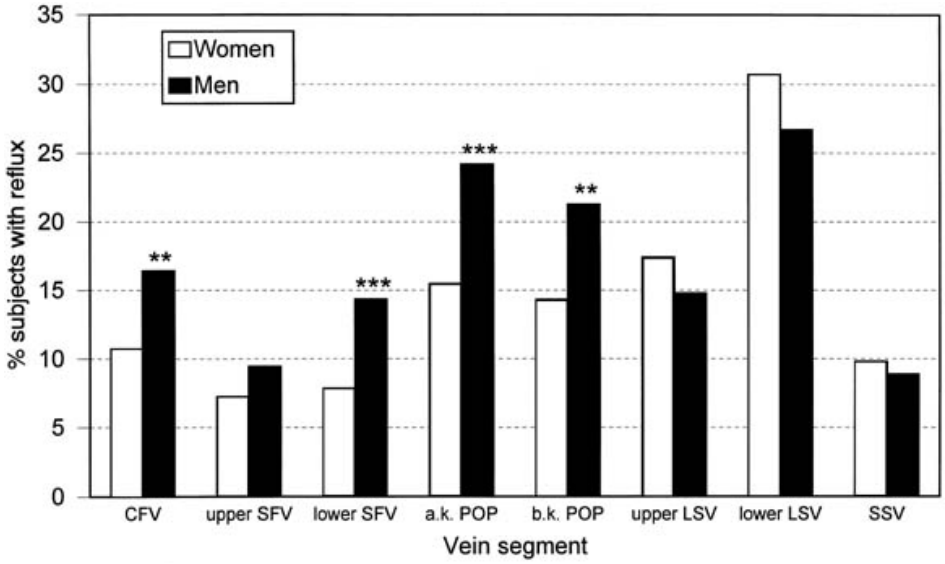

Figure 9 Shows the prevalence of DUPLEX confirmed venous reflux in a cross-sectional survey of 1566 subjects ranging in age from 18 to 64 years, randomly selected from 12 general practices. It remains unknown as to what percentage of these subjects would progress to clinical chronic venous disease.

The US National Health Survey of 1961 noted varicose veins in 24% of whites compared to 10.4% of colored people; Mekky assessed two groups of females in England and Egypt, finding 32% of English workers had varicose veins compared to 5.8% of Egyptians.[35] Similarly Maffei found a significant difference between whites

(49%) and non-whites (35.8%) in Brazil. Beaglehole, who concludes that varicose veins are commoner in Caucasian western populations., has summarized the available data[36].

Inheritance

It is widely believed that varicose veins have a familial bias. However, varicose veins are extremely common, and accordingly most sufferers are aware of other family members with varicose veins. Several studies have found a positive association between varicose veins and family history,' though all these studies are open to bias in that relatives were not examined. However, Belcaro found a positive association in a small study using Doppler examination in both parents and offspring.[37] According to one study the risk of developing varicose veins for the children (in later life) was 90% when both parents suffered from this disease, 25% for males and 62% for females when one parent was affected, and 20% when neither parent was affected.[124] Cytogenetic abnormalities have been described in familial varicose vein tissue. Cytogenetic investigation of primary cell cultures from fragments of varicose veins of patients showed metaphases with structural abnormalities, clonal trisomies of chromosomes 7, 12, and 18, and monosomy of chromosome 14 only in cases with the familial type, while the sporadic cases had no similar chromosome aberrations[38].

1.3 Applied Venous Physiology of the Lower Limbs.

60-75% of the blood in the circulatory system is located in the veins. Unlike the splanhnic veins the skeletal muscle veins are not known to have abundant sympathetic nervous supply. These are however responsive to circulatory catecholamines. There are considerable changes in the venous pressure in the lower limbs in the erect posture. The pressure remains fairly constant at the hydrostatic indifferential point just below the diaphragm. When upright, there is loss of approximately 500mls of blood into the venous circulation. The extra loss of interstitial fluid is collected by the lymphatic system. Reversal of flow in the vein is essential for venous valve closure. A velocity of approximately 30 cm/sec woul usually cause valve closure. Venous valve imaging by advanced US imaging has demonstrated pulsatile flow with regular opening and closure cycles. The cross-sectional area across the valve is 35% smaller even in the fully opened

state. Two flow streams are generated during forward flow. The first one is a proximally directed jet. The second is a vortex flow into the valve cusp. There is suggestion that valve closure occurs when the vortex flow exceeds the proximally directed jet flow.[39]

Pressure in the superficial and deep veins is similar during periods of ‘quiet standing. The 1 mm or so higher pressure in the deep veins may keep the perforator valves closed. It is thought that the perforator valves may protect the skin and underlying tissues from the extreme venous pressures generated during muscular contraction (100-130 mm Hg). Their exact role, however, is the subject of much debate.

In the heel-strike position the plexuses under the heel are compressed and blood finds its way into the calf. From there muscular contractions (aided by the function of the valves) drive the blood into the deep veins.

THE CALF MUSCLE PUMP & ORTHOSTATIC VENOUS PRESSURES

The principal participants in the pumping mechanism are the veins of the calf within the calf muscles, gastrocnemius and soleus. These usually contain about 250ml of blood. Additional muscle pumps are also recognised in the thigh and foot. The latter has a much smaller capacity (about 25ml), but is probably responsible for return of most blood from the foot. It may have a 'pump-priming' effect on the calf muscle pump. The calf pump is often referred to as the peripheral heart. If the calf muscles are not contracted periodically it may result in syncope in an erect subject.

Venous flow is affected by the contractile force of the heart, static filling pressure and gravity. The pressure in the venules being 15-20 mmHg and 0-6 mmHg in the right atrium During calf contraction, the pressure in and around all the structures contained within the deep fascia becomes raised resulting in all the intra-muscular veins becoming completely compressed resulting in the blood that is present in these veins being emptied by the calf muscle pump into the outflow tract which is the popliteal vein. The large veins within the gastrocnemius and soleus muscles form the main chamber of the pump but all the other deep veins participate. Distal deep venous valves prevent axial reflux and valves in the communicating veins prevent reflux from the deep venous system to the superficial venous system. (With continuous exercise, the calf blood volume is reduced by 1.5 - 2.0 ml/100ml mainly as a result of the compression of the

veins in the pump chamber and the average expelled volume is approximately 30 - 40 ml/100 ml, that is, a significant proportion of all the blood within the pump. The pump will normally expel this volume in four or five contractions though one single sustained contraction can expel as much). As the exercise rate increases, the muscle blood flow may increase to 20 - 30 ml/100ml/min, that is, an additional load of 600 ml/min on the calf pump. The calf must contract about 20 times every minute to expel this increased blood flow. At a normal walking pace of 80 steps/minute, each calf contracts about 40 times/minute so the calf muscle pump can easily deal with the high blood flow of exercise hyperaemia. The popliteal vein is a large bore vein which offers virtually no resistance to outflow. The gradient of 10 - 15 mmHg between the small veins and the heart is sufficient to ensure venous blood flow in the supine position. The increase in gradient produced by the calf muscle pump during contraction is sufficient to ensure an adequate rapid venous return to the heart during vigorous erect muscle exercise.

As the calf muscles relax, valves cephalad to the muscle pump close and prevent reflux of blood from more proximally in the axial veins. The valves in the communicating veins open and allow normal blood flow from the superficial to the deep venous system. In addition, inflow from the lower extremity capillary beds re-primes the calf muscle pump for a subsequent contraction. At the moment when the calf muscles relax their contained veins are empty, at zero pressure and as yet unfilled by arterial inflow. As the veins are collapsed, they are unaffected by hydrostatic pressure. On the other hand, the superficial veins are full and subjected to hydrostatic pressure plus the remnant of cardiac generated pressure. The pressure gradient between the two compartment may become 100 - 110 mmHg resulting in blood immediately flowing from the superficial to the deep compartment through the many communicating veins (Bjordal et al, 1970). This empties the superficial compartment and reduces its pressure (Pollack et al, 1949). Therefore the function of the calf muscle pump is vital in ensuring venous return from the lower limbs during exercise and the reduction of the superficial venous pressure thus removing the damaging effects of the hydrostatic pressure.

PHYSIOLOGICAL INVESTIGATION OF ‘VENOUS EMPTYING’ OF THE LOWER LIMBS

The effectiveness of the calf muscle pump can be investigated by several methods. The ambulatory venous pressure measurement technique measures deep venous pressure directly by placing a needle in a dorsal foot vein and connecting it to a standard pressure transducer and a recording device. The baseline dorsal foot vein pressure is approximately 80-90 mmHg in subject standing upright with no calf muscle movement. This pressure is predominantly hydrostatic and is directly related to the distance from the needle canulation site to the right atrium. The high resting distal venous pressures in normal subjects in the upright position is due to the lower extremity venous valves being partially open. The venous pressure may transiently rise further after calf muscle contraction reflecting the presence of physiologic reflux before valve closure. As the calf veins are emptied by the contraction of the calf muscle pump, the pressure steadily decreases and as the calf veins empty there is a functional shortening of the hydrostatic fluid column as valves close in the axial veins at progressively lower levels. When the hydrostatic fluid column is maximally shortened and the veins maximally emptied a new baseline pressure is obtained, the ambulatory venous pressure. This is generally reached after ten successive up and down tiptoe movements. This pressure reflects the point where the forces promoting venous return are balanced by arterial inflow and inflow from the superficial veins to the deep system. The ambulatory venous pressure is generally below 40 mmHg in normal subjects.

Other Aspects of the Venous Circulation

When the subject is in the supine position, the respiratory cycle plays an important part in the venous return from the lower extremities. In the supine position, the pressure in the foot vein of the subject represents the increment of pressure that remains after the dissipation of kinetic energy generated from the heart by the resistance of the arterioles and capillaries. This foot vein pressure in the supine position is approximately 15 mmHg. The right atrial pressure is normally between 0 and 2 mmHg so the venous

return to the heart in the supine position is generated by a pressure gradient of 13-15 mmHg. During inspiration, the volume of the veins of the thorax increases and the pressure decreases in response to reduced intrathoracic pressure. Expiration leads to the opposite effect, with decreased venous volume and increased pressure. The venous response to respiration is reversed in the abdomen, where the pressure increases during

inspiration because of the descent of the diaphragm, and decreases during expiration as the diaphragm ascends. Increased abdominal pressure during inspiration decreases pressure gradients between peripheral veins in the lower extremities and the abdomen, thus reducing flow in the peripheral vessels. During expiration, when intra-abdominal pressure is reduced, the pressure gradient from the lower limbs to the abdomen is increased and the flow in the peripheral veins rises correspondingly. The respiratory effects are usually associated with clear phasic changes in venous flow in the extremities. These can be detected by various instruments, including many forms of plethysmography and Doppler flow detectors. The respiratory changes in venous velocity may be exaggerated by respiratory manoeuvres, such as the Valsalva manoeuvre, which increases intra-thoracic and abdominal pressures and decreases, abolishes, or even reverses flow in some peripheral veins. Also, the respiratory effects on venous flow may be diminished in the lower limbs of individuals who are chest or shallow breathers and whose diaphragm may not ascend sufficiently to change intra-abdominal pressure.

In the fully upright position, the expiratory promotion of venous flow is not enough to overcome gravitational hydrostatic forces and promote adequate venous return. The contraction of the calf muscles is required to overcome this gravitational force. In the upright position, the column of blood between the heart and the foot exerts a gravitational force known as the hydrostatic pressure. The pressure in the foot vein then becomes the sum of (15 mmHg)the approximate foot vein pressure in the supine position plus the hydrostatic pressure. This hydrostatic pressure is exerted by the column of blood between the foot and the level of the manubrium sterni, the point used as the zero reference for CVP measurement.

1.5 Biology of Chronic Venous Disease

Endothelial/ leucocyte activation is being increasingly recognised as an important marker event that could be used to prognosticate the development of complications in many scenarios.[40]

The aetiology of skin damage in patients with chronic venous disease is incompletely understood. In 1987 Moyses *et al*[18] reported a 15 and 20% reduction in the number of white cells leaving the feet in eight healthy subjects during a 45 minute period of sitting. Thomas *et al*16 found a larger fall (of 24%) in a group of ten patients with chronic venous insufficiency compared to normal control subjects (5%) following 60 minutes of leg dependency. After the subjects were allowed to lie supine, the white cells returned to the circulation. Coleridge Smith et al. using video microscopy showed that the number of visible capillaries fell during a period of venous hypertension, suggesting that increased venous pressure reduced capillary perfusion and hence the capillary flow rate. The white cell trapping hypothesis proposed that raised venous pressure led to decreased flow, This causes leucocyte margination, activation, adhesion, and extra-vascular migration. It was proposed that the release of proteolytic enzymes and free radicals from activated leucocytes resulted in tissue damage. This is supported by histological evidence of white cell infiltration of the tissues[41] in skin biopsies.

Bollinger[42] using capillary fluorescence microscopy showed that there were areas of skin with no apparent blood flow in patients with chronic venous insufficiency, which was restored when elastic stockings were applied. He suggested that some of the capillaries were not functioning because of thrombosis. He also demonstrated increased diffusion of fluorescein out of capillaries in patients with chronic venous insufficiency, indicating that capillaries in chronic venous insufficiency are much more permeable than normal to this molecule, suggesting abnormal endothelial function. Using simultaneous fluorescence and light capillary microscopy Franzeck et al. described the appearances of capillary loops that did not appear to be perfused, suggesting capillary thrombosis to be a feature of chronic venous insufficiency[43]. Coleridge Smith et al also showed using video microscopy that the number of visible capillaries fell following a period of venous hypertension, suggesting that increasing venous pressure reduced the capillary perfusion pressure and hence the capillary flow rate.

It is well known that low capillary flow is a potent initiator of white cell adhesion[44 45-46] and Schmid-Schonbein has shown that the likelihood of a leucocyte adhering to a vessel wall is inversely related to the shear force exerted on it.[11] On the basis of these observations Coleridge Smith *et al*. put forward the white cell trapping hypothesis, [17] postulating that the fall in the count of capillaries in the leg skin of patients with chronic

venous insufficiency on dependency was due to reversible adherence of white blood cells to the endothelium. The hypothesis postulates that an increase in venous pressure during standing or walking causes a reduction in capillary flow rate, resulting in trapping of white blood cells in the leg. The trapped white cells plug the capillaries resulting in areas of ischaemia, and also become activated releasing proteolytic enzymes and toxic metabolites (free radicals) that damage the capillaries, and chemotactic substances that attract more white cells. Activated white cells have been shown to be mediators of tissue damage in a number of organs including the heart[47,48] brain, lung and kidney.[148,49] Monocytes release cytokines interleukin-l (IL-I) and tumour necrosis factor-alpha (TNF-alpha) when activated. These may also activate endothelial cells resulting in increased vascular permeability.[50] The latter may be the mechanism by which fibrinogen passes into the pericapillary spaces and forms fibrin cuffs.

Skin biopsies taken from three groups of patients with chronic venous disease (no skin changes; LDS only; LDS and ulceration) showed that there was a low number of white cells in the skin of patients without LDS, while in patients with LDS but no ulceration, the number of white cells in the skin was increased eight-fold, and in patients with ulceration, there was a 40-fold increase in the number of white cells in the skin.[51] . A more recent study has confirmed increased numbers of white cells in the skin of patients with LDS. Immuno-histochemical features suggest that they are mainly macrophages and lymphocytes.[52]

The molecular mechanism of leucocyte activation, leucocyte-endothelial adhesion and extra vascular migration has been the subject of much recent study. Various adhesion molecules are expressed by circulating leucocytes and endothelial cells in response to diverse physiological and pathological stimuli, leading to firm adhesion of leucocytes to the endothelium with subsequent degranulation or extra vascular migration.

Experimental work to study white cell activation in venous disease was undertaken by Shields who investigated neutrophil activation by measuring neutrophil degranulation products lactoferrin and elastase in both normal subjects following experimental venous hypertension and patients with chronic venous disease. Neutrophils posses two types of granules, primary and secondary, which contain various chemical substances.

Upon activation these are released into phagocytic vacuoles and some is spilled into the plasma. Lactoferrin is an iron binding glycoprotein found in secondary granules of neutrophils and has been used as an indicator of neutrophil activation in several disease states including various forms of arthritis, pneumonia[53] and sepsis.[54],[55] It is produced in response to various stimuli such as TNF-α, FMLP, opsonised bacteria, C5a coated bacteria/immunoglobulins and lipopolysaccharides, and has various functions Neutrophil Elastase is a 29KD protein which is stored in the primary granules[56] and released during the process of phagocytosis or when neutrophils are activated by other stimuli such as soluble immune complexes, C5a or endotoxins.[57] It is chemotactic, degrades fibrinogen[58] and has been implicated in the tissue destruction seen in venous disease by causing endothelial cell injury.[59] In a series of experiments Shields et al showed that there was a rise in plasma lactoferrin levels in normal subjects in response to venous hypertension. Patients with varying severity of venous disease all had raised lactoferrin levels compared to age and sex matched controls[161]. Similarly plasma elastase was noted to be higher in patients with venous disease compared to age and sex matched controls.[20] However, no difference was found between the various patient groups and it was concluded that neutrophils are probably responsible for acute inflammatory changes and for initiating vascular endothelial damage but unlikely to account for the skin damage seen in patients with chronic venous insufficiency.

1.6 ENDOTHELIAL CELL ACTIVATION (ECA)

The endothelium is extremely heterogenous and varies from one anatomical site to another. Not all endothelial cells exhibit histocompatibility locus A (HLA) class II molecules.[60] ECA was first used as a term in 1960 when it was noted that endothelial cells showed both a loss of integrity (leakiness, cellular oedema)as well as increased amounts of intracellular organelles.[61] Willms-Kretschmer et al implied in this paper that there were functional consequences to the altered morphology.

The functions of the endothelium normally include maintainence of selective permeability, transduction of blood borne signals, regulation of vasucular tone, antithrombogenic properties, regulation of leucocyte adhesion as well as regulation of vascular growth. Agents causing ECA are diverse. These include cytokines (notably IL-1 & TNF), Bacterial endotoxins (lipopolysaccharides) , complement, viral infections

and immune complex deposition. There are two distinct types of endothelial cell activation viz. type-I ECA and type-II ECA.

Type-I ECA (endothelial cell stimulation) does not require denovo protein synthesis or gene upregulation. This response may occur within seconds of a stimulus being applied. There is retraction of the endothelial cells from each other and the subendothelium is exposed. There is increased expression of P-selectin, release of Von Willebrand factor and secretion of platelet activating factor (PAF).

Type-II ECA requires a period of time (usually up to 4-6 hours in vitro) to be estalished. It involves gene activation, transcription and increased protein synthesis.. Increased transcription signalling involves leucocyte adhesion molecules (E-selectin, ICAM-1, and VCAM-1), cytokines (IL-1, IL-6, and IL-8), monocyte chemoattractant protein (MCP) and tissue factor. At the same time thrombomodulin and other significant molecules are lost from the endothelial surface.

Processes Involved In ECA

These are shown in Figure 12 and include loss of vascular integrity, expression of leucocyte adhesion molecules, secretion of cytokines, prothrombotic changes and upregulation of HLA molecules. Loss of integrity involves retraction of the cells from each other. This allows proteins and cells to pass from the lumen to the outside. The key leucocyte adhesion molecules are E-Selectin, P-Selectin and the integrins. The endothelium is both a source of cytokines as well as a target for these. The cytokines include IL-1, IL_6, IL-8, TNF, CSF and MCP-1 amongst others.

The prothrombotic changes seen in ECA include alteration of its normal anti-platelet, anti-coagulant and fibrinolytic effects. The decreased fibrinlytic effect is probably mediated through increased production of PAI-1 (Plasminogen activator inhibitor-1) that counters the normal tisssue plasminogen activator. VW factor is one of the molecules expressed in ECA; it is also an acute phase protein. Other coagulation pathways affected include expression of anti-thrombin III as well as heparan sulfate.

Integrin	Other Name	Ligand
α1β1	CD49a-CD29	Laminin (collagen)
α2β1	CD49b-CD29, gpIa-IIa	Collagen (Laminin)
α 3β1	CD49c-CD29	VCAM-1,fibronectin
α 5β1	CD49e-CD29,fibronectin receptor	Fibronectin
α 6β1	CD49f-CD29, laminin receptor	Laminin
α 8β1		
α vβ1	CD51-CD29	Fibronectin
α sβ2	CD11a-CD18,LFA-1	ICAM-1,ICAM-2,ICAM-3
α $_{M}$β2	CD11b-CD18, Mac-1	ICAM-1,ICAM-3,fibrinogen,C3bi
α xβ2	CD11c-CD18,p150,95	Fibrinogen,C3bi
α $_{HB}$β3	CD41a,gpIIb-IIIa	Fibrinogen,fibronectin, , , Von Willebrand Factor
α $_{V}$β3	CD51-CD61	Von Willebrand Factor,laminin,thrombospondin,fibronectin
α 6β4	CD49f-CD104	Laminin
α $_{v}$β5		Vitronectin,
α $_{v}$β6		fibronectin
α $_{4}$β7		VCAM-1,MAdCAM-1,fibronectin
α 1Elβ7		
α $_{v}$β8		

Table 1 shows the various integrin adhesion molecules along with their ligands.

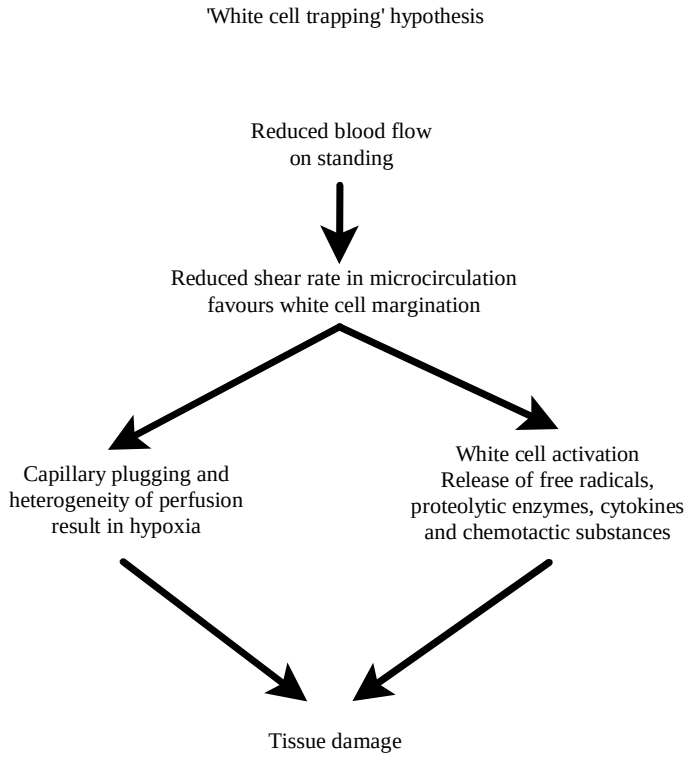

Figure 10 illustrates the white cell activation hypothesis. From Coleridge Smith PD. Microcirculation in venous disease. Landes Bioscience 1998

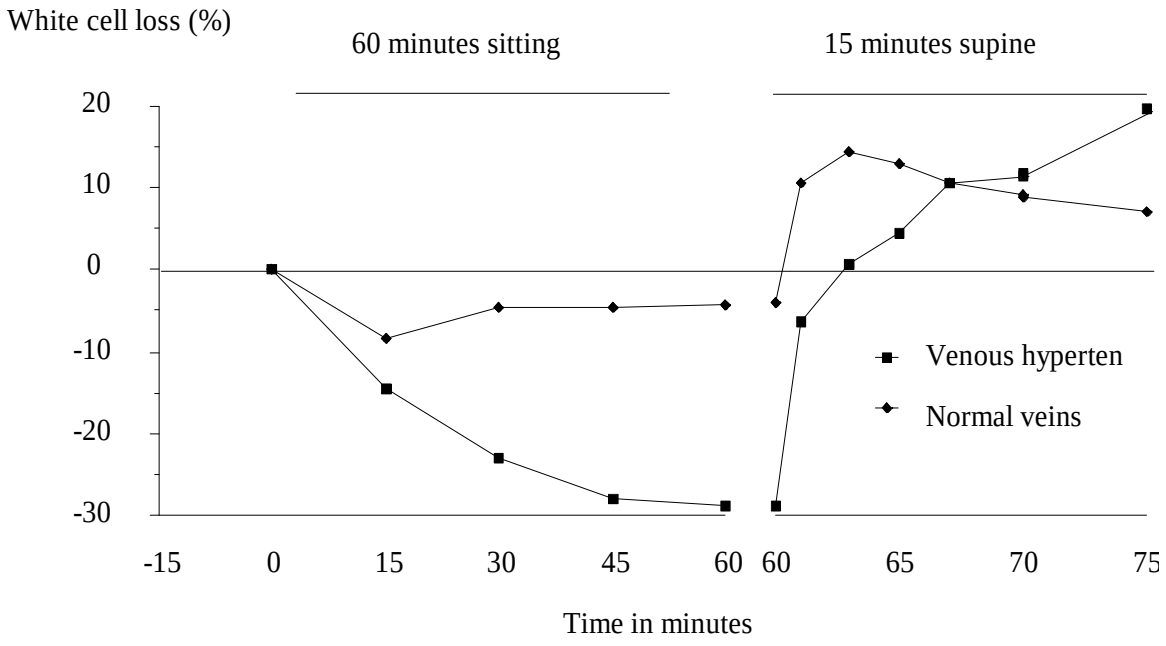

Figure 11 illustrates the white cell trapping hypothesis. It illustrates the trapping of the leucocytes in the venous side of the circulation on standing up on dependency of the legs and their subsequent release on assuming the supine position. From Coleridge Smith PD. Microcirculation in venous disease. Landes Bioscience 1998.a

Tissue factor and vesicles with increased prothrombinase acttivity are also affected.Human endothelial cells in culture normally exhibit Class I major histocompatibility complex (MHC-I) like HLA-A, B and C). Stimulation of cultured endothelial cells results in increased expression of class I but not class II molecules (HLA-DR, DQ and DP)[¥]. In vivo only certain endothelial cells (notably post-capillary venules, veins and some arteries) exhibit class II molecules. In chronic rejection and inflammatin, however, the expression of these molecules is increased in many tissues affected by these processes.

Intracellular processses in ECA

Altered gene transcription is involved in the transduction of signals in ECA. From the endothelial cell surface the message is carried intra-cellularly by a transcription factor nuclear factor κB (NF-κB). Gene upregulation during ECA include binding site for NF-

[¥] Human Leucocyte Antigen; DR DQ DP

κB in their promotor area. NF-κB is stored as an inactive form in the cytoplasm and is activated by removal of an inhibitory subunit IκB.

1.7 Adhesion Molecules in CVD

Leucocytes express adhesion-promoting receptors that mediate cell-cell and cell-matrix interaction. These interactions are crucial for the direction and control of leucocyte traffic, migration through tissues, and the development of immune and nonimmune inflammatory responses

There are four main groups of adhesion molecules viz. Integrins, Immunoglobulin super family, Cadherins and Selectins♣. The integrins are trans-membrane cell surface proteins that bind to cytoskeletal structures and communicate extra-cellular signals. They have α and β subunits, their classification being according to the β subunit. In association with different α subunits about 21 different combinations are known (see table 1). Their functions are varied. Certain of the integrins function as extra cellular matrix receptors, others mediate leucocyte–endothelial cell adhesion or bind primarily

to matrix proteins. They may be important in inflammation, wound healing and development. Table 2 gives the details of the immunoglobulin super family. Previous work suggests that the leucocyte population in the human consists of “primed" and "un-primed" group. Leucocytes most likely to bind to endothelium represent the "primed" group, which express the highest levels of CD11b and L-selectin. Therefore, the fall in the activation of the circulating cells is the result of the more activated cells adhering to the endothelium. Surface expression of CD11b and L-selectin may remain low even after the venous hypertensive insult is withdrawn. A rise in the soluble L-selectin levels in the plasma was noted, confirming it had been shed, a process that precedes CD11b expression and firm adhesion.

These findings, considered together, provide strong evidence of leucocyte activation and trapping in the microcirculation. Naturally occurring blocking antibodies to CD11b/CD18 receptors have been reported, and it may be that patients with long-

♣ Lectins (to select) are sugar binding proteins with high selectivity for the sugar moiety. First described in 1888 as agglutinins.

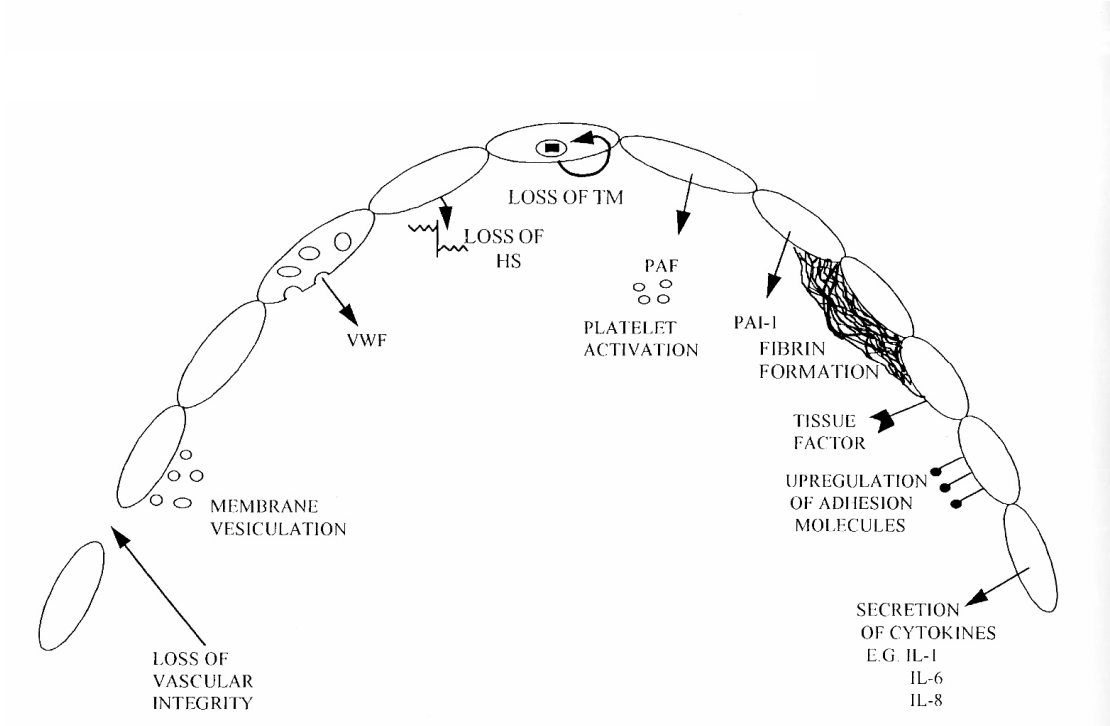

Figure 12 depicts the various changes invloved in endothelial cell activation. VWF=VonWillebrand's factor, HS=Heparan Sulfate, TM=Thrombomodulin, PAF=platelet activating factor, PAI-1=Plasminogen activator inhibitor-1. (From Hunt & Jurd by permission).[60]

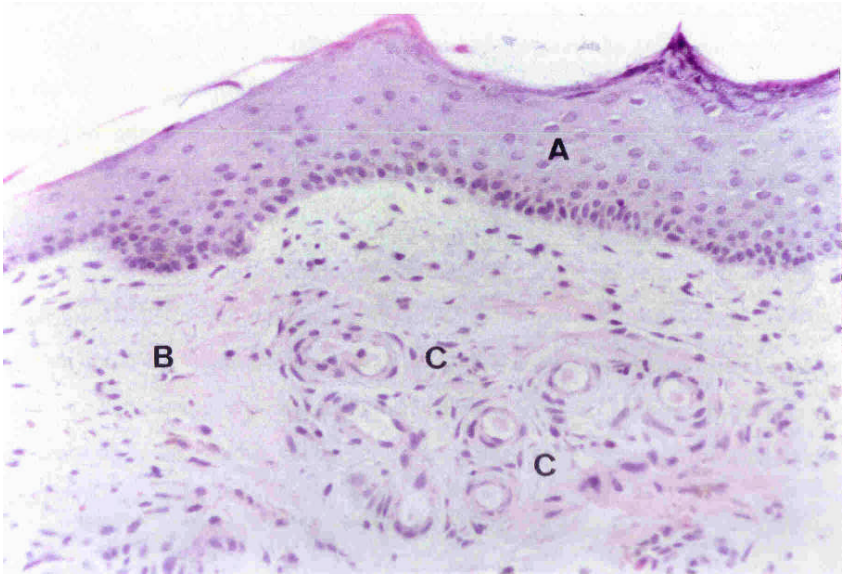

Figure 13 is a photomicrograph of the skin of the leg in LDS. It shows increased number of capillaries in the dermis (C). A= epidermis, B=sub-papillary dermis. (Photograph courtesy of H Pardoe MS FRCS)

Immunoglobulin	Other names	Ligand
ICAM-1	CD54	$\alpha_L\beta_2$ (CD11a–CD18) $\alpha_M\beta_2$ (CD11b–CD18)
ICAM-2	CD102	$\alpha_L\beta_2$ (CD11a–CD18)
ICAM-3	CD50	$\alpha_L\beta_2$ (CD11a–CD18) $\alpha_M\beta_2$ (CD11b–CD18)
VCAM-1	CD106	$\alpha_4\beta_1$ (CD49d–CD29) $\alpha_4\beta_7$
PECAM-1	CD31	PECAM-1
MAdCAM-1		L-selectin, $\alpha_4\beta_7$

ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet–endothelial cell adhesion molecule; MAdCAM, mucosal addressin cell adhesion molecule

Table 2 shows the adhesion molecules of the Immunoglobulin super family.

standing chronic venous disease release a circulating inhibitory factor that binds to and blocks CD11b/CD18 receptors on the activated leucocytes, thereby preventing adhesive interaction. There appears to be no difference in response to venous hypertension in the two groups (superficial and/or deep) of patients with venous disease. There is no definite explanation of why skin changes develop in one group of patients and not in others. Presumably, other aspects of the response of the tissues to leucocyte adhesion are involved in the development of skin damage.

1.8 Investigation of Chronic Venous Disease

Objectives Of Investigations

These tests are to confirm the presence of obstruction or reflux (or both), localise the sites of obstruction or reflux and quantify the abnormality. The investigations start out with a history and examination.

Duplex-Scan confirms the anatomic localisation of obstruction and reflux in the majority of cases. It detects the sites of deep to superficial reflux. Ascending or descending venography is rarely required. MR scan may be required to give the exact anatomic pattern in some cases.

Quantitative analysis of of reflux could be measured from duplex criteria which could be used to obtain para meters like reflux flow-volumes. Air plethysmography can add by information to differentiate between venous obstruction, venous reflux and calf muscle pump dysfunction. Some authorities use ambulatory venous pressure more than the other criteria.

Phlebography

The purpose of ascending venography is both to confirm the presence as well as the extent of outflow obstruction. *Ascending phlebography* is performed by injecting the dye into a foot vein after applying a tourniquet at the ankle. Proximal passage of the dye is delayed either by putting the patient in a semi upright position or by using a second

	Sensitivity	Specificity
CLINICAL TESTS		
Cough	0.59	0.67
Tap	0.18	0.92
Trendelenburg	0.91	0.15
Perthe's	0.97	0.20
HAND-HELD DOPPLER		
SFJ	0.97	0.73
GSV	0.82	0.92
SPJ	0.8	0.90

Table 3 Compares sensitivity and specificity of the various clinical tests to hand held Doppler examination (Kim et al)[Ref]

tourniquet at the thigh. Deep to superficial reflux may be shown by asking the patient to actively plantar flex the ankle (functional ascending phlebography). Descending phlebography is used to demonstrate both the extent as well as the site of reflux. Iso-osmotic dye is injected into the femoral vein with the patient in the 60° semi-upright (head-up) position. Repeated boluses of contrast are used. Reflux in the common femoral, superficial femoral, deep femoral and popliteal veins as well as the SFJ may be demonstrated. Asking the patient to do a valsalva and active plantar flexion are performed to demonstrate reflux. The reflux is graded according to the sites involved [62]. There has been no significant correlation between these grades and the development of skin changes/ leg ulceration. Direct varicography may be used for recurrent veins and/or veins in the region of the SPJ. Most workers utilize these techniques uncommonly presently.

Continuous Wave Doppler

This is perhaps the simplest most easily performed investigation at the bedside. Based on the principle first described by the Austrian physicist Christian Johann Doppler (1803-53).This test utilizes an ultrasound beam emitted by a piezoelectric crystal. Pocket versions comprising small unidirectional units can complement clinical examination.

The Doppler effect is governed by the equation fd=2v×f/c× cosd

Fd= frequency variation

F= reflected frequency

V= blood velocity

C= sound velocity in flow

D= angle of incidence of sound from the flow direction

The hand held instruments are designed so that the reflected sound is in the audible frequency range of about 20,000 Hz. The emitted frequencies are usually 5-8 MHz. See table 2 for comparison of handheld dopple with clinical examination. One of the main limiting factors for hand held Doppler is its lack of anatomic selectivity especially at the SPJ. Reflux in the Giaccomini or gastrocnemius veins can give false positive results[64].

Photoplethysmography

(Greek:"plethysmo", to increase, an "graphos" to write) is the recording of changes in the content of blood in the tissues. It is also known as ‘light reflection rheography’ and depends on the ability of blood to reduce the amount of light reflection form the tissues. It is difficult to quantitate the amount of blood and attempts to do so have shown no correlation.

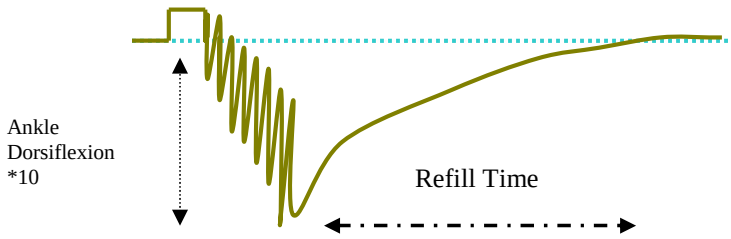

Figure 14 Shows principal of PPG and a typical trace obtained from the lower limb at base line and after ankle dorsiflexion.

Venous ‘refill time’ has a strong linear correlation with venous pressure measurements[63]. For testing usually 10 tiptoe or foot dorsiflexion movements are used. Normal venous refill time is >25 sacs. With venous insufficiency the reduced refill times may be corrected by placement of strategically placed tourniquets. This may provide anatomic localisatgion of sites of deep to superficial reflux. Digital PPG may

provide better interpretation of data. While the exact place for PPG continues to evolve in clinical practice, correction of prolonged refill times with a superficial tourniquet is indicative of a predominantly superficial vein issue. This may be correctible with an interventional procedure.

Strain gauge plethysmographs consists of mercury filled silastic loop placed around the leg with just enough stretch for good contact. The length of the strain gauge and hence its electrical resistance is increased by increase in the circumference of the leg. It has mainly been applied to the diagnosis of DVT and outflow obstruction. Air plethysmography is used by many workers to quantify some parameters of CVD.

Air Plethysmography

This allows quantitative non-invasive measurement of venous reflux and calf muscle ejection. It is performed using a PVC 'chamber' that surrounds all of the lower leg (capacity circa 5 L). After calibrating the machine the patient is asked to do tiptoes and

PARAMETERS OBTAINED BY AIR PLETHYSMOGRAPHY	UNITS
VV (Venous volume) measured after a baseline reading	ml
VFT 90(Venous Filling Time) time to reach 90% VV	sec
RV (Residual Volume) after 10 tiptoe movements	ml
VFI (Venous Filling Index)= 90%VV/ VFT 90	ml/sec
EV (Ejection Volume)	ml
EF (Ejection fraction)= (EV/ VV) × 100	%
RVF (Residual Volume Fraction)=(RV/ VV)× 100	%

various parameters are measured as depicted in the diagram. Air plethysmography allows non-invasive quantitative measurement of venous reflux and calf muscle pump ejection**.** The PVC chamber (5 L) covers the lower leg from ankle to knee. The system is primed to 6 mmHg and then calibration is performed using a 100 ml syringe. The various parameters assessesed in clude the following

A. VV is the increase in leg volume as a result of standing (100-150 mls normal).This is obtained following a stable baseline supine measurement. The subject is upright with the weight on the opposite leg and supporting himself on an orthopaedic frame.

B. VFT90 is time taken to achieve 90% of the filling

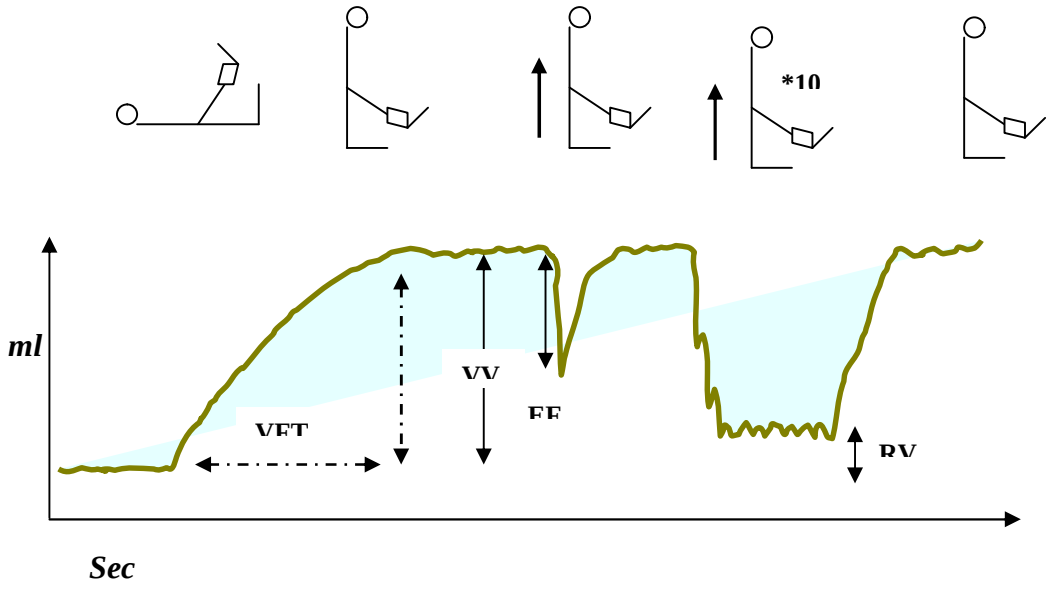

Figure 15 Illustrates the principal of Air-plethysmography and the various variables that can be calculated from this technique (Redrawn after Nicolaides AN, Sumner DS. 'Investigation of Patients with Deep Vein Thrombosis and Chronic Venous Insufficiency'.

C. Venous filling index (VFI) =90%VV/ VFT90. This is expressed in ml/sec. A VFI of less than 2 ml/sec confirms absence of significant venous reflux. A VFI of more than 7 mls/sec is associated with a high rate of skin changes and ulceration. Suitable tourniquet tests will point to the probable site of reflux.

D. EV (Ejected Volume) is determined by asking the patient to do a single tiptoe with the weight on both legs. The patient is then asked to return to the original postion when the reading is made.

E. EF (ejection fraction) = (EV/VV) ×100.

F. The residual volume (RV) and RVF (residual volume fraction) determines the amount of blood left behind after ten tiptoe movements. RVF= (RV/VV) ×100. The RVF shows a linear correlation with ambulatory venous pressure. It is an effective non-invasive test of the efficacy of the calf muscle pump mechanism[62].

An abnormal RVF (>40%) may be the result of i. reflux ii.decreased EF or iii. a combination of both of these. In recurrent deep vein thromboses obliteration of venous channels with decreased EV and EF may occur. In these patients valvuloplasty may not be very successful.

Type of Limbs	No ankle cuff	Ankle cuff inflated
Normal	**15-30**	**15-30**
Primary Varicose Veins	**25-40**	**13-30**
Varicose Veins/ incompetent perforators	**40-70**	**25-60**
Deep venous insufficiency/ proximal occlusion	**55-85**	**50-80**
Competent popliteal/ proximal occlusion	**25-60**	**10-60**

Table 4 Values of Ambulatory Venous Pressure P (mm Hg)[79]

Ambulatory Venous Pressure

AVP remain the gold-standard for measurement of venous function and to validate other investigational techniques. AVP have been used since the observation in the 1940s that foot venous pressures increase on standing still and drop dramatically on flexing the calf muscles.

The test is performed by canulating a foot vein with a 21G 'butterfly' needle. This is connected to a transducer. The patient holds himself upright with an orthopaedic frame without putting any weight on the limb from which the measurements are taken. This is done because the contraction of calf muscles can cause artifacts in the readings. 10 tiptoe movements are performed at the rate of 1/sec. The patient remains still again at the end of the exercise as resting pressures are again recorded. The exercise is repeated after inflating a tourniquet at the ankle level to occlude the superficial veins. The test may also be repeated with the tourniquet placed at successive higher levels on the lower limb. Pressure may actual rise following tip-toe movements in patients with venous outflow obstruction. The most useful measurements are the mean ambulatory pressure ((P) measured towards the end of the 10 tip-toe movements) in the steady state and the 90% refill time (90%RT)[64].

There is a strong direct relationship between the incidence of ulceration and the value of P. It is one of the important tests for measuring the efficacy of calf muscle pump. The 90% refill time (90% RT) is also very useful . In the presence of deep vein obstruction the value of P may increase during exercise (exercise hyperemia).

Duplex Scanning

Duplex scanning was first used in the 1980's for diagnosis of DVT. Duplex (incorporating B-mode imaging) and colour flow imaging are some of the most useful and commonly utilised investigations for CVD. Duplex imaging allows anatomical localisation of the reflux and also allows for its quantitation. Usually a 7.5 imaging MHz probe is used with a 3.0 MHz pulsed Doppler.

With the lower limb in a non-weight bearing position reflux velocities and the diameter of the vein are determined. The refluxing volume in ml/sec can be determined. For quantitative measurements a ''Hokanson device'' and a presssure cuff may be used to ensure unifrom inflation/ deflation and standard pressures (70mmHg). Because the subject is standing up the cross-section of the vein is taken as circular. Samples are taken at the common femoral vein, 2-4 cms distal to the junction at the femoral vein and 2-4 cms from the junction at the long saphenous vein with the vessel axis of about 45°. Compression of the calf muscles is used to detect the reflux. The short saphenous system is examined with the patient standing up and facing away. The probe is placed longitudinally after identifying the veins in the transverse axis. Longitudinal imaging of the popliteal vein, spaheno-popliteal junction and the short saphenous vein are obtained[64].

It has been shown that leg ulceration does not occur with refluxing volumes of <10ml/sec. Refluxing flow of >15ml/sec is associated with high incidence of skin changes/ ulceration[65]. This is irrespective of whether this reflux flow is in the superficial or in the deep part of the venous system.

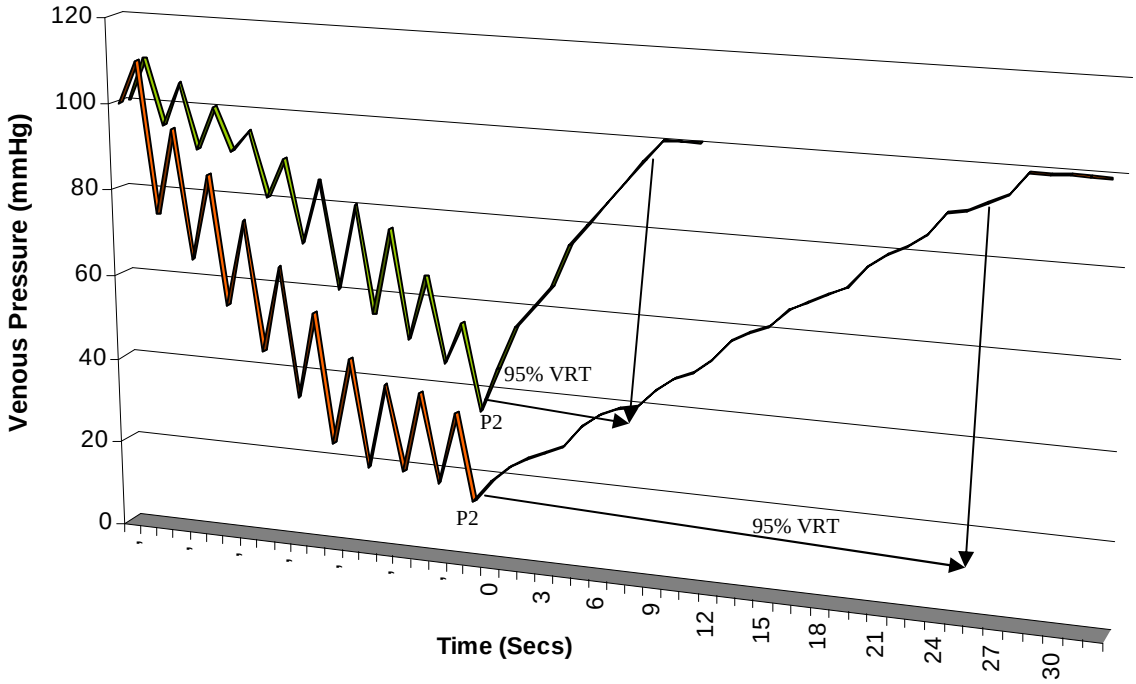

Figure 16: represents tracings of ambulatory venous pressure and its response to calf muscle contraction in normal (red) and venous insufficiency (green). The 90% refill time (VRT) is markedly different between the two sets. Decrease in pressure in venous insufficiency would be typically significantly lower than in subjects with no venous insufficiency.

% Change in pressure= (P1-P2/ P1) * 100
P1=initial pressure
P2=Pressure immediately after calf contractions

N	P (mmHg)	Incidence of ulceration (%)
34	<30	0
44	31-40	12
51	41-50	22
45	51-60	38
34	61-70	57
28	71-80	68
15	>80	73

Table 5: Incidence of leg ulceration in relation to AVP (Nicolaides)[64]

Number of patients	RVF (%)	Incidence of Ulceration (%)
20	<30	0
24	31-40	8
48	41-50	18
43	51-60	42
32	61-80	72
8	>80	88

Table 6: Incidence of ulceration in relation to the residual volume fraction (RVF) (Nicolaides)[64]

VFI (ml/sec)	Ulceration (%)	Skin Changes (%)
<3	0	0
3-5	0	19
5-10	46	61
>10	58	76

Table 7 incidence of sequelae of venous disease in relaion to VFI (Nicolaides) [64]

Colour Flow Imaging

The preceding measurements may be quite tedious since accuratged gating of the Doppler pulse is required and hence colour Duplex is often used instead to detect abnormal flow. Colour duplex is coded so that the hue and intensity are related to the direction and intensity of the Doppler shift. This allows immediate recognition of abnormal retrograde flow. Although this may be quantitated (to a degree) by software, colour is most often used for qualitative measurement of flow incontemporary practice..

For colour flow imaging (CFI) the grey-scale image is optimised by adjustments made for tissue attenuation ad then the colour is switched on. Using manual calf compression the detection of reflux both at the SFJ as well as the SPJ has become a relatively simple matter. Colour flow Doppler has been shown to be as accurate as conventional Duplex and in addition it can detect abnormal flow in the calf veins. Colour flow imaging has also shed much light on understanding the flow phenomena in the calf perforators. There is deep to superficial flow initially with muscular contraction in ‘incompetent perforators’. The perforating veins eventually close, however, with increasing muscle contration and occlude the flow more or less completely. During relaxation of the muscles the flow is from superficial to the deep system.

Figure 17 is a photograph of a leg with severe lipodermatosclerosis. There is evidence of an ulcer.

1.9 Pharmacotherapy in CVD

Currently no pharmacologic agent restores venous competence in CVD. It is even doubtful wether any medical treatment exerts major effects on the haemodynamics in CVD. Thus the aetiologic mechanisms in CVD i.e. venous hypertension cannot be reversed using the present paradigms.

Drugs that may act on the microcirculation in established CVD and ameliorate its effects may therefore be important. These may counteract some or all of the mechanisms that have been proposed at various times to act at the microcirculatory level. Thus drugs affecting increased capillary permeability, micro-vascular slugging, cytokine release and leucocyte activity has been utilized. Drugs affecting fibrin deposition in the pericapillary space have also been used. A wide variety of drugs have been used with varying results. These include the following

i. 'Phlebotropics'
    - a. Benzopyrones
        1. Alpha Benzopyrones (Coumarin)
        2. Gamma Benzopyrones (Flavonoids)
            - Flavones/ Flavonols (Diosmin, Rutosides)
            - Flavanes/ Flavonones (Hesperidin, Catechin)
    - b. Saponins (e.g. Horse Chestnut extract, Escin)
    - c. Other Plant Extracts (Ginkgo Biloba)
    - d.Synthetics e.g. Ca Dobesilate

ii. Rye-Ergot derivatives

iii. Heparin & heparinoids

iv. Dicoumarol anti-coagulants

v. Fibrinolytics

vi. Sclerosing agents

vii. Topical preparations

viii. Non-steroidal anti-inflammatory agents

ix. Diuretics

x. Anti-platelet agents

Pentoxifylline (Trental®, Hoechst, Hounslow, UK), which inhibits the effects of TNF-α and IL-1,[66] has been shown by some workers to have some effect on ulcer healing when used in conjunction with compression therapy.[67] A recent Cochrane review suggested some efficacy of Pentoxifylline treatment especially when used without compression therapy. Twelve trials involving 864 participants were included. The quality of trials was variable. Eleven trials compared pentoxifylline with placebo or no treatment; in seven of these trials patients received compression therapy. In one trial pentoxifylline was compared with defibrotide in patients who also received compression. Combining 11 trials that compared pentoxifylline with placebo or no treatment (with or without compression) demonstrated that pentoxifylline is more effective than placebo in terms of complete ulcer healing or significant improvement (RR 1.70, 95% CI 1.30 to 2.24). Significant heterogeneity was associated with differences in sample populations (hard-to-heal samples compared with "normal" healing samples). Pentoxifylline plus compression is more effective than placebo plus compression (RR 1.56, 95% CI 1.14 to 2.13). Pentoxifylline in the absence of compression appears to be more effective than placebo or no treatment (RR 2.25, 95% CI 1.49 to 3.39). A comparison between pentoxifylline and defibrotide found no statistically significant difference in healing rates.More adverse effects were reported in people receiving pentoxifylline (RR 1.56, 95% CI 1.10 to 2.22). Nearly three-quarters (72%) of the reported adverse effects were gastrointestinal. The authors' conclusions were that Pentoxifylline is an effective adjunct to compression bandaging for treating venous ulcers and may be effective in the absence of compression. The majority of adverse effects were gastrointestinal disturbances.[68]

Prostaglandin E1, which prevents activation of neutrophils, has also been shown to be of some benefit in patients with venous ulceration, as have free radical scavengers applied topically to ulcers.[69] In a recent report a randomized, placebo-controlled, single

blind study in which 87 patients who had venous leg ulcers homogeneous for dimensions and characteristics were treated for 20 days with an infusion of prostaglandin E-1 or placebo, in association with topical therapy. The dimension and the number of the ulcers were determined at the beginning of the treatment and then every 20 days up to 4 months, or until total recovery. The main outcome of the study was the recovery percentage of the ulcers at the end of the 120-day period of observation and the referred healing time. The reduction in the extension of ulcers from the baseline measurement to the last observation was also evaluated. It was observed that the the reduction in the size of the ulcers was faster in the patients treated with PGE-1. In this group, 100% of the ulcers healed < or = 100 days, whereas in the placebo group, only 84.2% did so by the end of the 120-day observation period ($P < .05$). The estimated healing times of 25%, 50%, and 75% of the patients treated with PGE-1 were 23, 49, and 72 days, respectively, compared with 52, 80, and 108 for the patients in the placebo group. Only one serious event occurred in the treated group. This study demonstrated some effectiveness of PGE-1 in reducing the healing time of venous ulcers.[70] (The effects observed were not large and longer term follow-up is lacking).

The effect of an analogue of PgE_1 has been investigated in a study in 44 patients with venous ulcers. PGE1 has been shown to cause reduction of leucocyte activation, platelet aggregation inhibition, and small vessel vasodilatation as well as reduction of vessel wall cholesterol levels. Patients were randomized to receive either the active compound or placebo by daily intravenous infusion over a period for 6 weeks [71]. Improved venous ulcer scores were observed in patients treated with the active compound at the end of the study period. The duration of the investigation was too short to be able to assess the influence of PgE_1 on the rate of ulcer healing. Unfortunately this method of treatment requires the use of an intravenous infusion and is quite intensive.

Two other studies have investigated the use pentoxifylline (Trental, Hoechst, Germany) in the management of patients with venous ulceration [72] [73]. Pentoxifylline is thought to inhibit cytokine mediated leucocyte activation, reducing leucocyte endothelial interaction and superoxide free radical production. Both studies have shown a beneficial effect on the rate of ulcer healing, but in the second where much more effective compression was applied; the influence of Trental was more modest. This is a treatment with a definite efficacy, but of small magnitude. Its exact role in the management of patients with venous ulceration remains unclear.

Prostacyclin analogues (Iloprost, Schering Berlin) have been used with some success in the systemic treatment of leg ulcers. Iloprost is thought to affect leucocyte activation, platelet aggregation, small vessel dilatation as well as fibrinolysis. In a double-blind randomized trial (with placebo controls) involving 44 patients, the healing was more (8 of 20 Vs 2 of 22) in the Iloprost group. In addition the clinical parameters improved in the non-placebo group significantly.[74] A more recent study of 98 consecutive patients confirmed these findings[75].

The flavonoids have been extensively studied and merit discussion. Antibiotics are often used for acute exacerbation of inflammation associated with infection in the ulcers. Drugs used for recanalisation of acute obstruction such as. Plasminogen activators and oral anticoagulants are not discussed here. Non-steroidal anti-inflammatory drugs are mainly used for thrombophlebitis and acute exacerbation of LDS. Diuretics are used only sparingly to control chronic oedema. Their haemoconcentrating effect may encourage thrombosis. Relatively recently objective microcirculatory criteria are increasingly used for assessment of both conservative and operative treatment.

i. Flavonoids in Venous Disease

Flavonoids are a broadly distributed class of naturally occurring plant products. Because of their widespread occurrence in edible plants such as fruits, vegetables and grains, they are an integral part of the human diet. Many compounds traditionally used for treating inflammatory conditions have flavonoids as active components. Daflon, which is a 'micronised' preparation of flavonoids, has been used in the treatment of varicose veins & haemorrhoids.[76]

The leaves of 'Baphia nitida ' have been used in folk medicine for the treatment of inflamed and infected umbilical cords in Nigeria. Phytochemical analysis of the leaves detected tannins, flavonoids and saponin glycosides. This anti-inflammatory effect has been tested experimentally. Flavonoid rich preparation of the leaf, obtained by a chromatographic process, was formulated into an ointment and tested at three dose levels for anti-inflammatory activity against croton oil and heat induced inflammation on ears of mice and depilated backs of rats, respectively. Both provide evidence for

the ability of this fraction to inhibit the inflammatory condition on the rodents. The activity was found to be dose related, and the mouse ear model was found to be the more sensitive of the two test s used. Hydrocortisyl cream was used as a positive control reference in this experiment. [77]

Pharmacokinetics

In a study involving Pharmacokinetic parameters of diosmin performed after oral administration to healthy volunteers, diosmin and its aglycone, diosmetin, were determined by HPLC (high performance liquid chromatography) and LC-MS (Liquid chromatography-mass spectrometry). At the level of sensitivity of this method, no parent compound was present in the plasma but only its aglycone, diosmetin. Analysis of the pharmacokinetic parameters showed that the drug was rapidly absorbed. Diosmetin presents a long plasma elimination half-life ranging from 26 to 43 hours. Data show the total absence of urinary elimination for both diosmin and its aglycone diosmetin, while its minor metabolites are eliminated in the urine, mainly as glucuronic acid conjugates. The presence of degradation products such as alkyl-phenolic acids confirms a metabolic pattern similar to other flavonoids. [78]

MECHANISM OF ACTION[164-168]

Flavonoids have several anti-inflammatory actions. In varicose veins increase in glycosaminoglycan levels in the vein wall is known in varicose disease. This reflects a deregulation of the normal matrix biosynthesis by the cells of varicose vein wall, especially smooth muscle cells. Some flavonoid drugs are capable of correcting these deviations by decreasing proteolytic attack on fibrous proteins and decreasing the accumulation of proteoglycans and hyaluronan.[79]

Changes in venous haemodynamics have not been convincingly demonstrated. It is possible that the clinical effects of Flavonoids are mainly at the microcirculatory level.[80]

Capillary permeability

In a hamster cheek pouch model, in comparison with vehicle, Flavonoids significantly inhibited the macromolecular permeability increasing effect of histamine, bradykinin (p

< 0.001) and LTB4 (Leukotriene B4; p < 0.001). At reperfusion, after 30 min ischaemia, Flavonoid significantly decreased the observed macromolecular permeability (p < 0.01). Flavonoid-treated animals also tended to have a lower number of leukocytes adhering to the venular endothelium (104.8 +/- 11.0 vs. 75.8 +/- 9.7/6 mm2; p > 0.05). These results demonstrate that oral administration of micronised purified flavonoid fraction for 25 days at 20mg/kg body weight/day has a protective effect on leakage of macromolecules after application of permeability-increasing substances and during ischaemia-reperfusion in the cheek pouch microvasculature of diabetic hamsters. Data illustrating the inhibitory effect of clinically relevant doses of micronised purified flavonoid fraction on the inflammatory processes may serve as a basis of its mode of action.[81]

Effect in clinical capillary fragility

The efficacy and safety of this flavonoid fraction consisting have micronized diosmin (90%) and hesperidin (10%) were studied in 100 patients with symptomatic capillary fragility in a double blind, randomised, placebo-controlled trial. Treatment lasted 6 weeks and consisted of 2 daily tablets of either flavonoid or placebo. Patients were examined at weeks 0, 2, 4 and 6. Compared to placebo, capillary resistance, assessed by the negative suction cup method, was significantly higher in the flavonoid group at week 4 (p < 0.001) and week 6 (p < 0.00 1). This resulted in a significant improvement of symptoms of capillary fragility (spontaneous ecchymosis, epistaxis, purpura, petechiae, gingivorrhagia, metrorrhagia and conjunctival haemorrhage) in S5682* treated patients (p < 0.001) flavonoid was well tolerated. The rate of side effects spontaneously volunteered by the patients was similar in both groups. It was concluded that flavonoid increase the capillary resistance in patients with abnormal capillary fragility to a significant extent without major side effects.[82]

Haemorrheological

An open pilot study performed to verify the variations in capillary packed cell volume in comparison with the velocity in 24 patients with third-stage chronic venous insufficiency before (day 1) and after (day 28) a 28-day treatment with Daflon 500 mg a micronized purified flavonoid fraction consisting of diosmin, 450 mg, and

* Flavonoid preparation

hesperidin, 50 mg, per tablet) 1 g/day, and then 14 days (day 42) after cessation of treatment. Ankle skin micro-circulation was evaluated by dynamic capillaroscopy. Values of relative capillary packed cell volume were calculated by a densitometric method, and red blood cell velocity was calculated using the cross-correlation simplified method. Relative capillary packed cell volume (mean +/- SD) significantly (p = 0.001) increased from day 1 (64.10 +/- 9.34%) to day 28 (72.89 +/- 5.74%) and then decreased until day 42 (66.84 +/- 7.48%). In the same patients, red blood cell velocity (mean +/- SD) significantly (p = 0.041) increased from day 1 (0.26 +/- 0.14 mm/sec) to day 28 (0.35 +/- 0.11 mm/sec) and then remained stable until day 42 0.33 +/- 0.16 mm/sec). Two possible explanations can account for this apparent discrepancy: first, dissociation between viscosity and velocity due to the Fahraeus-Lindqvist effect (sigma effect)[†]; and secondly, increased deformability of red blood cells leading to an increased red blood cell velocity despite an increased packed cell volume. It can be concluded that Daflon 500 mg seems to have a beneficial haemorheological effect, resolving the stasis with an increase in red blood cell velocity. A concomitant increase in relative packed cell volume and red blood cell velocity after therapy suggests an improvement of the flexibility of red blood cells.[83]

Free Radicals & Oxidation

Flavonoids have been used in the treatment of vascular endothelial damage. They are known to be excellent scavengers of oxygen free radicals. It has been suggested that Daflon 500 mg could interact with free radicals, which could have a deleterious effect in ischaemic tissues(particularly in the retina).[84].

The nitric oxide radical (NO) probably plays an important role in this mechanism. In studies of the NO scavenging effects of flavonoids, they were found to be very potent NO scavengers. The anthocyanidins were found to be more effective scavengers than the hydroxyethylrutosides, which correlated with their therapeutic activity. The values of their scavenging rate constants are only 30 times less active than that very potent

[†] Under most circumstances, blood flow can be modeled by the Navier-Stokes equations. Whole blood can often be assumed to be an incompressible Newtonian fluid. However, this assumption fails when considering flows within arterioles. At this scale, the effects of individual red blood cells becomes significant, and whole blood can no longer be modeled as a continuum. When the diameter of the blood vessel is slightly larger than the diameter of the red blood cell the Fahraeus–Lindqvist effect occurs and there is a decrease in wall shear stress. However, as the diameter of the blood vessel decreases further, the

endogenous NO scavenger, haemoglobin. It is speculated that NO scavenging plays a role in the therapeutic effect of the flavonoids. [85]

In an animal model intravenous injection of Daflon 500 mg (25 and 50 mg/kg) reduced the hyperglycaemia induced by injection of alloxan in rat. This effect of Daflon 5 00 mg was linked to its ability to scavenge active oxygen radicals, demonstrated in vitro using human neutrophils or mouse peritoneal macrophages stimulated by zymosan. The free radical scavenger effect of Daflon 500 mg is observed at concentrations ranging from 10(-7) M[‡] to 10(-4) M, with half-maximal effect between 10(-6) M and 10(-5) M. Thus, Daflon 500 mg behaves as a potent protective agent against inflammatory disorders. These properties may explain, at least in part, its clinical activity and justifies its therapeutic use. [86]

Xanthine Oxidase Inhibition

Flavonoids have been demonstrated to inhibit beef heart mitochondrial succinoxidase and NADH-oxidase activities. The primary site of inhibition is suggested to be in the complex I (NADH-coenzyme Q reductase) portion of the respiratory chain. The order of potency for inhibition of NADH-oxidase activity was robinetin, rhamnetin, eupatorin, baicalein, 7, 8-dihydroxyflavone, and norwogonin with IC50[♣] values of 19, 42, 43, 77, 277 and 340 nmol/mg protein, respectively. Flavonoids with adjacent tri-hydroxyl or para-dihydroxyl groups exhibited a substantial rate of auto-oxidation, which was accelerated by the addition of cyanide (CN-). Flavonoids possessing a catechol configuration exhibited a slow rate of auto-oxidation in buffer that was stimulated by the addition of CN-. The addition of superoxide dismutase (SOD) and catalase in the auto-oxidation experiments each decreased the rate of oxygen consumption, indicating that O2- and H2O2 are generated during auto-oxidation. In the CN[♦] (-)-stimulated oxidation experiments, the addition of SOD[ℜ] also slowed the rate of oxygen consumption. These findings demonstrate that the CN-/flavonoid interaction generated O2- non-enzymatically, which could have biological implications.

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red blood cells have to squeeze through the vessel and often can only pass in single file. In this case, the inverse Fahraeus–Lindqvist effect occurs and the wall shear stress increases.

[‡] M= Molar

[♣] IC50=Half maximal inhibitory concentration for experimental samples

[♦] Cyanide

[ℜ] SDO=Super Oxide Dysmutase

Leukocyte activation

Studies exist to suggest that pre-treatment with Daflon 500 mg prior to the induction of 4 h of tourniquet ischaemia significantly lowers the number of adherent leukocytes. This observation is linked to the protective effect of flavonoids in the treatment of oedema, as decreased activation is also associated with a decreased platelet and complement system activation, leading to a lowered release of histamine and decreased leukocyte-dependent endothelial damage.

Inflammatory Mediators

The arachidonic acid derivatives form part of the chemical/ biological factors associated with activation of the inflammatory reaction. The mediators include are (prostaglandin [PG], leukotrienes [LT], or thromboxanes [TX]), vasoactive amines (histamine or serotonin), and oxygen free radicals (superoxide ion, O2-, or hydrogen peroxide, H2O2). In peri-venous inflammation, these mediators play a prominent role in favouring vasodilatation (histamine),increasing membrane permeability (PGE2, histamine, free radicals) and providing a chemotactic signal for specialised cells, i.e., neutrophils, macrophages, lymphocytes (LTB4, free radicals). Flavonoids can act at various levels, possibly independently. Daflon 500 mg (100 mg/kg, orally) may reduce oedema formation and inhibit the synthesis for PGE2 (78.5%), PGF2 alpha (45.2%) and TXB2[§] (59.5%).

Effect on Cytokine Release

The growth of two human lymphoid tissue derived cell lines, IM-9 and Molt-4 cells[**] together with normal lymphocytes have been studied in the presence of several plant natural products. Amongst the 11 test compounds studied, the flavonoids (fustin, taxifolin, phloretin) and the polyphenol tannic acid were found to be potent inhibitors. They exerted varying degrees of inhibition on Molt-4 cell and normal lymphocyte cell proliferation but not on the non-malignant (IM-9) cells. . The IL-2 level was also enhanced in the Molt-4 but inhibited in normal lymphocytes. However, its level remained unchanged in the IM-9 cells. The amount of IL-2 secreted could be directly correlated to the percentage cell growth inhibition for only Molt-4 cells.

[§] TXB2=Thromboxane B2

[**] MOLT-4/ IM-9 =Experimental cell lines

Effect on arterial smooth muscle

Flavonoids relaxed the contractions induced by nor adrenaline, KCl orphorbol 12-myristate, 13-acetate in rat aortic strips, the order of potency being: flavonols (quercetin, kaempferol, pentamethylquercetin) >flavones luteolin, apigenin) > flavanols (+)-catechin, (-)-epicatechin) which correlates with the reported order of potency to inhibit protein kinase C. The relaxant effects were slightly potentate by isoprenaline and those of pentamethylquercetin, kaempferoland apigenin by sodium nitroprusside. It is concluded that the main vasodilatory mechanism of flavonoids seems to be the inhibition of proteinkinase C. Inhibition of cyclic nucleotide phosphodiesterases or decreased Ca2+ uptake may also contribute to their vasodilatory effects. It is not known if this effect contributes to the clinical effects in patients with CVD.

Effects on venous wall composition

Varicose vein walls differ from normal venous walls by an important loss of their collagen content and increase of their glycosaminoglycan content, essentially of hyaluronan. The decrease in fibrous protein content can be attributed to increased proteolytic (collagenolytic) activity as well as to free radicals. Glycosaminoglycan increase reflects a deregulation of the normal program of matrix biosynthesis by the cells of varicose vein wall, essentially smooth muscle cells. Some flavonoid drugs re capable of correcting these deviations by decreasing proteolytic attack on fibrous proteins and the accumulation of proteoglycans and hyaluronan. These effects, due to interactions between flavonoid rugs and the cells and fibrous proteins of the venous wall may differ according to the nature of such drugs. These differences are based on the conformation of these drugs and their interaction with the triple helical structure of collagen fibres as well as with the cell membranes.

Clinical Efficacy of Flavonoids

The wheal vanishing (WV) time has been used to assess the effect of therapy with Daflon ®on capillary filtration in subjects with mild-moderate venous hypertension. The WV time, which was comparable in the two groups at the beginning of the study decreased significantly in the treated group. No change was observed in the WV time in the placebo group. Subjective symptoms of venous disease measured with an analogue scale improved following treatment with hydroxyethylrutosides [foot oedema

($p < 0.005$), ankle oedema ($p < 0.001$), and paraesthesia ($p < 0.01$)]; only night cramps were reported less in patients receiving the placebo ($p < 0.05$). Pitting oedema and eczema also improved significanlty.[29]

Controlled Clinical Studies

The efficacy of Daflon 500 mg was investigated in three double-blind, randomized trials using strain gauge plethysmography to provide quantitative information on venous hemodynamics in patients with chronic venous insufficiency. In total, 183 patients were treated with Daflon 500 mg versus a control group of equal number of patients. Daflon 500 mg produced a significant decrease in venous capacitance, venous distensibility, and venous emptying time ($P < 0.001$). In addition, these changes were accompanied by improvement in clinical symptoms and a decrease in the supramalleolar circumference. Clinical side effects were rare and led to treatment withdrawal in only three patients. It was concluded that Daflon 500mg could be of benefit to patients with chronic venous insufficiency. [22]

A study, similar to one mentioned earlier was performed to evaluate the effect of hydroxyethylrutosides on capillary filtration in subjects with mild to moderate venous incompetence—superficial varicose veins and/or deep venous disease and ankle oedema—using the vacuum suction chamber (VSC) device applied to the internal peri malleolar region and the wheal vanishing (WV) time. Subjects entered in to the study were randomised to receive either hydroxyethylrutosides (1 g twice daily for 4 weeks) or placebo for fmy weeks. The two groups entering and completing the study were comparable. Microcirculatory parameters (laser-Doppler resting flux, the veno-arteriolar response, transcutaneous PO2 and PCO2) remained constant during the fmy week study in both groups. The WV time, which was comparable in the two groups at the beginning of the study decreased significantly [from a median 55 min (interquartile 95-50 min), to a median45 minutes (interquartile 65-40 min) in the treated group, $p < 0.01$]. No change was observed in the WV time in the placebo group. Subjective symptoms measured with an analogue scale improved following treatment with hydroxyethylrutosides [foot oedema ($p < 0.005$), ankle oedema ($p < 0.001$),and paraesthesia ($p < 0.01$)]; only night cramps were reported less inpatients receiving the placebo ($p < 0.05$). Furthermore, the changes observed in WV time correlate well with an improvement in symptoms.

Effects on Tco2 & Laser Doppler Parameters

A 3-month, double-blind, randomised, parallel-group study carried out in 104 patients who received Daflon® in varying doses. The parameters studied were transcutaneous tension of oxygen and Laser Doppler flow measurements. These patients were divided into 3 groups according to the daily dose: 1 tablet (group 1, n = 34), 2tablets (group 2, n = 33), on 4 tablets (group 3, n = 37). All patients (mean age 43.7 +/- 13.1 years; 100 females, 4 males) included in the study were affected by mild CVI. They were followed for 90 days with visits at 1month (day 28) and 3 months (day 90). At inclusion, there were no significant differences between groups as regards biometrics data, meantcpO2 (group 1, 62.7 +/- 4.5 mm Hg; group 2, 64.0 +/- 3.3 mm Hg; group 3,64.1 +/- 3.5 mm Hg), mean tcpCO2 (group 1, 40.7 +/- 2.5 mm Hg; group 2, 39.3 +/- 2.9 mm Hg; group 3, 40.0 +/- 2.5 mm Hg) and laser Doppler parameters. Fourteen patients withdrew from the study (group 1, n = 4; group 2, n = 3; group 3, n = 7): 9 for reasons not related to treatment, 3for adverse events, 2 because they were lost to follow-up. From day 0 today 90, mean tcpO2 significantly increased ($p < 0.001$) in each group (group 1, 3.0 +/- 2.1 mm Hg; group 2, 2.9 +/- 2.1 mm Hg; group 3, 2.5 +/-1.6 mm Hg), mean tcpCO2 significantly decreased ($p < 0.001$) in each group (group 1, 2.6 +/- 2.0 mm Hg; group 2, 1.7 +/- 1.9 mm Hg; group 3, 2.2 +/-1.5 mm Hg). No significant differences were observed between groups. Laser Doppler parameters remained unchanged from day 0 to day 90 in the 3groups. Symptoms (discomfort, pain, heaviness, burning sensation) and signs (oedema) of CVI as well as perimetric measurements of calf and supramalleolar area were significantly improved in all the 3 groups. During this 3-month study, Daflon 500 mg improved oximetric measurements and did not alter laser Doppler parameters. These data suggest that Daflon 500 mg, at the early stages of CVI can act favourably on the microcirculatory disturbances also involved in the patho-physiology of more severe stages of CVI.

ii.Benzopyrones

The gamma-benzopyrones include ‘flavones’ and ‘flavonols’ (diosmine, rutin). The alpha-benzopyrones include coumarin and dicoumerols among others.

iii.Hydroxyrutosides

Hydroxyrutosides are a class of drug derived from plant glycosides (chemically gamma-benzopyrones). They initially gained favour 20 years ago when experimental studies

indicated that they reduced capillary permeability following burns in dogs. A number of clinical studies evaluating their effect on a variety of symptoms associated with CVI followed. In general these indicated that hydroxyrutosides appeared to be marginally more effective than placebo in reducing aching, tiredness, muscle cramps and other symptoms, which one might imagine, are difficult to evaluate objectively. The drugs do appear to be more effective than placebo in reducing oedema [87]; the clinical relevance of this is uncertain. One study of venous ulcer healing seemed to show an increased number of healed ulcers after 2 weeks of treatment with hydroxyrutosides compared with placebo, but this difference disappeared after 4 weeks of treatment. A study on the effect of rutosides on symptoms in 112 patients with venous insufficiency included with ulceration. All took rutosides for eight weeks, only one showed any evidence of improvement[88] . The use of hydroxyrutosides in venous disease appears to have significant symptomatic value[89,90, 91,] but there is no objective evidence of a beneficial effect on venous ulcers.

The efficacy and tolerability of O-(beta-hydroxyethyl)-Rutosides (HR) in elderly patients (aged over 65 years) with chronic venous insufficiency or varicose veins was studied in a multi-centre, double-blind, randomised, placebo-controlled trial. Of the 104 patients entered into the trial, data from 102 were available for analysis of tolerability and from 86 for efficacy. Treatment as for 6 months, with monthly examinations. Three different dosages were used due to slight differences in the registered dosage in various countries: (1) 250 mg 4 times daily (1 g/day), UK, n = 19 patients; (2) 300 mg 3 times daily (900 mg/day), FRG and Belgium, n = 55, and (3) 300 mg 4 times daily (1,200 mg/day), The Netherlands, n = 30. Each centre had its own placebo control group. The HR-treated group (n = 41) showed a significantly greater reduction in the total symptom score, 5.7 +/- 2.4 to 2.3 +/- 1.8, than in the placebo group, 4.4 +/- 3.0 to 3.0 +/- 2.4 ($p < 0.01$). Of the 5 studied symptoms there was also a significant ($p < 0.05$) improvement in leg cramps, heavy legs and restless legs. No significant differences between the two groups were seen for aching pains and paraesthesia. A small reduction was also seen in ankle and calf circumferences, which became significant at the end of the trial ($p < 0.05$). Pitting oedema of the leg ($p < 0.01$) and eczema of the leg ($p < 0.05$) also improved significantly greater than in the control. The drug seemed to be well tolerated. Diosmin is chemically 3′.5.7trihydroxy-4′-methoxyflavone-7-rhamnoglucoside. This

has been used in continental Europe for some time. Reported effects include increased venous tone, lymphatic drainage and decreased micro-vascular permeability.

iv. Dicoumarols

These are powerful oral anticoagulants and unlike the gamma-benzopyrones have different and widespread pharmacological applications.

v.Coumarin

Coumarin has been used for lymphoedema in Europe. They may induce proteolysis of the high molecular weight proteins present in lymphoedema. Unlike the Dicoumarols they donot modify blood coagulation. Some preparations are hepatotoxic.

vi.Aescin (Horse chestnut extract)

Results with this compound have proved disappointing. In 125 female patients with CVD ankle circumference was not reduced after 2 months of treatment. [92] There is no published evidence of ulcer healing or prevention of recurrences with this compound.

vii.Calcium Dobesilate

In one study this synthetic drug decreased the calf circumference and leg volume significantly compared with placebo. However any published evidence for ulcer healing or prevention of recurrences is lacking.

viii.Fibrinolytic Therapy

The concept of an oxygen diffusion barrier causing skin hypoxia proposed by Browse and Burnand has influenced attempts at treatment of chronic venous insufficiency by pharmaceutical means. They demonstrated that fibrinolytic activity was markedly reduced in patients with venous disease [93], [94], [95]. These observations led to attempts to reverse the damaging cutaneous effects of ambulatory venous hypertension by enhancing fibrinolysis. The effect of stanozolol, an anabolic steroid with pro-fibrinolytic properties, was evaluated in 14 patients with long-standing LDS, without active ulceration [96]. After three months, all showed clinical improvement both subjectively and objectively (by mapping the area of LDS). Serum parameters of fibrinolytic activity improved in all cases. Subsequently a 6 month double-blind crossover trial on 23 patients with long-standing LDS, which had not responded to compression hosiery, was

conducted[97]. All patients continued with stockings during the trial. The area of liposclerotic skin fell during treatment with both stanozolol and placebo. Leg volume as measured by plethysmography increased on the steroid, presumably as a result of fluid retention. Skin biopsy analysis suggested but did not prove that tissue fibrin was reduced by stanozolol treatment; foot vein pressure reduction on exercise was improved to the same extent on both active and placebo treatment. All but one patient described subjective improvement during the trial but was unable to differentiate between the active and placebo periods. The exception to this was in pain relief that was significantly better while taking the active component.

Overall, the results seem to have been unspectacular, indicating a possible small benefit from fibrinolytic treatment rather than a major advance in therapy. This impression was confirmed in a further double blind crossover study of 60 patients performed in UCL [98]. Stanozolol combined with stockings caused a reduction of liposclerotic skin area of 28% over 6 months. However, when the separate contributions of the two treatment elements were calculated using multivariate analysis, the effect attributable to stanozolol alone was not statistically significant.

Fibrinolytic treatment for venous ulceration has been evaluated in one trial of 75 patients [99]. Patients were allocated to receive either stanozolol or placebo for up to 420 days, with conventional compression treatment in all cases. In an interim report, the authors found complete healing in 26 of 40 ulcers in the stanozolol group and 27 of 44 in the placebo group, indicating no benefit from active over placebo treatment.It appears thatone may say that fibrinolytic enhancement may be of minor benefit in the symptomatic treatment of LDS, but that it does not appear to improve ulcer healing.

ix. Diuretics

Diuretics have a lmited role in the treatment of chronic vein disease. They may be useful in the short term for controlling osedema mostly associated with other co-morbidities.

x. Anti-platelet agents

They have mainly a preventative role. Results of meta-analysis show that aspirin reduces the risk of pulmonary embolism and deep-vein thrombosis by at least a third throughout a period of increased risk. Hence, there is now good evidence for

considering aspirin routinely in a wide range of surgical and medical groups at high risk of venous thromboembolism.

1.10 Compression therapy - Mechanism of Action & the microcirculation

Compression treatment has been used for the treatment of venous leg ulcers since antiquity. Despite its successful and widespread use the exact mechanism of its action remains unknown. There are several relative and absolute contraindications for compression therapy. These may include local infection and sometimes significant cardiac failure. Fontaine type III and IV stage (rest pain, gangrenous changes) arterial occlusive disease is also a contraindication. In lesser degrees of arterial disease (Fontaine I and II i.e. asymptomatic, intermittent claudication) the policy is less clear with no scientific evidence to back up any recommendations. This assumes special importance in case of leg ulcers of mixed arterial and venous aetiology. Several factors contribute to the relative effectiveness of compression therapy. Ulcer size, duration, limb joint mobility and general mobility were significant independent factors in multivariate analysis[100]. In a related study the impact of socio-economic status was limited.[101]

Studies of the macro-circulation fail to provide adequate explanation for the mechanism of action of compression therapy in CVD. The microcirculation may be important in this regard. In this thesis a study of the microcirculatory response to compression therapy is included. The pressure exerted by the bandage is proportional to the tension of the bandage (T) and inverse of the radius of curvature (R) of the skin surface i.e. **Laplace's law**: P=T/R. Hence if even tension is maintained while applying a bandage the pressure exerted would be higher at the ankle and lower at the thigh level. It is important to blot out concavities e.g. those around the malleoli. The stretched bandage exerts the resting pressure while the working pressure is exerted by muscle contraction with change in size of the leg. The latter is intermittent and is exerted by walking.

Compression therapy may not make the valves competent at the lower pressures ordinarily used i.e. 30-40 mm Hg. It can increase the stroke volume and decrease the mean residual filling volume of the leg during walking. Refilling rates and times may

Class	UK **(mm Hg)**	EU (mm Hg)	France (mm Hg)	Switzerland (mm Hg)	Germany (mm Hg)
I	14-17	15-21	10-15	18-21	18.37-21
II	18-24	23-32	15-20	15-20	25.12-32.25
III	25-35	34-46	20-36	20-36	36.37-46.5
IV	>35	>49	>36	>36	>58.87

Table 8 Comparison of classifications of elastic support in Europe

also be improved.[102] It may increase intra-tissue pressure with increased resorption of oedema. The effect of this magnitude of changes in the macro-circulation on ulcer healing is unclear. Some studies have reported increased fibrinolysis following compression therapy.

It has been shown that compression actually increases flux of blood through the skin. This may increase the shear force between the leucocytes and the endothelium and thus provide a possible mechanism of action for the stockings. Hence Mondeville's suggestion in 1309 that compression works by driving out evil humors may not have been far off the mark.[103]

Various types of bandages may be used. Long stretch elastic bandages are easier to use and last longer they have a high resting pressure. The walking pressure however, is not high. Calibrated bandages may help the patient to check the compression by means of deformation of small built in ''squares''

Previous Studies Of Compression & The Microcirculation

Studies undertaken previously have established several facts regarding response of macro-circulation and the microcirculation to compression therapy. These include the following (i) compression restores venous valvular competence at a lower pressure i.e. circa 40 mmHg in about a quarter or less of patients. (ii) Higher pressures may simply lead to complete collapse and closure of the veins (iii) patients with CVD have a higher rate of flux of blood through their micro-circulations compared to controls (iv) Flux increases in the skin of patients with CVD with increasing compression. This is especially evident at pressures between 30-60 mmHg. In general higher pressures are needed to achieve this in the upright position as compared to the supine position.

Sarin et al studied the effect of compression on valvular competence in controls and patients.[104] They used a water-filled cuff around the knee with the long and short saphenous veins as well as the popliteal veins scanned through the cuff. Vein diameter, vein patency and reflux was determined at increasing pressures in increments of 10 mm Hg. This study suggested that the beneficial effect of compression might not after all be due to a simplistic correction of venous reflux. It may be that some of the answers lie in the microcirculatory response to compression.[105]

Effects of compression have been widely studied on the venous haemodynamics of the lower limb. In patients with CVD the ambulatory foot vein pressure is raised. It would be reasonable to assume that application of compression stockings will restore this index to normal. The time required for the foot vein pressure to return to resting levels after the end of exercise is an indicator of the degree of reflux in a limb. This may be measured either by direct vein pressure or indirectly by air plethysmography, strain gauge plethysmography, foot volumetry etc. Air plethysmography may permit a more detailed analysis of the calf muscle pump, however, this has not demonstrated reduced venous refilling times after the application of stockings. It seems probable that stockings and compression bandaging may benefit the overall haemodynamics of the limb to some degree.

Laser Doppler fluxmetry measures both the sub papillary (thermoregulatory) shunt flow as well as the nutritional flow in the papillary capillary loops. Its findings are broadly comparable to those of capillary microscopy. The technique is non-invasive and simple to use and is a useful tool in the assessment of diseases that affect the microcirculation.

Although the laser Doppler flux in subjects in the supine position was higher in liposclerotic skin, as has been reported previously, the blood cell velocity in patients with liposclerotic skin was substantially lower than that in control subjects. The increased laser Doppler flux was attributable to an increase in the combined cross-sectional area of the capillaries. Previous histological studies have shown that the number of capillary loops seen on histological section of liposclerotic

	Popliteal Vein		Long Saphenous Vein		Short Saphenous Vein	
	No.	**Median Diameter (mm)**	**No.**	**Median Diameter (mm)**	**No.**	**Median Diameter (mm)**
All refluxing vessels at 0 mm Hg (n=57)	17	10 (7.8-10.8)	19	6.7(5.3-7)	21	6.1(4.9-6.8)
Refluxing abolished at 40 mm Hg. (n=5)	0	-	5	3.8(3.4-4.8)	0	-
Refluxing not abolished at 40 mm Hg n=52)	17	9(7-10)	14	5.8(5-6.4)	21	4.6(3.9-5.9)

Table 9 shows diametrs of the veins at zero and at 40 mm Hg external presure with those still demonstrating reflux distinguished from those that did not. Reflux was abolished in only a proprtion of the samples at 40 mm Hg. The diameters following compressio were smaller than initial diameter in all the cases. Adapted from PD Coleridge Smith 'Microcirculation in Venous disease' 2nd edition 1998)

skin is increased compared with normal skin. This may partly represent multiple cross sectioning through elongated and distended capillaries.

It has been reported that the capillaries in liposclerotic skin are dilated and coiled. [35,36] It is therefore not surprising that there should be an increased volume of moving blood cells in the skin. It has been reported that plasma viscosity, erythrocyte sedimentation rate, and fibrinogen are increased in patients with post thrombotic syndrome, but the hematocrit level is not significantly different from control subjects. The increased volume of moving blood cells shown in this study is consistent with the observation that capillaries are dilated and coiled in liposclerotic skin as shown by studies with capillary microscopy and histological examination. The microangiopathy of liposclerotic skin seems to comprise not just of structural changes but also a disturbed microcirculatory flow pattern.

The decrease in blood cell velocity may increase the likelihood of white blood cells in capillaries or venules interacting with the endothelium. It has been shown both in vivo and in vitro that at low shear rates, interaction of leucocytes with the endothelium is much more likely than at higher shear rates. Because the shear rate is a function of the flow velocity, leucocyte adhesiveness is inversely proportional to the velocity of blood flow. It is possible that white blood cells play a significant role in the venules interacting with the endothelium.

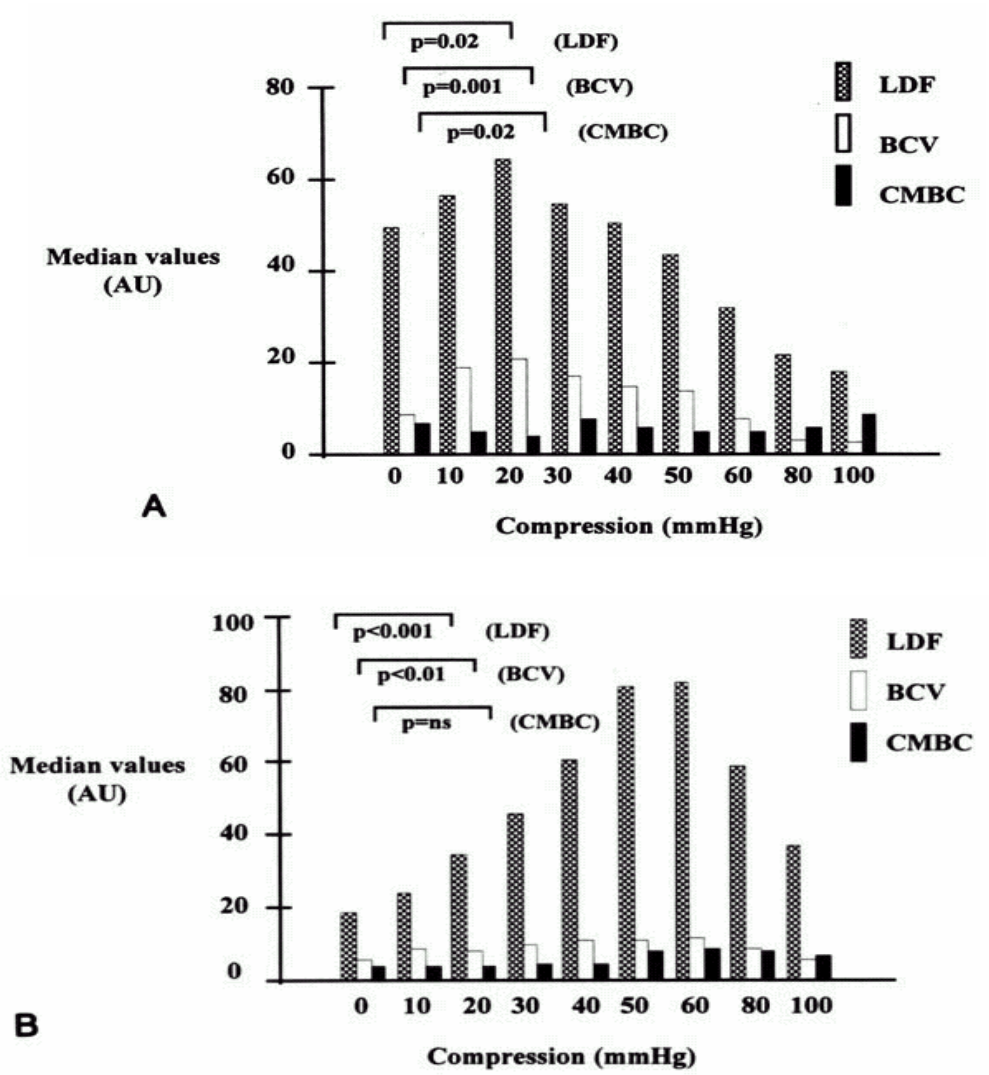

Figure 18 shows the effect of skin compression on laser doppler flux, velocity and concentration of blood cells in the microcirculation of the skin. The figure at the top shows data obtained in the supine position while that on the bottom shows data from upright position. From PD Coleridge-Smith 'Microcirculation in Venous disease 2nd edition 1998, with permission) ♣

♣ LDF=Laser Doppler flow, BCV= Blood Cell Velocity, CMBC=Concentration of Moving Blood Cells

On application of compression there is an increase in the laser Doppler flux in patients with liposclerotic skin. In the horizontal position, the peak flow occurs in the region of 10 to 20 mm Hg, which corresponds with what one might expect the capillary pressure to be with a patient lying in this position. When the patient moves to the sitting position, the peak flow occurs at 50 to 60 mm Hg, which again corresponds with the expected capillary pressure with a patient sitting erect because of the increased hydrostatic pressure.

Compression in the range of 20 to 30 mm Hg, commonly achieved by use of compression stockings, is sufficient to double the laser Doppler flux of a patient in the sitting position. In addition it is also suggested that still higher levels of compression (of the order of 40 to 60 mm Hg) may be effective in the treatment of patients with venous disease, without resulting in cessation of blood flow to skin in the patient in the sitting position. It has been shown that compression bandaging reaching pressures of 45 mm Hg results in rapid healing of venous ulcers, [45] and this is consistent with the suggestion that the findings of increased laser Doppler flux on compressing the skin are related to the mechanisms of the effect of compression on venous disease. Clearly there is no fundamental difference in the response to compression in liposclerotic skin compared with normal skin, although the effect is less marked in control subjects. It can be seen that local compression of the skin, without compression of the whole limb, results in significant alteration in the local microcirculatory blood flow. There is an increase in blood cell velocity in response to compression. Such an increase in velocity may reduce the likelihood of white blood cells interacting with the endothelium. This finding could have an important bearing on the mechanisms of action of compression.

One of the major shortcoming of these studies was thet the laser flux measures flow both in the nutritive as well as the ‘non-functional’ part of the microcirculation. The latter may be relatively unimportant in skin nutrition and leucocyte mediated damage. There is also the question of patients with mixed arterial and venous disease. The response in these patients may be very different.

1.11 Surgical/ Interventional Treatment of Chronic Venous Disease

Surgery for varicose veins involves tying off incompetent communications between the deep and the superficial venous systems, stripping of lengths of vein and multiple phlebectomies. 'Strategic' ligation of incompetent veins and operations to restore valve patency in the superficial venous system remain to be proven. Superficial vein disease alone is responsible for leg ulceration in about half of all cases. Studies have shown that surgery in these instances can lead to a high healing rate in the short to medium term[106]. No studies of the long-term outcome are available. There is some evidence of the efficacy of both laser vein ablation (& radiofrequency)[118] as well as foam sclerotherapy. More long term data is not available.

It has been shown recently that selecting patients for superficial vein surgery with AVP criteria can improve venous function significantly post-operatively[107]. The significance of perforator vein surgery remains to be proven conclusively. Part of the reason for this is the fact that perforator incompetence and indeed some DVI seem to improve with superficial venous surgery alone.

Surgery to the deep veins is still practiced in relatively few centres. More recent studies have suggested that both primary venous insufficiency, as well as post-thrombotic CVD may be suitable for deep vein reconstruction[108]. The procedures utilized are valvuloplasty (Open, trans-commissural, angioscopic guided), vein segment transposition and vein segment auto-transplantation. Bypass procedures for obstructive disease (circa 10% of CVD) include the Palma procedure and femoro-popliteal (venous) bypass operations as well as more proximal bypasses including the vena cava. However these procedures are suitable only for a relative minority.

Varicose Vein Surgery

Traditionally issues in varicose vein surgery have focused on the role of Duplex scans, whether or not to strip the GSV, method of stripping used, preventing recurrent varicose veins and medico-legal problems. More recent developments are laser vein surgery, foam sclerotherapy and other similar treatments.

The accuracy of DUPLEX and its comparison to other tests is discussed in section 1.4. Most surgeons would use DUPLEX scan for (i) Recurrent varicose veins (ii) Short saphenous varicosities (iii) suspicion of DVI (iv) Any other uncertainty about the origin of the varices.

The case for performing DUPLEX for all cases has been put forward. Studies have shown that leaving the GSV in situ leads to a higher rate of recurrence. The mid-thigh perforator has been implicated in many of these recurrences. The GSV is duplicate in many instances and reconnection to these may be responsible for other cases. Bruising of the thigh is a frequent sequel of GSV strip. Gentler stripping techniques including inversion techniques have been advocated. The perforate Invaginate stripper (PIN stripper) has been used in an effort to reduce exit wound scar length as well as to reduce the trauma of stripping. Studies have not, however shown any conclusive difference between PIN technique and conventional stripping.

LEG ULCERS AND THE ROLE OF SUPERFICIAL VENOUS SURGERY

Various studies have documented the role of the superficial venous system in aetiology of leg ulceration.). It has been shown that superficial venous surgery will heal the vast majority of leg ulcers. In sick patients this may even be performed under local anaesthesia. If there is no associated DVI then the use of post-operative compression stockings may not be necessary. There is no long-term data regarding what happens to

Author	Legs	Assessment	Perforators Alone %	SVI %	Primary DVI/(co-existing saphenous incompetence SVI)%	Post-Phlebitic %
Shami	59	DUPLEX	-	53	47 (32)	N.A.
Lees	25	DUPLEX	-	52	48 (12)	N.A.
Weingarten	148	DUPLEX	-	9	80 (55)	N.A.
Van Rij	120	DUPLEX/ APG	2	67	31 (28)	N.A.
Grabs	111	DUPLEX	-	51	43(38)	14

Table 10 Venous ulceration-morphology of the venous insufficiency [109]

these ulcers eventually. My own pilot study included in this thesis looks at some long term data. In one published study 122 limbs underwent surgery for SVI. Cumulative

healing rates at 6, 12 and 18 months were 57, 74 and 82 per cent respectively. It was observed that the larger ulcers also healed but took longer. None of these patients had DVI and none of them were prescribed post-operative leg compression. [106]

Thus direct treatment of the cause of venous ulceration in these often very infirm patients would avoid the morbidity and prolonged immobilization of procedures such as skin grafting. In this report the healing rates were equally good with either GSV or SSV incompetence causing leg ulceration. It is also reported in that a fixed ankle joint (surrogate for calf muscle pump dysfunction) as more common in patients with unhealed ulcers[110].

PERFORATOR REFLUX

The incidence of pure perforator incompetence is very low. Most often it is associated with superficial, deep or combined chronic venous disease. Reverse flow in these perforators may be restored to normal by correction of the SVI when there is no associated DVI. Hence most patients in this group have healed ulcers following saphenous surgery alone.

Distribution of Reflux	*Site of Ulcer* Medial Malleolus	Lateral Malleolus	Other*
Long Saphenous (n=93)	62 (67)	21 (23)	10 (11)
Short Saphenous (n=13)	7	6	-
Long and short spahenous (n=16)	10	5	1

*Bimalleolar/ Posterior calf

Table 11 Distribution of superficial venous reflux and ulceration in legs (per cent). [106]

There is uncertainty regarding the role of combined/perforator surgery in patients with simultaneous superficial, deep and perforator disease. No studies have shown conclusively that perforator surgery either on its own or in tandem with saphenous surgery is better than saphenous surgery alone. The role of perforator interruption

(surgery or other interventions) in patients with DVI but no obstruction continues to be investigated.

Sub-fascial Endoscopic Perforator Surgery (SEPS)

This is a technique for ligating calf perforators that avoids both a long incision as well as its placement in diseased skin of the leg in CVD. SEPS is technically effective at eradicating calf perforators and produces less complications than open surgery. Although SEPS and saphenous surgery produce good initial venous ulcer healing, there is no hard evidence to support its use in preference to conservative management or saphenous surgery alone. SEPS may actually worsen the situation if used in the presence of deep venous obstruction. In this situation the calf perforators are thought to act as safety valves for relieving the high pressures within the deep venous system.[111]

Chemical Ablation & Radiofrequency Ablation of Perforators

Ultrasound guided Radiofrequency ablation may give similar results to SEPS with experience.[112] Careful guided sclerotherapy may also yield good results in perforator vein closure.[113] Good quality long term data is lacking regarding these procedures.

DEEP VENOUS RECONSTRUCTION

These include valvuloplasty, vein valve transplantation/ implantation, valve support devices and vein segment transposition. Results of bypass surgery for iliac vein occlusion are reported to be favourable. Below the inguinal ligament, reflux is regarded as the primary pathologic problem leading to symptoms in patients afflicted with chronic venous insufficiency (CVI). In 1968 the first valve repair 1 was performed on a patient with incapacitating pain and oedema of the lower extremity and severe reflux of the femoral system. After repair of the incompetent superficial femoral vein and interruption of perforators, the patient was relieved of his CVI symptoms and remained free of them until his death 13 years later. The postoperative venogram confirmed patency and competence of the repair. The first series of valve reconstruction was reported in 1975, and good clinical results were achieved in 80% with a follow-up of 5 years. Since a technique for direct surgical correction of reflux was developed, other procedures have been introduced for reflux, including valve substitution by transposition and transplantation. Despite reports of encouraging clinical results by

several centres, considerable controversy and uncertainty persists regarding the durability and long-term effectiveness of valve reconstruction procedures. Furthermore, although haemodynamic studies show improvement after valve reconstruction, these tests do not normalize in all cases, even with successful relief of symptoms.[114]

Kistner and Masuda published their long term results (Mean follow-up 10 years) with venous reconstruction surgery[114]. One of the most striking findings in this long-term study is the strong correlation of valve competence, verified by descending venography or duplex scanning, with successful clinical outcome. All limbs that were converted to an asymptomatic state demonstrated either complete valve competence or mild incompetence. Valve competence was more frequently achieved in the valve repair procedure as opposed to the valve transposition procedure and correlated with the better clinical results found in the group undergoing repairs. Valve competence was found up to 16 years after valvuloplasty and confirms the long-term durability of the valve repair operation. The importance of having a competent proximal valve is also seen in those who had recurrence of symptoms, because the most frequent finding in them was recurrent reflux in the proximal valve.

In the patients where the results were poor, this occurred within 6 years in all patients but one and within 4 years in the PVI (primary venous insufficiency) group. This suggests that future studies of valve operations shoauld give long-term valid results after an observation time of 4 to 6 years. This long-term study reflects the importance of thorough workup of the patient with CVI to find patients with repairable proximal valves. Descending phlebography is necessary to discover which patients have repairable vessels caused by PVI. In those who have pure PVI incompetence, valvuloplasty can be expected to yield good long-term results in a high percentage of patients. The results of this series represent what has been accomplished with repair of a single valve in the SFV, or proximal transposition surgical procedure. Results may be improved by better techniques of repair or repair of multiple valves or reconstruction in different sites such as the popliteal vein. The overriding finding of the series was that the individual who is relieved of major amounts of axial reflux in te CVI extremity experiences dramatic clinical improvement. In these cases the surgeon is only able to provide a measure of improvement to try to restore the patient to a compensated state rather than a totally curative solution. The fact that one third of the entire group were

completely relieved of their CVI state, free of elastic support, for more than 10 years' follow-up is encouraging[114].

Neo-Valve Reconstruction

Maleti and colleagues have presented a series of 40 patients with neo-valve reconstruction creating flaps using the thickened vein wall. They report some encouraging early results especially with a modified technique. Further results are awaited.[115]

Clinical outcome after surgery	
+3	Asymptomatic, no symptoms of chronic venous disease, improvement of VRT to normal or at least +5 seconds, improvement in AVP to normal or at least -10 torr
+2	Moderate improvement, mild symptoms of chronic venous disease, improvement of VRT to normal or at least +5 seconds, improvement in AVP to normal or at least -10 torr
+1	Mild improvement, clinical improvement or improvement in vascular laboratory test results (VRT or AVP)
0	Unchanged, no change clinically or by vascular laboratory test results
-1	Mild worsening; worsening of symptoms of chronic venous disease or vascular laboratory tests (VRT or AVP)
-2	Significant worsening, worsening of symptoms and worsening of vascular laboratory test results (VRT or AVP)
-3	Marked worsening, same as - 2 accompanied by either new or worsening ankle claudication

AVP, Ambulatory venous pressure.

Table 12 depicts a standardized outcome of surgery for the purpose of study design and reporting. It is recommended that no clinical outcome grade be assigned until at least 6 months after operation.

1.12 REPORTING STANDARDS OF OUTCOME

The clinical outcome after surgery for CVD must be standardized for various procedures to be compared amongst themselves as well as comparison between different institutions. A venous severity score[116] has been validated and it was found that venous severity scores are significantly higher in advanced venous disease, demonstrating correlation with anatomic extent. Both venous clinical severity scores, VCSS and CEAP clinical score, were equally sensitive and significantly better for

measuring changes in response to superficial venous surgery than was the CEAP clinical class. Although the assignment of CEAP clinical class might be adequate for daily clinical purposes, venous severity scoring systems should be used in clinical studies to quantify venous outcome[117].

Comparison of CEAP clinical score and VCSS

CEAP clinical score*	***VCSS†***
Pain	Pain
Varicose Veins	
Edema	Venous edema
Venous claudication	
Pigmentation	Skin pigmentation
Lipodermatosclerosis	Induration
Inflammation	
Ulcer size	Ulcer size
Ulcer duration	Ulcer duration
Ulcer number	Ulcer number
Ulcer recurrence	
Compression	
therapy	
*Maximum score, 18.	
†Maximum score, 30.	

The venous severity scoring (VSS) system has been proposed by the American Venous Forum Ad Hoc Committee on Venous Outcomes Assessment as a useful and reliable method for outcome quantification, comparison of different management approaches, and reports in the management of chronic venous disease (CVD). The proposed system has three elements: venous clinical severity score (VCSS), which is a modification to replace CEAP clinical score; venous segmental disease score (VSDS), which is a (VDS), a modification of the original CEAP disability score. The purpose is to use these three components together as an integrated and improved method for assessing venous outcome[118].

PART II

HYPOTHESIS

Chronic venous disease (CVD) is associated with leucocyte and endothelial activation as well as a local growth factor response. This increased activation may respond favourably to various forms of therapy in CVD. The Hypothesis tested in this work is that there are measurable changes in leucocyte/endothelial activation and microcirculatory blood flow in CVD in response to medical, surgical and compression therapy. Thus these may act as objective measures of response to treatment.

It has been shown that there is increased expression of VEGF in skin tissue with CVD. Therefore it is possible that plasma vascular endothelial growth factor (VEGF) would also be raised in patients with CVD. If this is proven then plasma VEGF may be used in subsequent studies to monitor response to therapy. This may be accomplished by measuring plasma levels in both patients as well as normal subjects. There may be potential diagnostic, prognostic and therapeutic applications of this.

Physiological assessments following therapy have both limitations as well as logistical difficulties for more widespread use. Previous studies from this department have shown that plasma levels of VCAM, ICAM-1, vW factor & Lactoferrin are elevated in patients with CVD. Therefore treatment of the venous disease by a number of methods may normalise changed expression of leucocyte/ endothelial activation. Flavonoids (especially purified micronised fraction) have been shown to be of symptomatic benefit in CVD. They may ameliorate the increased neutrophil activation/ degranulation and endothelial activation. Therefore plasma levels of VCAM, ICAM-1 VW-factor and Lactoferrin may be significantly reduced following therapy with oral purified micronised flavonoid fraction.

Compression therapy is effective in CVD by means of an as yet unproven mechanism. There is no evidence that compression treatment renders incompetent valves competent. Compression may affect the microcirculation directly by changing the microcirculatory skin blood flow in patients with pure CVD as well as in those with mixed arterial and CVD. This may lead to change in shear at the venular/ capillary endothelial interface and thus possibly help in ameliorating the effects of CVD. Measuring the red cell velocity in microvasculature of patients with venous, arterial or mixed disease before and after compression may show demonstrable changes in this parameter.

In summary the hypothesis tested in this thesis is that alteration in microcirculatory flow; endothelial activation, VEGF activity & increased neutrophil degranulation activity described in CVD would show a measurable response to therapy.

PART III-

METHODOLOGY

3.1 **Protocol For Quantifying Lower Limb Venous Reflux Using Duplex Ultrasound Scanning**

Colour duplex ultrasonic mode imaging, B-mode (brightness mode) imaging and spectral Doppler mode measurements were performed using the Acuson 128XP/10v (Acuson, Mountain View, California, USA) computed sonography system with ART (acoustic response technology) using a 5- or 7.5- MHz linear array transducer.

Patient preparation

The ultrasound examination is done with the patient in the standing position so that the veins are under hydrostatic pressure as in pathological conditions. The limb to be examined should be externally rotated and completely relaxed and non-weight bearing with all the weight transferred to the contralateral limb.

Venous segments that are as close to the gaiter area as possible without interfering with the cuff position were examined: the SFV in the distal thigh, distal popliteal vein segment, proximal PTV segments, LSV at knee level and proximal SSV segment. Measurements at the valve cusps were avoided due to the presence of turbulent flow and all measurements were carried out on segments just above or below the cusp. In the quantification of blood volume flow within a vein, the size of the pulsed Doppler gate should insonate the entire vessel in order to account for all the blood cells which travel at different velocities with the slowest velocities present towards the vessel walls and the fastest present in the centre of the vessel.

The long saphenous vein and superficial femoral vein were examined with the patient in the standing position and facing the examiner, with the leg to be examined slightly flexed and externally rotated with the body weight supported by the contralateral limb. The sapheno-popliteal junction, short saphenous vein and popliteal vein are examined with the patient standing and facing away from the examiner with the knee on the side being examined slightly flexed so as to relax the popliteal fossa and the weight distributed on the contralateral limb. A pneumatic calf cuff with a width of 12cm that could be rapidly inflated and deflated automatically to a pressure of 120mmHg was placed around the calf and used to induce reverse flow. A standardised measuring method was adopted throughout in all the vein segments examined after augmentation

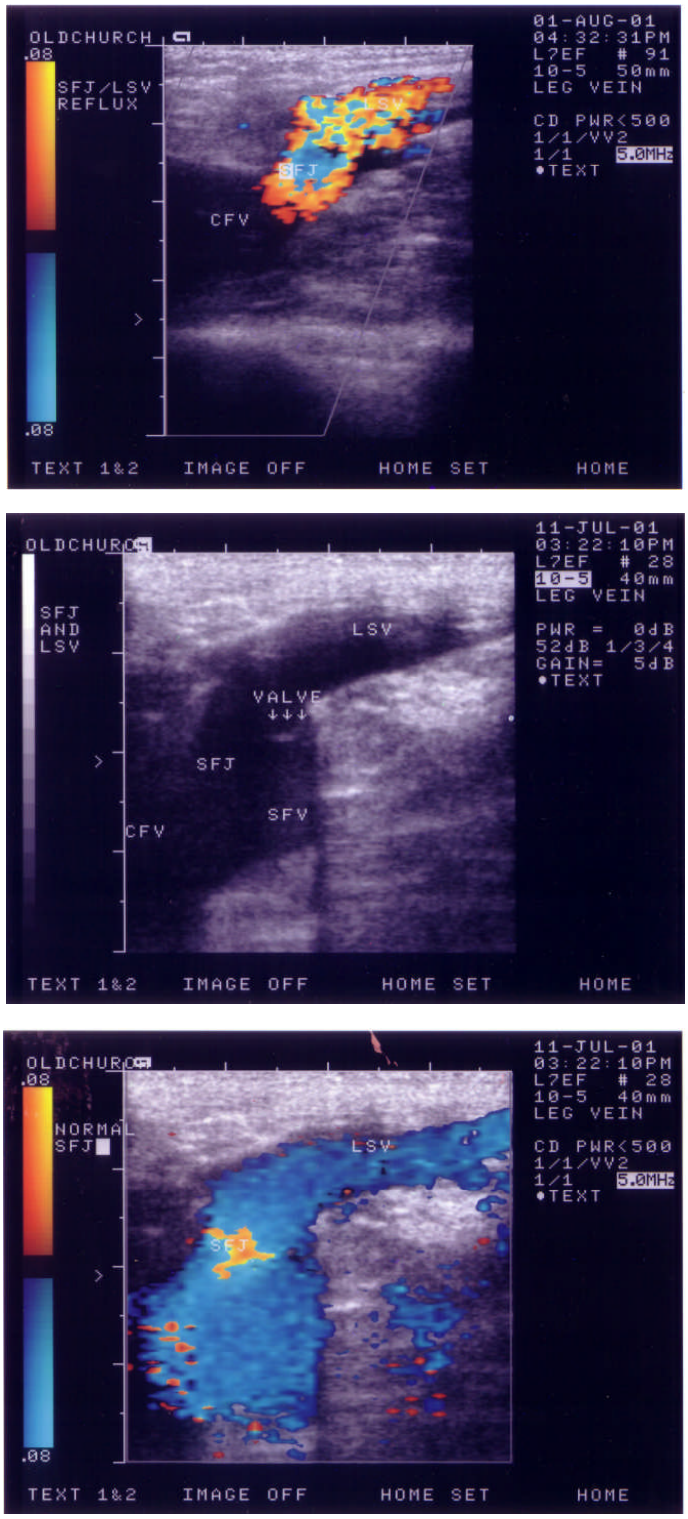

Figure 19 .The top photograph shows turbulent reflux at the SFJ. The middle photograph is a higher resolution B-Mode image depicting the valve cusps at the SFJ. The lower photograph shows a colour flow image of the SFJ.(All photographs courtesy of John Farrah PhD).

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of blood flow by the standardised cuff inflation method in the calf. A standardised rapid cuff inflation and deflation unit constructed in our laboratory (Middlesex Hospital Vascular Laboratory, 1995) with a calf cuff width of 12cm was used to augment flow in the vessels (see section 2.7). The cuffs were inflated for approximately 3 seconds with the deflation requiring only 0.3seconds. A B-mode 2D image of the vein segment of interest was obtained after insonation of the vessel with plenty of scanning gel applied between the transducer and the skin to avoid distortion of the underlying superficial vessels that may be compressed with the transducer.

In order to obtain the B-mode image and the frozen spectral Doppler display the patient is investigated in the standing position with the leg to be examined slightly flexed and externally rotated with the body weight supported by the contralateral limb and a pneumatic calf cuff with a width of 12cm that could be rapidly inflated and deflated to a pressure of 100mmHg placed around the calf to induce reverse flow as described by the standardised method above. The vessel of interest is insonated with the transducer and a frozen B-mode image is obtained to measure the vessel diameter followed by a frozen spectral Doppler display obtained after calf compression and release which has captured both the antegrade and retrograde flow.

To calculate reflux or retrograde volume blood flow

A reflux time of 0.5 secs or more in the popliteal vein was was taken as an abnormal reading.

The formula used to calculate the reflux volume blood flow is

$$Q = TAV \times area \times \Delta T$$

where, Q = reflux volume flow (ml), TAV = time averaged velocity (metres / sec), Area = cross-sectional area (mm^2) at the Doppler sample gate and ΔT = a multiplier to express flow within the duration of reflux (seconds).

The formulae ($\pi D^2/4$ or πr^2) where D is the cross-sectional diameter of the vein in mm obtained from the B-mode 2D imaging mode and r is the radius of the vein in mm is used to calculate the cross-sectional area of the vein and the TAV and ΔT can be calculated from the axial Doppler reflux velocity waveforms on the frozen spectral Doppler strip as described earlier.

Protocol for Venous reflux test by photoplethysmography (ppg)

The model PPG13 Vasculab® Photoplethysmograph (PPG) (Medasonics, Mountain View, California) was used in this thesis. It serves as a preamplifier and power supply and was used with the model PH77 PHOTOPULSE® photoplethysmograph sensor

Methodology

The subject was examined in the sitting position with the lower limbs dependent and with the transducers attached using a double-sided tape 5cm proximal to the medial malleolus. A stable trace was established, and the subject was asked to perform a series of 10 dorsiflexions and plantarflexions at the ankle to activate the calf muscle pump. This produced an emptying phase in which the falling content of haemoglobin resulted in increased amounts of light reaching the photodetector. The subject was then asked to rest and the refilling phase recorded. If the test showed an abnormal response, a tourniquet cuff (5 cm dia) was placed above the knee and inflated to about 80-110mmHg (depending on the size of the thigh) to occlude the superficial veins in the thigh and the test repeated as described above. If the test still showed an abnormal response, it is repeated with below knee cuffs inflated to 40-60 mmHg to occlude the calf veins. If a torunquet was required at the ankle level a 2.5 cm tourniquet was used.

Data Analysis

The time taken for the trace to return to the baseline level was referred to as the PPG refilling time. It was measured by placing the start cursor at the end of the exercise phase and the end cursor at the end of the refilling phase where the trace started to level off. In normal subjects, this refilling time is greater than 20 seconds whereas abnormal subjects with incompetent veins will have refilling times of less than 20 seconds.

Protocol for measuring resting ankle brachial pressure indices (ABPI).

The subject is examined in the supine position after lying relaxed in a comfortable ambient temperature room for 10 minutes.

Measuring arm pressures

A standard 12 inch sphygmomanometer cuff♣ or other suitable cuff (depending on size of limb) is placed around the arm just above the elbow. The brachial pulse is located

♣ Standard Adult cuff 16 ∗ 30 cms, small adult 12 * 22 cms, large adult 16 * 36 cms, thigh cuff 16 * 42 cms.

with a hand-held bi-directional Doppler (Dopplex®, Huntleigh Healthcare) by positioning the 8MHz probe over the brachial pulse after applying gel and holding and manoeuvring the Doppler probe at a 45 degree angle between the forefinger and thumb until a good Doppler signal is obtained. The cuff is inflated until the Doppler signal disappears and slowly deflated until the signal returns. The pressure at that point is noted as this is the brachial systolic pressure. The same procedure is repeated in the other arm.

Measuring ankle pressures

The sphygmomanometer cuff is placed around the leg just above the ankle and using the Doppler probe as described above, both the dorsalis pedis artery as well as the posterior tibial artery pulse is located. The cuff is inflated until the Doppler signal disappears and slowly deflated until it returns. The pressure at this point is noted as the ankle systolic pressure. The higher of any readings obtained are used for calculation. Both pedal ankle systolic pressures are measured and recorded.

Data Analysis

To calculate the ankle brachial pressure index (ABPI), the ankle systolic pressure reading is divided by the brachial systolic pressure using the higher of the brachial pressures and the higher of the ankle systolic pressures in the calculation. Normal ankle brachial pressure index is equal to or greater than 0.90 in a non-diabetic individual.

3.2.1 Human soluble ICAM-1 Immunoassay

Adhesion molecules mediate the interaction of cells with the extracellular matrix and with other cells. The immunoglobulin superfamily of proteins contains a large class of adhesion molecules with multiple immunoglobulin-like domains. ICAM-1 (CD54) is a member of this family (1, 2). It is a 90 kDa type-I transmembrane glycoprotein with five Ig-like extracellular domains. The most important ligands for ICAM-1 are the â2 integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) , which are expressed on leucocytes . ICAM-1 thus mediates the adhesion of leucocytes to ICAM-1-expressing cells. ICAM-1 also binds fibrinogen , hyaluronan , Rhinoviruses, Plasmodium

falciparum-infected erythrocytes and CD43 (sialophorin) . ICAM-1 is either a transmembrane protein (mICAM-1) or soluble (sICAM-1) . mICAM-1 is expressed on endothelial and epithelial cells, lymphocytes, monocytes, eosinophils, keratinocytes, dendritic cells, hematopoietic stem cells, hepatocytes and fibroblasts.

Regulation of ICAM-1 expression is cell specific. Up-regulation generally is by inflammatory cytokines (TNF-á, IFN-ã and IL-1) and down-regulation generally is by anti-inflammatory agents (e.g.glucocorticoids) . One important, well-characterized function of ICAM-1 is immune-cell trafficking. At sites of inflammation, inflammatory cytokines induce up-regulation of ICAM-1 expression on vascular endothelial cells and activation of leucocyte integrins (LFA-1 and Mac-1). This leads to adhesion of leucocytes to the local endothelium, an essential step in migration of leucocytes to the site of inflammation. sICAM-1 has been reported in serum (10), cerebrospinal fluid and bronchoalveolar lavage. sICAM-1 likely arises by proteolytic cleavage of mICAM-1; synthesis from an alternatively spliced message has not been found . In general, elevated levels of serum sICAM-1 appear to be associated with inflammatory conditions and certain malignancies . It has, however, been pointed out that in inflammatory conditions, where the ligands LFA-1 and Mac-1 are likely to be activated, binding and clearance of sICAM-1 might be enhanced, so that a reciprocal relationship between sICAM-1 levels and inflammation also is possible .

The Parameter human sICAM-1 Immunoassay is a 2 hour solid phase ELISA that is designed to measure sICAM-1 in cell culture supernatants, serum and plasma. It contains recombinant human ICAM-1 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantify recombinant and natural human sICAM-1 accurately. Results obtained using natural human sICAM-1 showed linear curves that were parallel to the standard curves obtained using the recombinant Parameter kit standards. These results indicate that the Parameter Immunoassay kit can be used to determine relative mass values for natural human sICAM-1.

Principle Of The Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sICAM-1 has been pre-coated onto a microplate. Standards, samples, Controls and Conjugate are pipetted into the wells and any sICAM-

1 present is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sICAM-1. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of sICAM-1 bound. The colour development is stopped and the intensity of the colour is measured.

Assay Procedure

All reagents and samples are brought to room temperature before use. It is recommended that all samples, Standards and the sICAM-1 Control be assayed in duplicate. The following protocol as recommended by the manufactures (R&D Systems Abingdon UK) was used

1. Prepare all reagents, working Standards, samples, and Control as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100 µL diluted Conjugate to each well.
4. Add 100 µL Standard, sICAM-1 Control*, or sample* to each well with sufficient force to ensure mixing.
5. Cover the plate with a plate sealer provided and incubate at room temperature for 1.5 hours.
6. Aspirate or decant each well and wash, repeating the process five times for a total of six washes. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid after each wash is essential to good performance. After the last wash, aspirate or decant the contents and remove any remaining Wash Buffer by tapping the inverted plate firmly on clean paper towels.
7. Immediately add 100 µL Substrate to each well. Cover the plate with a new plate sealer and incubate at room temperature for 30 minutes.
8. Add 100 µL of Stop Solution to each well. The Stop Solution should be added to the wells in the same order as the Substrate.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 620 or 650 nm. If wavelength correction is not available, subtract readings at 620 or 650 nm from the

readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation Of Results

The mean absorbance values for each set of duplicate Standards were calculated. A standard curve was constructed by plotting the mean absorbance for each Standard on the y-axis against the concentration on the x-axis and drawing a best-fit curve through the points on the graph. The concentration of each unknown sample was determined by calculating the concentration of sICAM-1 corresponding to the mean absorbance from the standard curve. For samples and the sICAM-1 Control, the concentration determined from the standard curve was multiplied by the dilution factor. The sICAM-1 Control was run in each assay. If the values obtained were not within the expected range, as stated on the Control vial label, the assay results were considered invalid.

3.2.2 Human sVCAM-1 Immunoassay

Human Vascular Cell Adhesion Molecule-1 (VCAM-1) is a 100 - 110 kDa, 715 amino acid (aa) type I transmembrane glycoprotein typically characterized by the presence of seven C2-type immunoglobulin (Ig) domains .Its extracellular region is 674 aa in length, followed by a 22 aa transmembrane segment and a 19 aa cytoplasmic tail . In the extracellular region, there are multiple N-linked glycosylation sites (the predicted molecular weight is 80 kDa), and each C2 domain is closed by a disulfide bridge. There is considerable interspecies VCAM-1 homology, with mouse and rat VCAM-1 showing approximatley 75% aa identity to human VCAM-1. Notably, the short 19 aa cytoplasmic tail is absolutely conserved, mouse to human to rat. Cells expressing mouse VCAM-1 bind both mouse and human leucocytes, and this reflects their high degree of aa identity. A number of variants of VCAM-1 are known to occur, all of which are likely the result of alternate gene splicing. In particular, a human six Ig domain molecule is known, and in rabbits, an eight Ig domain form has been identified.

There is also a three C2 domain, 43-kDa GPI-linked form of VCAM-1. Although it binds known VCAM-1 ligands (or co-receptors), its function is unclear. Cells known to express VCAM-1 include neurons, endothelial cells, smooth muscle cells, fibroblasts

and macrophages. Soluble VCAM-1 has been identified in culture supernatants, blood, and cerebrospinal fluid. The exact mechanism by which VCAM-1 is generated is unknown; it may, however, involve both proteolytic processing and alternate splicing. Functionally, VCAM-1 binds to both α4β1 (VLA-4) and α4β7 (LPAM-1) integrins. These integrins (or VCAM-1 ligands) are expressed on a variety of cells, with VLA-4 found on all leucocytes with the exception of neutrophils (17, 19, 20). Because of this, VCAM-1/VCAM-1 ligand interactions are undoubtedly key events in the rate and timing of leucocyte extravasation. Other roles proposed for VCAM-1 include the regulation of osteoclastogenesis via a cell-to-cell contact mechanism, and the induction of sickle cell adherence to vascular endothelial cells during hypoxemia. The Parameter human sVCAM-1 Immunoassay is a 2 hour solid phase ELISA that is designed to measure sVCAM-1 in cell culture supernatants, serum and plasma. It contains recombinant human sVCAM-1 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant and natural human sVCAM-1 accurately. Results obtained using natural human sVCAM-1 showed linear curves that were parallel to the standard curves obtained using the recombinant Parameter kit standards. These results indicate that this immunoassay kit can be used to determine relative mass values for natural human sVCAM-1.

Principle Of The Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sVCAM-1 has been pre-coated onto a microplate. Standards, samples, Controls and Conjugate are pipetted into the wells and any sVCAM-1 present is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sVCAM-1. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of sVCAM-1 bound. The color development is stopped and the intensity of the color is measured.

Assay Procedure

All reagents and samples are brought to room temperature before use. All samples, Standards and the sVCAM-1 Control were assayed in duplicate. The following protocol recommended by the manufacturers (R&D Systems Abingdon UK) was used.

1. Prepare all reagents, working Standards, samples, and Control as directed in the previous sections.
2. Remove excess microplate strips from the frame and store in the resealed foil pouch with the desiccant pack.
3. Add 100 µL diluted Conjugate to each well.
4. Add 100 µL Standard, Control*, or sample** to each well with sufficient force to ensure mixing.
5. Cover the plate with a plate sealer provided and incubate at room temperature for 1.5 hours.
6. Aspirate or decant contents from each well and wash by adding 300 µL of Wash Buffer per well. Repeat the process five times for a total of six washes. After the last wash, aspirate or decant the contents and remove any remaining Wash Buffer by tapping the inverted plate firmly on clean paper towels.
7. Immediately add 100 µL Substrate to each well. Cover the plate with a new plate sealer and incubate at room temperature for 20 minutes.
8. Add 100 µL of Stop Solution to each well. The Stop Solution should be added to the wells in the same order as the Substrate.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 620 nm. If wavelength correction is not available, subtract readings at 620 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of Results

The mean absorbance values for each set of duplicate Standards were calculated. A standard curve was constructed by plotting the mean absorbance for each Standard on the y-axis against the concentration on the x-axis and drawing a best-fit curve through the points on the graph. The concentration of each unknown sample was determined by calculating the concentration of sVCAM-1 corresponding to the mean absorbance from the standard curve. For samples and the sVCAM-1 Control, the concentration determined from the standard curve was multiplied by the dilution factor. The sICAM-1 Control was run in each assay. If the values obtained were not within the expected range, as stated on the Control vial label, the assay results were considered invalid.

3.2.3 Human VEGF Immunoassay[††]

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) or vasculotropin, is a homodimeric 34 - 42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. The amino acid sequence of VEGF exhibits primary structural, as well as limited amino acid sequence, homology with that of the A and B chains of PDGF. All eight cysteine residues involved in intra- and inter-chain disulfide bonds are conserved among these growth factors. A cDNA encoding a protein having a 53% amino acid sequence homology in the PDGF-like region of VEGF has been isolated from a human placental cDNA library. This protein, named placenta growth factor (P*l*GF), is now recognized to be a member of the VEGF family of growth factors. Based on its homology with VEGF, P*l*GF was also proposed to be an angiogenic factor. A gene encoding a polypeptide with homology to VEGF has been discovered in the genome of the orf virus (OV), a parapoxvirus that affects sheep, goats and sometimes humans.

VEGF is expressed by numerous rodent and human tumor cells, including human lung adenocarcinoma, bladder carcinoma, fibrosarcoma, HL60 promyelocytic leukemia, GS-9L glioma, and U937 lymphoma cells (6 - 10). In normal tissues, VEGF expression has been found in activated macrophages (11), keratinocytes (12), renal glomerular visceral epithelium and mesangial cells (13, 14), hepatocytes (15), smooth muscle cells (16), Leydig cells (17), embryonic fibroblasts and bronchial and choroid plexus epithelium (18, 19). The expression of VEGF is upregulated by phorbol ester, TGF- and in hypoxia (6 - 10, 18). In contrast to the widespread distribution of VEGF, the expression of PlGF mRNA is limited to placental tissue, choriocarcinoma cells and cultured endothelial cells .In the conditioned media of human choriocarcinoma cells (JAR and JE-3), the occurrence of VEGF/PlGF heterodimers has also been observed . The gene for human VEGF is organized into 8 exons. As a result of alternative splicing, at least 4 transcripts encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid

[††] The broad term 'VEGF' covers a number of proteins from two families, that result from alternate splicing of mRNA from a single, 8 exon, *VEGF* gene. The two different families are referred to according to their terminal exon (exon 8) splice site - the proximal splice site (denoted $VEGF_{xxx}$) or distal splice site ($VEGF_{xxx}$b). In addition, alternate splicing of exon 6 and 7 alters their heparin binding affinity, and amino acid number (in humans: $VEGF_{121}$, $VEGF_{121}$b, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}$b, $VEGF_{189}$, $VEGF_{206}$. An alternative classification is VEGF A,B,C and PIGF.

residues (VEGF121, VEGF165, VEGF189, and VEGF206), each preceded by a 26 amino acid residue signal peptide, have been detected. VEGF121 and VEGF165 are diffusible proteins that are secreted into the medium. VEGF189 and VEGF206 have high affinity for heparin and are mostly bound to heparin-containing proteoglycans in the extracellular matrix. VEGF contains a potential N-linked glycosylation site and the natural protein is a glycoprotein. E. coli-expressed, recombinant human VEGF is indistinguishable from natural VEGF in its in vitrobiological actions, suggesting that the carbohydrate moiety may not be required for activities. VEGF is a highly conserved protein that has cross-species activity. Between human, rat or bovine VEGF, 84 - 94% sequence identity has been observed. Two receptor tyrosine kinases have been described as putative VEGF receptors. Flt-1 (fms-like tyrosine kinase) , and KDR (kinase-insert-domain-containing receptor) proteins have been shown to bind VEGF with high affinity . The mouse homologue of KDR was named Flk-1, for fetal liver kinase-1. Mouse Flk-1 shares 85% amino acid sequence identity with human KDR Flt-1 and KDR/Flk-1 are members of the superfamily of RTKs (receptor tyrosine kinases) that also include the receptors for PDGF, M-CSF and SCF. In addition to the membrane-spanning Flt-1, a cDNA encoding a soluble truncated form of Flt-1 has been cloned from a human vascular endothelial cell library. The mRNA for the soluble receptor is apparently generated by alternative splicing .Recombinant soluble Flt-1 binds VEGF with high affinity and inhibits VEGF actions on vascular endothelial cells. Thus, it is possible that natural soluble Flt-1 may act as a VEGF antagonist in vivo. Using a dominant-negative Flk-1 mutant, Flk-1 has been shown to be involved in the VEGF-mediated transduction of signals that are important for angiogenesis and vasculogenesis. As assessed by Northern blot analysis and/or in-situ hybridization, Flt-1 was found to be expressed in both endothelial and non-endothelial cells, while KDR/Flk-1 expression was reported to be restricted to endothelial cells. In vitro, VEGF is a potent endothelial cell mitogen. In cultured endothelial cells, VEGF can activate phospholipase C and induce rapid increases of free cytosolic Ca2+. VEGF has been shown to stimulate von Willebrand factor release from endothelial cells and induce expression of tissue factor activity in endothelial cells as well as in monocytes. VEGF has also been shown to be chemotactic for monocytes and osteoblasts (31). In vivo, VEGF can induce angiogenesis as well as increase microvascular permeability. As a vascular permeability factor, VEGF acts directly on the endothelium and does not degranulate mast cells. It promotes

extravasation of plasma fibrinogen, leading to fibrin deposition that alters the tumor extracellular matrix.

The modified extracellular matrix subsequently promotes the migration of macrophages, fibroblasts and endothelial cells. Based on its in vitro and in vivo properties, VEGF is expected to play important roles in inflammation and during normal and pathological angiogenesis, a process that is associated with wound healing, embryonic development, and growth and metastasis of solid tumors. Elevated levels of VEGF have been reported in synovial fluids of rheumatoid arthritis patients and in sera from cancer patients.

Bioassays for VEGF, based on its proliferative effects on endothelial cells, are time-consuming and not completely specific for VEGF. The Quantikine VEGF Immunoassay is a 4.5 hour solid phase ELISA designed to measure VEGF165 levels in cell culture supernates, serum, and plasma. It contains Sf21-expressed, recombinant human VEGF165 and antibodies raised against the recombinant protein. Results obtained for naturally occurring human VEGF and recombinant human VEGF121 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human VEGF.

Principle Of The Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is stopped and the intensity of the color is measured.

Assay Procedure

All reagents and samples are brought to room temperature before use. All samples, Standards and the sVCAM-1 Control were assayed in duplicate. The following protocol recommended by the manufacturers (R&D Systems Abingdon UK) was used.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. For Serum/Plasma Samples: Add 100 µL of Assay Diluent RD1W to each well.
4. For Serum/Plasma Samples: Add 100 µL of Standard, control or sample per well. Cover with the adhesive strip provided and incubate for 2 hours at room temperature. A plate layout is provided to record the standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of VEGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Protect from light.
For Serum/Plasma Samples**:** Incubate for 25 minutes at room temperature.
9. Add 50 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate.

Calculation Of Results

Thee duplicate readings for each standard, control, and sample were averaged and the average zero standard optical density subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draws a best fit curve through the points on the graph. The data was linearized by plotting the log of the VEGF concentrations versus the log of the optical density (O.D.), and the best-fit line be determined by regression analysis. This procedure produces an adequate fit of the data. If samples were diluted, the concentration read from the standard curve was multiplied by the dilution factor.

3.2.4 Serum Assay for Lactoferrin

Human lactoferrin (LTF) is an 80 kDa glycoprotein which was first isolated from human milk. LTF is found in most body fluids and secretions, *e.g.*, in the nose, genital tract, and tears. In the blood, LTF is secreted by neutrophils and its plasma concentration is positively related to the total pool of neutrophils and to the rate of neutrophil turnover. Because of its ability to strongly bind iron, LTF is considered to be bactericidal. In a number of cases of inflammation, LTF is released into the extracellular medium from secondary granules of neutrophilic leucocytes. Its extracellular concentration can therefore be used as an index of neutrophil activation, especially in blood samples containing anti-myeloperoxidase antibodies.

Principle Of The Procedure

The BIOXYTECH® Lacto*f*-EIA™ method is an enzyme-linked immunosorbent assay (ELISA). Lactoferrin is captured by a monoclonal antibody (MAb) that is coated on wells of a sectional microplate. A second LTF-MAb labeled with biotin is added to the well and binds with the captured LTF forming a “sandwich.” A solution of streptavidin-peroxidase is then added. Streptavidin has a high affinity for biotin and once bound, its horseradish peroxidase (HRP) label is available for color development by addition of the substrate, *o*-phenylenediamine (OPD). This color development at 450 nm is proportional to the quantity of LTF in the sample.

Assay Procedure

The actual assay for LTF is performed in the following fashion

· Perform a serial dilution of the 100 ng/mL Standard to the following concentrations: 50, 25, 12.5, 6.2, 3.1 and 1.6 ng/mL.

· Pipet 100µL Standard or sample to the appropriate well.

· Cover the wells with a Plate Sealer and incubate at 37°C for one hour. (± 5 minutes).

Anti-LTF incubation

· Prepare the required volume of diluted Anti-LTF Solution (4) (table 2).

· Empty the microplate.

· Wash wells 5 times with diluted Wash Buffer (3).

· Blot residual liquid on absorbent paper.

· Pipet 100 µL of diluted anti-LTF Solution into each well.

· Cover the wells with Plate Sealer and incubate at 37°C for 1 hour. (± 5 minutes)

Streptavidin-HRP incubation

· Prepare the required volume of Streptavidin-HRP solution (5) (table 2).

· Empty the microplate.

· Wash wells 5 times with diluted Wash Buffer (3).

· Blot residual liquid on absorbent paper.

· Pipet 100 µL of diluted Streptavidin-HRP into each well.

· Cover the wells with Plate Sealer and incubate at 37°C for 15 minutes.

Colorimetric measurement

· Prepare the required volume of OPD solution (table 3).

· Empty the microplate.

· Wash wells 5 times with diluted Wash Buffer (3).

· Blot residual liquid on absorbent paper.

· Pipet 100 µL of prepared OPD solution into each well.

· Cover the wells with Plate Sealer and incubate at 37°C until the absorbance of the 100 ng/mL

Standard reaches about 1-1.5 (approximately 5 to 10 minutes).

· Add 50 µL of Stop Solution (8) to each well.

· Read the absorbance at 450 nm.

Calculations are performed in a standard fashion.

3.2.5 Serum Assay for VW Factor Activity

von Willebrand factor (vWF) is a complex multimeric adhesive glycoprotein synthesized by endothelial cells and megakaryocytes. It is a carrier for factor VIII♣ and acts as an adhesive protein in haemostasis. It mediates the adhesion of platelets to the subendothelium (glycoprotein Ib). It may also be involved in platelet/platelet interaction (glycoprotein IIb/IIa). It binds collagen and possibly other endothelial structures. Decreased levels of vWF are seen in Von Willebrand's disease. This may be caused by other coexisting diseases (Von Willebrand's Syndrome).

Increased levels of vWF antigen/activity indicate damage to the endothelium. However short term increase in vWF areseen after exercise, adrenaline or DDAVP infusion, pregnancy and venous hypertension. These may indicate endothelial activation rather than damage.

PRINCIPLE OF THE PROCEDURE

ELISA utilising a monoclonal antibody which recognises a functional epitope on the vWF molecule.This is a reproducible, easy-to-use, sensitive and cost effective assay. It detects low vWF activity to aid diagnosis of vW disease as well as elevated vWF activity to aid assessment of endothelial damage in cardiovascular disease. During the first incubation specific vWF antigen in the serum binds to the antibody coating the wells. The wells are washed to remove unbound components. A horseradish-peroxidase/-labelled anti-human vWF binds to the surface bound antigen in the second phase. After a further washing substrate solution is added and detects specifically-bound Anti-body. The stop solution is used to terminate the reaction. The amount of conjugate bound is measured in absorbance units.

The kit components include 12x8 well microtitre plate strips coated with monoclonal antibody, calibrator and controls, diluent concentrate (for samples, controls and conjugate), wash buffer concentrate, substrate, substrate buffer, stop solution.

ASSAY PROCEDURE

1. Add 100µl of standard or sample to wells

2. Incubate at room temperature for 60 minutes

3. Wash

4. Add 100µl of anti-vWF conjugate

5. Incubate at room temperature for 60 minutes

6. Wash

♣ vWF monomer is a 2050 amino acid protein. Every monomer contains a number of specific domains with a specific function; domains D'/D3 act as carrier for factor VIII. Domain A1 binds to collagen and

7. Add 100µl of substrate

8. Incubate at room temperature for 5 minutes

9. Wash

10. Add 100µl of stop solution. Read absorbance at 450nm

3.3 Laser Doppler Anemometer for Measuring Blood Cell Velocities In Perpendicular Capillary Loops

There are several methods available for measuring blood cell velocity within single capillaries they are all image-based techniques and can only measure flows in vessels lying parallel to the surface. Since the measurement is derived from the image, a good high contrast image is necessary. This is not always possible to obtain in many subjects and using standard video equipment restricts the maximum range of measurement to the order of 2 mm/s. Velocity measurement using the laser Doppler technique is well established and there are many applications in the study of fluid flow. The technique was first demonstrated in 1964. Because of the relatively high cost of the necessarily precise optics, its use is generally restricted to measurements that would otherwise be impossible or impracticable. Typical applications include studies of velocity and turbulence profiles of liquids and gases in complex systems.

The application of laser Doppler to measuring flows in microscopic biological vessels has been published by several groups. The first *in vivo* application was the measurement of the velocity of blood cells in an 80-micron diameter retinal artery of an albino rabbit, using laser light backscattered at a known angle. In 1974 Mishina *et al.* described a dual beam Laser Doppler Microscope[119]. This was used to demonstrate the measurement of velocity in a 70-micron diameter venule in the web of a frog's foot by transmission through the tissue. Another laser Doppler microscope anemometer was used to measure velocity profiles in arterioles 65-98 microns in diameter. This system used the beam scattered at a known angle through the tissue to detect blood cell velocity. The

---

Heparin & platelet GPIb-receptor. There are also C1 & ‘cysteine knot’ domains.

measurements compared well with those from a previously obtained calibrated particle velocity meter technique.

Later Einav et al [120] described a fringe mode transmittance laser Doppler microscope. Measurements of the velocity profile across a rectangular channel gave good accuracy and reproducibility better than 1% All the above techniques depend on the angle of incidence or the angle of scattering of the laser beam. Some of All the above techniques measure velocities parallel to the tissue surface, which like the imaging techniques restricts measurements to such sites as the nail-fold or forearm. Following on from the first instrument of Riva et al., was the development of laser Doppler perfusion monitors. In these systems the laser light is delivered and collected by two optic fibres. The laser light radiates out into the tissue is diffusely scattered and shifted by the flows in the multitude of vessels within the measuring volume of about 1 cubic mm. These systems produce an exponentially shaped Doppler shift spectrum that is processed to give arbitrary values proportional to average velocity and average blood cell concentration. The output requires linearisation to correct for changes in blood cell concentration. The linearisation is only effective for part of the physiological range typically encountered. The output is dependent on the morphology of the tissue, i.e. the distribution of vessel dimensions and directions.

The ‘’CAM1 Capillary Anemometer’’ was developed to overcome the limited velocity range, image quality, and limited tissue sites of the imaging techniques, the high cost and complexity of the dual beam laser Doppler microscopes, and the unknown probe volume, arbitrary values, and non-linearity of the laser Doppler perfusion monitors.

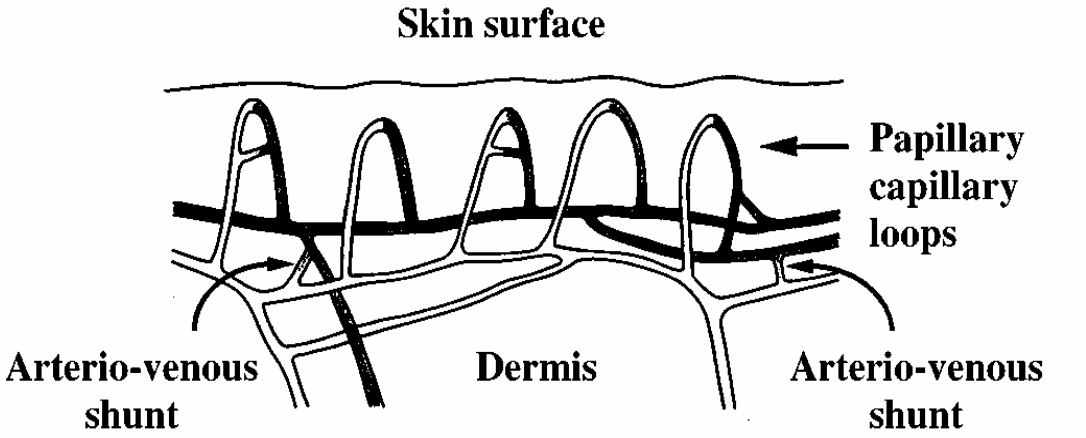


Figure 20 shows diagrammatically the anatomy of the skin capillaries that are assessed by capillary microscopy.

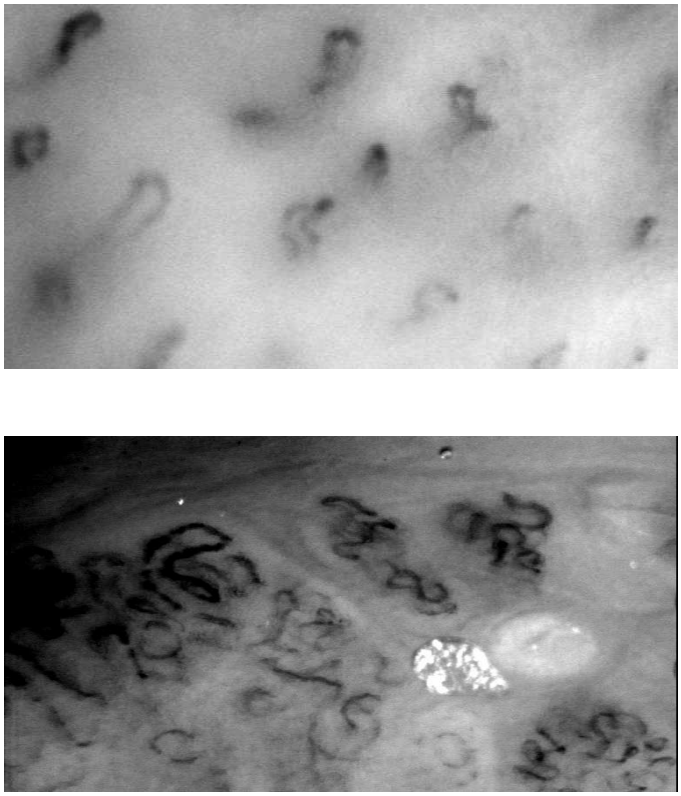


Figure 21 the lower photograph shows a view of the skin capillaries obtained at capillary microscopy. The capillaries are elongated, tortuous and glomeruloform. This is unlike th straight capillaries seen in normal skin. This is shown in the top photograph (Authors' own photographs)

3.4 DESCRIPTORS & STATISTICAL METHODS

Because of the relatively small number of patients the data was analysed using non-parametric tests. Advice was sought from experts regarding the method of analysis used. The median was used as descriptor of 'average'. The description of spread mostly used in the work in this thesis is by interquartile range. The analysis of paired data was by the Wilcoxon signed ranks test. Unpaired data was analysed using the Mann-Whitney U test (an equivalent test to the Wilcoxon rank two sample test).

Descriptors of Average

An average describes a 'typical' measurment. This representative value can summarise the data. Commonly used parameters used the mean, the mode, the median, the geometric mean & the weighted mean.

The Median

An ordered set is first created by arranging the values in increasing order of magnitude. The median would divide this set into two halves with equal values above and below it. If the number of observations is odd, the median is the

$$(n+1)/2 \text{ th}$$

value in the set of observations. If n=even number then there strictly no median. In practice the arithematic mean of the two middle observations is often used as the median. Symmetry of the data greatly affects the median. In symmetrical data, the median and the mean are the same. In data skewed to the right the median is lower than the mean, in data skewed to the left the median is higher than the mean. One of the advantages of the median is that it is not distorted by outliers.

The Arithmetic mean

This is calculated by adding all the values and dividing them by the number of observations

$$\bar{X} = \frac{x1+x2+x3+\ldots\ldots\ldots+xn}{n}$$

OR $\bar{x} = \frac{\Sigma x}{n}$

the arithmatic mean uses all the data values and is manageable algebraically. It is however, distorted bioth by outliers as well as by skewed data.

The Mode

This is the value that occurs most frequently in the set of observations. In continuous data the data is first grouped and then the modal group is dtermined. If each value occurs only once there is no mode. Similarly there may be more than one mode. The mode ignores most of the information and is not algebraically defined.

The Geometric Mean

In skewed data if logarithmic transformation makes the distribution symmetrical, the logarithmic mean is taken. This is then back transformed to the non-logarithmic value. This technique has obvious limitations.

Weighted Mean

If a variable x of interest has certaain values more important than others then a weight w_i is attached to each of the values x_i.

$$\frac{W1x1+w2x2+w3x3+\ldots\ldots\ldots..+wnxn}{W1+w2+w3+\ldots.+wn} = \frac{\Sigma wixi}{\Sigma wi}$$

This can be a very useful value provided the weights are known.

Descriptors Of Spread

These give an idea about the dictribution of the data. Most analyses require this along with an ‘average’ to describe data. Commonly used descriptors of spread include range, ranges derived from percentiles (e.g. interquartile ranges), the variance, and standard deviation. Like many other statistical parameters it is often best represented graphically.

The Range

This is stated as the difference between the largest and the smallest observations in the set of values. In many instances these values are quoted instead. This is distorted by outliers and increases with increasing sample size.

Ranges from Percentiles

The values of x that divide the data into 10 equally sized groups i.e. the 10th, 20th, 30th…..90th percentileas are called **deciles.** Similarly **quartiles** divide the data into four equal sets. These are the 25th, 50th and the 75th percentiles.

The interquartile range is the difference between the fisrt and the 3rd qaurtiles i.e. between the 25th and the 75th percentiles(see **Figure 22**). The interdecile range lies between the 10th and the 90th percentiles and contains the central 80% of the data. The **95% central range** is also a frequently used range. It ignores 2.5% of the data below the lower limit and 2.5% abovet the upper limit.

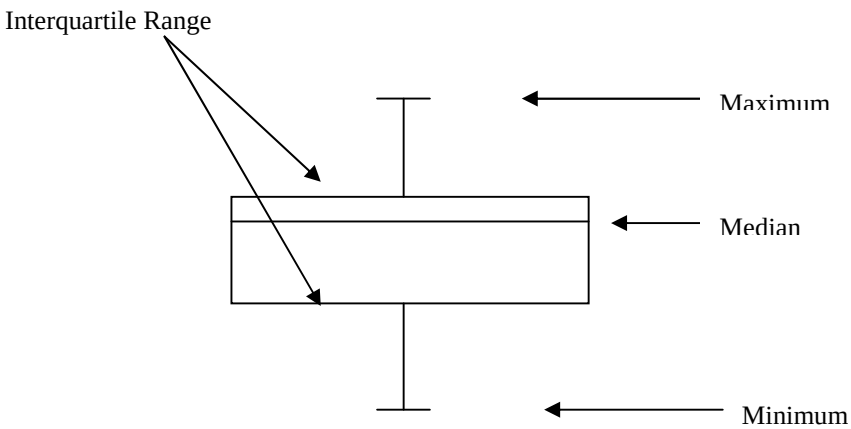


Figure 22 Box and whiskers method of representing the interquartile range relating to the median as well as the maximum and minimum values.

These ranges from percentiles are not algebraically defined and are difficult to use for smaller samples.

Variance

This depicts the degree to which the data deviates from the average. Because of mathematical reasons the calculation of variance (s^2) involves the sqaure of the deviation of all the values and the divisor is n-1 rather than n.

$$S^2 = \frac{\Sigma(x_i - \bar{x})2}{n-1}$$

The unit is the square of the original units. Variance is considered inappropriate for skewed data.

Standard Deviation

This is the square-root of variance. Although it has the same limitations as the variance it uses the same units as the original observations and is thus easier to interpret.

$$S = \sqrt{S^2 = \frac{\Sigma(x_i - \bar{x})2}{n-1}}$$

The coefficient of variation is obtained by dividng the standard deviation by the mean and expressing the quotient obtained as a percentage.

Paired Data Analysis For Numerical Data

This is used when the variable that is to be measured is on the same indiidual in different circumstances. This would happen in a crossover trial amongst other situations. In some case control studies different individuals may be mathced to each other and the data is therefore treated as paired data. Account of dependence of the two values is taken by taking the difference of the two paired values. This converts the data into a single set of differences.

The Wilcoxon signed ranks test takes account of both the sign of both the signs of the differences as well as their magnitude. It is therefore a much more powerful test than a simple sign test. After calculating individual differences for each pair, positive or negative signs are assigned and all zeros are left out. The differences are ranked according to their agnitude irrespective of their signs. The smallest value is assigned 1, the next smallest 2 and so on. Identical differences receive an average of the rank they would have otherwise received. The null hypothesis would requie the sum of positive and negative ranks to be the same.

To calculate the Wilcoxon statistic difference for all pairs of results is calculated first. All n' differnces are then ranked assiging 1 to the smallest and n' to the largest. Then the postive (T+) and the negative differences (T-) are summed up.

I. if n' </=25 then T, the test ststistic is the smaller of the two vlues T+ or T-
II. If n' >25 then the test statistic z has to be calulated using the following formula

$$z = \frac{\left| T - \frac{n'(n'+1)}{4} \right| - \frac{1}{2}}{\sqrt{\frac{n'(n'+1)((2n'+1)}{24}}}$$

z is then compared to a list of values from a list of known probability distributions.

Analysis Of Un-Paired Data (For Numerical Data)

This analysis involves samples from two different groups of individuals. There is one numerial variable of interest. The item of interest is whether the means or the distribution of the variable in the two groups is the same. The Wilcoxon rank sum (two-sample) test makes no assumption about the distribution and could be used here. An alternative test is the Mann-Whitney U test.

If there is a sample of n1 observations to be compared with an independent sample of n2 observations where n1 is the smaller sample size.

i. Rank all the (n1+n2) observations ignoring the grouping.
ii. Find the total (T) of the ranks in sample 1.

If each of the sample sizes is ten or more then the test statistic a Standardised Normal Distribution (SND) or z score, is

$$SND = \frac{T - n1\,(n1+n2+1)/2}{\sqrt{n1n2(n1+n2+1)/12}}$$

The test statistic has a normal distribution and a table is used to find out a p value for the test statistic.

PART IV

STUDIES

4.1 Vascular Endothelial Growth Factor (VEGF) in CVD

Vascular endothelial growth factor (VEGF, vascular permeability factor) is a member of the platelet derived growth factor (PDGF) family. It is a disulphide linked homodimeric glyco-protein. It is a potent mitogen for endothelial cells even at very low concentrations and significantly increases vascular permeability.[121] VEGF is important both in angiogenesis and oedema formation.[122] VEGF acts via two different tyrosine kinase membrane receptors, which have been identified on vascular endothelial cells[123]. Its angiogenic role in neoplasia is well established. Cells producing VEGF include vascular smooth muscle cells, fibroblasts, keratinocytes and histiocytes.[124] . VEGF is ubiquitous in wound repair and the inflammatory process has an important part to play in the healing process. However abnormally increased levels over a prolonged period of time can have local deleterious effects.

Burnand et al[125] reported on the existence of peri-capillary fibrin cuffs in patients with CVD. Although the morphology of the microangiopathy has since been well described, the factors that mediate its development remain unknown.

In the presence of lipodermatosclerosis there is great proliferation of the subepidermal/dermal capillaries in the skin. Capillary microscopy has shown that the capillaries are elongated and tortuous (Bollinger et al). [126] In advanced stages they may look glomerular (see Figure) compared to the normally pin-shaped capillary loops.[127] Recently many growth factors including VEGF have been identified which could be responsible for the angiogenic response in CVD [128]-[129]

Increased leucocyte activity may damage the skin and the vein wall itself in CVD.[130] In the chronic stage a reparative tissue response coexist with the inflammatory response.[131] Many growth factors including vascular endothelial growth factor (VEGF) are involved in this response. VEGF is a glycoprotein secreted by a variety of cells including smooth muscle cells, monocytic cells and keratinocytes[132]. Increased expression of VEGF is dependent on increased transcription.[133] It has been shown previously that there is up-regulation of VEGF expression in the skin of patients with CVD[172]. This is more so in

patients with clinical stage C4 disease[134]. VEGF has been shown to stimulate NO release in bovine, rabbit and human endothelial cells. [135] [136] NO may mediate the angiogenic effects of VEGF.[137] Excessive mounts of NO may lead to local tissue damage.

It has been shown previously that the leg skin in CVD shows increased expression of VEGF when studied by immunohistochemistry. The plasma levels of VEGF had not been studied before in CVD and I undertook a study to determine these levels in CVD. The aim of this study was to measure plasma VEGF levels in controls and patients with CVD before and after a period of experimental hypertension to investigate the role of this angiogenic factor in the development of chronic venous disease.

The highly tortuous capillaries in CVD are cut many times on histological sections and these account in part for apparent increase in numbers reported by Burnand. The resulting microcirculation is abnormal and shows many features suggesting impaired efficiency including a diminished cutaneous hyperemic response[138]. The proliferation of the capillary endothelium has not been fully explained, although recently production of VEGF in the epidermis has been demonstrated. [128]-[139] VEGF acting on the main capillaries in the underling papillary dermis causing them to proliferate is a likely mechanism.

Material & Methods

The UCL Medical School Committee for Medical Ethics considered and approved the protocol for this study. Patients attending the vascular clinic at the Middlesex Hospital for management of lower limb venous problems were invited to volunteer. The control group comprised 25 volunteers and included members of the staff of the Department of Surgery or patients being treated for unrelated conditions. They had no symptoms or signs of venous disease of the lower limb. *There was no duplex evidence of chronic venous disease*. Volunteers with a history or clinical evidence of arterial disease, diabetes mellitus, connective tissue disorders including rheumatoid arthritis, blood disorders, infection within the previous six weeks, or medication known to alter white cell activity were excluded from the study. All controls had CVD excluded by means of duplex venous scan. Patients and controls who gave informed written consent were

considered for inclusion in this study. The demographic details of subjects included are shown in **Table 13**. All subjects were examined clinically for signs of venous disease by a surgeon trained in the management of vascular diseases. Patients were divided into the two appropriate CEAP (Clinical, Etiologic, Anatomic, and Pathologic) [134]groups based on the clinical examination. There were 15 patients in the C3 group (varicose veins and oedema) and 15 patients in the C4 group (trophic skin changes). Patients underwent colour duplex ultrasonography by skilled vascular technologists and photoplethysmography (PPG) follow up. The extent of venous valvular incompetence and post-thrombotic vein damage in the deep and superficial venous systems was established and recorded systematically.

	No. of subjects	**Age**	**SVI**	**DVI + (SVI)**	**TOTAL**
C0 (Controls)	8F/7M	31(23-42)	-	-	15
C3	12M/3F	48 (29-73)	13	2 (2)	15
C4	7F/8M	51 (32-69)	3	12 (10)	15
TOTAL	45		16	14	

Table 13 Distribution of subjects according to sex and CEAP stage

A foot vein or the long saphenous vein at the ankle was canulated using an 18G canula (Vasculon 2, Viggo-Spectramed, Helsingborg, Sweden) and blood samples were taken from each subject before and after a period of experimental venous hypertension blood . The subject first lay supine for 20 minutes, to minimize venous pressure in the leg. Following this the subject stood supported, with minimal movement for 30 minutes. This was followed by a further 10-minute period of lying supine. Blood was collected at the end of each period, using EDTA as the anticoagulant. Plasma was separated by spinning the sample at 2000 rpm for 10 minutes. It has shown previously that standing produces pressures of 70 - 80 mm Hg in the foot veins[140].

Details of ELISA techniques are mentioned in the section on methodology. Assay of $VEGF_{165}$ protein was performed using a sandwich ELISA technique. A ‘Quantikine’ kit supplied by R&D systems (4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK) was used. Plasma levels were read from standard curves obtained by plotting

mean absorbance for standards. The kit used detects 25% of VEGF protein activity in the plasma. Detectable levels in serum are 100% by contrast. Thus the absolute levels of $VEGF_{165}$ may be higher than the levels depicted in this paper. However, detection in both serum as well as plasma is equally reliable and the manufacturers confirm that this kit is suitable for use with plasma samples. There are no reports to indicate significant differences in normal individuals in the respective age groups.

	CONTROL	**C3 (IQR)**	**C4 (IQR)**	**ALL PATIENTS**
SUPINE P= difference between controls & patients	52 (35-71)	92 (70-108) **p=0.01** (M-W)	82 (47-157) p=0.072 (M-W)	**81 (56-122)** **p=0.004** (M-W)
STANDING P= difference between controls & patients	60 (39-105)	102 (60-118) p=0.066 (M-W)	99 (48-200) p=0.14 (M-W)	**98 (63-153)** **p=0.03** (M-W)
P= difference between supine & standing	(Wilc) p=0.08	(Wilc)p=0.17	(Wilc)p=0.19	(Wilc) **p=0.008**

Table 14 median plasma vegf levels (pg/ml)

Values in parentheses are Inter-quartile ranges. M-W: Mann Whitney u test, Wilc: Wilcoxon matched pairs signed rank test.

The test used measures levels of VEGF165. This is one of the four 4 transcripts encoding mature monomeric VEGF (viz. $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{121}$ and $VEGF_{165}$ are diffusible proteins that are secreted into the medium. The other are mostly bound to heparin containing proteo-glycans in the matrix. Results obtained for naturally occurring human VEGF and the recombinant VEGF showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards.

RESULTS

The median plasma VEGF165 levels (IQR in parentheses) in controls and patients before and after venous hypertension is shown in **Table 14**

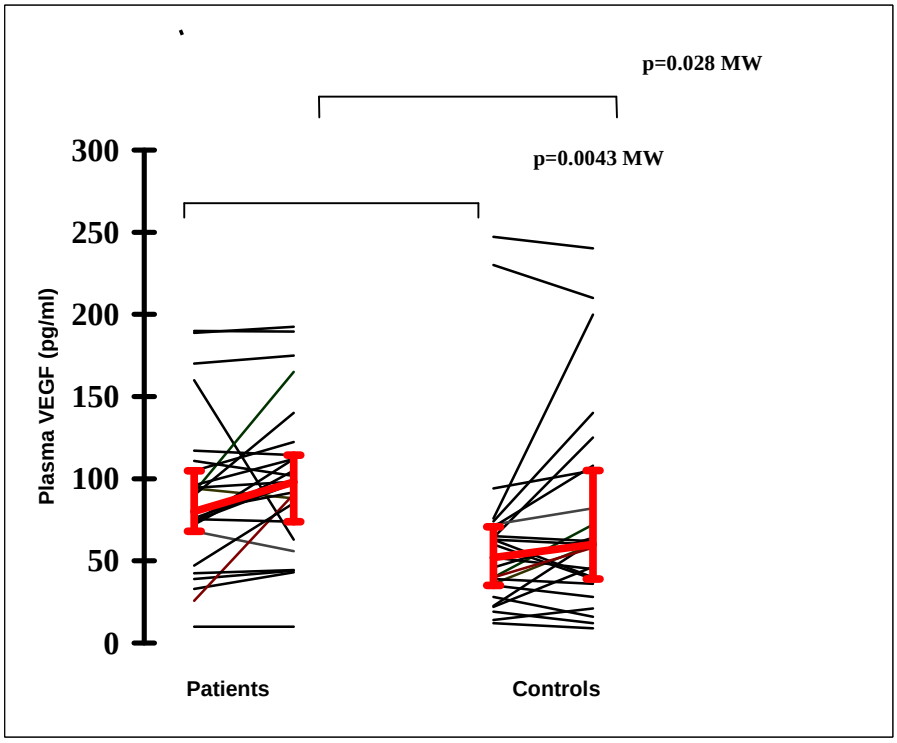


Figure 23 Plasma VEGF levels. Comparison between patients & controls. The horizontal lines at the top compare VEGF levels in the supine and standing positions between the two groups. values indicated by special symbols (♣,♦) are respective values for differences between supine & standing positions within the same group. Refer to the table for statistics.

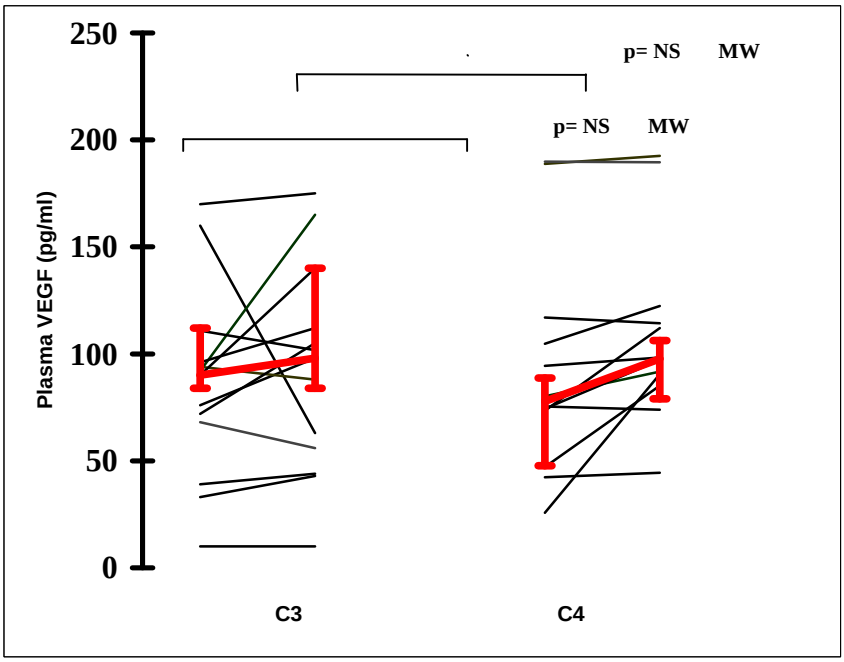


Figure 24 Plasma VEGF levels. Comparison in CVD between patients with & without skin changes. The horizontal markers at the top compare VEGF levels in the supine and standing positions between the two groups. P values indicated by special symbols (♣,♦) are respective values for differences between supine & standing positions

DISCUSSION

It is recognised that physiological indices of venous function (e.g. air plethysmography) predict the likely severity of venous disease but that there is considerable overlap between the impairment of venous function in patients from different clinical groups. This simply reflects the fact that the development of skin changes and venous ulceration is not simply a matter of ambulatory venous hypertension producing leg ulcers, but that the response of the tissues is also a major determinant of the severity of the clinical syndrome. It is specifically this point that I wished to investigate since it still reflects a poorly understood aspect of the disease that might eventually be a useful target for drug treatment.

My control subjects were somewhat younger than either of the study groups. I investigated the influence of age on the VEGF levels in my control subjects and could find no correlation. A MEDLINE search of the world literature did not reveal any studies that investigated the influence of age on plasma VEGF levels in adults. The median VEGF levels in my control subjects were similar to those measured by the manufacturers of the analysis kit in their human controls and I believe that these reflect those in a normal population of healthy subjects. Nevertheless the age differences between patient and control groups may leave the possibility that some of the differences I have observed may be accounted for by age differences.

Foot vein cannulation was in general very well tolerated. There were atwo patients who developed mild bruising and extravastion of blood after foot vein cannulation. No cases of thrombophlebitis or DVT were observed.

VEGF is strongly expressed by epidermal keratinocytes during wound healing, in psoriasis, in skin with ultra-violet burns, and in bullous diseases such as erythema multiforme and bullous pemphigoid. All of these disorders are characterized by increased micro-vascular permeability and angiogenesis. A large increase in VEGF mRNA and protein levels is seen following irradiation of quiescent keratinocytes with physiologically relevant doses of UVB. Over-expression of VEGF is

considereddependent on de- novo protein synthesis.[141] . It appears that tissue damage in general, especially hypoxic, can cause up-regulation of VEGF expression. [10] [142]

There are no large population studies of the plasma levels of VEGF measured using this assay. However data on record in the supplier's laboratory shows a median plasma levels at 62 pg/ml [143] in control subjects, which is very close to that in my control subjects (52 pg/ml lying, 60 pg/ml standing). In my study, levels of VEGF were higher in patients compared to controls in both supine and standing positions. Due to the large scatter of the data, statistical significance was not reached until the results of all patients with venous disease were considered together. Levels of VEGF were approximately 60% higher in the patient groups compared to the control group in both lying and standing positions. The two patient groups showed similar levels of VEGF (Figure 24). There was a small rise in VEGF level following 30 minutes of venous hypertension in the control group, which did not reach statistical significance. In the patient groups there was a similar modest rise in plasma VEGF that did reach significance due to the larger numbers when all patients were considered together.

In chronic venous disease there is proliferation of vessels in the skin[144]. This correlates with the severity of the clinical skin damage. Until recently, the growth factors, which mediate this, have remained unknown. It has been reported that vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) expression is increased in the skin in patients with venous disease and may be the mediator of vascular proliferation [20]

In this study my aim was to assess the association of the development of skin changes with plasma VEGF levels. I therefore used the clinical classification of the CEAP system to divide the patients into those with no clinically detectable skin changes and those with lipodermatosclerosis. This allowed us to investigate the factors, which are responsible for the development of the skin changes, which may not necessarily be the same factors that result in venous valvular incompetence of the deep or superficial veins.

The data from this study show that both groups of patients showed similar elevations in plasma VEGF compared to controls. This may reflect the fact that this growth factor is

induced in patients with venous disease in order to repair tissue damage caused by venous hypertension. However, measurements of plasma levels may not reliably reflect the actual tissue levels expressed in the region of tissue repair. It is unlikely that VEGF as reflected by these plasma levels is an important factor in explaining the differences between C3 and C4 patients.

Epidermally derived VEGF is likely to be a factor in the angiogenesis of lipodermatosclerosis. This increased expression of VEGF is seen even before any skin changes develop. I have shown increased serum levels of VEGF in my study in patients with venous disease. In my study the changes in serum levels were actually more prominent in the group with C3 disease. Studies that measure the expression of VEGF mRNA levels could more conclusively prove the actual site of origin of the protein.

VEGF seems to act both acutely over a period of a few minutes) and chronically (over a few hours) to increase micro-vascular permeability.[145] VEGF promotes the extravasation of fibrinogen and deposition in the tissues as fibrin. This could represent a mechanism for the formation of the 'Fibrin Cuffs' that are characteristically found in CVD .[146]

It has previously been shown that leucocyte activation occurs within 30 minutes of experimental venous hypertension using the same model as employed in this study. This can be shown both in control subjects as well as those with venous disease. This is associated with evidence of endothelial activation and I believe these are the mechanisms that initiate the skin damage caused in CVD. This may initiate a repair process that involves increased expression of VEGF by keratinocytes and vascular smooth muscle cells. This causes the neo-vascularization essential to any tissue repair process. The neo-vasculature is permeable to large molecules when compared to normal capillary endothelium and allows the perivasular accumulation of large molecules accounting for the 'fibrin cuff' originally reported by Browse and Burnand. Such perivascular cuffs are of course common in many inflammatory conditions.

It has been demonstrated that Hyaluron oligosaccharides (OHA) in the intercellular matrix modulate the invasive and proteolytic properties of bovine micro-vascular endothelial cells and synergize specifically with VEGF in the induction of angiogenesis

in vitro. The synergism between OHA and VEGF probably plays a role in the regulation of angiogenesis. This could be exploited therapeutically in situations that would benefit from modulation of new blood vessel growth. The signalling cascade is thought to involve inducible NO synthase, Guanylate cyclase and cGMP dependent protein kinase. Experimentally administration of VEGF was found to cause severe hypotension in animals. The hypotension is thought to be mediated by production of NO and is reversible with appropriate blocking therapy with N (G)-monomethyl-L- arginine (L-NMMA)[147148]. NO can also explain the hyper-permeability induced by VEGF. An excessive release of nitric oxide can be caused by this mechanism, and in contrast to its usual beneficial role, this free radical may contribute to local tissue damage.

To summarize all these observations a possible scenario for the sequence of events in skin injury in CVD may be the following. Damage to the tissues is caused by excessive leucocyte activation. The injury itself may cause either increase in VEGF protein synthesis or release of VEGF from depot sites. Activated platelets or macrophages present at the site of injury may release peptide factors that in turn stimulate VEGF release. The abundant macrophages in skin exposed to CVD are a potential source of VEGF as well. These could be within the basement membrane or extra-cellular matrix. Components of the extra-cellular matrix like heparin proteo-glycans are also exposed by injury. These are known to facilitate migration and tube formation by endothelial cells. -[149] Re-synthesis of extra-cellular matrix components and migration of pericytes could represent a mechanism of quenching the angiogenic stimulus.[150]

CONCLUSIONS

In my study plasma VEGF levels were elevated in both groups of patients with chronic venous disease, compared to control subjects. Experimental venous hypertension caused a further statistically significant rise in VEGF levels. Increased VEGF expression may play a role in causing tissue injury in CVD. Leucocyte mediated tissue damage could directly release VEGF from depots in the intima or intercellular matrix. Excessive neo-vascularization with hyper-permeable vessels could directly contribute to the skin changes seen with CVD. Increased NO production is a possible mechanism of local tissue damage. The source of the VEGF detected in this study is currently unclear and studies are being undertaken to determine localisation of VEGF mRNA expression to

confirm the origin of the protein. It remains to be seen whether early rise of plasma VEGF will serve as a marker for developing skin changes. Although VEGF may well be the cause of cutaneous capillary proliferation in venous disease, whether pharmacological inhibition of this process would achieve a clinically useful effect remains to be proven.

4.2 Endothelial Activation Response (& Plasma VEGF levels) in CVD to Oral Micronised Flavonoid Therapy

Endothelial cell activation by cytokines or thrombin leads to increased adhesion molecule expression. These include ICAM-1 (binds neutrophil/ lymphocytes) and VCAM-1 (binds lymphocytes/ monocytes). This can lead to MHC-II[‡‡] (T-cell response) and GMP-140[♣] (binds platelets) expression. Binding of platelets increases the availability of platelet activating factor and further accelerates the expression of adhesion molecules. The circulating levels of many of these molecules are known to be increased in patients with CVD.[151]

The physical interaction between the leucocyte and the micro-vascular endothelium is important for its function in the micro-vasculature.[152] Patients with chronic inflammatory and septic states show increased leucocyte priming. This is also observed in patients with CVD. The primed cells show increased surface CD62L and CD11B[130]. CD62L is involved in the initial 'rolling' movement of the leucocyte along the endothelium. CD11B is the molecule responsible for firmer adhesion and subsequent diapedesis. There is some evidence of inflammatory mechanisms being important in damage to the venous valves seen in CVD[153]. Following any successful treatment for CVD, a reduction in leucocyte activation would be expected

Pharmacological treatment is widely used to ameliorate the symptoms of venous disease in many European countries. It is hardly used in other places. One reason for these discrepancies is the lack of controlled scientific trials establishing the role of these compounds. At least one recent study shows increased ulcer healing rates in response to flavonoid treatment[154]. Unfortunately no 'phlebotonic' drugs exist that claim to correct venous valvular incompetence. However many of these do affect the inflammatory changes responsible for the skin damage.

In the chronic stages there is a tissue repair response alongside the inflammatory response[131] and many growth factors are involved[155]. Histological data suggested that VEGF (vascular endothelial growth factor) may be responsible for the vascular

[‡‡] MHC II-expressed on specialised cells including B-cells that interact with CD4+ T helper cells

proliferation seen in the cutaneous capillaries in patients with chronic venous disease. VEGF is one of the molecules involved physiologically in the angiogenesis of healing as well as in tumour angiogenesis. In addition, it is a potent factor for increasing vascular permeability [156]. It is a glycoprotein secreted by a variety of cells including smooth muscle cells, monocytic cells and keratinocytes[157]. Its expression is dependent on increased mRNA synthesis and increased transcription[158]. Any tissue injury, especially hypoxic injury could be a potent stimulus for its expression.

The response of the increased endothelial activation to any form of therapy remains unknown. Many pharmacologic compounds have been used for treating CVD. A purified micronised Flavonoid fraction (DAFLON®500, Servier Laboratories France) has been used in Europe for the treatment of CVD for some time. “DAFLON” consists of 90% Diosmin (3',5,7 trihydroxy -4'- methoxyflavone 7 rhamnoglucoside; C28 H32 O15) and 10% hesperidin flavonoids (3',5, 7 trihydroxy -4'- methoxyflavanone 7 rhamnoglucoside; C28 H34 O15). The use of pharmacological treatment is variable, mainly because of lack of good controlled trials. There is some evidence of increased healing rate of venous ulcers with flavonoid therapy.[159]

Daflon® comprises 450 mg of diosmin and 50 mg of hesperidin per tablet. The 'micronisation' of the effective components is claimed to increase its bio-availability. It has been reported to be a 'venotonic' (increases venous tone) and to decrease capillary 'leakage' in many scenarios. A double blind randomised trial published in 1994 had shown its efficacy in treating the clinical symptoms of CVD without any significant side effects.160

The aim of my study was to measure the effects of micronised purified flavonoid fraction (S5682, Daflon® 500 mg, Servier, France) on expression of soluble markers of endothelial activation in CVD[161].

♣ GMP-140= P-Selectin = CD 62L (Granulocyte Membrane Protein)

	SVI	SVI/ DVI	DVI	TOTAL
C2	3	-	-	3
C3	4	3	-	7
C4	7	2	1	10
TOTAL	14 (2M:12F)	5(3M:2F)	1 (M)	19 (20 inc. 1DVI)

Table 15 Distribution of the patients according to their CEAP stage and anatomical localisation of CVD. There is equal number of patients with and without skin changes (n=10). Two patients had reflux localised to 'minor' tributaries that did not relate to either of the two major superficial systems. Perforator incompetence is not listed separately because none of the patients had isolated perforator incompetence.

Material & Methods

Ethics committees' consent for the study was obtained from UCL Medical School Committee for medical ethics. This study was performed under the terms of a clinical trials exemption certificate (CTX). Twenty patients with chronic venous disease, clinical stage 2-4 were recruited from the vascular clinic at the Middlesex Hospital. Patients who consented were asked to attend the department on three separate occasions at two weeks before treatment, the day of starting treatment and after finishing treatment. A medical history was taken and clinical examination performed. This included a systemic as well as venous examination. Patients had a full blood count, serum urea and electrolyte/ liver function tests. All patients were assessed clinically according to the clinical classification of the CEAP system. People with (Duplex proven) varicose veins only (C2), with associated oedema (C3), skin changes (C4) and/ or healed ulceration (C5) of > 4weeks were included. Active ulceration (C6) would have interfered with activation of leucocytes and were thus not included.

All patients had a venous Duplex examination and photoplethysmography within the previous eight weeks to establish venous and to localise it anatomically. The minimum extent of Duplex examination in my patients included sapheno-femoral junction, long saphenous vein, sapheno-popliteal junction, short saphenous vein, anatomical

perforators and other reflux sites as picked up by colour Doppler. Patients were assessed for other possible diagnoses including arterial disease. Ankle-Brachial pressure ratios were performed whenever there was clinical suspicion. All patients wore class II support stockings and continued to do so during the study.

Patient Selection

The inclusion criteria for the study were (1) Age above 18 years (2) patients affected with CEAP stage C2 to C4 (3) C5 with a healed ulcer for at least 4 weeks (4) psychological stability & motivation.

The exclusion criteria were directed at (i) patients who have conditions that would change the status of leucocyte/ endothelial activation, (ii) subjects who were taking compounds similar to flavonoids to avoid bias and (iii) patients who had other major systemic illnesses.

These criteria were (1)History of alcohol or drug abuse (2) known history of allergy or intolerance to diosmin or any other venotonic agent (3)active venous ulceration (4)diabetes mellitus (5)impaired hepatic function (ALT or AST 3 fold above the normal limit) or Impaired renal function (serum creatinine > 120 µmol/l) (6) any concomitant active disease or abnormality in laboratory test (judged as clinically significant by the investigator) (7) patients treated with other vasoactive drugs within the 15 days prior to inclusion (8) patients with an acute/ chronic inflammatory or infectious disease (9) deep venous thrombosis within the past 12 months (10) superficial venous thrombosis within 3 weeks (11) patients using steroids, NSAIDs, other 'vaso-active' drugs, Vitamins A, C &E or anti-coagulants (12) previous poor compliance to treatment (13) participants of a trial within past 3 months were excluded. (14) pregnancy, breast-feeding or lack of active contraception also excluded the patient.

DAFLON® 500 mg BD (Servier France) was administered orally for 60 days. Evaluation of venous symptoms employing a visual analogue scale was performed before and after treatment. Specimens (30mls each) were taken from foot veins before and after standing the patient supported for 30 minutes.

Collection of Specimens

A 18G canula (Vasculon 2, Viggo-Spectramed, Helsingborg, Sweden) was placed in the distal long saphenous vein or dorsal foot vein of one leg. The canula was flushed with heparinized saline solution. The patient then stood supported against the side of the couch for 30 minutes without moving the calf muscles (it was previously shown by the authors by direct pressure measurement that this raises the venous pressure in the superficial veins of the leg to between 70 and 80 mm Hg. after which 2 ml of blood was taken and discarded from the canula, and a further 10 ml was collected into two tubes containing ethylenediamine<TK;4 tetraacetic acid and one tube containing citrate<TK;1 (Vacutainer, Becton Dickinson Vacutainer Systems Europe, BP No 37-38241 Meylan Cedex, France).Blood samples were carefully placed directly into the sample tubes after removing the stoppers to prevent excessive cell agitation.

ELISA tests for soluble markers

Details about the ELIS techniques are mentioned in the chapter on methodology. Blood for Elisa tests for soluble plasma markers was collected in EDTA bottles. The specimens were spun at 20,000 rpm for 10 minutes to separate the plasma and promptly frozen at -20°c before analysis. The Markers measured were E-selectin, P-selectin, VCAM, ICAM-1, VW Factor and Lactoferrin. Commercial kits (ICAM-1/ VCAM/ E-selectin/ P-selectin by R&D Systems 4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK; VW factor kit by Diagnostica Stago 9 rue des Freres Chausson 92600 ASNIERES-SUR-SIENE France; Lactoferrin assay by OXIS International Portland Oregon USA) were used for these analyses.

Assay of $VEGF_{165}$ protein was performed using a sandwich ELISA technique. A ‘Quantikine’ kit supplied by R&D systems (4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK) was used. Plasma levels were read from standard curves obtained by plotting mean absorbance for standards supplied with the kit. The test used measures levels of VEGF165. This is one of the four 4 transcripts encoding mature monomeric VEGF (viz. $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{121}$ and $VEGF_{165}$ are diffusible proteins that are secreted into the medium. The other two are mostly bound to heparin containing proteo-glycans in the matrix. Results obtained for naturally occurring human VEGF and the recombinant $VEGF_{121}$ showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards.

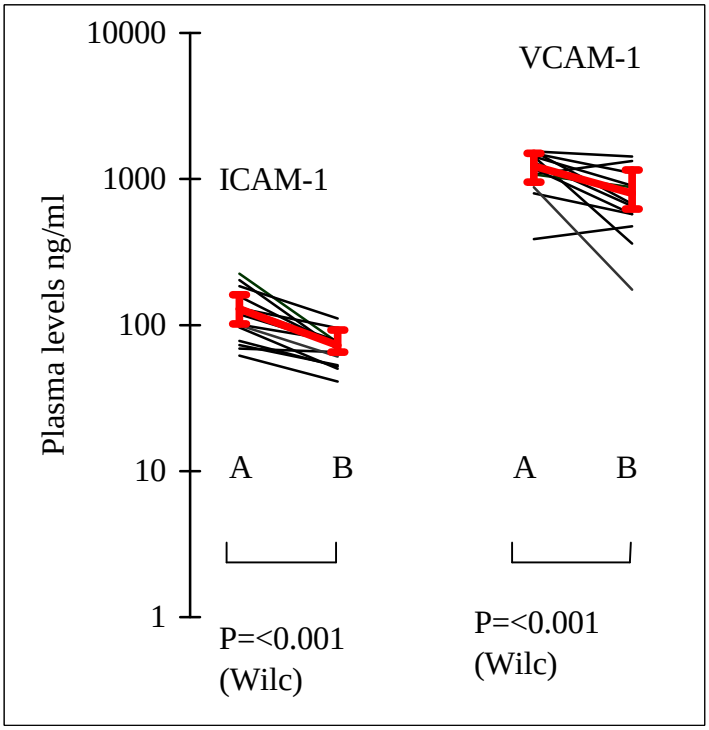


Figure 25. Changes in plasma levels of ICAM-1 and VCAM-1 in patients with CVD before (A) and after (B) sixty days treatment with micronised purified flavonoid fraction. A logarithmic scale has been used because of the differences in the absolute levels of the two molecules. P levels are Wilcoxon ranked sum test. The thick lines join the respective median levels. Vertical lines represent inter-quartile range.

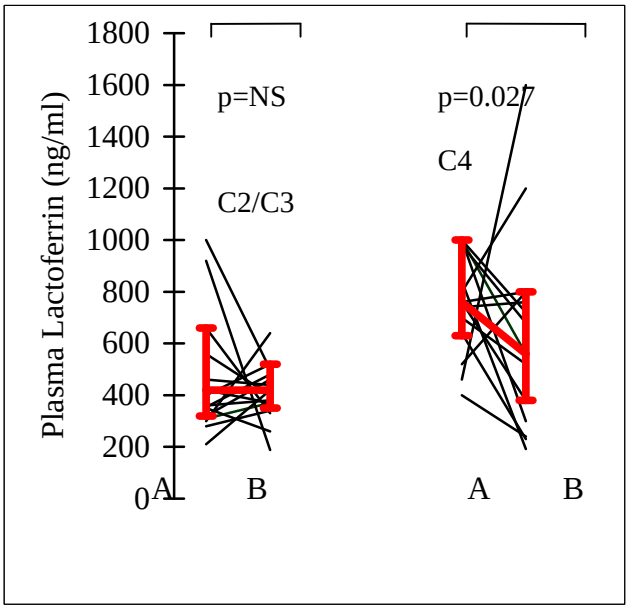


Figure 26 Shows the differing changes in plasma Lactoferrin in patients with (C4) and without (C2-3) skin changes before (A) and after (B) sixty days treatment with micronised purified flavonoid fraction. Probability levels are Wilcoxon ranked sum test. The much increased plasma Lactoferrin activity is lowered significantly in patients with skin changes

RESULTS

There were equal numbers of patients with and without skin changes (n=10). Seven patients in each group had superficial venous insufficiency (SVI) only. Of the remaining six patients the majority (5) had combined SVI and deep venous insufficiency (DVI). Most patients either had long saphenous (GSV) involvement (n=9) or combined GSV and short saphenous (SSV) involvement (n=7). Two patients had CVD involving neither of the two major superficial anatomic systems. These patients had other superficial veins (e.g. lateral superficial vein of the thigh) involvement without reflux in the mentioned major superficial systems. Median age was 58 years (range39-82). These are shown in Figure 25 Figure 26 and Table 18. The statistical tests are used as shown. Non-parametric tests were used because of the non-normal distribution of the data.

Significant reduction (p=<0.05) was seen in plasma levels of VCAM & ICAM-1 activity following therapy. Reduction in the level of ICAM-1 32% (141 ng/ml: 73 ng/ml) & VCAM, 29% (1292 ng/ml: 717 ng/mls were seen). A statistically significant reduction in plasma Lactoferrin (in C4 patients) was observed. The levels of reduction in endothelial markers ng/ml: 560 ng/ml) occurred in the skin-changes group. The levels of VW factor also decreased (p=<0.05)in patients with skin changes but not in simple venous disease. Changes (**p=>0.05**) in E-selectin levels (decrease) & P-selectin levels. Lactoferrin levels were appreciably reduced following therapy in patients with skin changes only. This probably represents damping of leucocytic activity.

Results for VEGF are shown in figures 4.5.1, 4.5.2 and tables 4.5.2 & 4.5.3. The lines in the figures represent the values of the concentration of the VEGF molecule. The bold lines represent the median values. The vertical lines represent inter-quartile ranges. The X-axes show the values before and after treatment. The Y-axes represent the value of the concentration of the respective molecule. The statistical tests are used as shown. Non-parametric tests were used because of the non-normal distribution of the data. The median compliance rate with DAFLON treatment was 98%, (count of returned tablets). Seven patients experienced minor side effects (loose motions 3, headache 2, and faintness 1). All were transient and preferred to complete the study.

Patients with clinically obvious skin changes (C4) had plasma levels of VEGF that were considerably greater than those in patients without skin changes (C2 & C3) (tables 1 and 2). There were two patients with consistently high levels of plasma VEGF in the C2/C3 group. I are following these up to look for development of clinically obvious skin changes. I found a decrease in the levels of plasma levels of VEGF in patients with CVD after treatment with purified. micronised flavonoid fraction treatment. The difference was only found in patients with clinical skin changes (C4) of CVD. (see Table 16).

	Before Treatment	**After Treatment**
C2/C3	9 (5-25)	10(5-26)
C4	**$^{\Phi}$98 (64-113)**	**$^{\Phi}$ 57(37-95)**
All patients	**47(17-101)**	**29(8-56)**

Φ p=<0.02 (Wilcoxon)

Table 16 Median plasma VEGF levels (pg/ml) in patients with (C4) and without skin changes (C2/C3). The respective values before and after sixty days treatment with flavonoids are shown. This table shows the combined values in each group before and after a 30-minute period of standing up. Values in parentheses are inter-quartile ranges. The statistical tests are used as shown. Non-parametric tests were used because of the non-normal distribution

		SE-Selectin **(ng/ml)**	**Sp-Selectin (ng/ml)**	**Lactoferrin (ng/ml)**
ALL PATIENTS	Before Treatment	49 (31-61)	74 (57- 97)	690 (373-760)
	After Treatment	36 (28-59)	90 (55-120)	494 (350-715)
	Significance (Wilcoxon)	0.156	0.09	0.231
C2- C3	Before Treatment	41 (33-61)	78 (58-97)	420 (32-660)
	After Treatment	36 (30-47)	80 (53-101)	420 (350-520)
	Significance (Wilcoxon)	0.1	0.74	0.59
C4	Before Treatment	50 (27-63)	77 (58-96)	**760** (635-1000)
	After Treatment	31 (25-60)	100 (72-125)	**560** (380-800)
	Significance (Wilcoxon)	0.61	0.54	**0.027**

Table 17 Median values of plasma levels of endothelial activation markers and plasma Lactoferrin in patients with CVD. Data obtained for patients with simple CVD (C2-3) and those with dermatological changes (C4) is shown. The values are before and after a sixty day treatment with oral purified micronised fraction (parenthesis=IQR).

		ICAM-1 (ng/ml)	VCAM (ng/ml)	VW Factor % activity
ALL PATIENTS	Before Treatment	141 (102-162)	1292 (950-1500)	65 (45-105)
	After Treatment	73 (65-93)	717 (625-1156)	65 (50-105)
	Significance (Wilcoxon)	**<0.001**	**<0.001**	0.88
C2- C3	Before Treatment	109 (91-136)	1300 (1140-1500)	50 ((40-77)
	After Treatment	68 ((62-78)	800 (625-11250	78 (51-109)
	Significance (Wilcoxon)	**0.001**	**0.001**	**0.012**
C4	Before Treatment	153 (120-186)	1075 (913-1513)	65 (51-105)
	After Treatment	78 (72-106)	880 (675-1231)	58 (50-75)
	Significance (Wilcoxon)	**0.001**	**0.003**	**0.039**

Table 18 Median values of plasma levels of endothelial activation markers and plasma Lactoferrin in patients with CVD. Data obtained for patients with simple CVD (C2-3) and those with dermatological changes (C4) is shown. The values are before and after a sixty day treatment with oral purified micronised fraction (parenthesis=IQR).

	Pain	Heaviness	Cramps	Paresthesiae	Oedema
Before Treatment	8.2 (3.3)	7.7 (2.6)	8.1 (2.8)	5 (3.3)	6.8 (3.2)
After Treatment	**8.2 (3.3)**	6.4 (2)	6.8 (3.4)	4.8 (2.8)	5.8 (2.4)
P Value (Wilcoxon)	0.094	**0.004**	0.07	0.19	0.057

Table 19 Comparison of mean visual analogue symptom scores in all patients before and after 60-day treatment with 'purified micronised Flavonoid Fraction'. Although decrease was seen in all the parameters, only the decrease in 'Heaviness' reached statistical significance. (Values in parentheses are standard deviation).

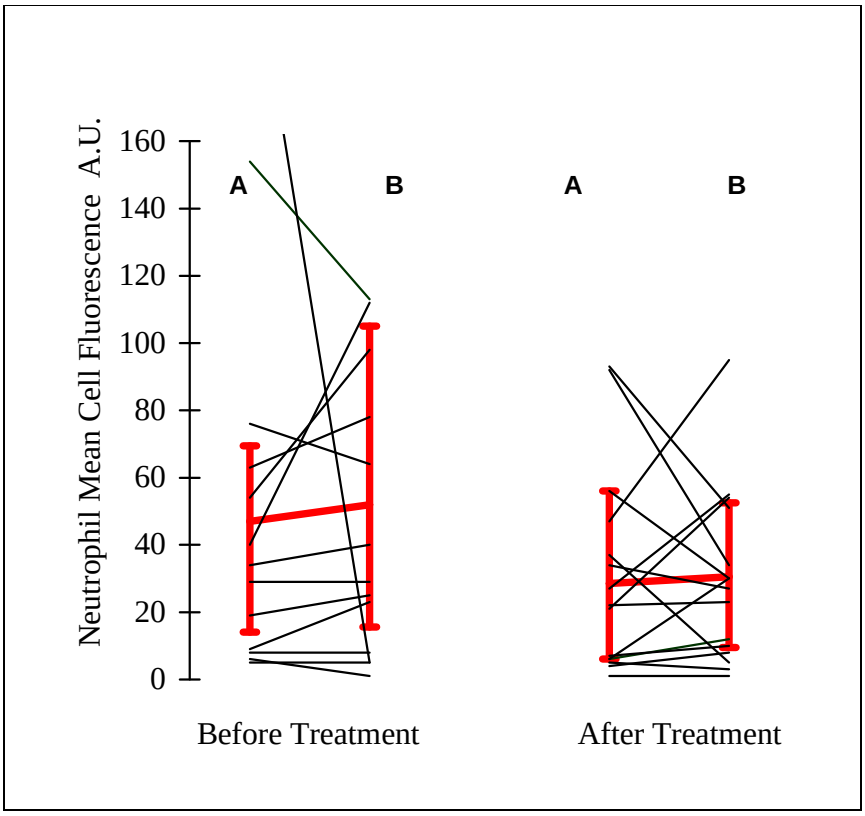


Figure 27 Figure 4.3.1 Shows comparisons of plasma VEGF levels in patients before and after 60 days of flavonoid treatment. Values shown are before (A) and after (B) a 30-minute period of standing. The bold lines join the two respective median values, differences between these values were not significant. Vertical bars represent inter-quartile ranges.

the much increased granulocyte activity in these patients compared to those with simple CVD. The effect on E-selectin levels was also seen more in the C4 group. Unknown effects on platelet activity might explain the slight rise seen in P-selectin following therapy. These effects may explain the differing phenomena seen with VW factor following therapy (minor rise in VW factor in C3 patients and fall in C4 group).

The reduction in endothelial/ leuocyte activation may block the initial adhesion of these cells to the endothelium and decrease the amount of tissue damage. The effect seems to be equivalent in patients with and without skin changes. (Several patients with skin changes reported partial resolution of their LDS. with 60 days of treatment). Improvement in clinical scores of all the parameters was seen in the patients treated with purified Flavonoid fraction. This study demonstrates the possibility of using leucocyte activation as a marker for therapy in CVD.

I did not make any distinction between patients with superficial venous insufficiency and those with deep venous insufficiency in interpreting the results. Previous studies from this department did not report on any difference between these two groups[130] for a similar clinical stage. I compared equivalent clinical stages (C) that are representative of the severity of CVD. Some of these markers especially VW-factor are not very sensitive indicators of endothelial activation. Some like P-selectin and VW factor are almost certainly affected by other local phenomena like platelet activation. The physiological significance of these soluble markers remains an issue of debate. They may represent protease cleavage from cell surface subsequent to endothelial stimulation. They may also represent molecules released to block receptors and to prevent their attachment to the endothelium. Despite these they are widely used as markers of endothelial activation.

It is only relatively recently that the effectiveness of therapy for CVD is being tested with objective parameters. It has been shown that colour duplex ultra-sonographic and air-plethysmographic criteria improve in patients one month following surgical therapy. This improvement was shown to be maintained at two years[162]. It would be interesting to measure the mid to long-term effect of therapy on endothelial markers. Another study of the effect of compression therapy/sclerotherapy on the microangiopathy (Leu &

Bollinger et al)[163] showed non-significant changes post therapy. The parameters studied were capillary microscopy, Doppler flux-metry and $tcPo_2$ measurement. Elevated levels of VCAM-1, VW Factor, E-selectin, ICAM-1 and Lactoferrin have been reported in patients with CVD compared to controls[151]. However my study is the first to demonstrate a measurable change in these parameters in CVD following therapy. This shows the feasibility of using the soluble markers of endothelial activation as a parameter to measure the response to various forms of treatment.

Experimental blockade of adhesion molecules either with soluble ligands or with specific monoclonal antibodies has a significant effect on inflammation and immune mediated tissue damage[164,165]. The observation that some markers are specifically lowered following therapy in patients with skin changes demonstrates the possibility of flavonoids to ameliorate the symptoms of CVD.

DISCUSSION

I observed a decrease in some soluble endothelial activation markers in patients with CVD following sixty days therapy with oral purified flavonoid fraction. In particular VCAM-1 and ICAM-1, adhesion molecules important in endothelial interaction with neutrophil, monocytes and and/ or arrest some of the skin damage that occurs in CVD. Inflammatory mechanisms have been implicated in the valvular damage seen in CVD[153] and these compounds may have a role in ameliorating this damage as well. These aspects merit further study.

Further studies are needed to assess further the role of soluble endothelial markers in prognosticating the clinical course of CVD. The role of purified micronised flavonoid fraction and similar compounds also needs to be re-examined in the context of non-surgical treatment for CVD.

Experimentally flavonoids have been shown to have several anti-inflammatory actions. My study is the first to demonstrate a mode of action for these compounds in the clinical setting. In the hamster cheek pouch Daflon® significantly inhibited the macromolecular permeability increasing effect of histamine, bradykinin and LTB4. Flavonoid-treated animals also tended to have a lower number of leucocytes adhering to

the venular endothelium.[166] In addition Flavonoids could decrease the production of free radicals [167], alter cytokine release [168]. They can alter the composition of the venous wall [169] and have some haemorrheological effects.[170] This study demonstrates the effect of micronised purified flavonoid fraction on decreasing leucocyte de-granulation and endothelial activation. The effects of these compounds on Duplex ultra-sonographic parameters and on resolution of lipodermatosclerosis need to be quantified in prospective studies. The lack of side effects of these compounds makes them especially attractive as medications for CVD.

These anti-inflammatory effects of purified micronised flavonoid may be important in their clinical effects in CVD. It is possible that different doses & treatment duration may be needed for various indications. The role of flavonoids in resolving skin changes needs to be quantified. I observed that it is feasible to use changes in the level of leucocyte surface adhesion molecules as markers of response to treatment in CVD.

Other modalities of treatment may also be assessed using these parameters. The finding of large differences between patients with clinically obvious skin changes and those without is of considerable relevance. I have previously reported many inflammatory processes that I have observed in the skin and blood of patients with venous disease, but none has shown major differences between patients with skin changes and those without. These findings mirror closely the observations of capillary proliferation originally made by Burnand [171]. He found that capillary proliferation correlated closely to the development of clinical skin changes referred to as lipodermatosclerosis (LDS). The fact that plasma VEGF levels are greatly elevated in patients with clinical skin changes of CVD implies that VEGF is involved in the development of the skin changes.

The proliferation of the skin capillaries is associated with the development of perivascular fibrin cuffs. The significance of these remains unclear, however, VEGF promotes extravasation of large molecules by its action on the capillary endothelium. This may be one of the factors leading to deposition of fibrin and other large molecules and provides an explanation for the mechanism of formation of fibrin cuffs.

Abnormally increased and prolonged exposure to VEGF could have local deleterious effects. VEGF is strongly expressed by epidermal keratinocytes during wound healing,

in psoriasis, in skin with ultra-violet burns, lesions of rheumatoid arthritis and in bullous diseases such as erythema multiforme and bullous pemphigoid. All of these disorders are characterised by increased micro-vascular permeability and angiogenesis. I have shown previously up-regulation of VEGF expression in the skin and plasma of patients with CVD[172]. The expression of this molecule in the skin is more marked in patients with CEAP clinical stage C4 disease than in less severe forms of venous disease without skin damage[134]. The clinical syndrome of lipodermatosclerosis comprises a wide spectrum ranging from hyperpigmentation to inflammatory changes and fibrosis. It is possible that growth factors may play different roles at various stages but this has not been studied in detail. The role of VEGF may be different after the onset of ulceration.

The large difference between VEGF levels in C2/C3 and C4 patients could represent different stages of development of the dermatological changes. In the initial stages leucocyte mediated damage could be the predominant event. Later on the repair processes may become more important and may co-exist with continuing skin damage. There were two patients with higher levels of VEGF in the C2/C3 group. One explanation for this could be that these had incipient skin changes that were more advanced than was clinically apparent or these patients may be particularly susceptible to the development of lipodermatosclerosis. I had previously shown increased plasma levels of VEGF in patients with CVD[15]. If further studies indicate that high plasma levels of VEGF indicate impending skin changes, it could provide us with a useful tool for establishing the prognosis. Similarly if it could be demonstrated that absence of a fall in VEGF levels following therapy predicts a higher chance of recurrence, this could be used as a marker of success of the treatment.

I acknowledge that the current data cannot demonstrate whether this finding is the cause or consequence of the skin changes. However, plasma VEGF levels might then be used as a surrogate for assessing lipodermatosclerosis by clinical means. Measurement of LDS in a quantitative way is notoriously difficult, and results in wide variance of the resulting data [173]. VEGF blocking has shown some promise experimentally in oncological therapy [174] [175]. Linomide and VEGF-blocking anti-bodies have been used for this purpose. Tamoxifen has been shown to have some anti-angiogenic activity. The role of this type of treatment in patients with CVD remains to be explored and will

depend upon the availability of anti-angiogenic drugs of substantially lower toxicity than those generally used in tumour my chemotherapy.

In this investigation I assessed the effect of a purified flavonoid fraction preparation (500) mg on the plasma VEGF levels. This drug is widely used in continental Europe for the treatment of the symptoms of chronic venous disease, including uncomplicated varicose veins where is ameliorates the symptoms of discomfort associated with varices [176]. Recent animal studies have suggested that it has an effect on leucocyte: endothelial adhesion, protecting the endothelium from damage in models of reperfusion injury. [177] I found that plasma VEGF decreases considerably in patients with skin changes following sixty days therapy with oral purified micronised fraction. The authors acknowledge that the significance of lower plasma VEGF following therapy remains unclear. No significant effect was seen in this study in patients without skin changes. However, the plasma levels of VEGF detected in these patients were at the lower level of the sensitivity of the assay, and biological variation and well as insensitivity of the test may have resulted in failure to find a difference.

Concluding Remarks

In conclusion Plasma VEGF levels are much higher in these patients as compared to those with uncomplicated CVD. These levels are higher than those in controls. Plasma VEGF levels decrease in patients with CVD induced skin shanges following treatment with purified micronised flavonoid fraction. The increase in some endothelial activation markers studied was dampened by the administration of purified micronised flavonoid fraction for sixty days in patients with CVD. This suggests that these compounds may down-regulate the activated endothelium in these patients. My study demonstrates the feasibility of using changes in levels of soluble endothelial markers as parameters for assessing the response to therapy in CVD.

4.3 Results of Operative Treatment for Leg Ulcers Due to Superficial Venous Disease

Many different treatment modalities could achieve healing in leg ulcers due to CVD. Recurrence of these ulcers is, however, a major problem. Theoretically leg ulcers due to superficial venous disease only may be cured permanently by appropriate surgery. Definitive diagnosis and therapy in venous disease is becoming increasingly possible.[178] There are few reports, however, of their long-term results.[179] Reports that do have a longer follow-up are lacking in the accuracy of the data, mainly because venous duplex ultrasound has not been widely used until relatively recently.

Sapheno-femoral junction (SFJ) ligation and stripping of the long saphenous vein (GSV) was the commonest procedure (>70%). Recurrent veins operations were 15%. Perforator incompetence was reported in only 4%. The mean follow-up period was 52 months (range 12-108 months). >40 % had to use stockings permanently for troublesome symptoms.

INTRODUCTION

The prevalence of leg ulcers in one study of 92,100 subjects aged > 40 years was 0.38%. [180] In another study of 463 legs with active ulceration venous insufficiency was detected in 332 patients (72%).[181] In these patients venous insufficiency was the dominant causative factor in 54% of cases. Until quite recently all legs with venous pigmentation/ulceration were dubbed 'post-phlebitic'. Many authors categorically denied the role of superficial venous disease in venous ulceration. [183] In fact a substantial proportion of leg ulcers are associated with superficial disease alone. [182]

Definitive diagnosis and therapy is becoming possible in many more cases than was previously the case.[183] Duplex scanning has revolutionised diagnosis of venous disorders affecting the lower limb. Measurements of the site and duration of venous reflux can be accurately and reliably charted.[184] Theoretically once the haemodynamic derangement is recognised it should be possible to cure the limb. In practice this is possible only in a limited number of cases[185].The reports of success with deep venous

reconstruction show mixed results. Disease confined to the superficial venous system on the other hand may be ideal for surgical correction.

It is widely accepted that patients with superficial incompetence and normal deep veins can achieve good outcomes.[186] There are few reports of the long term outcome of superficial venous surgery for leg ulceration.[187] Most of the reports include relatively short follow-up periods. Reports that do have a longer follow-up are lacking in the accuracy of the data, mainly because ultra-sound imaging was not available until relatively recently. Since 1987 all patients with suspected lower limb venous disease have been investigated with Duplex ultra-sound at the Middlesex Hospital Vascular Laboratory. I
carried out a retrospective study to assess the outcome of management based on duplex ultra-sound findings.

MATERIAL & METHODS

The vascular laboratory records were examined to locate patients who were examined for lower limb venous duplex investigation. Patients with venous ulceration attributable to superficial venous disease were selected for inclusion in the study. These included patients whose disease was predominantly superficial but who also had an element of deep disease which was not considered to be the dominant element.

I examined the Middlesex vascular laboratory records to locate patients with venous ulceration attributable to superficial venous insufficiency (SVI). Outcome of surgery was assessed for patients who had surgery performed at least one year previously. Out of 88 such patients, I was able to contact 52. Telephone interviews or questionnaires were used to assess the status of the ulcer, the use of stockings and the subjective symptoms from the affected leg.

Outcome of surgery for ulceration due to superficial venous disease was assessed for patients who had surgery performed at least **12** months previously. All of these patients had superficial venous disease confirmed by means of Duplex ultrasonography and Photoplethysmography (PPG). Patients with any deep venous problems were excluded from the study. Patients with perforator disease (above/ below knee) were included.

Non-parametric tests were used because of the non-normal distribution of the data. Probability statistics are those for differences between surgical and conservative groups. Out of 88 patients scanned before & after surgery for SVI with leg ulcers since 1989 at the vascular Laboratory of the Middlesex Hospital, London, I was able to contact 52. Telephone interviews or questionnaires were used to assess the status of the ulcer, the use of stockings and the subjective symptoms from the affected leg.

SFJ ligation and stripping of the GSV was the most frequently performed operation ((>70%). Recurrent veins were the indication for 15% of the operations. Perforator incompetence was reported in only 4%. Female patients accounted for 61% of the operations. The mean follow-up period was 52 months (range 12-108 months). Initial healing rates were >85% with surgical treatment. Only 40% initial healing was achieved with conservative treatment.

RESULTS

These results are shown diagrammatically in Figure 28 & in Table 21. Clinical recurrence rates are relatively high at >30% in the operative group. Nearly one third of the patients still had ulcers at varying intervals of 1-8 years following treatment. Both patients who healed on conservative treatment remained ulcer free. Around 50% of all the patients still had symptoms from their veins. These ranged from cosmetic recurrences to re-ulceration.

Most of the patients who had surgery (>80%) used graduated elastic stocking in the pre-operative and short-term post-operative period. >40 % had to use them permanently to avoid troublesome symptoms. Some patients could not use graduated elastic stockings because of the size of the limb or other factors.

	Anatomical Site						
	SFJ/GSV	**GSV Only**	**SSV**	**GSV/SSV**	**Perforators ♠**	**Recurrent GSV**	**Recurrent SSV**
Male (19) Mean Age (57-85)	13	1	4	1	1	2	-
Female (33) Mean Age (44-76)	25	4	2	2	1	3	-
Total (52)	38	5	6	3	2	5	-

Table 20 Distribution of the patients according to sex and anatomical site involvement

♠ Perforator reflux was not an isolated involvement in these patients.

	Healed ulcers	**Recurrence**	Stockings	**Persisting Symptoms**
Surgery (41)	37† (90%)	9‡ (22%)	35§ (85%)	19¥ (46%)
Conservative (11)	6† (55%)	2‡ (18%)	7§ (64%)	6¥ (55%)
All (52)	43 (83%)	11 (21%)	42(81%)	25(48%)
Significance	**† 0.01** (Fisher's)	‡ 0.32 (Fisher's)	§ 0.09 (Fisher's)	¥>0.05(chi-square)

Table 21 shows the rate of initial healing and persistence of ulcers at the time of surgery in patients with ulceration due to superficial venous disease only. There was a higher recurrence rate in the group without surgery. Mean follow-up period since initial duplex examination 52 months.

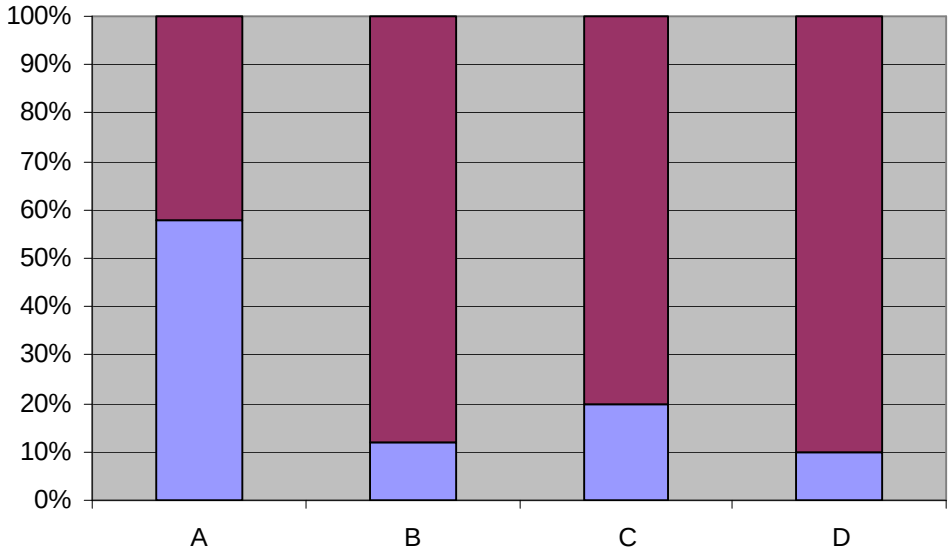


Figure 28 shows the outcome at the time of the study of the 41 surgically treated patients included in the study. This shows that circa 32% of patients had ulceration either from non-healing or from healing/recurrence/ non-healing. About 60% had primary healing without recurrence following surgery preceded by Duplex ultrasonography.

A=Maintained initial Healing
B=No Healing
C=Recurrence/ No Secondary Heealing
D= Recurrence/Secondary Healing

DISCUSSION

The primary haemodynamic derangement in SVI is reflux. The majority of DVI have reflux as the major mechanism as well. On the other hand the mechanism in some in secondary DVI is obstruction. Quoted results for deep venous reconstruction are better with primary DVI[188] than with secondary DVI.

The role of the superficial venous system has been underestimated in the past. In a study of 300 limbs in 153 patients were examined by Duplex ultrasonography with colour-flow imaging for the presence of venous reflux Ninety-eight limbs had skin changes, which included hyper-pigmentation, lipodermatosclerosis, atrophie blanche and ulceration. Of this group, 2 per cent had no evidence of venous reflux on duplex scanning, 39 per cent had deep vein incompetence, 57 per cent had superficial vein incompetence and 2 per cent had isolated medial perforating vein reflux. Of 25 limbs with ulceration, 13 had superficial and 12 deep vein reflux. A total of 202 legs, which included 20 normal control limbs, had no skin changes; 22.3 per cent of these had no venous reflux, 8.4 per cent had deep vein incompetence, 65.3 per cent had superficial incompetence and 4.0 per cent had isolated medial calf perforating vein incompetence[189]. Isolated deep venous reflux was present in only 12 limbs (15%). A combination of deep and superficial venous reflux was found in 25 limbs (32%), and in 42 limbs (53%) there was only superficial venous reflux.

There is some suggestion that superficial venous surgery may improve both the venous haemodynamics as well as the microangiopathy associated with CVI. A recent study confirmed improvement in physiological parameters following varicose vein surgery[107]. In 11 patients followed up prospectively the Venous filling index, ejection fraction, residual volume fraction and the venous filling index were shown to improve significantly at mean follow-up of 4 and 6 months post-operatively. In another study the laser doppler flux and $tcP0_2$ were shown to improve following sclerotherapy and compression treatment in 15 patients with moderate to severe disease. [190]

There were higher healing rates following surgery preceded by Duplex Ultrasonography compared conservative treatment. However the recurrence rates and the number of ulcers at the time of the study were not different. Previous experience with surgery has

been similar.[191] In another published paper [162] the results of surgical treatment of eleven limbs showed a complete and maintained response at 16.4 months mean follow up.

High initial rates of ulcer healing are obtainable with surgery for duplex ultrasound confirmed superficial venous disease. However, recurrence rates may be substantial in the long term. This suggests that either the altered venous haemodynamics are not corrected at the initial operation and/ or these patients develop an irreversible microangiopathy of the leg skin.

Results of Conservative Treatment[192]

Most venous ulcers can be expected to heal when patients are enrolled in a nurse-managed/physician-supervised ambulatory ulcer clinic. Strict compliance with the treatment protocol may significantly decreases the time to healing and prolonged the time to recurrence[193]. In another prospective study 105 consecutive patients with leg ulcers were recruited (aetiology: 77% venous, 4% arterial, 9.5% mixed, 9.5% other). 70 (67%) had a history of previous ulceration. 83 patients could be followed up for 1 year. The healing rate for the whole group was 41 (49%) after 3 months and 61 (73%) after 1 year. The corresponding figures for the 67 venous patients are 44 (66%) and 52 (78%) respectively. From 61 healed ulcers 18 (30%) reoccurred during the 1st year. At the primary examination several factors were investigated which might have influenced the healing rate. Age, ulcer-size, the duration of the ulcer, lateral localisation, absence of foot-pulses and lymphoedematous skin changes on the forefoot could be shown to have negative influence on healing.

Results of Surgical Treatment[191]

In one study of surgical treatment of leg ulcers the results of treatment of 159 consecutive limbs presenting with a clinical diagnosis of venous ulcer in 140 patients (70 male, aged 28-90 years, median 66 years) were reviewed. Of the patients, 61% were referred because of severe pain and 53% of the ulcers had been present > 2 years. Patients were evaluated clinically and by duplex ultrasound, with selective use of venography, photoplethysmography, arteriography and latterly duplex scanning. Seventy-one limbs had surgery to the superficial veins, 18 limbs had arterial reconstruction, and 10 limbs had skin grafting alone. There was one operative death after arterial reconstruction but none after venous surgery. Patients were followed up for

1-5 years (median 3 years). Of those who had been treated surgically, healing was achieved in 88%, and ulcers healed in 52% of those treated non-operatively. In all, 18% of the ulcers recurred in each group. These results show a favourable association between appropriate venous and arterial surgery and the healing of venous ulcers, with relief of pain. They support a policy of thorough evaluation and appropriate surgical treatment in these patients.

My study reports mid to long term results in a population of patients with venous ulcers who have Duplex confirmed superficial venous disease only. It would be informative to perform repeat Duplex examinations on these patients and compare those that are completely cured to those with recurrent ulceration and recurrent symptoms.

In one published paper [162] the results of surgical treatment of eleven limbs that had been subjected to Duplex scanning, venography and Air plethysmography (APG) were studied. All of these patients had GSV disease as the superficial component. All the patients had deep venous disease in addition. The mean follow up was 16.4 months. All the ulcers healed in this study and remained healed for the duration of the follow-up. The mean clinical score decreased from 10.1 to 1.45. However all the subjects in this study had evidence of deep venous insufficiency in addition. No mention was made of any conservative measures employed or if the patients continued to use support stockings post-operatively [194] In addition the vast majority of patients had reflux only in the proximal deep vein segments. These are now known to be unimportant unless >1.0 minute in duration[§§] and are probably of significance only if they are associated with distal reflux. Some deep venous reflux is associated as a secondary phenomenon with advanced superficial disease.[195] Thus evidence of any deep venous reflux should be critically evaluated and not taken as an absolute contra-indication to surgery per se[196].

Recurrence rates were high in my series. Larger series of conservative management have shown similar results to the operative management. In one series 188 patients with recently healed leg ulceration were followed for at least 18 months. Overall cumulative recurrence rate was 26% after 1 year and 31% at 18 months. [197] Some series had suggested that >80 % of patients do not require any long-term support stockings after

surgery. [186]It seems likely that patients who have non-healing or recurrent ulcers have either had an inadequate operation and/or have a microangiopathy locally that persists despite correction of the venous reflux. It would not be amiss to discuss here the problem of recurrent veins, which is intimately related to failure of ulcers to heal. Recurrent varicose veins are a common problem and can be difficult to deal with. In a recent study involving a survey of > 150 patients, more than 10 % had to visit their GP post-operatively with recurrent veins within six months of their operation.[198] Patients' satisfaction rates with varicose vein surgery are generally low and vary amongst different series.[199] This may be because different surgeons vary in the importance given to the meticulousness of operative technique of VV surgery.

Concluding Remarks

High initial rates of ulcer healing are obtainable with surgery for superficial venous disease. Although about a third recurred in my study and a similar number still had an ulcer more than a year after their operation. The ESCHAR study also showed significant recurrence rates although the healing rates had an advantage in the surgical group[196]. This suggests that either the altered venous haemodynamics may not have been corrected at the initial operation and/ or these patients had developed a microangiopathy not reversible with operative treatment alone. They may have had a predisposition to ulceration that is not changed by surgery alone. Careful technique is essential. The exact nature of the microangiopathy and the inflammatory mechanisms involved needs to be addressed simultaneously. Further studies to continue follow-up of patient prospectively using the CEAP classification are warranted.[200] Equally important are further studies into the micro-circulatory aspects of the disease. A study that would observe the effect of operative treatment on markers of leucocyte and endothelial activation may help in elucidating the effect on the microcirculation. The next section deals with this aspect.

4.4 The Effect of Operative Treatment on Leucocyte & Endothelial Activation in CVD – A Prospective Study

The inflammatory response seen in chronic venous disease (CVD) involves leucocyte and endothelial adherence, cell activation and cell migration. This involves adhesion molecule expression by endothelial cells, leucocyte activation and local cytokine activity. The endothelium is activated and histologically the capillaries are proliferative and convoluted (possible VEGF effect) in skin affected with changes of CVD.

Endothelial cell activation by cytokines or thrombin leads to increased adhesion molecule expression. These include ICAM-1 (binds neutrophil/ lymphocytes) and VCAM-1 (binds lymphocytes/ monocytes). This may lead to increased MHC-II (T-cell response) and GMP- 140 (binds platelets) expression. Binding of platelets increases the availability of platelet activating factor and further accelerates the expression of adhesion molecules.

Lactoferrin (LF), also known as lactotransferrin (LTF), is a globular multifunctional protein with antimicrobial activity (bacteriocide, fungicide) and is part of the innate defense, mainly at mucous membranes. Lactoferrin is found in milk and many mucosal secretions such as tears and saliva. Lactoferrin is also present in secondary granules of neutrophils and also is secreted by some acinar cells. Lactoferrin can be purified from milk or produced recombinantly. Human colostrum has the highest concentration, followed by human milk, then cow milk. Lactoferrin belongs to the transferrin family proteins (TF, melanotransferrin, ovotransferin, etc.). Its molecular mass is 80,000 u (80 kDa). Lactoferrin antimicrobial activity is due partly to its high affinity for Fe (ferric state). LF proteolysis produces lactoferricin, kaliocin-1 small peptides with antimicrobial activity.Lactoferrin receptors have been found on brush-border cells, PMN, monocytes, and activated lymphocytes. Lactoferrin is a good inducer of IL-6 and TNF-alpha production. It mayalso act as an opsonin.

Objective parameters are increasingly replacing purely subjective assessment of the outcome of these patients. Duplex scanning has revolutionised diagnosis of venous

disorders affecting the lower limb. Measurements of the site and duration of venous reflux can be accurately and reliably charted.[201] Theoretically once the haemodynamic derangement is recognised it may be possible to cure the limb. In practice this is possible only in a limited number of cases.[202].Many growth factors are important in the reparative response. Vascular endothelial growth factor (VEGF) has been shown to be up regulated in the patients with CVD. This molecule may be of importance in the perpetuation of the skin changes in CVD as is discussed later. Reports of success with deep venous reconstruction show mixed results. Disease confined to the superficial venous system on the other hand would be ideal for surgical correction.

The aim of this study was to assess the effect of removing varicose veins on patients with uncomplicated varicose veins as well as those with lipodermatosclerosis. Changes in circulating levels of soluble 'endothelial markers', Lactoferrin (surrogate for leucocyte activity) & VEGF were monitored to reflect changes in leucocyte and endothelial activation.

MATERIAL & METHODS

Ethics committee consent for the study was obtained from University College London (UCL) Medical School Committee for medical ethics. Clinical component of the CEAP (Clinical, etiologic, Anatomical, Pathological) classification was used to stratify patients.[134] Patients with CVD, clinical stage 2-4 were recruited from the vascular clinic at the Middlesex Hospital. These patients were given a full clinical examination by a surgeon experienced in managing patients with CVD. A medical history was taken and clinical examination performed. This included a systemic as well as venous examination.

All patients had a full blood count, serum urea and electrolyte/ liver function tests. All patients had a venous Duplex examination within the previous eight weeks to establish venous reflux and to localise it anatomically. All the deep and superficial veins in the lower limbs were examined. Venous reflux greater than 0.5 sec was considered significant. Patients who consented were asked to attend the department on three separate occasions. These were immediately pre-operatively and at four weeks and six months post-operatively.

Patient Selection

The inclusion criteria for the study were (1) Age above 18 years (2) patients affected with CEAP stage C2 to C4 (3) C5 with a healed ulcer for at least 4 weeks (4) Involvement of the superficial venous system only (5) consenting for operative treatment (6) consenting to the study.

Exclusion criteria for the study were used to eliminate factors that could distort leucocyte activation status. (1) History of alcohol or drug abuse (2) known history of allergy or intolerance to diosmin or any other venotonic agent (3) active venous ulceration (4) diabetes mellitus (5) impaired hepatic function (ALT or AST 3 fold above the normal limit) Impaired renal function (serum creatinine > 120 µmol/l) (6) any concomitant debilitating active disease or abnormality in laboratory test (judged as clinically significant by the investigator) (7) patients treated with other vasoactive drugs within the 15 days prior to inclusion (8) patients with an acute/ chronic inflammatory or infectious disease (9) deep venous thrombosis within the past 12 months (10) superficial venous thrombosis within 3 weeks (11) patients using steroids, NSAIDs, other 'vaso-active' drugs, or anti-coagulants (12) pregnancy or breast-feeding.

Blood was collected from the patients in the morning.No vigorous exercise was permitted prior to specimen collection. Patients with skin changes were fewer (n=5) than patients without skin changes (n=15). Most patients either had long saphenous (GSV) involvement with reflux at the groin (n=13) or short saphenous (SSV) involvement (n=4). Two patients had superficial venous disease (SVD) involving the GSV through other feeding sources like the mid-thigh perforator.

Collection of Specimens

Blood was collected via an 18G canula (Vasculon 2, Viggo-Spectramed, Helsingborg, Sweden) placed either in the distal long saphenous vein or dorsal foot vein of the affected leg. The canula was flushed with heparinized saline solution. 2 ml of blood was taken and discarded from the canula, and a further 10 ml was collected into two tubes containing ethylenediamine- tetraacetic acid and citrate (Vacutainer, Becton Dickinson Vacutainer Systems Europe, BP No 37-38241 Meylan Cedex, France) respectively. The stoppers were removed before adding blood to prevent excessive cell agitation.

The Markers measured were E-Selectin, VCAM, ICAM-1, VW Factor, Lactoferrin and VEGF. Commercial kits were used for these tests (ICAM-1/ VCAM/ E-selectin/ P-selectin/ Lactoferrin by R&D Systems 4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK; VW factor kit by Diagnosica Stago 9 rue des Freres Chausson

	GSV	**SSV**	**GSV/ SSV**	**OTHER**	**Total**
C2	3	2	-	1	6 (3F: 3M)
C3	7	2	-	-	9 (6F: 3M)
C4	3	-	1	1	5 (2F: 3M)
Total	13	4	1	2	20 (3F: 3M)

Table 22 shows the patients according to their CEAP stage and anatomical localisation of CVD. Most patients had stage C2 or C3 diseases (n=15). Two patients had reflux localised to mid-thigh perforators and localised segments of the GSV (‘Other’ category). Perforator incompetence is not listed separately because none of the patients had isolated perforator incompetence.

92600 ASNIERES-SUR-SIENE France; were used for these analyses). Assay of VEGF165 protein was performed using a sandwich ELISA technique. A ‘Quantikine’ kit supplied by R&D systems (4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, UK) was used. Plasma levels were read from standard curves obtained by plotting mean absorbance for standards. The VEGF test used measures levels of VEGF165. This is one of the four transcripts encoding mature monomeric VEGF (viz. VEGF121, VEGF165, VEGF189, VEGF206). VEGF121 and VEGF165 are diffusible proteins that are secreted into the medium. The other two are mostly bound to heparin containing proteo-glycans in the matrix. Results obtained for naturally occurring human VEGF and the recombinant VEGF121 showed linear curves that were parallel to the standard curves obtained using the ‘’Quantikine’’ kit standards. Please see methodology section for a more detailed description.

Statistical methods

The statistical tests are used as shown. Non-parametric tests were used because of the non-normal distribution of the data. Because of the non-Gaussian distribution the descriptors used are the median and interquartile range. The levels of statistical

significance were obtained using the Wilcoxon matched-pairs signed-ranks test for within group differences.

RESULTS

These results are shown diagrammatically in **Table 23** as well as in Figure 29,Figure 30, Figure 31. There was a statistically significant decrease in the plasma Lactoferrin levels at six months. This decreased from 864 (IQR 662-1260) to 519 (IQR 306-911) ng/ml (p=0.03)). This was an absolute decrease of 40% in the median levels compared to preoperative levels. There was no significant change in Lactoferrin levels at 2 months.

Siginificant increase in the levels of sVCAM-1 (43%) was observed at six months when compared to pre-operative levels. The change in levels was observed from 280ng/ml (IQR 200-390) to 399ng/ml (IQR 324-418) over the respective time periods.

Changes in plasma VEGF levels (65: 83:134 pg/ml) failed to reach statistical significance in this study. Although there was a trend towards rising levels, significant spread in the results may account for the lack of statistical significance. There were no siginificant changes in the levels of either VW factor or ICAM-1 levels (75: 78:79 [p= 0.12] & 208: 175: 199 [p=0.62]. This was true both at four weeks as well as at six months.

	VWF (% activity)	**sICAM-1** (ng/ml)	**VEGF** (pg/ml)	**E-Selectin** (ng/ml)	**sVCAM-1** (ng/ml)	**Lactoferrin (ng/ml)**
Pre-Operative	**75** (63-90)	**208** (176-223)	**65** (37-178)	**49** (34-57)	**280** (200-390)	**864(662-1260)**
Four Weeks	**78** (51-96)	**175**(133-307)	**83** (74-137)	**40** (38-49)	**240** (215-290)	**870(605-1717)**
P value[ə]	0.27	0.84	0.25	0.53	0.41	**0.7**
Six Months	**79** (67-87)	**199** (137-243)	**134** (38-2940)	**51**(40-72)	**399** (324-418)	**519(306-911)**
P value[f]	0.12	0.62	0.15	0.01	0.01	0.03

Table 23 Changes in the levels of plasma markers of endothelial activation, lactoferrin and VEGF at four weeks and six months following surgery. Bold values represent medians, values in parentheses are inter-quartile ranges.

Yellow=statistically significant changes. The actual differences in the E-Selectin levels are too small to be meaningful.

[ə] These values (Wilcoxon) are for the differences between the values obtained pre-operatively and at four weeks post-operatively.

[f] These values (Wilcoxon) are for the differences between the values obtained pre-operatively and at six months post-operatively.

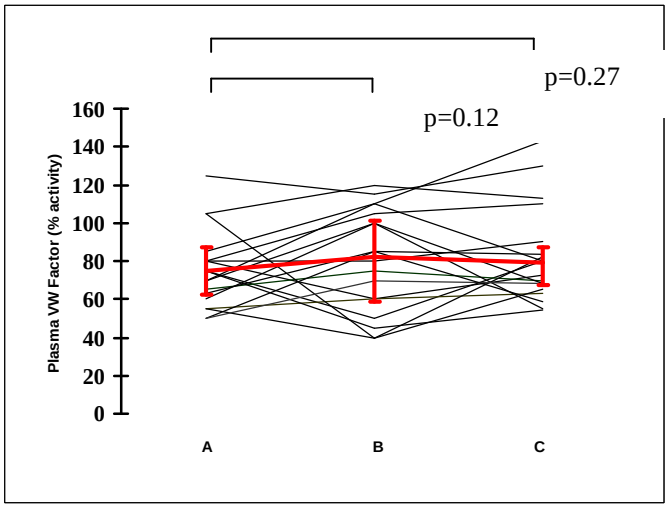


Figure 29 Shows changes in the plasma activity of VW factor before (A) four weeks (B) and six months (C) following surgery. VW factor is expressed by endothelial cells and is up regulated in endothelial activation in addition to its role as a pro-coagulant. There no statistically significant change at six months.

The lines in the figures represent the values of the concentration of the relevant molecule. The bold lines represent the median values. The vertical lines represent inter-quartile ranges. The X-axes show the values pre-operatively, 4 weeks post-operatively and six months post-operatively. The Y-axes represent the value of the concentration of the respective molecule.

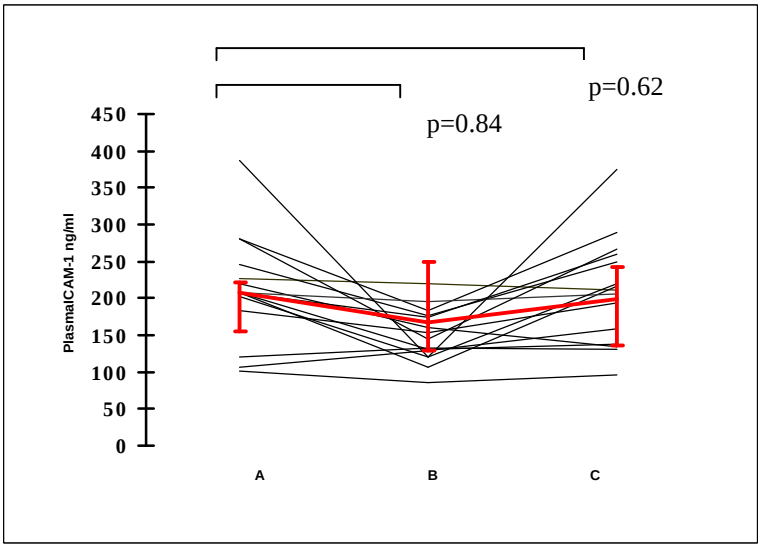


Figure 30 The response of plasma levels of sICAM-1 to operative therapy in the short to medium term. There is no significant change either at 4 weeks or 6 months post-operatively.

The lines in the figures represent the values of the concentration of the relevant molecule. The bold lines represent the median values. The vertical lines represent inter-quartile ranges. The X-axes show the values pre-operatively, 4 weeks post-operatively and six months post-operatively. The Y-axes represent the value of the concentration of the respective molecule.

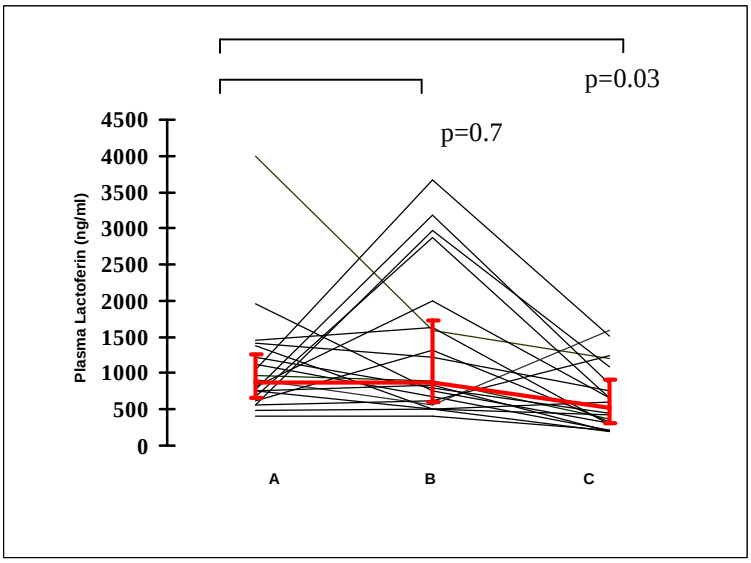


Figure 31 shows changes in plasma levels of lactoferrin at 4 & 6 months post surgery. Lactoferrin levels represent leucocyte degranulation. There is a statistically significant decrease at 6 months.

The lines in the figures represent the values of the concentration of the relevant molecule. The bold lines represent the median values. The vertical lines represent inter-quartile ranges. The X-axes show the values pre-operatively, 4 weeks post-operatively and six months post-operatively. The Y-axes represent the value of the concentration of the respective molecule.

DISCUSSION

I observed an initial rise and a subsequent decrease to below starting levels in the levels of lactoferrin in the plasma. On the other hand the markers of endothelial activation and VEGF continued to show a trend towards increase at six months post-operatively. The exact medical significance of this amount of change in endothelial activation is open to further work. This decrease may reduce the initial adhesion of these cells to the endothelium and thus decrease the amount of tissue damage. These effects seem to be equivalent in patients with and without skin changes. Other possible confounding effects (e.g. the time of the year the samples were taken) also have to be acknowledged.

Presently there are no reliable markers either to assess the effectiveness of treatment of chronic venous disease (CVD) or to help in its prognosis. Many patients are worried about the long-term sequelae of their venous disease and do not want surgery merely for appearance's sake. Markers of venous disease include clinical, hand held Doppler, duplex ultrasound, plethysmography (air, photo, strain gauge), capillary microscopy and tonometric criteria. Many assessments are subjective and may be unreliable. Duplex examination after therapy may indicate success in the short term but may not predict future recurrences or the course of skin complications.

Chronic venous disease (CVD) and its sequelae are a major cause of morbidity in the Western world.[203,] Previous studies from this department have shown increased leucocyte activation in patients with CVD. Leucocyte mediated inflammatory mechanisms have been a focus of attention in CVD This report is the first to show the response of this activation to any form of therapy. Moyses et al reported sequestration of leucocytes in the circulation of the lower limb following venous hypertension in 1978. [204]. Bollinger et al showed that there were areas of very low skin blood flow in legs affected with CVD. These areas diminished on application of compression stockings. [205] Blood flow in the microcirculation is dependent on i. The arterio-venous gradient ii. The viscosity of the blood and iii. The vascular resistance. The white cells contribute importantly to the vascular resistance by being about 2000 times less compliant than the red cells. Thus ‘sludging’ of the WBCs can seriously affect the

micro-circulatory haemodynamics. This is thought to be at least in part attributable to leucocyte-induced damage mediated through cytokines.

Coleridge-Smith et al put forward the hypothesis of leucocyte trapping due to reduction in capillary blood flow because of an increase in venous pressure in 1988.[206] A study published by the same group in 1997 showed that venous hypertension results in sequestration of the more activated population of neutrophils and monocytes in the microcirculation of the leg in patients with CVD. These cells bind to the endothelium and may not emerge from the limb when venous hypertension is reversed.[130] The physical interaction between the leucocyte and the micro-vascular endothelium is important for its function in the microvasculature.[207]

Patients with chronic inflammatory and septic states show increased leucocyte priming. This is also observed in patients with CVD. The primed cells show increased surface CD62L and CD11B[130]. CD62L is involved in the initial 'rolling' movement of the leucocyte along the endothelium. CD11B is the molecule responsible for firmer adhesion and subsequent diapedesis. There is some evidence of inflammatory mechanisms being important in damage to the venous valves seen in CVD[153]. Experimental blockade of adhesion molecules either with soluble ligands or with specific monoclonal antibodies has a dramatic effect on inflammation and immune mediated tissue damage[164]. The observation that some markers are specifically lowered following therapy in patients with skin changes demonstrates the possibility of flavonoids to ameliorate and/ or implicated in the valvular damage seen in CVD[153] and these compounds may have a role in ameliorating this damage as well. These aspects merit further study.

Inflammatory processes are regarded as important in the pathogenesis of skin changes seen in CVD[130]. Increased leucocyte presence has been shown in the skin and the vein wall itself. In the chronic stages there is a reparative tissue response that exists alongside the inflammatory response[131]. Many growth factors are involved in the reparative response208. Vascular endothelial growth factor (VEGF) is one of the molecules involved physiologically in the angiogenesis of healing. It is a very potent micro-vascular permeability-increasing factor initially associated with tumour

angiogenesis[209]. It is a glycoprotein secreted by a variety of cells including smooth muscle cells, monocytic cells and keratinocytes[210]. Its expression is dependent on increased mRNA synthesis and increased transcription[211]. Any tissue injury, especially hypoxic injury could be a potent stimulus for its expression.

Abnormally increased and prolonged exposure to VEGF could have local deleterious effects. VEGF is strongly expressed by epidermal keratinocytes in psoriasis, skin with ultra-violet burns, lesions of rheumatoid Arthritis and in bullous diseases such as erythema multiforme and bullous pemphigoid.[212] There is a characteristic increased micro-vascular permeability and angiogenesis.

I used a model of surgery for varicose veins to study the effect of operative management on CVD in general. The effects on microcirculation for any given clinical (CEAP) grade of severity are similar for superficial as well as deep disease. Superficial venous disease is currently considered to be of equal importance in the prevalence of CVD. In a study of 98 limbs with skin changes due to CVD (hyper-pigmentation, lipodermatosclerosis, atrophie blanche and ulceration) 57 per cent had superficial vein incompetence only[189]. Following any successful treatment for CVD, a reduction in leucocyte activation would be expected. Because of the paucity of the number of surgical procedures performed for deep venous insufficiency, it would not have been practical to include patients with DVI in this study.

The relatively short follow-up in this study also precludes any study of the effect of recurrence on endothelial/ leucocyte activation markers. The clinical effect of this amount of reduction in the levels of LAMs has yet to be elucidated scientifically. Despite no controls in the present study I were able to demonstrate the effect of operative treatment on the abnormal leucocyte activation seen in CVD in the short to medium term.

SVI is an important factor in many cases of chronic venous insufficiency. The long-term effects of SVI may be equally severe compared to DVI. The role of the superficial venous system has been underestimated in the past. Superficial venous surgery has been shown to improve venous haemodynamics associated with SVI. In a study of 11 patients followed up prospectively, ejection fraction, residual volume fraction and the

venous filling index were shown to improve significantly at mean follow-up of 4 and 6 months post-operatively. The microangiopathy associated with CVD may also improve following therapy. Laser Doppler flux and $tcP0_2$ were shown to improve following sclerotherapy and compression treatment in one study of 15 patients with moderate to severe SVI. [190]

In another study [162] the results of surgical treatment of eleven limbs with leg ulcers due to SVI was studied. The mean follow up was 16.4 months. All the ulcers healed in this study and remained healed for the duration of the follow-up. The mean clinical score decreased from 10.1 to 1.45. More recent studies of the efficacy of surgical treatment alone exist.[213] Longer follow-up results of such series are required to show many late recurrences.

Changes in the level of VEGF may have some clinical significance. Proliferation of micro-vessels in the skin and accumulation of peri-capillary fibrin cuffs in CVD has been recognised for some time[214]. The significance of these cuffs remains unclear. VEGF selectively promotes extravasation of Fibrinogen[215]. VEGF blocking has shown some promise experimentally in oncological therapy[216 217]. Linomide and VEGF-blocking anti-bodies have been used for this purpose. Tamoxifen has been shown to have some anti-angiogenic activity. The role of such therapy in CVD remains to be explored. Ancillary therapy in CVD to reduce recurrence or accelerate healing of skin lesions in selected cases may be a possibility in the future.

Evidence of decreased plasma lactoferrin may be explained on removal of segments of veins that were causing venous stasis and leucocyte activation. at six months further corroborates these findings. Increase in levels of plasma VEGF (if real) may be postulated to be a continuing process of vascular remodelling in these patients. It is unknown at the moment if clinical recurrences of veins or persistence/ worsening of skin changes are preceded by increase in the levels of these markers.

Concluding Remarks

In summary operative treatment of varicose veins leads changes in endothelial activation markers that are mostly difficult to interpret. Many of the markers of endothelial activation were increased at six months post-surgery. Plasma VEGF showed

a similar trend. This may be explainable on some form of vascular remodelling that may occur after operative treatment to varicose veins. These phenomena may or may not be related to the recurrence of varicose veins or the non-healing of venous hypertension induced skin changes.

4.5 Effect of Skin Compression On the microcirculation

The effect of compression on the microcirculation in patients with CVD as well as CVD combined with occlusive peripheral arterial disease is important. Laser Doppler 'flux' has been used in the past for this purpose. This measures flow in both the nutritional vessels of the skin as well as the deeper thermoregulatory plexuses.

Compression in patients with combined venous and arterial disease

More than 20% of patients with venous leg ulceration have associated arterial disease [218]. It is conventional practice to avoid the application of high levels of compression in patients with arterial disease because of the possibility of causing ischaemia of the leg resulting in gangrene. No systematic investigation of this problem has been undertaken and no simple measure is currently available which allows an assessment of a particular patient when considering the use of compression treatment, other than the measurement of Doppler ankle indices.

Conventional capillary microscopy is of limited use in the skin of the leg. It is usually employed in the nail fold where capillary loops run horizontally in the skin. Elsewhere the nutritional capillaries pass from deep to superficial, running perpendicularly to the skin surface, and flow velocities cannot be measured. A device termed a laser capillary anemometer uses Doppler shift in a laser beam to assess the flow in a single capillary. The device incorporates a capillary microscope through which capillary loops may be visualized. The laser beam is then employed to assess blood flow velocity. Skin capillaries may be counted and measured, as well as their flow estimated.

The aim of my study was to use laser Doppler fluxmetry and capillary microscopy / laser capillary anemometry to assess the leg skin microcirculation in patients and controls during the application of compression in order to measure the response of the nutritional circulation of the skin.

Sex	Age	SVI	DVI	`Perforator	C1-C3	C4	C5
Female (n=8)	76(62-86)	6	1	1	2	3	3
Male (n=4)	69(55-78)	2	1	1	2	1	1

Table 24 Characteristics of the patients with mixed arterial and venous disease. Most patients had SVI and proximal arterial occlusive disease.

MATERIAL & METHODS

Patients were investigated by colour doppler ultrasonography and photoplethysmography to confirm that venous disease was responsible for the skin changes and to define the extent of arterial disease. All patients had ankle and brachial pressures measured using Doppler ultrasound and the ankle: brachial pressure index was calculated. Patients with ischaemic rest pain were excluded from the study.

Initially the subjects lay supine. At least 5 representative capillaries were studied in each limb. Images of the capillaries were recorded on videotape for subsequent morph metric analysis. The laser anemometer and fibre-optic laser Doppler system was used to measure the mean flow velocity over a 1-minute interval. Compression was then applied in 10 mm Hg increments up to a pressure of 100 mm Hg. Further images and flow velocity readings were taken. The measurements were repeated with the subjects standing supported, using a high stool or couch to maintain comfort and balance. Recordings were made in the same region of the limb as before.

Blood flow velocity during compression

A capillary microscope/laser anemometer was modified so that the skin is viewed through an acrylic window. Details of the instrument are mentioned in the section on methodology. The microscope, which is of small dimensions, was mounted on a pivoted frame so that it may be applied to the lower limb with the patient lying supine or in the sitting position.

The frame was designed so that a variable force may be applied to the skin and measured with the aid of a strain gauge incorporated into the microscope mounting system. The system was designed to apply pressures of up to 100 mm Hg to the viewing window of the microscope, allowing the skin microcirculation to be viewed and measured whilst under compression. A conventional fibreoptic laser Doppler probe was incorporated into the system to assess the effect of this system on previously reported effects of compression using this measuring technology. All measurements were made in an environmental chamber at 22°C.

Testing of subjects

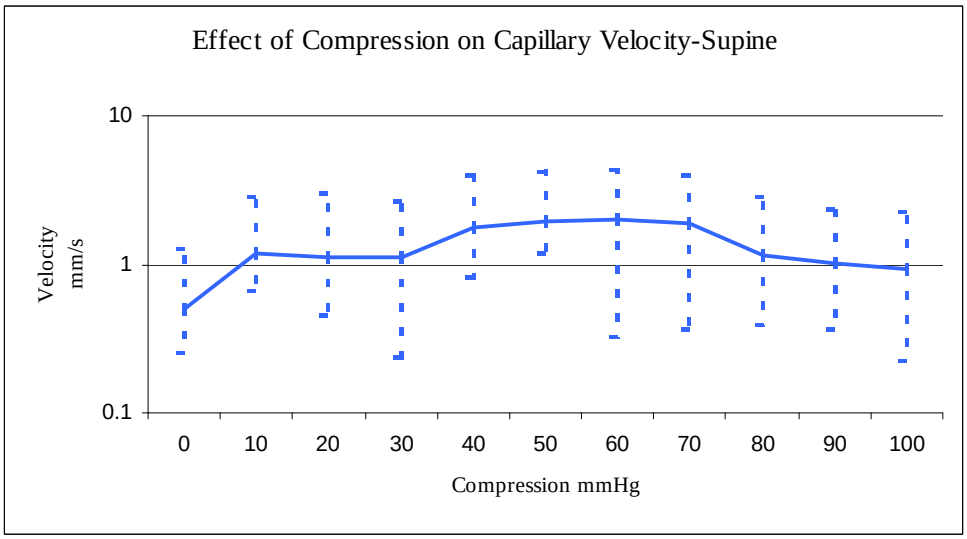
The study was conducted in an environmental chamber at 22°C. Patients were acclimatized for 20 minutes before the start of the experimental protocol. In each patient and volunteer the supra-malleolar skin 5 cm proximal to the medial malleolus was investigated.

RESULTS

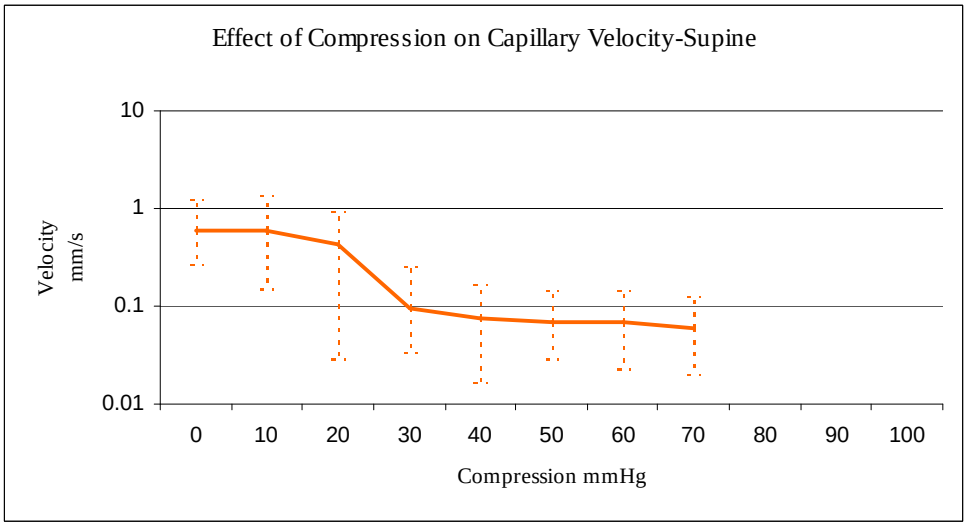
A group of 15 patients with skin changes due to venous disease (lipodermatosclerosis or haemosiderosis) or healed and unhealed venous ulcers (CEAP classification: clinical groups 4, 5 Proximal arterial= Ileo-Femoral disease, Distal Arterial= Popliteal and distal disease, C1-C4 etc refer to the CEAP clinical classification), 15 patients with moderate lower limb arterial disease (ABPI <0.8 and >=0.5), 12 patients with combined venous disease (CEAP classification: clinical group any) and moderate arterial disease (ABPI <0.8 and >=0.5) and 15 control subjects was studied. The results obtained from all three groups of patients are shown in the following figures.. Also show the data obtained from the control group. The most marked response to treatment was seen in the patients with CVD. The lines in the figures represent the median values of the relevant velocities. The vertical lines represent inter-quartile ranges. The X-axes show the velocities in the capillaries.

The Y-axes represent the value of the compression applied to the limb. The statistical tests used are non-parametric tests because of the non-normal distribution of the data. Maximal velocities were seen well into the 60-70 mm Hg compression levels. In patients with arterial disease only pressure above 20-30 mmHg was poorly tolerated with flows

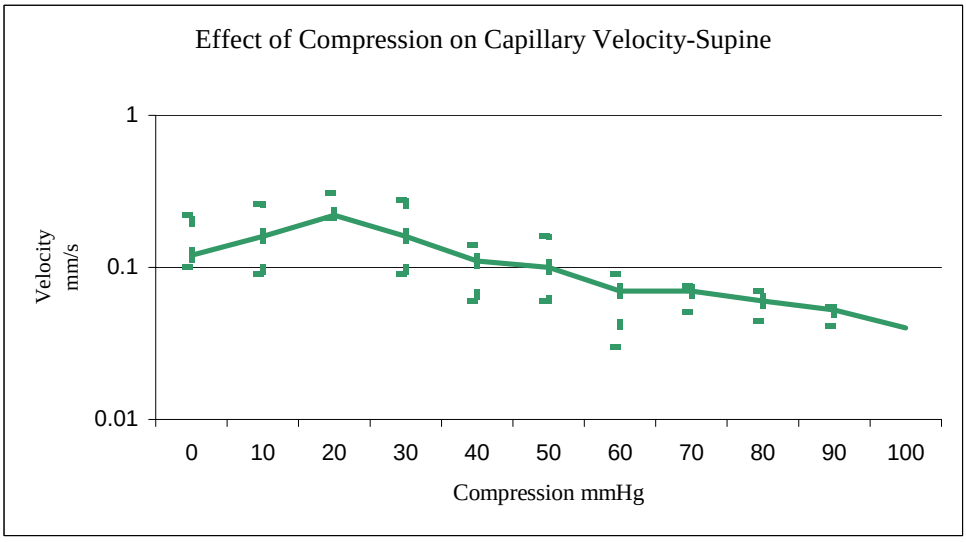
rapidly declining above these pressure levels. The patterns in the mixed disease sub-group did not strictly lie in between the arterial and venous groups. In the mixed group of patients reached a maximum at 20 mm compression. Blood flow was barely detectable at 100mmHg. Thus compression at higher levels than 30-40mm was poorly tolerated. Capillary velocity reached a maximum at 40mm compression. Although blood flow was still not abolished at 100mmHg, the velocity was greatly reduced. Higher pressures were tolerated with the patient in the upright position among all the groups.


Effect of Compression on Capillary Velocity-Supine
Velocity mm/s
10
1
0.1
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(a)


Effect of Compression on Capillary Velocity-Supine
Velocity mm/s
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(b)


Effect of Compression on Capillary Velocity-Supine
Velocity mm/s
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(c)

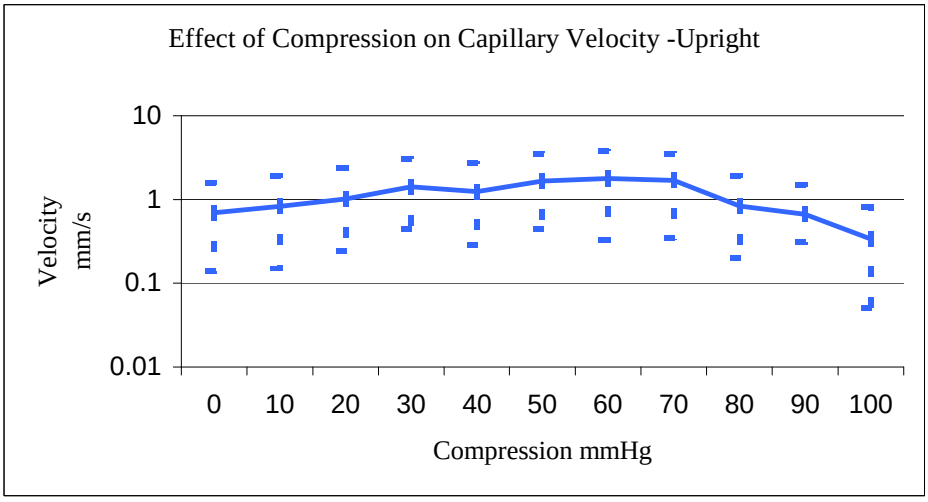
Figure 32 Shows a comparison between data from mixed disease with patients suffering from either arterial OR venous disease (all in supine position). Blue lines represent CVD; red lines represent arterial disease while green lines represent the group with mixed disease.

Patients with CVD (a) show a very marked and enhanced response to compression peaking at about 40-60mm of pressure

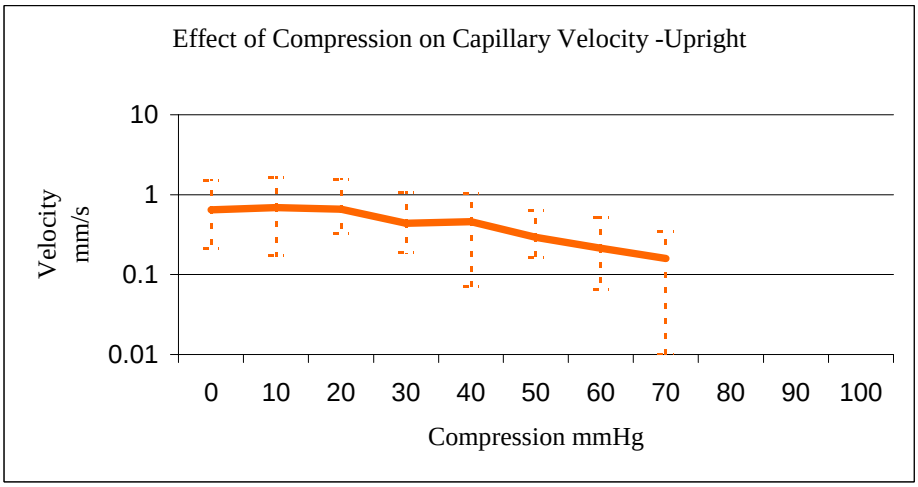
Patients with arterial disease (b) show a marked cut-off in velocity at just 30mm especially in the supine position. There is no tendency to peak but rather a decreasing trend in velocity with increasing pressure.

The line representing the mixed group (c) does not strictly lie between the arterial and the venous groups but does show a more sustained response to compression.

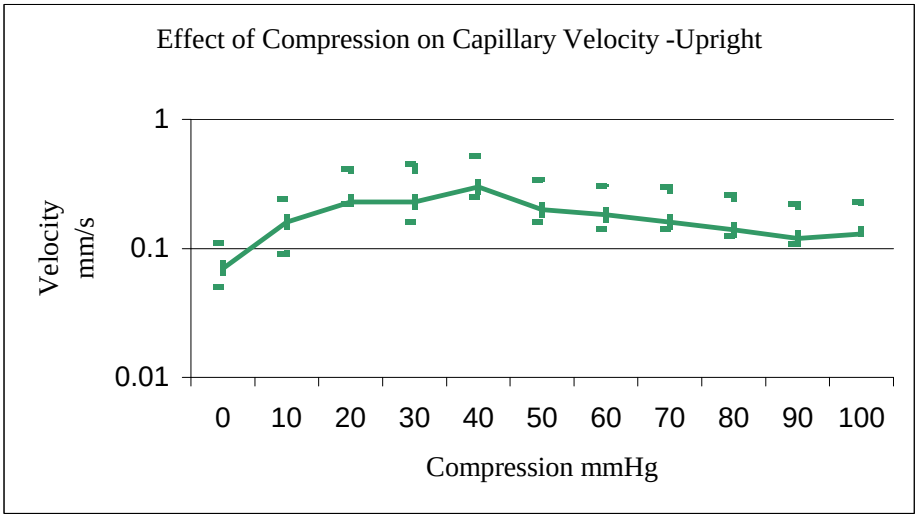
Velocity= mm/s


Effect of Compression on Capillary Velocity -Upright
Velocity mm/s
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(a)


Effect of Compression on Capillary Velocity -Upright
Velocity mm/s
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(b)


Effect of Compression on Capillary Velocity -Upright
Velocity mm/s
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Compression mmHg

(c))

Figure 33 Shows a comparison between data from mixed disease with patients suffering from either arterial OR venous disease (all in upright position). Blue lines represent CVD; red lines represent arterial disease while green lines represent the group with mixed disease.

Patients with CVD (a) show a very marked and enhanced response to compression peaking at about 60mm of pressure

Patients with arterial disease (b) show a cut-off in velocity at higher than the supine position. There is no tendency to peak but rather a decreasing trend in velocity with increasing pressure.

Velocity= mm/s

COMPRESSION Pressure	0 mm	10 mm	20 mm	30 mm	40 mm	50 mm	60 mm	70 mm	80 mm	90 mm	100 mm
Control	0.55 (0.48-.62)	0.613 (0.56-0.66)	0.66 (0.61-0.72)	0.71 (0.66-0.77)	0.673 (0.61-0.74)	0.755 (0.7-0.8)	0.893 (0.82-0.94)	0.71 (0.66-0.78)	0.815 (0.75-0.87)	0.23 (0.180-0.29)	0.145 (0.09-0.2)
Combined	0.07 (0.04-0.08)	0.16 (0.08-0.17)	0.23 (0.18-0.36)	0.23 (0.22-0.42)	0.3 (0.22-0.42)	0.2 (0.14-0.4)	0.18 (0.12-0.29)	0.16 (0.14-0.29)	0.14 (0.12-0.22)	0.12 (0.1-0.2)	0.13 (0.1-0.22)
Arterial	0.59 (0.32-0.68)	0.6 (0.45-0.76)	0.42 (0.39-0.54)	0.095 (0.06-0.16)	0.077 (0.06-0.09)	0.069 (0.04-0.08)	0.0685 (0.05-0.08)	0.06 (0.04-0.069)	-	-	-
Venous	0.7 (0.56-0.89)	0.83 (0.68-1.09)	1.02 (0.78-1.34)	1.42 (0.98-1.34)	1.25 (0.96-1.45)	1.67 (1.22-1.87)	1.78 (1.45-1.99)	1.69 (1.34-1.86)	0.84 (0.64-1.09)	0.67 (0.36-0.84)	0.34 (0.29-0.48)

Table 25 Shows a comparison between data from mixed disease with patients suffering from either arterial OR venous disease (all in upright position).

Patients with CVD show a very marked and enhanced response to compression peaking at about 60mm of pressure Patients with arterial disease show a cut-off in velocity at higher than the supine position. There is no tendency to peak but rather a decreasing trend in velocity with increasing pressure. The data representing the mixed group does not strictly lie between the arterial and the venous groups but does show a more sustained response to compression. Again higher levels of pressure are tolerated as compared to the supine position.

Velocity= mm/s

COMPRESSION	0 mm	10 mm	20 mm	30 mm	40 mm	50 mm	60 mm	70 mm	80 mm	90 mm	100 mm
Control	0.655 (0.52-0.89)	0.705 (0.63-0.96)	0.735 (0.68-0.88)	0.83 (0.73-1.07)	0.88 (0.7-0.98)	0.83 (0.71-1.09)	0.78 (0.7-0.97)	0.675 (0.63-0.79)	0.625 (0.59-0.75)	0.605 (0.59-0.74)	0.56 (0.53-0.68)
Arterial	0.65 (0.44-0.87)	0.7 (0.52-0.96)	0.66 (0.33-0.89)	0.44 (0.25-0.630	0.46 0.39-0.58)	0.3 0.13-0.35)	0.22 0.15-0.31)	0.16 0.15-0.19)	-	-	-
Venous	0.5 (0.25-0.77)	1.17 (0.0.52-0.1.66	1.13 (0.68-1.88)	1.12 (0.88-1.54)	1.77 (0.96-2.24)	1.96 0.79-2.22)	2.01 (1.69-2.3)	1.89 (1.52-2.09)	1.15 (0.76-1.63)	1.01 (0.64-1.36)	0.92 (0.7-1.35)
Combined	0.12 (0.1-0.16)	0.16 (0.06-0.14)	0.22 (0.15-0.32)	0.16 (0.15-0.25)	0.11 0.04*0.23)	0.1 0.05-0.13)	0.07 (0.03-0.13)	0.07 (0.03-0.09)	0.06 (0.04-0.07)	0.53 (0.04-0.06)	0.04 (0.03-0.04)

Table 26 Represents data obtained with the patient supine. Median flow velocities (mm/sec) in all fourgroups of patients are shown. Velocities at varying levels of compression from 0-100 mm Hg. Increments of 10 mm were used for this study. Values in parentheses represent inter quartile ranges.

Correlate arterial pressure with foot compression pressure

DISCUSSION

Previously it has been widely assumed that the application of compression to the lower limb restores valvular competence to venous valves[219,220,221,222]. Objective assessment of this assumption using duplex ultrasonography has failed to show that valvular competence is restored by the application of moderate levels of compression, known to be effective in the treatment of venous ulceration[223]. However studies suggest that moderate levels of compression do augment the calf pump mechanism and may lead to improvement in air plethysmographic criteria. On the other hand only moderate levels of compression may work in ulceration due to CVD. Stockings, which exert 20 - 30 mm Hg, heal venous ulcers and prevent their recurrence.

The method we used utilises pressure applied to an acrylic disc. Although these measurements may be not show pressure transducer measurements underneath a pressure garment. The method was used to allow direct measurement of the underlying capillaries. Nevertheless it appears that compression therapy at lower levels increases the flow of blood through the skin microcirculation. This was true even in patients with mixed disease and arterial disease. Lower levels of compression may be useful in patients with mixed arterial and venous disease.

In a previous study from this department the effects of compression on the microcirculation of the limb were investigated using laser Doppler fluxmetry. It was found that increased flow of blood through the microcirculation of the skin occurred with compression levels as low as 20 mm. Therefore compression stockings may work principally by increasing the blood flow velocity through the capillaries and venules skin. This may modify the interaction of the blood, in particular the leucocytes, with the endothelium of the microcirculation preventing stagnation, leucocyte adhesion and acting as an anti-inflammatory treatment. (It has been shown that WBC trapping in the leg was decreased by compression stockings).[224]

A study of the microcirculation using laser Doppler fluxmetry to assess skin blood flow found that compressing the leg skin in patients with venous disease resulted in a 50% increase in laser Doppler flux when only 20 mm Hg of compression was applied [105]. When patients sat with their limbs dependent up to 60 mm Hg compression could be applied

before laser Doppler flux began to decrease. This suggests that much of the efficacy of compression may be attributable to its effect on the microcirculation. It has been confirmed that compression stockings achieve the same effect on laser Doppler flux in the skin [225]. The fibre-optic method of laser Doppler fluxmetry used in these studies measured flow in both the nutritional vessels of the skin as well as the deeper thermoregulatory plexuses below, particularly from the sub-papillary plexus [226].

Direct measurement of blood flow in skin capillaries in my study permitted an assessment of whether compression influences these vessels, and whether where the efficacy of compression occurs and how treatment might best be optimised.

More than 20% of patients with venous leg ulceration have associated arterial disease. If the patient has arterial disease as well as venous incompetence it becomes difficult to gauge the extent of compression therapy that may be applied safely. Excessively high levels of compression may harm the patient by causing ischaemic necrosis of the skin over bony prominences. Although ABPI may dictate the maximum levels of compression that may be used it does not provide any guide to an optimum compression level.

Studies done by Abu-Own's study suggest that the increased flux associated with skin compression involves both increased velocity as well as increased concentration of moving blood cells. I did not measure the either change in diameter of the capillaries with increasing compression or the number of visible capillaries. These may be important parameters, however; the present study was concerned with the effect of compression on blood flow.

Concluding Remarks

Skin compression increases the capillary blood velocity in the sub-papillary plexus in patients with mixed disease. Thus compression therapy does seem to help in these patients, however, maximum velocities are reached at relatively smaller pressures especially in the supine position. The fact that maximum velocities in these patients are reached at 20 mm in supine and 40 mm in sitting positions suggests that lower levels of compression may be useful in these patients.

The outcome of this investigation may indicate the optimum levels of compression likely to be effective in the management of chronic venous disease and in combined venous and arterial disease. This may help in the design of more effective methods of compression for the treatment of patients with venous disease. It may indicate whether compression is helpful in the lying position as well as when the patient is standing or sitting. Laser anemometer as a research tool may help in the design of better methods of compression therapy for CVD.

PART V

DISCUSSION

Previously many inflammatory processes have been reported in the skin and blood of patients with venous disease. Burnand [227] found that capillary proliferation correlated closely to the development of clinical skin changes (lipodermatosclerosis, LDS). There are no studies showing major differences between patients with skin changes and those without. Reports of changes in the microcirculatory processes following therapy are also few.

VEGF & Inflammatory markers

I demonstrated high plasma levels of vascular endothelial growth factor (VEGF) among patients with chronic venous disease (CVD). The finding of large differences between patients with clinically obvious skin changes and those without may be of relevance. Although, measurements of plasma levels may not accurately represent the actual tissue levels expressed in the tissues. The data from this study show that both groups of patients showed similar elevations in plasma VEGF compared to controls. This suggests that this growth factor is induced in patients with venous disease as a response to tissue damage caused by venous hypertension

My studies showed for the first time that there is a decrease in some soluble endothelial activation markers in patients with CVD following sixty days therapy with oral purified flavonoid fraction. This demonstrated a quantifiable change in endothelial activation . VCAM-1 and ICAM-1 (adhesion molecules important in endothelial interaction with neutrophil, monocytes and lymphocytes) were decreased significantly following therapy. This effect was seen in all patients irrespective of their clinical stage. Lactoferrin levels were appreciably reduced following therapy mainly in patients with skin changes. Changes seen in endothelial activation markers following surgery are hard to interpret. This may represent damping of the much increased granulocyte activity in these patients compared to those with simple CVD. The effect on E-selectin levels was also seen more in the patients with skin changes in the leg. As yet unknown factors may be responsible for the differing phenomena seen with VW factor following therapy (rise in VW factor in C3 patients and fall in C4 group). A rise in VW factor expression may indicate both an acute inflammatory phase as well as a pro-thrombotic tendency. A decreased expression may imply the reverse.

Inflammatory markers & Surgery for Varicose veins

Despite advances in doppler ultrasound, surgery for varicose veins causing leg ulcers may not always result in leg ulcer healing. In my review of long-term outcome in 32 patients initial healing rates were >85% with surgical treatment. Only 40% initial healing was achieved with conservative treatment. Recurrence rates are relatively high at >30% in the operative group. About half of all the patients continued to be symptomatic from their veins. Most of the patients who had surgery (>80%) used graduated elastic stocking in the pre-operative and short-term post-operative period. >40 % had to use them permanently to avoid troublesome symptoms. Some patients could not use graduated elastic stockings because of the size of the limb or other factors.

I have shown that operative treatment led to an initial increase and a subsequent decrease to below starting levels of lactoferrin in the plasma. As acknowledge previously, changes seen in endothelial activation markers following surgery are hard to interpret. On the other hand ICAM-1, vWF and VEGF continued to show a trend towards increase at six months post-operatively. The significance of this amount of change in endothelial activation is open to further work. These effects seem to be equivalent in patients with and without skin changes.

Effects of Compression on the Micro-circulation

I showed that compression therapy at lower levels in patients with mixed venous and arterial disease increases the flow velocity of blood through the skin microcirculation. It may be reasoned that compression therapy at a lower level may be useful even in patients with mixed arterial and venous disease. Patients with CVD showed the most change in cpillary blood velocity in response to compression. Maximal velocities were seen well into the 60-70 mm Hg compression levels. In patients with arterial disease only pressure above 20-30 mmHg was poorly tolerated with flows rapidly declining above these levels.

In patients with mixed arterial and venous disease, capillary velocity reached a maximum at 20 mm compression. Thus compression at higher levels than 30-40mm was poorly tolerated. Capillary velocity reached a maximum at 40mm compression.

Higher pressures were tolerated with the patient in the upright position among all the groups.

Discussion of methodology

In the study that showed increased levels of VEGF in patients with CVD the control subjects were slightly younger than either of the study groups. I investigated the influence of age on the VEGF levels in my control subjects and could find no correlation. A Medline search of the world literature did not reveal influence of age on plasma VEGF levels at the range involved in my study. The median VEGF levels in my control subjects are similar to those measured by the manufacturers of the analysis kit in their human controls. Nevertheless I admit that the age differences between patient and control groups leave the possibility that some of the differences I have observed may be accounted for by age differences.

No distinction was made between patients with superficial venous insufficiency and those with deep venous insufficiency in interpreting the results. Previous studies from this department did not observe any difference between these two groups[130] (for a similar clinical stage). I compared equivalent clinical stages (C), which are representative of the severity of CVD. Although some of these markers like VW-factor are also acute phase reactants, they are widely used to estimate endothelial cell activation. Nonetheless they are widely used for estimating endothelial/ leucocyte activation. Various roles have been ascribed to circulating plasma levels of adhesion molecules. They represent protease cleavage from cell surface subsequent to endothelial stimulation.

The primary haemodynamic derangement in SVI is reflux. Most primary DVI has reflux as the major mechanism as well. On the other hand the mechanism implicated in some cases of secondary DVI may be obstruction. Models of surgical treatment of varicose veins causing ulcers have produced similar results even with segmental (& possibly) total deep venous insufficiency (ESCHAR trial).[228 196]

My study of mid to long-term results of surgery for varicose veins causing venous ulcers reports on the outcome in a cross-sectional study. Superficial venous disease is

currently considered to be as important as deep venous insuficiency in the pathogenesis of CVD. For example, in a study of 98 limbs with skin changes due to CVD (hyper-pigmentation, lipodermatosclerosis, atrophie blanche and ulceration) 57 per cent had superficial vein incompetence only[189].

Following any successful treatment for CVD, a reduction in endothelial/ leucocyte activation would be expected. Clinical studies performed recently of surgery for both superficial as well as superficial and partial/ total deep venous reflux have shown possible beneficial effect in all groups.[196] [229] [230] The relatively short follow-up in this study also precludes any study of the effect of recurrence on markers (or vice versa). The clinical significance of this amount of reduction that I have shown in the levels of markers has yet to be elucidated. Despite there being no controls in the present study I was able to demonstrate the effect of operative treatment on the abnormal leucocyte activation seen in CVD in the short to medium term. The results were compared with historical controls.

My study on effect of compression on the skin microcirculation suggested that compression therapy at lower levels increases the flow of blood in mixed arterial and venous disease. It may be reasoned, that lower levels of compression may be useful in patients with mixed arterial and venous disease.

Previous Studies

Increased VEGF expression has been shown previously in the skin of patients with CVD[172]. It was observed that VEGF upregulation is seen both in patients with as well as those without skin changes. The expression was more marked in patients with lipodermatosclerosis. It was thought that the VEGF was the main factor in causing the neo-angiogenesis in CVD. The precise stimulus to the increased VEGF stimulation was not studied. Local tissue hypoxia is a potent stimulus for the release of VEGF. Hypoxia, although not thought to be the man tissue damaging event in CVD, may nevertheless exist.[231] Mixed aetiology ulcer s may have more pronounced hypoxia. Non-hypoxic tissue damage may lead to release of VEGF as well through little understood mechanisms.

Recurrence of Venous Leg Ulcers

Most studies show that venous ulcers can be expected to heal when patients are enrolled in a nurse-managed/physician-supervised ambulatory ulcer clinic. Photoplethysmography-derived venous refill time of 10 seconds or less predicts delayed healing. Strict compliance with the treatment protocol significantly decreases the time to healing and prolonged the time to recurrence[232]. However recurrence of ulcers is a problem in a substantial proportion of patients.

In a prospective study 105 consecutive patients with leg ulcers were recruited (causes: 77% venous, 4% arterial, 9.5% mixed, 9.5% other). 70 (67%) had a history of previous ulceration. 83 patients could be followed for 1 year. The healing rate for the whole group was 41 (49%) after 3 months and 61 (73%) after 1 year. The corresponding figures for the 67 venous patients are 44 (66%) and 52 (78%) respectively. From 61 healed ulcers 18 (30%) reoccurred during the 1st year. At the primary examination several factors were investigated which might have influenced the healing rate.

In a recently published paper [162] the results of surgical treatment of eleven limbs that had been subjected to Duplex scanning, venography and Air plethysmography (APG) were studied. All of these patients had GSV disease as the superficial component. All the patients had deep venous disease in addition. The mean follow up was 16.4 months. All the ulcers healed in this study and remained healed for the duration of the follow-up. The mean clinical score decreased from 10.1 to 1.45. However all the subjects in this study had evidence of deep venous insufficiency in addition. No mention was made of any conservative measures employed or if the patients continued to use support stockings post-operatively. It was not mentioned if the contra-lateral limb was screened for deep vein reflux detectable without any apparent clinical consequences.[233] In addition the vast majority of patients had reflux in the proximal deep veins. The ESCHAR study does address many of these issues and is referred to in the introductory section.

Superficial venous surgery has been shown to improve venous haemodynamics associated with SVI. In a study of 11 patients followed up prospectively, the Venous filling index, ejection fraction, residual volume fraction and the venous filling index

were shown to improve significantly at mean follow-up of 4 and 6 months post-operatively. Laser doppler flux and $tcP0_2$ were shown to improve following sclerotherapy and compression treatment in one study of 15 patients with moderate to severe SVI.[190] In another published paper the results of surgical treatment of eleven limbs with leg ulcers due to SVI was studied. The mean follow up was 16.4 months. All the ulcers healed in this study and remained healed for the duration of the follow-up. The mean clinical score decreased from 10.1 to 1.45. More recent studies of the efficacy of surgical treatment alone exist.[234] Longer follow-up results of such series are required to show many late recurrences.

Leucocyte Activation

Previous studies from this department have shown increased leucocyte activation in patients with CVD. Leucocyte mediated inflammatory mechanisms have been a focus of attention in CVD. Moyses et al reported sequestration of leucocytes in the circulation of the lower limb following venous hypertension in 1978. [235]. Bollinger et al showed that there were areas of very low skin blood flow in legs affected with CVD. These areas diminished on application of compression stockings[205]. Blood flow in the microcirculation is dependent on i. The arterio-venous gradient ii. The viscosity of the blood and iii. The vascular resistance. The white cells contribute importantly to the vascular resistance by being about 2000 times less compliant than the red cells. Thus 'sludging' of the WBCs can seriously affect the micro-circulatory haemodynamics. This is thought to be at least in part attributable to leucocyte-induced damage mediated through cytokines.

Coleridge-Smith et al proposed the hypothesis of leucocyte trapping due to reduction in capillary blood flow because of an increase in venous pressure in 1988.[236] A study published by the same group in 1997 showed that venous hypertension results in sequestration of the more activated population of neutrophils and monocytes in the microcirculation of the leg in patients with CVD. These cells bind to the endothelium and may not emerge from the limb when venous hypertension is reversed.[130]

Effects Of Compression on the Microcirculation

The exact mechanism of action of compression therapy in CVD remains unknown. The increase in flux through skin associated with compression therapy in CVD has been put

forward its possible mechanism of action A study of the microcirculation using laser Doppler fluxmetry to assess skin blood flow found that compressing the leg skin in patients with venous disease resulted in a 50% increase in laser Doppler flux when only 20 mm Hg of compression was applied. [105] When patients sat with their limbs dependent up to 60 mm Hg compression could be applied before laser Doppler flux began to decrease. This suggests that much of the efficacy of compression may be attributable to its effect on the microcirculation. It has been confirmed that compression stockings achieve the same effect on laser Doppler flux in the skin. [237] The fiber-optic method of laser Doppler fluxmetry used in these studies measured flow in both the nutritional vessels of the skin as well as the deeper thermoregulatory plexuses below, particularly from the sub-papillary plexus. [238]

In my studies direct measurement of blood flow in skin capillaries permitted an assessment of whether compression influences these vessels, and where the efficacy of compression occurs This may have implications for optimsising and individualising treatment. More than 20% of patients with venous leg ulceration have associated arterial disease. If the patient has arterial disease as well as venous incompetence it becomes difficult to gauge the extent of compression therapy that may be applied safely. Excessively high levels of compression may harm the patient by causing ischaemic necrosis of the skin over bony prominences. Although ABPI may dictate the maximum levels of compression that may be used it does not provide any guide to an optimum compression level. My study attempted to rationalize the use of compression.

Abu-Own's study suggests that the increased flux associated with skin compression involves both increased velocity as well as increased concentration of moving blood cells. My study did not concern change in diameter of the capillaries with increasing compression or the number of visible capillaries. These may be important parameters, however; the present study was concerned with the effect of compression on blood flow.

It has been previously been assumed that the application of compression to the lower limb restores valvular competence to venous valves[239,240,241]. Objective assessment of this assumption using duplex ultrasonography has failed to show that valvular competence can be restored by the application of moderate levels of compression, known to be effective in the treatment of venous ulceration [242]. However studies suggest

that moderate levels of compression do augment the calf pump mechanism and may lead to some improvement in air plethysmographic criteria. It is known that stockings, which exert 20 - 30 mm Hg, heal venous ulcers and prevent their recurrence. A previous study from this department investigated the effects of compression on the microcirculation of the limb using laser Doppler fluxmetry. It was found that increased flow of blood through the microcirculation of the skin occurred with compression levels as low as 20 mm. Therefore compression stockings may work at least partly by increasing the blood flow velocity through the capillaries and venules skin. This modifies the interaction of the blood, in particular the leucocytes, with the endothelium of the microcirculation preventing leucocyte adhesion and acting as an anti-inflammatory treatment. (It has been shown that WBC trapping in the leg may be prevented by stockings).

Clinical Implications

VEGF

VEGF is important both in angiogenesis and oedema formation.[243] VEGF acts via two different tyrosine kinase membrane receptors, which have been identified on vascular endothelial cells[244]. VEGF has an important part to play in the healing process. However, abnormally increased levels over a prolonged period of time can have local deleterious effects. It leads to increased micro-vascular permeability and angiogenesis. Up-regulation of VEGF expression in the skin and plasma of patients with CVD[15] has been demonstrated previously.

The expression of this molecule in the skin is more marked in patients with CEAP clinical stage C4 disease than in less severe forms of venous disease without skin damage[134]. It is acknowledged that the current data cannot demonstrate whether this finding is the cause or consequence of the skin changes. There is a possibility of using plasma VEGF levels as a surrogate for assessing lipodermatosclerosis by clinical means. Measurement of LDS in a quantitative way is notoriously difficult, and results in wide ranging discrepancies of the resulting data. [245]

Epidermally derived VEGF is likely to be a factor in the angiogenesis of lipodermatosclerosis. This increased expression of VEGF is seen even before any skin changes develop. I have shown increased serum levels of VEGF in my study in patients

with venous disease. Studies that measure the expression of VEGF mRNA levels may more conclusively prove the actual site of origin of the protein.

VEGF seems to act both acutely over a period of a few minutes as well as chronically (over a few hours) to increase micro-vascular permeability.[246] VEGF promotes the extravasation of fibrinogen and deposition in the tissues as fibrin. This could represent a mechanism for the formation of the 'Fibrin Cuffs' that are characteristically found in CVD. [247] There is no concrete evidence that these cuffs have a causative role. They are probably an association in the chronic inflammatory process.

Previous work had shown that leucocyte activation occurs within 30 minutes of experimental venous hypertension using the same model as employed in this study. This can be shown both in control subjects as well as those with venous disease. This is associated with evidence of endothelial activation and I believe these are the mechanisms that initiate the skin damage caused in CVD. This may initiate a repair process that involves increased expression of VEGF by keratinocytes and vascular smooth muscle cells. This causes the neo-vascularization essential to any tissue repair process. The neo-vasculature is permeable to large molecules when compared to normal capillary endothelium and allows the perivasular accumulation of large molecules accounting for the 'fibrin cuff' originally reported by Browse and Burnand. Such perivascular cuffs are of course common in many inflammatory conditions.

The physical interaction between the leucocyte and the micro-vascular endothelium is important for its function in the microvasculature.[248] Patients with chronic inflammatory and septic states show increased leucocyte priming. This is also observed in patients with CVD. The primed cells show increased surface CD62L and CD11B[130]. CD62L is involved in the initial 'rolling' movement of the leucocyte along the endothelium. CD11B is the molecule responsible for firmer adhesion and subsequent diapedesis. There is some evidence of inflammatory mechanisms being important in damage to the venous valves seen in CVD.[153]

Experimental blockade of adhesion molecules either with soluble ligands or with specific monoclonal antibodies has a dramatic effect on inflammation and immune

mediated tissue damage[164,249]. The observation that some markers are specifically lowered following therapy in patients with skin changes demonstrates the possibility of flavonoids to ameliorate and/ or arrest some of the skin damage that occurs in CVD. Inflammatory mechanisms have been implicated in the valvular damage seen in CVD[153] and these compounds may have a role in ameliorating this damage as well. These aspects merit further study.

The clinical syndrome of lipodermatosclerosis comprises a wide spectrum ranging from hyperpigmentation to inflammatory changes and fibrosis. It is possible that growth factors may play different roles at various stages but this has not been studied in detail.

The large difference between VEGF levels in C2/C3 and C4 patients could represent different stages of development of the dermatological changes. In the initial stages leucocyte mediated damage could be the predominant event. Later on the repair processes may become more important and may co-exist with continuing skin damage. If further studies indicate that high plasma levels of VEGF indicate impending skin changes, it could provide us with a useful tool for establishing the prognosis. Similarly if it could be demonstrated that absence of a fall in VEGF levels following therapy predicts a higher chance of recurrence. This could potentially be used as a marker of success of the treatment.

It has been demonstrated that Hyaluron oligosaccharides (OHA) in the intercellular matrix modulate the invasive and proteolytic properties of bovine micro-vascular endothelial cells and synergize specifically with VEGF in the induction of angiogenesis in vitro. The synergism between OHA and VEGF probably plays a role in the regulation of angiogenesis. This could be exploited therapeutically in situations that would benefit from modulation of new blood vessel growth.

VEGF has been associated with stimulation of Nitric Oxide (NO) production. The signaling cascade is thought to involve inducible NO synthase (iNOS).The pathways involve Guanylate cyclase and cGMP dependent protein kinase. Experimentally administration of VEGF was found to cause severe hypotension in animals. The hypo tension is thought to be mediated by production of NO and is reversible with

appropriate blocking therapy with N (G)-monomethyl-L- arginine (L-NMMA)[250-251]. NO can also explain the hyper-permeability induced by VEGF. An excessive release of nitric oxide can be caused by this mechanism. In contrast to its usual beneficial role in small doses, this free radical may contribute to local tissue damage in larger amounts.

Ulcer Healing & Superficial Vein (Varicose Vein) Surgery

Superficial venous surgery has been shown to improve the venous haemodynamics associated with CVI. The majority of patients with combined superficial and deep incompetence can be selected for SVS on the basis of AVP measurement with tourniquets. SVS can improve segmental deep incompetence and PRI in those properly selected[107]. In a study of 11 patients followed up prospectively the Venous filling index, ejection fraction, residual volume fraction and the venous filling index were shown to improve significantly at mean follow-up of 4 and 6 months post-operatively. In another study the laser Doppler flux and $tcP0_2$ were shown to improve following sclerotherapy and compression treatment in 15 patients with moderate to severe disease. [190]

Larger series of conservative management have shown similar results to the operative management. In one series 188 patients with recently healed leg ulceration were followed for at least 18 months. Overall cumulative recurrence rate was 26% after 1 year and 31% at 18 months[197]. Some series had suggested that >80 % of patients do not require any long-term support stockings after surgery.

It is quite possible that patients who have non-healing or recurrent venous ulcers have either had an inadequate operation and/or have a microangiopathy locally that persists despite correction of the venous reflux.

Presently there are no reliable markers either to assess the effectiveness of treatment of chronic venous disease (CVD) or to help in its prognosis. Many patients are worried about the long-term sequelae of their venous disease and do not want surgery merely for appearance's sake. There are also no reliable markers to indicate which patients will develop skin changes/ ulceration. Presently available markers of venous disease include clinical, hand held doppler, duplex ultrasonography, plethysmography (air, photo, strain gauge), ambulatory venous pressure, capillary microscopy and tonometric criteria. Many assessments are subjective and may be unreliable. Duplex examination after

therapy may indicate success in the short term but may not predict future recurrences or the course of skin complications.

My study of the effect of operative treatment on markers attempted to answer some of these questions. The initial increased lactoferrin (at four weeks) in my study imay be due to the effect of operative trauma on the leucocytes. The subsequent decline in lactoferrin may be due to the corrected venous hypertension. Changes in the level of VEGF may have some clinical significance.

The decrease in lactoferrin seen at six months post-operatively may be explained on removal of segments of veins that were causing venous stasis and leucocyte activation. Changes in levels of plasma VEGF may be explained by a continuing process of vascular remodelling in these patients. It is unknown at the moment if clinical recurrences of veins or persistence/ worsening of skin changes are preceded by increase in the levels of these markers.

My study of the effect of compression on the microcirculation indicates the possible optimum levels of compression likely to be effective in the management of chronic venous disease and in combined venous and arterial disease. This may be helpful in the design of more effective methods of compression, especially for the treatment of patients with combined venous & arterial disease. It may indicate whether compression is helpful in the lying position as well as when the patient is standing or sitting.

Further Studies

Experimental blockade of adhesion molecules either with soluble ligands or with specific monoclonal antibodies has a dramatic effect on inflammation and immune mediated tissue damage[164, 252]. The observation that some markers are specifically lowered following therapy in patients with skin changes demonstrates the possibility of flavonoids to ameliorate and/ or implicated in the valvular damage seen in CVD[153] and these compounds may have a role in ameliorating this damage as well. Other modalities of modifying adhesion molecule activity may have a role in CVD. These aspects merit further study.

VEGF; Therpeutic Implications

VEGF blocking has shown some promise experimentally in oncological therapy[253 254]. Linomide and VEGF-blocking anti-bodies have been used for this purpose. Tamoxifen has been shown to have some anti-angiogenic activity. The role of such therapy in CVD remains to be explored. Ancillary therapy in CVD to reduce recurrence or accelerate healing of skin lesions in selected cases may be a possibility in the future.

Further studies are needed to assess further the role of soluble endothelial markers in prognosticating the clinical course of CVD. The role of purified micronised flavonoid fraction and similar compounds also needs to be re-examined in the context of non-surgical treatment for CVD.

Monitoring Response to Therapy

It is only relatively recently that the effectiveness of therapy for CVD is being tested with objective parameters. It has been shown that colour duplex ultra-sonographic and air-plethysmographic criteria improve in patients one month following surgical therapy. This improvement was shown to be maintained at two years[162]. It would be interesting to measure the mid to long-term effect of therapy on endothelial markers. Another study of the effect of compression therapy/sclerotherapy on the microangiopathy (Leu et al)[163] showed non-significant changes post therapy. The parameters studied were capillary microscopy, Doppler flux-metry and $tcPo_2$ measurement. Elevated levels of VCAM-1, VW Factor, E-selectin, ICAM-1 and Lactoferrin have been reported in patients with CVD compared to controls[130]. However my study is the first to demonstrate a measurable change in these parameters in CVD following therapy. This shows the feasibility of using the soluble markers of endothelial activation as a parameter to measure the response to various forms of treatment.

Role of Flavonoids in CVD

The effects of flavonoids on Duplex ultra-sonographic parameters and on resolution of lipodermatosclerosis need to be quantified in prospective studies. The lack of side effects of these compounds makes them especially attractive as medications for CVD. Experimentally flavonoids have been shown to have several anti-inflammatory actions.

My study is the first to demonstrate a possible mode of action for these compounds in the clinical setting.

Comments

To summarise all these observations a possible scenario for the sequence of events in skin injury in CVD may be the following. Damage to the tissues is caused by abnormal leucocyte activation. The injury itself may stimulate a VEGF response. Activated platelets or macrophages present at the site of injury may have a role in stimulating VEGF release. The abundant macrophages in skin exposed to CVD are a potential source of VEGF as well. These could be within the basement membrane or extra-cellular matrix. Components of the extra-cellular matrix like heparin proteo-glycans are also exposed by injury. These are known to facilitate migration and tube formation by endothelial cells. -[255] Re-synthesis of extra-cellular matrix components and migration of pericytes may represent a mechanism of dampening the angiogenic stimulus.[256]

I have shown that increase in some endothelial activation markers studied was dampened by the administration of purified micronised flavonoid. This suggests that these compounds may down-regulate the activated endothelium in these patients. This study demonstrates the feasibility of using changes in levels of soluble endothelial markers as parameters for assessing the response to therapy in CVD.

Plasma VEGF activity decreases in patients with skin changes dues to CVD following treatment with purified micronised flavonoid fraction. VEGF protein levels may be much higher in some of these patients as compared to those with uncomplicated CVD. The role of confounding factors like seasonal variations etc. has to be considered. The role of circulating VEGF as a marker for skin complications in these patients needs to be explored further.

In my study of ulcer recurrence following varicose vein surgery high initial rates of ulcer healing were seen with surgery. About a third recurred in my study and a similar number still had an ulcer more than a year after their operation. These findings have been corroborated by further clinical studies like the ESCHAR study. This suggests that either the altered venous haemodynamics were not corrected at the initial operation and/

or these patients had developed a microangiopathy not reversible with operative treatment alone. They may have a predisposition to ulceration that is not changed by surgery alone. Every effort should be made to make varicose vein surgery anatomically and haemodynamically precise. The exact nature of the microangiopathy and the inflammatory mechanisms involved may need to be addressed simultaneously. Further studies to continue follow-up of patient prospectively using the CEAP classification are warranted.[134] Equally important are further studies into the micro-circulatory aspects of the disease.

Operative treatment of varicose veins leads to an initial increase in lactoferrin followed by a decrease to below pre- operative levels at six months. This may indicate success of operative treatment in ameliorating the leucocyte/ endothelial activation responsible for the complications of CVD. Longer-term follow up is indicated to observe the effect of recurrent varicose veins on leucocyte activation (or vice versa) in these patients over a period. Many of the markers of endothelial activation were increased at six months post-surgery. These results are harder to interpret meaningfulluy. Plasma VEGF showed a similar, but more marked trend. This may be explained on some form of vascular remodelling that may occur after operative treatment to varicose veins. The change in VEGF levels may have a similar explanation. These phenomena may or may not be related to the recurrence of varicose veins and/ or the non-healing of venous hypertension induced skin changes.

The laser anemometer is a research tool that my help in the design of better methods of compression. Skin compression increases the capillary blood velocity in the sub-papillary plexus in patients with mixed disease. Thus compression therapy does seem to help in these patients, however, maximum velocities are reached at relatively smaller pressures especially in the supine position. The fact that maximum velocities in these patients are reached at 20 mm in supine and 40 mm in sitting positions suggests that lower levels of compression may be useful in these patients. Further work needs to be done to observe if compression pressures for patients for individualised therapy are deducible from this method.

PART VI

CONCLUSIONS

The studies included in this thesis support the hypothesis that there are measurable changes in leucocyte/endothelial activation and microcirculatory stasis in CVD in response to therapy. Increased plasma 'Vascular endothelial growth factor' (VEGF) in patients with CVD was also clearly demonstrated. Further studies may show if a correlation between clinical status/ prognosis and these parameters exists. These parameters may be used as objective measures of response to treatment. Increased endothelial/ leucocyte activation seems to respond favourably to therapy in CVD.

Plasma levels of VCAM, ICAM-1, vW factor Lactoferrin are markers of increased microcirculatory activity. The increased endothelial activation and neutrophil activation/ degranulation seem to be ameliorated by pharmacological compounds (purified flavonoid fraction) that have been shown to be of symptomatic benefit in CVD. Plasma levels of VCAM, ICAM-1 are significantly reduced following therapy with oral purified micronised flavonoid fraction. In addition levels of VW-factor and Lactoferrin are reduced significantly in patients with C4 disease.

Following varicose vein surgery a statistically significant change was seen in the level of plasma lactoferrin levels. However at 6 months plasma E-Selectin and VCAM were higher to some degree than pre-operative levels. Changes in VEGF levels failed to reach significant levels. As mentioned the latter results may be harder to interpret.

Compression therapy seems to increases the skin blood flow velocity in patients with pure CVD as well as in those with mixed arterial and CVD. This may lead to increased shear at the venular/ capillary endothelial interface. This may decrease leucocyte/ endothelial interaction and possibly help in ameliorating the effects of CVD. In my model of acrylic disc skin compression I demonstrated the increased velocity of blood in the subpapillary plexus. I also showed that lower levels of compression (20 mm Hg) are effective in increasing velocity in patients with mixed disease. I have demonstrated in this thesis that the sluggish microcirculatory flow, endothelial activation, VEGF activity & increased neutrophil degranulation activity been described previously in chronic venous disease show changes in response to various forms of therapy.

Critique of the Research Presented In the Thesis

Although the research presented in the thesis was performed some time ago the inflammatory aspects of CVD remain topical issues. They are the subject of much debate and ongoing discussion. CVD continues to be a cause of much morbidity as well as a drain on NHS resources.

The microcirculatory changes in chronic venous disease were described in the early 1980s and 1990s. Thus they are not new findings. However, there were relatively few studies recording the changes in these parameters in response to treatment. The techniques used for measurement of the markers of endothelial activation were well established ELISA techniques. The CAM capillary anaemometer was a new instrument looking at the velocity of blood in specific sub-papillary nutritive vessels.

It is acknowledged that the understanding of the VEGF molecule has progressed considerably recently. The original classification is VEGF A (the main VEGF), B, C and PlGF. The results of the study measure the main molecule (VEGF A) and are relevant[♣]. These were the first todemonstrate increased VEGF in chronic venous disease.

It is also acknowledged that larger studies may show differing seasonal temporal, sexual and age-related changes in plasma levels of VEGF in chronic venous disease. Many of the studies include relatively few patients. The scope of this work, however, was to perform initial studies to look at the levels in a population of subjects with/ without clinical chronic venous disease. Further larger studies would be useful and informative in this regard.

Positive Conclusions from this Work

I demonstrated a decrease in Lactoferrin levels at six months following surgery (36%). My report regardin increased plasma VEGF in patients with CVD was the first such

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[♣] In alternative classifications the term 'VEGF' covers a number of proteins from two families, that result from alternate splicing of mRNA from a single, 8 exon, VEGF gene. The two different families are referred to according to their terminal exon (exon 8) splice site - the proximal splice site (denoted VEGFxxx) or distal splice site (VEGFxxxb). In addition, alternate splicing of exon 6 and 7 alters their heparin binding affinity, and amino acid number (in humans: VEGF121, VEGF121b, VEGF145, VEGF165, VEGF165b, VEGF189, VEGF206).
PlGF= Placenta Growth Factor

published report. This may explain the link between the white cell activation and 'fibrin' cuff theories (i.e. white cell activation in inflammation triggers VEGF release that is a well known permeaboility factor allowing extravastion of Fibrin).

I demonstrated decrease in plasma leels of VEGF, ICAM-1, VCAM, vW Factor & Lactoferrin after 60 days treatment with oral Flavonoids. I also showed that the sub-papillary capillary blood velocity increases during the range of pressures used therapeutically.These velocities were highest at about 60 mm Hg in CVD and about 40mm Hg in mxed disease. These findings may help explain some of the mechanisms by which compression treatment may work.

Practical Clinical Applications

The study involving Flavonoid confirmed improvement in some markers of endothelial activation. It also confirmed some clinical benefit. Since these were initial studies, larger studies were awaited before there was more generalised prescription of Flavonoid. These compounds do continue to be prescribed by some clinicians in this country. Whether or not different (& stronger!) anti-inflammatory agent (e.g. NSAIDS) would be effective in this clinical setting remains to be proven[ℜ].

As suggested it may be a possible application of this data to determine whether the treatment (surgical/ pharmacological/ physical) being applied is effective. However the scope of these studies would not allow such conclusions to be made. It may also be possible to test devices that allow bespoke best compression levels for individual subjects.

The study of response of capillary velocity to skin compression is indicative that patients with mixed disease may indeed benefit from a lower level of compression

Publications & Citations

[ℜ] Both NSAIDS (non-selective/ COX-2) as well as corticosteroids are eicosanoid inhibitors. They may be ant-inflammatory at different stages. Systemic therapy with a useful benefit/ risk ratio has not been demonstrated in chronic venous disease. NSAIDS are currently mainly used for acute thrombophlebitis and sometimes in acute inflammatory exacerbations of lipodermatosclerosis. Local steroid creams are in widespread use for venous eczema.

Several presentations/ publications resulted directly from the work in this thesis. Three of the papers were published in widely circulated, high quality, peer reviewed journals (Journal of Vascular Surgery/ European Journal of Vascular & Endovascular Surgery). Copies of these have been appended at the end of the thesis. One other published article is also included. Several studies followed on upon the work in this thesis and resulted in published work[¥].

Further Studies

It is acknowledged that an added study of effect of compression on plasma markers of endothelial/ leucocyte activation would have been useful in comparing this response to that seen with pharmacotherapy and surgery. Constraints of the project did not allow that to happen but it would have been a very informative study to perform.

The study of the outcome of treatment was meant to try and quantify possible outcomes after surgical treatment of duplex proven varicose vein disease in subjects with leg ulcers. Because of the long-term results required a prospective study was not possible in the constraints of the project. Larger population longer term results may now become available through centrally maintained web based registeries.

Studies into the role of VEGF monitoring in patients with chronic venous disease looking at a larger population may be useful and informative.

As suggested it may be a possible application of this data to determine whether the treatment being applied is effective. However the scope of these studies would not allow such conclusions to be made.

[¥] See Appendix I

PART VIII

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PART IX

APPENDICES

APPENDIX I- PUB LISHED WORK & OTHER CITATIONS

ORIGINAL PAPERS

1. Shoab SS, J. Porter, J. H. Scurr, P. D. Coleridge.Plasma VEGF As A Marker Of Therapy In Patients With Chronic Venous Disease Treated With Oral Micronised Flavonoid Fraction-A Pilot Study. European Journal of Vascular & Endovascular Surgery Volume 17- issue 10- October 1999. 334-338
   Cited by 32 publications[¥]

2. **Shoab SS**, J. Porter, J. H. Scurr, P. D. Coleridge Smith. Endothelial Activation Response to Oral Micronised Flavonoid Therapy in Patients with Chronic Venous Disease a Prospective Study. **European Journal of Vascular & Endovascular Surgery** Volume 17 - Issue 4 - April 1999. *313-318*
   Cited by 28 publications

3. **Shoab SS**, Scurr JH, Coleridge-Smith PD. Increased Plasma Vascular Endothelial Growth Factor among patients with Chronic Venous disease. ***Journal of Vascular Surgery*** 1998; 28: 535-40
   Cited by 20 publications

4. **Shoab SS**, JH Scurr & PD Coleridge-Smith. Effect of induced venous hypertension on plasma VEGF in patients with chronic venous disease. **Scripta Phlebologica**.Oktober 1998: Vol 6; 55-57.

PRESENTATIONS

1. **Shoab SS/** P D Coleridge-Smith **Effect of Operative treatment on Leucocyte/Endothelial activation in Patients with Chronic Venous Disease**-A Prospective Study. Royal Society Venous Forum Meeting, Gloucester, April 2001

2. **Shoab SS**/ M Howlader/ P D Coleridge-Smith. The effect of skin compression on capillary blood velocity in patients with combined arterial & venous disease. ***European Venous forum*** meeting, Lyon France 29 Jun 2000. **Prize European Venous Forum.
   Cited by 1 publication

3. **Skin Compression Increases Capillary Blood Velocity In Patients With Chronic Venous Disease**. S Sulaiman-Shoab/ J H Scurr/ P D Coleridge-Smith. Presentation Venous Forum Royal Society of Medicine, Manchester April 1999.

4. **Levels Of Soluble Endothelial Adhesion Molecules Persist after Corrective Surgery for Superficial Venous Reflux**. S Sulaiman-Shoab/ J H Scurr/ P D Coleridge-Smith. Presentation Venous Forum Royal Society of Medicine, Manchester April 1999.

[¥] For an actual list of citations see www.scholar.google.com

5. **Increased Leucocyte Activation Persists After Corrective Surgery For Superficial Venous Reflux.** S Sulaiman-Shoab/ J H Scurr/ P D Coleridge-Smith. Presentation, American Venous Forum Dana Point California Feb 1999.

6. **Outcome of leg ulceration due to superficial venous disease (mid to long term results).;** S Sulaiman-Shoab/ J H Scurr/ P D Coleridge-Smith. Presentation Venous Forum of the Royal Society of Medicine. Oct 1997.

7. **Endothelial adhesion molecule expression in patients with chronic venous disease- Effect of oral purified micronised flavonoid fraction**
   Presentation

   International Union of Phlebology (UIP) Meeting Sydney Australia Sep 1998

8. **Increased Plasma Vascular Endothelial Growth Factor in Venous disease.**
   The 3rd North Sea Meeting on Venous Diseases. May 23-24, 1997 Bruges, Belgium.
   Venous Forum of the Royal Society of Medicine. Oct 1997.Abstract published in phlebology; 1997.

9. **Direct medial and lateral tributaries of the Femoral vein - A prospective study;**
   Venous Forum of the Royal Society of Medicine. Oct 1997.
   Sixth meeting of the Antyllus society, at The Royal Society. 1996.

   Abstract published in phlebology; 1997.

CORRESPONDENCE

1. **Role of superficial venous surgery in the treatment of venous ulceration** S S Shoab & PD Coleridge Smith: *correspondence section* Br J Surg. 1999; 86: 1475-6.
   Cited by 28 publications[¥]

2. **Saphenous Vein stripping and quality of outcome,** *Correspondence section. Br J Surg 1997; 84:139.*
   Cited by 7 publications

TEXT BOOK CITATION

Lees TA & Redwood NFW. Chronic Venous Insufficiency & Lymphoedema; In Eds. Beard JD & Gaines PA Vascular & Endovascular Surgery 2nd Edition, W.B. Saunders 2001:451-482.

[¥] Please refer to www.scholar.google.com for an actual list of citations

SUBSEQUENT STUDIES UTILISING FINDINGS IN THIS THESIS

1) Howlader MH, Smith PD. Correlation of severity of chronic venous disease with capillary morphology assessed by capillary microscopy. J Vasc Surg. 2006 ;43(3):563-9.
   The data from the CAM capillary anemometer lead to this work.

2) Howlader MH, Coleridge Smith PD. Relationship of plasma vascular endothelial growth factor to CEAP clinical stage and symptoms in patients with chronic venous disease. Eur J Vasc Endovasc Surg. 2004 ;27(1):89-93.
   This followed from my study that showed increased vascular endothelial growth factor among patients with chronic venous disease.

3) Howlader MH, Smith PD. Symptoms of chronic venous disease and association with systemic inflammatory markers. J Vasc Surg. 2003 ;38(5):950-4.
   My study of changes in endothelial/ leucocyte markers were precursors to this work

4) Howlader MH, Smith PD. Microangiopathy in chronic venous insufficiency: quantitative assessment by capillary microscopy. Eur J Vasc Endovasc Surg. 2003 ; 26(3):325-31.
   The morphological data from the CAM capillary anemometer lead to this study being performed.this is mentioned in my thesis and photographs from the CAM are included.

5) Howlader MH, Smith PD. Increased plasma total nitric oxide among patients with severe chronic venous disease. Int Angiol. 2002 ;21(2):180-6.
   NO and VEGF are closely related. My studies involving plasma VEGF in chrnic vein disease (the first reported series) were a precursor to this study.

APPENDIX II

Classification of chronic venous disease (C, E, A, P) (Original)

Limbs with chronic venous disease should be classified according to clinical signs (C), cause (E), anatomic distribution (A), and path physiologic condition (P). The classification system detailed below and summarized in Table I was developed in 1994 by an international consensus conference on chronic venous disease held under the auspices of the American Venous Forum.

Clinical classification (C_{0-6})...

It replaces clinical classes 0 to 3 outlined in the 1988 version of 'Reporting Standards in Venous Disease'. This updated method of classifying chronic venous disease is designed to provide the additional details necessary to accurately compare limbs in medical and surgical treatment trials.

Any limb with possible chronic venous disease is first placed into one of seven clinical classes (C_{0-6}) according to the objective signs of disease listed.

Venous-dilation

Mild chronic venous insufficiency (CVI) is signified by the occurrence of a submalleolar venous flare. Greater degrees of venous dilation are apparent by both observation and

palpation. Telangiectasiae are defined as dilated intradermal venules less than 1 mm in Size. Reticular veins are defined as dilated, nonpalpable; sub dermal veins 4 mm in size or less. Varicose veins are defined as dilated palpable subcutaneous veins generally larger than 4 mm.

Oedema.

The presence of oedema indicates more functionally advanced venous disease than the presence of venous dilation alone. In individual case reports and when otherwise applicable, the location and extent of oedema should be noted and objectively documented with circumferential limb measurements.

Classification of chronic lower extremity venous disease	
C	Clinical signs (grade$_{0\text{-}6}$), supplemented by ($_A$) for asymptomatic and ($_S$) for symptomatic presentation
E	Etiologic Classification (Congenital, Primary, Secondary)
A	Anatomic Distribution (Superficial, Deep, or Perforator, alone or in combination)
P	Path physiologic Dysfunction (Reflux or Obstruction, alone or in combination)

Table 27 Classification of chronic lower extremity venous disease

Skin

pigmentation

Pigmentation changes and other cutaneous manifestations of chronic venous disease (venous eczema, lipodermatosclerosis) are important signs of severe chronic venous disease. They should be described along with a subjective assessment of severity.

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Venous

ulceration

The location and measurements of any venous ulcer should be described, and the presence absence of granulation tissue should be noted. The presence of healed venous ulceration manifested by cutaneous scarring should be noted

Limbs in higher categories have more severe signs of chronic venous disease and may have some or all of the findings defining a less severe clinical category. Each limb is further characterized as asymptomatic (A), for example, $C_{0\text{-}6,A}$, or symptomatic (S), for example,

$C_{0\text{-}6,S}$. Symptoms that may be associated with telangiectacic, reticular, or varicose veins include lower extremity aching, pain, and skin irritation. Therapy may alter the clinical category of chronic venous disease. Limbs should therefore be reclassified after any form of medical or surgical treatment.

Clinical classification of chronic lower extremity venous disease	
Class 0	No visible or palpable signs of venous disease
Class 1	Telangiectasiae, reticular veins, malleolar flare
Class 2	Varicose veins
Class 3	Edema without skin changes
Class 4	Skin changes ascribed to venous disease (e.g., pigmentation, venous eczema, lipodermatosclerosis)
Class 5	Skin changes as defined above with healed ulceration
Class 6	Skin changes as defined above with active ulceration

Table 28 Clinical classification of chronic lower extremity venous disease

Anatomic classification ($A_{S,D,P}$).

The anatomic site(s) of the venous disease should be described as superficial (A_S), deep combination. For reports requiring greater detail, the involvement of the superficial, deep, and perforating veins may be localized by use of the anatomic segments listed .

Measurement by superficial venous canulation of the foot venous pressure at rest in the upright position and the change in pressure on walking has historically represented the "gold standard" for the overall objective assessment of chronic venous disease. [25] It is now recommended that reports of patients with chronic venous disease be accompanied by sufficient objective measurements of venous haemodynamics and anatomy to document adequately the individual patho-physiologic changes, reflux, obstruction, or both, accompanying chronic venous disease. Phlebographic or vascular laboratory studies can (A_D), or perforating (A_P) vein(s). One, two, or three systems may be involved in any objectively assess the presence of venous outflow obstruction (PO), as well as the presence of venous reflux (PR) in the superficial, communicating, and deep venous systems.

Table 1.8. ***Etiologic classification of chronic lower extremity venous disease***	
Congenital (E_C)	The cause of the chronic venous disease has been present since birth
Primary (E_P)	Chronic venous disease of undetermined cause
Secondary (E_S)	Chronic venous disease with an associated known cause (postthrombotic, posttraumatic, other)

Table 29 Etiologic classification of chronic lower extremity venous disease

Patho-physiologic classification (PR,O).

Clinical signs or symptoms of chronic venous disease result from reflux (PR), obstruction (PO), or both (PR,O). Ascending phlebography defines areas of obstruction, recanalization, and collateral vein formation. Descending phlebography demonstrates competency of venous valves by assessing the magnitude of contrast reflux. Comparisons of different reports of with the venous segments outlined in Table IV. Currently, duplex ultrasonography performed with the patient in an upright position and with the limb

examined in a non Wight bearing position, in combination with proximal deflation of a venous occluding blood pressure cuff, [24] is the best-documented noninvasive method of quantifying reflux, by measuring reflux duration in specific axial superficial or deep venous segments. Duplex scanning is also suggested as a means of identifying reflux in individual communicating veins.

Patho-physiologic classification of chronic lower extremity venous disease
Reflux (P_R)
Obstruction (P_O)
Reflux and obstruction ($P_{R,O}$)

Table 30 Patho-physiologic classification of chronic lower extremity venous disease

Segmental localisation of chronic lower extremity venous disease	
Segment no.	
	Superficial veins (A_{S1-5})
1	Telangiectases/reticular veins
	Greater (long) saphenous vein
2	Above-knee
3	Below-knee
4	Lesser (short) saphenous vein
5	Non-saphenous
	Deep veins (A_{D6-16})
6	Inferior vena cava
	Iliac
7	Common
8	Internal
9	External
10	Pelvic: gonadal, broad ligament
	Femoral
11	Common
12	Deep
13	Superficial
14	Popliteal
15	Tibial (anterior, posterior or peroneal)
16	Muscular (gastrointestinal, soleal, other)
	Perforating veins ($A_{P17,18}$)
17	Thigh
18	Calf

Table 31 Segmental localiszation of chronic lower extremity venous disease

The modified CEAP classification was proposed[257], the clinical classification according to this is presented in table form below. To improve the assignment of designations under E, A, and P a new descriptor, n, is now recommended for use where no venous abnormality is identified. This n could be added to E (En, no venous cause identified), A (An, no venous location identified), and P (Pn, no venous pathophysiology identified). The authors of the 'new' CEAP stress that CEAP is a descriptive

classification, whereas venous severity scoring and quality of life scores are instruments for longitudinal research to assess outcomes.

The authors also recommend that the level of investigation in CVD can be logically organized into 1 or more of 3 levels of testing, depending on the severity of the disease: They also suggest that this be reported in the staging.

Level I: office visit, with history and clinical examination, which may include use of a hand-held Doppler scanner.

Level II: noninvasive vascular laboratory testing, which now routinely includes duplex color scanning, with some plethysmographic method added as desired.

Level III: invasive investigations or more complex imaging studies, including ascending and descending venography, venous pressure measurements, computed tomography (CT), venous helical scanning, or magnetic resonance imaging (MRI).

C_0 No visible or palpable signs of venous disease.
C_1 Telangiectasies or reticular veins.
C_2 Varicose veins; distinguished from reticular veins by a diameter of 3 mm or more.
C_3 Edema.
C_4 Changes in skin and subcutaneous tissue secondary to CVD, now divided into 2 subclasses to better define the differing severity of venous disease:
- **C_{4a}** Pigmentation or eczema.
- **C_{4b}** Lipodermatosclerosis or atrophie blanche.

C_5 Healed venous ulcer.
C_6 Active venous ulcer.

The C clinical stages of the ‘new’ CEAP classification

APPENDIX III

Enzyme-Linked Immunosorbant Assays (ELISA)

ELISA is an alternative solid-phase readout system (and mostly took over from radio immunoassay) in which antibodies or antigen are covalently coupled to an enzyme instead of a radioisotope so that bound enzyme activity is measured instead of radioactivity. Some of the enzyme labels are shown in table1. In practice the safety and convenience of non-radioactive materials and the commercial availability of plate readers that can measure the absorbance of 96 wells in 1 minute account for ELISAs growing popularity. Since both ELISA and RIA are governed by the same thermodynamic constraints, and the enzyme can be detected in the same range of molarity as commonly used radioisotopes. The sensitivity and specificity are comparable. I consider four basic strategies for using FLISA assays to detect specific antibody, antigen or cross-reacting antibodies.

As shown in figure 26, the indirect antibody method is the simplest way to detect and measure specific antibody in an unknown antiserum. Antigen is non-covalently attached to each well of a plastic micro titre dish. For this purpose it is fortunate that most proteins bind nonspecifically to plastic. Excess free antigen is washed off and the vials are incubated with an albumin solution to block the remaining nonspecific protein binding sites. The test antiserum is then added, and any specific antibody binds to the solid-phase antigen. Washing removes unbound antibodies. Enzyme-labelled anti-immunoglobulin is added. This binds to specific antibody already bound to antigen on the solid phase bringing along covalently attached enzyme. Unbound antiglobulin-enzyme conjugate is washed off; then substrate is added. The action of bound enzyme on substrate produces a coloured product that is detected as increased absorbance in a spectrophotometer.

Although this method is quick and very sensitive it is often difficult to quantify. Within a defined range the increase in optical density is proportional to the amount of specific antibody (added in the first step). However the amount of antibody bound is not measured directly. Instead, the antibody concentration of the sample is estimated by comparing it with a standard curve for a known amount of antibody. It is also difficult

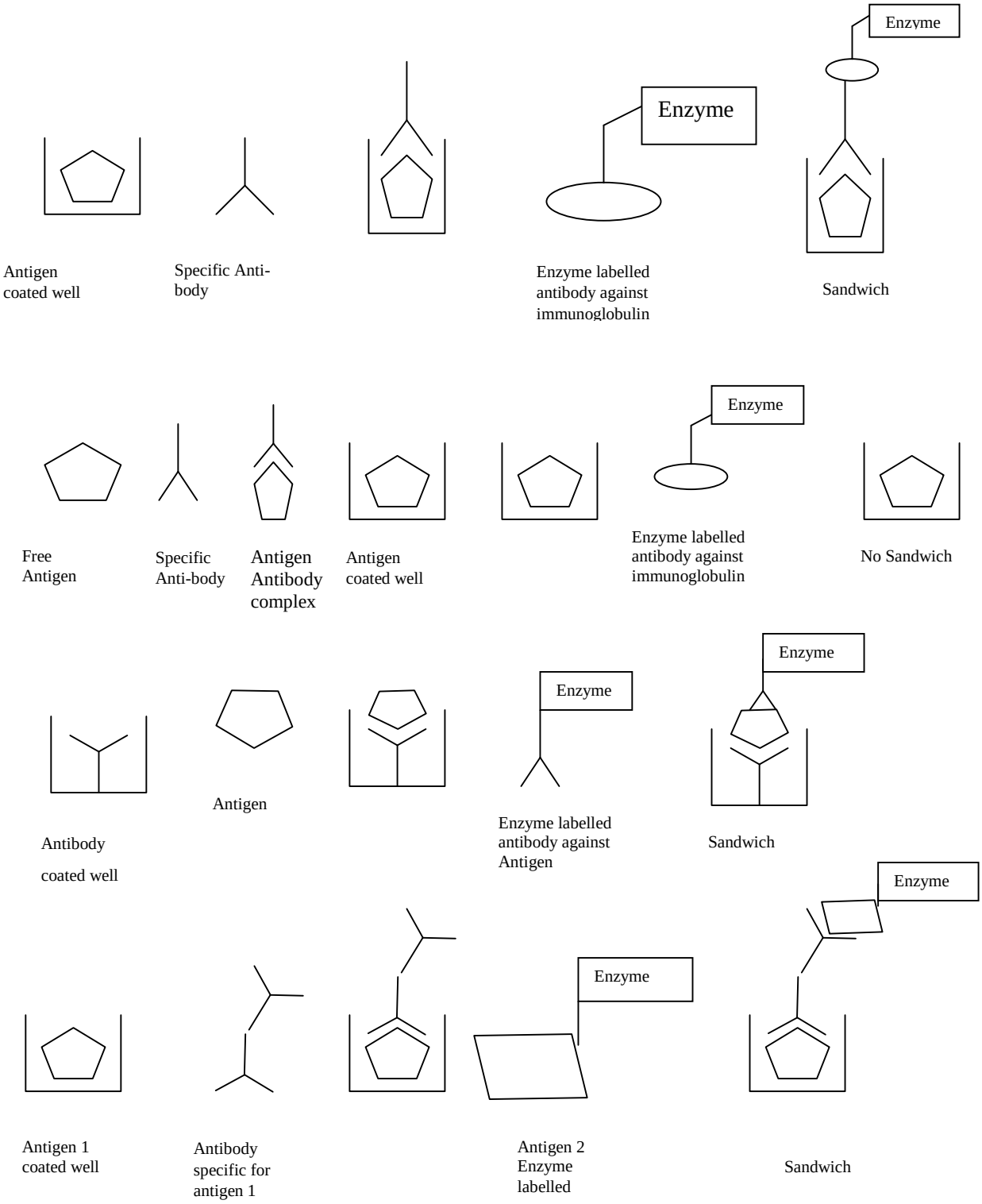


Figure 34 shows diagrammatically various types of ELISA tests. Each of the four rows depicts a different test.

to determine affinity by this method, since the solid-phase antigen tends to increase the apparent affinity. A single preparation of enzyme-linked antiglobulin can be used to detect antibodies to many different antigens. Alternatively class specific antiglobulins can be used to detect how much of a specific antibody response is due to each immunoglobulin class. Obviously reproducibility of the assay depends on uniform antigen coating of each well that can vary. The specificity depends on using purified antigen to coat the wells.

Figure **34** also shows the competition technique for detecting antigen. Soluble antigen is mixed with limiting amounts of specific antibody in the first step. Then the mixture is added to antigen-coated wells and treated is described. Any antigen-antibody complexes formed in the first step will reduce the amount of antibody bound to the plate and hence will reduce the absorbance measured in the final step. This method permits the estimate of affinity for free antigen which is related to the half-inhibitory concentration of antigen. In addition, some estimate of cross-reactivity between the antigen in solution and that on the plate can be obtained.

The figure also shows the sandwich technique for detecting antigen. Specific antibody is used to coat the micro-titre wells. Antigen is then bound to the solid-phase antibody. Finally, a second antibody linked to enzyme, is added. This binds to the solid-phase antigen-antibody complex carrying enzyme along with it. Excess second antibody is washed off and substrate is added. The absorbance produced is a function of the antigen concentration of the test solution, which can be determined from a standard curve. Specificity of the assay depends on the specificity of the antibodies used to coat the plate and detect antigen. Sensitivity depends on affinities as well as the (amount of the first antibody bound to the well that can be increased by using affinity-purified antibodies in the coating step. The binding of both antibodies of the sandwich depends on divalency of the antigen, or else the two antibodies must be specific for different antigenic determinants on the same antigen molecule. If the antibodies are two different monoclonal antibodies that both bind to the same monomeric antigen. This technique can be used to ascertain whether

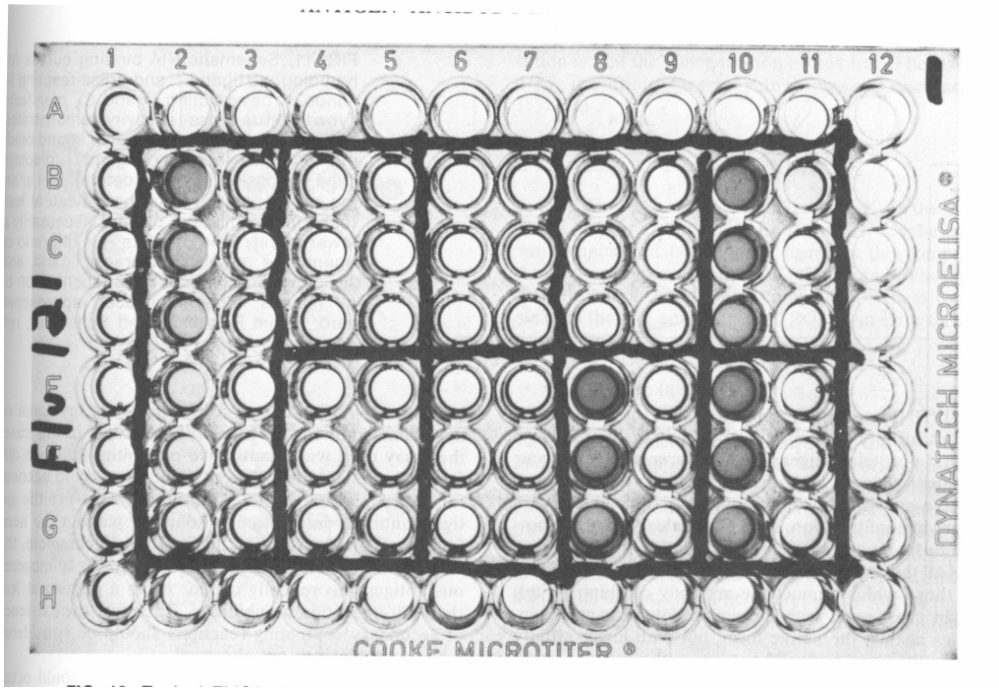


Figure 35 is a photogtraphhotograph of a typical 96 well ELISA plate.

Enzyme	Source	Molecular Weight	Turnover rate*
Alkaline Phosphatase	Calf Intestine	140,000	420,000
13-Galactosidase	E Coli	540,000	324,000
Glucose Oxidase	Aspergillus Niger	186,000	53,000
G6PD	Leuconostoc mesenteroides	130,000	93,600
Peroxidase	Horseradish	40,000	220,000
Urease	Black Bean	540,000	450,000

*Turnover rate is the number of moles of product released per munite per mole of enzyme at the designated temperature

Figure 36 Enzymes used in ELISA tests

the two antibodies can bind simultaneously to the same molecule or whether they compete for the same site or sites close enough to cause steric hindrance. The sandwich technique for detecting antibody cross-reactivity is also depicted. The wells are coated with antigen I, followed by the adsorption of specific antibody for antigen 1. Excess antibody is washed off, and enzyme-labelled antigen 2 is added. If bound antibody cross-reacts with both antigen I and antigen 2 then enzyme-labelled antigen 2 will adsorb onto the solid phase. This will be measured as increased absorbance of the enzyme-catalyzed reaction. This method has been used to assay the cross-reactivity of anti-idiotype antisera for two preparations of idiotype or for detection of anti anti-idiotype. Other arrangements of antibody and antigen are also possible. Extra layers of detecting agents can amplify sensitivity but also tend to raise the background and introduce variability.

An example of the first method described above is the election of human antibodies to influenza virus. Alternate columns were coated with influenza virus or bovine 'albumin. Serum is added at 1/10 dilution to the top two wells of each box and serially diluted in four fold steps from top to bottom. The last coloured well indicates the titre, whereas the absence of colour in the albumin-coated wells indicates the specificity. A second use of this method is for screening culture supernatants in the production of hybridoma antibodies. The sensitivity and peed of the ELISA method make it possible to screen large numbers of wells for the production of specific antibody. (Clones selected by this method tend to have high antigen affinities, perhaps due to dissociation of low-affinity antibodies during the wash steps.

An important caution when using native protein antigens to coat solid phase surfaces (Figure 34) is that binding to surface can alter the conformation of the protein. For instance using conformation specific monoclonal antibodies to myoglobin it was found that binding of myoglobin to a surface altered the apparent affinity of some antibodies more than others.

APPENDIX IV

CAM 1 Capillary Anemometer

Optical System

A schematic for the CAM1 is shown in Figure 37. A 7 mW 780 nm laser diode is focused by lenses l1 and objective l2. The position of l1 is adjusted so that the waist of the laser beam is in the object plane of the CCD camera and the photodetector. The position of mirror m1 is adjusted so that the laser beam is in the centre of the image. The position is set so that the photodetector is aligned with the image of the laser beam focal point. For maximum contrast between red blood cells and the surrounding tissue, green light of 525 nm is used for illumination for the CCD camera. Beam splitter bs1 separates the laser light from the green ccd image. The 50:50 beam splitter bs2 splits the backscattered laser radiation via mirror m2 onto the detector. Laser power at the focal point is typically 0.8 to 1 mW with an elliptical spot of about 5 m by 10 m. zw

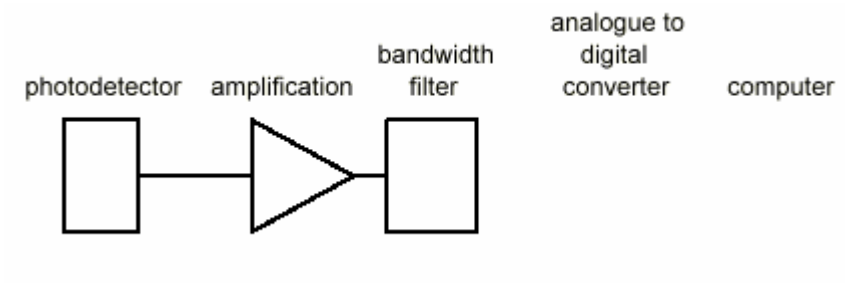


Figure 37 Schematic drawing of signal processing in the CAM-1

small fraction of the laser light will be backscattered by blood cells and collected by lens l1. Lens l1 will also collect laser light reflected from the surrounding tissue. Laser light backscattered by a moving blood cell will shift the frequency of the light. The magnitude of the shift will depend on the angle of scatter and the angle of the blood cell velocity. Since the CAM1 collects the reflected light along the same path as the incident light, the frequency shift will only depend on the relative angle, q, between the incident beam and the velocity, V.

dwg #

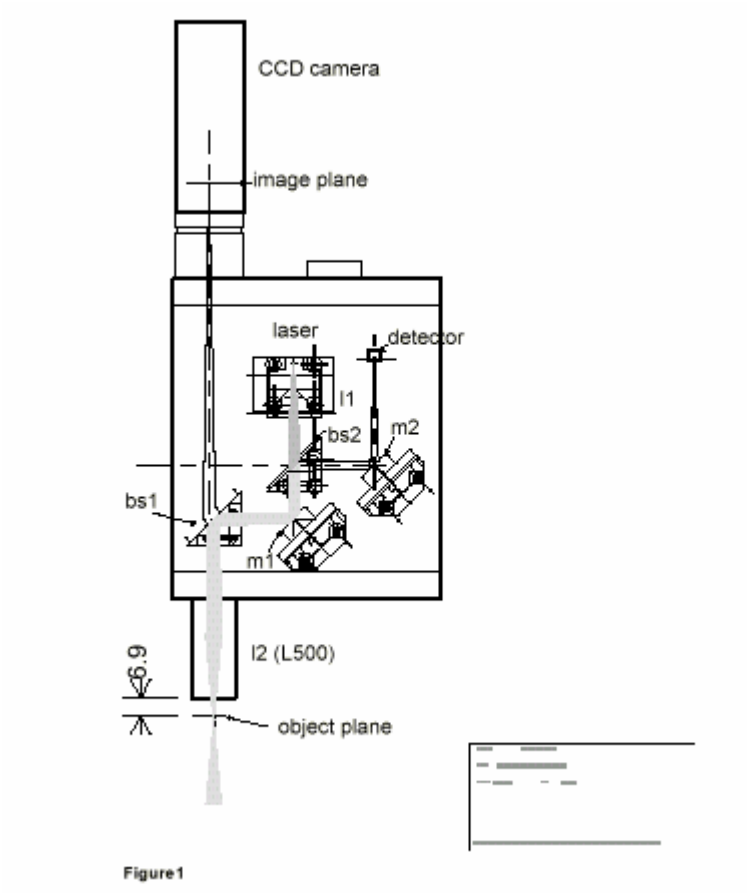


Figure 38 A schematic presentation of the CAM-1 capillary microscope/ laser Doppler anemometer

--***Electronic system***

The CAM1 electronics is shown in Figure 37. Output from the photo-detector is amplified then bandwidth limited. The bandwidth is limited to 68 Hz to 50 kHz, which corresponds to a velocity range of 0.02 to 14.6 mms -1. An analogue to digital converter (ADC) on an interface card in a personal computer, digitses the signal for processing by the CAM1 software. The ADC samples the signal with 12 bit resolution. The software allows the Doppler 'bandwidth' to be set to 6.25, 12.5, 25, or 50 kHz, corresponding to an ADC sample rate of 12.5, 25, 50, and 100 kHz, and velocity full scale ranges of 1.8, 3.7, 7.3, and 14.6 mm.s -1 . The bandwidth into the ADC is fixed, only the ADC sample rate is changed. The 'bandwidth' is therefore determined by the ADC sample rate. Since the 'bandwidth' will always be set to eliminate clipping of the calculated velocity signal, anti-aliasing filters are not necessary. Data is captured in 512 x 12 bit sample blocks. The interval between blocks can be adjusted but is set by default to 20 blocks

per second. The maximum rate is determined by the processing power of the computer, although it is not possible to faster than 24.4 Hz at the lowest bandwidth setting (6.25 kHz) due to the time required to acquire the 512 samples.

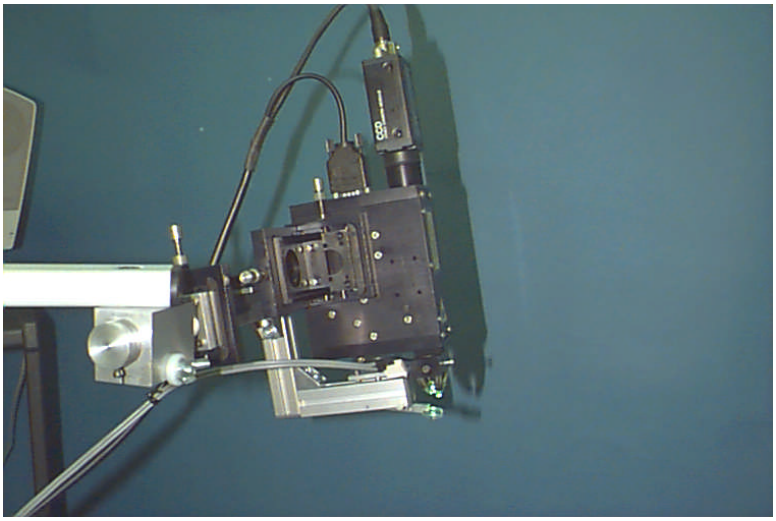
Signal Processing

A radix-4 fast Fourier transform (FFT) is performed on each block of 512 samples. When squared this produces a power spectrum with 256 points. On the lowest 'bandwidth' this gives a resolution of 7.2 m.s -1, and 0.057 mm.s -1 on the highest bandwidth. Two methods are provided for determining the velocity from the Doppler shift power spectrum (DSPS). The large low frequency components are from the amplitude and phase variations as the blood cells pass through the laser beam. For larger vessels, in which the blood flow may have a velocity profile, there will be a wide range of shifts from the low velocities at the edge, to the maximum velocity at the centre.

The first method works well with small capillaries that usually produce a well defined narrow peak in the DSPS. In this method the software searches for a peak in the DSPS. A user-defined threshold is used to ignore the background noise. ft is set to the maximum frequency component that exceeds the threshold. The maximum power is then searched for in a section of the PSDS between ft/2 and ft. The capillary blood cell velocity (CBV) can then be calculated from fp and equation 1♣.

The second method detects the highest frequency component ft that exceeds the user defined threshold. This is more suitable to the DSPS of larger vessels as shown. However this later method can be sensitive to the threshold level set by the operator, and the signal strength, if the cut off at fp is not steep.

♣ $\delta = \frac{2nV}{\lambda} \cos\theta$ (where n is refractive index here taken as 1.33; λ is wavelength here taken as 780nm)



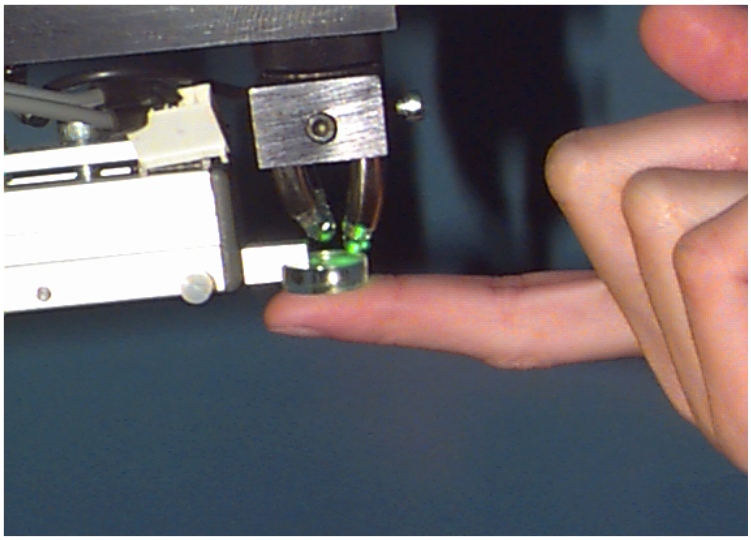


Figure 39 shows a photograph of the CAM-1 capillary anemometer. The lower photograph shows a detail of the acrylic disc used for exerting graduated pressure on the skin.

PART VIII

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