THE ROLE OF INFLAMMATORY CYTOKINES AND HYPOXIA IN VARICOSE VEIN WALL REMODELLING

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by

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STATEMENT OF ORIGINALITY

All the experiments described in this report have been performed by me, and are, to the best of my knowledge, original research, which has not been carried out elsewhere.

ABSTRACT

Varicose Veins (VV) affect 10-40% of 30-70 year olds. They have a prominent twisted appearance caused by changes in vein wall thickness. Primary weakening of the vein wall, due to an imbalance in matrix metalloproteinase enzymes (MMP) and their inhibitors (TIMP), is thought to have a major role in homeostasis of extracellular matrix. This could cause dilatation of the vessel wall and dysfunction of the valve cusps resulting in reflux. This could be contributed to by other factors such as an inflammatory response involving cytokine-mediated cellular infiltration and/or hypoxia.

My study examined mRNA expression in VVs, compared with veins from other sources (VOS), of MMP and TIMP with pro-inflammatory and pro-angiogenic cytokines (TNF-α, IL-1β, IL-6 and VEGF). In parallel I examined expression of hypoxia-regulated transcription factors (HIF). Cell culture was performed on Human Saphenous Vein Endothelial Cells (HSVEC) and HMEC-1 cells exposed to differing degrees of hypoxia.

I have shown a significant difference between VVs and VOS in expression of TIMP-2, -3 and IL-1β. I have demonstrated a significant correlation between VEGF and TIMP-3, TNF-α and MMP-2, MT1-MMP, TIMP-2 and TIMP-3 and IL-1β and MMP-2, MT1-MMP and TIMP-2. This study has also described, for the first time, HIF-1α and HIF-2α expression in VV, and shows that mRNA for both HIFs is significantly reduced in VVs when compared to VOS. HIF-1α significantly correlates with MMP-2, TNF-α and IL-1β, whereas HIF-2α significantly correlates with MT1-MMP and TNF-α. Cell culture studies revealed little difference between different times of hypoxic exposure on mRNA expression of MMP-2, MT1-MMP and TIMP-2 in either cell type. However there was a significant increase in TIMP-3 as the degree of hypoxia increased.

These data suggest that alterations in MMP/TIMP, possibly favouring matrix degradation leading to vein wall weakness and dilatation, may be mediated both by changes in inflammatory signals and/or by alterations in oxygen tension.

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LIST OF ABBREVIATIONS

ARP	Acidic ribosomal protein
DEPC	Diethylpyrocarbonate
cDNA	Complementary DNA
CVI	Chronic venous insufficiency
CVD	Chronic venous disease
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DVT	Deep vein thrombosis
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSV	Greater saphenous vein
HDAC	Histone deacetylase enzyme
HIF	Hypoxia inducible factor
HRE	HIF-responsive elements
HSVEC	Human saphenous vein endothelial cells
HUVEC	Human umbilical vein endothelial cells
MMLV	Moloney murine leukaemia virus
mRNA	Messenger RNA
MMP	Matrix metalloproteinases
MT MMP	Membrane type matrix metalloproteinases
NAD	Nicotinamide adenine dinucleotide
NTC	Non-template controls
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHD	Prolyl 4-hydroxylases
RCC	Renal cell carcinomas
RNA	Ribonucleic acid
RT	Reverse transcriptase
SMC	Smooth muscle cells
SSV	Small saphenous vein
TACE	TNF-α converting enzyme
TGF	Transforming growth factor
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF	Tumour necrosis factor

VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
pVHL	von Hippel-Lindau tumour suppressor protein
VOS	Veins from other sources
2ME2	2-methoxyestradiol

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CHAPTER 1

1 INTRODUCTION

This project aimed to identify potential explanations for the pathophysiology of varicose veins. Varicose veins pose a major clinical problem in western society. This study concentrated on finding the effect in changes in matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP), and assessed their potential relationship with inflammatory cytokines and growth factors. Furthermore the potential relationship between these factors and low oxygen concentration was addressed.

This introduction will have two approaches; a brief description on the clinical features and medical management of varicose veins and a more in-depth report focussing on the scientific attributes and literary evidence related to varicose veins.

1.1 DEFINITION OF VARICOSE VEINS

A varicose vein is a vein that is permanently dilated and has become enlarged. They are described as dilated, twisted, tortuous and elongated and are usually blue or dark purple. The term commonly refers to veins on the leg although varicose veins can occur anywhere. It is often assumed that small intradermal subcutaneous veins are varicose veins; however this is a misconception.

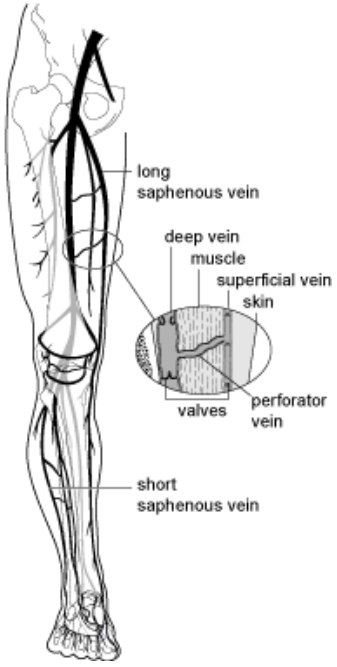
1.1.1 ANATOMY OF LOWER LIMB VEINS

Veins are the conduits for the transport of blood from the tissues back to the heart. Within the lower limbs, the venous system is classified as deep and superficial, with communicating veins connecting the two. There are two main points where these 2 systems meet, namely the saphenopopliteal and the saphenofemoral junctions.

The superficial veins of the lower limb begin as the dorsal venous arch of the foot. This is continuous with two primary veins, the greater and the small saphenous vein. Both of these veins contain valves which prevent the reflux of blood and aid the return of blood to the body. The valves are bicuspid in shape and more abundant distally. The greater saphenous

vein (GSV) is on the medial side. It receives blood from the dorsal venous arch which then heads upwards, travelling anterior to the medial malleolus, up the leg, through the knee region on the posterior aspect of the medial condyle of the femur, then turns anteriorly and laterally as it travels up the thigh. The GSV travels through the saphenous opening, a passageway in the fascia of the femoral triangle, to drain into the femoral vein. Along its course the GSV receives tributaries from the dorsum of the foot, the heel, the front of the leg, and anterior, medial and lateral portions of the thigh. Before emptying into the femoral vein, the GSV receives the superficial epigastric, superficial circumflex iliac and superficial external pudendal veins. The small saphenous vein (SSV) is on the lateral side. It runs posterior to the lateral malleolus and up the middle of the back of the leg. It usually pierces the crural fascia about halfway up the leg, running the rest of the way deep to the fascia. In most cases, the SSV terminates in the popliteal vein. This is displayed pictorially in Figure 1.1

Figure 1.1 Anatomy of the venous system in the lower limb



Perforating veins direct blood from the superficial to deep veins. The deep veins are surrounded by muscles and have valves as well. As the muscles contract, they actually pump the blood back toward the heart because of these valves. Valves in the perforating veins

prevent backflow to the superficial veins. When the valves in the perforators fail, all of the superficial blood and some of the blood from deep structures is forced into the superficial veins. Because of the hydrostatic pressure and the blood being forced by the muscles into the superficial veins, the valves in the superficial veins then fail and the veins become varicose. The deep veins of the lower limb consist of paired anterior tibial, posterior tibial and peroneal veins that parallel the course of the arterial system. These veins join into the popliteal vein. Once the popliteal vein passes through the adductor canal, it becomes the femoral vein. The deep femoral vein is a branch of the femoral vein. After receiving branches from the saphenous vein, the femoral vein continues as the common femoral vein. Veins from the gastrocnemius and soleus muscles are also part of the deep venous system.

The incompetence of these veins can be due to a failure of the deep venous system, superficial venous system and/or the perforating veins, all of which can lead to raised venous pressure in the lower limb. Varicose veins can further be divided into primary (no identifiable cause) or secondary (arise as a consequence of another disease process).

1.1.2 VARICOSE VEIN MILESTONES IN HISTORY

The origin of the word "varicose" probably comes from the Greek meaning "grapelike". It is thought to have been first used as a medical description by Hippocrates in 460BC.

The first written record and treatment suggestions for varicose veins were found in ancient Egypt in the Ebers papyrus around 1550 BC. They described early attempts at surgery and suggested that an operation on these "serpentine windings" would cause fatal haemorrhage.

The first illustration of a varicose vein was discovered in Greece at the foot of Acropolis. It is a commonly reproduced votive tablet that shows a large leg with a tortuous varicose vein on its medial aspect and dates back to the 4th Century BC. The Hippocratic Treatises (460BC) describes a surgical treatment of multiple punctures followed by compression. Hippocrates also describes the use of cautery in surgery and is quoted to have

said “what cannot be cured by medicaments is cured by the knife, what cannot be cured by the knife is cured by the searing iron, whatever this cannot cure must be considered incurable”.

The first ligation of blood vessels was described by the Alexandrian School of medicine in Egypt around 270BC. Celsus, a roman physician in the 1st century AD treated varicose veins by excision and cauterisation and described ligation of bleeding veins and division of veins between ligatures. Galen used silk ligatures to tie off blood vessels and suggested that varicose veins should be treated by incision and avulsion with the use of hooks (phlebectomies), a technique that is still used today.

By the 13th century there was a lot of progress in anatomy, physiology, surgical procedures and techniques which have expanded greatly and become more refined. Surgeons in this era were beginning to perform routine operations.

The 15th century brought new interest in venous anatomy because of Leonardo da Vinci’s drawings of the human body and in the 16th century the anatomy of the venous system was presented in great detail in the works of Andreas Vesalius.

The 16th century brought the discovery of valves. Venous valves were probably first mentioned by J B Canano who described the valvular fold in the azygos vein in 1547. Others have been recognised for their drawings of valves but the first full description of valves was given by Hieronymus Fabricus de Aquapendente, Professor of Anatomy of the medical school in Padua in 1603. In 1628 William Harvey published the discovery of circulation. He wrote “blood moves in the veins from parts below to those above and to the heart, and not in the opposite way”.

The 1800s lead to further understanding of the venous system but more with regards aetiologies and consequences of an acute venous thrombosis. However, John Gay’s Lettsomian Lectures published in 1866 contain accurate descriptions of perforating veins in patients with varicosities and ulcerations and also suggests that treatment of varicose veins results in the healing of ulcers.

In the early 20th century stripping of the GSV was performed in addition to the proximal ligation that was originally described by Trendelenburg in the 1890's. In 1905 Keller describes the use of an internal stripper and Mayo describes an external ringed vein stripper in 1906. Babcock's contribution was the development of a flexible internal saphenous stripper slightly later. Later on around the middle of the century suggestions were made for perforator vein surgery as a management for varicose ulcers due to incompetent communicating veins and approximately 30 years after this venous valve reconstructions were also successful in the deep venous system for management of deep venous incompetence.

In the past 3 decades thrombolysis, endovascular intervention methods, venous angioplasty and stents are rapidly improving the ability to treat the deep venous system. The earlier methods as described above, until very recently, were still common place in the practice of managing varicose veins. However treatment now readily consists of minimally endovenous procedures which is making the operation originally described by Trendelenburg a thing of the past (Rutherford).

1.2 EPIDEMIOLOGY OF VARICOSE VEINS

1.2.1 INCIDENCE AND PREVALENCE OF VARICOSE VEINS

Prevalence of varicose veins in the Western population over the age of 15 years has been estimated at between 10-15% for men and 20-25% for women (Callam 1994). Prevalence has been reported to vary widely by geographical location, with the highest reported rates in Western countries (Beebe-Dimmer *et al.* 2005). This could possibly be explained by environmental factors in the western world such as low fibre diet and lack of exercise. Carpentier *et al* compared the prevalence of varicose veins and chronic venous disease within France at 4 different locations. Within this one country he showed a high prevalence of both varicose vein disease and chronic venous disorders but this showed no significant geographical variation (Carpentier *et al.* 2004).

1.2.2 RISK FACTORS FOR VARICOSE VEINS

Evidence linking many assumed risk factors to the development of varicose vein disease is limited. Although it is repeatedly reported that the incidence of varicose veins is more common in women it has been found that being female is not a strong risk factor (Jawien 2003). The general consensus is that the most apparent risk factors for this disease are Parity and Weight. Studies suggest that women who have had 2 or more pregnancies were at increased risk of developing varicose veins (da Silva *et al.* 1974; Maffei *et al.* 1986; Brand *et al.* 1988). It is commonly assumed that the enlarged uterus impairs venous return and therefore increases the venous pressure in the lower limbs. This does not explain why the majority of varices appear very early on in pregnancy so other factors must play a key role such as alterations in hormone balance or increased circulating blood volume. Both the Framingham study as well as a community survey in West Jerusalem confirmed that people with a higher Body Mass Index were more likely to have venous disease (Abramson *et al.* 1981; Brand *et al.* 1988). The Basle Trial also reports this but only in females (da Silva *et al.* 1974).

There is conflicting evidence as to whether age, family history, constipation and prolonged sitting or standing/lack of exercise may play a key role in the development of varicose veins.

Again evidence is inconclusive but trauma to the lower limb in the form of a deep vein thrombosis or fracture would understandably cause stress on the venous system and increase the risk of varicose vein formation.

1.3 SYMPTOMS AND DIAGNOSIS

For most individuals, varicose veins do not inflict any symptoms and never cause any harm. The most common complaint, especially in females, is that they are not aesthetic. Campbell *et al* performed a questionnaire study on a population of patients who had been referred for hospital assessment of uncomplicated primary varicose veins. They found that

many people were anxious of the potential harm their veins could inflict on them. Specifically, fears included thrombosis, trauma, ulcers and other more general concerns about the future. In this group of patients, many of these worries are inappropriate and with good management including specific enquiry and reassurance about these concerns, unnecessary treatment may be avoided (Campbell *et al.* 2006).Varicose veins can cause a wide range of symptoms but the most common complaints include discomfort, heaviness, achiness and itching, mainly in the lower limb. This was confirmed by the Edinburgh Vein Study however the relationship between the distribution of the incompetent vein and the symptoms were inconsistent; specifically in males (Bradbury *et al.* 1999). The symptoms are often exaggerated after prolonged standing, walking or towards the end of the day. Patients usually report an element of relief on elevation of the legs. Leg swelling is another complaint but this is much less common (Campbell 2006). A proportion of patients with varicose veins will develop serious complications including superficial and deep vein thrombosis (DVT), superficial thrombophlebitis, skin thickening and staining (lipodermatosclerosis), haemorrhage and in the most severe scenario, ulceration. These changes in the skin and the subcutaneous tissues are often referred to clinically as chronic venous insufficiency (CVI) or chronic venous disease (CVD) and this warrants serious consideration for treatment as it associated with increased venous pressure. These complications together with the fact that varicose veins are one of the commonest reasons for surgical intervention in the Western world explains why venous diseases absorb approximately 1% of hospital in patient costs in major EU countries.

1.3.1 CLINICAL EXAMINATION AND INVESTIGATION

Clinical examination alone, in straightforward cases, can reveal all the information required to make a clear diagnosis and compile a management plan. Inspection, both lying and more specifically standing and visualising both the front and the back of the leg, is vitally important. This will provide the clinician with the extent, size and distribution (e.g. GSV and/or SSV involvement) of the disease. It is important to look at the skin, especially the

lower leg on the medial aspect (the gaiter area) for venous eczema, skin discolouration, ulcers or swelling, and also in the groin for scars indicating previous surgery.

Palpation of the saphenofemoral junction will inform the clinician of whether or not a sapheno varix is present. If it is, the cough test may be positive (Cruveilhier's sign).

Traditionally specific examinations include the Trendelenburg test and the tourniquet test which both involve lying the patient down with the leg lifted so as to drain the venous system of blood (the leg may require some gentle squeezing to facilitate this) and then palpating and occluding the saphenofemoral junction with gentle pressure using either a hand or a tourniquet. The patient is then asked to stand up. If there is rapid filling of the varicosities with the saphenofemoral junction controlled, either by pressure or tourniquet, then this suggests there are incompetent perforator veins. If no filling is seen then the pressure can be released. If there is rapid filling of the varicosities at this stage then this is suggestive of saphenofemoral junction incompetence. The advantage of using the tourniquet is that it can be placed in the mid-thigh region in order to examine different levels of perforator veins. It is more likely these days that a clinician would use a hand held Doppler to identify saphenofemoral or popliteal fossa reflux by holding the Doppler over the respective junctions and squeezing the thigh or the calf and listening to the proximal blood flow up the leg and waiting to see whether there is a second "whoosh" that occurs with incompetent veins as blood refluxes back down the leg.

Duplex ultrasound is a painless procedure that combines Doppler flow information and conventional imaging information in order to see the anatomy of blood vessels. Duplex ultrasound shows how blood is flowing through vessels and measures the speed of the flow of blood. It can also be useful to estimate the diameter of a blood vessel as well the presence of an occlusion. It is performed by specialists within that field called Vascular Scientists. Some specialists would prefer all patients to have duplex imaging, however, other units limit the scanning for specific indications like a previous history of DVT, ulceration, recurrent varicose veins or differentiating whether the disease is in the greater saphenous or short saphenous region, as occasionally this can be ambiguous. All patients with SSV disease must be imaged

by this modality as the anatomy varies greatly in this area, so before operation must be marked.

1.4 TREATMENT OF VARICOSE VEINS

1.4.1 CONSERVATIVE METHODS

For patients who are mildly symptomatic from varicose vein disease it is reasonable to offer conservative management. Good explanation, reassurance that their condition is unlikely to cause them harm and that operative treatment is not essential, will often suffice. Conservative approaches may improve mild symptoms. These include wearing graduated compression stockings with pressure of 30-40mmHg, leg elevation, regular exercise (although there is no evidence to support this (Chant *et al.* 1985) and weight loss.

1.4.2 TRADITIONAL SURGICAL TREATMENT

However for patients with significant symptoms, who have failed to get relief from conventional efforts or have substantial venous insufficiency, open surgery has been the treatment of choice for many years. It involves saphenofemoral (see Section 2.1.4 for in-depth description of the operation) or saphenopopliteal disconnection and stripping of either the greater or SSV and removal of superficial varicosities. By doing this venous reflux is eliminated and superficial venous pressure is reduced to near normal levels.

1.4.3 ENDOLUMINAL TREATMENT METHODS

1.4.3.1 Sclerotherapy

Sclerotherapy is considered the treatment of choice for small intradermal subcutaneous veins, however, it is now also considered an effective treatment for the significant reflux associated with varicose vein disease (Berridge *et al.* 2009; Coleridge Smith 2009). Despite this it still tends to be only used in patients that require further intervention for residual varicosities after having an alternative surgical procedure or in those who are unfit or do not want surgery.

It involves injection of a chemical, in either liquid or foam form, directly into the vein. The chemical irritates and scars the vein from the inside out so that the abnormal veins can then no longer fill with blood. Blood that would normally return to the heart through these veins returns to the heart through other veins. The body will eventually absorb the redundant vein.

Results are better if the procedure is performed under Ultrasound guidance, if a foam agent is used as the sclerosant and if the vein is smaller rather than larger.

1.4.3.2 Radiofrequency Ablation

Radiofrequency ablation uses a thin, flexible tube with a tiny bipolar electrode at its tip that is inserted into a varicose vein. When the high frequency alternating current is triggered it inflicts ionic agitation and local heating resulting in venous vasospasm and intimal destruction. This produces a fibrotic luminal seal.

The procedure is usually performed under general, regional or tumescent local anaesthesia. This technique cannot be performed on veins that are too small to cannulate (<2mm), tortuous veins or veins with thrombus in them.

1.4.3.3 Endovenous Laser Treatment

This ablation technique is very similar to radiofrequency ablation excepting the catheter allows insertion of a laser fibre. The laser energy generates steam bubbles that boil the blood in the lumen of the varicose vein and delivers heat injury to the vein wall (Proebstle *et al.* 2002). This procedure is usually performed under perivascular tumescent local anaesthesia.

There is no single appropriate method for treating the range of manifestations in venous disease (Subramonia and Lees 2007; Berridge *et al.* 2009). However, endovenous venous ablation techniques are becoming more and more popular and the traditional operation is being performed less often in clinical practice.

1.5 AETIOLOGY

1.5.1 POTENTIAL GENETIC LINK IN THE CAUSATION OF VARICOSE VEINS

No clear genetic link has been found in varicose vein disease. However there has been some evidence suggesting a familial susceptibility, thereby warranting future genetic studies (Lee *et al.* 2003).

Studies have shown a direct correlation between the severity of reflux and the clinical symptoms of CVI such as skin colour changes and lipodermatosclerosis (Christopoulos *et al.* 1988). Skin staining and lipodermatosclerosis causes iron in the form of haem to be deposited into the subcutaneous tissues. Iron deposition is known to increase the concentration of free radicals in the surrounding tissue and these results in tissue injury (Zamboni *et al.* 2005; Zamboni *et al.* 2006). Haemochromatosis is a genetic disorder causing the body to absorb an excessive amount of iron from the diet which is then deposited in various organs, mainly the liver, but also the pancreas, heart, endocrine glands, and joints. Factor VIII is a cross linking protein that is key in ulcer healing. It has been found that gene mutation of the haemochromatosis C282Y (HFE) gene and genetic variations of the factor VIII V34L gene are present in patients with CVD and varicose veins, thus predisposing them to this condition. These patients may have long term implications for increased risk of more severe forms of CVD as well as venous ulcers (Zamboni *et al.* 2005; Tognazzo *et al.* 2006).

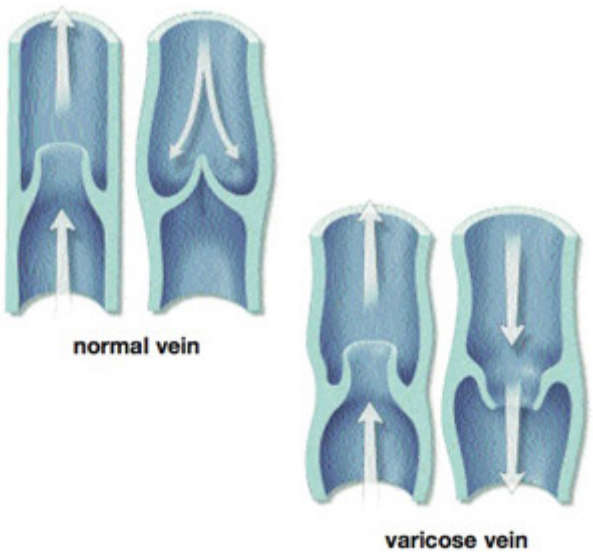
There are also clinical conditions that in theory would support a genetic link. Klippel-Trenaunay Syndrome is characterized by a triad of dermal capillary haemangiomas, varicose veins, and bony and soft tissue hypertrophy involving an extremity. This syndrome can have a number of congenital venous anomalies associated with it such as atresia, agenesis of the deep venous system, valvular insufficiency, venous aneurysms and embryonic veins. These patients have a significant impairment of venous muscle pump function and valvular incompetence (Delis *et al.* 2007). Another example is Ehlers-Danlos syndrome which is a group of ten different types of inherited connective tissue disorders, caused by a defect in the synthesis of collagen. Type IV predisposes to vascular pathology and can present with

varicose veins. In contrast to this evidence suggesting a genetic link, Marfan's syndrome is a autosomal dominant genetic disorder of the connective tissue that is carried on the FBN1 gene which encodes a connective protein called fibrillin-1. This is an essential protein for elastic fibre synthesis. However, this disorder mainly affects the aorta and the heart valves and is not associated with venous disease or varicose veins (McKusick 1991).

1.5.2 VALVULAR DEFECTS AS A CAUSE FOR VARICOSE VEINS

Blood reflux and valve dysfunction are central to the progression of venous disease. Reflux can occur within many of the venous systems of the lower limb, namely the superficial venous system (GSV and SSV), tributary veins, the saphenofemoral and saphenopopliteal junctions, the deep venous system and perforator veins (as shown in figure 1.2). Peak vein velocity has been found to have no significance in determining reflux. The method of choice is the duration of retrograde flow across a valve. The duration of retrograde flow across a diseased valve in the superficial and deep calf veins is greater than 0.5 seconds and for the femoropopliteal veins should be greater than 1 second. Outward flow in the perforating veins should be considered abnormal at greater than 0.35 seconds (Labropoulos *et al.* 2003).

Figure 1.2 Valvular function in normal and varicose veins
In a normal vein, the valve permits blood to flow in only one direction. In a varicose vein, defective valves never fully close, allowing backflow.
http://hopkins.portfolio.crushlovely.com/reference/article/varicose-veins



Labropoulos *et al* have done a number of studies investigating the location of reflux, the tributaries of the main superficial veins and the progression of reflux. They have concluded that primary venous reflux can occur in the superficial and the deep venous system of the lower limb. It is more common to have reflux below the knee and that this can be the case for asymptomatic patients, patients with prominent veins but not varicose and varicose vein disease. Because of this anatomical prominence it implies that progression of the disease is ascending. Reflux tends to be a local process. However, it can be multifocal which is more likely to be associated with worsening disease (Labropoulos *et al.* 1997). In parallel, they performed a study assessing tributary reflux and found that both the greater saphenous system and small saphenous system had tributaries which could be affected by reflux and that this could occur in the tributaries independently from the GSV, SSV, deep veins or perforator veins. This suggests that the tributaries do not have to have a feeding source and can develop reflux independently. It did show that the tributaries of the GSV were affected more often than the SSV tributaries (Labropoulos *et al.* 1999). A later study by the same group found that approximately a third of patients with confirmed venous reflux will have progression of their disease. 15% showed extension of the pre-existing reflux while 12% presented with new areas of reflux. On average the progression time to notice this change by Doppler ultrasound, and in some cases because of clinical changes, was 6 months. Again, progression was more common in the GSV, its tributaries and perforators (Labropoulos *et al.* 2005).

To summarise the evidence so far, reflux is suggested to occur segmentally and occur in an ascending fashion. Retrograde flow in the lower limb venous system occurs due to failure of the valves to function sufficiently. This could occur as primary valve dysfunction, in which the aetiology is uncertain or there may be factors occurring within the vein wall that could cause the valve function to fail. Several studies have indeed shown significant changes in the organisation of the extracellular matrix (ECM) and architecture of the smooth muscle cells (SMC) within the varicose vein vessel wall.

Mashiah *et al* supports the "weak vein wall" theory concerning the aetiology of varicose veins. They examined samples of varicose veins under a scanning electron

microscope in 29 women who had undergone surgery under local anaesthesia for symptomatic varicose veins. All had been previously diagnosed as having incompetence of the valve at the saphenofemoral junction. They found no changes in the intima or adventitia and no changes in any of the upper valves of the saphenous vein. There was, however, varying degrees of thinning of the media of the venous wall, which coincided with the areas of varicose dilatation between areas that retained their normal configuration (Mashiah *et al.* 1991).

Gandhi *et al* performed a study to evaluate the connective tissue constituents of greater saphenous varicosities and also the proteolytic activity, a factor that can lead to matrix degradation and cause weakening and dilation of the venous wall. They found a significant increase in the collagen content and a significant reduction in the elastin content of varicose veins. There was also an increase in the collagen/elastin ratio which is an indication that there is an imbalance within the connective tissue matrix. The biochemical profile of potential varicose veins was similar to varicose veins and both of these were significantly different from normal saphenous vein. When looking at the enzymatic activity there was no increase in the proteolytic activity which indicates that there is potentially no essential enzymatic matrix degradation in the formation of venous varicosities (Gandhi *et al.* 1993). Venturi *et al* aimed to find a biochemical explanation to why varices develop. They did this by evaluating sensitive biochemical markers of collagen (4-L-Hydroxyproline) and elastin (desmosine and isodesmosine) in the varicose vein wall of 20 patients. This was then compared with samples taken from 14 patients with normal saphenous veins in whom the vein was removed to be used for graft procedures. 4-L-Hydroxyproline and collagen content were similar in varicose and normal veins. There was a significant reduction of both desmosine and isodesmosine in the dilated segments of varicose veins indicating a reduction in elastin. The ratio of elastin to collagen was lower in varicose than normal veins, and this reduction was most significant in the dilated segments. These results suggest that dilatation of the varicose vein wall may be related to some defect of elastin metabolism (Venturi *et al.* 1996). Psaila *et al* measured the physical strength and elasticity of varicose veins and compared them to “normal” veins. They

found that varicose veins had a lower breaking strength and breaking energy than normal veins and that elastic stiffness was less in normal veins than in varicose veins. In the same samples they also determined that the collagen content was significantly higher in normal vein specimens in all sites examined. Visually, normal veins displayed distinct viscoelastic features in the wall around the valves which were absent at other sites. These differences were not found in varicose veins. They concluded that significant structural changes are seen in varicose veins (Psaila and Melhuish 1989). The objectives of Travers *et al* were to compare collagen and elastin content in varicose and control GSVs by histological staining. Varicose veins were significantly larger in wall area and contained increased amounts of collagen. There was no difference in wall thickness or elastin content between the two groups (Travers *et al.* 1996). Sansilvestri-Morel *et al* found an imbalance in collagen subtypes synthesis by SMC in varicose vein wall. Their data illustrates that SMC cultured from varicose veins deposit less collagen type III and fibronectin than control cells despite comparable levels of messenger RNA (mRNA) for these proteins suggesting dysregulation of posttranslational steps in the synthesis of both proteins by SMC from varicose veins (Sansilvestri-Morel *et al.* 1998). They also evaluated cultured dermal fibroblasts to see whether the changes observed in venous SMC were similar. Both these cell types had increased synthesis of type I collagen but a decrease in type III (which is important for elasticity and distensibility). This finding is evidence that varicose vein patients may have a systemic problem affecting collagen and may imply a genetic cause for the disease (Sansilvestri-Morel *et al.* 2002). They also measured the content of hydroxyproline in these cells and quantified the amount of collagen types I, III and V. There was an increase in hydroxyproline content which would normally indicate an increase in collagen, however, there was a decrease in type III collagen (Sansilvestri-Morel *et al.* 2003).

There have been many studies investigating the changes in wall matrix proteins and their associated components. This is explained in more detail in Section 1.5.3. These studies provide further evidence to suggest that alterations in the vein wall precede changes in the venous valve during varicose vein formation.

1.5.3 WEAKNESS OF THE VEIN WALL AS A CAUSE OF VARICOSE VEINS

It would make sense that the increase in venous hypertension in the lower limb would cause endothelial cell damage/dysfunction (discussed in Section 1.5.3.2) and cause a local inflammatory response (Section 1.5.3.4). However, significant inflammation is likely to occur at a later stage of the disease and is preceded by other actions on the endothelium and smooth muscle (Section 1.5.3.1) e.g. an increase in MMP expression/activity (Section 1.5.3.3) (Elsharawy *et al.* 2007).

1.5.3.1 Venous Wall Smooth Muscle Changes

Cultured SMC from varicose veins tissue have been used in a number of studies in order to determine whether or not ECM changes are associated with SMC. Sansilvestri-Morel *et al* cultured SMC from varicose veins and also control veins for assessment by immunofluorescence. The immunostaining for both collagen type III and fibronectin was weaker in cells from varicose veins than in those of control veins despite their transcriptional products being similar. Collagen type I deposition was similar in both types of cells while collagen type III was decreased in varicose vein cells. They also quantified MMP-1, -2 and -9 and TIMP-1, by enzyme immunoassays in the supernatants from smooth muscle cell cultures postconfluence. No significant difference was observed in the synthesis of any of the MMP or their inhibitors tested. This result only reflects what was found in the supernatant and does not exclude alterations in expression or activity of MMP or TIMP in whole tissues. These results would suggest that the regulation for collagen type III and fibronectin in SMC are post-transcriptional events (Sansilvestri-Morel *et al.* 1998). The same authors furthered their investigations by adding Marimastat, a non-selective MMP inhibitor, to the SMC. This caused a partial restoration in the production of collagen type III. In addition to this, MMP-3 which degrade fibronectin, was elevated in both transcription product and protein expression. They concluded that collagen III, and probably fibronectin, are degraded extracellularly in SMC from varicose veins by a mechanism involving MMP, and maybe MMP-3 by a direct or an indirect pathway (Sansilvestri-Morel *et al.* 2005). So in conclusion, the evidence suggests that in varicose vein SMC there is an imbalance in collagen production with dysregulation and

increased type I collagen production, but a decrease in type III. The reduction in type III collagen is likely to be post transcriptional as it is associated with normal mRNA production. Also, it could occur as a result of degradation/inhibition by MMP-3.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Programmed cell death involves a series of biochemical events that can occur in response to intrinsic signals or extrinsic stimuli. Examples of these apoptosis factors are Tumour necrosis factor-α (TNF-α), neurotransmitters, Growth Factor withdrawal, IL-2 withdrawal, FAS ligands, Radiation, Chemotherapeutic agents, reactive oxygen metabolites, viral infection and toxins. Ascher *et al* hypothesized that abnormal cell cycle events in the vein wall may contribute to changes in its structural integrity predisposing to varicosity development, and that possibly apoptosis is a contributing factor in the pathophysiology of varicosities. They designed a study to investigate whether programmed cell death varies in varicosities as compared to normal veins by staining samples immunohistochemically for the expression of bcl-2 and cyclin D1 and by detecting apoptosis by the TUNEL assay. The apoptotic index was 15% for varicose veins and 48% for control veins. Varicose vein specimens demonstrated increased nuclear expression of cyclin D1 whereas its cytoplasmic expression was significantly diminished which suggests that programmed cell death is inhibited in varicose veins. There was no difference in Bcl-2 protein for either study groups (Ascher *et al.* 2000). They then went on to examine the expression of bax protein and of poly ADP-ribose polymerase, which is involved in the repair of DNA damage and is inhibited by the activation of caspases 3 and 6. Both bax and poly ADP-ribose polymerase were decreased in the distal portion of varicose veins when compared to distal control vein segments. Varicose veins had no expression of bax or poly ADP-ribose polymerase in their adventitial layer although their expression was detected in this layer of the control group (Ascher *et al.* 2001). Together these studies suggest a role of abnormal apoptosis that may in turn lead to venous wall changes and dilatation.

1.5.3.2 Venous Endothelial Cell Dysfunction

The role of MMP in varicose veins has largely been attributed to their proteolytic effects on the ECM, degradation of the valve leaflets and weakening of the venous wall (Herouy *et al.* 1999; Pascarella *et al.* 2005). It is now apparent the MMP are present in venous SMC as well as the venous endothelium and this suggests the possibility of additional roles for MMP in the pathophysiology of venous disease (Thulesius 1996; Woodside *et al.* 2003). Rafetto *et al* performed studies on the rat inferior vena cava and demonstrated that MMP-2 causes inhibition of phenylephrine-induced venous contraction. They then wanted to establish whether or not MMP-2 induced inhibition of contraction involved the endothelium. They concluded that MMP-2 causes a significant inferior vena cava relaxation that is potentiated in the absence of endothelium or during blockade of endothelium-mediated nitric oxide and prostacyclin synthesis. They also suggested that MMP-2 may induce smooth muscle hyperpolarisation and activation of calcium dependant potassium channels that may play role in the early stages of venous dilatation and varicose vein formation (Raffetto *et al.* 2007).

1.5.3.3 Matrix Metalloproteinases, Their Inhibitors And Their Role In Veins

Matrix metalloproteinases (MMP or matrixins) are a group of zinc- and calcium-dependent endopeptidases that play an important role in degrading components of the ECM and thereby regulate formation, remodelling and maintenance of tissue. MMP also have a key role in migration of normal and malignant cells through the body. These mechanisms are important in both normal physiological repair processes as well as disease processes such as arthritis and metastasis. Most MMP are extracellular enzymes e.g. collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9) and stromelysins (MMP-3 and -10). There are, however, a group of membrane type MMP (MT-MMP). The basic MMP structure consists of a prodomain, catalytic domain, hinge region and hemopexin domain. An example of their structure is shown in Figure 1.3. All of these are expressed mainly by connective tissue cells and in cells of bone marrow origin as inactive pro-proteins (pro-MMP) which then get cleaved by extracellular proteinases that activate them. MMP activity is controlled by regulation at the transcriptional level, as well as by zymogen activation and inhibition by

specific tissue inhibitors of metalloproteinases (TIMP). So far 4 TIMP (TIMP-1, -2, -3, -4) have been identified. The two-domain TIMP are of relatively small size but have been shown to exhibit several biochemical and physiological/biological functions including inhibition of active MMP, pro-MMP activation, cell growth promotion, matrix binding, inhibition of angiogenesis and induction of apoptosis (Brew *et al.* 2000). An example of their structure is shown in Figure 1.4. As is the case for matrixins, the expression of TIMP in tissue is also controlled during tissue remodelling and physiological conditions to maintain a balance in the metabolism of the ECM (Das *et al.* 1997; Gomez *et al.* 1997).

Figure 1.3 History and Domain Structure of MMPs
Compilation of MMPs. Date of discovery of MMP and amino acid sequence listed in parentheses (http://www.abcam.com/index.html?pageconfig=resource&rid=11034)

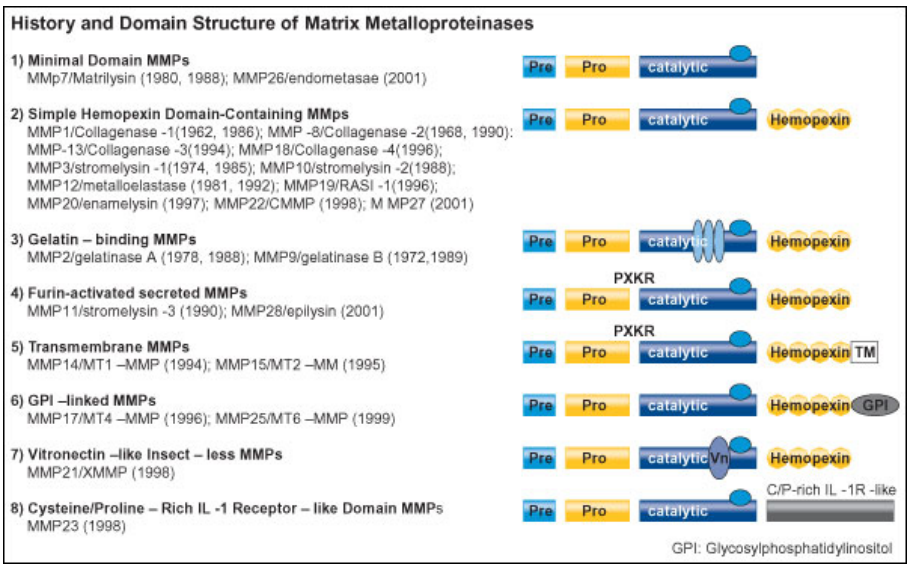
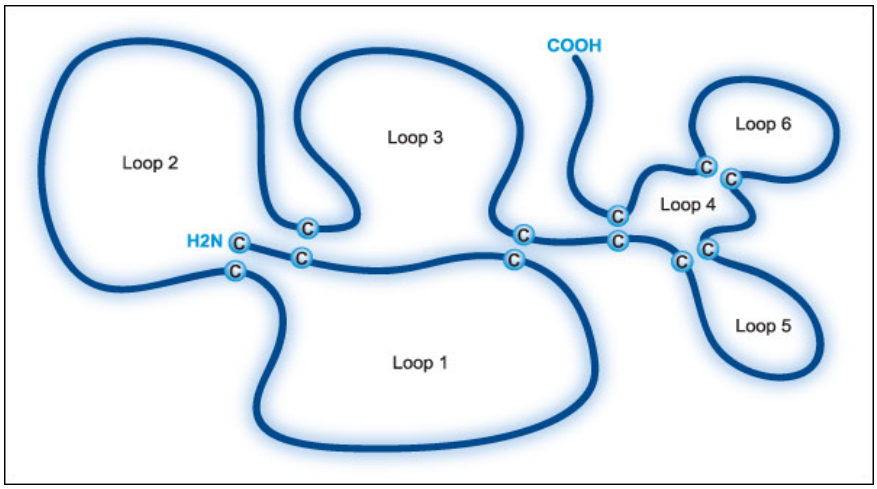


Figure 1.4 TIMP Structure
Schematic representation of TIMP-1 structure. TIMP-1 contains 12 cysteine residues which form six loop structures through disulfide bonds. The N-terminus of TIMPs 1-4 binds to the catalytic domain of most activated MMPs and inhibits function. The C-terminus of TIMP-1 and TIMP-2 binds to the hemopexin domain of proMMP-2 and proMMP-9, respectively; this binding regulates MMP function. (http://www.abcam.com/index.html?pageconfig=resource&rid=11034)



Remodelling of the ECM is an important cause of the changes in the walls of varicose veins, and as a result MMP/TIMP have been proposed to play a key role. Studies have confirmed the presence of MMP-1, -2, -3, -9, -12, -13 and TIMP-1 and -2 in both normal and varicose vein tissue (Gillespie *et al.* 2002; Woodside *et al.* 2003). Not all the evidence reports the same findings, for example, the presence of MMP-3 in varicose veins is inconclusive. Gillespie *et al* attempted to isolate MMP-3 mRNA in both healthy and varicose veins but were unable to get a positive result (Gillespie *et al.* 2002). However, Kowalewski *et al* have showed that MMP-3 is present in veins, and in increased amounts in varicose veins when compared to non-varicose veins. They also isolated MMP-1 MMP-2 and MMP-9 (Kowalewski *et al.* 2004). Gillespie *et al* continued his study into MMP and varicose vein tissue and demonstrated that there is an increase in MMP-1 protein in varicose veins segments when compared to control vein segments. A post transcriptional cause is likely for this observation as both tissue types showed similar mRNA expression. They also showed that there is a tendency for regional variation of both MMP-1 and MMP-13 expression in varicose veins, in that both these matrixins are more abundant in proximal segments (Gillespie *et al.* 2002).

In order to assess the localisation of MMP within the varicose vein wall, and therefore the potential role in the development of varicosities, immunohistochemistry has been performed by a number of groups. Woodside *et al* confirmed the presence of MMP-1, MMP-2, MMP-9, and MMP-12 as well as TIMP-1 and TIMP-2 were confirmed in varicose vein tissue as well as control vein tissue by Western blot analysis and then proceeded onto looking at the venous architecture and protein localization by histology and immunohistochemistry. They observed that varicose veins were not uniformly distributed along the circumference; areas of intimal thickening were often interspersed with focal areas of dilatation. Fragmentation of elastic lamellae and loss of circular and longitudinal muscle fibres were evident in the varicosities. Focal aggregates of macrophages were detected within the media and adventitia of both normal and varicose veins. MMP-1 and MMP-9 were expressed in both types of vein segments but with different localisation. Normal vein expressed MMP-1 in endothelial cells, occasional SMC, and adventitial microvessels whereas varicose vein MMP-1 expression was localized to fibroblasts, SMC and endothelial cells. MMP-9 was localized to endothelial cells, medial SMC, and adventitial microvessels in both normal and varicose veins, although varicose veins demonstrated increased medial smooth muscle cell staining. MMP-12 was found in SMC and fibroblasts in both normal and varicose veins. Neither TIMP-1 nor TIMP-2 was detected with immunohistochemistry in any specimens examined. This confirms that there is a distinct difference in the structural architecture and localization of MMP expression in normal and varicose veins (Woodside *et al.* 2003).

A number of studies have examined at the relationship between TIMP-1 and MMP-2. Badier-Commander *et al* examined 12 varicose veins and ten control veins for proteolytic enzyme/inhibitor balance using western blotting, gelatine or plasminogen-casein zymography and ELISA techniques. A high TIMP-1 level and a low MMP-2 level/activity were found in varicose veins resulting in a three-fold increase in the TIMP-1/MMP-2 ratio in varicose *versus* control veins (Badier-Commander *et al.* 2000). The same conclusion with reported by Parra *et al* who, with similar methodology al found an increased TIMP-1/MMP-2 ratio in

varicose veins (Parra *et al.* 1998). This may partially explain why there is ECM accumulation in varicose veins. The mechanisms involved in the regulation of the MMP/TIMP balance are hitherto unknown, but potential candidates include cytokines and/or alterations in oxygen tension which are discussed in their own sections.

I previously stated that Rafetto *et al* had performed studies on the rat inferior vena cava and concluded that MMP-2 inhibits venous contraction potentially through inducing smooth muscle hyperpolarisation and activation of calcium dependant potassium channels. They went on to test whether or not prolonged increases in venous wall tension causes over-expression of MMP and decreased contractility which in turn would promote dilation. They found that in Rat inferior vena cava, increases in the magnitude and the duration of wall tension causes reduced contraction and over-expression of MMP-2 and MMP-9. This would suggest that the progression of events entail increased venous pressure and wall tension increases that MMP expression which in turn reduced venous contraction and leads to progressive venous dilatation (Raffetto *et al.* 2008).

1.5.3.4 Inflammatory Changes In The Pathology Of Varicose Veins

It is thought that leukocyte trapping and activation in the microcirculation of the leg skin due to venous hypertension causes lipodermatosclerosis and ulceration in CVD.

Takase *et al* performed immunohistochemistry on proximal saphenous vein and subterminal valve, which were removed during surgical treatment, in order to detect adhesion molecules and cytokines. Their results showed an increase in monocyte/macrophage infiltration in the vein wall and valve. They also found an increase in intracellular adhesion molecule-1 in the specimens. This molecule promotes adherence of leukocytes to the endothelium. Other cytokines were not elevated that not only luminal venous endothelium but also endothelium in the vasa vasora of refluxing saphenous veins is activated, as indicated by the up-regulation of intracellular adhesion molecule-1. However, there were no consistent observations of the cytokines IL-1α and TNF-α (Takase *et al.* 2000). Sayer *et al* also looked at the localisation of inflammatory infiltrate in varicose veins in comparison to normal controls and, similarly to Takase, found that varicose veins had significantly more

inflammatory infiltrates in the form of mast cells and macrophages/monocytes in comparison to controls. There was also a trend of increased numbers of T-lymphocytes in varicose vein (Sayer and Smith 2004). Saharay *et al* investigated leukocyte endothelial adhesion in patients who were subjected to short-term venous hypertension. Blood samples were taken from a foot vein before and after standing for 30 minutes to raise the venous pressure in the lower limb, and after lying supine again for 10 minutes in 2 groups of patients. The first had varicose veins with skin changes and the second varicose veins without skin changes. The samples were analyzed for leukocyte surface CD11b and L-selectin (CD62L) expression as well as plasma-soluble L-selectin. Their results were very similar within the 2 groups. Both neutrophil CD11b and monocytes CD11b levels fell after 30 minutes of venous hypertension. The expression of L-selectin also fell but soluble plasma L-selectin levels increased during venous hypertension suggesting adhesion of these molecules to the endothelium (Saharay *et al.* 1997). This study suggests there in leukocyte interaction with the endothelium in patients with venous disease and that this could progress to inflammation.

Powell *et al* took 2 samples of blood, one from the distal leg vein and the other from the antecubital fossa, in patients with varying forms of CVD and also healthy control group. They found that patients with CVD had increased percentages of both platelet-monocyte aggregates and platelet-neutrophil aggregates throughout the circulation when compared to the healthy control group. They concluded that the presence of more of these aggregates and the increased propensity to form aggregates in the presence of platelet agonists in all classes of CVD suggests an underlying state of platelet activation and increased reactivity that is independent of the presence of ulceration. They also found an increase in the expression of monocyte CD11b throughout the circulation in all classes of CVD that suggests that although systemic monocyte activation occurs in CVD, its presence is independent of the presence of ulceration as well (Powell *et al.* 1999).

There is increasing evidence that suggest that inflammation plays a key role in modulating MMP expression in systemic vessels. For example, in atherosclerosis MMP co-localise with macrophages within atherosclerotic plaques, and cytokines like TNF-α, that

induce production of MMP, may be involved in initiating a cascade of events leading to plaque rupture and occlusion. In addition, mechanical injury to the wall of a blood vessel in animal models also induces MMP (Thakker-Varia *et al.* 1998).

1.5.3.5 Cytokines And Their Role In Varicose Vein Disease

Cytokines (Greek *cyto-*, cell; and *-kinos*, movement) are a category of signalling molecules that are used extensively in cellular communication. They are proteins, peptides, or glycoproteins. Cytokines are small secreted proteins which mediate and regulate immunity, inflammation and haematopoiesis. They are generally produced de novo in response to an immune stimulus, usually act over short distances, have a short half life and work at very low concentrations. Responses to cytokines include increasing or decreasing expression of membrane proteins, proliferation and secretion of effector molecules.

Badier-Commander *et al* compared levels of transforming growth factor (TGF) β, fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF) in control and varicose veins by a combined histological, immunohistochemical, and biochemical approach. This study found that both TGFβ1 and FGF-2 were present in control veins and also varicose veins, although they were more abundant in the latter. There was no significant difference in VEGF between varicose vein and normal veins. There were also no inflammatory cells present, suggesting that an inflammatory mechanism is unlikely to account for cytokine modulation (Badier-Commander *et al.* 2001). Murphy *et al* also confirmed the presence of cytokines in CVD and varicose vein disease in serum samples taken from the leg and measuring the level of TNF-α and VEGF. Both groups of patients were found to have elevated levels of these cytokines. The CVD group were then managed with 4-layer graduated compression to attempt to reverse the venous hypertension and then had regular follow up to further assess cytokine levels. It was found that there was a reduction in both cytokines to below control values with treatment (Murphy *et al.* 2002). Hollingsworth *et al* attempted to induce blood stasis by application of a tourniquet for 10 minutes on both a control group and also a group with primary varicose veins to measure change in levels of VEGF. Baseline

levels of VEGF were similar in both groups as well as between arm and foot samples. However after induction of blood stasis the control group showed a clear increase in the mean levels of VEGF. In contrast, the varicose vein group, showed no significant alteration in VEGF suggesting the tourniquet was insufficient to stimulate the response required to produce VEGF (Hollingsworth *et al.* 2001). Signorelli *et al* also induced venous stasis in patients with varicose veins and patients with non-varicose veins using the tourniquet method. They reported an increase in TNF-α, IL-1β and IL-6 in varicose vein patients in comparison to non-varicose vein patients and this became even more marked when induced stasis had been performed (Signorelli *et al.* 2000).

MMP and TIMP have been shown to be stimulated by inflammatory cytokines. Among the agents that induce MMP and TIMP production are the inflammatory cytokines TNF-α and IL-1β (Borden and Heller 1997). This is especially true for TIMP-1 although TNF-α appears to have little effect on MMP-2. It is thought that various other cytokines and soluble factors such as angiotensin II, PMA and retinoids may also play a key role. There is presently no data specific to varicose veins to support this.

1.5.4 BLOOD STASIS AND HYPOXIA AS A CAUSE FOR VARICOSE VEINS

1.5.4.1 Blood Stasis As A Cause For Varicose Vein Disease

The lack of the "calf muscle pump" action, as seen in varicose veins, contributes to a lack of venous return. This in turn may lead to increased venous filling and subsequently a rise in intraluminal venous pressure within the veins of the legs. The resultant blood stasis, relative hypoxia, reduction in pH (Cheatle *et al.* 1998) and associated increase in oxidative metabolites may all contribute to vein wall damage.

It is thought standing, alone, can promote blood stasis in the lower limbs and that such stasis will promote ischemia decreasing the oxygen availability to the tissues. Gaylarde *et al* has supported this hypothesis by showing there is a link between stasis and hypoxia, reporting improvement in skin blood flow by application of external compression stockings in standing subjects thus preventing microcirculatory stasis secondary to venous hypertension

(Gaylarde *et al.* 1993). Alterations in the microcirculation in CVI are thought to be due to a mixture of venous stasis, high capillary pressure and anoxic tissue. Matic *et al* has reported that the partial pressure of oxygen is higher in ulcerated limbs than those without ulceration which they suggest is due to blood passing directly from arterioles to venules over arterio-venous temperature-regulating shunts in the dermis (Matic *et al.* 2000). However another study found significant skin hypoxia in patients with venous disease and skin ulcerations (Clyne *et al.* 1985). Jacob *et al* studied the relationship between blood stasis and MMP-9. Blood was taken at rest (supine) from varicose vein and brachial vein and then again after 30 minutes of stasis. After 30 minutes of blood stasis the partial pressure of oxygen decreased although thrombomodulin, vWF, VEGF and MMP-2 were not modified. However, there were significant increases in endothelial intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and angiotensin converting enzyme. Leukocyte markers lactoferrin, myeloperoxidase and IL-8 were not modified whereas L-selectin shed from leukocytes increased, as did pro-MMP-9 (Jacob *et al.* 2002). This study also shows that MMP are important proteolytic enzymes in patients with CVD and are involved in the interaction occurring between leukocytes and the endothelium in varicose veins.

Animal studies have helped illustrate the role of MMP as well as valve and venous wall changes. A feasible *in vivo* model appears to be a rat with induced venous hypertension due to the formation of a femoral arteriovenous fistula and the control as the contralateral femoral vein in the same rat. Studies have shown that the formation of a fistula induces significant venous hypertension in femoral vein leading to dilatation and progressive reflux. The veins become dilated with subsequent valve damage including reduction in the height and width of the valve and fibrosis (Takase *et al.* 2004; Pascarella *et al.* 2005). One study noted significant inflammatory response with an infiltration of leukocytes into the entire vein wall with an increase in P-selectin and intercellular adhesion molecules. Interestingly this group reported an increase in the number of apoptotic cells found in the vein wall and valves which is contradictory to previous findings by others. They also found no differences in MMP-2 or MMP-9 at 3 weeks (Takase *et al.* 2004). Another study with an identical model noted

complete valve obliteration and an increase in both MMP-2 and MMP-9 at day 21 and 42 (Pascarella *et al.* 2005).

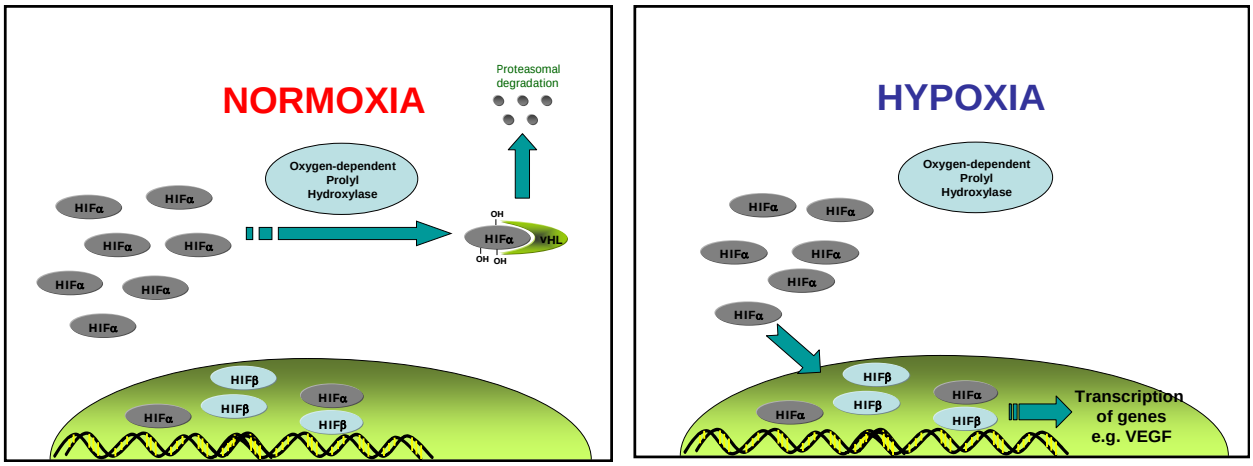
1.5.4.2 Hypoxia As A Cause For Varicose Vein Disease

Although hypoxia has been suggested as being involved in varicose vein disease, there has been very little work concentrating on the possibility and perhaps subsequent effects of hypoxia in either the venous macrocirculation or microcirculation of the leg, although there has been some investigation of the role of hypoxia in severe venous disease resulting in ulceration.

Hypoxia has been sub-divided by time span. Acute hypoxia activates endothelial cells to release inflammatory mediators and growth factors, which together with platelet-activating factor promote neutrophil adherence to the endothelium. Chronic hypoxia is thought to increase the expression of specific genes that encode for cytokines as well as growth factors e.g. VEGF, platelet-derived growth factor. These genes are modified by transcription factors, the most important being hypoxia inducible factor (HIF) (Hollingsworth *et al.* 2001). The proteins of these factors are activated in response to periods of hypoxia-induced physiological stress (a decrease in the concentration of molecular oxygen) and this adaptive pathway regulates changes in gene expression that then mediates adaptation and survival of the cells.

HIF transcription factors are a complex of two (α and β) sub-units: the hypoxia regulated α subunit and the oxygen insensitive β subunit. Under normal oxygen conditions the α subunit has a short half life and is rapidly degraded by hydroxylation of proline residues by prolyl 4-hydroxylases (PHD), which requires oxygen as a co-substrate. This allows binding of the von Hippel-Lindau (VHL) ubiquitin ligase complex, which targets HIF-α for proteasomal destruction. This means that no HIF protein accumulates. However, when the conditions change and become hypoxic the degradation of the a subunit is blocked and it therefore accumulates within the nucleus where it binds to the HIF-1β and this complex recognises and binds to HIF-responsive elements (HRE's) in the promoter or enhancer regions of various hypoxia-responsive target genes, which include VEGF (Figure 1.5).

Figure 1.5 Regulation of HIF-1 transcription factor
In normoxia, HIF-1α is modified by HIF-prolyl hydroxylases, triggering HIF-1α recognition by pVHL and subsequent degradation. Hypoxia blocks this mechanism allowing HIF-1α to accumulate and bind to p300 promoting the transcription of HIF-1 target genes



In the context of varicose vein development, there is no clear evidence of how the initial insult of hypoxia could be brought about. Possibilities include: 1) blood stasis causing macrocirulatory ischemia damaging the endothelial cells of the intima, 2) hypoxia to the endothelial wall caused by insufficient supply by the vasa vasorum in the adventitia and 3) tissue hypoxia due to alterations in the microcirculation (Nicolaides *et al.* 2008).

Taccoen *et al* measured oxygen tension *in vivo* of 31 varicose veins and 7 normal veins using a computerized polarographic system Kimoc 6650. This allowed blood samples to be taken by a needle probe in a microdriven stepwise fashion. The oxygen tension profile was similar in both groups of patients. Oxygenation of the outer two thirds of the venous wall is provided by vasa vasorum. The average minimum values in the media was significantly reduced in varicose veins compared to non-varicose veins which would indicate that there is good reason to believe that there is a deficiency in the oxygen supply in varicose veins and that potentially the vasa vasorum may be affected as suggested above (Taccoen *et al.* 1996).

As explained above, VHL plays an important role in the regulation of HIF. VHL has been linked to the regulation of cell cycle cessation (G(0)) and to the control of expression of various mRNAs via HIFs e.g. VEGF. Loss of function of the VHL tumour suppression gene occurs in familial and most sporadic renal cell carcinomas (RCC). Koochekpour *et al*

compared VHL-positive RCC cells with VHL-negative RCC cells. They found that the VHL-negative cells expressed reduced levels of TIMP-2 relative to VHL-positive cells. This would indicate the VHL plays a role in the regulation of TIMP-2 (Koochekpour *et al.* 1999) and given their similarity, potentially may be involved in the regulation of other MMP and TIMP. There is another genetic condition that also involves the VHL gene. Chuvash polycythaemia is an autosomal recessive disorder with increased serum erythropoietin levels and haemoglobin concentrations in normoxia that is endemic in Chuvashia. It is characterised by homozygous mutation (598C>T) of the VHL gene resulting in elevated normoxic levels of HIF-1α. Because of this germline mutation in the VHL gene it is thought that homozygotes for this mutation may develop certain vascular tumours similar to those associated with classic vHL syndrome. Gordeuk *et al* performed a matched cohort study and showed that VHL 598C>T homozygosity is associated with varicose veins, lower blood pressures, elevated serum VEGF, and premature mortality related to cerebrovascular events and peripheral thrombosis (Gordeuk *et al.* 2004).

Following on from the evidence written above, research has shown that hypoxia may regulate MMP and their inhibitors. HIFs are the transcription factors that mediate the adaptive response to hypoxia. As previously described above, in order to prevent the accumulation of HIF protein, VHL ubiquitin ligase binds to the prolyl hydroxylated HIF (the alpha subunit of either HIF-1 or HIF-2) complex, and promotes destruction via the proteasome pathway. Petrella *et al* described a connection between the loss of the VHL tumour suppressor function and the up-regulation of MT1-MMP gene and protein expression, which was medicated by HIF-2. They also identified a functional HIF-binding site in the proximal promoter of MT1-MMP. This suggests that as HIF accumulates, MT1-MMP gene expression and protein production is increased (Petrella *et al.* 2005). There have also been reports of an increase in MMP-2 mRNA expression and protein in endothelial cells (Ben-Yosef *et al.* 2002). MMP-2 is secreted as a pro-enzyme and undergoes activation through interaction with MT1-MMP and TIMP-2 forming a ternary complex. In another study using endothelial cells, hypoxia was shown to increase both MT1-MMP and MMP-2 mRNA thereby up-regulating MMP-2

activation (Ottino *et al.* 2004). This finding was also supported by Chen *et al* who exposed rat hepatic stellate cells to hypoxia and noted that the expression of MMP-2 was enhanced (Chen *et al.* 2001) . However there is one study by Ye *et al* that reports down regulation of protein content and activity of MMP-2, in response to hypoxia, in porcine pulmonary artery endothelial cells (Ye *et al.* 2005). Similarly, TIMP have also been reported to be regulated by hypoxia. There are conflicting reports in one study endothelial cells exposed to hypoxia suppressed TIMP-2 mRNA (Ben-Yosef *et al.* 2002) but in another study hypoxic endothelial cells increased TIMP-2 mRNA expression (Ottino *et al.* 2004). Another study analysing VHL-negative RCC cells (which reproduce the hypoxic milieu since HIF degradation is prevented) expressed reduced levels of both TIMP-1 and TIMP-2 when compared to VHL-positive cells. This implies that VHL is involved in the regulation of these molecules. Interestingly when analysing MMP they found that MMP-2 and MMP-9 elevated (Koochekpour *et al.* 1999). There is very little data on HIFs and TIMP-3. Yamaguchi *et al* looked at the effect of hypoxia alone and also the effect of hypoxia together with IL-1β stimulated temporomandibular joint disc cells. Together hypoxia and IL-1β caused a significant decrease in TIMP-3. In the same cells they also found a significant increase in MMP-2 in group that were only exposed to hypoxia (Yamaguchi *et al.* 2005).

There is increasing evidence that HIF-1 may play a role in immune reactions suggesting an association with cytokines. Scharte *et al* investigated the effects of cytokines on HIF-1 in the presence of normoxia (21% oxygen) and hypoxia (1% oxygen) using human transformed intestinal cells. They found that IL-4 significantly increased HIF-1 protein levels during hypoxic episodes and not in normoxia. There was no upregulation of HIF-1 DNA-binding activity or HIF-1 dependent gene expression. IL-10 had no effects on HIF-1 protein levels (Scharte *et al.* 2006).This had been shown by Cramer *et al* who examined the inflammatory response in conditional HIF-1α knockout mice (myeloid-specific knock-outs). They proved that HIF-1α is essential for the chief cellular agents of innate immunity - myeloid cell infiltration and activation (Cramer *et al.* 2003). This would strengthen the

suggestion of a link between cytokines and HIFs. It is known that VEGF is a downstream target gene of HIF-1α. Li *et al* investigated the expression of HIF-1α and VEGF in pulmonary arteries of rats with hypoxia induced pulmonary hypertension. They found that HIF-1α and VEGF were both involved in the pathogenesis of hypoxia induced hypertension in rats. HIF-1α tended to peak at 7 days exposure to hypoxia and remained elevated until day 14. VEGF protein however peaked later at day 14 (Li and Dai 2004). Ozaki *et al* also showed an increase of HIF-1α expression when exposed to hypoxia and a corresponding increase of VEGF in ischemic retinal vessels (Ozaki *et al.* 1999). In contrast, presently there is little literature to suggest a link between HIFs and TNF-α. In fact it has been commented that hypoxia-induced TNF-α expression occurs via hypoxia-activated signals independent to those of the HIF-1 pathway (Li *et al.* 2005). Hellwig-Burgel *et al* suggest that inflammatory cytokines, like TNF-α and IL-1β, actually stimulate HIF-1-dependent gene expression themselves, even in normoxic cells. It is thought that both hypoxic activation and the cytokine-induced activation of HIF-1, use signalling pathways that involve phosphatidylinositol-3-kinase and mitogen-activated protein kinase (Hellwig-Burgel *et al.* 2005). This implies that the HIF-1 pathway is still used but a reduced oxygen concentration does not have to be present in order for the pathway to be activated. Zhang *et al* commented on the fact the IL-1β shares common features with other known HIF-1α regulated genes. In fact IL-1β genes carry multiple binding sites in their promoter regions. They report that there is transcriptional activation of IL-1β in astrocytes exposed to hypoxia and that this occurs through the HIF-1 pathway (Zhang *et al.* 2006). It has also been shown in desferrioxamine-stimulated cochlear auditory cell lines that IL-6, another inflammatory cytokine, production can be increased in hypoxic conditions and this is associated with the activation of HIF-1 and MAPK (Jeong *et al.* 2005).

1.6 HYPOTHESIS AND OBJECTIVES

To summarise, varicose veins are a common problem that for a multitude of reasons often require surgical intervention. Their aetiology is poorly understood. Literature is limited and inconsistent. There are a number of theories as to whether the pathological process occurs at the valves or the vein wall, whether it is inflammatory or genetic and if and how hypoxia may be involved.

My hypothesis in this study was that varicose veins result from vein wall remodelling due to alternation in the MMP/TIMP balance as a consequence of stimulation by inflammatory cytokines and/or hypoxia.

In order to explore the above, my objectives were:

1) to determine the relationship between inflammatory cytokine expression and matrix-degrading MMP and their inhibitors in varicose veins
2) to investigate whether hypoxia is a feature of varicose veins and leads to alterations in MMP
3) To assess the effect of hypoxia on human saphenous endothelial cell expression of MMP.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 PATIENT SELECTION AND TISSUE SAMPLING

2.1.1 PATIENT SAMPLING

The patients were assessed in the Outpatient Department at Charing Cross Hospital in a Consultant led clinic for varicose veins (VV), to determine whether or not they required surgery for definitive management of their condition. Patients who were undergoing operations for primary varicose vein of the greater saphenous system of the superficial veins were chosen for the study. Those undergoing operation for disease in the short saphenous system and/or recurrent varicose vein disease were excluded. The patients then attended either as a day case or as an inpatient and were informed of the study and consent obtained. Assurance was provided regarding data protection and tissue disposal.

A full medical history, including drug history was obtained from the patient. The ideal criteria for this study would be to exclude patients with various co-morbidities such as a history of DVT, diabetes, rheumatoid arthritis, connective tissue disorders, known arteriopaths and cancers. This is because these conditions affect the structure of the vein wall. For example DVT causes an early increase in neutrophil infiltration in vein walls which is associated with inflammation and is likely to contribute to vein wall injury (Downing *et al.* 1996). Connective tissue disorders are known to affect the quality and quantity of collagen and elastin present in the dermis (Holbrook and Byers 1982). Collagen and elastin are also abundant in the venous wall so patients who have a connective tissue disorder are likely to have similar alterations in their vein walls. There is also evidence that malignancy may be associated with changes in proteinase expression, either because of the potential cause, like a virus (Katori *et al.* 2006), or due to the medications used to treat them (Iovieno *et al.* 2009) Other conditions will also require the patient to take medication, for example arteriopaths and patients receiving statins, which may influence MMP (Turner *et al.* 2005; Wilson *et al.* 2005). However, these criteria could not be met in the veins from other sources group of patients, as

the subjects were most likely to be arteriopaths or patients that have chronic renal failure. Both of these conditions involve the patients regularly taking a number of medications and predisposing the patients to a number of co-morbidities such as DVT etc. Therefore, these patients could not be excluded, otherwise a comparative group of sufficient size would not have been available. A very small number of patients in the varicose vein group also experienced some of the factors that ideally would be excluded from the study. The severity of the pathology that they displayed was very minor with respect to the veins from other sources group, as open varicose vein surgery is generally only performed on healthy individuals due to the risk of anaesthesia. I have therefore included these patients in this study. Throughout analysis, any changes noted in MMP/TIMP expression have been considered with caution.

Clinical examination was carried out, and all patients who had varicose vein operations had duplex scans to assess their lower limbs pre-operatively. A duplex scan is a combination of ultrasound and Doppler signals. Ultrasound allows visualisation of the veins while Doppler signals help to identify the direction of the blood flow. This aids, together with the clinical picture, confirmation of the site or sites of the disease showing whether or not valves are competent or incompetent.

Veins from other sources (VOS) were obtained from patients undergoing arterial bypass, carotid endarterectomy operations or arterio-venous fistula formation at the Charing Cross Vascular Surgical Department and used for comparison (see Sections 2.1.5, 2.1.6 and 2.1.7 below).

Pre-operatively, full written consent was obtained. This usually occurred on the day of the operation. The patient was informed what kind of tissue and the amount that would be taken, and it was carefully explained that the tissue would otherwise have been discarded. The informed consent was obtained by the doctor who was to perform the operation. Once the specimen had been taken in the operating theatre it underwent collection and anonymisation which was performed by a dedicated member of staff in liaison with the tissue bank at Charing Cross Hospital. Once the experiments had been completed, anonymity was reversed

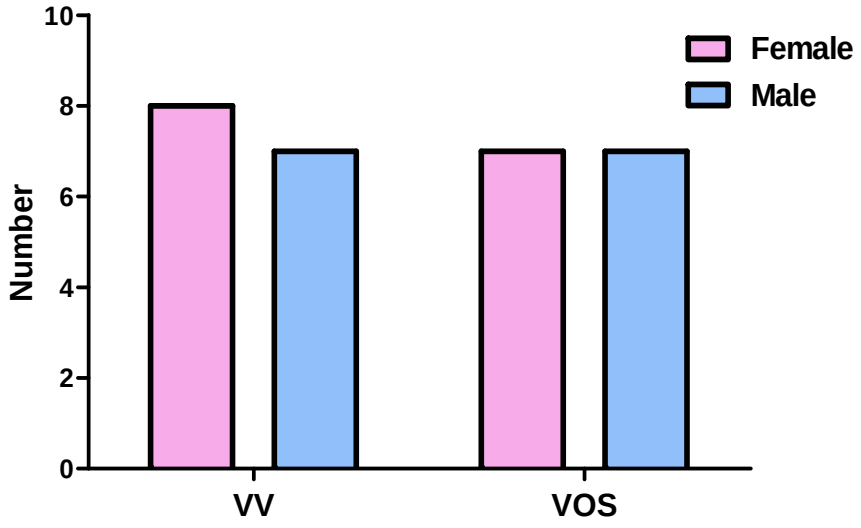
and the clinical case notes of the patients recalled from the medical records department. They then underwent systematic review looking at the following: Gender, Age, Race, Parity, BMI, co-morbidities and medications.

2.1.2 DEMOGRAPHICS OF THE STUDIED POPULATION

The studied population were taken from patients that underwent operation under the guidance of Professor Alun H. Davies, Consultant Vascular Surgeon, in Charing Cross Hospital. A total of 29 patients were enrolled and included those undergoing varicose vein surgery, leg bypass operations from whom GSV segments were harvested, Carotid Endartarectomy from whom facial vein was harvested and fistula formation from whom arm vein was harvested.

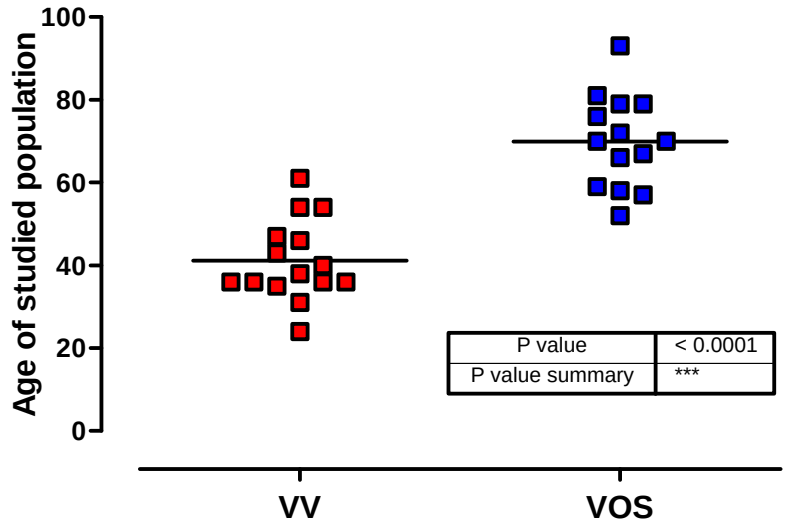
There were 7 males (47%) in the varicose vein group and also 7 males (50%) in the veins from other sources group, compared with 8 females (53%) in the varicose vein group and 7 females (50%) in the veins from other sources group (no significant difference using Fishers exact test) (Figure 2.1).

Figure 2.1 Gender distribution in the studied population
Bar chart to represent 29 patients who underwent surgery. 8 females and 7 males had varicose vein surgery and 7 females and 7 males underwent an alternative operation in which VOS were retrieved



The mean age of the varicose vein patients was 41.13 years (range 24-61), while the patients who supplied veins from other sources had an average age of 69.93 (range 52-93). This difference was highly significant using an unpaired t-test (p=<0.0001) (Figure 2.2)

Figure 2.2 Age of the studied population
Scatter plot to show the ages of the 29 patients who underwent surgery divided by the type of operation. Data are shown with mean values.



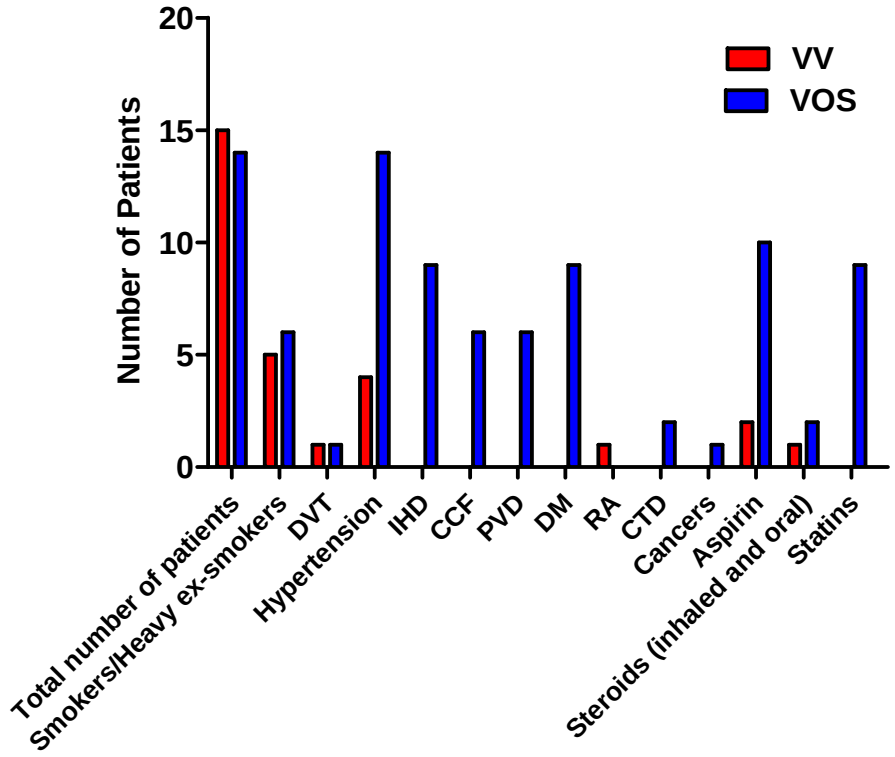
A total of 33% of the varicose vein group were smokers, compared with 71% in the veins from other sources. I included patients that had claimed to have recently given up smoking, i.e. the last 6 months, and also those that had given up in the last 5 years that smoked over 20 cigarettes a day (Figure 2.3).

When comparing the 2 groups, 27% of the varicose vein group suffered with hypertension, whereas it affected 100% of the patients in the veins from other sources group, as might be expected. The patients in veins from other sources group, 64 % had some form of ischaemic cardiac compromise and 43% had peripheral vascular disease. There were no arteriopaths in the varicose vein group. 1 patient from each group had experienced a DVT.

Although there was a larger number of patients taking Aspirin, Steroids and Statins in the veins from other sources group there was still a small proportion of patients on regular aspirin and inhaled sreroids in the varicose vein group.

Figure 2.3 Comparison of the co-morbidities and other characteristics of patients included in the study

Bar chart to show the number of patients with co-morbidities amongst the 2 specimen groups.



2.1.3 ETHICAL APPROVAL

Ethical approval has been granted for the project (Riverside Research Ethics Committee: RREC3092: The Role Of Matrix Metalloproteases And Their Inhibitors In The Development Of Varicose Veins; RREC3199: Pro-Inflammatory And Pro-Thrombotic Profile Of Aneurismal And Occlusive Atherosclerotic Disease – A Novel Ex Vivo Approach; RREC2989: An Investigation Into Factors Influencing Atheromatous Plaque Instability; REC 06/Q0407/27: Arteriovenous Fistulae: Factors Predicting Outcome). All the specimens were anonymised according to the rules set out in the Human Tissue Bill (2004).

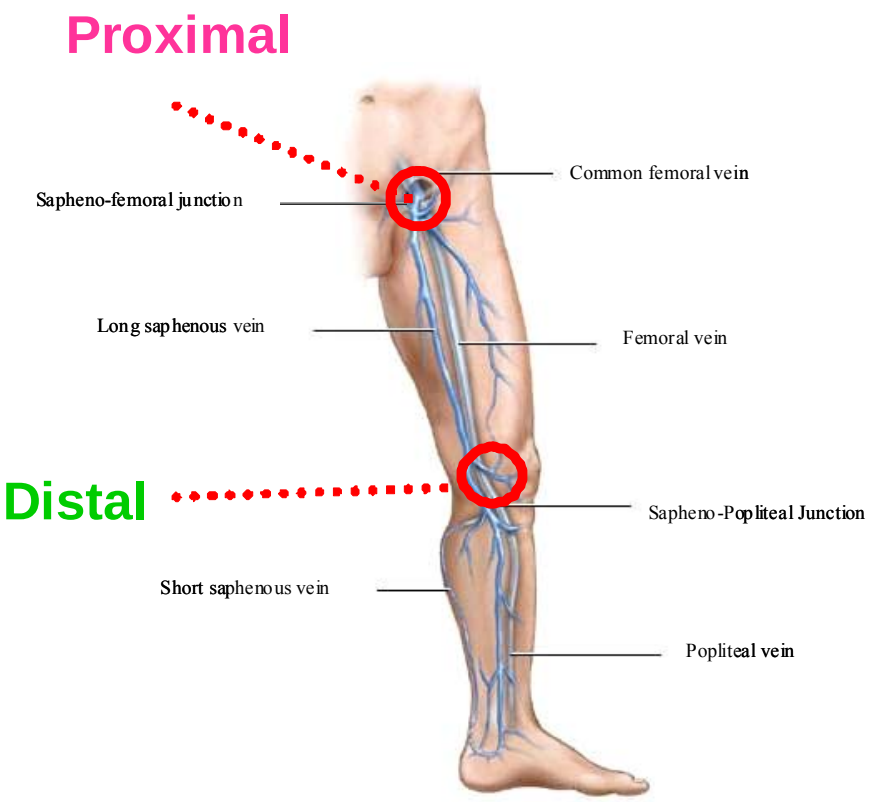
2.1.4 OPERATION FOR VARICOSE VEINS AND SPECIMEN COLLECTION

The procedure commonly performed for this type of surgery is known as "High tie of the GSV and multiple avulsions" or traditionally Trendelenburg's Operation. This is usually

performed under general anaesthesia. It involves a 5cm oblique groin incision, just below and parallel to the inguinal ligament in the groin crease, to expose the saphenofemoral junction. Once the saphenofemoral junction has been dissected, along with its tributaries, the tributaries must all be ligated. The junction is then ligated flush with the deep vein and the length of the vein then stripped to below the knee using a plastic stripper. Skin wounds were closed using absorbable sutures. The operation is concluded by avulsions. Avulsions are the removal of small prominent veins under the skin and are removed by small incisions placed directly over them and pulled out using a vein hook. Compression bandaging, to avoid haematoma formation, is applied at the end of the operation. The recorded blood loss for this operation is around 500ml; it rarely requires an overnight stay and patients can return to their normal work within a week, or less depending on their age and co-morbidities.

The tissue for the research was obtained at 2 stages. Initially after the proximal end of the vein was flush ligated with the deep vein, a segment of 2-3cm was excised from the main vein segment. This specimen would usually have been discarded after the operation. At the stage when the vein was stripped, a piece of similar dimension was also excised from the distal end, around the knee (Figure 2.4). Comparison of these two different anatomical sites is important as it has been shown that ambulatory venous pressure of the lower extremities differs between proximal and distal segments (Neglen and Raju 2000). Each of the specimens were marked as “proximal” and “distal”. The specimens were retrieved by a designated tissue collector in accordance with the Human Tissue Act (2004). Specimens were then snap frozen in liquid nitrogen.

Figure 2.4 **Lower limb showing the venous system and the site of vein retrieval during varicose vein surgery**



2.1.5 COLLECTION OF GREATER SAPHENOUS VEIN TISSUE FROM BYPASS OPERATIONS

These patients have experienced a blockage of the main arteries in the legs. Occasionally it is possible to unblock these arteries using a balloon in a procedure called angioplasty. However when this is not possible, in order to improve the blood flow to the foot, these patients would require an operation called a bypass. This involves bypassing the blockage by either attaching a vein, taken from the same patient, or a piece of artificial material, PTFE or Dacron. Vein bypass is considered superior to prosthetic grafts as they are durable with longer patency rates. Veins are preferred to be taken from the GSV; however if for some reason these are not available or suitable, then an arm vein can be used (see Section 2.1.7 below). This procedure needs a preoperative Duplex assessment in order to assess to suitability of vein. During this scan the course of the vein was marked on the skin surface with indelible ink pen for easy salvage and placement of incision. This also helps in ruling out the presence of varicose veins as using a varicose vein for bypass can result in early failure

and aneurysmal dilatation of the bypass (Soury *et al.* 1999). At operation a skin incision was made along this marking and the vein dissected out. The vein was then reversed so that the existing valves do not prevent blood from flowing down the leg and sutured to the arteries in order to re-establish blood flow. If there was any vein left surplus to the requirement for the procedure, this was used for research. These segments were treated in exactly the same way as varicose veins, retrieved by the designated tissue collector, snap frozen and stored for experiments in the -80°C freezer.

2.1.6 COLLECTION OF NECK VEINS

The brain relies on oxygen supplied in blood from the heart via the carotid arteries. These arteries can become blocked by atheromatous plaques which therefore reduce the blood flow to the brain. In order to re-establish this flow and reduce the potential threat of small bits of plaque breaking off and travelling up to the brain causing a stroke, a carotid endarterectomy can be performed. This involves coring the narrowing plaques out from the artery. This operation is prophylactic, in that it will not improve the symptoms that have occurred due to a previous stroke.

At operation, an incision was placed along the side of the neck and tissue dissected along the artery to gain access to them above and below the blockage. The artery needs to be completely exposed in order for success. This complete exposure is often hindered by the facial or jugular vein which travels across part of the artery and hence one of the important steps of the operation is to ligate and separate them. These excised segments were used for research as neck veins, they were immediately retrieved by the designated tissue collector, snap frozen and stored for experiments in the -80°C freezer. Neck veins are very tiny and in order to be able to extract RNA from them I had to accumulate four specimens and treat it as one. The samples were amalgamated at the time of pulverisation.

2.1.7 COLLECTION OF ARM VEINS

Arm veins were collected from two types of patients. The first were patients already described in Section 2.1.5, who underwent arterial surgery on the lower limb using arm veins as grafts, and the second are chronic renal failure patients requiring haemodialysis.

Chronic renal failure is a common condition which leads to gradual and complete shutdown of the kidneys. The kidney has the role of filtering toxic materials out of the body. When it shuts down dialysis helps to support bodily functions. There are 2 types of dialysis, peritoneal and haemodialysis. Peritoneal dialysis depends on fluid inserted in to the abdominal cavity to remove the toxic materials, whilst haemodialysis involves the removal of blood from a vein; filtering it outside the body in a machine called a dialyser and re-infusing it back in to the body through the arm vein. This is a cyclical process that takes 3-4 hours. One of the crucial requirements for this procedure is access to a vein with a good blood flow. This was done by the formation of "Cimino" fistulas which are a communication between an artery and the vein in an accessible part of the body – usually the arm. The arterial component makes sure that there is good blood flow whilst the veins, which are superficial, give easy access. A classic example is a radio-cephalic fistula which is formed between the radial artery and the cephalic vein. Patients who require fistula formation were referred from different hospitals to Charing Cross Vascular Surgery Department. The patients were seen in the clinic and underwent pre-operative assessment in the form of Duplex scan in order to assess suitability of the vein depending on calibre and accessibility and at the same time underwent marking.

The surgery was mostly under local anaesthetic. The vein segment was dissected out followed by the artery to which it was then connected in order to form the fistula. It was a usual precaution to dissect out extra length on the vein segment in case the previous determined length was not sufficient to approximate the vein to the artery to form the fistula. If there was any excess length of vein segment after formation of fistula it was used for research purposes. This was treated as in the cases of the other veins, retrieved by the designated tissue collector, snap frozen and stored for experiments in the -80°C freezer.

2.2 QUANTITATION OF mRNA FOR GENES OF INTEREST

2.2.1 EXTRACTION OF mRNA FROM VARICOSE VEIN TISSUE AND VEINS FROM OTHER SOURCES

The following section describes isolation of total RNA from tissue, which ensures that the integrity of RNA is maintained. The technique utilised varicose vein tissue, either proximal or distal, or veins from other sources, which were rapidly snap frozen (liquid nitrogen at -196°C and then stored at -80°C).

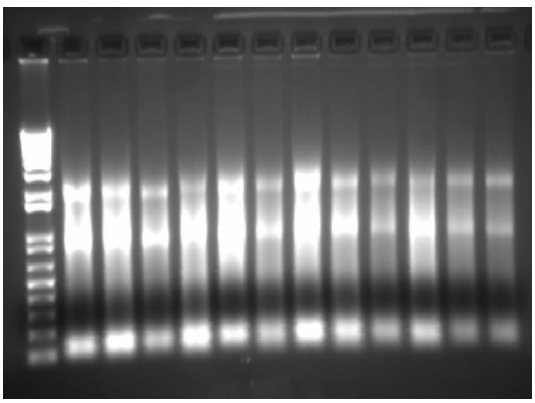
The vein tissue was pulverised to a fine powder using a mechanised pestle and mortar cooled with liquid nitrogen in order to keep the tissue frozen, and then transferred into bijou bottles and kept frozen. The RNA had to be preserved so pulverisation had to be fast and thorough, and it was imperative to keep the tissue frozen until it came in contact with the denaturant. Prior to homogenisation, the tissue was weighed and 1ml TRIZOL® Reagent (Invitrogen Life Technologies, Paisley, UK) per 100mg tissue was added. TRIZOL® is a mono-phasic solution of phenol and guanidine isothiocyanate that contains a combination of denaturants and RNAase inhibitors. During sample homogenization, TRIZOL® reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Homogenisation was carried out until tissue slurry was apparent. For phase separation, the slurry was centrifuged (12,000 x g, 10 minutes), the supernatant removed, leaving pelleted debris which was discarded. 200µl chloroform (VWR International Ltd, Lutterworth, Leicestershire, UK) was added to the supernatant and left to stand for 2-3 minutes, prior to centrifugation (12,000 x g, 15 minutes at 4°C). This allowed separation of the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase.

RNA was precipitated by pipetting off the clear aqueous phase and 550µl acid phenol chloroform added, followed by vortexing for 1 minute and centrifugation (12,000 x g, 5 minutes at 4°C). 500µl propan-2-ol (VWR International Ltd, Lutterworth, Leicestershire, UK) was then added to the aqueous phase, inverted and left to stand for 10 minutes before finally centrifuging (12,000 x g, 10 minutes at 4°C). The RNA pellet was washed by the addition of

1ml 75% ethanol, which was removed by centrifugation (12,000 x g, 5 minutes). After air drying for 5 minutes, the pellet was re-suspended in 50µl of diethylpyrocarbonate (DEPC, an irreversible RNase inhibitor)-treated water (QIAGEN, GmbH, Germany). When the pellet was difficult to re-suspend this step was aided by heating the pellet to 55°C. 1µl of RNasin (a broad spectrum RNase inhibitor; Promega, Southampton, UK), 5µl of 10xDNase buffer and 2µl of turbo DNase (to remove trace quantities of DNA; Ambion, Huntingdon, Cambridgeshire, UK) was added. This was then incubated at 37°C for 30 minutes before a further 1µl of turbo DNase was added. The mixture was incubated at 37°C for a further 30 minutes. 10µl of the DNase inactivation agent was then added to stop further reaction and mixed by vortex before being incubated at room temperature for 2 minutes. The final mixture was centrifuged at 10,000 x g for 2 minutes and the RNA was transferred to a fresh eppendorf tube.

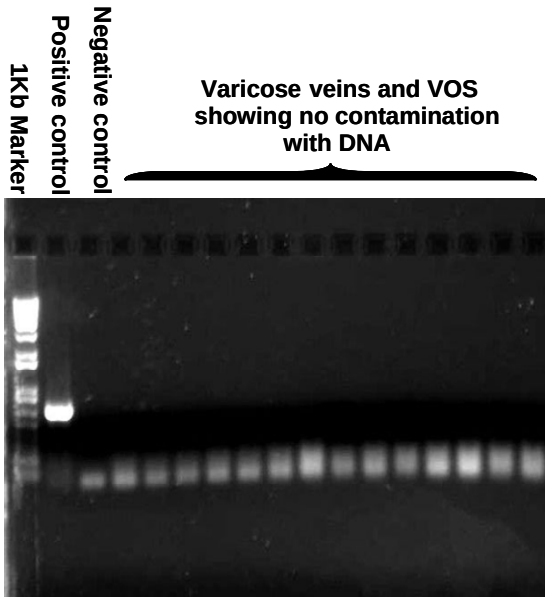
The extracted RNA was run on a gel to confirm the presence of RNA (as 18S and 28S ribosomal RNA bands) as shown below in Figure 2.5 (see Section 2.2.5 for gel electrophoresis methodology).

Figure 2.5 Gel electrophoresis confirming the presence of mRNA



Subsequently, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR was carried out at this time point in order to confirm the absence of DNA in the sample. An example of the gel electrophoresis is shown in Figure 2.6 (for PCR and gel electrophoresis method please refer to Sections 2.2.3 and 2.2.5).

Figure 2.6 Gel electrophoresis of GAPDH PCR confirming the absence of genomic DNA in RNA sample



2.2.2 PREPARATION OF CDNA FROM MRNA – REVERSE TRANSCRIPTION

Once the absence of DNA was confirmed in all the samples, the RNA was quantified by absorbance using a spectrophotometer, which measures absorption of light at 260nm. The spectrophotometer was allowed to warm up and the setting of DNA/RNA selected. The spectrophotometer was calibrated to zero using a cuvette of 70µl DEPC-treated water. For sample readings, 7µl of sample RNA was added to 63µl of DEPC-treated water and then transferred to the spectrophotometer machine in order to measure the A260. By using the calculation below it was possible to find of the amount of RNA in micrograms per millilitre:

A260 reading x 40 x dilution factor (1:10) = µg/ml RNA

500ng of RNA in a volume of 11µl were added to 1µl of random primers (Invitrogen Life Technologies, Paisley, UK) to make a total volume of 12µl. Random primers, as opposed to specific primers, were used as they non-specifically transcribe all mRNA present within the reaction mix, thus, maximising the number of molecules that can be analysed from a small sample of RNA. This was incubated at 70°C for 10 minutes to denature the RNA. In order to keep the RNA single-stranded the sample was then placed immediately on ice for 5 minutes.

Direct amplification of RNA is limited due to the inability of Taq polymerase to recognise RNA as a template. To overcome this, a retroviral reverse transcriptase (RT) enzyme was used to produce a complementary strand of DNA (cDNA) for the RNA template. Reverse transcription was carried out using Moloney murine leukaemia virus (MMLV) RT (Promega, Southampton, UK). This required the use of the following components added to each sample in the form of a master mix:

- 4µl of 5x MMLV Reaction Buffer (Tris hydrochloride, potassium chloride and magnesium chloride, all essential for the reaction; Promega, Southampton, UK)
- 1µl RNasin 40 Units/µl; to inhibit any RNA contamination in the cDNA (Promega, Southampton, UK)
- 1µl dNTP; this adds the nucleotides required for the building of cDNA from RNA by reverse transcription and is a combination of dATP, dCTP, dGTP and dTTP (Promega, Southampton, UK)
- 0.5µl of MMLV 200 Units/µl (RNAse H minus point mutant; Promega, Southampton, UK)
- 1.5µl of water

The samples, with the added mastermix, were incubated at 25°C for 10 minutes in order to allow the primers to anneal to the RNA. They then underwent 42°C for 60 minutes,

and incubation at 70°C for 15 minutes, for the reverse transcription reaction, before being placed on ice for 5 minutes. A summary of the complete reaction is shown in Table 2.1.

Table 2.1 Summary of RT reaction using a thermocycler

Temperature	Time	Process
70°C	10 mins	Denatures secondary structure of RNA
4°C	5 mins	
Addition of Mastermix		
25°C	10 mins	Anneal Primers
42°C	60 mins	Reverse Transcription
70°C	15 mins	
4°C	Forever	

1µl of the RT reaction was used in a GAPDH PCR in order to confirm the presence of cDNA, as described below. An example of the gel is shown in Figure 2.7.

2.2.3 POLYMERASE CHAIN REACTION

In order to investigate if the genes of interest were expressed in the venous samples, the cDNA was amplified using Polymerase Chain Reaction (PCR) using specific primers for the genes of interest and then electrophoresed on a Sybr Green agarose gel and visualised by an ultraviolet transilluminator in a Biodoc cabinet.

A master mix was prepared using the components in Table 2.2 for the required number of samples, including a positive (cDNA prepared from RNA known to contain gene of interest) and negative control (only containing 24µl of mastermix). 24µl of the master mix was transferred in to the 200µl PCR tubes and subsequently 1µl of the appropriate cDNA then also added to the pre-marked tubes.

Table 2.2 Contents of PCR master mix

PCR ready kit provided 10xPCR Buffer, MgCl2 and Taq ((Invitrogen Life Technologies, Paisley, UK). Primers (Eurofins MWG Operon, Germany) were sourced separately as was the dNTP (Promega, Southampton, UK)

	Concentration	Final	Amount (μl)
10 x PCR Buffer (Invitrogen Life Technologies)	10x	1x	2.5
dNTP (Promega, Southampton, UK)	10mM	200μM	0.5
Forward Primer (MWG)	12.5μM	0.25μM	0.5
Reverse Primer (MWG)	12.5μM	0.25μM	0.5
$MgCl_2$ (Invitrogen Life Technologies)	50μM	1.5μM	0.75
Taq Polymerase (Invitrogen Life Technologies)	5 units/μl	0.5 units	0.1
Water			19.15
Total			24μl

PCR is a three step process, referred to as a cycle, that is repeated a specified number of times in order to replicate DNA *in vitro*. The process takes place in a thermocycler; this controls and alternates temperatures for programmed periods of time for the appropriate number of PCR cycles – usually between 30-40 cycles. One cycle consists of the following stages: i) denaturation ii) annealing iii) extension.

Denaturation by heat (94°C) separates the double stranded DNA into two single strands. This occurs as the hydrogen bonds that link the base pairs are weak and therefore break easily at high temperatures. The bonds between the deoxyribose and phosphates are strong covalent bonds and so remain intact.

The annealing process allows the primers specific to the gene of interest to bind. Two primers are included in the PCR (see Table 2.3 and Table 2.4 for reaction details); one for each of the single strands that was produced during the denaturation process. The primers flank the sequence of interest that is to be replicated. The annealing temperature is usually between 40-65°C.

Extension starts once the primers are annealed the temperature raised to 72°C and the enzyme Taq DNA polymerase is used to replicate the DNA strands. Taq DNA polymerase is a recombinant thermostable DNA polymerase from the organism Thermus aquaticus and unlike normal polymerase enzymes is active at high temperatures. Taq recognises primers bound to single stranded DNA and polymerises the reverse component strand using DNA as a template. It does this by facilitating the binding and joining of the complementary nucleotides that are free in the solution (dNTPs). Extension always begins at the 3' end of the primer and continues towards the 5' end of the template DNA making a double strand out of each of the 2 strands. Taq DNA polymerase synthesises exclusively the 3'-5' direction.

At the end of the cycle there are two new strands of DNA identical to that of the original target. The DNA polymerase does not recognise termination sequences and carries on until it falls off. The new strands have a beginning which is defined by the 5' end of the primer, but the 3' end is not precisely defined. As the number of cycles increases a strand with more defined length is formed and serves as a template for the newly synthesised sequence. The DNA strand that is produced from the template then has a precisely defined length that is limited at either end by the 5' end of each of the two primers. These DNA strands are called an amplicon. After a few cycles DNA strands that correspond to the target sequence are present in larger numbers than the variable length sequences.

Table 2.3 PCR primers

Gene of interest	Primer	Gene product size
GAPDH	Fwd: atgaggtccaccaccctgtt	671
	Rev: catggagaaggctggggctc	
ARP	Fwd: ctctggagaaactgctgcc	378
	Rev: tgtagatgctgccattgtcg	
MMP-2	Fwd: caagtggtccgtgtgaagtatg	496
	Rev: cgtcatcgtagttggctgtg	
MT1-MMP	Fwd: gtcttcaaggagcgctggttctg	488
	Rev: tagcccggttctaccttcag	
TIMP-2	Fwd: gagtgcaagatcacgcgcgctgcc	548
	Rev: tgggagatccctaagtgctg	
TIMP-3	Fwd: gtaactgcaagatcaagtcctgc	469
	Rev: cctcattctttctggcatgg	
VEGF	Fwd: cttgctgctctacctccacc	354
	Rev: ctgcattcacatttgttgtgc	
TNF-α	Fwd: ccatgttgtagaaaccctcaag	357
	Rev: ctgagtcggtcacccttctc	
IL-1β	Fwd: gagcaacaagtggtgttctcc	347
	Rev: gacacaaattgcatggtgaag	
IL-6	Fwd: caattcgttctgaagagccc	587
	Rev: ctgcgtccgtagtttccttc	
HIF-1α	Fwd: ttctctctagtctcacgagggg	458
	Rev: accataacaaaaccatccaagg	
HIF-2α	Fwd: gctgacaaggagaagaaaagga	443
	Rev:ttgagactcaggttctcacgaa	

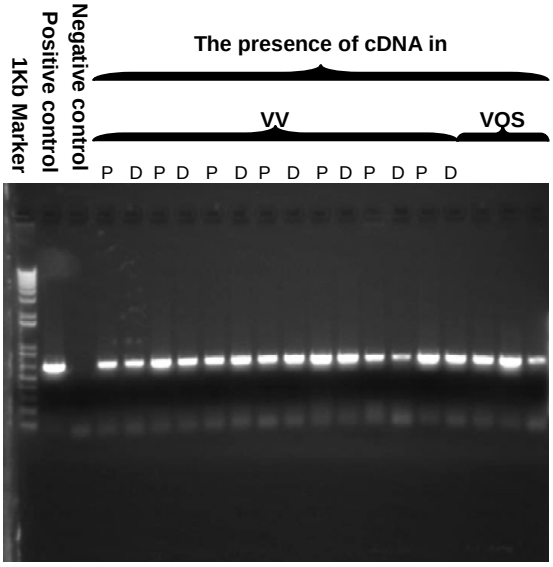
Table 2.4 PCR cycle

Example of PCR reaction: where X is the annealing temperature that is determined by the primers' melting point.

Step	Temperature	Time	Cycles
Denaturation	94^{o}C	5 minutes	
	94^{o}C	30 seconds	For 36 cycles
Annealing	X	30 seconds	
Extension	72^{o}C	40 seconds	
	72^{o}C	5 minutes	
	4^{o}C	forever	

GAPDH PCR was used not only to confirm absence of DNA in RNA samples (Figure 2.6), but also to confirm the presence of cDNA after reverse transcription in the vein samples at various stages of their processes (Figure 2.7). GAPDH catalyzes an important energy yielding step in carbohydrate metabolism – the reversible oxidative phosphorylation of glyceraldehydes-3-phosphate in the presence on inorganic phosphate and nicotinamide adenine dinucleotide (NAD). Since this is an essential process in energy production, all cells express the enzyme at a relatively constant rate and it is therefore termed a "housekeeping" gene, as it is consistently detected in cellular DNA.

Figure 2.7 Gel electrophoresis of GAPDH PCR confirming the presence of cDNA following reverse transcription



2.2.4 PRIMER VALIDATION

Primer3 software (http://frodo.wi.mit.edu/) was used to design the primers which were synthesized by MWG Biotech AG, UK. A validation assay was performed to monitor the difference in amplification efficiency on both the housekeeping genes and the genes of interest prior to quantitative PCR analysis (Livak and Schmittgen 2001). All the primers were validated with a reaction efficiency of between 92%-108%. If values were outside this range then the primers were redesigned and validated again.

2.2.5 GEL ELECTROPHORESIS

In order to visualise the products of the PCR, agarose gel electrophoresis was performed in order to separate the amplified DNA. This technique separates macromolecules that differ in size. By placing the molecules on an electric field they migrate to the positive pole. Agarose gels are advantageous as they are non-toxic and simple to make, as well as allowing a large range of separation. A 100ml 1.2% agarose gel (polysaccharide extract from seaweed) was prepared by mixing 1.2g agarose (Invitrogen Life Technologies, Paisley, UK) with 100ml of an electrophoresis buffer, 1xTAE (Tris-acetate-EDTA, containing 40mM Tris base, 20mM acetic acid and 1mM EDTA). 10µl of SYBR safe DNA gel stain, a fluorescent dye used to stain nucleic acids, (Invitrogen Life Technologies, Paisley, UK) was added (1:10000 dilution in DMSO). The mixture was heated for approximately 90 seconds in a microwave, or until all the agarose was fully dissolved in the TAE. The solution was left to cool slightly and then poured in to the casting tray with the appropriate combs in place in order to make wells to place the PCR products in. This was then left to solidify. The gels were then placed into the electrophoresis chamber and just covered with buffer, 1xTAE. 4µl of loading dye (50mg Bromophenol blue (VWR International Ltd, Lutterworth, Leicestershire, UK), 8mls water and 12mls glycerol (VWR International Ltd, Lutterworth, Leicestershire, UK) was added to each sample in the PCR tube. 1µg of 1Kb marker (Invitrogen Life Technologies, Paisley, UK) was loaded for size reference into the first lane. 29µl of mixed PCR product and loading dye was then pipetted into subsequent wells. The positive and negative leads were then attached. The power supply was switched on at 140 mV for approximately 30 minutes. Electronic connection is confirmed as bubbles can be observed from the electrodes. The distance the DNA migrated was monitored by observing the tracking dye. When adequate migration had occurred the power was switched off and the gel removed from the tank and placed on an ultraviolet transilluminator. Sybr Green dye binds double-stranded DNA and fluoresces at ultraviolet wavelengths. This highlights whether or not the gene of interest is present in the samples and also confirms the molecular weight of the gene.

2.2.6 QUANTITATIVE PCR

Real-time or quantitative PCR is a powerful and rapid technique for nucleic acid amplification in a reaction which continuously monitors changes in fluorescence during cycling within a PCR tube. Quantitative PCR is based on the detection of a fluorescent reporter molecule that increases as the PCR products accumulate with each cycle of amplification. Quantitative PCR exceeds the limitation of traditional end-point PCR methods by allowing either an absolute or relative quantification of PCR product at the end of each cycle.

Sybr Green Jumpstart Taq Readymix (Sigma, St Louis, MO, USA) (excitation and emission maxima of 494nm and 521nm respectively) was used for quantitation. This contained SYBR Green I dye, Jumpstart Taq DNA polymerase antibody which prevents non-specific DNA amplification), deoxynucleotides (dATP, dCTP, dGTP, dTTP), and reaction buffer made up of 20mM Tris-HCl (pH8.3), 100mM KCl and 7mM $MgCl_2$. It exhibits little fluorescence when in solution but emits a strong signal when bound to double stranded DNA. As each cycle proceeds, more double stranded DNA is produced and therefore more fluorescence can be detected. Sybr Green is inexpensive, simple to use and sensitive. However it will bind to any double stranded DNA in the reaction, including primer-dimers and misprimed products, which can result in an overestimation of the target concentration. Thorough testing of the primers was done to ensure no primer dimer or non-specific products were produced. Specific primers for the genes of interest were used for quatitative PCR and the primer pairs were validated to ensure amplification efficiencies of housekeeping gene and gene of interest were equal (Livak and Schmittgen 2001).

The Corbett robot (Robotics CAS-1200) was used to prepare the quantitative PCR reagents, as specified in Table 2.5 below, in order to minimise the pipetting errors due to the relative small reaction volume. Each sample was duplicated and placed into a 100µl eppendorf. Two non-template controls (NTC's) were also pipetted using water for control. These values were helpful in identifying and excluding any vein samples with threshold cycle (CT value) values that were either equal or greater than those of the NTC's. After preparation

the tubes were then transferred to the RotorGene 6000, a 72 closed-tube centrifugal thermal cycler designed by Corbett Research (Mortlake, Sydney, Australia) for analysis.

Table 2.5 Quantitative PCR mastermix reagents

Reagents	Concentration	Volume
SYBR Green Ready Mix (Sigma)		7.5μl
Forward Primer (MWG)	12.5μM	0.5μl
Reverse Primer (MWG)	12.5μM	0.5μl
cDNA		2μl
DEPC Water		4.5μl
Total		**15ml**

ARP (Acidic Ribosomal Protein; see Table 2.3 for primer details), a house keeping gene, was used to normalise for well to well variation and differences in extraction efficiency and cell number. Typical curves are shown in Figures 2.8 and 2.9.

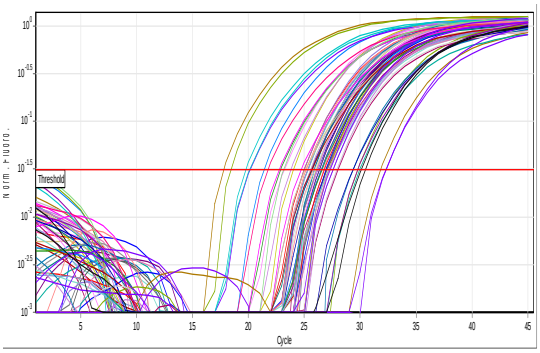
The data was collected on the Rotor-gene 7 program in order to set a threshold and gain the Ct values. These were then collated on to an Excel spread sheet and calculations made using comparative Ct method, also called $2^{-\Delta\Delta Ct}$ method. This involved normalising the Ct values of the genes of interest (for example, MT1-MMP) to an endogenous housekeeping gene, ARP, resulting in the value ΔCt. Thereafter, the ΔCt values of the samples of interest (that is, varicose veins) were compared with the ΔCt values of reference or calibrator:

$$\Delta\Delta C_t = \Delta C_{\text{t sample}} - \Delta C_{\text{t reference}}$$

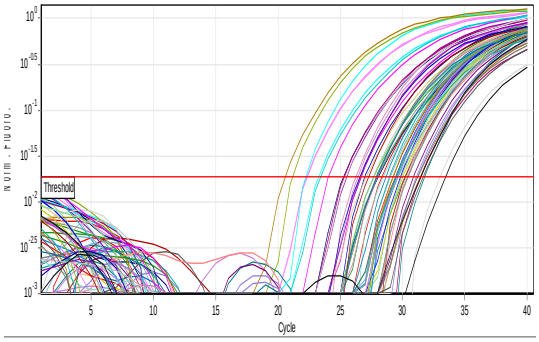
The mRNA levels were expressed as $2^{-\Delta\Delta Ct}$, and were first calculated for all samples (proximal and distal) relative to ΔCt of veins from other sources as reference.

Figure 2.8 The quantitation curves obtained for each gene and sample of interest, including the threshold at which the Ct values were calculated: MMP, TIMP and cytokines

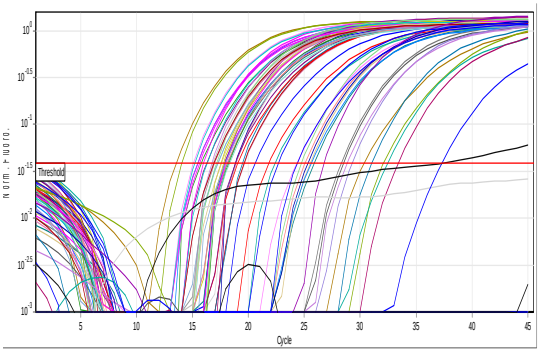
MMP-2



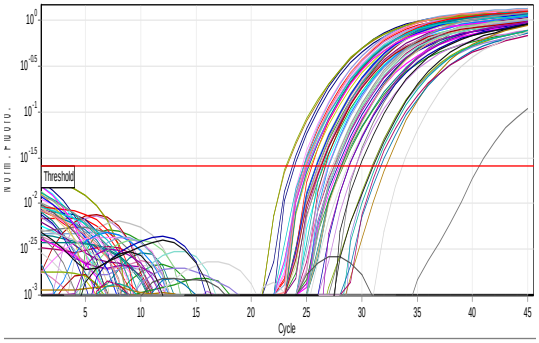
MT1-MMP



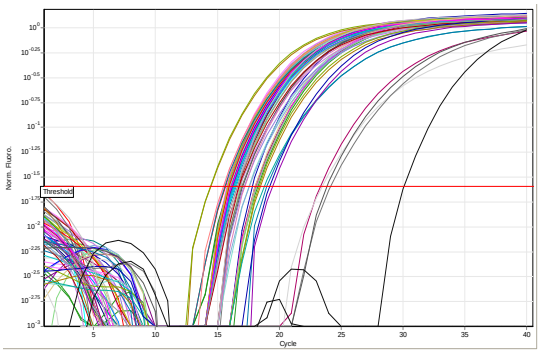
TIMP-2



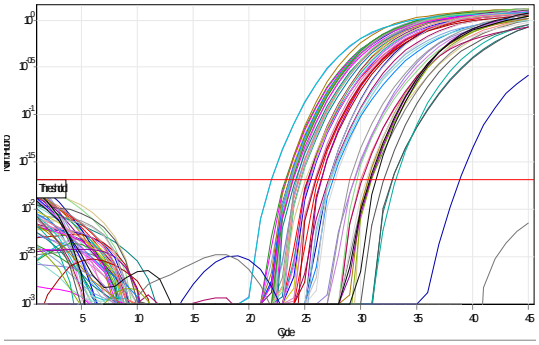
TIMP-3



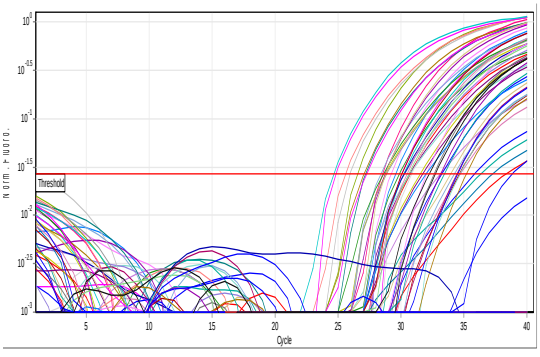
VEGF



TNF-α



IL-1β



IL-6

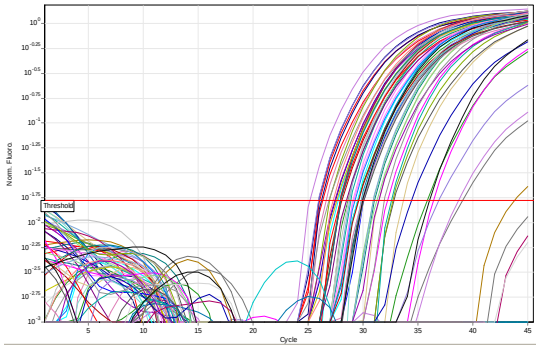
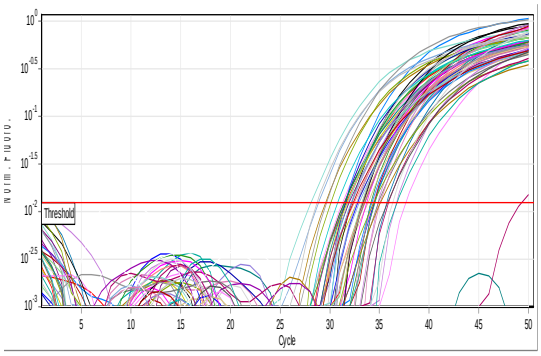
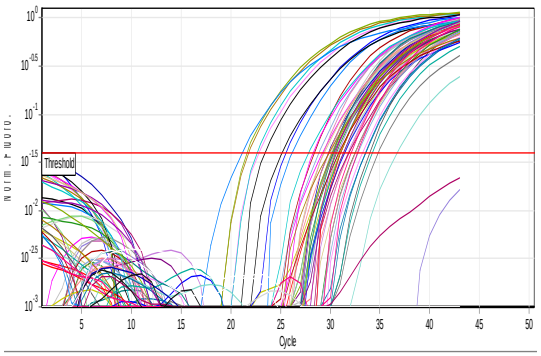


Figure 2.9 The quantitation curves obtained for each gene and sample of interest, including the threshold at which the Ct values were calculated: HIF and ARP

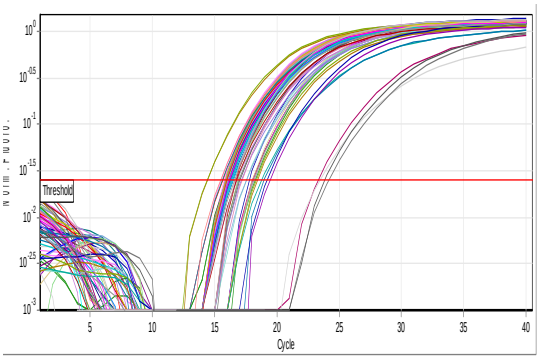
HIF-1α



HIF-2α



ARP



2.3 EXPOSURE OF HMEC-1 AND HSVEC TO ACUTE AND CHRONIC HYPOXIA

2.3.1 HSVEC EXTRACTION AND CULTURE

Human saphenous vein endothelial cells (HSVEC) had already been harvested by predecessors in the Department. They were obtained by enzymatic release from saphenous veins harvested, some 10 years before, during high ligation of varicose veins or bypass surgery. The residual external connective tissue was removed, the vein was opened longitudinally and the luminal surface placed face down in a shallow Petri dish containing Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS; 150mM NaCl, 2mM NaH_2PO_4 and 2mM Na_2HPO_4) and 1 mg/ml collagenase (Type II, Sigma, Poole, UK). They were incubated

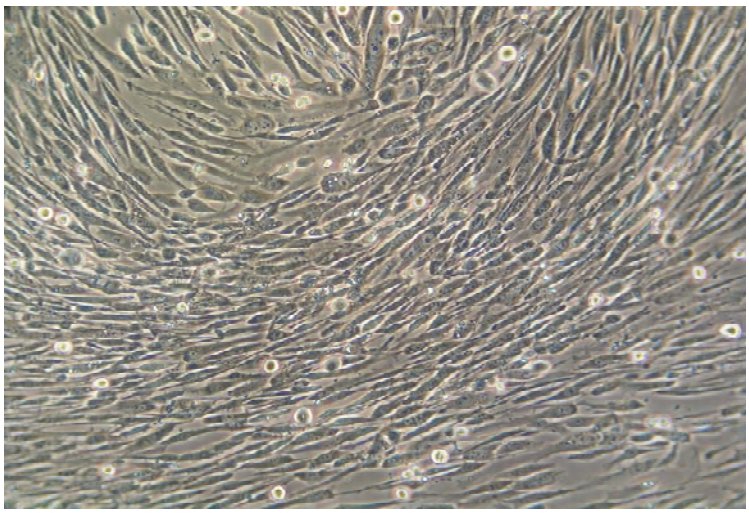
at room temperature (20-22°C) for 30 minutes. Cells were placed in culture on fibronectin-coated flasks and grown in M199 medium supplemented with heparin, endothelial cell growth supplement, antibiotic solution (200 units/ml penicillin and 200μg/ml streptomycin) and 10% heat inactivated human serum (from either non-diabetic patients with peripheral vascular disease greater than 65 years of age or from healthy donors less than 30 years of age) and maintained in humidified atmosphere of 5% CO_2 in air at 37°C (Gosling *et al.* 1998).

In order to immortalise the HSVEC they were taken at passage 2, cultured in 75cm^2 flasks until they reached 50% confluence. They were infected with two retroviral vectors, pZipSVtsA58-U19 which expresses the SV40 large T-antigen and pLXSN16E6E7 containing the E6 and E7 genes from HP16 in the presence of 8mg/ml polybrene. After 24 hours the virus-containing medium was discarded and fresh medium added. Cells were grown to confluence and then selected by supplementing the medium with 1mg/ml of the selective antibiotic G-418. The medium was changed twice weekly until the cells became confluent. The cells were detached using trypsin-EDTA solution and subcultured in 1:3 ratio in 25cm^2 flasks. Cell aliquots from each passage were frozen in medium containing 10% (v/v) DMSO and stored in liquid nitrogen (Scoumanne *et al.* 2002).

The cells were removed from the liquid nitrogen, and defrosted in a water bath of 37°C. They were placed in 5mls of the culture medium EGM 2 (Lonza Group Ltd, Switzerland) supplemented with 100U/ml penicillin, 100μg/ml streptomycin (BioWhittaker, Wokingham, UK) and 5% (v/v) heat inactivated foetal calf serum (FCS; Sigma UK) and then seeded onto 25cm^2 gelatin coated flasks. The flasks were then checked daily under the microscope for cell confluence. The culture medium was changed twice weekly to provide continuing nourishment. Once confluent they were split. This involved the addition of 3mls 0.25% trypsin/EDTA (Invitrogen Life Technologies, Paisley, UK) in order to cover the whole flask. 2mls of the trypsin/EDTA is then almost immediately removed before the cells detach. The flask was then left to incubate for a short while with the remaining 1ml of tryspin/EDTA while regularly checking for cell detachment under the microscope. Once the cells were

released from the base of the flask the mixture of the cells and the trypsin/EDTA were then split into 3 and placed in to new 25cm^2 gelatin coated flasks and grown using the above culturing method until three identical confluent flasks were obtained (Figure 2.10) which were then exposed to differing degrees of hypoxia as explained in Section 2.3.3.

Figure 2.10 Confluent HSVEC cells



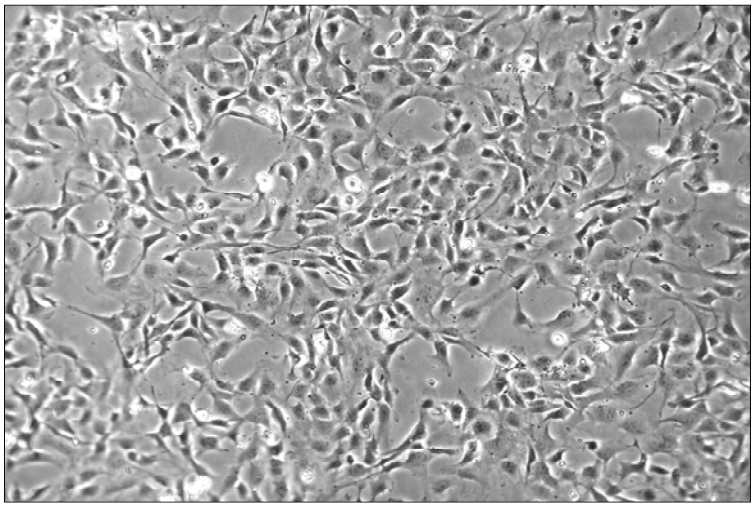
2.3.2 CULTURING HMEC-1

HSVEC, obtained as described above, are venous endothelial cells and therefore a good model for the response of large vein endothelial cells to hypoxia (as might expected if venous stasis were the predominant cause of hypoxia in varicose vein disease). However, it is also possible that hypoxia might occur at the level of the vasa vasorum, due to venous wall hypertrophy and/or venous hypertension, small vessels (i.e. vasa vasorum) might also be exposed to hypoxia in patients varicose veins. Therefore, I also used micro vascular endothelial cells and studied their responses to hypoxia.

HMEC-1 were obtained from Centers For Disease Control and Prevention (CDC)/National Centre for Infectious Diseases, Atlanta, Georgia USA. HMEC-1 (SV40 T antigen transformed dermal endothelial cells derived from human foreskin, pulmonary and

hepatic endothelium, which retain microvascular endothelial cell characteristics; Figure 2.11) have been cultured in 25cm^2 non-coated flasks as these cells are adherent. They were fed 2-3 times a week with 4mls RPMI-1640 medium supplemented with 100U/ml penicillin, 100μg/ml streptomycin (BioWhittaker, Wokingham, UK) and 5% (v/v) heat inactivated foetal calf serum (FCS; Sigma UK). HMEC-1 cells were split after reaching confluence using the same method as previously described for HSVEC in Section 2.3.1. However, the HMEC-1 cells were very sticky and took a little more time and vigor to detach them. The mixture of the cells and the trypsin/EDTA were then split into 3 and placed in to new 25cm^2 flasks and grown using the above culturing method until three identical confluent flasks were obtained (Figure 2.11) which were then exposed to differing degrees of hypoxia as explained in Section 2.3.3.

Figure 2.11 Confluent HMEC-1



2.3.3 HYPOXIC EXPOSURE

The cells were exposed to differing degrees of oxygen concentration in the RS Biotech Galaxy R incubator over a 24 hour period. Each cell line i.e. HSVEC and HMEC-1 had 3 identical flasks of confluent cells. One of the identical flasks of cells underwent incubation for 24 hours in a 21% oxygen chamber - equivalent to normoxia. In parallel, another flask underwent 6 hours exposure to 1% oxygen and then moved back to normoxia to

complete the 24 hours, in order to create an acute hypoxia model. To represent chronic hypoxia, a flask from each cell line was exposed to 24 hours of 1% oxygen. Triplicates were completed for each experiment.

On completion of the hypoxia experiment 350µls of a mixed solution of Buffer RLT and betamecaptaethanol (1ml Buffer RLT (QIAGEN GmbH, Germany) with 10µl betamecaptaethanol (Merck & Co, N.J., USA)) was placed in each flask in order to lyse the cells and they were placed in the –80°C freezer until ready to proceed to RNA extraction.

2.3.4 RNA EXTRACTION USING THE QIAAMP RNA BLOOD MINI KIT

RNA was extracted from the cells using the QIAamp RNA blood mini kit (QIAGEN). The QIAamp RNA blood mini kit (QIAGEN GmbH, Germany) is a purposely designed kit with specialised shredder and spin columns together with collection tubes and reagents to ease RNA extraction from blood, tissues and cells. The suspension of cells was centrifuged at 1200 rpm for 10 minutes and a cell pellet was obtained after discarding the supernatant. 350µl of a cell lysis solution composed of 10µl of beta-mercaptoethanol per 1ml of Buffer RLT (QIAGEN) was added to the pellet of cells and dispersed by gently pipetting. The lysate was homogenised in a QIA shredder column by centrifuging for 2 minutes at maximal speed and collected in a 2 ml collection tube. After re-suspending the homogenised lysate in 350µl of 70% ethanol the sample was transferred into a QIAamp spin column containing a selective binding RNA silica-gel based membrane and spun for 15 seconds at 8000 x g. The spin column was transferred into a new 2ml collection tube and 700µl of buffer RW1 (QIAGEN) was pipetted into the spin column and centrifuged for 15 seconds at 8000 x g. Contaminants were washed away with 500µl of buffer RPE by centrifuging the spin column for 15 seconds at 8000 x g, followed by a further 500µl washing at maximal centrifuging speed for 3 minutes. The final RNA product was obtained by washing the spin column with 50µl of RNase free water and centrifuging at 8000 x g for 1 minute and collecting the solute in a 1.5ml collection tube. Using the turbo DNase (Ambion, Austin,

USA) the 50µl of RNA was incubated with 1µl of DNase and 5µl of buffer at 37°C for 30 minutes. The DNase enzyme was then inactivated with 5µl of DNase inactivation reagent by gently swirling for 2 minutes to optimise binding of the enzyme to the neutralising beads. The sample was then centrifuged at 10000g for 1 minute and the 55µl effluent pipetted out.

Once mRNA was extracted and it was confirmed that there was no DNA present, the samples underwent reverse transcription in order to make cDNA which was then quantified by quantitative PCR. The results of were then analysed as previously described. All these methods have been previously described in full above.

2.4 STATISTICAL ANALYSIS

Data was compared and statistically analysed using the Graph Pad Prism software package (Graph Pad Software, CA, USA). Wilcoxon rank test was used to compare non-parametrically distributed paired data, and paired Student's t-test for normally distributed paired data. Mann-Whitney test was used to compare non-parametrically distributed unpaired data, and unpaired Student's t-test for normally distributed unpaired data.

Correlations were assessed using Spearman's co-efficient for non-parametrically distributed data. Correlation displays a broad class of statistical relationships between two or more random variables or observed data values. Correlations are useful because they can indicate a predictive relationship that can be exploited in practice. Correlations can also suggest possible causal, or mechanistic relationships; however statistical dependence is not sufficient to demonstrate the presence of such a relationship. Rank correlation coefficients, such as Spearman's rank correlation coefficient as used in this thesis, measure the extent to which, as one variable increases, the other variable tends to increase, without requiring that increase to be represented by a linear relationship. If, as the one variable increase, the other decreases, the rank correlation coefficients will be negative. Power calculations were not performed, as I was not able to predict at the start where differences would occur. However, future studies can use my data as a basis for further assessing potential differences.

CHAPTER 3

3 ASSESSMENT OF THE RELATIONSHIP BETWEEN MMP AND CYTOKINES IN VARICOSE VEINS

3.1 INTRODUCTION

There is a large amount of evidence to support the current theory that the balance between MMP and their inhibitors, TIMP, in a particular environment may determine the degrading potential of MMP (Borden and Heller 1997; Gomez *et al.* 1997; Nagase 1997). Within varicose veins this refers to the remodelling of the ECM. Previous studies have confirmed the presence of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, MMP-13 and TIMP-1 and TIMP-2 in both normal and varicose vein tissue (Gillespie *et al.* 2002; Woodside *et al.* 2003), although results have not always been consistent. A number of studies have examined at the relationship between TIMP-1 and MMP-2. It is has been widely shown that TIMP-1 activity tends to increase whilst MMP-2 activity decreases, resulting in an increased TIMP-1/MMP-2 ratio (Parra *et al.* 1998; Badier-Commander *et al.* 2000). This may partially explain why there is ECM accumulation in varicose veins. The mechanisms involved to regulate the MMP/TIMP balance are unknown, but potential candidates include cytokines and/or alterations in oxygen tension.

Rafetto *et al* has shown that prolonged increases in vein wall tension had lead to increased expression of MMP-2 and MMP-9 and reduced contraction in the rat vena cava. This increased expression of MMP results in decreased contractility within the vessel and therefore promotes dilation. This study supports the theory that venous hypertension/stasis may play a very important role in the formation of varicose veins by mechanisms influencing MMP expression (Raffetto *et al.* 2008).

Cytokines are small proteins that are secreted in response to an immune stimulus, that act quickly and locally, by mediating and regulating immunity, inflammation and haematopoiesis. They have been shown to increase or decrease the expression of membrane proteins, and to affect proliferation and secretion of effector molecules. Badier-Commander *et*

al compared levels of TGF-β, FGF-2 and VEGF in control and varicose veins. This study found that both TGFβ and FGF-2 were present in control veins and also varicose veins, although these cytokines were more abundant in the latter. No inflammatory cells were present which would indicate that there was no inflammatory mechanism responsible for the cytokine changes observed (Badier-Commander *et al.* 2001). Murphy *et al* also confirmed the presence of the cytokines, TNF-α and VEGF, in CVD and varicose vein disease. In fact the cytokine levels were raised when compared to a control group and then reversed with compression treatment. (Murphy *et al.* 2002). A similar study, looking at VEGF levels, by Hollingsworth *et al* attempted to induce blood stasis by application of a tourniquet for 10 minutes. After induction of blood stasis the control group showed a clear increase in the mean levels of VEGF. In contrast, the varicose vein group showed no significant alteration in VEGF (Hollingsworth *et al.* 2001). Signorelli *et al* investigated levels of TNF-α, IL-1β and IL-6. The baseline of these were increased in varicose vein patients and became even more augmented when induced stasis was applied (Signorelli *et al.* 2000).

MMP and TIMP have been shown to be stimulated by inflammatory cytokines, such as TNF-α and IL-1β (Borden and Heller 1997).

3.2 OBJECTIVES

There is a suggestion that MMP may be linked with inflammatory cytokine activity. The purpose of this component of my research was to investigate and determine the relationship between inflammatory and angiogenic cytokines with that of selected MMP/TIMP within varicose vein specimens, and addressed the question of whether vein wall remodelling is caused by cytokine-driven alterations in the MMP/TIMP balance.

3.3 MATERIALS AND METHODS

3.3.1 PATIENT SELECTION AND TISSUE SAMPLING

Varicose vein samples were obtained from patients from Charing Cross Hospital who required surgery for definitive management of their condition. Veins from other sources were used for comparison and these were obtained from patients undergoing arterial bypass, carotid endartarectomy operations, or arterio-venous fistula formation. Samples gained were immediately transferred to the laboratory and frozen using liquid nitrogen and then stored in the freezer (-80^{o}C) until required.

3.3.2 RNA EXTRACTION AND QUANTITATION

Varicose vein tissue, including proximal and distal samples for each vein, and the veins from other sources was pulverised in a frozen state in order to preserve the RNA. The tissue was weighed and homogenised using TRIZOL Reagent (Invitogen Life Technologies, Paisley, UK). This maintains the integrity of the RNA while disrupting cells and dissolving cell components. RNA was then extracted using the method previously described in Section 2.2. GAPDH PCR was performed in order to confirm the absence of genomic DNA. RNA was then quantified using a spectrophotometer and 500ng of RNA underwent reverse transcription reaction to prepare cDNA. Subsequently, RT-PCR was performed for each of the genes of interest in order to ensure amplification of a single discrete gene of interest band with no primer-dimer formation. Quantitative PCR using a Sybr Green Jumpstart Taq Readymix allowed sequential quantitative PCR runs, including non-template controls, to analyse the genes of interest and allow calculation of a Ct value from the quantitation curves. The genes of interest included the matrixmetalloproteinases MMP-2, MT1-MMP, TIMP-2 and TIMP-3 and the cytokines VEGF, TNF-α, IL-1β and IL-6.

3.3.3 STATISTICAL ANALYSES

The data, in the form of a quantitation curve, were collected on the Rotor-gene 7 program in order to set a threshold and obtain the Ct values. These were then collated on to an

Excel spread sheet and calculations made using comparative Ct method, also called $2^{-\Delta\Delta Ct}$ method (see Chapter 2). The mRNA levels were then compared and statistically analysed using the Graph Pad Prism software package (Graph Pad Software, CA, USA). Wilcoxon rank test was used to compare non-parametrically distributed paired data, and paired-test for normally distributed paired data. Mann-Whitney test was used to compare non-parametrically distributed unpaired data, and unpaired-test for normally distributed unpaired data. Correlations were assessed using Spearman's co-efficient.

3.4 RESULTS

The following analyses were performed

i) total varicose vein mRNA, expressed as fold change relative to a human cDNA sample, and compared to veins from other sources

ii) comparison of total proximal *versus* total distal population of varicose veins (using data from fold change relative to a human cDNA sample)

iii) comparison of paired proximal and distal varicose vein samples (using data from fold change relative to a human cDNA sample)

3.4.1 MMP-2 EXPRESSION IN VARICOSE VEINS

MMP-2 mRNA levels were quantified by PCR in the varicose vein samples and veins from other sources. MMP-2 mRNA levels in the varicose veins (median=2.29) were found to be similar to but if anything slightly elevated relative to veins from other sources (median=1.76; Figure 3.1). This was more apparent when comparing the range of data between the 2 populations; varicose veins (0.64 – 135.80) and veins from other sources (0.17 – 31.56). This difference was not statistically significant (p=0.4629) using Mann-Whitney test. When data from the comparison of proximal and distal samples were examined, it appeared that MMP-2 mRNA was higher in distal varicose vein segments relative to the proximal varicose vein segments. However when the medians were compared, there was little difference, with the proximal (median=2.14) being similar to the distal (median=2.45; Figure 3.2). Similar data was found when the samples were analysed as pairs (Figure 3.3). This difference was not statistically significant using Mann–Whitney test (p=0.5755) or by Wilcoxon signed rank test (p=0.4543) respectively.

Figure 3.1 MMP-2 mRNA levels in varicose vein segments and VOS
MMP-2 mRNA levels in 30 varicose vein segments and 8 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

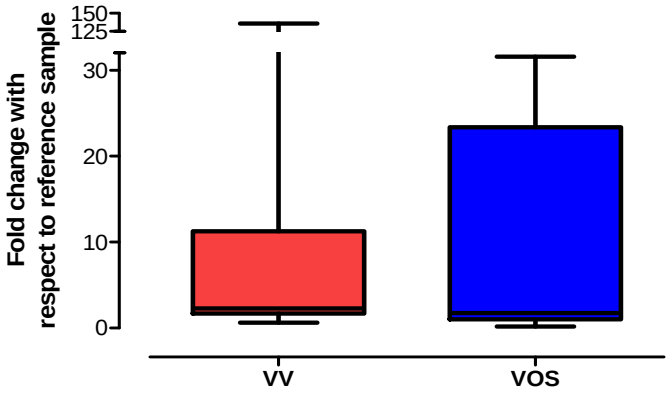


Figure 3.2 MMP-2 mRNA levels in total proximal and distal varicose vein segments
MMP-2 mRNA levels in 15 proximal and 15 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

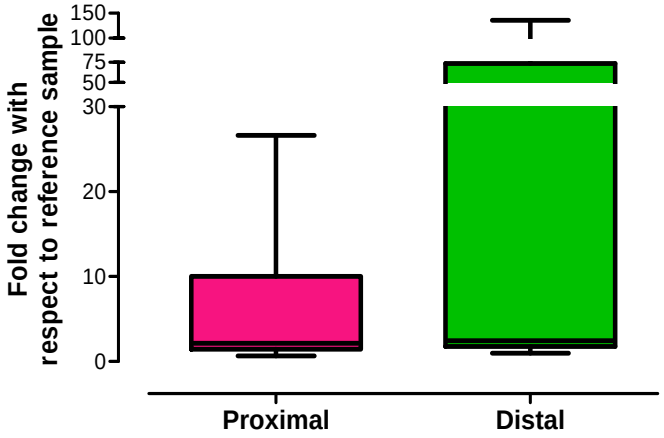
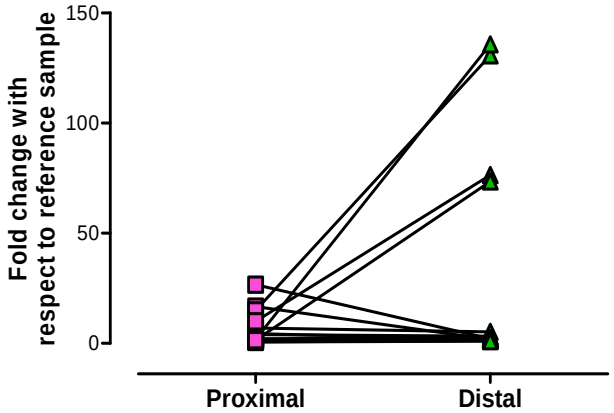


Figure 3.3 MMP-2 mRNA levels in paired proximal and distal vein segments
MMP-2 mRNA levels in 15 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.2 MT1-MMP EXPRESSION IN VARICOSE VEINS

Furthermore, MT1-MMP mRNA levels were quantified by PCR in the varicose vein samples and veins from other sources. When comparing median data, MT1-MMP mRNA levels in the varicose veins were found to be decreased (median=0.99) relative to the veins from other sources (median=7.56; Figure 3.4). However, this could be due to a larger population range in varicose veins (0.11 – 43.71) than that of veins from other sources (0.24 – 20.18). This difference between veins from other sources and varicose veins was non-significant (p=0.5040) using Mann-Whitney test. When data from the comparison of proximal and distal samples were examined, MT1-MMP mRNA was found to be higher in distal varicose vein segments (median=2.21) relative to the proximal varicose vein segments (median=0.97;Figure 3.5). A similar ratio was found when analysing paired proximal and distal segments (Figure 3.6). This difference was not statistically significant using Mann Whitney test (p=0.5614) and Wilcoxon signed rank test (p=0.2769).

Figure 3.4 MT1-MMP mRNA levels in varicose vein segments and VOS
MT1-MMP mRNA levels in 30 varicose vein segments and 4 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

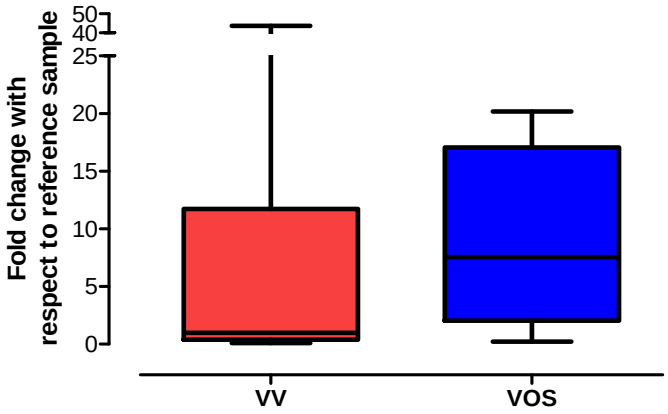


Figure 3.5 MT1-MMP mRNA levels in total proximal and distal varicose vein segments
MT1-MMP mRNA levels in 15 proximal and 15 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

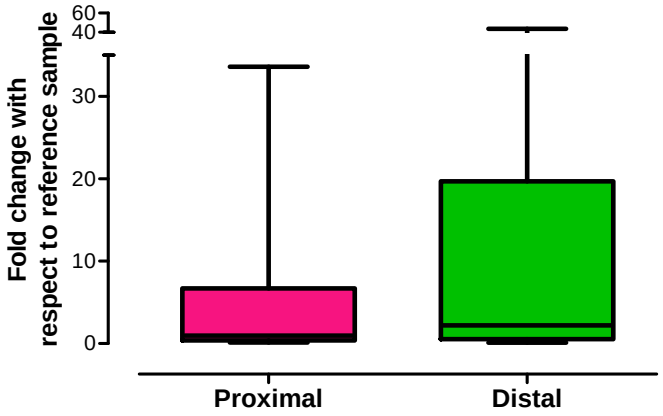
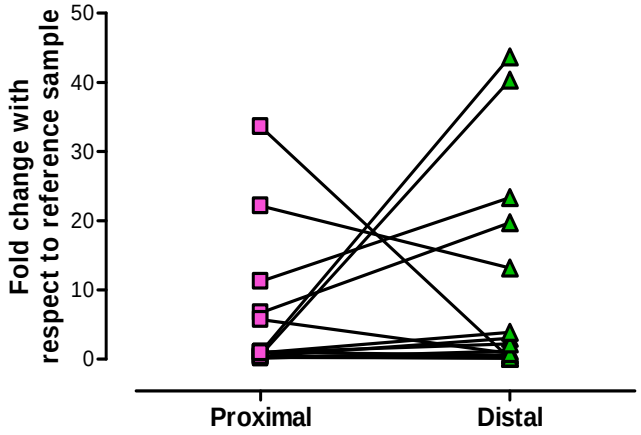


Figure 3.6 MT1-MMP mRNA levels in paired proximal and distal vein segments
MT1-MMP mRNA levels in 15 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.3 TIMP-2 EXPRESSION IN VARICOSE VEINS

TIMP-2 mRNA levels were quantified by PCR in the varicose vein samples and veins from other sources. TIMP-2 mRNA levels in the varicose veins were found to be grossly elevated (median=1148.00) relative to the veins from other sources (median=1.61; Figure 3.7). This data was statistically significant different using Mann Whitney test (p=0.0016). When data from the comparison of proximal and distal samples were examined, either alone or as paired samples, the range appeared to be equivalent, nevertheless TIMP-2 mRNA was found to be higher in distal varicose vein segments (median=1808.00) relative to the proximal varicose vein segments (median=754.80; Figure 3.8, Figure 3.9). This difference was not statistically significant using Mann-Whitney test (p=0.8357) or the Wilcoxon signed rank test (p=0.8904) respectively.

Figure 3.7 TIMP-2 mRNA levels in varicose vein segments and VOS
TIMP-2 mRNA levels in 30 varicose vein segments and 7 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

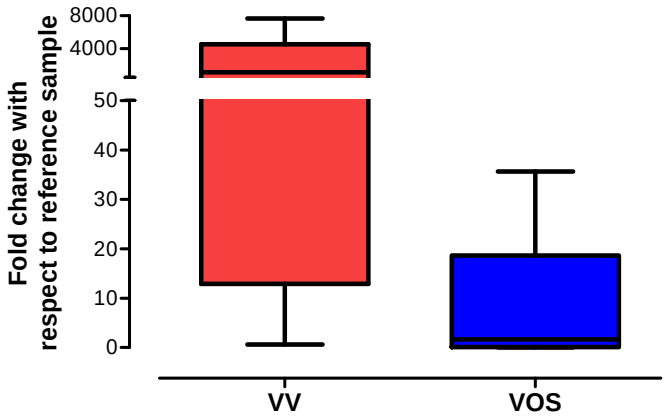


Figure 3.8 TIMP-2 mRNA levels in total proximal and distal varicose vein segments
TIMP-2 mRNA levels in 15 proximal and 15 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

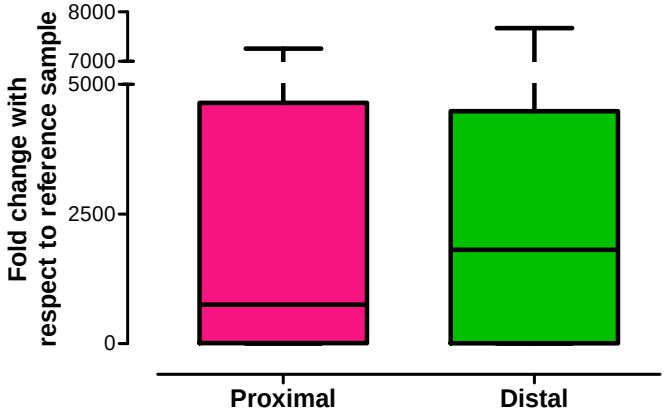
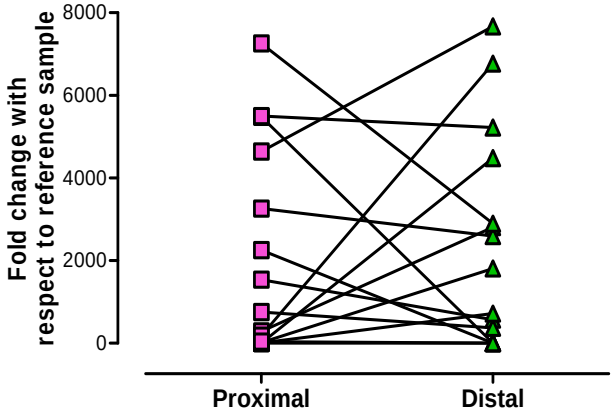


Figure 3.9 TIMP-2 mRNA levels in paired proximal and distal vein segments
TIMP-2 mRNA levels in 15 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.4 TIMP-3 EXPRESSION IN VARICOSE VEINS

TIMP-3 mRNA levels were quantified by PCR in the varicose vein samples and veins from other sources. TIMP-3 mRNA levels in the varicose veins (median=11.00) were also found to be grossly elevated relative to the veins from other sources (median=0.99; Figure 3.10). This was highly significant using Mann Whitney test (p=0.0002). When data from the comparison of proximal and distal samples were examined, TIMP-3 mRNA was found to have broader range in distal varicose vein segments (2.02 – 58.89) relative to that of the proximal varicose vein segments (1.00 – 17.75). However comparison of their medians showed little difference with proximal (median=10.65) being equivalent to distal (median=12.73; Figure 3.11). Data were also available from 13 varicose vein matched pairs and again this showed little difference between distal varicose vein segments (median=12.73) relative to that of the proximal varicose vein segments (median=10.59; Figure 3.12). This difference was not statistically significant using Mann Whitney test (p=0.3693) and the Wilcoxon signed rank test (p=0.2734) respectively.

Figure 3.10 TIMP-3 mRNA levels in varicose vein segments and VOS
TIMP-3 mRNA levels in 27 varicose vein segments and 7 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

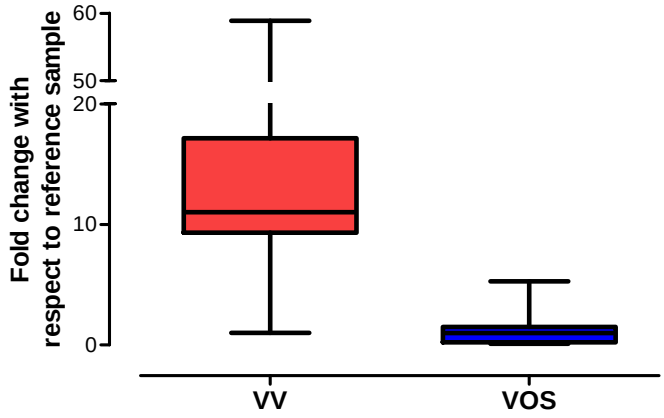


Figure 3.11 TIMP-3 mRNA levels in total proximal and distal varicose vein segments
TIMP-3 mRNA levels in 14 proximal and 13 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

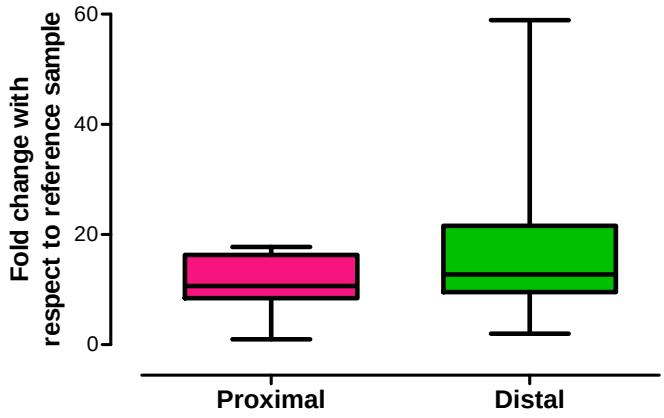
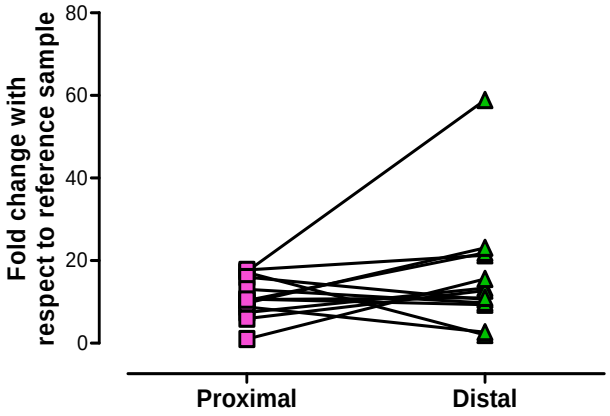


Figure 3.12 TIMP-3 mRNA levels in paired proximal and distal vein segments
TIMP-3 mRNA levels in 13 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.5 VEGF EXPRESSION IN VARICOSE VEINS

VEGF mRNA levels were quantified by PCR in the varicose vein samples and the veins from other sources. VEGF mRNA levels in the varicose veins were found to be slightly more abundant (median=0.81) than in veins from other sources (median=0.34; Figure 3.13). The range of data was similar in varicose vein segments (0.09 – 2.46) compared to veins from other sources (0.03 – 1.88). This data was not significant (p=0.2685) using the unpaired t-test. When examining the data from the comparison of proximal and distal samples, VEGF mRNA was found to be equivalent in distal varicose vein segments (median=0.73) relative to the proximal varicose vein segments (median=0.83; Figure 3.14). The range was also very similar in distal varicose vein segments (0.07 – 3.82) in comparison to that observed in proximal segments (0.09 – 2.24). Data were also available from the varicose vein matched pairs and again this showed that there was little difference in VEGF mRNA levels in distal varicose vein segments (median=0.73) relative to that of the proximal varicose vein segments (median=0.835; Figure 3.15). However both these comparisons were non-significant using unpaired t-test (p=0.9483) and paired t-test (p=0.8216) respectively.

Figure 3.13 VEGF mRNA levels in varicose vein segments and VOS
VEGF mRNA levels in 29 varicose vein segments and 6 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

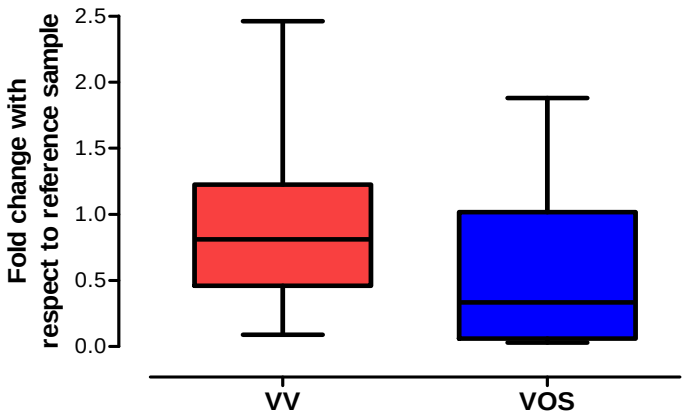


Figure 3.14 VEGF mRNA levels in total proximal and distal varicose vein segments
VEGF mRNA levels in 15 proximal and 14 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

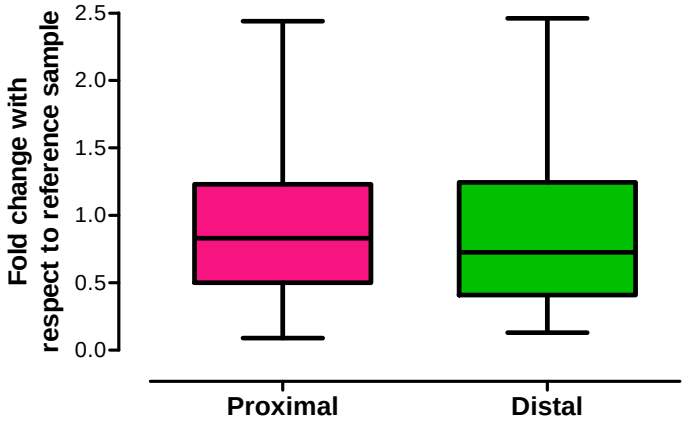
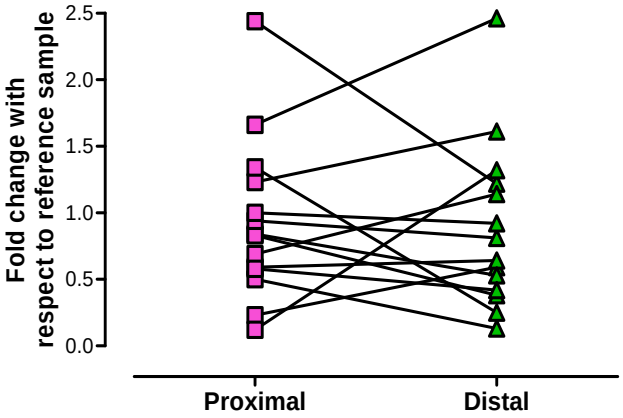


Figure 3.15 VEGF mRNA levels in paired proximal and distal vein segments
VEGF mRNA levels in 14 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.6 TNF-α EXPRESSION IN VARICOSE VEINS

TNF-α mRNA levels were quantified by PCR in the varicose vein samples and the veins from other sources. TNF-α mRNA levels in the varicose veins (0.32 – 104.70) were found to be elevated relative to the veins from other sources (0.13 – 33.71; Figure 3.16). This is also reflected when comparing the medians, with there being more TNF-α mRNA in varicose vein samples (median=11.79) than in veins from other sources (median=1.97). These data were not significant (p=0.1317) using Mann-Whitney test. When data from the comparison of proximal and distal samples were studied, the trend seemed to suggest that TNF-α mRNA may be slightly increased in distal varicose vein segments relative to that of the proximal varicose vein segments (Figure 3.17). However when the medians were analysed it showed that there was a lower mRNA level in distal veins (median=10.28) than proximal (median=13.09). Data was also available from the varicose vein matched pairs and again this showed that TNF-α was lower in distal varicose vein segments (median=10.28) relative to proximal varicose vein segments (median=13.16; Figure 3.18). However both these comparisons were non-significant using Mann Whitney test (p=0.9826) and Wilcoxon signed rank test (p=1.00) respectively. Overall this equates to similar TNF-α mRNA expression in distal varicose veins and proximal varicose veins.

Figure 3.16 TNF-α mRNA levels in varicose vein segments and VOS
TNF-α mRNA levels in 31 varicose vein segments and 6 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

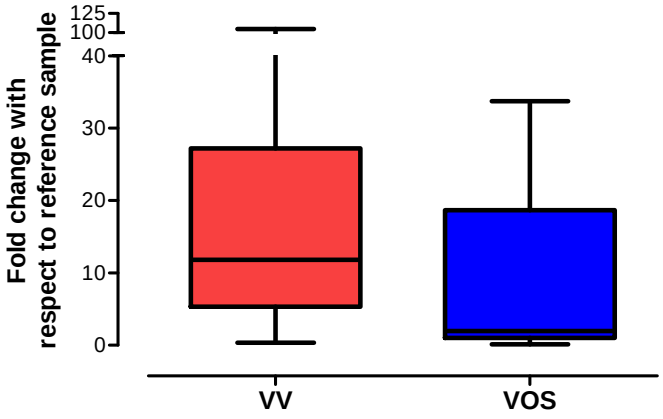


Figure 3.17 TNF-α mRNA levels in total proximal and distal varicose vein segments
TNF-α mRNA levels in 15 proximal and 14 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

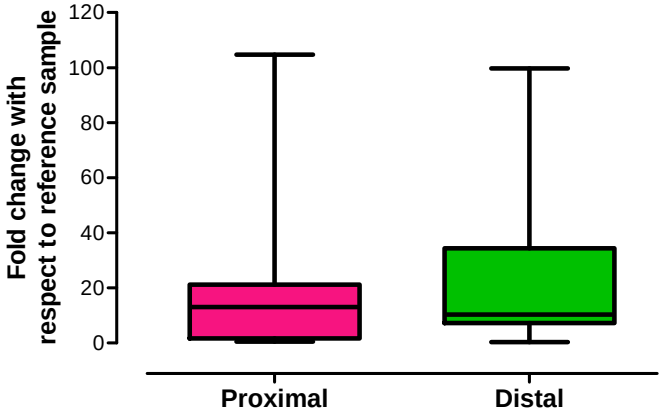
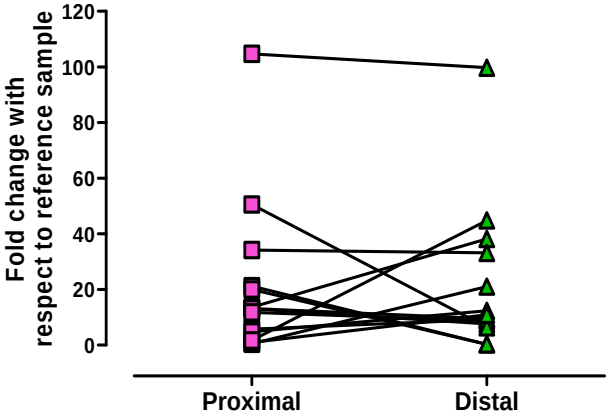


Figure 3.18 TNF-α mRNA levels in paired proximal and distal vein segments
TNF-α mRNA levels in 14 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.7 IL-1β EXPRESSION IN VARICOSE VEINS

IL-1β mRNA levels were quantified by PCR in the varicose vein samples and were found to be significantly underexpressed relative to veins from other sources (p=0.0111, Figure 3.19) using Mann-Whitney test. This is not obviously reflected by the graph as the data revealed a wider range of mRNA expression of IL-1β in varicose vein segments (0.01 – 8.49) compared to veins from other sources (0.93 – 5.31). However, the varicose vein samples have a lower median (median=0.22) in comparison to veins from other sources (median=1.68). When examining the data from the comparison of proximal and distal samples, it appears that proximal and distal levels are equivalent despite the increased range of levels found in distal varicose vein segments. This is confirmed by comparing the median and inter-quartile ranges. Distal varicose vein segments (median=0.36) are similar to proximal varicose vein segments (median=0.19; Figure 3.20). Data was also available from the varicose vein matched pairs and again this showed little difference between distal varicose vein segments (median=0.25) relative to that of the proximal varicose vein segments (median=0.19; Figure 3.21). Both these comparisons were non-significant using the Mann Whitney test (p=0.5222) and Wilcoxon signed rank test (p=0.2969) respectively.

Figure 3.19 IL-1β mRNA levels in varicose vein segments and VOS
IL-1β mRNA levels in 21 varicose vein segments and 5 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

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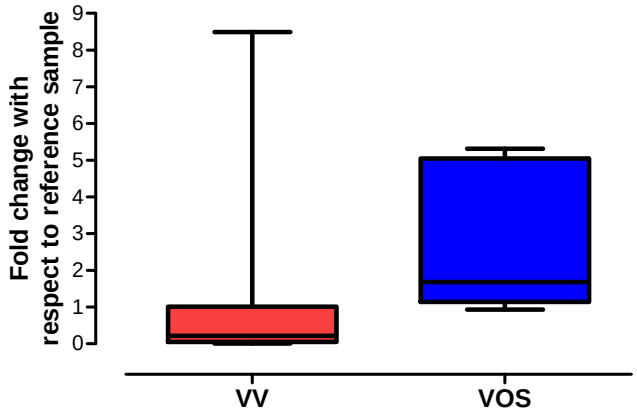


Figure 3.20 IL-1β mRNA levels in total proximal and distal varicose vein segments
IL-1β mRNA levels in 12 proximal and 9 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

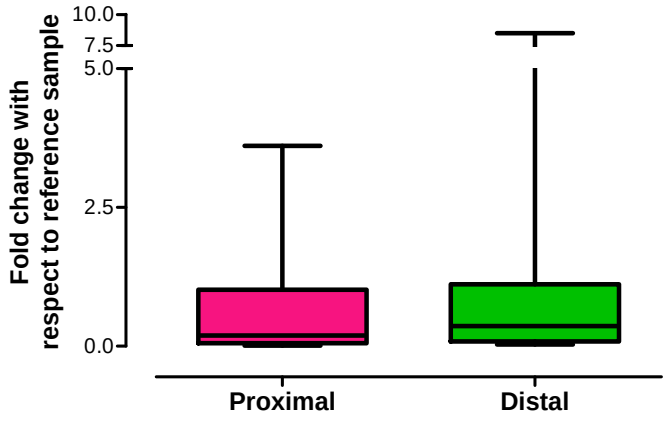
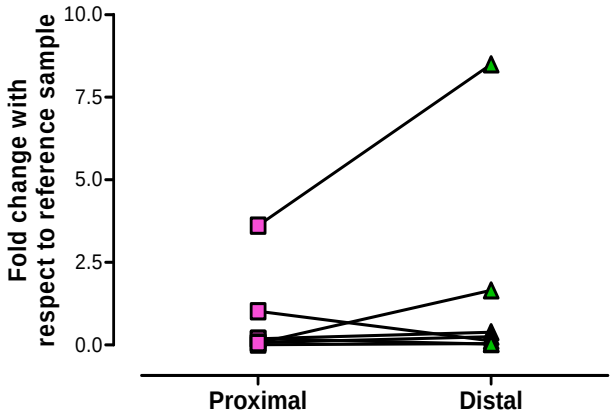


Figure 3.21 IL-1β mRNA levels in paired proximal and distal vein segments
IL-1β mRNA levels in 7 paired varicose vein segments (distal and proximal expressed relative to a human cDNA sample.



3.4.8 IL-6 EXPRESSION IN VARICOSE VEINS

IL-6 mRNA levels were quantified by PCR in varicose vein samples and veins from other sources. Varicose vein samples (median=3.12) appeared to be decreased relative to the veins from other sources (median=5.98; Figure 3.22). This trend also occurred with the ranges of the two sets of results. The range was a lot smaller in the varicose vein segments (0.04 – 30.06) when compared to the veins from other sources (1.00-320.70). Despite this the data was non-significant (p=0.5510) using the Mann-Whitney test. When looking at the data from the comparison of proximal and distal samples, IL-6 mRNA appeared to be slightly lower in distal varicose vein segments relative to proximal segments. However, when the medians were compared distal varicose vein segments (median=3.07) were equivalent to proximal varicose vein segments (median=3.19; Figure 3.23). Data was also available from the varicose vein matched pairs and again this showed that IL-6 levels were similar in distal varicose vein segments (median=3.67) relative to the proximal varicose vein segments (median=3.07; Figure 3.24). Both these comparisons were non-significant using the Mann Whitney test (p=0.5441) and Wilcoxon signed rank test (p=0.7334).

Figure 3.22 IL-6 mRNA levels in varicose vein segments and VOS
IL-6 mRNA levels in 27 varicose vein segments and 7 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

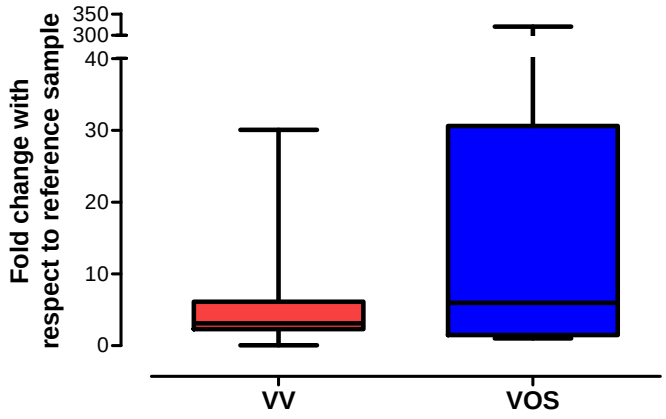


Figure 3.23 IL-6 mRNA levels in total proximal and distal varicose vein segments
IL-6 mRNA levels in 13 proximal and 14 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

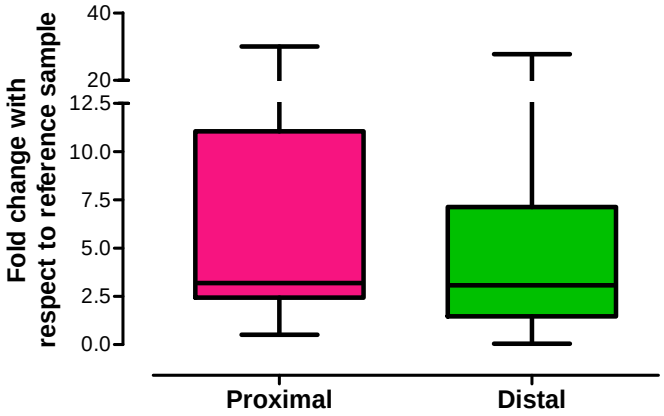
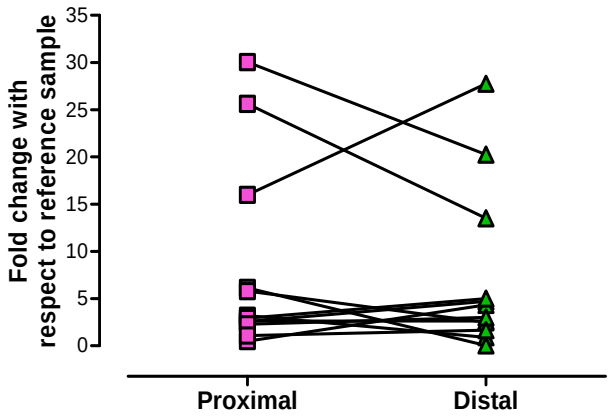


Figure 3.24 IL-6 mRNA levels in paired proximal and distal vein segments
IL-6 mRNA levels in 12 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



3.4.9 Correlation Of Cytokines To Matrix Metalloproteinases And Their Tissue Inhibitors

To analyse the relationship between cytokines and MMPs and their inhibitors, mRNA levels for cytokines were compared to mRNA of MMP and TIMP in the same samples. Significance was analysed using the Spearman correlation co-efficient (r), and p values of less than 0.05 were considered statistically significant.

VEGF expression was compared with the expression of MMP-2 (r=0.0116), MT1-MMP (r=-0.0692), TIMP-2 (r=-0.0214) and TIMP-3 (r=0.4647; Figure 3.25). The former 3 values showed no significant correlation; however there was a significant correlation between VEGF expression and TIMP-3 expression (p=0.0146).

Similarly, TNF-α expression was compared with the expression of MMP-2 (r=0.6499), MT1-MMP (r=0.3777), TIMP-2 (r=0.5658) and TIMP-3 (r=0.0425;Figure 3.26). TNF-α showed highly significant correlation with MMP-2 ($p<0.0001$) and TIMP-2 (p=0.0014), and significant correlation with TIMP-3 (p=0.0272) and MT1-MMP (p=0.0434).

IL-1β expression was compared with expression for MMP-2 (r=0.5406), MT1-MMP (r=0.4392), TIMP-2 (r=0.5854) and TIMP-3 (r=-0.0369; Figure 3.27). IL-1 β showed significant correlation with MMP-2 (p=0.0114), MT1-MMP (p=0.0464) and TIMP-2 (p=0.0053). In contrast, there was no significant correlation between IL-1 β and TIMP-3.

Finally, IL-6 expression was compared with the expression of MMP-2 (r=0.1566), MT1-MMP (r=0.1169), TIMP-2 (r=0.2277) and TIMP-3 (r=0.2123; Figure 3.28). There was no significant correlation between IL-6 and any of these parameters.

Figure 3.25 Correlation of VEGF and MMP/TIMP expression in varicose veins
VEGF expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.

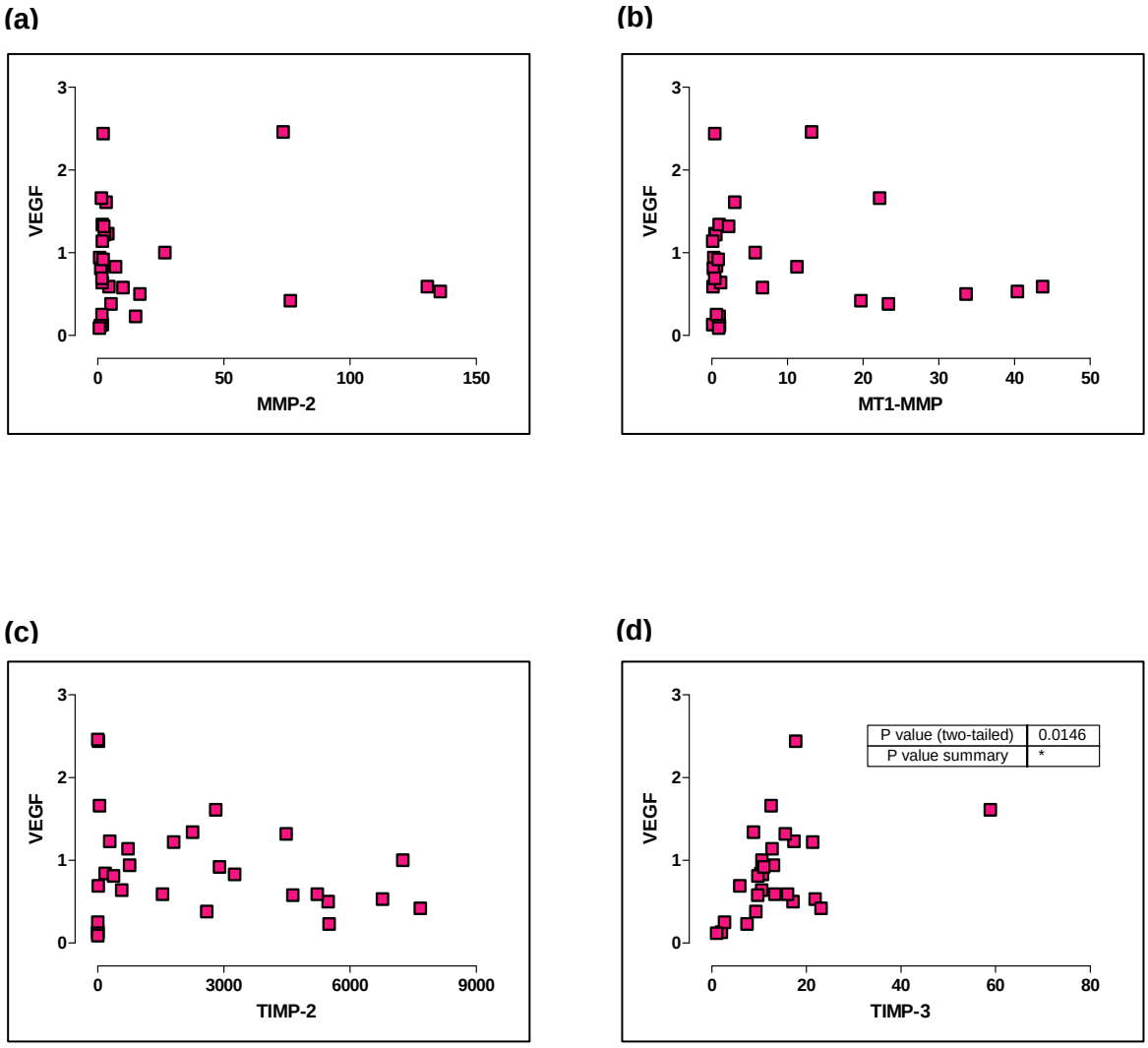


Figure 3.26 Correlation of TNF-α and MMP/TIMP expression in varicose veins
TNF-α expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.

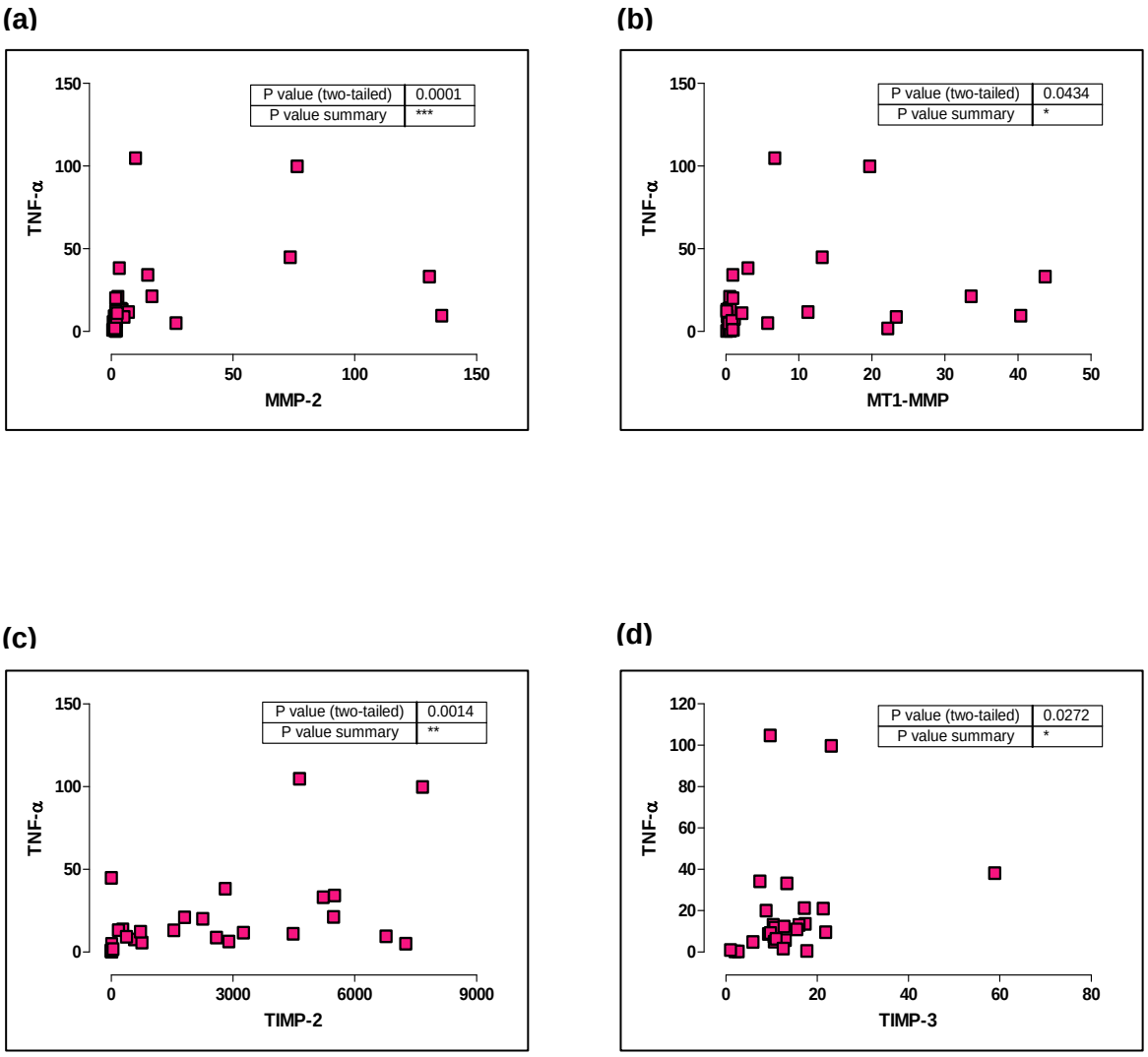


Figure 3.27 Correlation of IL-1β and MMP/TIMP expression in varicose veins
IL-1β expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.

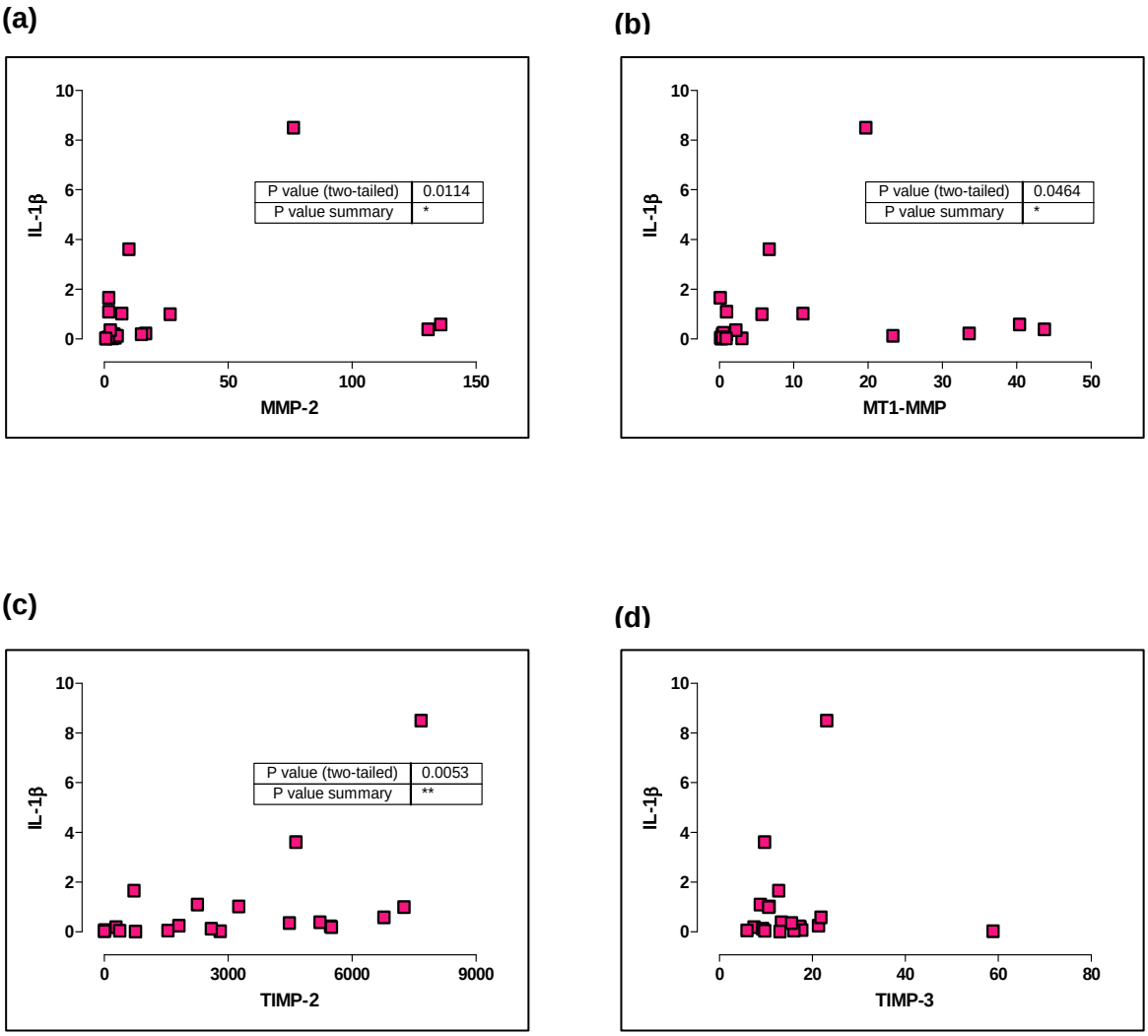
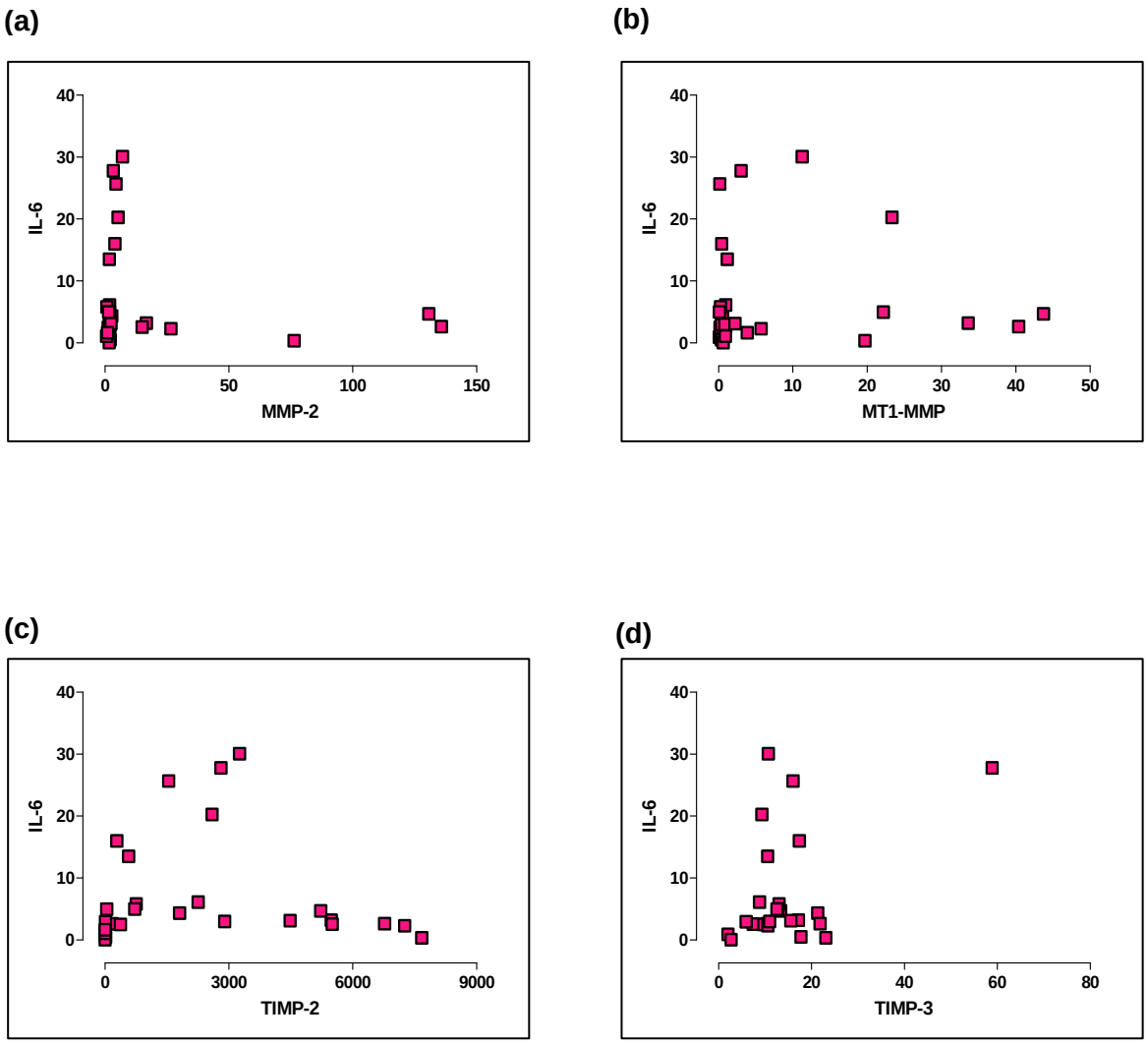


Figure 3.28 Correlation of IL-6 and MMP/TIMP expression in varicose veins
IL-6 expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.



3.5 DISCUSSION

This chapter set out to assess the relationship between inflammatory cytokine expression and matrix degrading MMP and their inhibitors in varicose veins. This was addressed by quantifying and comparing mRNA expression for specific genes of interest in proximal and distal portions of varicose vein tissue and veins from other sources. The genes of interest chosen were MMP and their inhibitors MMP-2, MT1-MMP, TIMP-2 and TIMP-3, and the cytokines VEGF, TNF-α, IL-1β and IL-6.

I have confirmed that the mRNA for MMP-2, MT1-MMP, TIMP-1 and TIMP-2 are all expressed in varicose vein tissue. When these were compared to veins from other sources there was no difference in the mRNA expression for the MMP, MMP-2 or MT1-MMP. Present literature is conflicting. Norgauer *et al* would suggest in biopsies taken from patients with venous leg ulcers there is increased expression of MMP-2 and MT1-MMP (Norgauer *et al.* 2002). Rafetto *et al* would also agree that MMP-2 expression increases and they suggest that this is because of the effect of venous hypertension on the vein wall (Raffetto *et al.* 2008). In contrast, Takase *et al* induced venous hypertension in femoral veins of rats and used the contralateral vein as a control and was unable to generate any change in MMP-2 expression (Takase *et al.* 2004). There was however, a significant increase in my study in the amount of mRNA expressed for TIMP-2 and TIMP-3 in varicose vein tissue when compared to veins from other sources. Again there is no consensus on the literature. Woodside *et al* demonstrated by Western blot analysis that only small quantities of TIMP-1 and TIMP-2 protein isolates were present in varicose veins and veins from other sources, neither of which could be detected by immunohistochemistry (Woodside *et al.* 2003). In contrast Bader-Commander *et al* found high TIMP-1 levels and low MMP-2 level/activity in varicose veins resulting in a three-fold increase in the TIMP-1/MMP-2 ratio in varicose *versus* control veins (Badier-Commander *et al.* 2000). Herouy *et al* also support Bader-Commander *et al*. They concluded from the work they have performed on venous leg ulcers that MMP-2 was imbalanced by the locally reduced expression of TIMP-2 (Herouy *et al.* 1998). Our results

would echo these thoughts. I found no change to MMP-2 and MT1-MMP expression when compared with veins from other sources and a significant increase in TIMP-2 and TIMP-3 expression. This would suggest that the MMP/TIMP imbalance is disrupted by the over-expression of TIMP.

Regional variation in the literature has only commented on MMP-1 and MMP-13 where both matrixins are more abundant in the proximal segment (Gillespie *et al.* 2002). When analysing our data comparing proximal varicose vein tissue and distal varicose vein tissue, MT1-MMP mRNA was found to be more abundantly expressed in distal VV segments relative to proximal varicose vein segments. This is consistent with the protein and preliminary data obtained by my predecessor (Aravind 2007). However, despite an increase in expression and an obvious trend, this data was not statistically significant. TIMP-2, and also TIMP-3, is obviously abundantly expressed in varicose vein tissue. On analysis of the TIMP-2 graphs, either alone or paired, there was no obvious regional variation. However, comparison of the medians between these sample groups indicate that TIMP-2 mRNA levels were 2-fold higher in distal varicose vein segments relative to proximal segments. This result is non-significant. MMP-2 and TIMP-3 consistently showed no difference between the two anatomical sites. It is interesting that MT1-MMP and TIMP-2 both show a tendency to be more abundant in the distal portion of varicose vein and that there appears to be no regional variation in MMP-2. We know that the activation of MMP-2 is promoted by TIMP-2 binding to the membrane bound receptor MT1-MMP. The fact that there is no change in MMP-2 levels would support some recent research by D'Alessio *et al* that the interaction of TIMP-2 and MT1-MMP controls cell functions by a mechanism independent of ECM degradation (D'Alessio *et al.* 2008). It also of interest that distal segments of varicose veins tend to be thinner which suggests that MMP/TIMP balance may favour matrix degradation in these areas.

I have also confirmed that mRNA for the cytokines VEGF, TNF-α, IL-1β and IL-6 are expressed in varicose vein tissue. When varicose vein samples were compared to veins from

other sources, there was increased mRNA expression for VEGF and TNF-α in varicose vein tissue, 2-fold and 5-fold respectively. This data was not significant but can be supported by Yasim *et al*, who took blood from the brachial vein in patients with primary varicose veins and compared the results with healthy volunteers. They compared the plasma levels of VEGF and found that they were statistically higher in the study group than in the control group (Yasim *et al.* 2008). Again, the literature is inconsistent. Signorelli *et al* also found elevated production of TNF-α in varicose vein disease that became further increased with an artificial increase in venous hypertension by tourniquet application. They also found that IL-1β and IL-6 increased under the same conditions (Signorelli *et al.* 2000). In contrast, Pappas *et al* measured the systemic, soluble, serum cytokine levels between healthy controls and patients with CVI. IL-6 was elevated in patients with CVD, while TNF-α and IL-1β, demonstrated no significant difference between the groups (Pappas *et al.* 1995). My results showed that the interleukins IL-1β and IL-6 both had a reduction in mRNA expression in varicose vein tissue when compared to veins from other sources. For IL-1β this result was significant.

When analysing data comparing proximal *versus* distal varicose vein samples, VEGF, IL-1β and IL-6 consistently showed no difference between the two anatomical areas. TNF-α, on the other hand, displayed more expression in the proximal portion of the varicose vein that in the distal portion when comparing the median values, but this data was not significant. There is no data supporting regional variations of cytokines in varicose veins.

When correlating the cytokines with MMP and their inhibitors, VEGF displayed a statistically significant correlation with TIMP-3. It has been reported that TIMP-3 blocks the binding of VEGF to VEGF receptor type 2 and inhibits downstream signalling and angiogenesis (Qi *et al.* 2003). Thus an increase in TIMP-3, to counter-act the possible pro-angiogenic effects of VEGF, may be a mechanism to regulate angiogenesis in varicose vein adventitia. In contrast, TNF-α showed a significant correlation with MT1-MMP and TIMP-3 and a very significant correlation with MMP-2 and TIMP-2. IL-1β also significantly correlated with MMP-2, MT1-MMP and TIMP-2. These data are of potential interest, since

active MT1-MMP and TIMP-2 are thought to bind pro-MMP-2 forming a ternary complex, which permits pro-MMP-2 activation by TIMP-2-free MT1-MMP. TIMP-2 bound to MT1-MMP on the cell surface is thought to act as a receptor for proMMP-2, with homophilic complex formation by MT1-MMP (Itoh *et al.* 2001). The fact that both TNF-α and IL-1β levels correlate with the expression of the 3 members of this complex may provide evidence that in varicose veins cytokine-mediated assembly of this complex could be important in regulating matrix turnover. TNF-α also correlated with TIMP-3. This is not all together surprising, TNF-α and TIMP-3 have a strong link via TNF-α converting enzyme (TACE). TACE is a membrane bound disintegrin metalloproteinase that processes the membrane bound cytokine pro TNF-α into an active, soluble form, TNF-α. TACE was found to be well inhibited TIMP-3 but not by TIMP-1, -2 and -4. These results suggest that TIMP-3 may be important in the modulation of pathological events in which TNF-α secretion is involved (Amour *et al.* 1998), such as apoptosis. IL-6 did not correlate with any of the MMP or their inhibitors.

There were some limitations during my research period. Due to the surgical reformation of varicose vein treatment, less and less pathological tissue was available as my project progressed. The frequent "open high tie and strip" operation was rapidly replaced by the more minimally invasive endovenous surgery. This meant that tissue from the saphenofemoral junction was no longer available for my studies. Tissue was only available from phlebectomies or avulsions. This in itself has implications for interpretation of my data. In my introduction (Chapter 1) I explained that varicose veins were considered to be a multi-segmental problem, with potential for regional variations along the vein length. When tissue was taken from the saphenofemoral junction (proximal segment) together with a portion of the GSV just below the knee (distal segment) during the traditional stripping method, this was by definition from patients that have some form of varicose vein disease. However, the "distal" and "proximal" segments utilised in this study were not necessarily diseased. It would be interesting to analyse in a comparable study avulsed segments which are by definition

veins that are prominent, dilated and tortuous.

Furthermore, it would also have been ideal to have been able to have been more selective about patients that were included in the study with regards to co-morbidities. Unfortunately, the co-morbidities of patients who could provide veins from other sources were plentiful and I was unable to exclude them from the study, otherwise the population of patients for comparison would have been non-existent due to ethical constraints. The ideal "control" patient would be a young healthy patient, who potentially required an operation for trauma in order to extract a sample of vein. These patients were not readily available to me in this study. It would have also been preferable to age match the varicose vein group and the controls. However, the patients from whom veins from other sources were obtained were of necessity an older group, although both groups were matched by gender. Much of the data compared proximal *versus* matched distal segments, which are not affected by these factors. Nevertheless, to confidently compare varicose vein tissue and veins from other sources, a larger and better matched "control" group would have been preferable.

From a technical perspective, RNA extraction from tissue itself was difficult and I needed to establish and validate this method in the laboratory. Many methods were attempted before RNA was successfully extracted using the method explained in Section 2.2. It appears that venous tissue is very tough and that breaking it down into smaller components was only possible when in frozen form. Of particular importance, it was challenging to obtain RNA from some of the control tissue. The facial veins retrieved during carotid surgery were very small and did not provide sufficient tissue alone to get a satisfactory yield of RNA. This was overcome by combining 4 individual specimens together.

When performing the quantitative PCR the housekeeping gene (ARP) that I used was itself relatively low in abundance. ARP does not change under conditions of hypoxia, and is therefore appropriate for this study. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative amounts of mRNA, which compares the gene of interest to the housekeeping gene (ARP). Low abundance of the housekeeping gene could therefore result in artificially large changes in gene expression. Nevertheless, initial data from a subsequent study on varicose veins using

18S ribosomal RNA as the housekeeping gene confirms many of my findings.

In summary, this chapter has shown that cytokines, growth factors and MMP/TIMP are expressed in varicose veins. Furthermore, the upregulation of TIMP-2 and TIMP-3 may cause an imbalance in the MMP/TIMP ratio, thereby contributing to varicose vein development. Since growth factors such as VEGF and MMP (particularly MT1-MMP and MMP-2) are potentially affected by hypoxia, the next part of my study (Chapter 4) aimed to assess HIF expression in varicose veins.

CHAPTER 4

4 ASSESSMENT OF HIF EXPRESSION IN VARICOSE VEINS

4.1 INTRODUCTION

Hypoxia has been suggested as being involved in varicose vein disease, but there is little work concentrating on the possibility and perhaps subsequent effects of hypoxia in either the venous macrocirculation or microcirculation of the leg. As previously mentioned in detail in Chapter 1, hypoxia has been sub-divided by time span, acute and chronic hypoxia.

Michiels *et al* has proven that HSVEC respond like other endothelial cells when exposed to hypoxia. They incubated human saphenous veins in normoxic and hypoxic conditions and observed the interactions between the endothelium and neutrophils using electron microscopy and histological observation to confirm the results. They concluded that when hypoxia occurs, endothelial cells get activated causing them to release inflammatory mediators which aid the neutrophils to adhere, and subsequently activate themselves. The activated leukocytes then release free radicals and proteases which directly degrade the ECM. Activation of endothelial cells also directly trigger smooth muscle cell proliferation and synthesis of the extracellular components causing an imbalance. If this process is frequently repeated it is likely to lead to alterations in the venous wall similar to that seen in varicose veins (Michiels *et al.* 1997).

There is no clear evidence of how the initial insult of hypoxia occur in the venous system in order to promote varicose vein development but potential possibilities include: 1) blood stasis causing macrocirulatory ischemia damaging the endothelial cells of the intima, 2) hypoxia to the endothelial wall caused by insufficient supply by the vasa vasorum in the adventitia and 3) tissue hypoxia due to alterations in the microcirculation. It is controversial as to whether or not blood stasis is present in varicose vein disease and, if it does, how it causes its insult. Potentially, inflammatory mediators will recruit and activate neutrophils which infiltrate the venous wall and damage components of the ECM. It could also be that

growth factors induce smooth muscle cell migration, proliferation and de-differentiation in to the synthetic phenotype leading to the formation of the neointima. Both of these processes over time would eventually cause the alterations of the venous wall that have been observed in varicose veins (Michiels *et al.* 2002).

It is thought, by some, that standing promotes blood stasis in the lower limbs and that such stasis will promote ischemia decreasing the oxygen availability to the tissues. This is logical as due to gravitational pull and the lack of the "calf muscle pump" action, as seen in varicose veins, there is a lack of venous return. Gaylarde *et al* has supported this hypothesis by showing there is a link between stasis and hypoxia, reporting improvement in skin blood flow by application of external compression stockings in standing subjects thus preventing microcirculatory stasis secondary to venous hypertension (Gaylarde *et al.* 1993). Alterations in the microcirculation in CVI are thought to be due to a mixture of venous stasis, high capillary pressure and anoxic tissue. Matic *et al* has reported that the partial pressure of oxygen is higher in ulcerated limbs than those without ulceration which they suggest is due to blood passing directly from arterioles to venules over arterio-venous temperature-regulating shunts in the dermis (Matic *et al.* 2000). However another study found significant skin hypoxia in patients with venous disease and skin ulcerations (Clyne *et al.* 1985).

4.2 OBJECTIVES

This part of the research focused on correlating the expression at the mRNA level of hypoxia-inducible transcription factors, HIF-1α and HIF-2α, in varicose vein specimens. As for the studies of MMP/TIMP and cytokine expression, this analysis was performed for varicose veins and veins from other sources, as well as for regional variation (proximal *versus* distal varicose vein segments in the same patient).

4.3 MATERIALS AND METHODS

4.3.1 PATIENT SELECTION AND TISSUE SAMPLING

Varicose vein samples were obtained from patients as well as veins from other sources that were used for comparison. This has been previously described in material and methods Section 2.1. Samples gained were immediately frozen using liquid nitrogen and then stored in the freezer (-80^{o}C) until required.

4.3.2 RNA EXTRACTION AND QUANTITATION

Similarly to Chapter 3, Varicose vein tissue, including proximal and distal samples for each vein, and veins from other sources were pulverised in a frozen state in order to preserve the RNA. The tissue was weighed and underwent homogenization, RNA extraction and reverse transcription. Quantitative PCR was finally used analyse the genes of interest and allow calculation of a Ct value from the quantitation curves. The genes of interest included the MMPs HIF-1α and HIF-2α. Comparisons were made to the data collected in chapter 3 for the MMPs MMP-2, MT1-MMP, TIMP-2 and TIMP-3 and the cytokines VEGF, TNF-α, IL-1β and IL-6.

4.3.3 STATISTICAL ANALYSES

The data was collected on the Rotor-gene 7 program, a threshold set and Ct values obtained. These were collated on to an Excel spread sheet and calculations made using comparative Ct method (see Chapter 2). The mRNA levels were then compared and statistically analysed using the Graph Pad Prism software package (Graph Pad Software, CA, USA). Wilcoxon rank test was used to compare non-parametrically distributed paired data, and paired-test for normally distributed paired data. Mann-Whitney test was used to compare non-parametrically distributed unpaired data, and unpaired-test for normally distributed unpaired data. Correlations were assessed using Spearman's co-efficient for non-parametrically distributed data.

4.4 RESULTS

The following analyses were performed

i) total varicose vein mRNA, expressed as fold change relative to a human cDNA sample, and compared to veins from other sources

ii) comparison of total proximal *versus* total distal population of varicose veins (using data from fold change relative to a human cDNA sample)

iii) comparison of paired proximal and distal varicose vein samples (using data from fold change relative to a human cDNA sample)

4.4.1 HIF-1α EXPRESSION IN VARICOSE VEINS

HIF-1α mRNA levels were quantified by PCR in the varicose vein samples and the veins from other sources. HIF-1α mRNA levels in the varicose veins (median = 0.42) were found to be much less to those in the veins from other sources (median = 32.46; Figure 4.1) This data was significant using the Mann Whitney test (p=0.0121). When data from the comparison of proximal and distal samples were examined, it appeared that HIF-1α mRNA was equivalent in distal varicose vein segments (median = 0.41) relative to the proximal varicose vein segments (median = 0.43; Figure 4.2) despite a slightly wider range of data in the distal varicose vein segments. This difference was not statistically significant using Mann Whitney test (p=0.8786). This was confirmed when the samples were analysed as paired samples (Wilcoxon signed rank test p=0.9515; Figure 4.3).

Figure 4.1 HIF-1α mRNA levels in varicose vein segments and VOS
HIF-1α mRNA levels in 29 varicose vein segments and 4 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

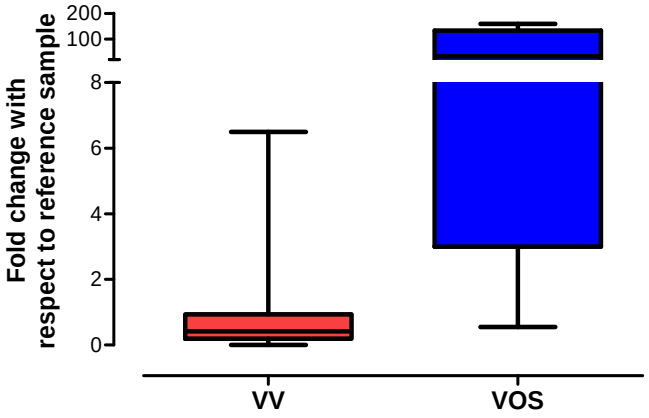


Figure 4.2 HIF-1α mRNA levels in total proximal and distal varicose vein segments
HIF-1α mRNA levels in 14 proximal and 15 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

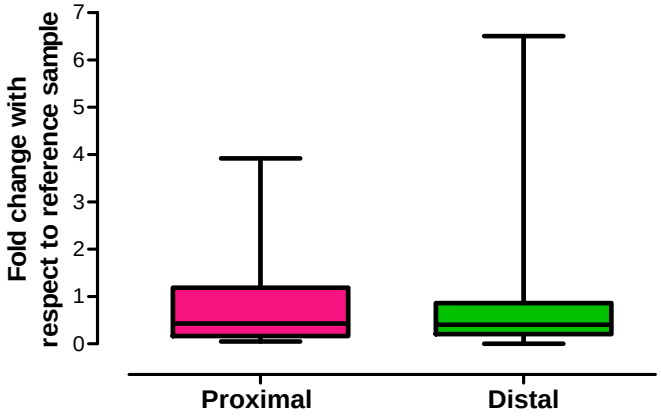
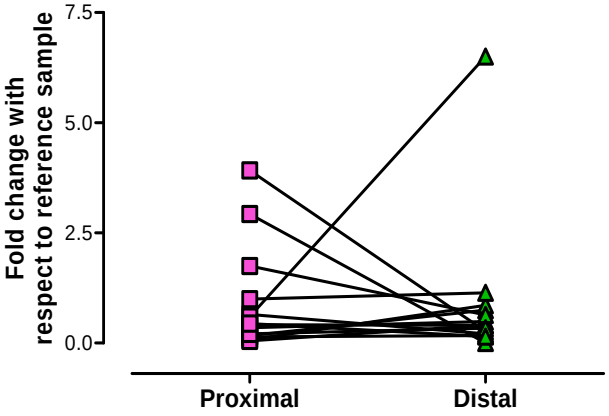


Figure 4.3 HIF-1α mRNA levels in paired proximal and distal vein segments
HIF-1α mRNA levels in 14 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



4.4.2 HIF-2α EXPRESSION IN VARICOSE VEINS

HIF-2α mRNA levels were quantified by PCR in the varicose vein samples and veins from other sources. Similarly to HIF-1α, HIF-2α mRNA levels in the veins from other sources were found to be significantly elevated (median = 370.90) relative to the varicose vein samples (median = 3.42) using the Mann Whitney test (p=0.0372; Figure 4.4). When data from the comparison of proximal and distal samples was reviewed it appeared, again similarly to HIF-1α, that there was little difference between the two areas. However, when comparing the medians there is an apparent difference, with HIF-2α being more abundant in proximal samples (median = 8.02) in comparison to distal samples (median = 2.27; Figure 4.5). When comparing paired samples the medians are in a much closer range (proximal = 3.66, distal = 2.41; Figure 4.6). Neither sets of data showed significance using Mann Whitney (p=0.3012) for the former and Wilcoxon signed rank test (p=0.8926) for the latter.

Figure 4.4 HIF-2α mRNA levels in varicose vein segments and VOS
HIF-2α mRNA levels in 28 varicose vein segments and 5 VOS segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum.

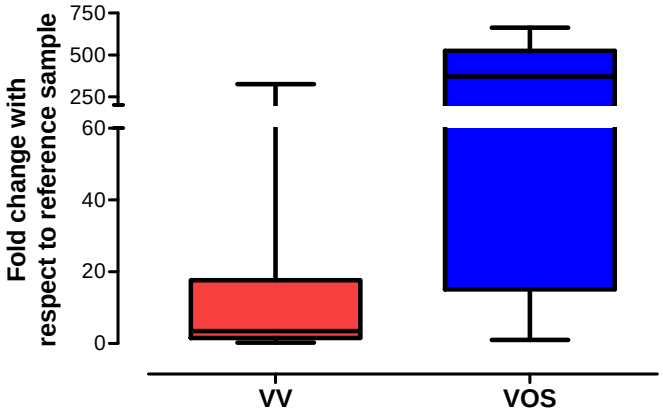


Figure 4.5 HIF-2α mRNA levels in total proximal and distal varicose vein segments
HIF-2α mRNA levels in 14 proximal and 14 distal varicose vein segments, expressed relative to a human cDNA sample. Data are medians plus inter-quartile (IQ) range, and maximum/minimum

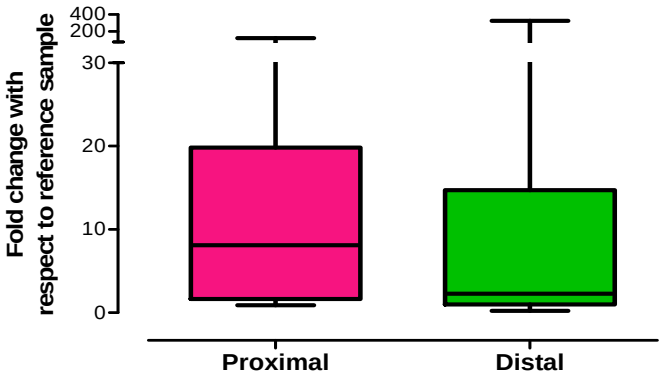
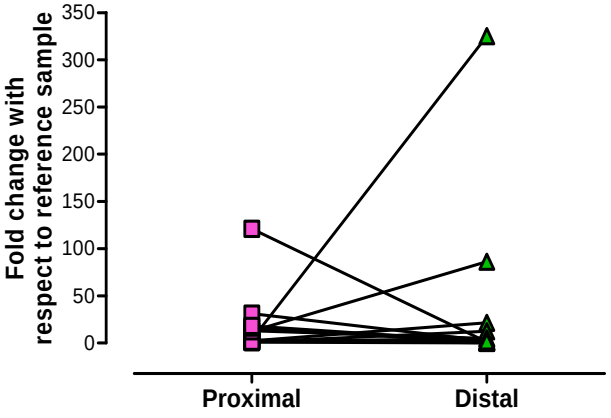


Figure 4.6 HIF-2α mRNA levels in paired proximal and distal vein segments
HIF-2α mRNA levels in 13 paired varicose vein segments (distal and proximal), expressed relative to a human cDNA sample.



4.4.3 CORRELATION OF HIF-1α AND HIF-2α TO MATRIX METALLOPROTEINASES, THEIR TISSUE INHIBITORS AND TO CYTOKINES

To analyse the relationship between HIF-1α and HIF-2α with MMP, their inhibitors and cytokines, the mRNA levels of HIFs were compared to the mRNA of MMP, TIMP and the cytokines, VEGF, TNF-α, IL-1β and IL-6. Significance was analysed using the Spearman correlation co-efficient (r) , and p values of less than 0.05 were considered statistically significant.

HIF-1α expression was compared with expression of MMP-2 (r=0.4456), MT1-MMP (r=0.3242), TIMP-2 (r=0.3920) and TIMP-3 (r=0.3563; Figure 4.7). HIF-1α showed significant correlation with MMP-2 (p=0.0154) and TIMP-2 (p=0.0355).

HIF-1α expression was also compared with the expression of VEGF (r=0.3652), TNF-α (r=0.4412), IL-1β (r=0.5900) and IL-6 (r=-0.0113; Figure 4.8). HIF-1α showed significant correlation with TNF-α (p=0.0188) and IL-1β (p=0.0049).

HIF-2α expression was compared with expression of MMP-2 (r=0.2543), MT1-MMP (r=0.5188), TIMP-2 (r=0.3027) and TIMP-3 (r=0.1556; Figure 4.9). HIF-2α showed significant correlation with MT1-MMP (p=0.0047). There was no significant correlation between HIF-2α and MMP-2, TIMP-2 or TIMP-3.

HIF-2α expression was compared with the expression for VEGF (r=-0.0089), TNF-α (r=0.4151), IL-1β (r=0.2937) and IL-6 (r=0.2259; Figure 4.10). There was significant correlation between HIF-2α and TNF-α (p=0.0313). There was no significant correlation between HIF-2α and any of the other cytokines analysed.

Figure 4.7 Correlation of HIF-1α and MMP/TIMP expression in varicose veins
HIF-1α expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.

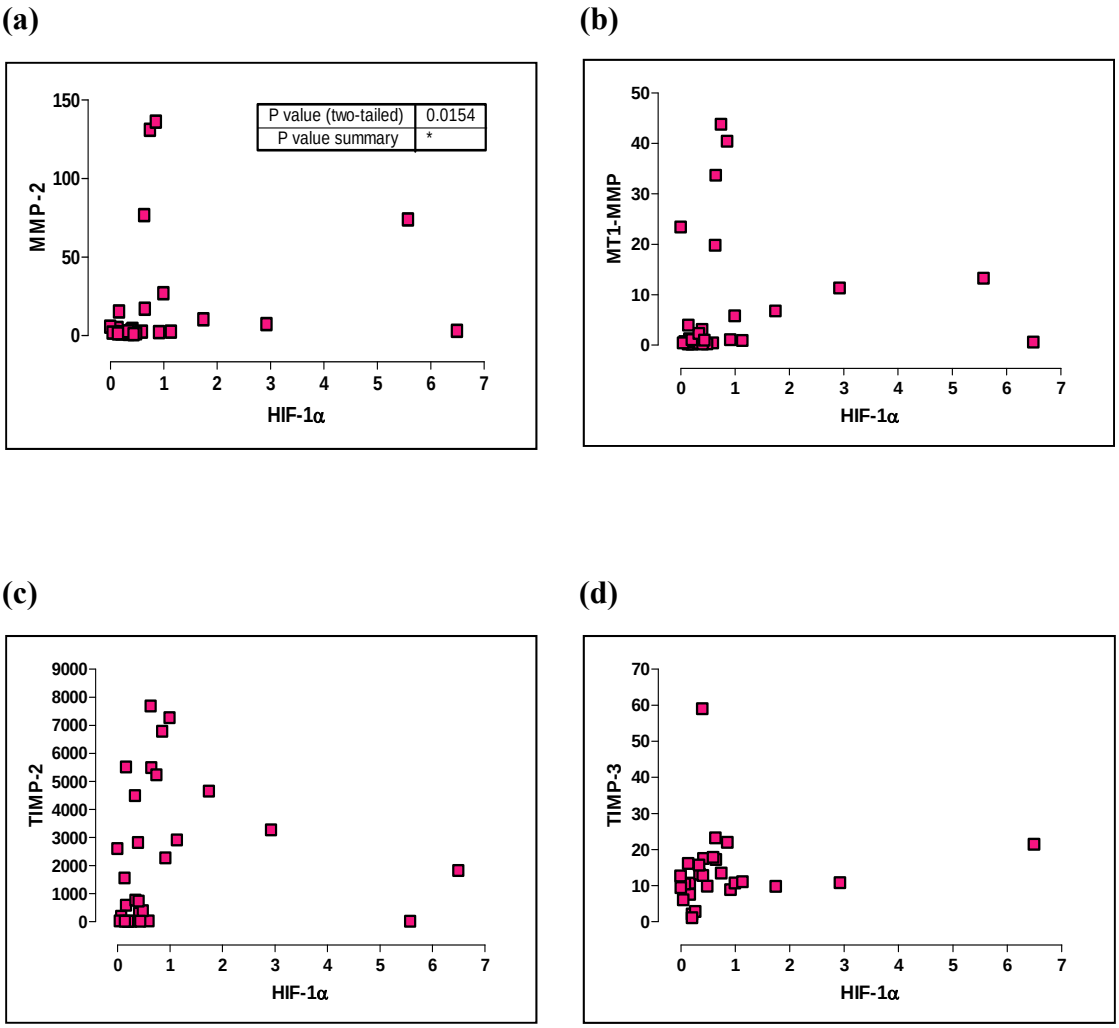
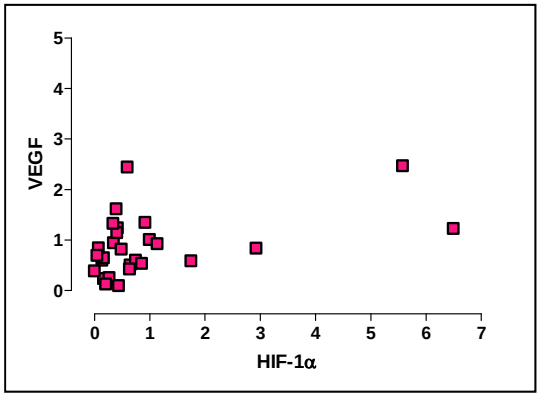
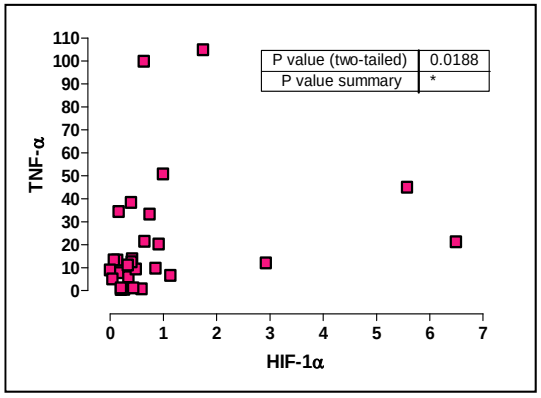


Figure 4.8 Correlation of HIF-1α and cytokine expression in varicose veins
HIF-1α expression *versus* (a) VEGF expression; (b) TNF-α expression; (c) IL-1β expression; (d) IL-6 expression.

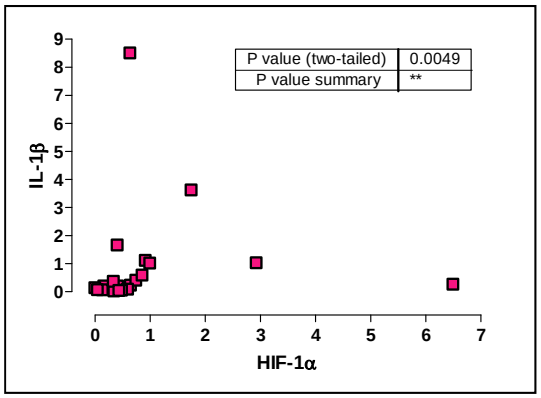
(a)



(b)



(c)



(d)

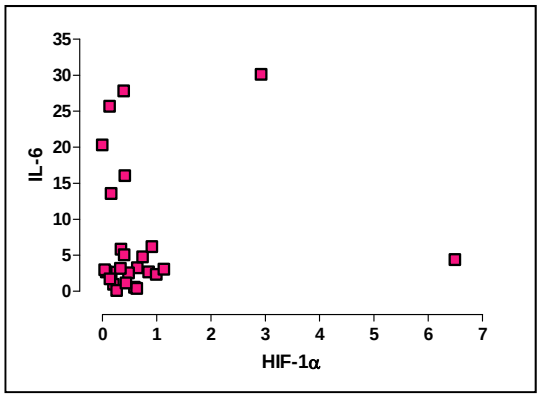
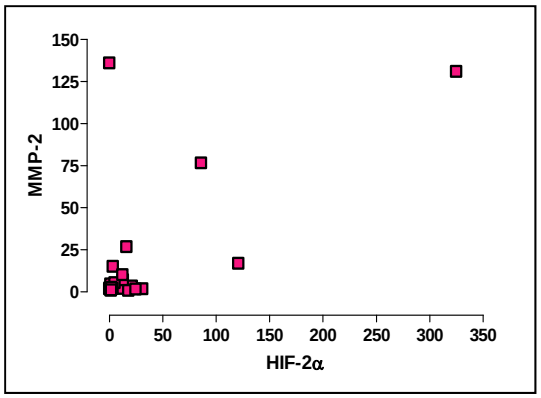
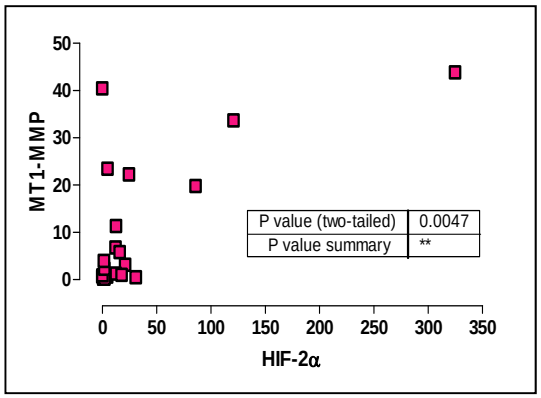


Figure 4.9 Correlation of HIF-2α and MMP/TIMP expression in varicose veins
HIF-2α expression *versus* (a) MMP-2 expression; (b) MT1-MMP expression; (c) TIMP-2 expression; (d) TIMP-3 expression.

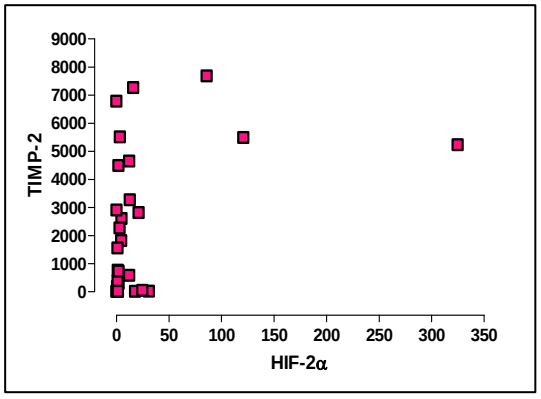
(a)



(b)



(c)



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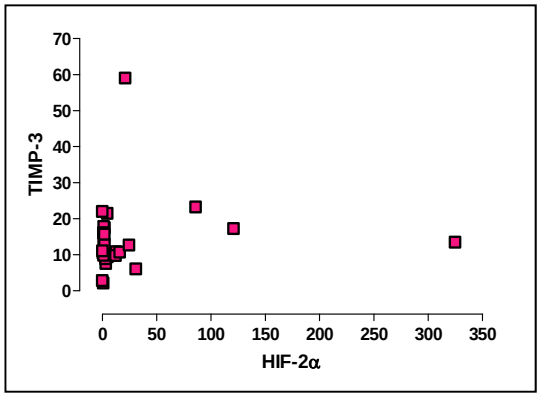
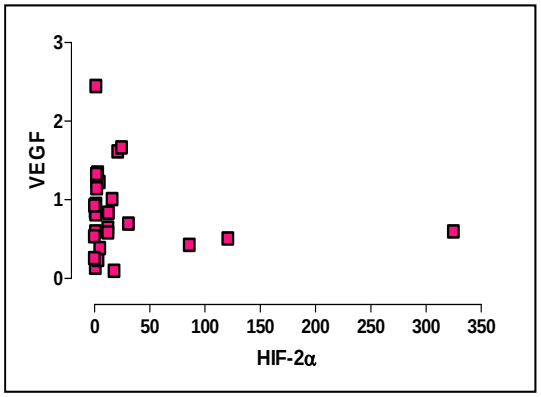
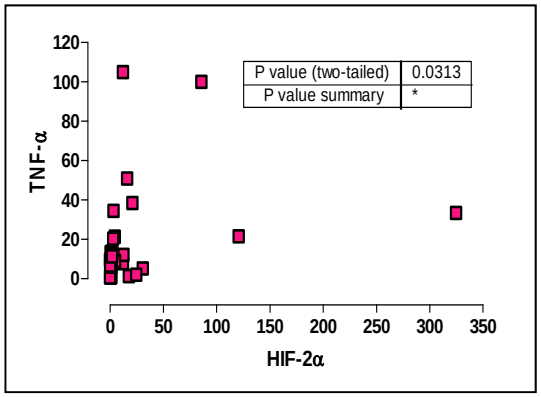


Figure 4.10 Correlation of HIF-2α and cytokine expression in varicose veins
HIF-2α expression *versus* (a) VEGF expression; (b) TNF-α expression; (c) IL-1β expression; (d) IL-6 expression.

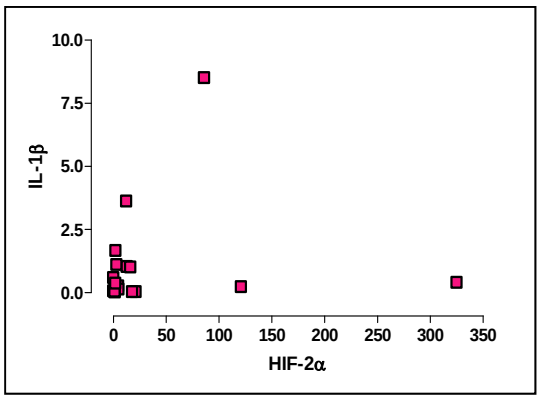
(a)



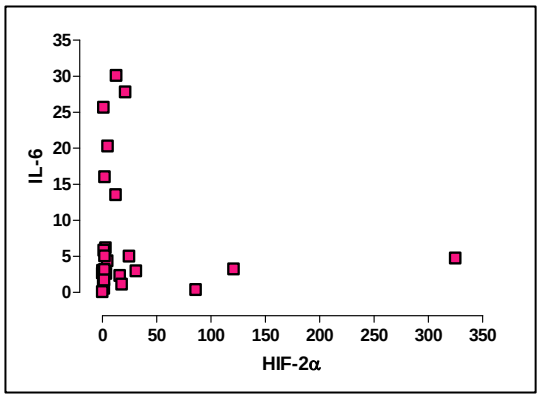
(b)



(c)



(d)



4.5 DISCUSSION

Research has shown that hypoxia may regulate MMPs and their inhibitors. HIFs are the transcription factors that mediate the adaptive response to hypoxia. As previously described in Section 1.5.4.2, in order to prevent the accumulation of HIF protein, VHL ubiquitin ligase binds to the prolyl hydroxylated HIF (the alpha subunit of either HIF-1 or HIF-2) complex, and promotes destruction via the proteasome pathway. In the context of varicose vein development, there is no clear evidence to suggest a possible mechanism of how the initial insult of hypoxia could be brought about. Blood stasis secondary to an increase in venous hypertension may cause microcirculatory ischemia damaging the endothelial cells of the intima, insufficient oxygen supply by the vasa vasorum in the adventitia direct to the endothelial wall could cause hypoxic insult or surrounding tissue hypoxia could occur due to alterations in the microcirculation.

This chapter set out to investigate if hypoxia is a feature of varicose veins and whether there is any effect or association with MMP or cytokine expression. This was addressed by quantifying and comparing mRNA expression for HIF-1α and HIF-2α in proximal and distal portions of varicose vein tissue and veins from other sources. The results were then compared to the MMPs and their inhibitors, MMP-2, MT1-MMP, TIMP-2 and TIMP-3, and the cytokines VEGF, TNF-α, IL-1β and IL-6 (described in Chapter 3). There have been few studies that have examined the contribution of changes in oxygen tension to varicose vein disease. This is somewhat surprising, since varicose veins by nature involve alterations in blood flow and venous stasis which must compromise tissue oxygenation, and hence involve alteration in HIF expression. However, there is presently no data relating to HIF expression in varicose vein disease.

I have confirmed, for the very first time that, that mRNA for HIF-1α and HIF-2α are expressed in varicose vein tissue. When these were compared to veins from other sources the mRNA expression in varicose vein tissue was significantly decreased for both HIF-1α and HIF-2α. This is an unexpected result. If hypoxia is a feature of varicose vein disease one

would have expected levels of HIF, a transcription factor that are activated in response to periods of hypoxia-induced physiological stress, to have increased in comparison to veins from other sources. This obscure result can only be explained by the fact that all the expression levels for HIF-1α and HIF-2α were very low. The only way to confirm this result is to measure the HIF-1α and HIF-2α protein levels in varicose veins and veins from other sources.

When analysing data comparing proximal *versus* distal varicose vein samples, HIF-1α consistently showed no difference between the two anatomical areas. On the other hand, despite a similar range of results, HIF-2α displayed more expression in the proximal portion of the varicose vein than the distal portion when comparing median values, but this data was not significant. However, there is a relatively large and similar range of HIF-2α expression in the proximal and distal segments and once the samples were paired the medians became closer suggesting that there is no regional variation.

When correlating HIFs with MMP and their inhibitors, HIF-1α displayed a statistically significant correlation with MMP-2. In contrast, HIF-2α showed a very significant correlation with MT1-MMP. These results would suggest that MMP-2 regulation is more likely to be mediated by HIF-1α, whereas MT1-MMP may be regulated by HIF-2α. Fan *et al* induced chemical hypoxia by cobalt chloride on rat hepatic stellate cells and measured the expression of MMP-2 and HIF-1α in rat hepatic stellate cells. The expression of MMP-2 and HIF-1α were increased gradually with the increase of the $CoCl_2$ concentration. This would support my correlation and suggest that HIF-1α may play a part in the regulation of MMP-2 transcription under hypoxic conditions (Fan *et al.* 2007). Petrella *et al* have shown direct regulation of MT1-MMP by HIF-2α as well as identifying a functional HIF-binding site in the proximal promoter of MT1-MMP (Petrella *et al.* 2005). Together with my findings this is strong evidence to suggest an association between HIF-2α and MT1-MMP.

When correlating HIFs with the cytokines, HIF-1α showed significant correlation with TNF-α and IL-1β, whereas HIF-2α showed very significant correlation with TNF-α. As

mentioned previously there is little data available but this result implies that there is a link between HIF-1α and HIF-2α suggesting that the TNF-α expression in likely to involve the HIF pathway. Coimbra *et al* support the suggestion from Hellwig-Burgel *et al* that the cytokines actually stimulate HIF gene expression themselves. They showed that in human chondrocytes experiencing up to 16 hours of hypoxic conditions there was a modest stabilization and/or an increase of HIF-1α expression. In parallel, treatment of the articular chondrocytes with TNF-α resulted in an increase in HIF-1α protein steady state levels under normoxic conditions (Coimbra *et al.* 2004). This would suggest that TNF-α may influence the HIF pathway but a reduced oxygen concentration does not have to be present in order for the pathway to be activated. HIF-1α also correlated with IL-1β.

There is no doubt from the literature that HIF-1α is linked with IL-1β. It is unclear whether IL-1β stimulates HIF-1α gene dependant expression or whether changes in HIF-1α expression affects the expression of IL-1β. An example of the former theory is Jung *et al*, who found that even under normoxic conditions IL-1β up-regulates functional HIF-1α protein through a classical inflammatory signalling pathway involving Nuclear Factor Kappa Beta and COX-2, consequently causing an up-regulation of VEGF (Jung *et al.* 2003). In contrast, Zhang *et al* reports that IL-1β shares common features of other known HIF-1α–regulated genes and that human IL-1β genes carry multiple HIF-1-binding sites in their promoter regions. They suggest that IL-1β is up-regulated by hypoxia and $CoCl_2$ in human and mouse astrocytes in parallel with the up-regulation of HIF-1α mRNA and protein (Zhang *et al.* 2006). It could be that both these pathways occur independently and are activated separately depending on whether the environment is under inflammatory or hypoxic stress. Either of these conditions could contribute to the formation of varicose veins. The fact that HIF-1α correlated with both TNF-α and IL-1β maybe of interest as these two pro-inflammatory cytokines have been documented by more than one to work in synergy with each other (Martensson *et al.* 2004; Gurantz *et al.* 2005).

It is surprising that neither HIF-1α nor HIF-2α correlated with VEGF as there is a multitude of data to support the dependence of VEGF expression on HIF-1α (Ozaki *et al.* 1999; Hewitson and Schofield 2004; Li and Dai 2004; Dery *et al.* 2005; Zhang *et al.* 2009). It is known that VEGF is a down-stream target gene of HIF-1α. The fact that there was no correlation between HIF-2α and VEGF is less surprising and supports the findings of Carroll *et al* who in addition to reporting that HIF-1α primarily regulates transcriptional activation of VEGF in response to hypoxia, they also observed a reciprocal relationship between HIF-1α and HIF-2α expression in hypoxia in renal cell carcinoma cells, in that HIF-2α enhanced HIF-1α-mediated VEGF (Carroll and Ashcroft 2006). Once again, since HIFs are predominantly regulated at the level of protein stability, it may have been informative to also measure HIF protein expression and correlate this with VEGF. IL-6 showed no correlation to either of the HIFs.

The frustrations and limitations relating to specimen quantity, type and the difficulty with RNA expression discussed in Chapter 3 apply also to this chapter. HIF, especially HIF-2α, was expressed at very low levels in venous tissue. On a practical level this could be due to the primers that were available to me at the time.

In order to examine further whether hypoxia was indeed a feature of disease and potentially contribute to changes in the vessel wall I performed immunohistochemistry of varicose vein tissue (proximal and distal specimens) and veins from other sources that were stored in formalin. This part of the study has not been included in this thesis as although HIF staining in renal cell carcinoma has previously been successful, the staining techniques used for venous tissue were still in experimental stages in our department and despite exploring a number of methods, the slides were substandard and unable to be analysed. For future studies, I would recommend that tissue is also stored in a snap frozen form in order to minimalise the potential for the possibility of HIF degradation.

In summary, I have successfully shown that both HIF-1α and HIF-2α are expressed at the mRNA level in varicose veins and that there is a relationship between HIFs and

MMP/TIMP, and between HIFs and cytokines. This work suggests an inter-play between hypoxia and cytokines in regulating the MMP/TIMP balance. The next part of my project (Chapter 5) will again address whether hypoxia alters the expression of MMP or their inhibitors on saphenous vein endothelial cells but it will also differentiate the effects of acute or chronic hypoxic episodes on these genes of interest.

CHAPTER 5

5 REGULATION OF MMP/TIMP BY HYPOXIA IN SAPHENOUS ENDOTHELIUM

5.1 INTRODUCTION

The endothelium lines the lumen of all blood vessels and provides an anti-thrombotic surface to protect the underlying smooth muscle cell layer from the potential action of leukocytes and platelets (Mantovani *et al.* 1992), as well as acting as a selectively permeable barrier regulating the flow of nutrients, biologically active substances and blood cells across the vessel wall (Cines *et al.* 1998). Endothelial cells can be activated by biochemical or biomechanical stimuli, including changes in the environmental oxygen tension, leading to the release of factors regulating important functions e.g. vessel diameter, vascular growth and repair, leading to changes in the ECM and in immune and inflammatory responses (Gimbrone *et al.* 1997). These responses occur in part via the transcriptional induction of genes, specifically, in the case of changes in matrix composition, in genes encoding matrix proteins or remodelling molecules such as the MMPs and their inhibitors. It is thought that short term exposure of endothelial cells to low oxygen tension results in the elaboration of predominantly vasoconstricting effectors whereas longer-term or more severe hypoxia generates factors that can induce smooth muscle proliferation and irreversible remodelling of the vasculature (Faller 1999).

The results described so far in my thesis suggest a relationship between hypoxia-inducible transcription factor family member HIF-1α and MMP-2, and between HIF-2α and MT1-MMP. There have certainly been reports of alterations in selected MMP/TIMP expression following exposure of endothelial cells to hypoxia. Ottino *et al* reported changes in gene expression for MMP-1, MMP-2, MMP-9, MT1-MMP and TIMP-2 in monkey endothelial cells exposed to hypoxia. MMP-2 protein was further evaluated by zymography. These authors found that hypoxia triggered a 3-fold increase in the gene expression of MT1-MMP, MMP-2 and TIMP-2, and a 10-fold increase in MMP-2 levels. Expression of MMP-1

and MMP-9 mRNA was not detected (Ottino *et al.* 2004). Ben-Yosef *et al* performed a number of studies investigating the effect of hypoxia and re-oxygenation on endothelial cells, using both human umbilical vein endothelial cells (HUVEC) and human macrovascular endothelial cells (EAhy 926), and studying MMPs and their inhibitors. Interestingly they have shown that an acute hypoxic episode of 6 hours decreased the expression of MMP-2, MT1-MMP and TIMP-2. This also caused a reduced latent and active MMP-2 protein. However chronic hypoxia further suppressed MT1-MMP and TIMP-2 expression but resulted in a marked increase in MMP-2 expression and enzyme secretion was observed. This suggests that MMP-2 interactions with ECM during episodes of hypoxia are likely to occur independently of MT1-MMP and TIMP-2 (Ben-Yosef *et al.* 2002; Ben-Yosef *et al.* 2005).

In contrast, there have been other studies on different types of endothelial cells that do not support the above findings. For example, Ye at al found that a 24 hour period of hypoxia caused a significant decrease in the protein content and activity of MMP-2 (Ye *et al.* 2005) in porcine pulmonary artery endothelial cells. Noda *et al* reported a 2.3-fold increase in MT1-MMP expression in rabbit retinal glial cells under hypoxic conditions (Noda *et al.* 2005). As briefly discussed, hypoxia could affect endothelial cells in varicose vein disease as a result of haemodynamic changes causing macrocirulatory ischemia damaging the endothelial cells of the intima and/or hypoxia to the endothelial wall caused by insufficient supply by the vasa vasorum in the adventitia.

5.2 OBJECTIVES

Evidence has shown that the degree of hypoxia effects endothelial cells in a differential manner. I aimed to investigate the effects of hypoxia on venous endothelial cells in terms of the expression of MMP and their inhibitors. In this study, potential alterations in MMP/TIMP expression were examined under differing hypoxic conditions, namely acute and chronic hypoxia.

5.3 MATERIALS AND METHODS

5.3.1 CELL EXTRACTION AND CULTURE

Human Saphenous Vascular Endothelial Cells (HSVEC) were obtained by enzymatic release from saphenous veins harvested during high ligation of varicose veins or bypass surgery and then immortalised and cultured as described previously in Materials and Methods. HMEC-1 (SV40 T antigen transformed dermal endothelial cells derived from human foreskin, pulmonary and hepatic endothelium, which retain microvascular endothelial cell characteristics) were also cultured as explained in the same section.

5.3.2 HYPOXIC EXPOSURE

The cells were exposed to differing degrees of oxygen concentration in the RS Biotech Galaxy R incubator over a 24 hour period. The protocol for this is explained in full in Chapter 2. HSVEC and HMEC-1 had three identical flasks of confluent cells. One flask under went 24 hours in normoxic conditions, the other acute hypoxia (6 hours 1% oxygen followed by 18 hours normoxia) and the last chronic hypoxia (24 hours 1% oxygen). Triplicates were completed for each experiment.

5.3.3 RNA EXTRACTION AND QUANTITATION

On completion of hypoxic exposure, RNA was extracted from the cells using the QIAamp RNA blood mini kit (QIAGEN) method. GAPDH PCR was performed in order to confirm the absence of genomic DNA. RNA was then quantified using a spectrophotometer and 500ng of RNA was used from each set of cultures and subjected a reverse transcription reaction to prepare cDNA. GAPDH PCR was then performed in order to confirm the presence of cDNA. Quantitative PCR using a Sybr Green Jumpstart Taq Readymix allowed sequential quantitative PCR runs to analyse the genes of interest. These included MMP-2, MT1-MMP, TIMP-2 and TIMP-3.

5.3.4 STATISTICAL ANALYSIS

The data was collected on the Rotor-gene 7 program in order to set a threshold and obtain the Ct values. These were then collated on to an Excel spread sheet and calculations made using comparative Ct method, also called $2^{-\Delta\Delta Ct}$ method (see Chapter 2). The mRNA levels were then compared and statistically analysed in the HSVEC populations and the HMEC-1 using GraphPad Prism version 4.03 software.

5.4 RESULTS

The following analyses were performed

i) Total mRNA levels in each individual population of HSVEC expressed relative to normoxia

ii) Total mRNA levels of all 3 HSVEC populations expressed relative to normoxia

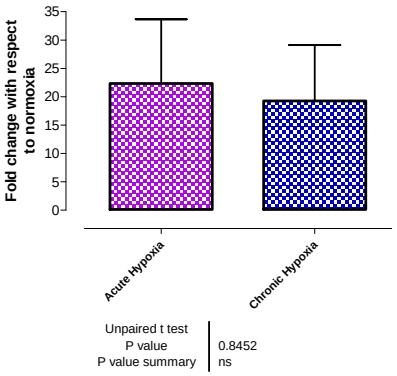
iii) Total mRNA levels of all 3 HMEC populations expressed relative to normoxia

5.4.1 MMP-2 EXPRESSION IN DIFFERENT ENDOTHELIAL CELL LINES

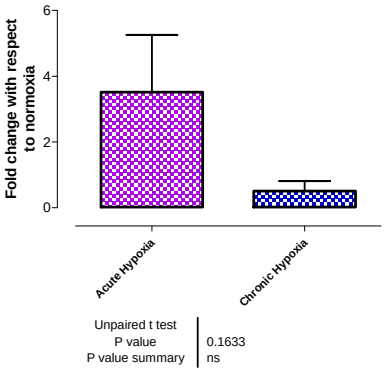
MMP-2 mRNA levels were quantified by PCR in HSVEC and HMEC-1. MMP-2 mRNA was present in both HSVEC and HMEC-1. Analysing the 3 individual populations of HSVEC, it was apparent that MMP-2 mRNA was more abundant in the acute hypoxic models and chronic hypoxic models than those that were maintained in a normoxic state. However, there was no significant difference between responses induced by acute *versus* chronic hypoxia when analysed using unpaired t-test ($p=0.8452$, $p=0.1633$, $p=0.2067$ respectively for Figure 5.1 i, ii and iii). When all data were compared, it was also clear that there was an increased expression of MMP-2 mRNA in cells that were exposed to acute hypoxia when compared to cells that underwent the chronic hypoxic protocol (Figure 5.2). However, this also was not significant using a paired t-test ($p=0.0750$). When comparing values for MMP-2 mRNA levels in the HMEC-1, cell line experiencing acute or chronic hypoxia, these appeared to follow a similar pattern to the HSVEC (Figure 5.3), in that there was an increased amount of MMP-2 mRNA expression in acute hypoxia (mean fold increase=472.837) when compared to chronic hypoxia (mean fold increase=55.629), but this difference was not significant using an unpaired t-test ($p=0.3019$).

Figure 5.1 MMP-2 mRNA levels in HSVEC during differing exposures to hypoxia
MMP-2 mRNA levels in the 3 different populations of HSVEC exposed to either acute or chronic hypoxia, expressed relative to normoxia. Data are expressed as mean +/- mean.

(i)



(ii)



(iii)

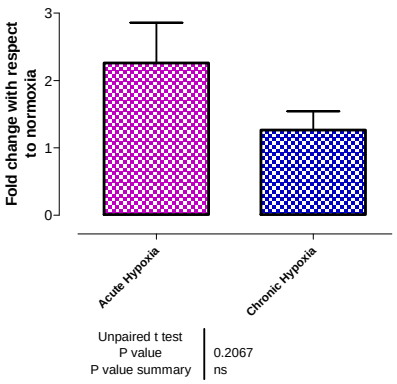


Figure 5.2 MMP-2 mRNA levels in paired HSVEC during exposures to acute and chronic hypoxia

MMP-2 mRNA levels in 3 populations of HSVEC exposed to either acute or chronic hypoxia, expressed relative to normoxia.

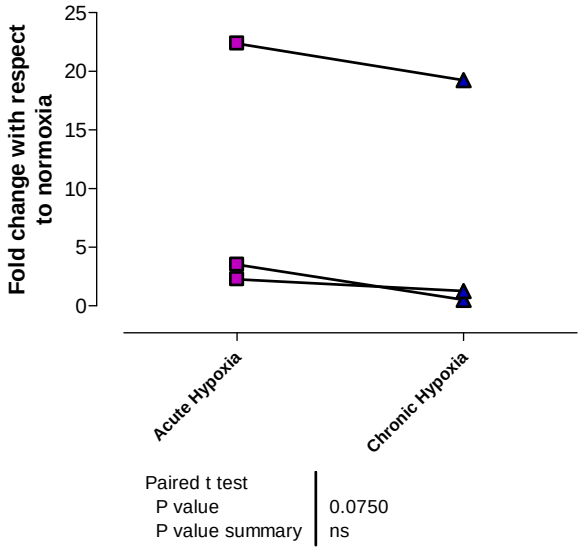
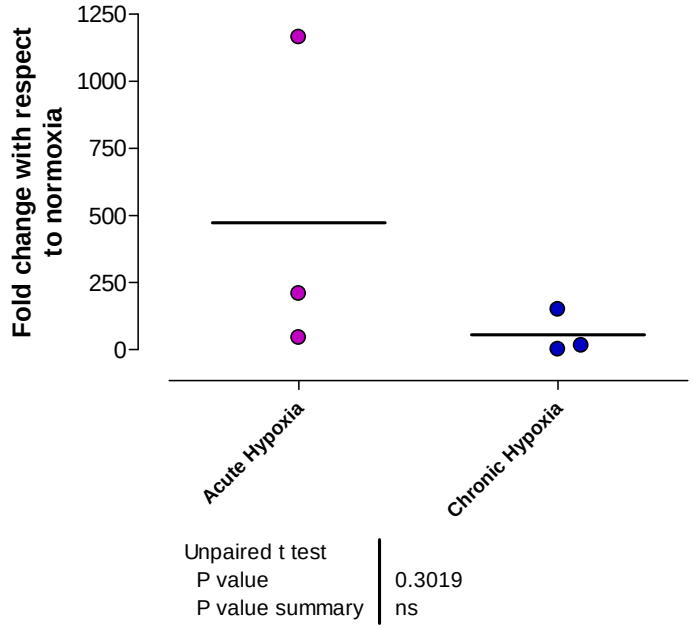


Figure 5.3 MMP-2 mRNA levels in HMEC-1 during differing exposures to hypoxia

MMP-2 mRNA levels in the 3 populations of HMEC-1 cells exposed to either acute or chronic hypoxia, expressed relative to normoxia. Data are mean and individual values.



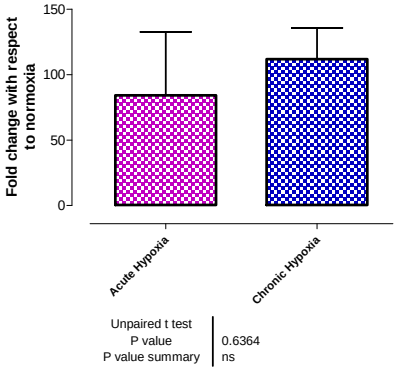
5.4.2 MT1-MMP EXPRESSION IN DIFFERENT ENDOTHELIAL CELL LINES

MT1-MMP mRNA levels were quantified by PCR in the HSVEC and HMEC-1. MT1-MMP mRNA was present in both HSVEC and HMEC-1. Analysing the 3 individual populations of HSVEC, it was apparent that MT1-MMP mRNA was more abundant in acute hypoxia than under normoxic conditions (Figure 5.4). However, the chronic hypoxic model showed a variable response. In one population there was more MT1-MMP mRNA expression under chronic hypoxia than in acute hypoxia (Figure 5.4i), although both showed more MT1-MMP expression than under normoxia. This data was not significant using an unpaired t-test ($p=0.6364$ for acute *versus* chronic hypoxia). The other 2 populations showed a large reduction in the amount of MT1-MMP mRNA expression under chronic hypoxia when compared to acute hypoxia, and indeed even a reduced amount when compared to normoxic model. This data was significant using unpaired t-test (for acute *versus* chronic hypoxia $p=0.0187$, $p=0.0480$ for Figure 5.4 ii and iii respectively). These trends are also apparent in Figure 5.5 when all data are compared. However, when data was paired, it was statistically non-significant ($p=0.5042$). When comparing the mean values for MT1-MMP mRNA levels in the HMEC-1, experiencing acute or chronic hypoxia, these follow a similar pattern to the HSVEC (Figure 5.6). There was an increased amount of MT1-MMP mRNA expression in the acute hypoxic model (mean fold change relative to normoxia=2.748) when compared to the chronic model (mean fold change relative to normoxia=0.0690). This data was not significant using an unpaired t-test ($p=0.2650$).

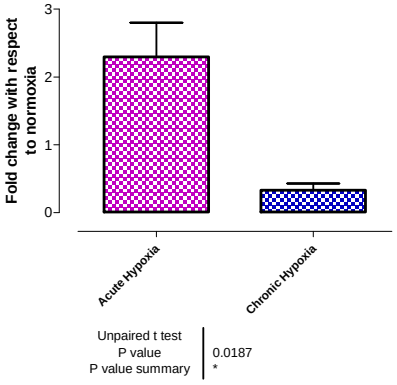
Figure 5.4 MT1-MMP mRNA levels in HSVEC during differing exposures to hypoxia

MT1-MMP mRNA levels in the 3 different populations of HSVEC exposed to either acute or chronic hypoxia, expressed relative to normoxia. Data are expressed as mean +/- mean.

(i)



(ii)



(iii)

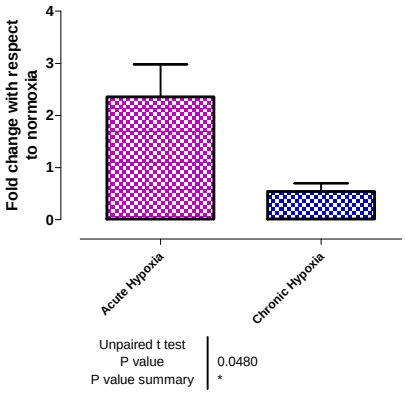


Figure 5.5 MT1-MMP mRNA levels in paired HSVEC during exposures to acute and chronic hypoxia

MT1-MMP mRNA levels in 3 populations of HSVEC, expressed relative to normoxia.

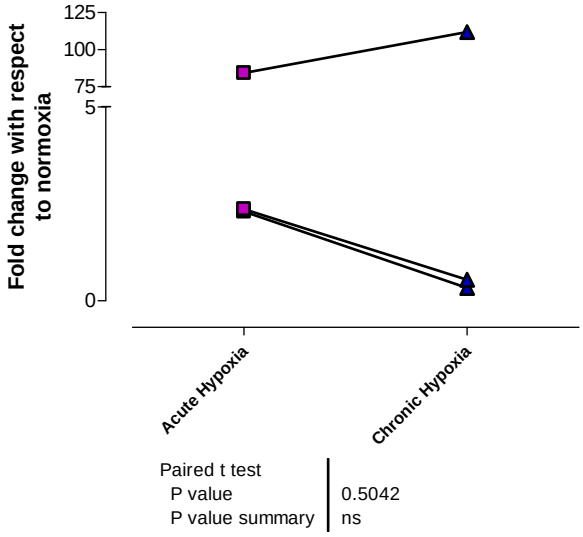
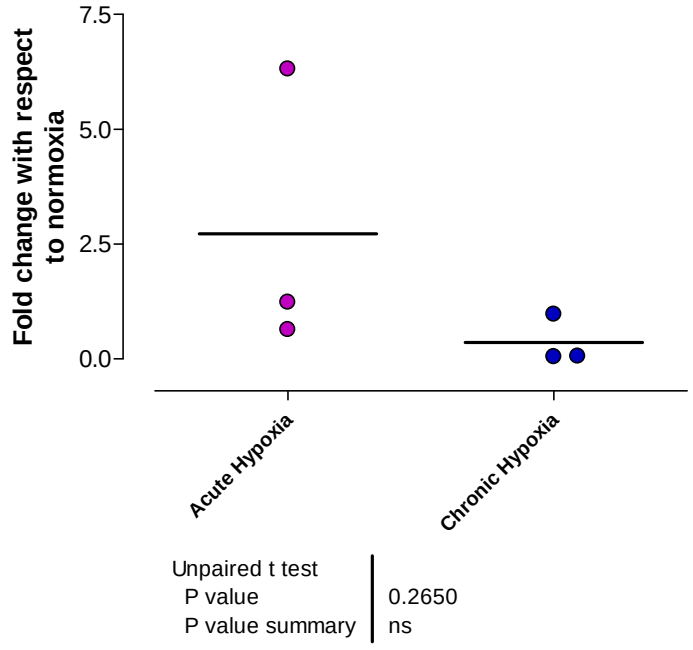


Figure 5.6 MT1-MMP mRNA levels in HMEC-1 during differing exposures to hypoxia

MT1-MMP mRNA levels in the 3 populations of HMEC-1 cells, expressed relative to normoxia. Data are mean and individual values.

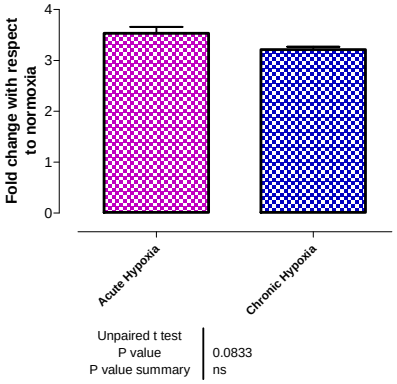


5.4.3 TIMP-2 EXPRESSION IN DIFFERENT ENDOTHELIAL CELL LINES

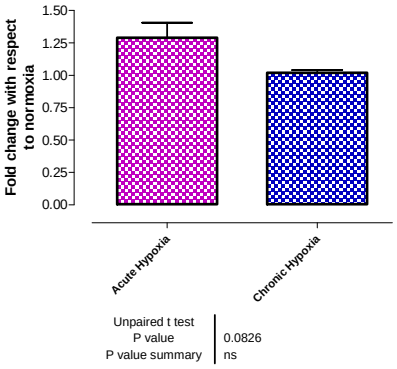
Similarly, TIMP-2 mRNA levels were quantified by PCR in the HSVEC and HMEC-1. TIMP-2 mRNA was present in both HSVEC and HMEC-1. Analysing the 3 individual populations of HSVEC there appeared to be little difference in TIMP-2 mRNA expression between the 3 different models (Figure 5.7). Statistical analyses were performed on all 3 populations using unpaired t-test and unsurprisingly none of the tests were significant (for acute *versus* chronic hypoxia p=0.0833, p=0.0826, p=0.3754 for Figure 5.7 i, ii and iii respectively). When this data was paired a subtle trend was visualised, in that there tended to be slightly more TIMP-2 mRNA in acute hypoxia than under chronic hypoxia (Figure 5.8). This trend was not significant using a paired t-test (p=0.0833). When comparing the mean values for TIMP-2 mRNA levels in the HMEC-1, experiencing acute or chronic hypoxia, it showed a similar, but more convincing, pattern to the HSVEC (Figure 5.9). There was an increased amount of TIMP-2 mRNA expression in the acute hypoxic model (mean fold change relative to normoxia=26.906) when compared to the chronic model (mean=5.470). This data was not significant using an unpaired t-test (p=0.1999).

Figure 5.7 TIMP-2 mRNA levels in HSVEC during differing exposures to hypoxia
TIMP-2 mRNA levels in the 3 different populations of HSVEC exposed to either acute or chronic hypoxia, expressed relative to normoxia. Data are expressed as mean +/- mean.

(i)



(ii)



(iii)

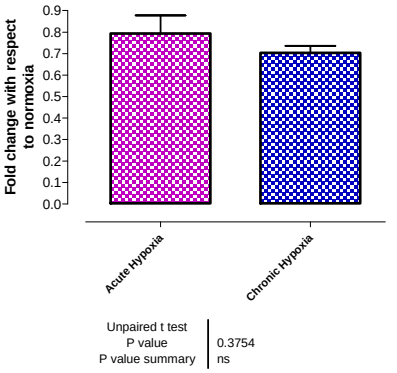


Figure 5.8 TIMP-2 mRNA levels in paired HSVEC during exposures to acute and chronic hypoxia

TIMP-2 mRNA levels in pooled 3 populations of HSVEC, expressed relative to normoxia.

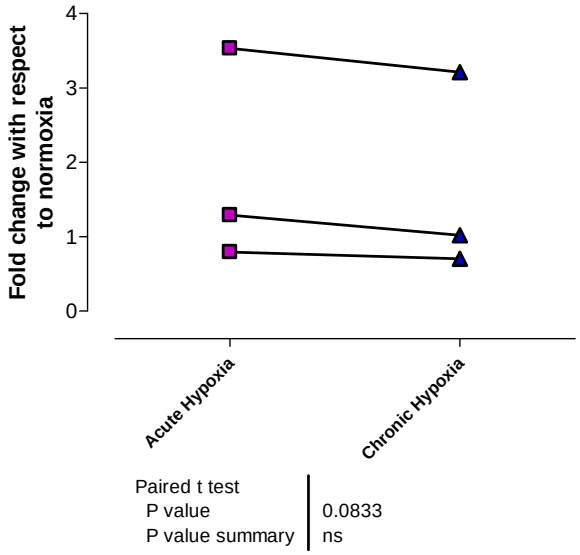
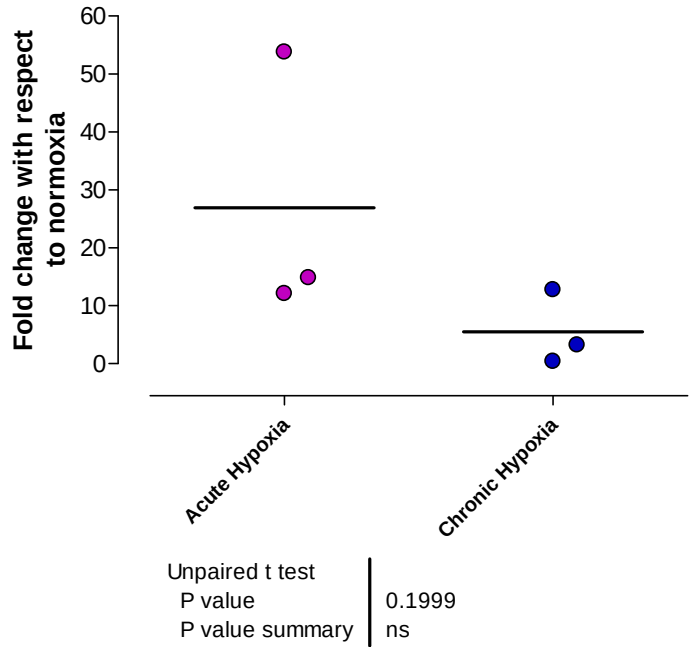


Figure 5.9 TIMP-2 mRNA levels in HMEC-1 during differing exposures to hypoxia

TIMP-2 mRNA levels in the 3 populations of HMEC-1 cells, expressed relative to normoxia. Data are mean and individual values.

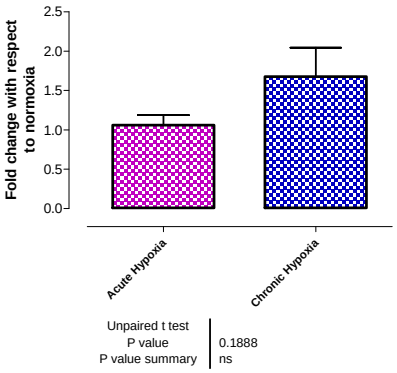


5.4.4 TIMP-3 EXPRESSION IN DIFFERENT ENDOTHELIAL CELL LINES

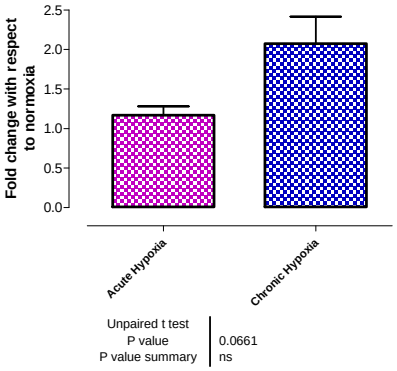
Finally, TIMP-3 mRNA levels were quantified by PCR in the HSVEC and HMECs. MMP-2 mRNA was present in both HSVEC and HMEC-1. Analysing the 3 individual populations of HSVEC it was interesting that TIMP-3 mRNA was more abundant in the chronic hypoxic models than in acute hypoxic models (Figure 5.10). This is a reverse trend in comparison to the other genes of interest investigated. When performing statistical analysis on the individual populations using unpaired t-test, none of this data was significant (for acute *versus* chronic hypoxia p=0.1888, p=0.0.661, p=0.5149). However, when pairing the samples (Figure 5.11) there was a clear trend, in that TIMP-3 mRNA expression was increased with increasing hypoxic conditions and this was found to be statistically significant using paired t-test (p=0.0120). When comparing the mean values for TIMP-3 mRNA levels in HMEC-1 experiencing acute or chronic hypoxia, this followed a similar pattern to the HSVEC (Figure 5.12). In contrast to the previous genes of interest examined, there was an increased amount of TIMP-3 mRNA expression in the chronic hypoxic model (mean fold change relative to normoxia=1.170) when compared to the acute model (mean fold change relative to normoxia=0.402). However this data was not significant using an unpaired t-test (p=0.5110).

Figure 5.10 TIMP-3 mRNA levels in HSVEC during differing exposures to hypoxia
TIMP-3 mRNA levels in the 3 different populations of HSVEC exposed to either acute or chronic hypoxia, expressed relative to normoxia. Data are expressed as mean +/- mean.

(i)



(ii)



(iii)

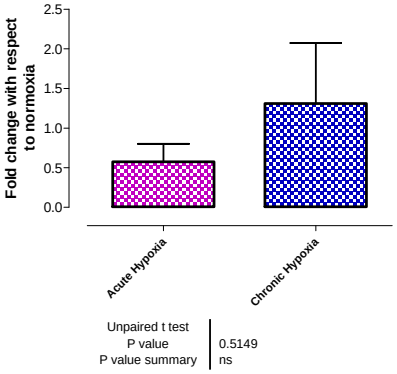


Figure 5.11 TIMP-3 mRNA levels in paired HSVEC during exposures to acute and chronic hypoxia

TIMP-3 mRNA levels in 3 pooled populations of HSVEC, expressed relative to normoxia.

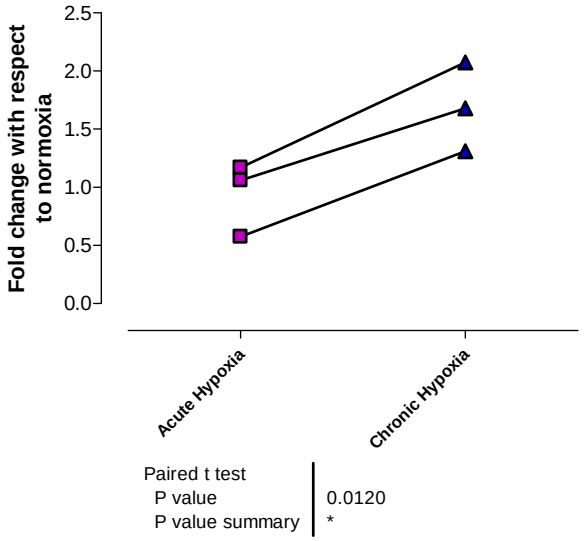
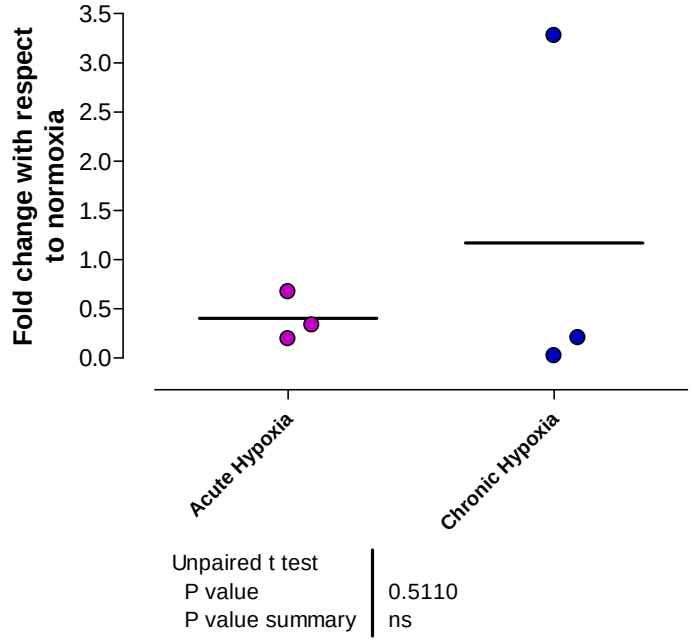


Figure 5.12 TIMP-3 mRNA levels in HMEC-1 during differing exposures to hypoxia

TIMP-3 mRNA levels in the 3 populations of HMEC-1 cells, expressed relative to normoxia. Data are mean and individual values.



5.5 DISCUSSION

This chapter set out to assess the effect of hypoxia on human saphenous endothelial cell expression of MMP and their inhibitors. This was addressed by culturing HSVEC and HMEC-1 and exposing them to differing hypoxic environments over a 24 hour period, extracting RNA and quantifying and analysing the expression levels for MMP-2, MT1-MMP, TIMP-2 and TIMP-3.

The venous wall is composed of an endothelial lining surrounded by an ECM composed of collagen, elastin, SMC, and proteoglycans. The integrity of the structure and function of such capacitance vessels is maintained by a delicate balance between the synthesis and degradation of these proteins and the ECM (Naoum *et al.* 2007). Endothelial cells are the major determinant of vascular tone, leukocyte adhesion and smooth muscle proliferation and under normal circumstances the endothelium maintains a vasodilator, antithrombotic and anti-inflammatory state. However, when endothelial cells undergo activation, an increase in the expression of adhesion molecules such as selectins, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 promotes the adherence of the inflammatory cells monocytes, neutrophils, lymphocytes, and macrophages and recruitment of additional cytokines, growth factors and MMP (Sprague and Khalil 2009). Taking this into consideration it is highly likely that endothelial cells play a large role in the inter-play that is likely to occur in the pathogenesis of varicose vein disease.

The vascular response to hypoxia has multiple components. Short term exposure of endothelial cells to low oxygen tension results in the elaboration of predominantly vasoconstricting effectors. The endothelium is known to be involved in vasoconstriction during hypoxic episodes which suggests that the vascular smooth muscle response during hypoxic episodes in regulated by interaction with adjacent endothelial cells. Long term and more severe hypoxic exposure generates factors that induce irreversible smooth muscle proliferation, remodelling and fibrosis. It is thought that the endothelial cells detect changes in oxygen tension through a biochemical sensor and respond by regulating the transcription of

genes encoding paracrine vasoactive and mitogenic growth factors (Faller 1999).

My predecessors reported, in 2001, the successful generation of human saphenous vein endothelial cell lines by infection with SV40 large T-antigen or HP16 E6 and E7 genes. This cell line provided me with a novel resource as primary cultures of human endothelial cells have a short life span *in vitro* and tend to de-differentiate after 3–4 passages reverting to a fibroblastic phenotype (Scoumanne *et al.* 2002). Previous studies have used endothelial cell lines generated from human umbilical vein. Although widely used, human umbilical vein endothelial cell lines may have characteristics associated with the limited life span and specialised functions of umbilical endothelial cells *in vivo*. They are commonly thought to be a poor model for adult vessel endothelial cells (Garlanda and Dejana 1997) and are therefore unlikely to be an appropriate model for the more clinically relevant saphenous vein endothelial cells. The only disadvantage to this library of cells was that some cells were derived from specimens taken from the GSV harvested during lower limb arterial surgery and other cells were cultured from specimens taken during varicose vein surgery. The most compatible comparison for our previously prepared HSVEC was HMEC-1 cells. HMEC-1 are the first immortalized human microvascular endothelial cell line that has retained the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells (Ades *et al.* 1992).

My results in this chapter have shown that there appears to be a trend of upregulation for the MMPs, MMP-2 and MT1-MMP, in endothelial cells when exposed to an acute episode of hypoxia. These results were not significant. The expression for these genes was less in the chronic model when compared to the acute model. For MMP-2 this result was not significant however, when comparing the individual HSVEC populations for MT1-MMP, 2 cell lines gave a significant result. This significance was lost when the data was paired. These results conform with the findings of Ottino *et al* who researched acute hypoxic exposure on monkey choroid-retinal endothelial cells. They found that MMP-2 activity increased 10 fold and MT1-MMP expression increased 3 fold when experiencing low oxygen tension for up to a maximum of 8 hours (Ottino *et al.* 2004). In contrast, Ben-Yosef *et al* found that endothelial

cells exposed to a short (6 hour) hypoxic episode inhibited the mRNA expression of MMP-2 and MT1-MMP, and TIMP-2 and that prolonged (24 hour) hypoxia further suppressed MT1-MMP mRNA (as shown by my results), whereas it enhanced MMP-2 mRNA (Ben-Yosef *et al*. 2002).

Although TIMP-2 expression was consistently detected, its abundance during normoxic, acute hypoxic and chronic hypoxic conditions remained unremarkable. Riches *et al* had a similar finding when examining the expression of TIMP-2 in normoxic and hypoxic myofibroblasts (Riches *et al.* 2009). TIMP-3 expression, on the other hand, showed little change in the acute hypoxic environment but displayed a significant increase in expression under chronic hypoxic conditions. The only evidence to support this is from Norman *et al* who examined the effects of hypoxia on MMP/TIMP expression in human renal fibroblasts. They found that hypoxia differentially affected expression of the mRNAs for the three TIMPs, increasing expression of TIMP-1 and -3 mRNA, while TIMP-2 mRNA levels were unaffected (Norman *et al.* 2000), thus echoing my results.

This chapter aimed to assess if hypoxia could affect venous endothelial cells and subsequently contribute to changes in the vessel wall, thus resulting in pathology. I have shown that hypoxic insult on endothelial cells potentially causes alterations in MMP and TIMP mRNA expression, to differing degrees. As a comparison, it would have been of interest to also assess the effect of hypoxia on cytokine expression in these populations of cells. In order to draw a sturdier conclusion and to further investigate the objective of this chapter, assessment of protein levels and activity of the MMPs, TIMPs and cytokines would be of interest and would give a better indication of alterations that could occur under hypoxic conditions. This could be done using Western Blotting or immunohistochemistry.

HIFs, when stabilized by hypoxic conditions, activate a signalling cascade that results in the upregulation of several genes to promote cell survival in low-oxygen conditions. Instead of measuring HIF directly, a different approach to studying the effects of hypoxia, is to quantify the expression of HIF target genes. Examples of these in endothelial cells would

be glucose transporter GLUT-1, carbonic anydrase (CA)-9 and enolase (ENO)-1 and their expression could be measured by similar methods to the techniques used in this thesis.

As discussed previously I have shown that hypoxic insult potentially causes alterations in MMP and TIMP mRNA expression in venous endothelial cells. Obviously by culturing endothelial cells, the results only portray a small part of what could be happening within the vein wall. In order to replicate *in vivo* conditions, a similar hypoxic experiment could be performed using a vein culture model (including venous endothelial cells, fibroblasts and SMC) and measurements for mRNA expression or activity levels of MMPs, TIMPs and cytokines taken and assessed. However, the pattern of responses between HSVEC and HMEC-1 in terms of response to hypoxia was similar, suggesting that whether hypoxia occurs in large veins and/or microvessels of the vasa vasorum, a comparable effect in terms of MMP/TIMP would be seen.

Another technique that would be helpful in investigating the pathogenesis of varicose vein disease are PCR-based arrays. PCR arrays are the most reliable tool for analyzing the expression of a focused panel of genes, in this case hypoxia-related genes. PCR arrays are a highly sensitive and specific method, with high amplification efficiencies and high reproducibility that profiles the expression of a panel of genes relevant to a particular pathway or disease state.

CHAPTER 6

6 CONCLUSIONS

The primary cause of varicose veins is still unknown. Varicose veins are common, affecting up to 40% of the population (Golledge and Quigley 2003). Although there are many options for treatment, including conservative methods such as support hosiery, surgical striping of the GSV is by far the commonest form of treatment for varicose veins, with more than 60,000 operations each year in England alone. The reasons for treatment are vast ranging from, unsightliness to bleeding or ulceration. This condition is therefore of great importance in surgery, as it is a common problem with frequent hideous complications that not only physically affect patients lives but impinge greatly on their quality of life.

This MD (Res) thesis, using gene expression techniques, aimed to assess whether or not metalloproteinase (MMP)/tissue inhibitor of MMP (TIMP) contribute to the potential changes in the varicose vein disease process by disrupting the normal turnover of the ECM, and whether cytokines are also involved in regulating this process. The aim was also to investigate whether hypoxia may be a feature of varicose vein disease and this was assessed at the mRNA level within varicose vein samples and also by visualising the relationship between markers of hypoxia with the MMPs, their inhibitors and cytokines. Endothelial cell reaction to acute and chronic hypoxic stress was also assessed with regards to expression of MMPs and their inhibitors.

6.1 PREVIOUS WORK

Research from the Kennedy Institute of Rheumatology (KIR, Faculty of Medicine, Imperial College), in collaboration with the Department of Vascular Surgery (Division of Surgery, Anaesthetics and Intensive Care, Faculty of Medicine, Imperial College), has demonstrated regional variation in varicose vein wall morphology, in the form of hypertrophic and atrophic changes. Atrophic changes appear to be more common in the distal end of GSVs. Previous studies have been conducted examining the imbalances of MMP/TIMP in varicose veins. My predecessor, Mr Biju Aravind, aimed to achieve this

objective as well as further analyse the cellular markers on the vein wall which could be viewed as possible triggers for the protease imbalance. He collected varicose veins as well as veins from other sources. The vein segments were immunostained for MMP-2, MT1-MMP, TIMP-2 and TIMP-3. The immunopositivity was then further quantified using image analysis. The expression of MT1-MMP and TIMP-3 was shown on the varicose vein wall for the very first time. These along with MMP-2 and TIMP-2 appeared to be mostly localised in the SMC. When proximal and distal varicose vein wall thickness were compared in the same patients, the distal vein walls tended to be thinner. He reported a significantly higher expression of MT1-MMP and TIMP-2 in varicose veins in comparison to veins from other sources by image analysis. When analysed further there was positive correlation in the expression of MT1-MMP and MMP-2 in varicose vein wall (Aravind 2007).

Furthermore, infiltration of the vein wall in both varicose veins and veins from other sources by inflammatory cells that stain positive for CD45 has been demonstrated. CD45 and Mast cell tryptase immunopositive cell counts were significantly higher in varicose veins than in veins of other sources. CD45 and Mast cell tryptase immunopositive cell counts showed a positive correlation with each other.

To complete his work, Mr Aravind also preliminary performed RNA extraction, quantitative PCR and activity assays for MT1-MMP in a very limited number of varicose vein specimens, which showed higher MT1-MMP activity in distal varicose veins than proximal segments. However there was no significant difference in the MT1-MMP gene expression between proximal and distal varicose vein segments.

Taken together, these data suggested that distal areas of varicose vein wall tended to be thinner, and were associated with greater MT1-MMP activity, and reduced inhibitor expression (TIMP-2 and TIMP-3) than thicker areas of vein wall, supporting the concept that changes in the MMP/TIMP balance may influence varicose vein wall morphology.

6.2 DATA DESCRIBED IN THIS THESIS

I have confirmed that mRNA is expressed for the MMPs and their inhibitors, MMP-2, MT1-MMP, TIMP-1 and TIMP-2, in varicose vein tissue. When these were compared to veins from other sources there was no difference in the mRNA expression for the MMPs, MMP-2 or MT1-MMP. This finding goes against my predecessors image analysis data that showed a significantly higher expression of MT1-MMP in varicose vein tissue. However there was a significant increase in the amount of mRNA expressed for TIMP-2 and TIMP-3 in varicose vein tissue when compared to veins from other sources. These results taken together indicate that with no change to MMP-2 and MT1-MMP expression and a significant increase in TIMP-2 and TIMP-3 expression there would be an alteration in the MMP/TIMP ratio. This would suggest that the MMP/TIMP imbalance could be disrupted by the over-expression of TIMP.

MT1-MMP mRNA was found to have higher mRNA expression levels in distal varicose vein segments relative to proximal varicose vein segments although this data was not found to be significant. This is consistent with the protein and preliminary data obtained by my predecessor. TIMP-2 and TIMP-3 are abundantly expressed in varicose vein tissue. TIMP-2 showed a non-significant trend to having increased expression levels (2-fold) in distal varicose vein segments than in proximal segments. MMP-2 and TIMP-3 showed no regional variation.

Degradation of the ECM is regulated by proteinases, including MMP, their activators, and their inhibitors. Activated MMPs can degrade all ECM components. MMP can be regulated at three levels: MMP production in the pro-MMP state, MMP activation and inhibition of the activated form of MMP. MMPs are inhibited by TIMPs, which form high-affinity, essentially irreversible, non-covalent complexes with the active forms of MMP. It is important that the activity of MMP is kept under tight regulation to maintain homeostasis and therefore keep a healthy ECM. Even though I did not observe increased expression of MMP-2, like other researchers have reported, upregulation of TIMP-2 and TIMP-3 in varicose vein

tissue maybe enough to upset the MMP/TIMP ratio and in turn disrupt homeostasis. This could partially explain the ECM accumulation in varicose veins.

It is well documented that reflux, a major feature of varicose vein disease, is suggested to occur segmentally and progress in an ascending fashion. As previously mentioned, my predecessor found that distal varicose vein walls were thinner in diameter than proximal samples retrieved from the same patient. This conclusion was not reached, however, by Travers *et al* (Travers *et al.* 1996). My mRNA data reiterates the results of Mr Aravind. I found a difference between MT1-MMP expression in proximal and distal varicose vein (reduced expression in the distal portion) and a trend of increased expression of TIMP-2 in distal varicose vein segments despite non-significance. Together this would imply that thinner varicose vein segments are associated with greater MT1-MMP activity and reduced TIMP-2 activity that thicker areas of wall supporting the concept that changes in the MMP/TIMP ratio may influence varicose vein morphology.

I have also confirmed that the cytokines VEGF, TNF-α, IL-1β and IL-6 are expressed in varicose vein tissue. The mRNA expression for VEGF and TNF-α, was upregulated in varicose vein tissue when compared to veins from other sources, 2-fold and 5-fold respectively. This data was not significant. In contrast the interleukins IL-1β and IL-6 both had a reduction in mRNA expression in varicose vein tissue when compared to veins from other sources. For IL-1β this result was significant.

The TNF-α data hinted that there may be more expression in the proximal portion of the varicose vein that in the distal portion when comparing the median values, but this data was not significant. The other cytokines showed no evidence of regional variation.

It is undecided whether inflammation may play a role in the formation of varicose vein disease. There have been a number of reports that cross-sectional histological studies have revealed monocytes/macrophage infiltrations in both the wall and the valves of the vein. Despite this Badier-Commander *et al* looked at pro-angiogenic cytokines in varicose veins by a combined histological, immunohistochemical and biochemical approach. They found that

TGFβ1 and FGF-2 were increased in varicose veins but there was no difference in VEGF when compared to control specimens. They also noted no inflammatory cells and suggested that the cytokine modulation seen was unlikely to be due to an inflammatory reaction (Badier-Commander *et al.* 2001). Various cytokines, both pro-angiogenic and pro-inflammatory have been shown to be increased in varicose veins or in simulated varicose vein models by inducing venous hypertension. My results indicate that cytokines are likely to be involved in the pathogenesis of varicose vein disease. The expression of the pro-angiogenic cytokine VEGF and pro-inflammatory cytokine TNF-α are increased in varicose vein tissue. However, the pro-inflammatory cytokines, IL-1β and IL-6, which, like TNF-α, tend to be involved in acute phase inflammatory reactions are decreased in varicose vein tissue when compared to veins from other sources.

There is no literature to suggest that cytokines display any regional variation in varicose vein tissue. My results also show no conclusive evidence that regional variation may be present.

VEGF displayed a statistically significant correlation with TIMP-3. It has been reported that TIMP-3 blocks the binding of VEGF to VEGF receptor type 2 and inhibits downstream signalling and angiogenesis (Qi *et al.* 2003). Thus an increase in TIMP-3, to counter-act the possible pro-angiogenic effects of VEGF, may be a mechanism to regulate angiogenesis in varicose vein adventitia. TNF-α showed a significant correlation with MT1-MMP and TIMP-3 and a very significant correlation with MMP-2 and TIMP-2. IL-1β also significantly correlated with MMP-2, MT1-MMP and TIMP-2. As explained above in detail, MMP-2, MT1-MMP and TIMP-2 are linked by forming a ternary complex. The fact that both TNF-α and IL-1β levels correlate with the expression of the 3 members of this complex, may provide evidence that in varicose veins cytokine-mediated assembly of this complex could be important in regulating matrix turnover. TNF-α also correlated with TIMP-3. This was not surprising as they have a strong link through TACE.

I have confirmed, for the very first time that, that mRNA for HIF-1α and HIF-2α are

expressed in varicose vein tissue.

When these were compared to veins from other sources the mRNA expression in varicose vein tissue was significantly decreased for both HIF-1α and HIF-2α. This was an unexpected result because logically if hypoxia is a feature of varicose vein disease one would have expected levels of HIF to have increased in comparison to veins from other sources. It is known that HIF protein levels increase in hypoxia. The fact that my results showed a reduction in HIF mRNA expression could be explained by HIF protein levels increasing under the hypoxic conditions but conversely HIF mRNA levels also being actively degraded at the same time. This is supported by the existence of aHIF, a natural antisense transcript derived from HIF-1α gene sequences that encodes the 3' untranslated region of HIF-1α mRNA (Thrash-Bingham and Tartof 1999)). aHIF is thought to cause degradation of HIF mRNA. During prolonged hypoxia, HIF-α proteins negatively regulate HIF-1α expression through an increase in aHIF and destabilization of HIF-1α mRNA (Uchida *et al.* 2004). As a consequence, elevated HIF protein levels (due to conditions of hypoxia) may be somewhat counter-intuitively be accompanied by decreased HIF mRNA levels. This has been shown to be the case in our laboratory in a range of other cell types, particularly after prolonged exposure to hypoxia, as might be expected to be the case in varicose veins, and could well be a mechanism to control HIF levels *in vivo* and prevent excessive HIF-mediated gene transcription.

When analysing data comparing proximal *versus* distal varicose vein samples, neither HIF-1α nor HIF-2α showed any difference between the two anatomical areas.

When correlating HIFs with MMP and their inhibitors, HIF-1α displayed a statistically significant correlation with MMP-2. This would suggest that MMP-2 regulation is likely to be modulated by HIF-1α. In contrast, HIF-2α showed a very significant correlation with MT1-MMP. It is thought that not only does MT1-MMP have a functional HIF-binding site in its proximal promoter region but MT1-MMP is under direct regulation by HIF-2α.

When correlating HIFs with the cytokines, both HIFs correlated significantly with TNF-α. Cytokines appear to stimulate HIF gene expression and it is likely that TNF-α does it independently to that of the HIF pathway. It is surprising that neither HIF-1α nor HIF-2α did not correlate with VEGF as there is a multitude of data to support the dependence of VEGF expression on HIF. Once again, since HIFs are predominantly regulated at the level of protein stability, it may have been informative to also measure HIF protein expression and correlate this with VEGF.

There appears to be a trend of upregulation for the MMPs, MMP-2 and MT1-MMP in endothelial cells when exposed to an acute episode of hypoxia. These results were not significant. The expression for these genes was less in the chronic model when compared to the acute model. For MMP-2 this result was not significant however, when comparing the individual HSVEC populations for MT1-MMP, 2 cell lines gave a significant result. This significance was lost when the data was paired. Although TIMP-2 expression was consistently detected, its abundance during normoxic, acute hypoxic and chronic hypoxic conditions remained unremarkable. TIMP-3 expression, on the other hand, showed little change in the acute hypoxic environment. However, TIMP-3 displayed a significant increase in expression under chronic hypoxic conditions. Taken together, these results suggest that endoluminal hypoxia may participate in the pathogenesis of varicose vein disease. Venous hypertension, blood stasis and alterations in blood flow may have a direct influence on the endothelial cells that in turn alter the configuration of MMPs/TIMPs and potentially have consequential effects in the deeper layers of the vein wall.

6.3 CLINICAL IMPLICATIONS

Hypoxia induces activation of HIF in almost all cell types, which results in induction of several HIF target genes, involved in cell metabolism, survival, angiogenesis and invasion. It is well established that the hypoxia/HIF-VEGF-angiogenesis pathway plays a very important role in tumour development and progression. Therefore the development of small molecule

inhibitors are targeting hypoxia and HIF, often in combination with conventional chemotherapy, has become more and more attractive. In the last years many studies have characterised agents which inhibit HIF-1 and angiogenesis, and a number of these compounds are currently in clinical trials, or are already approved for the treatment of cancer (reviewed in (Semenza 2007). Some of these might be useful for treatment of varicose vein disease.

For example, In 2002, Rapisarda *et al* developed a cell-based study to identify small molecule inhibitors of the HIF-1 pathway, which may have anti-angiogenic and anti-cancer activities. The investigators genetically modified a human glioma cell line to express a luciferase reporter gene under control of three canonical copies of HRE, and screened 2000 small molecule chemotherapeutic agents of the National Cancer Institute Diversity Set. This screen identified topoisomerase inhibitors, such as topotecan and camptothecin analogues, as being capable of repressing HIF activity and inhibiting transcription of well characterized HIF target genes, including VEGF (Rapisarda *et al.* 2002). Many studies have followed and topotecan and camptothecin analogues are currently approved agents for the treatment of cancer. Similarly, histone deacetylase enzyme (HDAC) 1 has been reported to be hypoxia-inducible and to stimulate angiogenesis *in vitro* and *in vivo*, suggesting a significant role for HDAC in hypoxia-induced angiogenesis. The acetylation of histones by histone acetyltransferase and the deacetylation by HDAC has a significant role in regulation of gene transcription in many cell types (Grunstein 1997). The deregulation of HDAC activity and recruitment to promoters appears to be one of the mechanisms by which these enzymes contribute to tumourigenesis (Cress and Seto 2000). Therefore, HDAC inhibitors with different chemical structures have been developed as novel anti-tumour agents.

Finally, in 2003 Mabjeesh *et al* reported that the endogenous oestrogen metabolite 2-methoxyestradiol (2ME2) downregulates HIF at the post-transcriptional level and inhibits HIF-1-induced transcriptional activation of VEGF expression, establishing 2ME2 as a small molecule inhibitor of HIF-1 (Mabjeesh *et al.* 2003). 2ME2 is currently being evaluated in clinical trials for the treatment of solid tumours and is undergoing pre-clinical evaluation for inflammatory conditions.

6.4 FUTURE WORK

There have been very few studies examining the contribution of changes in oxygen tension to varicose vein disease. This is somewhat surprising, since varicose veins by nature involve alterations in blood flow and venous stasis, which must compromise tissue oxygenation, and hence is likely to involve alteration in HIF expression.

I have successfully shown that both HIF-1α and HIF-2α are expressed at the mRNA level in varicose veins and that there is a relationship between HIFs and MMP/TIMP, and between HIFs and cytokines. My research provided some surprising results. Both HIF-1α and HIF-2α showed a significant reduction in expression in varicose vein tissue when compared to veins from other sources. However, as described earlier, the major pathway regulating HIF-1 activity is not through changes in mRNA, but via the oxygen-dependent degradation domain in the α-subunit of HIF protein which is the target of specific HIF-α PHD enzymes. These enzymes require both O_2 and Fe^{2+}, and they have been considered to function as direct cellular oxygen sensors, with no intermediate signalling required for HIF regulation. In the presence of O_2, HIF-α subunits are prolyl hydroxylated on Pro-402 and Pro-564 (in the case of HIF-1) and these hydroxyl-proline residues are required for the interaction with the von Hippel-Lindau tumour suppressor protein (pVHL), which subsequently guides ubiquitin E3 ligases to polyubiquitinate HIF-α. Once polyubiquitinated, HIF-α-subunits are targeted for proteasomal degradation by the 26S-proteasome. During hypoxia the decline in oxygen levels decreases the activity of the PHDs, thereby preventing proline and asparagine hydroxylation of HIF-1α. Consequently pVHL cannot target HIF-α for degradation, allowing HIF-α to accumulate and dimerise with HIF-1β in the nucleus. This complex recognises and binds to HRE in the promoter or enhancer regions of various hypoxia-responsive target genes (Figure 1.5). Thus, investigation of the mRNA levels of HIF-1α and HIF-2α should be complemented by studies of HIF-1α and HIF-2α protein using either immunohistochemistry or Western blotting techniques, to conclusively assess whether hypoxia in varicose veins leads to upregulation of HIF protein.

This thesis used whole vein tissue to assess gene expression. In addition to this data, gene expression of specific cells, such as venous endothelial cells, would have been useful as this would have provided specific information about the differing features of the vein wall. A suitable method to isolate specific cells is laser capture microdissection, which during sample collection does not alter or damage the morphology or chemistry of the cells (Orba *et al.*2003). Alternatively, in situ hybridisation could have been used to study gene expression in endothelial cells using whole tissue samples.

Furthermore, as mentioned above, aHIF induction by hypoxia may contribute to decreased HIF mRNA levels, and it would therefore be of interest to also measure aHIF mRNA levels in vein tissue. During hypoxia, the HIF-1α proteins that are produced, as a subsequent effect of increased expression of HIF-α, cause an increase in aHIF production in order to negatively regulate HIF. aHIF then encodes the 3' untranslated region of HIF-1α mRNA causing it to degrade. Interpretation of HIF mRNA expression is, therefore, not a fair representation of what may truly be occurring in varicose vein tissue. There are a number of techniques that could be performed in order to verify this preliminary data.

Importantly, as mentioned above, the accumulation of HIFs results in the activity of various hypoxia-responsive target genes. In the case of varicose veins these include VEGF, GLUT-1, ENO-1 and CA-9. A more reliable way of assessing HIF expression in varicose veins could be to quantify the expression of HIF target genes. In parallel, the protein expression and activity levels for HIF could also be evaluated This could then be compared to protein analysis for MMP/TIMP and cytokines.

Hypoxic stress appears to cause alterations in MMP and TIMP mRNA expression in venous endothelial cells. While my study described in Chapter 5 focused on endothelial cells, the endothelium is just a small component of the vein wall. In order to reproduce a more accurate model in order to simulate varicose vein conditions, a similar hypoxic experiment could be performed on a vein culture model (including venous endothelial cells, fibroblasts

and SMC) followed by measurements for mRNA expression or activity levels of MMPs, TIMPs and cytokines taken and assessed.

Finally, there is no evidence to suggest where the hypoxic insult may occur in the vein. Venous hypertension and alterations to blood flow obviously contribute largely to the multi-factorial pathology of this disease. It may be that this blood flow modification changes the oxygen tension within the blood itself resulting in a direct effect on the endothelial cells. A clinical experiment could directly measure venous oxygen levels in varicose vein patients and controls. Blood could be taken from the distal segment of GSVs at the medial malleolus in both varicose vein patients and controls (matched demographically and selected randomly from patients requiring acute surgical assessment). It would be transferred into an arterial blood gas syringe and analysed on a blood gas machine for the partial pressure of oxygen and pH, to allow determination of oxygen levels.

6.5 SUMMARY OF FINDINGS PRESENTED IN THIS THESIS

In summary, the aetiology of varicose veins remains unclear. There is increasing evidence that alteration of the vein wall is the primary abnormality of the disease. Numerous changes in the cellular and ECM components of the vein wall have been demonstrated including intimal hyperplasia, SMC proliferation, changes of collagen and elastin content, and imbalances of MMPs and their endogenous tissue inhibitors TIMPs. The overall effects include relaxation and weakening of the vein wall leading to venous dilatation and reflux However, the cause of these changes remains unknown and several factors including vein wall hypoxia have been proposed to contribute to the development of varicose veins. The following summarise the main findings and conclusions presented in this thesis:

- I found a significant increase in the amount of mRNA expressed for TIMP-2 and TIMP-3 in varicose vein tissue when compared to veins from other sources. This overexpression of TIMP may be accountable for disruption of the MMP/TIMP ratio and in turn affects homeostasis and the ECM.

- I would suggest that TIMP-2 and MT1-MMP have an association with each other that is independent of MMP-2 and the formation of a ternary complex between these 3 molecules. Potentially TIMP-2 binding to MT1-MMP activates an extracellular pathway which up-regulates cell proliferation and migration.
- HIF-1α and HIF-2α are expressed in both varicose and non-varicose veins and show no regional variation.
- HIF-1α significantly correlated with MMP-2 as well as with TNF-α and IL-1β. In contrast, HIF-2α showed significant correlation with MT1-MMP and TNF-α suggesting an inter-play between hypoxia and cytokines in regulating the MMP/TIMP balance.
- Endothelial cells under hypoxic stress show alterations in MMP/TIMP expression might not only damage the cells directly, for example by induction of apoptosis, but could potentially have consequential effects in the deeper layers of the vein wall.

BIBLIOGRAPHY

Abramson, J. H., C. Hopp and L. M. Epstein (1981). "The epidemiology of varicose veins. A survey in western Jerusalem." J Epidemiol Community Health **35**(3): 213-7.

Ades, E. W., F. J. Candal, R. A. Swerlick, V. G. George, S. Summers, D. C. Bosse and T. J. Lawley (1992). "HMEC-1: establishment of an immortalized human microvascular endothelial cell line." J Invest Dermatol **99**(6): 683-90.

Amour, A., P. M. Slocombe, A. Webster, M. Butler, C. G. Knight, B. J. Smith, P. E. Stephens, C. Shelley, M. Hutton, V. Knauper, A. J. Docherty and G. Murphy (1998). "TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3." FEBS Lett **435**(1): 39-44.

Aravind, B. (2007). Matrix Metalloproteinases (MMPs) and Tissue Inhibitors Of MMP (TIMPs): Role Of MMP/TIMP Balance In The Pathogenesis Of Varicose Veins. SORA/Kennedy Institute of Rheumatology Imperial College

Ascher, E., T. Jacob, A. Hingorani, Y. Gunduz, F. Mazzariol and S. Kallakuri (2000). "Programmed cell death (Apoptosis) and its role in the pathogenesis of lower extremity varicose veins." Ann Vasc Surg **14**(1): 24-30.

Ascher, E., T. Jacob, A. Hingorani, B. Tsemekhin and Y. Gunduz (2001). "Expression of molecular mediators of apoptosis and their role in the pathogenesis of lower-extremity varicose veins." J Vasc Surg **33**(5): 1080-6.

Badier-Commander, C., A. Couvelard, D. Henin, T. Verbeuren, J. B. Michel and M. P. Jacob (2001). "Smooth muscle cell modulation and cytokine overproduction in varicose veins. An in situ study." J Pathol **193**(3): 398-407.

Badier-Commander, C., T. Verbeuren, C. Lebard, J. B. Michel and M. P. Jacob (2000). "Increased TIMP/MMP ratio in varicose veins: a possible explanation for extracellular matrix accumulation." J Pathol **192**(1): 105-12.

Beebe-Dimmer, J. L., J. R. Pfeifer, J. S. Engle and D. Schottenfeld (2005). "The epidemiology of chronic venous insufficiency and varicose veins." Ann Epidemiol **15**(3): 175-84.

Ben-Yosef, Y., N. Lahat, S. Shapiro, H. Bitterman and A. Miller (2002). "Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation." Circ Res **90**(7): 784-91.

Ben-Yosef, Y., A. Miller, S. Shapiro and N. Lahat (2005). "Hypoxia of endothelial cells leads to MMP-2-dependent survival and death." Am J Physiol Cell Physiol **289**(5): C1321-31.

Berridge, D., T. Lees and J. J. Earnshaw (2009). "The VEnous INtervention (VEIN) Project." Phlebology **24 Suppl 1**: 1-2.

Borden, P. and R. A. Heller (1997). "Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases." Crit Rev Eukaryot Gene Expr **7**(1-2): 159-78.

Bradbury, A., C. Evans, P. Allan, A. Lee, C. V. Ruckley and F. G. Fowkes (1999). "What are the symptoms of varicose veins? Edinburgh vein study cross sectional population survey." BMJ **318**(7180): 353-6.

Brand, F. N., A. L. Dannenberg, R. D. Abbott and W. B. Kannel (1988). "The epidemiology of varicose veins: the Framingham Study." Am J Prev Med **4**(2): 96-101.

Brew, K., D. Dinakarpandian and H. Nagase (2000). "Tissue inhibitors of metalloproteinases: evolution, structure and function." Biochim Biophys Acta **1477**(1-2): 267-83.

Callam, M. J. (1994). "Epidemiology of varicose veins." Br J Surg **81**(2): 167-73.

Campbell, B. (2006). "Varicose veins and their management." BMJ **333**(7562): 287-92.

Campbell, W. B., H. Decaluwe, J. B. Macintyre, J. F. Thompson and A. R. Cowan (2006). "Most patients with varicose veins have fears or concerns about the future, in addition to their presenting symptoms." Eur J Vasc Endovasc Surg **31**(3): 332-4.

Carpentier, P. H., H. R. Maricq, C. Biro, C. O. Poncot-Makinen and A. Franco (2004). "Prevalence, risk factors, and clinical patterns of chronic venous disorders of lower limbs: a population-based study in France." J Vasc Surg **40**(4): 650-9.

Carroll, V. A. and M. Ashcroft (2006). "Role of hypoxia-inducible factor (HIF)-1alpha *versus* HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway." Cancer Res **66**(12): 6264-70.

Chant, A. D., P. Magnussen and C. Kershaw (1985). "Support hose and varicose veins." Br Med J (Clin Res Ed) **290**(6463): 204.

Cheatle, T., H. Scott and E. Stibe (1998). Microcirculation in venous disease. Texas, USA, Landes Bioscience.

Chen, P. S., W. R. Zhai, X. M. Zhou, J. S. Zhang, Y. E. Zhang, Y. Q. Ling and Y. H. Gu (2001). "Effects of hypoxia, hyperoxia on the regulation of expression and activity of matrix metalloproteinase-2 in hepatic stellate cells." World J Gastroenterol **7**(5): 647-51.

Christopoulos, D., A. N. Nicolaides and G. Szendro (1988). "Venous reflux: quantification and correlation with the clinical severity of chronic venous disease." Br J Surg **75**(4): 352-6.

Cines, D. B., E. S. Pollak, C. A. Buck, J. Loscalzo, G. A. Zimmerman, R. P. McEver, J. S. Pober, T. M. Wick, B. A. Konkle, B. S. Schwartz, E. S. Barnathan, K. R. McCrae, B. A. Hug, A. M. Schmidt and D. M. Stern (1998). "Endothelial cells in physiology and in the pathophysiology of vascular disorders." Blood **91**(10): 3527-61.

Clyne, C. A., W. H. Ramsden, A. D. Chant and J. H. Webster (1985). "Oxygen tension on the skin of the gaiter area of limbs with venous disease." Br J Surg **72**(8): 644-7.

Coimbra, I. B., S. A. Jimenez, D. F. Hawkins, S. Piera-Velazquez and D. G. Stokes (2004). "Hypoxia inducible factor-1 alpha expression in human normal and osteoarthritic chondrocytes." Osteoarthritis Cartilage **12**(4): 336-45.

Coleridge Smith, P. (2009). "Foam and liquid sclerotherapy for varicose veins." Phlebology **24 Suppl 1**: 62-72.

Cramer, T., Y. Yamanishi, B. E. Clausen, I. Forster, R. Pawlinski, N. Mackman, V. H. Haase, R. Jaenisch, M. Corr, V. Nizet, G. S. Firestein, H. P. Gerber, N. Ferrara and R. S.

Johnson (2003). "HIF-1alpha is essential for myeloid cell-mediated inflammation." Cell **112**(5): 645-57.

Cress, W. D. and E. Seto (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

D'Alessio, S., G. Ferrari, K. Cinnante, W. Scheerer, A. C. Galloway, D. F. Roses, D. V. Rozanov, A. G. Remacle, E. S. Oh, S. A. Shiryaev, A. Y. Strongin, G. Pintucci and P. Mignatti (2008). "Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism." J Biol Chem **283**(1): 87-99.

da Silva, A., L. K. Widmer, H. Martin, T. Mall, L. Glaus and M. Schneider (1974). "Varicose veins and chronic venous insufficiency." Vasa **3**(2): 118-25.

Das, S. K., S. Yano, J. Wang, D. R. Edwards, H. Nagase and S. K. Dey (1997). "Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period." Dev Genet **21**(1): 44-54.

Delis, K. T., P. Gloviczki, P. W. Wennberg, T. W. Rooke and D. J. Driscoll (2007). "Hemodynamic impairment, venous segmental disease, and clinical severity scoring in limbs with Klippel-Trenaunay syndrome." J Vasc Surg **45**(3): 561-7.

Dery, M. A., M. D. Michaud and D. E. Richard (2005). "Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators." Int J Biochem Cell Biol **37**(3): 535-40.

Downing, L. J., R. M. Strieter, A. M. Kadell, C. A. Wilke, S. L. Brown, S. K. Wrobleski, M. D. Burdick, M. S. Hulin, J. B. Fowlkes, L. J. Greenfield and T. W. Wakefield (1996). "Neutrophils are the initial cell type identified in deep venous thrombosis induced vein wall inflammation." ASAIO J **42**(5): M677-82.

Elsharawy, M. A., M. M. Naim, E. M. Abdelmaguid and A. A. Al-Mulhim (2007). "Role of saphenous vein wall in the pathogenesis of primary varicose veins." Interact Cardiovasc Thorac Surg **6**(2): 219-24.

Faller, D. V. (1999). "Endothelial cell responses to hypoxic stress." Clin Exp Pharmacol Physiol **26**(1): 74-84.

Fan, R. H., P. S. Chen, D. Zhao and W. D. Zhang (2007). "[Hypoxia induced by CoCl2 influencing the expression and the activity of matrix metalloproteinase-2 in rat hepatic stellate cells]." Zhonghua Gan Zang Bing Za Zhi **15**(9): 654-7.

Gandhi, R. H., E. Irizarry, G. B. Nackman, V. J. Halpern, R. J. Mulcare and M. D. Tilson (1993). "Analysis of the connective tissue matrix and proteolytic activity of primary varicose veins." J Vasc Surg **18**(5): 814-20.

Garlanda, C. and E. Dejana (1997). "Heterogeneity of endothelial cells. Specific markers." Arterioscler Thromb Vasc Biol **17**(7): 1193-202.

Gaylarde, P. M., I. Sarkany and H. J. Dodd (1993). "The effect of compression on venous stasis." Br J Dermatol **128**(3): 255-8.

Gillespie, D. L., A. Patel, B. Fileta, A. Chang, S. Barnes, A. Flagg, M. Kidwell, J. L. Villavicencio and N. M. Rich (2002). "Varicose veins possess greater quantities of

MMP-1 than normal veins and demonstrate regional variation in MMP-1 and MMP-13." J Surg Res **106**(2): 233-8.

Gimbrone, M. A., Jr., T. Nagel and J. N. Topper (1997). "Biomechanical activation: an emerging paradigm in endothelial adhesion biology." J Clin Invest **99**(8): 1809-13.

Golledge, J. and F. G. Quigley (2003). "Pathogenesis of varicose veins." Eur J Vasc Endovasc Surg **25**(4): 319-24.

Gomez, D. E., D. F. Alonso, H. Yoshiji and U. P. Thorgeirsson (1997). "Tissue inhibitors of metalloproteinases: structure, regulation and biological functions." Eur J Cell Biol **74**(2): 111-22.

Gordeuk, V. R., A. I. Sergueeva, G. Y. Miasnikova, D. Okhotin, Y. Voloshin, P. L. Choyke, J. A. Butman, K. Jedlickova, J. T. Prchal and L. A. Polyakova (2004). "Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors." Blood **103**(10): 3924-32.

Gosling, M., S. L. Harley, R. J. Turner, N. Carey and J. T. Powell (1998). "Human saphenous vein endothelial cells express a tetrodotoxin-resistant, voltage-gated sodium current." J Biol Chem **273**(33): 21084-90.

Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." Nature **389**(6649): 349-52.

Gurantz, D., R. T. Cowling, N. Varki, E. Frikovsky, C. D. Moore and B. H. Greenberg (2005). "IL-1beta and TNF-alpha upregulate angiotensin II type 1 (AT1) receptors on cardiac fibroblasts and are associated with increased AT1 density in the post-MI heart." J Mol Cell Cardiol **38**(3): 505-15.

Hellwig-Burgel, T., D. P. Stiehl, A. E. Wagner, E. Metzen and W. Jelkmann (2005). "Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions." J Interferon Cytokine Res **25**(6): 297-310.

Herouy, Y., A. E. May, G. Pornschlegel, C. Stetter, H. Grenz, K. T. Preissner, E. Schopf, J. Norgauer and W. Vanscheidt (1998). "Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: implications for venous ulcer formation." J Invest Dermatol **111**(5): 822-7.

Herouy, Y., P. Nockowski, E. Schopf and J. Norgauer (1999). "Lipodermatosclerosis and the significance of proteolytic remodeling in the pathogenesis of venous ulceration (Review)." Int J Mol Med **3**(5): 511-5.

Hewitson, K. S. and C. J. Schofield (2004). "The HIF pathway as a therapeutic target." Drug Discov Today **9**(16): 704-11.

Holbrook, K. A. and P. H. Byers (1982). "Structural abnormalities in the dermal collagen and elastic matrix from the skin of patients with inherited connective tissue disorders." J Invest Dermatol **79 Suppl 1**: 7s-16s.

Hollingsworth, S. J., C. B. Tang, M. Dialynas and S. G. Barker (2001). "Varicose veins: loss of release of vascular endothelial growth factor and reduced plasma nitric oxide." Eur J Vasc Endovasc Surg **22**(6): 551-6.

Iovieno, A., A. Lambiase, C. Moretti, E. Perrella and S. Bonini (2009). "Therapeutic effect of topical 5-fluorouracil in conjunctival squamous carcinoma is associated with changes in matrix metalloproteinases and tissue inhibitor of metalloproteinases expression." Cornea **28**(7): 821-4.

Itoh, Y., A. Takamura, N. Ito, Y. Maru, H. Sato, N. Suenaga, T. Aoki and M. Seiki (2001). "Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion." Embo J **20**(17): 4782-93.

Jacob, M. P., M. Cazaubon, A. Scemama, D. Prie, F. Blanchet, M. C. Guillin and J. B. Michel (2002). "Plasma matrix metalloproteinase-9 as a marker of blood stasis in varicose veins." Circulation **106**(5): 535-8.

Jawien, A. (2003). "The influence of environmental factors in chronic venous insufficiency." Angiology **54 Suppl 1**: S19-31.

Jeong, H. J., S. H. Hong, R. K. Park, T. Shin, N. H. An and H. M. Kim (2005). "Hypoxia-induced IL-6 production is associated with activation of MAP kinase, HIF-1, and NF-kappaB on HEI-OC1 cells." Hear Res **207**(1-2): 59-67.

Jung, Y. J., J. S. Isaacs, S. Lee, J. Trepel and L. Neckers (2003). "IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis." FASEB J **17**(14): 2115-7.

Katori, H., A. Nozawa and M. Tsukuda (2006). "Increased expression of matrix metalloproteinase-2 and 9 and human papilloma virus infection are associated with malignant transformation of sinonasal inverted papilloma." J Surg Oncol **93**(1): 80-5.

Koochekpour, S., M. Jeffers, P. H. Wang, C. Gong, G. A. Taylor, L. M. Roessler, R. Stearman, J. R. Vasselli, W. G. Stetler-Stevenson, W. G. Kaelin, Jr., W. M. Linehan, R. D. Klausner, J. R. Gnarra and G. F. Vande Woude (1999). "The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells." Mol Cell Biol **19**(9): 5902-12.

Kowalewski, R., K. Sobolewski, M. Wolanska and M. Gacko (2004). "Matrix metalloproteinases in the vein wall." Int Angiol **23**(2): 164-9.

Labropoulos, N., A. D. Giannoukas, K. Delis, M. A. Mansour, S. S. Kang, A. N. Nicolaides, J. Lumley and W. H. Baker (1997). "Where does venous reflux start?" J Vasc Surg **26**(5): 736-42.

Labropoulos, N., S. S. Kang, M. A. Mansour, A. D. Giannoukas, J. Buckman and W. H. Baker (1999). "Primary superficial vein reflux with competent saphenous trunk." Eur J Vasc Endovasc Surg **18**(3): 201-6.

Labropoulos, N., L. Leon, S. Kwon, A. Tassiopoulos, J. A. Gonzalez-Fajardo, S. S. Kang, M. A. Mansour and F. N. Littooy (2005). "Study of the venous reflux progression." J Vasc Surg **41**(2): 291-5.

Labropoulos, N., J. Tiongson, L. Pryor, A. K. Tassiopoulos, S. S. Kang, M. Ashraf Mansour and W. H. Baker (2003). "Definition of venous reflux in lower-extremity veins." J Vasc Surg **38**(4): 793-8.

Lee, A. J., C. J. Evans, P. L. Allan, C. V. Ruckley and F. G. Fowkes (2003). "Lifestyle factors and the risk of varicose veins: Edinburgh Vein Study." J Clin Epidemiol **56**(2): 171-9.

Li, Q. F. and A. G. Dai (2004). "Hypoxia-inducible factor-1 alpha regulates the role of vascular endothelial growth factor on pulmonary arteries of rats with hypoxia-induced pulmonary hypertension." Chin Med J (Engl) **117**(7): 1023-8.

Li, X., H. Kimura, K. Hirota, H. Sugimoto and H. Yoshida (2005). "Hypoxia reduces constitutive and TNF-alpha-induced expression of monocyte chemoattractant protein-1 in human proximal renal tubular cells." Biochem Biophys Res Commun **335**(4): 1026-34.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-8.

Mabjeesh, N. J., D. Escuin, T. M. LaVallee, V. S. Pribluda, G. M. Swartz, M. S. Johnson, M. T. Willard, H. Zhong, J. W. Simons and P. Giannakakou (2003). "2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF." Cancer Cell **3**(4): 363-75.

Maffei, F. H., C. Magaldi, S. Z. Pinho, S. Lastoria, W. Pinho, W. B. Yoshida and H. A. Rollo (1986). "Varicose veins and chronic venous insufficiency in Brazil: prevalence among 1755 inhabitants of a country town." Int J Epidemiol **15**(2): 210-7.

Mantovani, A., F. Bussolino and E. Dejana (1992). "Cytokine regulation of endothelial cell function." FASEB J **6**(8): 2591-9.

Martensson, K., D. Chrysis and L. Savendahl (2004). "Interleukin-1beta and TNF-alpha act in synergy to inhibit longitudinal growth in fetal rat metatarsal bones." J Bone Miner Res **19**(11): 1805-12.

Mashiah, A., S. S. Rose and I. Hod (1991). "The scanning electron microscope in the pathology of varicose veins." Isr J Med Sci **27**(4): 202-6.

Matic, M., V. Duran, M. Ivkov-Simic, M. Poljacki, Z. Gajinov and M. Begenisic (2000). "[Microcirculatory changes in chronic venous insufficiency]." Med Pregl **53**(11-12): 579-83.

McKusick, V. A. (1991). "The defect in Marfan syndrome." Nature **352**(6333): 279-81.

Michiels, C., T. Arnould, R. Thibaut-Vercruyssen, N. Bouaziz, D. Janssens and J. Remacle (1997). "Perfused human saphenous veins for the study of the origin of varicose veins: role of the endothelium and of hypoxia." Int Angiol **16**(2): 134-41.

Michiels, C., N. Bouaziz and J. Remacle (2002). "Role of the endothelium and blood stasis in the development of varicose veins." Int Angiol **21**(2 Suppl 1): 18-25.

Murphy, M. A., W. P. Joyce, C. Condron and D. Bouchier-Hayes (2002). "A reduction in serum cytokine levels parallels healing of venous ulcers in patients undergoing compression therapy." Eur J Vasc Endovasc Surg **23**(4): 349-52.

Nagase, H. (1997). "Activation mechanisms of matrix metalloproteinases." Biol Chem **378**(3-4): 151-60.

Naoum, J. J., G. C. Hunter, K. J. Woodside and C. Chen (2007). "Current advances in the pathogenesis of varicose veins." J Surg Res **141**(2): 311-6.

Neglen, P. and S. Raju (2000). "Ambulatory venous pressure revisited." J Vasc Surg **31**(6): 1206-13.

Nicolaides, A. N., C. Allegra, J. Bergan, A. Bradbury, M. Cairols, P. Carpentier, A. Comerota, C. Delis, B. Eklof, N. Fassiadis, N. Georgiou, G. Geroulakos, U. Hoffmann, G. Jantet, A. Jawien, S. Kakkos, E. Kalodiki, N. Labropoulos, P. Neglen, P. Pappas, H. Partsch, M. Perrin, E. Rabe, A. A. Ramelet, M. Vayssaira, E. Ioannidou and A. Taft (2008). "Management of chronic venous disorders of the lower limbs: guidelines according to scientific evidence." Int Angiol **27**(1): 1-59.

Noda, K., S. Ishida, H. Shinoda, T. Koto, T. Aoki, K. Tsubota, Y. Oguchi, Y. Okada and E. Ikeda (2005). "Hypoxia induces the expression of membrane-type 1 matrix metalloproteinase in retinal glial cells." Invest Ophthalmol Vis Sci **46**(10): 3817-24.

Norgauer, J., T. Hildenbrand, M. Idzko, E. Panther, E. Bandemir, M. Hartmann, W. Vanscheidt and Y. Herouy (2002). "Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers." Br J Dermatol **147**(6): 1180-6.

Norman, J. T., I. M. Clark and P. L. Garcia (2000). "Hypoxia promotes fibrogenesis in human renal fibroblasts." Kidney Int **58**(6): 2351-66.

Orba, Y., S.Tanaka, H. Nishihara, N. Kawamura, T. Itoh, M. Shimizu, H. Sawa and K. Nagashima (2003). "Application of laser capture microdissection to cytologic specimens for the detection of immunoglobulin heavy chain gene rearrangement in patients with malignant lymphoma." Cancer **99**(4): 198-204.

Ottino, P., J. Finley, E. Rojo, A. Ottlecz, G. N. Lambrou, H. E. Bazan and N. G. Bazan (2004). "Hypoxia activates matrix metalloproteinase expression and the VEGF system in monkey choroid-retinal endothelial cells: Involvement of cytosolic phospholipase A2 activity." Mol Vis **10**: 341-50.

Ozaki, H., A. Y. Yu, N. Della, K. Ozaki, J. D. Luna, H. Yamada, S. F. Hackett, N. Okamoto, D. J. Zack, G. L. Semenza and P. A. Campochiaro (1999). "Hypoxia inducible factor-1alpha is increased in ischemic retina: temporal and spatial correlation with VEGF expression." Invest Ophthalmol Vis Sci **40**(1): 182-9.

Pappas, P. J., S. R. Fallek, A. Garcia, C. T. Araki, T. L. Back, W. N. Duran and R. W. Hobson, 2nd (1995). "Role of leukocyte activation in patients with venous stasis ulcers." J Surg Res **59**(5): 553-9.

Parra, J. R., R. A. Cambria, C. D. Hower, M. S. Dassow, J. A. Freischlag, G. R. Seabrook and J. B. Towne (1998). "Tissue inhibitor of metalloproteinase-1 is increased in the saphenofemoral junction of patients with varices in the leg." J Vasc Surg **28**(4): 669-75.

Pascarella, L., A. Penn and G. W. Schmid-Schonbein (2005). "Venous hypertension and the inflammatory cascade: major manifestations and trigger mechanisms." Angiology **56 Suppl 1**: S3-10.

Pascarella, L., G. W. Schmid-Schonbein and J. Bergan (2005). "An animal model of venous hypertension: the role of inflammation in venous valve failure." J Vasc Surg **41**(2): 303-11.

Petrella, B. L., J. Lohi and C. E. Brinckerhoff (2005). "Identification of membrane type-1 matrix metalloproteinase as a target of hypoxia-inducible factor-2 alpha in von Hippel-Lindau renal cell carcinoma." Oncogene **24**(6): 1043-52.

Powell, C. C., M. J. Rohrer, M. R. Barnard, B. D. Peyton, M. I. Furman and A. D. Michelson (1999). "Chronic venous insufficiency is associated with increased platelet and monocyte activation and aggregation." J Vasc Surg **30**(5): 844-51.

Proebstle, T. M., H. A. Lehr, A. Kargl, C. Espinola-Klein, W. Rother, S. Bethge and J. Knop (2002). "Endovenous treatment of the greater saphenous vein with a 940-nm diode laser: thrombotic occlusion after endoluminal thermal damage by laser-generated steam bubbles." J Vasc Surg **35**(4): 729-36.

Psaila, J. V. and J. Melhuish (1989). "Viscoelastic properties and collagen content of the long saphenous vein in normal and varicose veins." Br J Surg **76**(1): 37-40.

Qi, J. H., Q. Ebrahem, N. Moore, G. Murphy, L. Claesson-Welsh, M. Bond, A. Baker and B. Anand-Apte (2003). "A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2." Nat Med **9**(4): 407-15.

Raffetto, J. D., X. Qiao, V. V. Koledova and R. A. Khalil (2008). "Prolonged increases in vein wall tension increase matrix metalloproteinases and decrease constriction in rat vena cava: Potential implications in varicose veins." J Vasc Surg **48**(2): 447-56.

Raffetto, J. D., R. L. Ross and R. A. Khalil (2007). "Matrix metalloproteinase 2-induced venous dilation via hyperpolarization and activation of K+ channels: relevance to varicose vein formation." J Vasc Surg **45**(2): 373-80.

Rapisarda, A., B. Uranchimeg, D. A. Scudiero, M. Selby, E. A. Sausville, R. H. Shoemaker and G. Melillo (2002). "Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway." Cancer Res **62**(15): 4316-24.

Riches, K., M. E. Morley, N. A. Turner, D. J. O'Regan, S. G. Ball, C. Peers and K. E. Porter (2009). "Chronic hypoxia inhibits MMP-2 activation and cellular invasion in human cardiac myofibroblasts." J Mol Cell Cardiol **47**(3): 391-9.

Rutherford, R. B. Vascular Surgery, Saunders.

Saharay, M., D. A. Shields, J. B. Porter, J. H. Scurr and P. D. Coleridge Smith (1997). "Leukocyte activity in the microcirculation of the leg in patients with chronic venous disease." J Vasc Surg **26**(2): 265-73.

Sansilvestri-Morel, P., I. Nonotte, M. P. Fournet-Bourguignon, A. Rupin, J. N. Fabiani, T. J. Verbeuren and P. M. Vanhoutte (1998). "Abnormal deposition of extracellular matrix proteins by cultured smooth muscle cells from human varicose veins." J Vasc Res **35**(2): 115-23.

Sansilvestri-Morel, P., A. Rupin, C. Badier-Commander, J. N. Fabiani and T. J. Verbeuren (2003). "Chronic venous insufficiency: dysregulation of collagen synthesis." Angiology **54 Suppl 1**: S13-8.

Sansilvestri-Morel, P., A. Rupin, S. Jaisson, J. N. Fabiani, T. J. Verbeuren and P. M. Vanhoutte (2002). "Synthesis of collagen is dysregulated in cultured fibroblasts derived from skin of subjects with varicose veins as it is in venous smooth muscle cells." Circulation **106**(4): 479-83.

Sansilvestri-Morel, P., A. Rupin, N. D. Jullien, N. Lembrez, P. Mestries-Dubois, J. N. Fabiani and T. J. Verbeuren (2005). "Decreased production of collagen Type III in cultured smooth muscle cells from varicose vein patients is due to a degradation by MMPs: possible implication of MMP-3." J Vasc Res **42**(5): 388-98.

Sayer, G. L. and P. D. Smith (2004). "Immunocytochemical characterisation of the inflammatory cell infiltrate of varicose veins." Eur J Vasc Endovasc Surg **28**(5): 479-83.

Scharte, M., K. Jurk, B. Kehrel, A. Zarbock, H. Van Aken and K. Singbartl (2006). "IL-4 enhances hypoxia induced HIF-1alpha protein levels in human transformed intestinal cells." FEBS Lett **580**(27): 6399-404.

Scoumanne, A., T. Kalamati, J. Moss, J. T. Powell, M. Gosling and N. Carey (2002). "Generation and characterisation of human saphenous vein endothelial cell lines." Atherosclerosis **160**(1): 59-67.

Semenza, G. L. (2007). "Evaluation of HIF-1 inhibitors as anticancer agents." Drug Discov Today **12**(19-20): 853-9.

Signorelli, S. S., M. G. Malaponte, L. Di Pino, M. P. Costa, G. Pennisi and M. C. Mazzarino (2000). "Venous stasis causes release of interleukin 1beta (IL-1beta), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFalpha) by monocyte-macrophage." Clin Hemorheol Microcirc **22**(4): 311-6.

Soury, P., C. Peillon, J. Watelet, M. Planet, D. Plissonnier, G. Del Gallo and J. Testart (1999). "Prosthetic reinforcement of varicose saphenous vein grafts for infrainguinal bypass." Ann Vasc Surg **13**(3): 290-3.

Sprague, A. H. and R. A. Khalil (2009). "Inflammatory cytokines in vascular dysfunction and vascular disease." Biochem Pharmacol **78**(6): 539-52.

Subramonia, S. and T. A. Lees (2007). "The treatment of varicose veins." Ann R Coll Surg Engl **89**(2): 96-100.

Taccoen, A., C. Lebard, H. Borie, J. C. Poullain, F. Zuccarelli, I. Gerentes, S. Stern and M. Guichard (1996). "[Measurement of oxygen tension in normal and varicose vein walls]." J Mal Vasc **21 Suppl C**: 259-65.

Takase, S., J. J. Bergan and G. Schmid-Schonbein (2000). "Expression of adhesion molecules and cytokines on saphenous veins in chronic venous insufficiency." Ann Vasc Surg **14**(5): 427-35.

Takase, S., L. Pascarella, J. J. Bergan and G. W. Schmid-Schonbein (2004). "Hypertension-induced venous valve remodeling." J Vasc Surg **39**(6): 1329-34.

Thakker-Varia, S., C. A. Tozzi, G. J. Poiani, J. P. Babiarz, L. Tatem, F. J. Wilson and D. J. Riley (1998). "Expression of matrix-degrading enzymes in pulmonary vascular remodeling in the rat." Am J Physiol **275**(2 Pt 1): L398-406.

Thrash-Bingham, C. A. and K. D. Tartof (1999). "aHIF: a natural antisense transcript over-expressed in human renal cancer and during hypoxia." J Natl Cancer Inst **91**(2): 143-51.

Thulesius, O. (1996). "The venous wall and valvular function in chronic venous insufficiency." Int Angiol **15**(2): 114-8.

Tognazzo, S., D. Gemmati, A. Palazzo, L. Catozzi, S. Carandina, A. Legnaro, G. Tacconi, G. L. Scapoli and P. Zamboni (2006). "Prognostic role of factor XIII gene variants in nonhealing venous leg ulcers." J Vasc Surg **44**(4): 815-9.

Travers, J. P., C. E. Brookes, J. Evans, D. M. Baker, C. Kent, G. S. Makin and T. M. Mayhew (1996). "Assessment of wall structure and composition of varicose veins with reference to collagen, elastin and smooth muscle content." Eur J Vasc Endovasc Surg **11**(2): 230-7.

Turner, N. A., D. J. O'Regan, S. G. Ball and K. E. Porter (2005). "Simvastatin inhibits MMP-9 secretion from human saphenous vein smooth muscle cells by inhibiting the RhoA/ROCK pathway and reducing MMP-9 mRNA levels." FASEB J **19**(7): 804-6.

Uchida, T., F. Rossignol, M. A. Matthay, R. Mounier, S. Couette, E. Clottes and C. Clerici (2004). "Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha." J Biol Chem **279**(15): 14871-8.

Venturi, M., L. Bonavina, F. Annoni, L. Colombo, C. Butera, A. Peracchia and E. Mussini (1996). "Biochemical assay of collagen and elastin in the normal and varicose vein wall." J Surg Res **60**(1): 245-8.

Wilson, W. R., J. Evans, P. R. Bell and M. M. Thompson (2005). "HMG-CoA reductase inhibitors (statins) decrease MMP-3 and MMP-9 concentrations in abdominal aortic aneurysms." Eur J Vasc Endovasc Surg **30**(3): 259-62.

Woodside, K. J., M. Hu, A. Burke, M. Murakami, L. L. Pounds, L. A. Killewich, J. A. Daller and G. C. Hunter (2003). "Morphologic characteristics of varicose veins: possible role of metalloproteinases." J Vasc Surg **38**(1): 162-9.

Yamaguchi, A., I. Tojyo, H. Yoshida and S. Fujita (2005). "Role of hypoxia and interleukin-1beta in gene expressions of matrix metalloproteinases in temporomandibular joint disc cells." Arch Oral Biol **50**(1): 81-7.

Yasim, A., M. Kilinc, M. Aral, H. Oksuz, M. Kabalci, E. Eroglu and S. Imrek (2008). "Serum concentration of procoagulant, endothelial and oxidative stress markers in early primary varicose veins." Phlebology **23**(1): 15-20.

Ye, H., Y. Zheng, W. Ma, D. Ke, X. Jin, S. Liu and D. Wang (2005). "Hypoxia down-regulates secretion of MMP-2, MMP-9 in porcine pulmonary artery endothelial and smooth muscle cells and the role of HIF-1." J Huazhong Univ Sci Technolog Med Sci **25**(4): 382-4, 407.

Zamboni, P., M. Izzo, S. Tognazzo, S. Carandina, M. De Palma, L. Catozzi, A. Caggiati, G. Scapoli and D. Gemmati (2006). "The overlapping of local iron overload and HFE mutation in venous leg ulcer pathogenesis." Free Radic Biol Med **40**(10): 1869-73.

Zamboni, P., G. Scapoli, V. Lanzara, M. Izzo, P. Fortini, R. Legnaro, A. Palazzo, S. Tognazzo and D. Gemmati (2005). "Serum iron and matrix metalloproteinase-9 variations in limbs affected by chronic venous disease and venous leg ulcers." Dermatol Surg **31**(6): 644-9; discussion 649.

Zamboni, P., S. Tognazzo, M. Izzo, F. Pancaldi, G. L. Scapoli, A. Liboni and D. Gemmati (2005). "Hemochromatosis C282Y gene mutation increases the risk of venous leg ulceration." J Vasc Surg **42**(2): 309-14.

Zhang, J., A. Liu, R. Hou, X. Jia, W. Jiang and J. Chen (2009). "Salidroside protects cardiomyocyte against hypoxia-induced death: A HIF-1a-activated and VEGF-mediated pathway." Eur J Pharmacol.

Zhang, W., J. M. Petrovic, D. Callaghan, A. Jones, H. Cui, C. Howlett and D. Stanimirovic (2006). "Evidence that hypoxia-inducible factor-1 (HIF-1) mediates transcriptional activation of interleukin-1beta (IL-1beta) in astrocyte cultures." J Neuroimmunol **174**(1-2): 63-73.

PRESENTATIONS AND AWARDS

PRESENTATIONS

By Beverley Saunders nee Sharp

Role of MT1-MMP in morphological changes in Varicose veins B Aravind, B Sharp, E Paleolog, C Monaco, A H Davies. European Venous Forum June 2005, Crete, Greece.

MT1-MMP in varicose veins B Aravind, B Sharp, E Paleolog, C Monaco, A H Davies. International Union of Phlebology Oct 2005. Rio de Janeiro, Brazil

TIMP-3 induced apoptosis has a role in the morphological variation of varicose veins. B Sharp, B Aravind, T Navin, C Monaco, E Paleolog, AH Davies. RSM, London for the Norman Tanner/MIA prize in Dec 2005

MMP 2 and MT1-MMP expression is abundant in smooth muscle cell-rich areas in varicose veins. B Aravind, B Sharp, T Navin, C Monaco, E Paleolog, AH Davies. RSM, London for the Norman Tanner/MIA prize in Dec 2005

TIMP-3 induced apoptosis has a role in the morphological variation of varicose veins. B Sharp, B Aravind, T Navin, C Monaco, E Paleolog, AH Davies. 18th Annual Meeting, American Venous Forum, February 2006, InterContinental Hotel, Miami, Florida

MMP 2 and MT1-MMP expression is abundant in smooth muscle cell-rich areas in varicose veins. B Aravind, B Sharp, T Navin, C Monaco, E Paleolog, AH Davies. 18th Annual Meeting, American Venous Forum, February 2006, InterContinental Hotel, Miami, Florida

Role of TIMP-3 in causation of varicose veins. B Sharp, B Aravind, T Navin, C Monaco, E Paleolog, AH Davies. Tripartite meeting of the European Venous Forum, Venous Forum of the Society of Medicine and the American Venous Forum. June 2006, Royal Society of Medicine, London.

Inhibitory Influence of TIMP Contributes to Morphological Changes of Varicose Vein Wall. B Sharp, B Aravind, T Navin, C Monaco, E Paleolog, AH Davies. 19th Annual Meeting, American Venous Forum, February 2007, Rancho Bernardo Inn, San Diego, Califonia.

Is hypoxia a feature of varicose vein disease? B Sharp, T Navin, C Monaco, E Paleolog, A H Davies. European Venous Forum, June 2007,Istanbul, Turkey.

Arm and neck veins are better controls for vein studies than long saphenous veins from bypass B Aravind, B Sharp, T Navin, C Monaco, E Paleolog, AH Davies. European Venous Forum, June 2007, Istanbul, Turkey.

PRIZES

2006: **Sigvaris Traveling Fellowship Award** Best paper presented at the American Venous Forum, Miami, Feb 2006.

TIMP-3 induced apoptosis has a role in the morphological variation of varicose veins. B Sharp, B Aravind, T Navin, C Monaco, E Paleolog, AH Davies. 18th Annual Meeting, American Venous Forum, February 2006, InterContinental Hotel, Miami, Florida

2007: **2nd Prize for best oral presentation** at the European Venous Forum, Istanbul, Turkey, June 2007.

Is hypoxia a feature of varicose vein disease? B Sharp, T Navin, C Monaco, E Paleolog, A H Davies. European Venous Forum, June 2007,Istanbul, Turkey.