Anatomical and physiological characterization of a novel porcine model for varicose veins

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Abstract

Varicose veins constitute a significant financial and social burden. Despite being well documented since Hippocrates, there is still a lack of understanding concerning both the underlying pathogenesis and the rationale of treatments of varicose veins. The management of varicose veins is marked by confusion and debate, which could benefit from well-designed experiments in a suitable animal model. Especially as the current available therapies for the treatment of varicose veins is limited by their high recurrence rates.

While studying neointimal hyperplasia in arterial ends of common femoral AVF created in pig models we, at the Department of Surgery in Dunedin Hospital, observed that the pigs also developed progressive enlargement and tortuosity of the superficial veins over the medial aspect the thigh and groin. These changes in superficial veins developed into what appeared to be an extensive network of varicose veins. This novel finding has not been described to the same extent in other animal models of venous disease. Consequently, the aim of this thesis is to further characterize a porcine model of varicose veins.

Eleven female domesticated large white Duroc cross pigs, aged 13-14 weeks and weights 25.1- 35 kg, were used in this research project (control pigs [n=2], surgery animals [n=9]). The first animal was used to assist in the characterisation the normal anatomy of pigs’ hind limb, the nominal saphenous vein and its relations and the sapheno-femoral junction. Each of the remaining animals had a right common femoral side to side arteriovenous fistula (AVF) fashioned by a vascular surgeon. Post-operatively the animals were assessed to document of macroscopic changes of venous vasculature, characterise the physiological changes that occur within the superficial venous system and to document any histological changes in the vessel wall.
Venous hypertension was demonstrated within the superficial varicosities, $23 \pm 11.4$ mmHg at the mid point of the study, and $20 \pm 8.3$ mmHg at termination, while in the control it was $4.5 \pm 3.5$ mmHg. In all but one pig with an AVF there was variation in pressure, associated with the cardiac cycle. The flow velocities also demonstrated a degree of pulsatility.

Overall there was both an intra-vessel and inter-animal heterogeneity to the vessel walls changes seen in the veins sampled. Finding which were consistently seen included: variable neointimal formation, medial hypertrophy, fragmentation of the medial and adventitial elastic tissue, and medial and mural atrophy. Areas of greatest intimal thickness were associated with disruption of the internal elastic lamina. A variety of valvular defects were also observed with the specimens including bilateral and unilateral elongation of cusps, and valve cusp tearing.

In conclusion we describe a stable and chronic porcine AVF model of venous insufficiency, which is simple to create and closely replicates the human condition of venous insufficiency and superficial varicose veins. The formation of an AVF between the femoral vessels leads to the progressive loss of venous valve competence and the development of superficial varicosities, which demonstrated elements consistent with the human disease including venous reflux and hypertension and pathological remodeling of the vein wall. The mechanism by which these varicosities are formed appears to be a combination of retrograde flow accompanied by increased pressures within the superficial veins. With further characterisation and refinement, including assessment of ambulatory venous pressures in particular the model may contribute to both the quantitative and qualitative evaluation of the underlying disease process, including its initial onset.
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Chapter 1: Introduction

1.1 A history of varicose veins

Venous disorders of the human lower limb, in particular varicose veins are a frequently encountered problem. They range in severity from asymptomatic incompetence of venous valves to chronic leg ulcerations. Incompetence of venous valves of the lower limb commonly present as varicose veins. These can be described as dilated, tortuous superficial veins.

Throughout history varicose veins in the lower limbs, have been the subject of many medical texts. The papyrus of Ebers (c. 1550 B.C.), is one of the earliest medical texts that refers to varicose veins. One section contains a description of three types of lump, and the author advises against operation for “certain serpentine windings” because that would be “head on the ground”. Medical historians have suggested that the term “serpentine windings” refer to varicose veins, which should not be incised because a fatal hemorrhage may occur (head on the ground).

It appears Hippocrates had been, among other things, practicing phlebology 2400 years ago. For the treatment of varicose veins (varix), he suggested: “when a varix is on the fore part of the leg and is very superficial or below the flesh, and the leg black, and seems to stand in need of having blood evacuated from it, such swellings are not by any means to be cut open, for generally large ulcers are the consequence of the incision, owing to the influx from the varix. But the varix itself is to be punctured in many places as circumstances may indicate” (as quoted by Adams in 1844). The limb was then elevated and compressed post procedure. Suggesting that phlebotomy and compression was performed for the treatment of
varicose veins over 2000 years ago. He also noted that varicose veins of the leg do not occur before puberty.

Figure 1.1 Votive table from the Asklepieion in Athens, illustrating a varicose vein. It was found on the site of the temple of the hero; Doctor Amynos. It is estimated to date from the end of the 4th century BC.

The Sushruta Samhita was the main textbook for Indian surgery around 200 BC. In it there are descriptions of the use of both maggots to clear away the necrotic tissue in ulcers and their treatment with inelastic Chinese cloth bandages. This may have generated compression, similar to what is practiced today.

During Roman times much was written about these conditions. Aurelius Cornelius Celsus appears to have described the first true Phlebectomy. He was a prominent Roman surgeon between 56 B.C and 40 A.D. In works describing his venous surgery it was written that: “the varicose veins were treated by exposure followed by avulsion with a blunt hook or by the touch of cautery”. It is also written that he described the ligation of veins that were bleeding, the double lamping and division of veins between ligatures. He also advised the use of plasters and linen roller bandages to pull leg ulcers together. Galen, (A.D. 130-
Aetius de Amida, a Byzantine physician, and Paulus Aegineta first described ligation of varicose veins and subsequent limb compression in the years 502 – 690 A.D.\(^3\)

The Humoral theories lead to a dark age in medical advancement, in particular the treatment of venous ulceration. It was believed that “bandaging varicose legs would reintroduce black bile into the circulation and lead to madness, and that they could be left alone as they were outside the main venous channels and thus the deleterious humors would be safely side tract unless pressed back by bandaging.\(^3\) During this period it was also widely believed that allowing an ulcer to heal would prevent the expulsion of evil humors. In fact if an ulcer healed it was advised that it should be deliberately opened once more.\(^1\)

Albucasis (A.D. 1013-1106) borrowed heavily from the writings of Aegineta and favored a form of ligation and stripping, or dissection of the superficial varicosities. Therefore it can be said that by the eleventh century the major principles of treatment of varicose veins were well outlined.\(^5\)

By the sixteenth century several risk factors for varicose veins had been elucidated. Marianus Snactus of Barletta (1555) believed the chief causes were childbearing and “standing too much before kings”. Ambroise Pare (1510-1590) described the ligation of varicose veins and the great saphenous vein in the thigh. His works on the healing of limb ulcerations are well documented. In particular while a prisoner after the siege of Hesdin in 1553 he attended Lord Vandeville. He treated his chronic ulcer, which he had had for 6 - 7 years, with debridment and regular bandaging from the foot to the knee. He also advises “…. do not forget to put little compress on the varicose vein to prevent the reflux of fluid to the said ulcer.” The ulceration was apparently healed in two weeks.\(^6\)
Leonardo da Vinci documented the anatomy of the veins of the peripheral system in 1452. However, Saloman Alberti published the first recorded drawing of a valve in a vein in 1585.

Figure 1.2 First recorded drawing of a valve in a vein by Saloman Alberti.¹

Hieronymus Fabricus subsequently re-described the valves in 1579 once he was able to demonstrate them at dissection. He also became aware that they prevented retrograde flow of blood.

In the seventeenth century, William Harvey’s discovery of circulation changed the face of ulcer management. He first demonstrated the understanding that venous valves ensured unidirectional flow. Consequently, the humoral theory lost favor as these valves would prevent the to-and-fro movement of the blood. Richard Wiseman, surgeon to Charles II described the association between varicose veins and ulceration. He appreciated the effect of venous dilation had on the valves and used the term “Varicose Ulcer”.¹ He also designed a leather lace-up stocking for the treatment of the lower limb venous disease.
During the eighteenth century varicose veins fell out of favor as a cause of ulceration, but were still treated with compression. There was a resurgence of interest in varicose veins in the nineteenth century, and the term “varicose ulcer”, seldom used since Wiseman’s day, became established once more. There were advances in both the understanding of venous disease and physiology. Briquet (1824) described the calf musculo-venous pump. While Brodie (1846) described the presence and prevention of Great saphenous vein reflux by digital pressure. Suggesting an awareness of incompetence of venous valves. Verneuil (1855) later went on to describe the presence of valves in the communicating veins between the deep and superficial system, even progressing to note that varicose veins may be due to deep venous incompetence.

In 1815 Hodgson believed ulcers that were situated near the ankle and intractable were due to “varicose conditions of the veins, for when the later is relieved, the ulcers are readily cured.” However, John Gay did not agree with this opinion and to support his argument he observed that the severity of a varicose vein does not correlate with the presence of ulceration. He observed that a severe varicosity may exist without ulceration. He was more in agreement with Spender (1868) that venous thrombosis of the deep venous system played an important part. They noted at autopsy that thrombosis may in fact damage
valves and Gay subsequently introduced the term “venous ulcer”. Gay was also known for his work describing incompetent perforating veins.¹, ⁶

Martin introduced compression bandages, made with the use of India rubber, in the 1870’s. He was also able to describe the manner in which they generated their compression.

For the first time, during the 1890’s, surgeons began describing surgery directed at correcting the underlying venous insufficiency present in patients with limb ulceration. Trendelenburg ligated the great saphenous vein in the upper third of the thigh, while Parona advocated ligation of the popliteal vein for the treatment of superficial varicosities.¹

The 1930’s were a prolific decade for research in to varicose veins with, John Homans deriving a classification of primary and secondary varicose veins based on the absence or presence of post thrombotic damage to the deep venous system. He also introduced the concept of a “post- phlebitic syndrome, in which the venous stasis caused by post thrombotic deep vein damage was the true causal agent for venous ulceration. While the work of Edwards and Edwards, in 1937, confirmed that venous thrombosis resulted in the destruction of valves but frequently was followed by recanalisation of the vessel. In 1931 Turner Warick described the “bleed back” test to assess the competency of communicating and perforator vein intra-operatively, which still has a place in venous surgery today. In 1938 both thrombectomy of deep venous thrombosis and subfascial ligation of the medial leg communicating veins were described. The first description of clinical phlebography, by Dos Santos, was also produced at this time.¹

Subsequently surgical venous bypass procedures for post thrombotic deep vein occlusion were described by Warren and Thayer in the 1950’s, with subsequent modifications in the 1970’s.⁶
So it can be seen through this brief account. The progression of our knowledge of venous disease has been slow, stalling on many occasions through our history. We are currently amidst a further resurgence in interest in venous disease in particular varicose veins and chronic venous insufficiency.

1.2 The epidemiology of varicose veins: The size of the problem

Since the first major epidemiologic study of varicose veins was performed in the United States National Health Survey of 1935 the epidemiology of varicose veins and chronic venous insufficiency (CVI) has been debated. This study found that a diagnosis of varicose veins was the seventh most common reason for medical referral a finding supported by recent a more French study. The cost to society is huge, exceeding 10 million Euros per million inhabitants per year in Belgium, France, Italy, and the United Kingdom for direct costs only.

Prevalence estimates for both CVI and varicose veins vary widely, CVI: in females <1% to 40% and from < 1% to 17 % in males. While the prevalence estimates for varicose veins are higher, <1% to 73% in females and 2% to 56% in males. The origin of this variation in reported ranges is probably multi-factorial:

1) **Method of assessment**: initial studies relied on self administered questionnaires thereby relying on self-diagnosis. Recent studies have used clinical, photographic and ultrasound examinations.

2) **Sample selection bias**: a large number of older studies were hospital or clinic based, while in the more recent population-based studies there are cultural, ethnicity and age
differences. For example: 0.1% in women living in rural New Guinea to 68% in female chemical workers in Basle, Switzerland, and studies investigated samples of clinic patients or specific occupational groups.\textsuperscript{10}

3) Temporal differences: Many studies were performed more than 20 – 30 years ago so possibly reflecting a difference in point prevalence or changing lifestyle factors.

4) Lack of clear definition: Until recently there have been no universally adopted definitions or consistency in application of the definitions used. Beaglehole\textsuperscript{11} quotes differing but overlapping descriptive definitions of varicose veins from four different studies ranging from ‘any prominent superficial vein in the lower extremity’ to ‘a vein which has permanently lost its valvular efficiency and as a result of continuous dilation under pressure, in the course of time becomes elongated, tortuous, pouched and thickened’.\textsuperscript{7, 12, 13}

1.3 Clinical picture

Varicose veins are not only associated with cosmetic disfigurement caused by their presence and ulceration. They are also reported to produce a variety of symptoms and complications, which can result in a reduced quality of life for patients with varicose veins and subsequent referrals to vascular services for possible treatment and advice. What follows is description of the clinical patterns associated with varicose veins and the relevant investigations and treatments of this condition.
1.3.1 Symptoms

The symptoms associated with varicose veins are variable and well documented.\textsuperscript{1,14,15}

These include:

1. Aching
2. Itching
3. Heaviness or tension
4. Swelling
5. Cramps

There is little evidence directly supporting their association with varicose veins as patients complain of a varying combination of these symptoms and removing them does not seem to ameliorate these symptoms. The Edinburgh vein study \textsuperscript{14} compared the prevalence of symptoms in men and women with and without varicose veins. All these symptoms increased with age and were significantly more common in women. In men there was no significant relation between trunk varices and any symptoms except itching. Even though there was a strong relationship between aching, heaviness, itching, and trunk varices in women, aching was present in 45\% of women with out varices and 63 \% of those with grade 2 and 3 varices. Consequently they report that not only do many asymptomatic patients have trunk varices on examination, but others experience a whole range of symptoms despite having little or no clinical evidence of venous disease.

Baker \textit{et al}\textsuperscript{15} also report that there is little evidence that operating on simple varicose veins significantly improves these symptoms.

Several surveys have reviewed the quality of life in patients with varicose veins. The results indicated that there was an impairment in their quality of life, but this was only
associated with underlying venous disease such as ulceration, rather than the varicose veins alone.\textsuperscript{16,17}

### 1.3.2 Complications

Recognized complications of varicose veins can be divided into 2 groups.

1) Venous hypertension which has been shown to cause:

- Venous ulceration
- Oedema
- Lipoderatosclerosis
- Skin pigmentation
- Atrophic blanche
- Varicose eczema

2) Those which occur less frequently and arise directly from varicose veins themselves:

- Superficial thrombophlebitis
- Haemorrhage

### 1.4 Investigations

Investigations of peripheral veins can be performed by invasive and non-invasive methods. Invasive methods include venography and venous pressure measurements, while the non-invasive methods are: Doppler ultrasound, air or strain gauge plethysmography and duplex scanning.
1.4.1 Plethysmography

Plethysmography is derived from the Greek word "plethysmo", to increase, and "graphos" to write. It is the recording of changes in the volume of a limb as blood moves in and out of it with each cardiac cycle.

1.4.2 Air plethysmography

This is based on volume changes detected by an air containing 14-in.-long polyvinyl chloride air chamber, which surrounds the entire limb from the knee to the ankle. The cuff is calibrated with the patient supine and inflated to a low pressure so that fluctuations of calf volume are reflected by pressure changes in the cuff. Once the cuff is calibrated venous incompetence is assessed by: standing the patient and allowing the cuff to fill in order to determine the venous volume and rate of venous inflow. VFT\textsubscript{90} is the time taken for 90\% filling of calf venous volume (VV). The venous filling index (VFI) is regarded as a index of the severity of venous reflux\textsuperscript{18} and is equal to: 90\% VV/ VFT\textsubscript{90}. Calf muscle pump function was measured by ejection volume (EV), ejection fraction (EF), residual volume (RV) and residual volume fraction (RVF). The EV is volume of blood which is ejected from the calf after one tiptoe exercise, while the EF = (EV/VV) x 100. It has been shown that after 10 tiptoe movements the venous compartments of the calf are nearly completely empty. The residual volume (RV) refers to the remaining volume in the calf after 10 tiptoes and the residual volume fraction (RVF) = (RV/VV) x100. RVF is considered to be analogous to ambulatory venous pressure. The severity of venous disease correlates with ejection and residual volume fractions.\textsuperscript{19} Patients with increased severity of
venous disease have been shown to have small ejection fractions and large residual volume fractions.

1.4.3 Hand-held Doppler (HHD)

HHD flow detection has become used increasingly during the last 15 years, as it is quick to perform and provides a more accurate assessment of reflux than clinical examination.\textsuperscript{20, 21}

The application of ultrasound techniques to the investigation of venous disease has had a significant impact. Doppler flow detection depends upon the principle that the frequency of a sound wave reflected from a moving object is changed in proportion to the speed of movement of the reflecting object. A crystal in the transducer receives the reflected ultrasound and converts the alteration in the frequency to an audible sound. Consequently the use of Doppler ultrasound also requires an understanding of venous haemodynamics. Unlike arterial blood flow, venous blood flow is irregular and significantly effected by respiration, abdominal pressure and skeletal muscle contraction.

1.4.3.1 Effects of respiration

Femoral vein blood flow at rest changes as intra-abdominal pressure varies with respiration. During inspiration IAP rises and the diaphragm contracts resulting in reduced flow, which increases again during expiration. The flow changes in upper limb veins are reversed as they depend on thoracic pressure changes. A deep breath or Valsalva manoeuvre (a deep breath followed by forced expiration against a closed glottis) causes much higher rise in IAP, which can stop or even reverse flow in peripheral veins. If the veins between the heart and the point of examination are patent then the blood flow will
stop in response to a Valsalva manoeuvre. Consequently, the absence of this response indicates an obstruction between the probe and the heart. If the valves within the vein being examined are incompetent a Valsalva manoeuvre will result in retrograde flow followed by a sharp increase in flow as the manoeuvre is stopped.

1.4.3.2 Effects of venous compression

Squeezing the calf or thigh or applying a tourniquet increases flow in the veins contained within the limb. In the presence of competent valves this flow is towards the heart. Therefore, manual compression of the calf augments the flow signal in the common femoral vein. Venous obstruction may reduce the flow response to such manoeuvres and is useful in diagnosing this problem. Compression of a vein down stream to the probe causes a response comparable to a Valsalva manoeuvre. There will be retrograde flow if the valves are incompetent, whereas as if they are competent then the flow will stop.

1.4.4 Duplex ultrasound

Duplex scanning is a combination of real-time B-mode (greyscale) Doppler and flow detection. The use of duplex ultrasound in the management of varicose veins is now common practice and its efficacy has been well reported.\textsuperscript{22-24} It has become regarded as the ‘gold standard” for the assessment of leg veins providing both anatomic and haemodynamic information that can be used in the treatment and investigation of varicose veins. In B mode scanning the ultrasound is passed across the area scanned and echoes are placed on the display in a position that corresponds to their anatomic position, thereby building up an image. Colour-flow ultrasound imaging superimposes a blood flow image on a standard
grey-scale. Even if a vessel is too small to be determined on the grey-scale image, colour flow Doppler highlights the vessel in vivid colour, so they may be easier to locate and follow. It also enables immediate interpretation of simple aspects of blood flow such as: presence; direction of flow and any localised areas of disturbance in flow. Using B-mode imaging individual veins can be identified and further investigated for reflux or stenosis with spectral or colour Doppler imaging. Valves may not be seen but reflux is and incompetence is detected by the presence of retrograde flow in response to down stream pressure, 95% of normal valves will close within 0.5 seconds. Therefore, retrograde flow of greater than this is said to be a sign of incompetence.25

1.4.5 Venography

It wasn’t until the 1980s that safety of venography had improved sufficiently to gain widespread use. However it was during the same period that the aforementioned non-invasive modalities also began to gain acceptance. Venography now only truly has a role in the management of venous disease where the findings of Duplex ultrasound are equivocal. Venography provides more accurate anatomical information than duplex ultrasound and as a consequence it has been suggested that it is indicated where reconstructive venous surgery is planned.6
1.5 Treatment options

Treatment of any condition should be aimed at reducing the symptoms and prevent the complications associated with that condition. In the case of varicose veins the current treatment options aim to prevent skin changes, oedema, superficial thrombophlebitis and external bleeding and to treat venous ulceration and minimize recurrence.

There are several treatment options for varicose veins, which include: Compression therapy, sclerotherapy and surgical treatment. The available surgical treatments include:

1) Vein ligation
2) Saphenous vein high ligation and stripping
3) Stab avulsion
4) Sclerotherapy
5) Endoluminal occlusion of the saphenous trunks
6) Vein valve transplant
7) Vein valve cuffing

It is not in the scope of this thesis to describe the technical details of each treatment option. What follows is a summary of the indications and efficacy of each surgical intervention.

1.5.1 Vein ligation

Trendelenburg first introduced great saphenous vein (GSV) ligation in 1891, while Homans described flush ligation with the femoral vein, to prevent reflux in any tributary
joining the femoral and GSV, in 1916. Flush ligation alone has a high recurrence rate of visible varicosities (39 – 83\%\textsuperscript{26-29}) and sapheno-femoral incompetence (46-71\%).\textsuperscript{27,30}

1.5.2 Saphenous vein high ligation and stripping

Most studies support stripping of the great saphenous vein because stripping has a lower 5 year recurrence (6 - 35\%\textsuperscript{26-28}) and higher patient satisfaction rates than high ligation alone. Clinical advantages after great saphenous vein stripping include disconnection of the mid-thigh perforating vein and reduced groin recurrence either through tributaries or neovascularization.\textsuperscript{26}

1.5.3 Stab avulsion

Stab avulsion, in which clusters of tortuous varicose veins that are not stripped are removed using a hook through a 3-5mm incision. It is usually used as an adjuvant treatment for varicose veins.

1.5.4 Sclerotherapy

Sclerotherapy has been available as an alternative to traditional surgery for a number of years. However, in 1974 Hobbs reported a randomized trial comparing the two and found that conventional sclerotherapy was inferior to surgery for truncal saphenous varicose veins. In recent time the sclerosant has been prepared as foam with a 20\% 5 year recurrence rate.\textsuperscript{31}
1.5.5 Endoluminal occlusion of the saphenous trunks

Modern methods of saphenous ablation include its exposure to radiofrequency energy generated by a high-frequency alternating current or an endovenous laser delivering light energy to the interior of the vessel. Both cause thermal damage of the vein wall, resulting in destruction of the intima and collagen denaturation of the media with eventual fibrotic occlusion of the vein. Both are limited by the fact that they only treat truncal varices in the thigh as in other areas the vessel is in close proximity to nerves. Both techniques are in their infancy, with limited reports. Of the series that have been reported radiofrequency ablation has a recurrence rate of 7% at 12 months and that of laser ablation is approximately 6.6% at 17 months.

1.5.6 Vein valve transplant

This procedure aims to restore competency to post-thrombotic veins (primarily involving the deep system). In which the valves have been destroyed or scarred by recanalisation. A native vein segment containing a competent valve, which is usually harvested from the axillary-brachial vein, is transplanted into the popliteal or the femoral vein. Results are variable, but in the majority of the reported series the success rate at two years is below 50%.

As can be seen the currently available options for the treatment of varicose veins still have high recurrence rates.
1.6 The anatomy of the veins of the lower limb

In this review aspects of the venous anatomy of the veins of the lower limb that are relevant to this thesis will be briefly discussed, these include:

1. The venous drainage of the lower limb.
2. Fascial layers of lower leg
3. The structure of the vessel wall
4. The valves of these veins

1.6.1 The venous drainage of the lower limb

The venous system of the lower limb consists of two channels separated by the deep fascia: one within the muscular system (the deep veins), and one superficial to it (the superficial veins). The superficial veins, the great and small saphenous veins and their tributaries, drain the skin and subcutaneous fat. While the deep veins, popliteal and femoral veins drain the muscles and structures deep to the deep fascia. The two systems are connected by perforating veins which permit unidirectional flow of blood from the superficial to the deep system.

The superficial veins lie in the subcutaneous fat where they are divided into three layers:

1. The subcuticular plexus consisting of thin walled venules.
2. Subcuticular tributaries of the saphenous trunks formed by the unison of the subcuticular venules.
3. The main trunks of the saphenous veins which lie on the deep fascia, except as it crosses the knee joint. The great saphenous vein (GSV) is the longest vein in the body its origin is at the medial end of the dorsal venous arch of the foot. It passes upwards, anterior to the medial malleolus, obliquely crossing the lower quarter of the medial surface of the tibia, enclosed in its own fascial sheath. From here it ascends towards the knee, posterior to the medial border of the tibia. At the level of the knee it lies close to the medial condyle of the femur. From this position it spirals forward round the medial convexity of the thigh and into the foramen ovale to join and empty in to the anteromedial side of the femoral vein.

The deep system below the knee forms the calf pump. It consists of the intramuscular soleal sinuses and gastrocnemius veins, and the intermuscular posterior, anterior tibial and peroneal veins. All these veins join to form the popliteal vein, which is the outflow tract of the calf pump.

1.6.2 The fascia of the lower limb

Both the superficial and deep fascia is important in the venous return of the lower limb. The superficial fascia has two layers, the superficial fatty layer and the dense fibrous deep layer. This deep layer extends up both the leg and thigh, covering the main saphenous trunks, but not their respective tributaries which lie superficial to it. It has been suggested that the function of this deep layer of the superficial fascia is to provide support to the saphenous veins, as they are less likely to become varicosed compared to their superficial
tributaries. The deep fascia plays an important role in the musculo-venous pump of the lower limb as it relatively inextensible and surrounds the muscles of the lower limb.

1.6.3 Venous wall structure

The structure of the venous system conforms to the general three-layered arrangement of adventitia, media and intima found elsewhere in the circulatory system.

1. Tunica adventitia: the outer supporting layer of the vessel. It consists of loose connective tissue and thick longitudinal collagen fibres. As the vessel size increases so does the elastic content and in larger vessels, such as the femoral vein, there are bundles of longitudinal smooth muscle in close apposition to the junction with the media. It also contains the vasa vasorum which supply the vessel with nutrition.

2. Tunica media: consists of collagen, elastin, which provide the passive tone of the vein wall, and smooth muscle fibres arranged circumferentially. The smooth muscle cells are arranged in close proximity to each other in regular whorls in a fine connective tissue matrix with minimal amounts of extracellular fibrous tissue. The smooth muscles of this layer mediate changes in the capacity of the venous system, and are influenced by autonomic nerves and circulating stimulants. This middle muscular layer varies between veins of different caliber. In the saphenous trunks the muscle fibres are well developed compared to their smaller tributaries, which as a consequence are at increased risk of varicosis.
3. Tunica intima: In saphenous and femoral trunks this innermost layer consists of endothelial cells supported by a thin basement membrane of connective tissue, which is absent in the venules and small veins. In medium superficial veins smooth muscle cells are present, arranged in a regular longitudinal pattern parallel to the intimal surface. It has a regular and smooth surface. The endothelium secretes factors VIII, prostacyclins and fibrinolytic activator. The normal ratio of intima to media is approximately 1:2.

1.6.4 Venous valves

Functioning valves dictate the direction of blood flow (William Harvey). Vein valves are bicuspid. The cusps of the superficial veins lie with their free edge parallel to the skin surface. The distribution of these valves is not even throughout the limb. As is shown in Table 1.1 the number of valves within vessels increase as you descend the limb. Valves have been found in veins as small 1 mm.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Number of valves and observed frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inferior vena cava</td>
<td>None</td>
</tr>
<tr>
<td>Common Iliac</td>
<td>None</td>
</tr>
<tr>
<td>External Iliac</td>
<td>1 valve in 22 – 33%</td>
</tr>
<tr>
<td>Common femoral</td>
<td>1 valve in 67 – 80 %</td>
</tr>
<tr>
<td>Superficial femoral</td>
<td>1 – 4 valves in 100%</td>
</tr>
<tr>
<td>Great saphenous</td>
<td>2 – 13 valves</td>
</tr>
<tr>
<td>Popliteal</td>
<td>1 in 100%</td>
</tr>
<tr>
<td>Short saphenous</td>
<td>1 - 12 valves</td>
</tr>
</tbody>
</table>
A diagrammatic representation of a venous valve can be seen in figure 1.4. Each cusp consists of a thin layer of collagen, containing a variable amount of collagen and an endothelial layer covering both its surfaces. Despite this they still are very strong with the valve cusps being more elastic and stronger than the vein wall. The valve sinus is wider than the vein above and the cusp below. The vein wall is also thicker at the base of the cusp due to an increased amount of muscle fibres in the media at that level. These muscle fibres extend further into the central portion of the cusp than at its edges. Immediately above the valve root in the sinus region there is a reduced amount of muscle in the media, and here the wall consists only of collagen and elastic fibres. Elastic fibres extend along the whole length of the cusp. Valve function is complex and it is still debated exactly how they close. It is thought to involve cusp movement and valve sinus distension. The valve cusp is much longer than the diameter of the lumen of the vessel and has an elliptical attachment to the vein wall. The cusps do not lie flat against the vein wall when the valve is open, but float in the blood stream parallel to the longitudinal axis of the vein. They fill and balloon out into the blood stream as reflux begins. As a consequence a considerable portion of the central surface of the upper part of the cusp presses against its counterpart of the opposing cusp. With further sinus distention the edges of the cusps tighten by separation of the commissures. If this does not occur and the valves edges remain loose there may be altered flow across the cusps and the valve can become incompetent.
1.7 Venous pressure and the peripheral venous pump

Normal venous function is to return blood from the peripheral capillary beds to the heart, while only allowing unidirectional flow which is ensured by functional valves. Flow within the limb venous system is created by a pressure gradient. This gradient is generated by a number of factors:

1. Gravitational force (hydrostatic pressure)
2. Muscular venous pump
3. Arterial inflow
4. Intra abdominal pressure
5. Venous tone

Hydrostatic pressure can be defined as: the pressure at a given depth in a static liquid as a result of the weight of the liquid acting on a unit area at that depth. At rest the normal venous pressure of the veins in the lower limb is dependant on position and is a reflection of this hydrostatic pressure. In the supine position venous pressures measured in
the foot are approximately 15mm Hg. Conversely, when standing, provided this column of blood is not interrupted at any point the pressures measured in the veins of the lower limb at rest will be equal to 15 mm Hg plus pressure exerted by the height of the column of blood between the foot and the right atrium.\textsuperscript{5, 37}

When a vein is relaxed it is collapsed flat. As it fills its shape alters from elliptical to finally become circular. This pressure-volume profile of veins is dependant on this change and the vessel wall compliance. Once the cross-section of the vein is circular, further increases in volume are associated with a disproportionate increase in pressure. In the erect position the superficial leg veins are full. Consequently any reflux through an incompetent vein causes significant increase in superficial venous pressure.

Blood from the lower limb is returned to the heart against gravity by a number of muscle pumps:

1. The planter or foot pump.
2. The calf pump.
3. The thigh pump.
4. The abdominal pump.

The musculo-venous pumps have one major function. They reduce the mean hydrostatic pressure in the lower limb below the knee through reduction of the volume of blood contained in the capacitance vessels of the lower limb during exercise. The calf muscle pump is a feature unique to humans and their erect posture.\textsuperscript{6} Important features of the calf pump include:
1. The deep fascia.

2. The large valveless venous sinusoids of the soleus and gastrocnemius muscles, the approximate volumes of these sinusoids are between 100 and 140 mls. The soleus sinus is U-shaped with both ends emptying towards the heart.

3. The direct perforating veins and their valves directing flow from the superficial to deep veins. The valves of the perforating veins prevent transmission of the high pressure, generated by the calf pump contractions (systole) to the superficial system. They also prevent any reflux during pump relaxation (diastole).

The energizing mechanism of the calf pumps is the contraction of the surrounding muscles, which develop pressures up to 250 mm Hg in the leg and 115 mm Hg in the thigh. This pressure is transmitted directly to the soleus sinusoids causing ejection of blood upwards into the popliteal vein. Pressures in the posterior tibial vein of healthy volunteers are approximately 140 mm Hg, but pressures within the sinusoids have yet to be measured.

During exercise this ejection of blood and subsequent diastole results in a reduction of the mean venous pressure in the foot from approximately 90 mm Hg to 25 mm Hg, while that in the posterior tibial vein can be reduced from 80 mm Hg to 40 mm Hg. On cessation of exercise the foot venous pressure takes approximately 25-35s to return to its high pre-exercise levels. Consequently, the most dramatic changes, which occur in lower limb venous pressure, take place with exercise, and these pressure profiles are represented in the diagram below.
As is described later loss of this function of the musculo-venous pump plays an important role in venous disease and the development of ambulatory venous hypertension.

In order to correctly treat a condition both the aetiology and pathophysiology of that condition must first be delineated and understood.

![Figure 1.5 Pressure profiles in the veins of the foot, calf, popliteal fossa and upper thigh at rest and on walking. The heavy black arrows indicate intramuscular pressures.](image)

### 1.8 Aetiology

The aetiology of varicose veins is controversial and still to be determined. A number of factors have been implicated suggesting a possible multifactorial origin. These factors can loosely be grouped into:

1. Risk factors.
2. Systemic factors.
3. Local or directly implicated deficiencies.
1.8.1 Risk Factors

Many cross-sectional epidemiological studies implicated the following variables as risk factors, for varicose veins, each offers information which influences our understanding of the aetiology and pathophysiology of varicosities.

They include:

- Age
- Family History/ Heredity
- Gender
- Pregnancy
- Ethnicity
- Body habitus
- Occupation

1.8.1.1 Age

The prevalence of varicose veins increasing with age is well documented. Both the Edinburgh Vein Study\textsuperscript{10} and Widmer\textsuperscript{41} demonstrated a near linear increase in prevalence of varicosities with age, ranging from 11.5\% in the 18- 24 year olds to 55.7 \% in the 55 – 64 year olds. Using photoplethysmography Schultz-Ehrenburg performed a prospective longitudinal study of 518 children of the same age group in 11 secondary schools of the town of Bochum, to investigate the early and preclinical stages of varicose veins. In the children aged between 10 and 12 years, isolated reflux was demonstrated at the saphenofemoral junction in 3.1\%, and discrete reticular varicose veins were found in 10.2\% while no varicose veins were yet visible. In the adolescents aged between 14 and 16 years,
the number of refluxes at the saphenofemoral junction had greatly increased (12.3%), and isolated varicose veins were visible in 3.7%. These findings suggest that there is a possible degenerative component to the development of varicose veins, though the onset of changes at such a young age raise the possibility of 2 separate disease processes at work.

1.8.1.2 Family History

As is demonstrated in table 1.2 a number of studies have documented a reported positive family history in patients with varicose veins when compared with those with out. Hirai et al report a study of 541 Japanese women. A positive family history was documented in 42% of females with varicose veins compared to only 14% of the varicosity-free subjects. It is difficult to assess the accuracy of these data as varicose veins are common and sufferers are likely to know of family members also affected by this condition, so biasing any questionnaire survey towards a positive association. None of the relatives were examined and the patients themselves reported the presence of a positive family history for varicose veins. Even with this in mind it is a consistent finding, with a reported family history of varicose veins increasing a persons likelihood of developing varicose veins as high as 21.5 fold.

<table>
<thead>
<tr>
<th>Study</th>
<th>Absent (%)</th>
<th>Present (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hirai et al (1990)</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>Scott et al (1995)</td>
<td>22.5</td>
<td>85</td>
</tr>
<tr>
<td>The Basle study</td>
<td>52</td>
<td>63</td>
</tr>
<tr>
<td>Lee et al (2003)</td>
<td>47.6</td>
<td>66.4</td>
</tr>
</tbody>
</table>
1.8.1.3 Genetics

Due to this possible association it has lead investigators to suggest a possible genetic basis for this disease. In 1969 Hauge and Gunderson tried to explain the wide variation in disease severity/phenotype, by hypothesizing a multi-factorial autosomal dominant inheritance of the disease. They proposed that varicogenesis was dependant on the expression of more than one autosomal dominant gene, and that clinical presentation and severity was dependant on the number of these genes inherited by the proband.\(^49\) Reidal et al\(^50\) examined patients with primary varices for 41 HLA antigens A, B, C, and their frequencies were compared to those in controls. They demonstrated a significant association between HLA-B7 and primary varicosities. This association was most pronounced in patients whose fathers had also suffered from primary varices. No relationship was found between HLA antigens and the sex of the patients or the age at the onset of varices.\(^50\)

Scappaticci\(^51\) performed cytogenetic investigations on primary cell cultures from varicose veins fragments of patients with supposed familial varicosities. This revealed the presence of metaphases with structural abnormalities, clonal trisomies of chromosomes 7, 12, and 18, and monosomy of chromosome 14, while the sporadic cases had no similar chromosome aberrations. These results suggest that karyotypic variations in familial varicose vein tissue cultures could in some way be associated with the genotypic constitution responsible for the familial type varicosities.\(^51\)

1.8.1.4 Gender and Pregnancy

With two major exceptions\(^10, 41\) most studies have reported a female preponderance toward developing varicose veins.\(^8, 9, 11, 40, 52\) With females also developing this condition at
an earlier age, with a 6:1 female to male in Israeli people aged 20-34 years old.\textsuperscript{52} It has been suggested that the greater incidence of varicose veins in women may be due to the effects of pregnancy on the venous system. This may be a cumulative effect as the frequency of varicose veins seems to be higher in multiparous women.\textsuperscript{53} The quoted incidence of varicose veins in pregnancy is between 11 and 20%. Dindelli and colleagues also noted that a family history and increasing age, but not the extent of weight gain were independent risk factors.\textsuperscript{53}

Several reasons have been suggested for the increased frequency of varicose veins during pregnancy:

1. In early pregnancy there is a significant increase in blood volume due to plasma expansion, 2/3 of which is contained within the venous system.\textsuperscript{6, 54, 55}
2. Increased intra-abdominal pressure during pregnancy.\textsuperscript{56}
3. Direct pressure of the of the gravid uterus on iliac veins, impairing venous return.\textsuperscript{56, 57}
4. Disordered calf pump function.\textsuperscript{58}
5. Hormonal influence (see System influences)

However, the above are theories with limited evidence to support them, for example the varicosities of pregnancy often develop in the first six weeks before the uterus is large enough to cause back pressure.

Of interest is the well documented apparent reversibility of varicosities occurring during pregnancy,\textsuperscript{59, 60} the cause of which is also debated. This occurrence had been thought to be secondary to incompetent valves becoming competent again postpartum and secondary to changes in levels circulating hormones or release of venous obstruction. More recent
studies have suggested that pregnancy possibly exacerbates rather than causes varicose veins. Using duplex ultrasound, Cordts\textsuperscript{60} demonstrated an increase in the diameter of GSV during pregnancy. These changes were greatest between weeks 32 - 34 and returned to normal postpartum. However neither study was able to demonstrate new reflux in limbs without preexisting venous disease.

1.8.1.5 Body habitus

There is conflicting evidence on the effects of body composition. Many studies have demonstrated a significant link between obesity in females and varicosities,\textsuperscript{41, 42, 52} but few have supported this observation in men.\textsuperscript{40, 42, 61} There is no evidence as to the nature of this relationship. Obesity has been shown to be associated with raised intra abdominal pressure.\textsuperscript{62, 63} Using duplex ultrasound it can be demonstrated that the raised intra abdominal pressure produced by a Valsalva manoeuvre or insufflation of gas during laparoscopic surgery\textsuperscript{64} is sufficient to stop and even reverse flow within the legs. Stepniakowski \textit{et al}\textsuperscript{65} also documented an association between obesity and reduced venous distensibility, which may in turn predispose to worsening valve dysfunction as the vessel may be unable to accommodate any the increases in pressure or volume. Whether obesity has a causative role or whether it increases the severity of the disease is uncertain. There is also a possibility that the observed relationship between obesity and development of varicose veins is explained by the confounding effect of parity, supported by the findings of Dindelli \textit{at el}.\textsuperscript{53}
1.8.2 Systemic Factors

1.8.2.1 Hormonal influence

The increased incidence of varicose veins in women and pregnancy, have led to the speculation that hormones play a role in the development and progression of varicose veins. Evidence supporting their role includes:

1. Estrogen has been shown to increase venous capacitance.⁹, ⁶⁶
2. Relaxin, secreted by the corpus luteum in early pregnancy, has been implicated as a potent vasodilator.⁶⁷
3. Increased progesterone is thought to weaken vessel walls.⁹, ⁶⁶
4. Increased expression of estrogen receptor β within hypertrophied circular muscle in varicose veins.⁶⁸
5. Serum estradiol values greater > 9.7 pg/ml in post menopausal women has been demonstrated to be associated with increased incidence of varicose veins.⁶⁹
6. Higher levels of estrogen receptors have been demonstrated in the nuclei of cells from the intima and media of varicosed segments compared with non-diseased segments of the same vein.⁷⁰

Several studies have also suggested that the observed association between obesity, female sex and varicosity may reflect the above hormonal impact on the disease. When the serum concentrations of sex hormones in white postmenopausal women were studied, the degree of obesity was a major determinant of estrone and estradiol. The estrone levels of
obese women were about 40% higher than the levels of non-obese women.\cite{Kaye2000} Kaye et al\cite{Kaye2000} assessed the associations of body mass and body fat distribution, with serum concentrations of sex hormones and sex hormone binding globulin in postmenopausal women. Body mass index (BMI) was significantly positively associated with both total and free estradiol. These results suggest that in obese postmenopausal women there are significantly higher concentrations of circulating estrogens. Therefore, the increasing endogenous estrogen coupled with increased central adiposity would expand the intravascular volume and subsequently impede peripheral venous return. Accounting for the increased incidence of varicose veins seen in this portion of the population.

1.8.2.2 Vasoactive agents

In patients, with a positive family history of varicosities, mast cell infiltration has been demonstrated to be a significant feature of their varicosed GSV compared to varicosed GSV of patients without a positive family history.\cite{Schuller-Petrovic2000} Schuller-Petrovic\cite{Schuller-Petrovic2000} postulated an imbalance between vasoconstrictive agents such as angiotensin-II and endothelin, and the vasodilatory agents: nitric oxide and prostacyclin in the pathogenesis of varicose veins. They showed that angiotensin-II levels were significantly lower while endothelin, bradykinin and c-AMP were unchanged between varicose veins and controls. Intracellular c-GMP/NO increased more in varicose veins in response to histamine/nitroprusside stimulation, though basal levels were similar. It was concluded that endothelial cells from diseased saphenous veins secrete less constrictor mediators than cells from healthy veins and that in diseased veins the nitric oxide/cyclic GMP system is up-regulated which may shift the balance of vasoactive factors towards vasodilatation and contribute to the development of primary varicosis\cite{Schuller-Petrovic2000}.
1.8.3 Local aetiological factors

There are three prevailing theories for the aetiological cause of primary varicose veins:

1) Valve incompetence.
2) Vessel wall defect.
3) Haemodynamic influences.

There is a body of evidence supporting each theory. The degree to which each of these purported aetiologic factors contributes to the development of varicose veins is difficult to ascertain, as the theories are based on observation made from patients with established disease. It is these observations that can be used to aide the characterization of an animal model for varicose veins. The strengths and weakness of each possible local aetiological local factor will not be discussed.

1.9 Current clinical definitions

In 1994, a new classification system was developed for the evaluation of the severity of venous disease and has been widely accepted in the clinical and scientific communities. The Clinical, Etiologic, Anatomic, Pathophysiologic (CEAP) classification includes not only the clinical symptoms of CVI, but also considers the etiology, anatomic distribution and position, and the pathogenic mechanism and produces a score based on severity of disease.9, 75 The CEAP system was developed to standardize evaluation for comparison of outcome across clinical studies.9
A recent revision of the *CEAP* classification\textsuperscript{76} recommended the following definition:

**Varicose vein**: subcutaneous dilated vein, 3 mm in diameter or larger measured in the upright position. This may involve the: saphenous veins, saphenous tributaries, or non-saphenous leg veins. Varicose veins are usually tortuous, but tubular saphenous veins which demonstrated reflux may be classified as varicose veins.

It is this definition, which will be adhered to when describing varicosities in this thesis.

1.10 **Histopathological changes seen in varicose veins**

The basic structure of vein walls and valves has been previously described (see section 1.63 & 1.64). The following changes are dependant on the severity and duration of the disease process.

1.10.1 **Macroscopic changes**

Phenotypic changes seen in varicosed veins include:

1. Elongated, tortuous and dilated veins.

2. Localized lateral blowouts/ focal varicosities.(figures 1.6 &1.7)

3. Both vessel wall hypertrophy and thinning.(figure 1.7)
Figure 1.6. Macroscopic specimen of a human focal varicosity. Note the lateral blow out of the vessel wall resulting in the focal varicosity and the variable wall thickness (as shown by transillumination). Scale bar equals 10mm.

1.10.2 Microscopic changes

Microscopically there are marked changes in the structure of the vessel wall, but even these changes are debated and they include:

1. Increased amount of fibrous tissue especially in the media.  
2. Disruption and separation of the regular circular pattern of the muscle bundles resulting in a disorganized muscle layer.  
3. Loss of muscle cells in areas of abnormal dilation. Typically the wall consists of thinned out collagen lined by endothelial cells and sub-endothelial tissue.  
4. Marked sub-intimal deposition of collagen.  
5. Hypertrophy of the intima, secondary to fibrous tissue infiltration, with reported reversal of the intima to media ratio 2:1.  
7. In the adventitia there is an increase in the number and size of capillaries and small arterioles.  
8. Reduced elastic and muscle tissue in media.  
9. Medial hypertrophy and atrophy.
10. The reported changes in extracellular matrix accumulation is more evident distally in the vessels.\textsuperscript{80}

The changes described are variable, between patients and within lengths of varicose vein. Woodside et al\textsuperscript{84} document its segmental nature, reporting the above changes but also noting the presence of areas of deceased cellular and matrix components.

\textbf{Figure 1.7. Human focal varicosity.} (magnification x12.5). Demonstrating areas of medial hypertrophy (M), intimal hyperplasia associated with medial atrophy (I) and the disrupted valve leaflet resulting in the unilateral focal varicosity (V).
Figure 1.8. Sections through the same superficial human varicosity (x72). A) Transverse section, demonstrating focal intimal hyperplasia(I) and medial hypertrophy(M) B) longitudinal section. Demonstrating the intimal hyperplasia(I) with disruption of the internal elastic lamina(IEL), and loss of the external elastic lamina (EEL).
Figure 1.9. Transverse section through varicose human saphenous vein. Demonstrating medial hypertrophy(M) with increased extracellular matrix (pink) deposition and separation of smooth muscle fibres and intimal (I) hyperplasia (magnification x64).

1.10.3 Changes in extracellular matrix

A number of abnormalities in the structural proteins of varicose veins have been demonstrated. These include:

1. A reduction in both collagen and elastin content of varicose veins.$^{85}$

2. In contrast Gandhi et al.$^{86}$ demonstrated a reduction in elastin content but an increase in collagen. This increased collagen content is also supported by Rose et al.$^{77}$ and Maurel.$^{87}$
1.10.4 Changes in valve structure

There have been remarkably few pathological studies of valves in venous disease, the reported findings include:

1. Decrease in the number of functioning valves.  
2. Valve cusp perforation.  
3. Cusp elongation and tearing.  
4. Valve disappearance.  
5. Dilatation of the valve annulus.  
6. Cusp hypertrophy.

1.11 Calf muscle pump dysfunction and venous hypertension

The function and nature of the calf muscle pump has been described previously (see section 1.7). Dysfunction of this pump is not unlike causes of heart failure. It may be the result of muscle weakness such as that which occurs with paraplegia, multiple sclerosis and muscle disorders. More frequently it is the result of venous reflux but can occur with other venous disorders such as deep system outflow obstruction or incompetence. Reflux is a retrograde flow within an incompetent vein connecting both poles of an ambulatory pressure gradient. This pressure gradient develops during the activity of the calf muscle pump between the veins of the thigh and leg. Therefore, in the standing position the hydrostatic pressure, which causes venous hypertension in the lower extremity, is equal in both the deep and superficial system and no reflux occurs in this situation. Arnoldi reported a pressure difference of 33 ± 11.8 mm Hg between the posterior tibial and the popiteal vein during ambulation. This ambulatory pressure gradient forces blood to
flow in the retrograde direction normally prevented by competent valves in the deep and superficial systems. During pump diastole an incompetent saphenous vein permits retrograde flow of between 300 and 600mL/min along its length. As a consequence in these patients the calf pump is like a heart with a normal stroke volume but increased preload because the reflux circuit partially or totally replaces the blood expelled from the deep veins with shunted reflux volume. Inhibiting the physiological reduction of the mean venous foot pressure following exercise, by only 40 -70 %, can also result in pressure swings about the mean venous pressure with little reduction in the mean foot venous pressure (figure 1.6). This situation is termed ambulatory venous hypertension. This high venous pressure and flow is transmitted to the venules draining the skin capillaries raising the interstitial tissue pressures in patients with varicose veins, and causing the microcirculatory changes seen in venous ulceration and lipodermatosclerosis.

Figure 1.10. Normal and abnormal foot venous pressure profiles. 6
1.12 A need for a model of varicose veins

Animal models have been of particular use in furthering the understanding of disease aetiology, pathogenesis and progression by recreating pathological conditions. The axiom “before testing a new treatment in man, test it first in animals if possible” has been part of drug development for the past 50 years or so. Animal models are often used to test the effectiveness of a drug or procedure before proceeding to clinical trials since they allow researchers to focus on particular pathological processes without the confounding effects of other medical conditions.\textsuperscript{94} This then increases the chances of identifying drugs that are sufficiently promising to justify the effort and expense of further clinical development. Animal models have also allowed experiments investigating therapeutic options, not possible in human subjects such as the evaluation of prosthetic heart valves and intra aortic balloon pumping devices in calves.\textsuperscript{95} Furthermore, there is no doubt that the ability to engineer the mouse genome has become an invaluable experimental tool for modeling genetic disorders, assigning functions to genes and evaluating drugs and toxins.\textsuperscript{96}

Varicose veins constitute a significant financial and social burden. Despite being well documented since Hippocrates, there is still a lack of understanding concerning both: the underlying pathogenesis and the rationale of treatments of varicose veins. The management of varicose veins is marked by confusion and debate, which could benefit from well-designed experiments in a suitable animal model. Especially, as the current available therapies for, the treatment of varicose veins are limited by their high recurrence rates.

The ideal animal model for superficial varicose veins would recreate the human condition and allow the investigator to enable understanding of the underlying disease process and options for treating it.
Any model should also be:

1) Simple.
2) Economical.
3) Reproducible.
4) Have an animal morbidity and mortality rate that is appropriate for the condition being reproduced.
5) Able to demonstrate physiological and histological changes seen in the human condition.
6) Allow ready evaluation of the disease and its intervention.

Specifically for varicose veins an ideal model should demonstrate:

1) Progressive development of varicose veins.
2) Progressive loss of venous valve competence.
3) Evidence of elements consistent with the human disease such as venous reflux and/ or impaired muscle pump function and venous hypertension.
4) Histological features comparable to those in the tissues of patients with the established disease.
5) Clinical signs of venous hypertension including skin changes and ulcer formation.

The lessons of comparative medicine are most useful when the model has sufficient similarities to suggest its relevance to the human disease. What follows is a more detailed description of those features that help define what constitutes varicose veins and what should be reproduced in a model.
1.13 Literature review of previously reported animal models of varicose veins

An extensive literature review was performed in order to identify all animal models previously used for the investigation of varicose veins and chronic venous insufficiency. Medline and PubMed were the two search engines employed. Key words searched were: animal model, canine model, venous hypertension, venous insufficiency, varicose veins, chronic venous hypertension, and experimental model. A number of animal models have been developed. The majority have been designed in order to generate venous pressures above those normally seen, and to investigate factors that influence the progression and severity of the condition. They can be categorized into four groups:

1) Occlusive models.
2) Venous valve incompetence models.
3) Arterio-venous fistula driven models.
4) Combined models.

1.13.1 Occlusive models

In this thesis occlusive models are defined as those which employ ligation or obstruction of proximal veins such as the femoral or iliac systems, to recreate either acute or chronic venous thrombosis/obstruction (CVO). The secondary goal of many of these models was the generation of raised venous pressures similar to those seen in the human condition. In 1965 Harris sought to mimic CVO by ligation of the common iliac vein in unselected
mongrel dogs. Despite his initial finding, that there was no subsequent venous distension or oedema associated with ligation, he persisted deciding to perform the ligation of the right common iliac vein at the same time as a right to left femoral–femoral vein bypass as he felt this simulated the clinical situation. This model failed to demonstrate any signs of venous hypertension or even its presence. In fact Harris\textsuperscript{98} documents that there was no stigmata of peripheral venous hypertension secondary to extensive collateralization.

Wright \textit{et al}\textsuperscript{99} while investigating the haemodynamics of venous repairs in an acute canine occlusive model noted that despite ongoing occlusion there was a significant fall within 30 minutes in initial venous hypertension generated by common femoral vein ligation. They did not, however, continue to monitor after this period. The same phenomenon was noted by Hobson \textit{et al}\textsuperscript{100}. Lalka \textit{et al}\textsuperscript{101} also employed iliofemoral occlusion to reproduce a hypertensive model, but they reported a mortality rate of 30\% at 24 hour. Stallworth\textsuperscript{102} used an acute canine occlusive model to study \textit{Phlegmasia Cerula Dolens}. This is a clinical picture of a painful, blue (cyanotic), swollen leg secondary to thrombosis of the external and common iliac veins. This may progress to small petechial lesions and venous gangrene.\textsuperscript{1} In this model a tourniquet, placed circumferentially around the left thigh, passing beneath and excluding the femoral artery and vein, was used to cause venous obstruction. Signs of \textit{Phlegmasia Cerula Dolens} developed within 60 minutes, however this model had a mortality rate of 33\% at 24 hours, and histopathological examination did not reveal the cause of death. All of the aforementioned models have employed a variety of mongrel dog.

Burnand’s\textsuperscript{103} initial attempts to create a canine hypertensive model by ligating and dividing the common femoral vein at the groin failed to generate venous hypertension, since the paw vein pressure remained normal, even during exercise. Unfortunately they do not
mention what these normal values were, or whether they were referring to normal ambulatory changes in pressure or absence of venous hypertension. They felt that this failure was due to rapid development of large collateral veins around the divided vein, which were demonstrated on phlebograms.

Despite the lack of success using ligation models, Lalka et al\textsuperscript{101} persevered creating a model of chronic venous hypertension by ligating both the external and internal iliac veins and the associated branches above the common femoral vein in the left hind limb of eight greyhounds. This model was used to assess the efficacy of a cross-femoral venous bypass augmented by an adjuvant arterio-venous fistula. The result was to produce a modest increase in venous hypertension at rest and persistent hypertension with ambulation. The resting pressures in the ligated limbs were: 12.5±1.9 mm Hg at two weeks and 10.0 ± 3.0 mm Hg at 15 weeks, compared to control values of 5.5 ± 2.1 mm Hg. After 300 seconds of ambulation the pressures in the ligated limb increased significantly to 18.2 ± 4.5 mm Hg compared to that in the controls, 5.7 ± 2.3 mm Hg. These animals were described to develop prominent superficial venous pattern on the ligated hind limb and abdominal wall. Unfortunately these changes were not quantified other than to demonstrate their presence with a single venogram. This model illustrates several benefits: larger animal (22-28kg); group of genetically similar animals compared to the mongrel population used in previous studies; no morbidity or mortality reported; the venous hypertension produced is still documented fifteen weeks into the study, both at rest and ambulation, which to date was the only model to generate sustained venous hypertension not at arterial levels. This was the first model to generate venous hypertension with reproducibility, acceptable morbidity and in an animal of sufficient size to enable the procedure to be performed with standard surgical techniques. However, this model does not produce any of the associated morbid
sequelae of CVI (i.e., no persistent oedema, no ulceration, and no other trophic skin changes). With the presence of effective collaterals one would not expect the oedema present at six weeks to persist. Consequently, though it allowed the efficacy of a revascularization procedure to relieve venous hypertension, it may not be an appropriate model for the assessment of the progression of superficial varicose veins or the pharmacological interventions directed at these processes.

It has been widely accepted that chronic venous hypertension is the pathological mechanism that results in the cutaneous stigmata of CVI, but there is still debate on the pathway that propagates these changes. The sole aim of the occlusive models discussed has been the study of revascularization techniques and investigating macrovascular characteristics of venous hypertension. In recent times the body of research, both human and animal has been directed at investigating the microvascular dysfunction that may mediate the cutaneous stigmata of CVI. Again occlusive models have been utilized but in this circumstance small animals have been the main focus.

Naschitz et al.\textsuperscript{104} designed a rodent occlusive model, ligating the common femoral and saphenous veins, with the contralateral limb as a control. They were trying to produce a model of lipodermatosclerosis. They postulated that the injury to subcutaneous tissues, compromised by chronic venous stasis, caused by application of lipases might replicate lipodermatosclerosis in man. Rats were sacrificed at three days and three and six weeks. At both three days and three weeks after ligation, the subcutaneous fascia of the operated limb was thickened and composed of disunited collagenous fibers, when compared with the fascia of the control limb. The fascial thickening was greatest at three weeks and the parafascial tissues appeared to be hypervascularised when compared to the control limb. All
these histological changes are suggestive of an inflammatory process that had resolved and were not present at six weeks.

It has been postulated that leukocytes play a causative role in the pathogenesis of chronic venous disease.\textsuperscript{105, 106} To determine if leukocyte tissue accumulation occurred primarily in response to venous pressure elevation or to the arterial flow reduction associated with the acute venous hypertension, Lalka et al.\textsuperscript{106} generated venous hypertension acutely in a rodent model through ligation of the inferior vena cava, common iliac and femoral veins. This model’s strengths are that it is reproducible and it demonstrated similar patterns of leukocyte infiltrations seen in human tissues affected by CVI. It also allows the body’s short-term response to sudden venous hypertension to be evaluated. However, it does have several limitations:

1) It is an acute model. The study period only lasted 135 minutes, where as varicose veins and CVI are both chronic diseases.

2) It generates gross sustained venous hypertension 2.5 – fold higher than normal venous limb pressures: normal pressures 9.91 ± 0.94 mmHg and post ligation 29.83 ± 2.46 mmHg.

3) Requires microsurgical techniques, with cannulation of carotid and epigastric vessels, and transperitoneal access to major vessels. Thereby becoming a complex and extensive procedure in comparison to others previously described.

4) It is a non survival model.
In subsequent studies they applied the same technique bilaterally to produce a chronic model for a seven-day period. Both models described by this group, though they generate venous hypertension they do so by preventing all venous drainage from the hind limbs. Neither model demonstrated either the micro-vascular or the macro-vascular remodeling that occurs in the human condition. Consequently it is difficult to argue their usefulness as a model for varicose veins.

It has been suggested that at a cellular level, recurrent bouts of ischaemia and reperfusion accompany the development of venous disease\textsuperscript{107} since they lead to an inflammatory cascade with cell activation and injury\textsuperscript{108} their impact may be enhanced by the elevation of venous pressure. Takase \textit{et al}\textsuperscript{108} used rat mesentery to design a study to examine the influence of high versus low micro-vascular pressure on the level of inflammation produced. Single unbranched venules with diameters ranging from 35 to 70 µm and lengths > 350 µm were occluded using a micropipette. This group used intravital fluorescence to directly visualize leukocyte infiltration and quantify tissue injury. The strengths of this model include: ability to quantify haemodynamics, cell death as well as leukocyte kinetics. Rapid and repeated measurements can be obtained. Again this model is limited by being an acute model of venous hypertension, so it has no long-term application. Only small amounts of tissue can be visualized due to limitations of trans-illumination. The tissue of the mesentery is unlike that of the lower limb effected by CVI so histological comparisons will be difficult.

All these models for venous occlusive disease have their strengths and limitations. Most are one dimensional, only designed to allow examination of individual facets of CVI at any one time, limiting their use. Many, in fact, failed to demonstrate sustained venous hypertension that was comparable to the human disease. The more recent rodent models
have been useful in confirming clinical suspicions about the role leukocytes play in the development of the cutaneous stigmata of CVI. However no occlusive model has shown to be adaptive enough to allow understanding of the macro-vascular changes occurring or assessment of appropriate interventions.

1.13.2 Valvular insufficiency models

Whether it is due to deep venous thrombosis with subsequent recanalisation or idiopathic in origin, venous valve incompetence has been found in up to 90% of patients with CVI. It has been postulated that this venous valve incompetence in the lower limb results in the venous hypertension found in CVI. Sheep and dogs have been used to create large animal model for venous valve insufficiency.

Mclachlin et al sought to develop a canine model of recanalised incompetent human superficial femoral vein. Using a needle driver they applied direct trauma to a collapsed segment of superficial femoral vein held between two clamps, the vein was allowed to fill and the lower clamp reapplied. The distended vein was again compressed between the jaws of the needle driver. A 48 hour period of venous stasis was then simulated by closing the skin over the vein. The recanalised vessels were then transplanted with vein segments with a competent valve to see if they would remain competent over time. They reported difficulty in producing a fixed venous thrombus in this model, but gave no figures on success rates. There was also no mention of changes in anatomical or physiological parameters, making it difficult to assess this models efficacy.

Waddell et al described a canine recanalisation model of valve destruction for experimental valve transplants. They used mechanical trauma, by clamping the femoral vein, to induce venous stasis, and a sclerosant (5% ethanolamine) to cause chemical
phlebitis. Within the sclerosant group less than 50% were usable due to erratic recanalisation, while in the traumatic group of the 83% that had stable usable recanalisation, there was a morbidity/mortality rate of 14%. There was also no haemodynamic evidence that venous hypertension was produced.

In 1988 Jessup\textsuperscript{113} used the fact that the valves of the external jugular vein (EJV) of crossbred merino sheep are frequently partially or completely incompetent. This was thought to be due to vessel dilation and subsequent leaflet separation. The study was principally designed to assess the efficacy of an implantable device to restore venous valve competence. The EJV was mobilized, and incompetent valves identified, and the device applied. The competencies of the valves were then reassessed 12 to 41 weeks later. This model has limited use beyond the assessment of such devices. This model is not comparable to the human pathology in a number of ways: it represents a normal variant with no adverse sequelae; there is no alteration in valve morphology associated with venous disease and no documented venous hypertension is generated.

A group involving Lalka and Dalsing\textsuperscript{114} has subsequently described a canine valvular insufficiency model. Greyhound dogs were used in the initial model described. They caused unilateral (left) hind limb valvular incompetence through the use of a valve cutting apparatus passed in a retrograde fashion though the iliac, femoral, and lateral saphenous veins.\textsuperscript{114} Pressure measurements were taken in the left lateral saphenous vein. These measurements were taken with the animal in the supine position, at 80 degrees of head elevation and pre and post muscle contraction induced by a peripheral nerve stimulator. Then repeated at fortnightly intervals for a 14-week period. In the experimental limb, in the supine position immediately after lysis the pressures were elevated to 14.7±1.74 mm Hg compared to 9.8 ± 1.38 mm Hg in the control limb. For the remainder of the
study period the pressure returned to the baseline, pre-lysis, levels for both the supine and elevated positions. They were also able to produce ambulatory venous hypertension, which had not previously been achieved, as valve lysis also caused an immediate elevation in peripheral venous pressure post muscle stimulation, 46.5± 1.46 mm Hg compared to 26.2 ± 1.45 mm Hg in the control. This elevated pressure persisted at approximately the same level until the fourteenth week of the study. This was a simple and reproducible model of the postphlebitic situation of valve destruction, having a low morbidity and mortality. In follow up studies in the long term, greater than six months, the model remained stable consistently producing venous hypertension. However, as with previous models, it does have its limitations as a model for varicose veins. It is a traumatic model, so there maybe resultant traumatic alterations to the vessel wall and endothelium. This may thereby limit its ability to assess histological changes within the vessel. It is a model in which the hypertension is generated acutely eliminating or altering the vessels’ and surrounding tissues natural response to the progressive development of hypertension. As the valve incompetence produced is acute and en masse, it would seem that the potential for vessel remodeling as seen in humans is limited. There is no mention of varicose veins being produced by this model or raised resting venous pressure. Dalsing also commented on this model’s deficiencies:

1. Small animal compared to humans and as a consequence there is only a short hydrostatic column of fluid present in its hind limb.
2. Solely a model superficial venous insufficiency rather than the combined deep and superficial process often seen in humans.
3. The muscle pump in the canine model is different to that of human leg.
1.13.3 Arteriovenous fistula (AVF) models

The last of the methods used to generate peripheral venous hypertension, is the creation of an arteriovenous fistula between the femoral artery and vein. The principle of this is the arterio-venous fistula shunts used to dialyze uraemic patients.

In 1966 Dart et al.\textsuperscript{115}, sought to investigate the acute haemodynamic effects of venous occlusion on femoral artery blood flow before and after the introduction of a femoral AVF in mongrel dogs weighing 12-25kg. After mobilization the femoral vessels were cannulated with a pressure probe and the femoral vein was occluded, proximal to the proposed AVF site, and readings taken. Subsequently, in the same animal, a 1.5 cm AVF was constructed between the common femoral artery and vein. Once stable the proximal fistula limb was temporally occluded and further measurements taken. Venous pressures were elevated by venous occlusion in the absence of an AVF from mean of 5 mm Hg to 35 mm Hg. Whilst in the presence of an AVF the mean venous pressures distal to the AVF was increased to 60 mm Hg before occlusion and 122 mm Hg with occlusion. The venous hypertension produced by the fistula in conjunction with the AVF was close to the arterial pressures measured (mean 131 mm Hg). This model was solely designed to assess the acute changes seen so they do not report any other data. It is also of insufficient length of time to allow any stigmata of varicose veins or CVI to develop. From this paper it is difficult to assess this models suitability for the study of varicose veins; however the magnitude of the rise in venous pressure would seem to preclude its use.

In 1982 in order to investigate the hypothesis that sustained venous hypertension during exercise produces an enlargement of the dermal capillary bed and subsequently
disturbs capillary function Burnand et al. created a model of unilateral venous hypertension. They did so using a 1 cm side-to-side AVF between the common femoral artery and femoral vein in the hind limb of a greyhound. To measure capillary permeability they implanted small Guyton capsules into the subcutaneous tissues of the thigh and mid-calf to observe the movement of three isotopically labeled substances from the blood to the capsule. This model succeeded in producing sustained venous hypertension three months after the creation of the AVF. The mean resting (standing) pressure in the control limb was 35.9 mm Hg ± 3, compared with 47.2 mm Hg ± 12 in the experimental limb. Along with ambulatory hypertension demonstrated by a significant reduction in the fall in paw pressure produced by exercise, 18.9 mm Hg ± 8 (52 %) in the control limb compared with 2.1 mm Hg ± 11 (4 %). Skin biopsies were taken at three months and it was found that the skin of the animals with demonstrated venous hypertension had an increased number of capillaries compared to the control limbs in the affected hind limb. With the exception of fibrinogen there was no statistical difference seen in the movements of the isotopes, when comparing the fistula and control limbs. The benefits of this model were its ability to create both resting and ambulatory hypertension in an animal large enough to perform surgical interventions on. The end organ changes developed associated with the hypertensive insult are in the tissues affected in the clinical condition. Its limitation include that even though it is a larger animal than the rodent it still is utilises relatively small animal which has no calf muscle pump. Moreover, it does not develop varicose veins but does produce the capillary changes seen in the clinical situation.
1.13.4 Combined models

Van Bemmelen et al. sought to characterize morphological changes of valves as they developed incompetence induced by a haemodynamic stress. The model they developed was a combination of a side-to-side AVF with venous outflow obstruction caused by proximal ligation of the femoral vein and the superficial epigastric vein in rats. There was a mortality of 25% at 36 hours due to cardiac failure, and two of the fistula failed, resulting in a 70% overall success rate. Corrosive resin casts of the venous vasculature were performed at intervals ranging from one to 120 days. Incompetent valves without visible structural defects were found within 24 hours.

After two months structural changes including elongation of valve cusps, with separation and leakage along their free border were demonstrated. At four months the valve areas were difficult to recognize. Despite phlebograms being performed prior to the casting there is no mention of venous dilation or elongation as is seen in the human condition. The combination of casting and phlebography allowed characterization of valves defects. A second advantage is that the small size of this model enables many subjects with progressive degrees of venous damage to be studied. Pressure profiles were not generated and the feasibility of doing so was not commented on, and also no histology of the venous wall performed. From this it is unclear whether the documented changes were due to venous hypertension or grossly increased velocities over the valves inconsistent with the human disease. Also, no varicose veins were produced in the rat AVF model.

In more recent times Bergan’s group has used this rat AVF model to further investigate venous hypertension and valve remodeling. Significantly they were able to measure pressures within the femoral vein 3 cm distal to the AVF, and blood back flow through the most proximal valve after division of the femoral vein distal to the fistula was
used as a measure of valve insufficiency. Along with the above physiological measurements they also examined valve morphology at: 1, 7, 21 and 42 days. They only report the mean pressure in the femoral vein distal to the fistula which was ten fold greater than in the control (96 ± 9 mm Hg vs 9 ± 9 mm Hg), and did not report how this changed over the study period. The reflux rate (ml/min) increased over the study period (2 ml/min at day 1 and 11 ml/min at day 42). The most dramatic change occurring between days 7 and 21, with a marked increase in reflux occurred from 3.8 to 9.2 ml/min. During the first postoperative week macroscopic observation of the terminal valve in the saphenous vein and of the valve just distal to the fistula in the femoral vein demonstrated bulging of the valve and dilation of the commissures. Ultimately the complete disappearance of the valves was observed in the 21-and 42-day groups. Microscopically the vein wall showed disappearance of the media associated with massive venous wall fibrosis, most prominent at 21 and 42 days. This model’s similarity to the human condition is still limited as previously mentioned. The rats did develop ipsilateral limb oedema and progressive gross morphological valve changes similar to those seen in saphenous veins removed at surgery. The increased pressures generated are significantly out of proportion and at near arterial levels, whether they are persistent or fluctuant it is not clear. Transmission of arterial pulsations to the venous wall is also described which may have had an impact on any histological or immunological data collected. This study did not report any mortality or morbidity rates, but as it is the same model used by van Bemmelen,\textsuperscript{91} a rate close to 25% may be expected. It is again an acute model and has not been demonstrated past 42 days. Moreover, it is restricted by the absence of varicose veins while its size may limit its uses in the assessment of surgical modifications.
Of the animals previously selected the canine has shown the most versatility. Being large enough to enable the variety of procedures to be performed in order to recreate different causes of venous hypertension and simulate ambulatory venous hypertension, which none of the small animal models were able to do. Canine models also appear able to be sustained over a longer period of time than rodent models. The actual model designs themselves have been limited. They each have only looked at isolated aspects of CVI and haven’t been a true reflection of the insidious onset of the condition. Despite this, insights into the pathogenesis of CVI have been gained through their use, particularly at a microvascular and immunological level.

An effective and simple animal model for CVI is still required. Particularly one in which varicose vein in conjunction with venous hypertension and some, if not all of the stigmata of CVI are recreated.

1.14 Development of the porcine model

While studying neointimal hyperplasia in arterial segments of common femoral AVF created in pig models we, at the Department of Surgery, University of Otago Medical School, observed that the pigs also developed progressive enlargement and tortuosity of the superficial veins over the medial aspect the thigh and groin. These changes in superficial veins developed into what appeared to be an extensive network of varicose veins. Interestingly, after a lag period of between six to eight weeks, a similar progressive venous alteration was observed in the superficial territories of the contralateral limb well away from the fistula. These venous alterations in the contralateral limb were less severe. No other phenotypic changes associated with varicose veins or CVI developed during the 14 week
study period. The formation of gross superficial varices makes this model novel compared with other animal models of venous disease describe previously in this chapter.

A technique of corrosive resin casts (as described in the methods chapter of this thesis) of the superficial venous system was used to investigate whether or not the gross venous alterations described above were also associated with morphological changes in the venous valves in these limbs. From valve casts of the superficial veins of the right (A-V fistula) limbs of these animals, the valve sinus height to vein diameter ratio was increased to 0.71±0.20 (n=104 valves), significantly greater than the control animals 0.54±0.08 (n=26 valves) (Mann-Whitney U test, p<0.0001). In the left (contralateral) limbs of the same animals, the ratio was 0.90±0.14 (n=121 valves). This was significantly different from both the control animals, and the right (A-V fistula) limbs of the surgical animals (Mann-Whitney U tests, p<0.0001 for each comparison).

The ipsilateral venous dilatations and associated valve damage may be directly due to immediate flow changes associated with the AVF. It is postulated that the delayed development of varicosities in the contralateral thigh allows time for the valves to remodel, coupled with the fact that they occur in the limb with out an AVF possibly driving the observed changes this may mean that the contralateral limb of these pigs could more closely mimic the progression of valve failure seen in humans with varicose veins.

Given these gross morphological observations this thesis was undertaken to determine the physiological and histological characteristics of this novel model. A comparison of these changes with those known to occur in human varicose veins will be made to determine the utility of this model for future varicose vein research.
Chapter 2: Materials and Methods

In this chapter the methods and materials utilized during the course of this thesis will be described.

2.1 Ethical Approval

Ethical approval to use live animals in this research project was given by the University of Otago Animal Ethics Committee, Dunedin campus.

2.2 Animals

Eleven female domesticated large white Duroc cross pigs, aged 13-14 weeks and weights 25.1-35 kg, were used in this research project (control pigs, n=2 surgery animals, n=9). These animals were supplied by a commercial pig breeder and subsequently housed in an open pasture with covered housing. Their diet consisted of water *ab libitum* and Reliance commercial stock pellet chow (15% crude protein, 4% crude fibre, 3% fats 1.5% calcium, 0.4% salt, and premixed vitamins and minerals).

On arrival at the research facility each animal underwent a comprehensive clinical examination by a qualified veterinarian. A nose ring and unique numbered ear tag was inserted under a general anesthetic, prior to any vascular surgical intervention.
2.3 Surgery

The first animal in this series was a control. This animal was used to assist in the characterisation the normal anatomy of pigs’ hind limb, the nominal saphenous vein and its relations and the sapheno-femoral junction. The remaining animals were treated in pairs and operated on sequentially. Each of the animals had a right common femoral side to side arteriovenous fistula (AVF) fashioned by a vascular surgeon. An appropriately qualified veterinary assistant anaesthetised each animal. The standard anaesthetic protocol described below was used throughout:

1. Premedication as an intramuscular injection (IM)
   - Ketamine - 10 mg/Kg.
   - Medetomidine - 0.1 mg/Kg.
   - Atropine – 0.05 mg/Kg.

2. Induction
   - 0.1 ml/1% of Lidocaine - subcutaneous injection for intravenous catheter placement in ear or leg.
   - Thiopentone - 5 -10 mg/Kg via intravenous line (IV).
   - Oxytetracycline – 400mg IM, prophylactic antibiotic.

3. Maintenance, (once intubated)
   - Halothane and oxygen 0.5 - 1%, inhaled as required.
   - 0.9% saline via IV line for maintenance fluid.
   - A Bain or Magill anaesthetic circuit was used.
2.4 Control procedure

A 13 week old animal weighing 25.1 kg was anaesthetised in accordance with above protocol. Full venous and duplex ultrasound assessments were then performed in order to characterise the venous system and the normal venous anatomy of the nominal sapheno-femoral junction in particular. Bilaterally a superficial tributary of the saphenous vein was cannulated with a 21 gauge luer. The animal was anti-coagulated with 1000IU intravenous heparin, injected via the ear vein cannula, to prevent thrombus formation and vasospasm. The animal was then euthanased with an overdosed of sodium pentobarbital (0.225ml/kg, IV). Death of the animal was confirmed by absence of a cardiac rhythm and no audible breath sounds.

The abdominal aorta and Inferior Vena Cava (IVC) were accessed through a midline laparotomy incision. Batson’s # 17 casting resin was perfused into the limb vasculature through the cannula previously inserted. As the resin reached the IVC, it was clamped in order to prevent resin escaping. The resin was left to cure over night.

2.5 Corrosion Resin Casting

2.5.1 Procedure

The aim of corrosion casting was to visualise the venous vasculature by producing a luminal mold. This was achieved by cannulating the distal ends of the right and left GSV (equivalent), and flushing these vessels with sufficient normal saline to remove any remaining blood clots. The vessels were then injected with sufficient resin to restore the vessels to their normal, in situ, calibre and shape.

The Batson’s #17 resin kit consists of a “base solution A” (a partially polymerised monomer), a catalyst, and a “promoter C” to allow curing at room temperature after
injection. Batson’s # 17 casting resin (Polysciences Inc.) is freshly mixed in a fume hood according to the manufacturer’s recommendations. The required pigment is added to the Base Solution A (blue for venous system, and red for arterial). In order to allow optimal control of the polymerisation the base solution A is then divided into two parts. The catalyst solution is the added to one part, while the Promoter C is added to the other and carefully mixed. The resin polymerisation requires all three components and therefore will not begin until the contents of the two beakers are mixed.

Immediately prior to injection these two solutions are mixed together and left to rest for approximately five minutes to allow any bubbles trapped with in the resin to rise to the surface. The working time is approximately 30 to 45 minutes, with the specimen becoming fully cured in 2 to 3 hours. Polymerisation is an exothermic reaction and consequently it is recommended that the specimen is kept in cold water or ice during the curing process.

Vascular casts from superficial tissue lack supporting structure and are thus potentially fragile, in particular at their narrowest points such as competent valves, which cause disruption of the resins continuity, especially in the control specimens. For this reason casts were filled in an anterograde position whenever possible to ensure that the valve cusps were pushed against the vein wall during the setting of the resin.

2.5.2 Cast examination

The vascular casts were initially examined over an x ray box as a light source. The venous valves were recognised by their characteristic appearance as seen in figure 2.1. When the proximal and distal segments of a venous valve were separated, the venous valve was recognised by either the proximal cast of venous valve sinus or distal “V-shape” cast of the lumen. The total number of valves was counted within each of the postulated GSV and
the luminal diameter of the associated vein was measured immediately proximal to the valve.

Figure 2.1 Vascular cast demonstrating characteristic structure of the A) vein valves (arrowheads), B) Magnified view of valve. The arrow in A indicates direction of blood flow. Both scale bars equal 3mm.

2.6 Anatomical dissection

The animal’s hind limb was transected at the level of the level of the lower lumbar vertebrae. It was placed in 10% formalin to preserve the tissue. The superficial venous system and associated structures were dissected out and documented photographically. Particular attention was paid to the nominal great saphenous vein and its junction with the femoral vein. Once the venous system was fully documented the limb was macerated in order to retrieve resin cast for closer examination.
2.7 **Tissue Maceration**

A standard tissue maceration protocol was used throughout. The tissue was rinsed through with H\textsubscript{2}O at 50 °C, and then placed in a plastic container. Sufficient 15% NaOH was freshly prepared to completely cover the tissue. The H\textsubscript{2}O was removed from the maceration container and replaced by the NaOH. The container was then sealed and placed in a 50 °C oven (time required dependant on volume of tissue). The NaOH was carefully removed and the tissue was rinsed through with warm water to remove any excess debris. The container was refilled with H\textsubscript{2}O and returned to the 50 °C oven. This process was repeated until all of the soft tissue was removed. Once maceration was completed, the resin cast was rinsed several times in warm H\textsubscript{2}O and placed in a clean dry container on filter paper to dry. The entire cast was then photographed with a 3.34 mega pixel digital camera.

2.8 **Arteriovenous fistula surgical procedure**

A standardised operative procedure was utilised for each surgical animal. Once anaesthetised and stabilised, in the supine position, the right groin (surgical site) was prepared with 70% alcohol and chlorhexidine. The animal and surgical site was draped with the usual aseptic surgical technique. Through a right oblique groin incision the nominal femoral artery and vein were dissected out and mobilised with preservation of all tributaries and branches, excepting a small anteromedial branch of the femoral artery, which is located proximal to the nominal sapheno-femoral junction (SFJ). Throughout the dissection these vessels were bathed in 1% lignocaine, in order to reduce vasospasm of said vessels. The femoral artery and vein were then controlled with silicone vascular loops (Surg-I-loop, Scanlan International, Saint Paul USA). An 8-12 mm incision was made in both the femoral vein and artery, the distal end of which was approximately 10 mm proximal to the SFJ and
incorporated the small antero-medial branch ligated beforehand. Both the femoral artery and vein were flushed with heparin (50 IU/ml) distally, to prevent any clot formation. An ‘H’ side-to-side AVf was then fashioned using a double armed 6-0 prolene (Davis & Geck) suture. (fig.2.1). Once haemostasis was secured, the wound was closed in layers, a continuous 3-0 monocryl for the fascia and continuous 3-0 monocryl subcuticular suture for the skin.

The patency of each fistula was confirmed immediately after surgery, either by using transcutaneous duplex ultrasound (7-12MHz) (ATL, Philips Medical Systems) or clinically through auscultation for an audible bruit or palpable thrill.

2.9 Recovery

Each pair of animals was recovered (indoors) from the surgical procedure for 2-5 days in a large animal facility. They were examined daily to assess the general animal health and to confirm normal healing of the surgical site, which was documented photographically on day five post operatively, before being relocated to a free-range pasture with covered shelter.

2.10 Post-surgery assessment

Post-operative assessment had three aims:

1. Assessment and documentation of macroscopic changes of venous vasculature.
2. Characterisation of the physiological changes that occur with the superficial venous system.
Figure 2.2 Surgical fashioning of a side to side femoral arteriovenous fistula. (A) Looped artery (blue) and vein (red), (B) vessels opened longitudinally prior to anastomosis, (C) completed anastomosis, notice the intact side branches of the femoral vein
2.11 Macroscopic changes

At weekly intervals post-surgery, each animal was examined for evidence of superficial varicose veins, their size and distribution and any related cutaneous stigmata of CVI on the skin overlying the inner and lateral thigh, groin, and abdominal wall. To facilitate this, conscious animals were restrained, and the upper body supported to bring the animal into a bipedal standing position. This allowed the areas of interest to be cleaned, examined, and digitally photographed.

This technique allowed assessment and documentation of any macroscopic changes that subsequently occurred and possible comparison between animals. The postural effect of lifting the animal to a near vertical position reduced venous return distending the superficial venous system of the groin, thigh, and abdomen. Also, the animals’ exceedingly vocal attempts to oppose this manoeuvre produced what appeared to be a valsalva-like response. This method of assessment produces a postural test of venous reflux, comparable to that used in an air plethysmography (APG) test performed on human chronic venous insufficiency patients.

2.12 Physiological characterisation

In order to perform physiological investigations animals were sedated and intubated at 6 and 12 weeks post-operatively.

This was performed in two stages once the position superficial varicosities had been marked on the skin:

1. Full venous ultrasound scans in order to characterise the venous system and any subsequent changes. Duplex ultrasound assessment of venous blood flow velocities, direction and where possible vessel diameters were performed at predetermined sites,
as illustrated in figure 2.2. The ultrasound results were analysed off-line using the HDI-lab software package in the Section of Surgery’s Vascular Assessment Laboratory.

2. Intravenous pressure measurements were then performed by inserting an 18 gauge intravenous cannulae into a superficial varicosity. The varicosity used was consistently seen in the area marked as “B” (Figure 2.3A) and was the largest. A Mikro-Tip® catheter pressure transducer (Millar Instruments, Inc) was then passed through the cannula into the varicosity. ECG leads were attached to the animals’ chest in order to assess the relationship of any pressure variations within the cardiac cycle. Both ECG and venous pressure were recorded simultaneously using the MacLab data acquisition system attached to a G3 PowerMac computer. At six weeks only the right side was cannulated, but at 12 weeks vessel “B” was cannulated bilaterally.

3. Pressure variation and venous reflux were assessed in the both supine position and with 45% head elevation (using a tilt table). They were also augmented using controlled posterior thigh compression, and regulated elevation of both intra-thoracic (gentle compression of the full anaesthetic airflow reservoir bag) and abdominal pressure.

2.13 Histological assessment

After the completion of the final physiological assessment, at between 12 – 16 weeks post fistula formation, the animals were anti-coagulated with 2000IU intravenous heparin, injected via the ear vein cannula. The animals were then euthanased with an overdose of sodium pentobarbital (0.225ml/kg, IV). Death of the animal was confirmed by absence of a cardiac rhythm and no audible breath sounds.
From the previously demarcated areas tissue was then excised *en masse*, so both the vessel and the overlying fascia and skin were removed and pinned to a board, epidermis down, ensuring that the underlying varicose vessels were retrieved undamaged and kept in an *in-situ* state. The tissue was then placed in 0.9% saline for transportation back to the vascular laboratory. The tissue was then examined macroscopically and any excess subcutaneous fat and tissue removed with care to ensure the vessels remained intact. The dissected tissue was subsequently placed in 10% formalin overnight, before being placed in 70% ethanol. After at least 24 hours in the 70% ethanol the vein samples were removed from the distal, mid and proximal saphenous vein and from superficial varicose tributaries at standard sites from both limbs. Histological specimens were embedded in paraffin and once set sections were cut on to microscope slides and stained with Verhoeff-Van Gieson preparation. They were then examined to assess connective tissue failure and wall thickness especially neointimal formation and medial remodeling.

The control animal was also physiologically assessed at the same time points as the animals with AV fistulae then euthanased and tissue recovered at the equivalent age to that of a sixteen-week post-operative AV fistula animal.

Histological findings were also compared with tissue retrieved from human patients with superficial varicosities and the one control animal.

### 2.14 Human Tissue

Human tissue used in this thesis to compare the histological features of clinically diseased tissue was received via the vascular surgeons in the Section of Surgery, Department of Medical and Surgical Sciences, Dunedin Public Hospital. The vessels were received within two hours of extraction from a living subject. Resin casts and histology was
prepared using similar methods to that for animal tissue described above. Consent for examination of this material was obtained from the patients by their Consultant Surgeon or member of the vascular research team.
Figure 2.3 Physiological examination. (A) Identification of the sapheno-superficial junction (↑) and the medial superficial vein territories (B) Ultrasound examination, (C) vein cannulation with pressure probe inserted (D) Mac-lab system for ECG and pressure monitoring.
Chapter 3: Results

3.1 Macroscopic anatomy

3.1.1 Superficial dissection

A superficial layer of dense connective tissue (superficial fascia) extended across the hind limb and was continuous with the superficial fascia of the abdominal wall above. Macroscopically there was no obvious variability in its thickness over its length. It was separated from the dermis by a variable amount of adipose tissue, within which were found superficial vessels. Beneath this layer is a second (deep) fascial layer that is in close apposition to its superficial relation and only separated from it by the main superficial venous drainage of the hind limb which ran between the two layers. Medially the deep fascia was thin, while laterally and around the leg it became macroscopically denser. The largest muscle on the medial side of the hind limb was the gracilis (figure 3.1).

3.1.2 Superficial vessels

The main superficial drainage of both the anteromedial and anterolateral aspects of the pig’s hind limb was a pair of veins accompanying, in close approximation to an, artery (figure 3.1) and their tributaries. In the literature these vessels have been referred to as the saphenous artery and vein. This nomenclature will be used for this thesis and the term: saphenous bundle will be used when referring to the veins and artery collectively. The saphenous veins are connected by bridging veins, which are unevenly distributed along their length (figure 3.2), suggesting that they may in fact be venae comitantes of the saphenous artery. In this dissection there were 13 in the medial saphenous vein and 14 in the lateral, up to the entry of the tributary which was only approximately 6 cm from the SFJ.
The saphenous veins originated anterior to the medial malleolus of the pig’s tibia, passing upwards and forwards along the medial border of the tibia to lie medial to the patella. The veins continued posterior-medially along the inside of the thigh. The saphenous bundle lies in a compartment, which could be a delineated using duplex ultrasound examination (Figure 3.3.B). This compartment appeared to be formed by the distinct separation of the deep and superficial fascia. The saphenous bundle lay on the deep fascia (in a deep plane of the hypodermis) and was also enclosed by a second distinct connective tissue layer formed by the saphenous fascia (figure 3.3. A.). The saphenous fascia was observed along the entire length of the saphenous bundle from its origin at the ‘ankle’ until it passes through the deep fascia between sartorius and gracilis muscles (figure 3.1).

Along their length the saphenous veins received tributaries draining the cutaneous regions of the leg and the anteromedial and anterolateral thigh. The saphenous fascia did not enclose these tributaries, except at their insertion to the saphenous veins. At the level of the patella, the laterally placed of the two veins (SVL) received the largest and most consistent of these tributaries (figure 3.1). It appeared as a confluence of cutaneous veins on the anterolateral aspect of the saphenous bundle, which drained the leg and the anterior and medial aspects of the thigh and may therefore correspond to an accessory lateral saphenous vein rather than the post axial vein of the calf (figure 3.4). The saphenous veins did not appear to receive any direct perforating vessels from the deep system along their length. However, duplex ultrasound examination did reveal that at least one of the superficial venous tributaries of the saphenous veins, vein C (described later), did receive perforating vessels from the deep system (figure 3.5).

Three superficial vessels were consistently observed in all the animals (Figure 3.6.A). It was along these vessels that the physiological, histological and duplex studies were
performed at standard points along their lengths given numerical references e.g.: B1-segment of vein closest to tributary and B3 furthest away.

The superficial veins A and B converged to form the major tributary described previously. Vein A received cutaneous veins draining the inferior portion of antero-lateral aspect of the pig hind limb (figure 3.7.A), while the superior portion drained into a large superficial flank vein that in turn communicated with the superficial epigastric vein of the pig.(figure 3.7.B). Vein B and its tributaries drained the medial aspect of the hind limb above the knee, while jointly veins B and C also appeared to drain the gluteal region. Veins B and C joined and passed superiorly to form the superficial epigastric vein above the inguinal ligament along the nipple line where they were joined by the larger flank vein. Below the inguinal ligament Veins A, B and C drained back towards the major tributary and saphenous vein, while above the inguinal ligament they drained superiorly into the superficial epigastric vein as did the flank vein.
Figure 3.1 Superficial dissection of the anteromedial aspect of the swine left hind limb demonstrating the saphenous bundle (SB). Note the paired saphenous veins (blue resin) of which one is usually larger than the other and saphenous artery (red resin). M indicates the medial aspect of pig hind limb where the tail and reflected skin flap can be seen. Laterally is the major tributary of the saphenous vein (T) formed by a confluence of superficial veins draining into the saphenous system.

Figure 3.2 Close up view of the saphenous bundle. Demonstrating the relationship of the saphenous vessels (with the artery seen as pink structure located between the two blue veins), and the presence of the bridging vessels.
**Figure 3.3 Saphenous fascia**

A) Resin cast demonstrating the saphenous bundle of the saphenous fascia (SF) of the saphenous bundle

B) Duplex ultrasound demonstrating: the medial (SVm) and lateral saphenous veins (SVL); artery (SA) and also the superficial fascia (SF) and the deep fascia (DF).

**Figure 3.4 Cutaneous skin flap with superficial facia removed.** Note the cutaneous veins and the confluence forming the largest tributary of the saphenous veins. It is these cutaneous vessels that become varicosed.

**Figure 3.5 Duplex ultrasound demonstrating a non-saphenous perforator to superficial vein C.** Note that vein C lies above superficial fascia.
Figure 3.6 Superficial tributaries of porcine saphenous vein. A) Photograph of hind limb demonstrating the positions of the three major superficial veins and the confluence of veins A and B. B) Low magnification image shows the extensive network of tortuous varicose veins in this retrograde filled corrosive resin venous cast mirroring that observed through the skin of the living animal (A). Note the dilated but straight saphenous veins (SV). C) Close up of tributary (T). D) Posterior view of the saphenous vein and tributary. Note how veins A and B converge to form the major tributary (white arrow) which is also dilated and tortuous, with a valve present where it joins the saphenous vein, while C drains into the saphenous vein (SV) more distally. Box in panel B indicates the region of the AVF.
Figure 3.7 Lateral and medial aspects of the pig’s hind limb. A) Demonstrates tributaries of vein A and the large flank vein (FV). B) Large flank vein (FV) and vein A. C) Superficial epigastric veins (E). Note how veins A and B pass up wards and converge to form the epigastric veins in the region of the inguinal ligament.
3.1.3 Saphenofemoral junction

Once the saphenous bundle pierced the deep fascia it passed obliquely backwards between the sartorius and gracilis muscles. Along this intermuscular course the saphenous veins were still within the saphenous fascia. The saphenous veins terminated on the anterolateral aspect of the femoral vein, inferior to the inguinal ligament (Figure 3.6). The femoral vein lay posterior to the artery at this point (figures 3.5 and 3.6) and the saphenous veins passed behind the lateral aspect of the femoral artery. On the right the lateral saphenous vein terminated more superiorly on the femoral vein than its medial counter part, but the reverse was observed on the left. On both sides a femoral valve was observed between the terminations of the saphenous veins. Within one centimetre of their termination, proximal to the last saphenous valve, each of the saphenous veins received a tributary that drained the deep structures of the thigh. In the region of the saphenofemoral junction the femoral vein received a number of deep tributaries on its medial and lateral sides (3.6.A). In this animal the superficial epigastric vein did not communicate with either the femoral or saphenous veins. As described above it was formed by a confluence of superficial veins B and C (figure 3.7.C).
3.2 Microscopic Anatomy

3.2.1 The saphenous bundle

As has been described previously the three vessels which constituted the saphenous bundle were surrounded by a well defined connective tissue fascia. Tributaries of the saphenous vein ran outside this sheath (figure 3.10.B). Closely-knit connective tissue sheets formed the fascia, which appeared to originate as fibrous strands from the superficial fascia above and deep fascia below (figure 3.10.A).

3.2.2 The Saphenous vein

The porcine saphenous vein appeared to be a muscular vein (figure 3.11), with the vein wall consisting of three distinct layers. An inner intimal layer which, in the control saphenous vein, consisted of a narrow layer of endothelium that lay on an elastic membrane (figure 3.11.B). The next layer was a well developed media, made up of closely arranged smooth muscle cells with interspersed elastic and collagen fibres. The smooth muscle fibres were arranged in a circular fashion and there did not appear to be any longitudinally arranged fibres. The outer most adventia layer consisted largely of fibroblasts, collagen, and elastic and smooth muscle fibres (figure 3.11.B).

3.2.3 Superficial veins

The superficial veins also had a three layered structure similar to that observed in the saphenous vein. The media of these vessels were less muscular than their saphenous counterparts.
Figure 3.8 A) Close up of left saphenous bundle (SB). B) A rotated close up view of right saphenous bundle passing between the reflected gracilis and sartorius muscles, note saphenous fascia (SF). C) Left SFJ note femoral vein (FV) lies medial and posterior to the artery (FA) and the saphenous vessels arise from the lateral aspects the respective femoral vessels. L denotes lateral aspect of hind limb. D) A-P view of the right saphenofemoral junction (SFJ) with femoral artery (FA) retracted medially away from the femoral vein; note the multiple tributaries of the femoral vein (FV) and an absence of a superficial epigastric vein joining the saphenous in both C and D.
Figure 3.9 Close up of SFJ. A) Medial view of resin cast of right SFJ. Note the femoral tributaries at the SFJ. B) Lateral view of a corrosion resin cast of the left SFJ demonstrating the anterolateral insertion of the saphenous veins into the femoral vein (SV) and the relationship of the femoral artery (FA) and vein (FV).
Figure 3.10 Saphenous bundle. A) Wax block cross section of thigh hypodermis and B) Microscopic sections of the same block demonstrating the relationship of both the superficial (SF) and deep fascia (DF) to the saphenous fascia (arrows) and also the saphenous compartment, SA, the saphenous artery, V the saphenous vein and * the tributary. The reference bar in A is 3 mm. Note how the saphenous fascia completely surrounds the saphenous bundle. Verhoeff’s elastic tissue stain with van Gieson’s counter stain.
Figure 3.11 Histology of porcine saphenous vein. A) Transverse section with three distinct layers (magnification x211). Note the large muscular component of the vein wall. B) Longitudinal section (magnification x239). Tunica intima (I), tunica media (M) and tunica adventia (A) are of uniform thickness. Note the well defined elastic tissue present in the adventia interspersed with smooth muscles cells and an outer layer of fibroblasts (FB) and collagen (pink regions) and the densely packed smooth muscle cell (blue grey pigmentation) with in the media. Verhoeff’s elastic tissue stain with van Giesons counter stain
Figure 3.12 Superficial vein B from right (A) and left (B) hind limbs of the control pig (magnification x77). Demonstrating the similar wall structure and vessel size. Verhoeff’s elastic tissue stain with van Giesons counter stain.
3.3 Animal demographics

Nine right femoral side to side arteriovenous fistulas (AVF) were created in ten female domesticated large white Duroc cross pigs, aged 13-14 weeks (control pig n=1, surgery animals, n=9). The surgically manipulated animals were examined in two groups. Group I pigs had AVF fashioned with an anastomosis of between 8-10mm in length and were studied between February – June 2006, and included the following pairs of pigs: AVF#1/control, AVF#2/AVF#3 and AVF#4/AVF#5. Group II had AVF with slightly larger anastomoses > 10mm and were studied between May – August 2006. Group II included: pigs AVF#6/AVF#7 and AVF#8/AVF#9. The respective weights of each group are shown in Table 3.1.

**Table 3.1.A** Weights of group I pigs during study period.

<table>
<thead>
<tr>
<th>Pigs</th>
<th>Weight (kg) and weeks post operation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre-op</td>
</tr>
<tr>
<td>Control</td>
<td>29</td>
</tr>
<tr>
<td>AVF#1</td>
<td>33.2</td>
</tr>
<tr>
<td>AVF#2</td>
<td>31</td>
</tr>
<tr>
<td>AVF#3</td>
<td>32</td>
</tr>
<tr>
<td>AVF#4 *</td>
<td>32</td>
</tr>
<tr>
<td>AVF#5</td>
<td>30</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>31.2±1.5</strong></td>
</tr>
</tbody>
</table>

**Table 3.1.B** Weights of group II pigs during study period.

<table>
<thead>
<tr>
<th>Pigs</th>
<th>Weight (kg) and weeks post operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-op</td>
</tr>
<tr>
<td>AVF#6</td>
<td>33</td>
</tr>
<tr>
<td>AVF#7</td>
<td>36</td>
</tr>
<tr>
<td>AVF#8</td>
<td>35</td>
</tr>
<tr>
<td>AVF#9</td>
<td>35</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>34.8±1.3</strong></td>
</tr>
</tbody>
</table>

* - not recorded as pig died
All the animals met the minimum operative weight of 30 kg. Between the two groups there was no significant difference in the operative or week 6 weights (p<0.4). At termination, however this difference reached statistically significance (p<0.03).

### 3.4 Complications

#### 3.4.1 Group I

At the pre-interventional health check two pigs (originally planned to be paired together) were noted to have systolic murmurs by the institutional veterinarian. Soon after, and prior to any manipulation, one pig collapsed and died. At postmortem it was diagnosed with infective endocarditis by a veterinary pathologist. It was decided that the surviving animal would not be subjected to surgery but was retained as an un-manipulated control.

Of the five animals with AVF fashioned in this group (AVF # 1-5), one did not survive to the end of the study period (between 13 – 16 weeks). Two AVF failed during the same period in pigs AVF #4 and AVF #5; these were detected on or before the six-week assessment. The fatality occurred in AVF #4, which also had a failed fistula. At 11 days it was noted that AVF #4 had forcibly extracted its nose ring and in the process severely injured its nasal septum. Under sedation both the fistula and the nasal septum were examined. At this time there was no audible bruit at the fistula site and a decision was made to euthanase the animal. AVF #3 was also examined at this time point and again the fistula was not functioning clinically. Of note, during the formation of the AVF in pig AVF #5, the initially large femoral vein under went considerable vasospasm despite the liberal use of lignocaine.
At the one-week check AVF #1 was observed to have developed a mass at the operation site (figure 3.13.A). This was observed for 3 days, but did not resolve. The differential diagnoses were: abscess, seroma, pseudo aneurysm or haematoma. On day 14 post operation the animal was sedated and the mass examined both clinically and using ultrasound (figure 3.13.C). As can be seen in figure 3.13.B there was an area of ulceration at the 8 o’clock position on the mass, but no erythema associated with the suture line or the skin overlying the mass. The ultrasound examination revealed a well-circumscribed complex cystic mass, with multiple areas of loculation but no obvious sedimentation suggestive of haematoma. A turbid white fluid was aspirated from the mass and sent for microbiology. Gram stain did not demonstrate any bacteria and the mass was diagnosed as a sterile abscess. No antibiotics were given and the abscess resolved with two weeks.

3.4.2 Group II

In the second group four fistulae (AVF# 6 - 9) were fashioned. One out of the four AVF failed in this group. AVF #8 was found moribund on the thirteenth day post operation. The right hind limb was noticeably larger than the left and discoloured. On post mortem dissection of the limb it was apparent that the AVF had ruptured and an extensive haematoma was present, which had tracked through out the limb. Cardiac examination was normal.

During creation of the fistula in pig AVF#7 it was noted that a dissection of the femoral vein occurred. At the six-week check it was noted that the AVF was not functioning and the direction of blood flow was towards the saphenofemoral junction in the opposite
direction to the flow in the saphenous artery (figure 3.14). There were no signs of obstruction with the superficial or deep venous systems.

Wound swellings similar to that found in AVF#1 occurred in all the animals post operatively. They were all to a lesser extent except in AVF#9 and they all resolved spontaneously without any intervention.

There were two postoperative deaths (pigs AVF#4 and AVF#8). A total of three fistulas failed, in pigs AVF#4, AVF#5 and AVF#7 resulting in a 33.3% failure rate.
Figure 3.13 Right groin mass 2 weeks post-operatively. A) Location of Mass  B) Close up of mass  C) USS image of mass.

Figure 3.14 Duplex USS of right saphenous bundle of a failed AVF 6 weeks post-operatively. Demonstrating different colour signals seen in the in the saphenous vein and artery, indicating normal opposing directional flow within these vessels.
3.5 Superficial varicose change

The macroscopic changes in the superficial vasculature were documented on a weekly basis. As the weight of the pig neared 90 kg, usually by approximately week 9, it became hazardous to the handlers to elevate them onto their hind limbs. There appeared to be two distinct periods of venous alteration. Gross changes in superficial veins of the right hind limb began to appear between weeks 1 and 2, post AVF formation. These changes consisted of increasing tortuosity and progressive enlargement of the superficial veins. These changes were established by weeks 3 - 6 (figure 3.15 and 3.16). Resulting in what appeared to be an extensive network of varicose veins. There was a degree of variability in the extent of the network produced and this appeared to be maximal at between six to eight weeks. A similar progressive venous alteration was also observed within the contralateral (left) limb. These changes developed following a delay of between three to five weeks and were established by week six. Figure 3.15 demonstrates the macroscopic changes seen in the hind limbs of the pigs from the original study mentioned in the introduction. As is seen in figures 3.15, 3.17 and 3.18, the venous alterations in the left limb were less pronounced compared to the right limb and in group II.

At termination it was noted that in each group there was an inflammatory response affecting the dermis overlying the medial aspect of the hind limb (figure 3.16.C, 3.17.D and H), this was a diffuse process consisting of erythema and macular elevations. Both limbs appeared to be equally effected with no associated ulceration or signs of infection.
Figure 3.15 Time series of the development of varicose veins in the hind limb of pig with an A-V fistula from pilot study. A - D. (Right limb), E – F (Left limb) at: (A) one, (B,E) three, (C,F) six and (D,E) ten weeks post-operative respectively.
Figure 3.16 Time series of the hind limb of control (non-manipulated) pig. (A) one, (B) six and (C) sixteen weeks.
Figure 3.17 Time series of the development of varicose veins in the hind limb of a pig with an A-V fistula from group I. A - D. Right limb, E – H Left limb at: (A, E) one, (B,F) three, (C,G) six and (D,H) ten weeks post-operative respectively.
Figure 3.18 Time series of the development of varicose veins in the hind limb of a pig with an A-V fistula from group II. A – D Right limb, E – H Left limb at (A, E) one, (B, F) three, (C, G) six and (D, H) ten weeks post-operative respectively.
3.6 Physiology: pressure profiles and duplex velocities

3.6.1 Pressure profile

Pressure in the superficial venous territory was recorded in vein B as this was the most consistent in position, size and easiest to cannulate. In the supine position the pressure profiles of the control pig and AVF#7 (failed AVF, normal duplex flow) demonstrated no variation with the cardiac cycle, and were in the range of 1 to 5 mmHg. The greatest change in pressure was seen in response to abdominal augmentation. Calf compression had minimal effect on the recorded superficial pressure increasing it from 3 to 5 mmHg. As is demonstrated in figures 3.19 and 3.20 the magnitude of pressure elevation was dependent on the amount of force applied which may have led to inconsistencies in the values recorded.

The profiles of the pigs with AVF differed from those described above. The pressures recorded were higher at both week 6 (23±11.4 mmHg) and at termination (20±8.3 mmHg) than that of the control (4.5± 3.5 mmHg) (table 3.2). In all but one pig with an AVF there was variation in pressure, associated with the cardiac cycle. As is illustrated in figure 3.21 the pressure probe was able to accurately demonstrate the appropriate variations of pressure in an arterial system. It can also be seen that the pressures recorded in the superficial veins are not of the same magnitude as those of the arterial system nor do they demonstrate the same degree of variation. In group I, where present, the degree of pulsatility was greatest at six weeks at between 2.0 – 4.5 mm Hg about the mean, while at termination it was only 1.5 – 2 mm Hg about the mean. In group II the magnitude of pulsatilaty remained unchanged over the study period at approximately 0.5 – 1.5 mm Hg about the mean. As is seen in table 3.3 abdominal augmentation produced a similar increase in pressure in vein B at weeks 6.
(10± 1.4 mm Hg) and at termination (9± 6.0 mm Hg) in the pigs with functional AVF. In the animals in which calf augmentation was achieved a greater response was noted in the pigs with functioning AVF.

**Table 3.2. Pressures in right superficial vein B (ipsilateral).**

<table>
<thead>
<tr>
<th>Pig</th>
<th>Week 6 (mmHg)</th>
<th>Termination (week 12 – 16)(mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>AVF#5*</td>
<td>7.5±0.5</td>
<td>-</td>
</tr>
<tr>
<td>AVF#7**</td>
<td>1.5±0.5</td>
<td>1</td>
</tr>
<tr>
<td>Mean</td>
<td>4.5±3.5</td>
<td>2</td>
</tr>
<tr>
<td>AVF#1</td>
<td>18±2.0</td>
<td>22</td>
</tr>
<tr>
<td>AVF#2</td>
<td>18</td>
<td>20±2.5</td>
</tr>
<tr>
<td>AVF#3</td>
<td>42.5±4.5</td>
<td>28.5±1.5</td>
</tr>
<tr>
<td>AVF#6</td>
<td>15.5±0.5</td>
<td>17±1.0</td>
</tr>
<tr>
<td>AVF#9</td>
<td>18.5±1.5</td>
<td>22±1</td>
</tr>
<tr>
<td>Mean</td>
<td>23±11.4</td>
<td>20±8.3</td>
</tr>
</tbody>
</table>

* Occluded fistula
**Failed fistula with normal venous flows on duplex

**Table 3.3. Variation in pressures in superficial right vein B with abdominal augmentation.**

<table>
<thead>
<tr>
<th>Pig</th>
<th>Week 6 (mmHg)</th>
<th>Termination (week 12 – 16)(mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>4 – 23</td>
</tr>
<tr>
<td>AVF#5*</td>
<td>7.5±0.5</td>
<td>-</td>
</tr>
<tr>
<td>AVF#7**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AVF#1</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>AVF#2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>AVF#3</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>AVF#6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>AVF#9</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>10±1.4</td>
<td>9±6.0</td>
</tr>
</tbody>
</table>

* Occluded fistula
**Failed fistula with normal venous flows on duplex
Figure 3.19 Pressure profiles of control animal demonstrating calf augmentation. A) Soft compression. B) Hard compression. Note the differing magnitude of the response, approximately 1 mm Hg with soft compression and 3 mm Hg with hard compression.
Figure 3.20 Pressure profile of control animal demonstrating abdominal augmentation. A) Soft compression. B) Hard compression. Note the difference in magnitude, only 1 mm Hg with minimal compression but nearly 20 mm Hg with harder compression.
Figure 3.21 Pressure profile in (A) distal saphenous artery and (B) superficial vein B. Note the differences in the scales on the pressure traces and that the pressure variations within superficial vein B are not of a similar magnitude to that seen in the arterial system.
Figure 3.22 Example of pressure profiles from a pig with a functioning AVF. A) Week 6 and B) Week 13 (termination). Note minimal change in profile pattern and magnitude of the pressure variation seen over study period, indicating that the model is stable and consistent when functioning.
3.6.2 Duplex velocities.

Duplex examination of the superficial veins was limited in several cases by the small caliber of the vessels, their close approximation to the skin with minimal supporting adipose tissue and velocities too small to detect. Consequently, in the majority of animals no velocities were recorded for the contra lateral limb or in the control at 6 weeks. As was observed with the pressure profiles, the flow velocities also demonstrated a degree of pulsatility, which reduced in magnitude with increasing distance from the AVF (figure 3.23). In the majority of animals the velocities recorded in the superficial veins dropped off with increasing distance from the AVF. Over the study period the velocities both increased and decreased. (table 3.4). At termination the mean peak flow velocity recorded in the superficial varicosities was $29.3 \pm 15.7 \text{cm/s}$ while in the control and failed fistula it was $7.6\pm5.2 \text{cm/s}$.

The previously described calf and abdominal augmentation procedures were also used to assess changes in flow within the veins of the hind limb at the same standard points. Abdominal augmentation reduced flow in all the superficial vessels examined with a subsequent rebound in velocity to pre-insult levels once the pressure was released (figure 3.24 C and D), while the opposite was observed with calf augmentation (figure 3.24 A and B). In both instances the level of response was governed by the force applied, i.e.: increased force = increased response as can be seen in figure 3.19. There was difficulty in standardizing the amount of force used in each instance.
Figure 3.23 Duplex Ultrasound of A) Right proximal saphenous vein, B) Right distal saphenous vein, and C) Right vein B. Note measurements of velocity are in bottom left corner of each frame and there is a reduction in magnitude of pulsations with distance from AVF.
Figure 3 24 Duplex ultrasounds demonstrating responses to augmentation procedures in pigs with functioning AVF. (A) and (B) Calf augmentations with B being the harder of the two. Note the rise in velocity and different magnitude of response. (C) and (D) Abdominal augmentation with D being the harder of the two. Note fall in pressure and again the variation in magnitude of response.
Table 3.4 Mean peak duplex flow velocities, at 6 weeks and termination (cm/s).

<table>
<thead>
<tr>
<th>Pig</th>
<th>A</th>
<th>Vein</th>
<th>B</th>
<th>Vein</th>
<th>C</th>
<th>Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 6</td>
<td>Termination</td>
<td>Week 6</td>
<td>Termination</td>
<td>Week 6</td>
<td>Termination</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>5.2±2.0</td>
<td>-</td>
<td>4.8±1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AVF#5*</td>
<td>8.3±6.6</td>
<td>18.9±3</td>
<td>3.8±0.5</td>
<td>4.2±1.4</td>
<td>3.5</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>AVF#7**</td>
<td>5.7±0.1</td>
<td>13.5±9.0</td>
<td>10.1±4.8</td>
<td>6.5±2.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>7.0±4.1</td>
<td>9.9±5.9</td>
<td>6.6±4.5</td>
<td>5.9±2.7</td>
<td>-</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>AVF#1</td>
<td>13.7±0.4</td>
<td>53.2±8.3</td>
<td>31.9±9.2</td>
<td>38.8±3.2</td>
<td>28.4±20.5</td>
<td>19.3±1.8</td>
</tr>
<tr>
<td>AVF#2</td>
<td>60.0±12.5</td>
<td>10.6±6.4</td>
<td>21.4±6.7</td>
<td>21.8±4.9</td>
<td>39.1±12.0</td>
<td>24.8±6.0</td>
</tr>
<tr>
<td>AVF#3</td>
<td>5.4±1.1</td>
<td>10.8±1.9</td>
<td>8.11±2.2</td>
<td>22.2±4.7</td>
<td>17.7±5.1</td>
<td>35.7±7.5</td>
</tr>
<tr>
<td>AVF#6</td>
<td>60.0±13.3</td>
<td>48.4±9.9</td>
<td>42.0±26.8</td>
<td>24.2±13.1</td>
<td>29.0±8.1</td>
<td>23.6±10.9</td>
</tr>
<tr>
<td>AVF#9</td>
<td>20.1±23.7</td>
<td>36.9±21.2</td>
<td>22.4±17.6</td>
<td>22.5±9.8</td>
<td>21.7±7.5</td>
<td>28.3±13.3</td>
</tr>
</tbody>
</table>

mean ± 1 SD
* Occluded fistula
**Failed fistula with normal venous flows on duplex
Table 3.5 Mean peak duplex velocities in all superficial varicosities, at termination (cm/s).

<table>
<thead>
<tr>
<th>Pig</th>
<th>Termination (week 12 – 16)(cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6±1.6</td>
</tr>
<tr>
<td>AVF#5*</td>
<td>7.4±4.6</td>
</tr>
<tr>
<td>AVF#7**</td>
<td>10.6±6.9</td>
</tr>
<tr>
<td>Mean</td>
<td>7.6±5.2</td>
</tr>
<tr>
<td>AVF#1</td>
<td>22.8 ± 11.5</td>
</tr>
<tr>
<td>AVF#2</td>
<td>40.4 ± 13.7</td>
</tr>
<tr>
<td>AVF#3</td>
<td>34.8 ± 16.1</td>
</tr>
<tr>
<td>AVF#6</td>
<td>21.4 ± 12.3</td>
</tr>
<tr>
<td>AVF#9</td>
<td>30.1 ± 14.0</td>
</tr>
<tr>
<td>Mean</td>
<td>29.3 ± 15.7</td>
</tr>
</tbody>
</table>

mean ± 1 SD
* Occluded fistula
**Failed fistula with normal venous flows on duplex

3.7 Histology of porcine and human varicosed veins

3.7.1 Porcine varicose vein histology

Irrespective of the functional status of the fistula, vessel wall morphology of the superficial vessels (A – C) was examined after post mortem in all the animals, except AVF#4 that had been euthanased, after extracting its nose ring. What follows is a summary of the histological findings, in particular changes in wall morphology and valves where encountered on the ipsilateral (AVF) side, as the normal (control animal) histology has previously been described.

Overall there was both an intra-vessel (figures 3.25 and 3.32) and inter-animal heterogeneity to the vessel walls changes seen in the veins sampled. With the changes noted being more pronounced on the right (AVF) limb compared to the left (contra lateral) (figures 3.34 and 3.35).
Finding which were consistently seen included:

1. Tortuous and elongated veins (figure 3.26).

2. Intimal thickening/neointimal formation. The extent of neointimal formation was variable, even within the same transverse section of vein (figures 3.27 and 3.32).

3. Both the medial and adventitial elastic tissue, which in the control veins had present as continuous distinct layers, appeared as discontinuous fragments (figures 3.27 and 3.31).

4. Areas of greatest intimal thickness were associated with both disruption of the internal elastic lamina and a reduction in the elastic content of the adventia (figure 3.27).

5. Medial hyperplasia/hypertrophy (figures 3.27 to 3.29 and 3.31).

Other features that were less consistently observed include:

1. Mural hypertrophy, i.e.: increase in vessel wall thickness as a result of both neointimal formation and medial hypertrophy. (figures 3.27 to 3.29 and 3.31)

2. Medial and mural atrophy, i.e.: vessel wall thinning. (figures 3.25.A and 3.30).

3. Uniform medial and intimal hyperplasia was observed in regions where the vein was relatively straight. (figure 3.31).

This variability was generated by a combination of medial hypertrophy or atrophy and intima hyperplasia that were present within the same vessel wall.
In a large number of specimens the intimal hyperplasia consisted of diffuse infiltrate of collagen fibres and smooth muscle cells, which were not organized in a regular pattern and showed no associated change in depth of the endothelial layer lining it (figure 3.29).

A variety of valvular defects were also observed with the specimens ranging from:


2. Valve cusp tearing (figure 3.33. B).


In the original study it had been noted that the contra-lateral limb developed what also appeared to be varicosities. On histological examination, veins from the contra lateral limb demonstrated normal histological appearances compared to the right. They were of smaller caliber than the equivalent vessels from the AVF limb with minimal disruption of vessel wall architecture (figures 3.34 – 3.35).
Figure 3.25 Comparison of varicosed and control superficial veins

A) Transverse section of right superficial vein C at 16 weeks (magnification x 23). Note the variable vein wall thickness: normal wall superiorly (between 10 and 2 o’clock), associated with medial and intimal hyperplasia inferiorly between 3 and 5o’clock and 7-10 o’clock in the specimen.

B) Superficial vein B from right hind limb of the control pig (magnification x 77). Note the difference in vessel and lumen size, and the homogeneous appearance of the control vein wall compared with the AVF vessel. Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.26 Longitudinal section through a porcine right superficial varicosity. The tortuosity of the vessel is clearly demonstrated. This histological section was orientated parallel to the epidermis. Verhoeff’s elastic tissue stain with van Giesons counter stain.

Figure 3.27 Transverse section of right flank veins of AVF# 7 (magnification x76). The double arrow demonstrates the neointima (I) of variable thickness, with associated loss of internal elastic lamina (IEL) and medial elastic tissue (MET.) Note the reduction in the adventitial elastic tissue beneath the area of greatest intima/medial alteration. Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.28 **Transverse section through right vein B (x42).** Demonstrating medial (M) hypertrophy, consisting of densely packed smooth muscle cells, along with variable intimal hyperplasia (I). Verhoeff’s elastic tissue stain with van Gieson’s counter stain.

Figure 3.29 **Transverse section right B vein** (magnification x197). Demonstrating collagen (pink regions) and cellular infiltration of intima, loss of internal elastic laminar (IEL) seen in the inferior portion of the slide and a disruption of the external elastic laminar (EEL). The associated medial hypertrophy is also demonstrated. Verhoeff’s elastic tissue stain with van Gieson’s counter stain.
Figure 3.30 Transverse section of vein C. A) Demonstrates the dilated thin walled vein (magnification x28). B) Magnified section (magnification x130) of same vein, demonstrating intimal hyperplasia, which is associated medial atrophy. Verhoeff’s elastic tissue stain with van Giesons counter stain.

Figure 3.31 Longitudinal section of right vein B. Demonstrates the relative uniform intimal and medial changes in the opposing walls of this relatively straight segment of vein. Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.32 Transverse section of a right vein B. A) Demonstrating focal varicose dilatation (magnification x57), secondary to mural atrophy seen in slide B. B) Close up of box in A, demonstrating the loss of internal elastic laminar (IEL) and intima at the point of dilatation (magnification x69). C) Close up of box in B, demonstrating intimal and mural atrophy (magnification x117). Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.33 Valvular pathology. (A) Elongated and incompetent valve leaflets. (B) Torn valve leaflet. (C) Neointimal formation on valve leaflets. (D) Apparent unilateral elongation and disruption of the left valve leaflet. Note that the arrows are indicating the direction of flow within the vessels.
Figure 3.34 Transverse section of vein B from pig 21. A) Left limb. B) Right (AVf) limb. Arrow demonstrates respective vein B. Note calibre difference between vessels. Scale bar: 2 mm

Figure 3.35 Comparison of histological changes seen in both the right and left vein B in an animal with functioning AVF. Transverse sections of (A) left (magnification x153), and (B) right (magnification x116) demonstrating the size discrepancy between left and right veins, and also intimal hyperplasia and the disruption of internal elastic lamina seen on the right.
3.7.2 Human Histology

For comparison with the observed histopathological alterations observed in this porcine model, varicose veins from two human subjects were examined. The first was from a 55 year old male with recurrent varicosities post sapheno-femoral junction ligation and patch repair. While the second sample was taken from a 36 year old male with primary varicose veins. Both sets of specimens demonstrated the variable histological features seen typically in patients with varicose veins (as reviewed in section 1.10).

**Macroscopic changes**

1. Elongated, tortuous and dilated veins.
2. Localized vessel dilation (figure 3.38).
3. Both vessel wall hypertrophy and atrophy (figure 3.38).

**Histological changes**

Microscopically there were marked changes in the structure of the vessel wall including:

1. Intimal hyperplasia, which was the most dominant feature, varying from uniform circumferential changes to pronounced focal hyperplasia (figures 3.36 and 3.37.A).
2. Disruption and separation of the regular pattern of the muscle bundles secondary to collagen infiltration, resulting in a disorganized muscle layers (figure 3.38.D).
3. Areas of abnormal dilation where there was loss of medial smooth muscle cells and the wall consisted of thinned out collagen lined by endothelial cells (figure 3.37.C).

5. Disruption of internal elastic lamina (figure 3.37.D and E).

6. In the adventitia there was loss of elastic tissue and smooth muscle cells (figures 3.37.D and 3.38.D).

7. Reduced elastic and muscle tissue in media (figures 3.37.C and D).

8. Increased deposition of extracellular matrix, mostly in the form of collagen in both the media and intima (figure 3.38.D and E).


Figure 3.36 Transverse section through a human superficial varicosity (x72). Demonstrating: focal intimal hyperplasia (I); medial atrophy (M) and medial hyperplasia (MH). Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.37 A) Longitudinal section of a saphenous varicosity (magnification x29.5). Slides B) to D) are close up views of different sections of the vein wall. Demonstrating a range of intra vessel variation in wall histopathology. (B) Thin wall due to medial atrophy, along with neointimal formation and intact internal elastic laminar (IEL), but an absent external elastic laminar (EEL) (magnificationx148). (C) Contrasting histology (magnificationx74). The superior wall demonstrates medial hyperplasia with some intimal hyperplasia, but on the inferior wall there can be seen extensive intimal hyperplasia and medial atrophy. (D) Close up of box in slide (C) demonstrating the intimal hyperplasia with medial atrophy and disruption of the internal elastic lamina, and loss of the external elastic lamina (EEL) (magnification x148). Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.38 Focal varicosity. (A) Macroscopic specimen. Note variable thickness as seen by transillumination (scale bar equals 10mm). Histology demonstrating (B) degenerate valve leaflet (V) and variable intimal hyperplasia (I) (magnification x14) (C) Medial hypertrophy with increased extracellular matrix (pink) deposition and separation of smooth muscle fibres and intimal (I) hyperplasia (magnification x48). (D) The opposing wall to C. Note the thinner wall with no obvious IEL or neointima formation but increased ECM (magnification x78). Verhoeff’s elastic tissue stain with van Giesons counter stain.
3.7.3 Comparable human and porcine slides

The histological changes observed in both the porcine and human tissue used in this study, have been discussed. The following slides are examples of the similarities observed between human and porcine varicosities. As can be seen in figure 3.39 transverse sections of both human and porcine varicosities can demonstrate a variety of pathological changes, ranging from intimal hyperplasia, medial hypertrophy and mural atrophy. While both porcine and human longitudinal sections of varicose vein shown in figure 3.40 demonstrate the uniform changes that can occur on opposing walls of the same length of vein. With both showing medial hypertrophy on the superior wall and intimal hyperplasia on the inferior wall in the slide. The transverse sections shown in figure 3.41 demonstrate the increased extracellular matrix (pink) deposition and separation of smooth muscle fibres of both the human and porcine tunica media.

![Figure 3.39 Transverse sections through (A) Human (magnification x56) and (B) Porcine superficial varicosities (magnification x19.5). Both demonstrate focal wall changes including intimal hyperplasia, medial hypertrophy and mural atrophy. Verhoeff’s elastic tissue stain with van Giesons counter stain.](image)
Figure 3.40 Longitudinal sections through A) Human (magnification x74) and B) Porcine superficial varicosities (magnification x128). Contrasting histology is seen in both slides. The superior walls both demonstrate medial hypertrophy with some intimal hyperplasia, but on the inferior wall there can be seen extensive intimal hyperplasia. Verhoeff’s elastic tissue stain with van Giesons counter stain.
Figure 3.41 Transverse sections through both A) Human (magnification x87) and B) Porcine (magnification x197) superficial varicosities. Demonstrating both intimal hyperplasia and medial hypertrophy, and collagen infiltration of media (pink regions). Verhoeff’s elastic tissue stain with van Giesons counter stain.
Chapter 4: Discussion

According to our knowledge this AVF driven porcine model of venous insufficiency is the first to report the progressive development of enlarged and tortuous superficial veins in the limb of the animal used. As shown in this thesis these changes in the superficial venous system progressed into what appeared to be an extensive network of varicose veins. This novel finding has not been described to the same extent in any other animal models of venous disease previously described by others.89, 91, 98-103, 105, 106, 109, 111, 114, 116-119

Animal models have been of particular use in furthering the understanding of disease aetiology, pathogenesis and progression by recreating pathological conditions. The axiom “before testing a new treatment in man, test it first in animals if possible” has been part of drug development for the past 50 years or so. Animal models, when used to test the effectiveness of a drug or procedure before proceeding to clinical trials, allow researchers to focus on specific pathological processes without the confounding effects of other medical conditions.94

As has been previously mentioned the lessons of comparative medicine are most useful when the model has sufficient similarities to suggest its relevance to the human disease.97 The ideal experimental model for superficial varicose veins would recreate the human condition ideally in an animal, which spontaneously developed the same condition. There is unfortunately no such animal. If one was to artificially induce varicose veins in an animal then it should at least have some anatomical, histological and physiological similarities to the human in both the normal and induced disease process if it is to have relevance.
Any model should if possible also be: simple, economical, and reproducible, while allowing ready evaluation of the disease and any interventions.

Specifically for varicose veins an ideal model should demonstrate:

1. Progressive development of varicose veins.
2. Progressive loss of venous valve competence.
3. Evidence of elements consistent with the human disease such as venous reflux and/or impaired muscle pump function and venous hypertension.
4. Histological features comparable to those in the tissues of patients with the established disease.
5. Show clinical signs of venous hypertension including skin changes and ulcer formation.

With the above parameters in mind what follows is a discussion of this porcine model.

4.1 Reproducibility

4.1.1 Mortality

The overall mortality in the group of animals in which AVFs were formed was high at 22.2% (two out of nine AVFs). However, only one of these deaths (11.1%) was related to the fistula, the second animal being euthanased after it extracted its’ nose ring. If the four animals from the pilot study are also included the mortality rate related to the AVF decreases to 7.7%. Of the previously developed animal models the reported mortality ranged from 0 – 33%, with the most frequently used model (Rodent AVF model) reporting the highest mortality rate which was due to cardiogenic shock. Consequently this porcine model has a comparable if not better mortality rate than the other described models.
4.1.2 Morbidity

Apart from the 7.7% mortality rate there was no other significant morbidity associated with the creation of the side-to-side femoral AVF in these animals. This is comparable with the other models.

4.1.3 Reproducibility

Three of the nine AVF failed (33.3% failure rate). The failure of these fistulae appeared to be associated with technical problems. Namely significant vasospasm at time of AVF formation despite liberal use of 1% lignocaine as an antispasmodic. While in one animal it was noted that what appeared to be a dissection of the vein wall occurred. Of the models which successfully generated venous hypertension one reported the failure rate of their model. Van Bemmelen et al\textsuperscript{91} report a 30% failure rate in their combined AVF/occlusive model, and it is also this model that Bergan’s group\textsuperscript{116} has used to generated near arterial levels of venous hypertension. Consequently, this model appears to be very susceptible to fistula failure, but the rate of failure is similar to that of a commonly utilised model.
4.2 Gross venous anatomy: A comparison of human and porcine

4.2.1 Anatomical similarities

There are a number of similarities between the superficial venous anatomy of the human lower limb and porcine hind limb.

1. The porcine saphenous vein traverses a course along the hind limb comparable to that of the human counterpart.

2. The porcine saphenous vein is duplex while in humans the saphenous vein is routinely described as a continuous single trunk. However, detailed studies of the surgical anatomy of the human saphenous vein have reported duplication to occur in 10 - 52%.[120-122] The reported incidence of complete duplication of the saphenous vein is between 1 - 10 %, while duplication isolated to the thigh is 23 – 35% of preoperative phlebograms. Consequently, in humans duplication of the saphenous vein is common and the observed duplication of the corresponding porcine saphenous vein is in keeping with this observed anatomical variation. The reason for this appears to be due to the embryological development of venous trunks from a venous network by coalescing of smaller channels.

3. The fascial arrangement of the porcine saphenous veins and their tributaries is similar to that of the human. As has been described the porcine saphenous bundle lies supported within a compartment formed by the superficial and deep fascia giving rise to an ultrasound appearance not unlike that described by Caggiati[123] in humans. Furthermore superficial tributaries lie unsupported in loose adipose tissue in the hypodermis above the superficial fascia, also described by Caggiati[123] and
Thomson.\textsuperscript{124} There is an interesting potential difference between the two species which is the presence of a separate dense connective tissue fascia surrounding the entire porcine saphenous bundle along its entire length which appears to be distinct from both the superficial and deep fascia. This has not been described in the human literature but clinically it may be present, seen contiguous with the adventitia when isolated saphenous vein is prepared for anastomosis.

4.2.2 Anatomical differences

There are several key anatomical differences:

1. The presence of a saphenous artery may have acted as a further supporting structure preventing the accompanying veins from elongating and becoming tortuous.

2. Corrosive resin casts of the porcine saphenous veins from the pilot project and the direct dissections described in this study revealed that the porcine saphenous veins have a greater number of valves than the human counterpart which has between 2 – 13 valves.

3. The calf muscle pumps of the human plays a significant role in both normal and abnormal lower limb venous pressures. In resin casts no deep venous sinuses were seen within the control or varicosed animals though this may in fact reflect a methodological problem. The pressures generated in the deep system of this model on ambulation were not assessed. An absence of overt calf musculature and calf sinuses in the pig may imply that the porcine calf pump does not play a similar role, as the human calf, in regulating ambulatory venous pressure in the porcine
hind limb. This may represent a significant anatomical difference since it has been demonstrated that loss or over load of the calf pump in humans plays an important role in the generation of ambulatory venous hypertension seen in chronic venous insufficiency. It should be noted that the porcine thigh pump may play a similar role in generation of pressure gradients in the porcine hind limb. Moreover, we suggest that the presence of a AVF may in fact overload this pump in a similar fashion to that seen in the calf pump in humans.

4.3 Comparison of microscopic venous anatomy

4.3.1 Normal wall histology

Histological studies of the porcine saphenous vein and its tributaries revealed three layered thick walled muscular veins. The most prominent layer being the muscular tunica media predominantly composed of densely packed smooth muscle cells which appeared to be arranged in a single circular layer. The human saphenous vein is also a muscular vein, with its media frequently being reported in the literature as having two distinct layers, an inner longitudinal and outer circular. In our human specimens this arrangement was not a consistent finding with the two layers in fact merging into one another. This absence of an obvious longitudinal layer of smooth muscle cells in the media of the porcine vessels may be a contributing factor for these pigs forming varicosities in the presence of an AVF. Reported histology of the canine saphenous also reported a similar configuration of the tunica media.125 There are, however no reports of the normal histological appearance of rodent veins, with particular reference to the muscular configuration of the tunica media.
4.3.2 Normal valve structure

The venous valves observed in this model shared a similar valve structure to the human valves described in the literature. The similarities include:

1. Both porcine and human venous valves are bicuspid.
2. In both species the venous cusps consist of a thin layer of collagen, and an endothelial layer covering both its surfaces.
3. The valve sinus is wider than the vein above and the cusp below.
4. The vein wall is also thicker at the base of the cusp due to an increased amount of muscle fibres in the media at that level.
5. The valve cusp is much longer than the diameter of the lumen of the vessel and has an elliptical attachment to the vein wall.

4.4 Anatomical comparison of human varicose veins and those of this porcine model and other animal models

4.4.1 Macroscopic changes

As has been described previously in this thesis the CEAP classification\textsuperscript{76} defines a varicose vein as a subcutaneous dilated vein, 3 mm in diameter or larger measured in the upright position. Varicose veins may involve the saphenous vein, its tributaries, or non saphenous leg veins. Varicose veins are usually tortuous but tubular saphenous veins which demonstrate reflux may also be classified as varicose veins.

It was planned that the above definition would have been applied when determining what did and did not macroscopically constitute varicosity formation in both the porcine and
animal models. However, none of the reviewed papers reported varicosity formation, so this definition will only be applied to the porcine model.

Though the extent of the varicose networks varied between this and our pilot study, the distribution remained the same with straight dilated saphenous veins (GSV) associated with elongated tortuous superficial tributaries. In humans changes typical of varicosis occur more frequently and extensively in incompetent tributaries of the saphenous vein. In fact the saphenous veins are rarely or minimally dilated in most varicosed limbs.\textsuperscript{123, 126-128} In both the porcine model and human disease the tributary veins are surrounded only by loose adipose tissue which cannot counteract dilative forces acting on their wall. In contrast the GSV’s are supported by the bilaminar saphenous fascia formed by the muscular and superficial fascia which appears to act as a mechanical shield.

4.4.2 Microscopic changes

As there has been no mention of varicosity formation or venous histological assessment in any other animal model, this section will again be limited to a comparison of human and porcine varicosities.

The histological appearances in human varicose veins are extremely heterogeneous depending on sampling even on different aspects of the circumference and within very short distances of the same vein. This has compounded the difficulty of characterizing the disease in any single vessel let alone between vessels in different patients with varying degrees of venous insufficiency. As a consequence the histological features of varicose veins reported in the literature is variable and at times contradictory thus making it difficult to accurately characterise what histological features are required in the perfect animal model, therefore,
generating a model that mimics the disease should also reflect this. This heterogeneity shown in the human setting was also seen in the porcine varicose veins. Where the vessels demonstrated:

1. Intimal hyperplasia, which was the most dominant feature.
2. Disruption of the regular pattern of the muscle bundles.
3. Areas of abnormal dilation.
4. Marked sub-intimal deposition of collagen.
5. The areas of intimal hyperplasia were frequently associated with disrupted elastic tissues networks both in the inner elastic membrane and media which is not only in agreement with the human veins examined as part of this project but also with previous published reports. 78, 129
7. Reduced elastic and muscle tissue in the media. 78
8. Areas of both medial hypertrophy and atrophy. 78 In the porcine vessels the medial hypertrophy was the result of increased smooth muscle and collagen content of the media, but the sections did not demonstrate the extensive separation and disruption of the normal muscle cell architecture caused by the increased collagenous infiltration seen in the literature 77, 87, 130 and our human specimens.

The porcine varicosities did not demonstrate the focal dilations or varicosities associated with valves commonly seen in the human disease.

The heterogeneity of the histological findings in this model is consistent with the heterogeneity of the reported literature. In particular the variable degree of intimal
hyperplasia and medial thickening seen within individual vessels is a feature seen in both the human and porcine varicosities.

4.5 Physiological comparison of human varicose veins and those in this porcine model and other animal models

The successful development of an upright, large animal model of venous insufficiency is one that can be verified by functional tests of hind limb venous haemodynamics.

4.5.1 Pressure profiles

As has been discussed in section 1.11 of the thesis the presence of venous hypertension is an important feature of venous insufficiency which should be replicated in an animal model of varicose veins, both at rest and more importantly on ambulation. The mean resting pressure in the superficial veins of the pigs with functional AVF was increased to $23 \pm 11.4$ mm Hg at week six and $20 \pm 8.3$ mm Hg at termination, compared to pressures in the control and those without functioning AVF ($4.5 \pm 3.5$ mmHg, 2 mmHg). In comparison, the mean systemic pressure was: $95 \pm 25$ mmHg (in an animal with a failed fistula). The six and 14 week intervals were chosen for physiological profiles, because in the pilot study it was at 12 – 14 weeks that the varicosities appear to be most prominent and consequently we wanted to ensure that the model was stable over this time period and produced a consist elevation in venous pressures. As is demonstrated in the results chapter the 6 week and termination profiles have a similar pattern and magnitude suggesting
stability in the model over time. The raised venous pressures observed in this model are in the supine position at rest and not augmented by addition of a hydrostatic component which occurs on standing. This may infer that pressures within these vessels could in fact be greater in the animal’s usual position of standing on all four limbs.

Four previous animal models of venous insufficiency have documented “venous hypertension”. The first of these is the rodent model employed by John Bergan’s group\textsuperscript{116} which also utilises an AVF fashioned between the femoral vessels of a rat. This model generates a persistent pressure of $96 \pm 9$ mmHg close to those recorded in the systemic system ($126 \pm 5$ mmHg). Such pressures infer that any documented changes especially histological, are likely to represent changes seen within arterialized vein and are not a true comparison for the human venous disease. This assumption is further supported by studies that have shown that under arterial conditions of high flow and pressure there is a total abolition of venous intimal proliferation.\textsuperscript{131} Other groups have also utilised a similar model (AVF between saphenous vessels or between saphenous artery and femoral vein) to investigate the arterialization of the venous system in a rat lower limb.\textsuperscript{132} The vessel walls of these veins exhibited thickening and muscle hypertrophy, such that the veins resembled arteries. The second model is a canine model which employed the use of a valve lysis to generate venous incompetence.\textsuperscript{114} At rest venous pressures in the experimental limb were significantly elevated ($14.7 \pm 1.74$ mmHg) compared with the control ($9.8 \pm 1.38$ mmHg) immediately after lysis. However, for the remainder of the experiment there was no difference between the two limbs.

The third model is an out flow obstruction model\textsuperscript{101} created by iliofemoral ligation in greyhound dogs. This model established a stable and reproducible elevation in venous blood pressure. By the 15th week of observation the pressure within the hind limb was still
significantly greater than in the control limb (10.0±3.0 mmHg compared with 5.5±2.1 mmHg).

The final model to produce venous hypertension was the canine AVF. The mean resting (standing) pressure in the AVF limb was significantly raised at 47.2±12 mmHg compared to 35.9±3 mmHg in the control. They were also able to demonstrate ambulatory venous hypertension, with a fall in of only 2.1±11 mmHg in the AVF limb on walking compared to 18.9±12 mmHg in the control limb.

In this porcine model of venous insufficiency there was a demonstrable long-term 4.4–11.5 fold increase in the resting pressure above that of the control at both 6 and 14 weeks. It should also be noted that in all the above-described models the pressures were recorded in the saphenous or femoral veins, whereas in the porcine model they were recorded in the tributaries of the saphenous vein. This may be why they are lower than those reported in the canine AVF model.

Recording pressures within these vessels while the animal is tilted to as near vertical as possible as was performed by Lalka et al was contemplated. However this would not be a true physiological position for the animal and not truly represent the models true hemodynamic parameters. Of more importance in understanding and accurately characterising the model would be assessing the haemodynamic changes that occur with the animal standing and ambulating on all four limbs, since this would represent the changes that occur outside of the controlled environment of the laboratory.

In the group of pigs with functioning AVF the pressure varied with the cardiac cycle. This variation was on average only of the magnitude of 2 mmHg about the mean. Though there was a degree pulsatility associated with the formation of an AVF we conclude
that its effect was likely to be minimal given the normal variations in venous pressures about the mean observed in human patients associated with ambulation.

Rapid increases in intra-abdominal pressure resulted in reciprocal rises in superficial venous pressure of: $10 \pm 1.4$ mmHg at week six and $9 \pm 6.0$ mmHg on termination.

### 4.5.2 Flow dynamics

Duplex scanning is currently the gold standard assessment of leg veins providing hemodynamic information that can be used in the investigation of varicose veins. Reflux and venous valve incompetence is detected by the presence of retrograde flow in response to down stream pressure, 95% of normal valves will close within 0.5 seconds. Therefore, retrograde flow of greater than this is said to be a sign of venous incompetence. When interpreting flow dynamics in the porcine model it should be remembered that the normal direction of flow within the superficial tributaries of the saphenous veins is down towards the saphenous veins themselves, with veins A and B uniting at the confluence to form the major saphenous tributary as described previously (section 3.1.2 and figure 3.4) and not at the sapheno-femoral junction. At rest, with the AVF acting as the down stream driving force within the porcine superficial varicosities, there is continuous phasic retrograde flow, away and upwards from the saphenous vein and the confluence of veins A and B. Therefore, duplex scanning was both a simple, reproducible and non invasive means to confirm that these superficial varicosities were incompetent by demonstrating the presence of continuous retrograde flow within them.

The majority of animal models did not report investigations into the presence of reflux. Of the models which successfully generated venous hypertension and also assessed
for the presence of reflux invasive techniques were utilised including: bleeding time, pressure catheters and strip tests to confirm the presence of reflux/retrograde flow. The presence of reflux in the rat AVF model developed by the Bergan group\textsuperscript{116} was demonstrated by, sectioning the femoral vein distal to the proximal valve. Reflux was determined for each rat by a timed collection of the back flow in the presence of native femoral pressure. This procedure had to be repeated at 2 days, one week and three weeks to determine the time interval to produce reflux, with the animals being euthanized at each time interval preventing their use for further studies. Burnand et al’s\textsuperscript{103} canine AVF model, the model which most closely mirrors the porcine model did not document the presence or absence of retrograde flow. They used greyhounds which typically are similar in size to the 30 kg pigs we used in our model. In the pig it was difficult to detect the superficial veins using duplex ultrasound at this weight and the same may be true for the dog. Thereby, limiting the use of duplex ultrasound in the assessment of reflux in this model. As expected Lalka et al’s\textsuperscript{114} greyhound valve lysis model did demonstrate retrograde flow which they confirmed utilising changes in venous pressures recorded using transducers placed in catheters inserted into the saphenous veins of the hind limbs.

In humans the normal venous flow is also phasic demonstrating changes in velocity of flow in response to quiet respiration and cardiac pulsation which are evident by duplex examination. However the continuous phasic flow present in the porcine vessels does not appear to be related to respiration, but did demonstrate features similar to an arterial wave form (figure 3.18). With rapid elevations and reductions in venous velocities which occurred at a regular intervals in keeping with the porcine arterial pulse rate. As the porcine veins were prone to significant vasospasm the calibre of the veins was not measured during the assessment of velocities. Consequently we were unable to quantify the volume of
flow through these vessels and thereby the degree of reflux, as had been reported in other animal models. Duplex scanning in this study, was used solely to confirm the presence of retrograde flow with in the superficial varicosities.

The valsalva manoeuvre in a human, with a competent peripheral venous system, increases intra-abdominal pressure (IAP) sufficiently to cause cessation of blood flow in the large and medium abdominal veins. As a consequence the velocity of the blood flow in the lower limb veins is reduced, whilst in an incompetent system the response to an IAP is the increase in reversed blood flow through the veins involved. In this porcine model abdominal augmentation produced an abrupt reduction in the velocity of retrograde flow in the superficial varicosities and on release the velocities rapidly returned to a pre-stimulus level. This is the converse of what is observed in the human condition.

During calf augmentation the rapid increase in signal, away from the saphenous veins, is an indirect indicator of incompetence between the site of the probe and compression, again confirming the presence of venous insufficiency in the superficial varicosities.

4.6 Mechanics of the model

It would be expected that the greater number of valves in the porcine saphenous veins would increase the likelihood that the vein distal to these competent valves would thrombose as there should be no or reduced flow. However, this does not appear to be the case. At least 50 percent of the flow from the AVF will be directed deep down the femoral vein since the fistula is formed between the femoral vessels proximal to the origins of the saphenous veins. The deep system may communicate with the saphenous veins distally by
undetected perforators and as a result allow continued flow within these veins and their more superficial tributaries. This may protect the tributaries from the sudden increase in flow and pressure that is associated with the acute phase after the formation the AVF in that limb. This in turn may provide the vessel walls and valves of these superficial tributaries time to remodel in response to the hemodynamic changes.

4.6.1 Variability of the macroscopic changes

In both the pilot and this study all the animals with functioning AVF developed macroscopic changes in the superficial veins consistent with the CEAP definition of varicose veins. In the initial study, the superficial varicosities that developed, were much more dramatic not only in the AVF limb but even more so in the non-AVF limb (figure 3.12), compared to the changes observed in this set of pigs. The only overt differences between the two groups were that in the pilot study larger AVF were fashioned and it was conducted over the summer months. The first group of pigs in this study did have smaller AVF fashioned which may explain the difference in appearance of the varicosities formed. However the second set of animals had larger AVF formed, but still their macroscopic changes were similar to the first set in this study and were consequently not as extensive as those seen in the pilot study. It must be mentioned that the second group were studied over the winter months and that their weights were significantly lower than their counterparts observed over the warmer summer months. It may be that the temperature difference associated with the two seasons may in fact have a confounding effect on the extent of varicose changes seen, as prolonged exposure of mammals to a cold environment has been thought to effect their body composition and organ size.\textsuperscript{133} Ingram and Weaver\textsuperscript{134}
demonstrated that the vascularity of porcine skin was significantly reduced in pigs reared at 5°C compared to those reared at 35 °C. While Heath\textsuperscript{133} demonstrated significant increase in the length of limb bones of the warm (35°C) reared pigs and the mass of the intra-abdominal organs of the cold (5° C) reared pigs compared to each other and the controls. This suggested a diversion of blood and nutrients away from the periphery and instead to organs and structures vital for survival. These reported findings would support the observation in this study that the seasonal rearing temperature affected not only the body weight of the animals but also the vasculature and size of their hind limbs, consequently impairing the formation of the extensive networks of superficial varicose veins that were seen in the pilot study.

4.6.2 Physiological changes

The raised venous pressures and velocities documented in this thesis are a result of the driving force of the AVF. The AVF is a direct communication between the arterial and venous systems of the lower limb allowing transmission of the greater arterial pressures and flow into the proximal peripheral venous circulation bypassing the distal networks of capillaries and venules which would, in the normal situation, buffer and equilibrate the two sides of the circulation. This explains why both the pressure and flow profiles varied with the cardiac cycle and why the flow profile exhibited an arterial wave form.

The increases in venous pressure and reductions seen in retrograde flow with abdominal augmentation may be the result of a number of factors:

1. Transmission of the increased intra abdominal pressure to either inferior vena cava or iliac veins with resultant retrograde flow through and increased circulating
volume and venous capacitance, in the incompetent saphenous veins, thereby increasing the measured superficial venous pressures.

2. The relationship of the porcine superficial epigastric to the saphenous vein. In humans the superficial epigastric vein is one of a number of tributaries expected to join the GSV in the region of the saphenous opening. However, in the porcine model it is formed by a confluence of the flank vein and superficial veins B and C before it passes up along the cutaneous surface of the anterior abdominal wall. How this vessel communicates with the deep system is not known, or if it does at all. If there is a communication it is possible that with the increase in intra-abdominal pressure there may be reflux down the epigastrics into the superficial varicosities opposing the flow induced by the AVF in veins A, B and C. As a consequence reducing flow and increasing the pressure within these veins.

3. Increased skin tension over the hind limb and abdomen caused by compression and stretching of the anterior abdominal wall. This may result in caudal occlusion of the superficial veins of the limb and or the epigastric vessels reducing or ceasing flow within the superficial varicosities, while they are still being filled distally by the active AVF increasing the intra-luminal pressure. Once the pressure is released there is a sudden increase in flow cause by the fistula.

4.6.3 Susceptibility

As was demonstrated in the histological examination of non-manipulated porcine veins there appears to be an absence of longitudinal smooth muscle fibres within the tunic
media. Using bovine mesenteric veins McConnell et al.\textsuperscript{135} demonstrated that longitudinally arranged contractile cells play a prominent role in counteracting haemodynamic stresses. Consequently the absence of longitudinal musculature suggests a reduced resistance of the porcine veins to increased intravascular pressure. As a result they may be unable to actively resist the longitudinal stresses induced by the increased reversed flow. Reversed flow against initially competent valves results in the proximal vein segments being placed under traction until the valve fails. This may also be the case in the previously described canine models however the presence or absence of varicosities is not mentioned. As a consequence we are unable to explain if there are any histological differences between pigs and these other animals which may explain their susceptibility to forming varicosities in the presence of AVF.

4.6.4 Microscopic changes

Uniform intimal hyperplasia has been reported as the normal haemodynamic response of a vein to arterial pressure as seen in patients with saphenous vein coronary bypass grafts\textsuperscript{136, 137} and arterio-venous fistula.\textsuperscript{138} Autologous saphenous veins have been utilised for conduits for lower limb arterial reconstruction and coronary artery bypass grafting. Following implantation, the vein graft is subject to arterial pressure, increased wall tension, shear stress and pulsatile blood flow. Uniform intimal hyperplasia accompanied by hypertrophic remodeling of the media is often reported in these venous conduits\textsuperscript{136, 138-140} resulting in thickened muscular veins and conduit stenosis. No histology was performed on porcine saphenous veins in this model due to their direct proximity to the AVF and the subsequent changes would represent arterialisation of this vein. However, macroscopically
they appeared as dilated thin wall vessels and did not exhibit the gross luminal narrowing or wall thickening seen in human venous conduits.

The pathogenesis of the intimal and medial changes observed in venous conduits has been investigated in a number of animal models. The findings of these models may help to explain the changes seen in this porcine model of venous insufficiency. Porcine saphenous vein to common carotid artery interposition grafts have been used to investigate the effects of external sheath supports on medial and intimal thickening in venous conduits. Mehta et al observed that externally supported veins exposed to arterial conditions had no medial thickening and the neointimal area was reduced by 97% compared to the non-supported grafts.

These observations can be explained by findings reported by Dobrin et al. They investigated mechanical factors predisposing to intimal hyperplasia and medial thickening in canine autogenous femoral vein grafts which demonstrated an inverse correlation between graft flow and neointimal formation. They also found that medial thickening occurs most markedly in regions of the wall subjected to increased circumferential deformation and tension. This observation is better understood when the law of Laplace is considered.

The law of Laplace states that tension in the wall of a cylinder (T) is equal to the product of the transmural or distending pressure (P) and the radius (r) divided by the wall thickness (W) (T = Pr/W). Therefore a vein that is maximally dilated as a result of persistently increased transmural pressure will be stimulated to undergo medial hypertrophic remodeling thus increasing its wall thickness in order to reduce wall tension. Consequently, the findings of the above external sheath study may be explained by the sheath providing support for the vessel wall thereby reducing circumferential diameter and wall tension and as a result limiting the stimulus for medial hypertrophic remodeling. The macroscopic
observations in the saphenous veins in our study may be explained by the presence of the fascial sheath seen around the porcine saphenous bundle acting in a similar fashion to the sheath used in the above study. Gusic et al.\textsuperscript{139} examined the effects of shear stress and pressures modulating the porcine saphenous vein remodeling \textit{ex vivo}. They cultured porcine GSV explanted under five different \textit{ex vivo} hemodynamic conditions, including one mimicking an arterial bypass graft for one week. They noted that the degree of medial hypertrophy correlated with the average pressure under which the veins were cultured. Veins subjected to the greatest pressure in culture displayed the greatest medial change, while veins cultured under lowest levels of pressure displayed the least, again demonstrating the law of Laplace in effect.

### 4.6.5 Variations in medial remodeling

The heterogeneity of medial histological changes observed in this model may be better understood when both Bernoulli’s principle and the law of Laplace are applied together. Bernoulli’s principle describes the relationship between the velocity and pressure exerted by a moving liquid and states that: as the velocity of a fluid increases, the pressure exerted by that fluid decreases.\textsuperscript{141} As is seen in figure 3.4 the porcine superficial veins exhibit a degree of tortuosity prior to manipulation. As a consequence patterns through the various segments will not be uniform or predictable as the tortuosity of these veins will result in disruption of laminar flow. Changes in the velocity of blood occur as flow navigates a sudden curvature in the vein with the blood on the greater curvature having greater velocity than that on the lesser curvature. This is because a corresponding volume of blood must travel a greater distance on the greater curvature than the lesser and thus have a
greater speed. Such a speed differential may lead to a difference in pressures exerted on the two walls. Which, if law of Laplace holds true, the opposing walls will also experience differing wall tensions and stimulus for medial remodeling. Though a more important factor maybe the increased shear stress that will also be present.

The role of pulsatility in the medial hypertrophy of vein grafts has not clearly been defined. In a study of mechanical factors predisposing to intimal hyperplasia and medial thickening Dobrin et al\textsuperscript{137} concluded that medial thickening is not associated with pulsatile pressures. Though they only modulated pulsatility and did not eliminate it while Gusic \textit{et al}\textsuperscript{139} observed medial thickening under non pulsatile conditions.

\subsection*{4.6.6 Variations in neointimal formation}

An important factor for vascular remodeling appears to be alterations in blood flow. As a result of their unique location endothelial cell experience three primary mechanical forces: pressure generated by the hydrostatic forces of blood within the vessel, circumferential tension and shear stress and the tractive force created by flowing blood on the endothelium that is parallel to the long axis of the vessel. Shear stress has been shown to be a particularly important hemodynamic force because it stimulates the release of vasoactive substances and changes in gene expression, cell metabolism, and cell morphology.\textsuperscript{142} Morinaga \textit{et al}\textsuperscript{143} demonstrated that endothelium plays a central role in the detection of shear stress and its ensuing conversion into vessel wall responses. In particular, remodeling since the normalisation of wall shear stress to physiological values (which in humans is between 10 to 20 dyn/cm\textsuperscript{2}) appears to be an important physiological response.\textsuperscript{144}
According to Hagen-Poiselle formula shear stress is proportional to flow velocity (Q) and blood viscosity (µ), and inversely proportional to the third power of the internal radius of the vessel (Shear stress = \(4\mu Q/\pi R^3\)). The nature and magnitude of shear stress plays an important role in the long-term maintenance of the structure and function of blood vessels. Changes in blood flow patterns arising from pathological or developmental phenomena can disrupt the normal haemodynamic cues detected by vascular endothelial cells and lead to adverse remodeling of the vessel wall. Experimentally a number of authors have reported an inverse relationship between the magnitude of shear stress and the extent of neointimal hyperplasia.\textsuperscript{137, 139, 147}

Along with the noted variable changes in media, there was a similar heterogeneity in the degree of neointimal formation in this porcine model of superficial varicose vein. The cause of this observed variability is likely to be due, at least in part, to the presence of branching veins and the tortuous nature of the superficial veins. These factors may cause alterations in the normal blood flow by the development of slower turbulent flow as fast laminar flow is disrupted by the branch points and at areas of abrupt curvatures in the vasculature where again the laminar flow is disrupted and separated flow patterns result, which, as described above, causes opposing vessel walls to experience differing shear stresses and endothelial cell activation. This inconsistency in shear stress and endothelial cell activation is exhibited as the irregularity in the degree neointimal formation observed in the porcine varicose veins.
4.7 Suggested modifications

4.7.1 Operative

1. Both intra-operatively and during assessment of physiological parameters significant vasospasm was frequently encountered and was likely to be due to high smooth muscle content of the tunica media of the porcine veins. Vasospasm alters blood flow through a vessel increasing the risk of thrombosis and it should ideally be avoided. Therefore intra-operatively the femoral vessels were bathed liberally in lignocaine (2%) in order to prevent or at least reduce the degree of vasospasm with marginal success with 33.3% of the AVF failing in this study. Gherardini et al\textsuperscript{148} investigated the efficacy of papaverine versus various doses of lignocaine in relieving vasospasm. They reported that lignocaine (2%) alone did not significantly alter blood flow after microvascular anastomosis while papaverine and lignocaine (20%) did demonstrate a significant increase in blood flow. In light of these findings in further experiments either papaverine or lignocaine (20%) should be used in preference to lignocaine (2%) to reduce vasospasm.

2. It was noted that the most dramatic macroscopic changes in the superficial vasculature appeared to occur by week eight post procedure. This may be the result of the veins having remodeled sufficiently to normalise the abnormal shear stress induced by the AVF. It may be possible to overcome this through occlusion of femoral vein proximal to the fistula at the initial operation to drive flow into superficial territory and generate more dramatic changes in both limbs. Though it may be more appropriate to place a vascular tie around the proximal femoral vein
at the primary operation and perform a delayed occlusion of the femoral vein at 6 – 8 weeks to induce further remodeling.

3. Timing: Any other planned models should be either performed during the same season (i.e.: preferably summer), or the animals should be housed inside with thermostatic control to reduce the impact of temperature on vasculature growth patterns.

4. No stigmata of chronic venous insufficiency such as lipodermatosclerosis, ulcers or oedema were generated in this model despite the presence of venous hypertension. This may be due to either the short time period of the study or the fact that the porcine dermis appeared to have a much great density of collagen than that of the much older human patient commonly affect by varicose veins. A means to try and instigate these changes may be to apply a dermal abrasive process to both limbs of control animals and assess if there was ulcer development and if there was any difference between the control and fistula group or correlation with the severity of the varicosities present in the AVF group.

4.7.2 Quantification of varicose network

In this study the extent of varicose vein formation was documented photographically by elevating the animals onto their hind limbs at weekly intervals. This was not a very accurate method. A more accurate and reproducible means to assess and document the degree of superficial varicose vein formation may be to perform descending venogram at 6 and 12 weeks with contrast being administered via a catheter passed
percutaneously through the external jugular vein and positioned in the external iliac vein on the right than subsequently on the left. This method is suggested as it would reduce the chance of thrombosing the femoral or saphenous veins if they were accessed. This may also reveal communications between left and right that may explain changes observed in the contralateral limb noted in the pilot study.

4.7.3 Physiological quantification

The physiological characterisation of this model was limited to resting pressures and velocities. In order to accurately characterise this model the presence or absence of ambulatory venous hypertension needs to be ascertained. There are two possible methods of doing this. The first is to simulate ambulation in an anaesthetised pig as was described by Lalka et al.\textsuperscript{114} In their canine model they utilised a peripheral nerve stimulator (Grass, Inc, Quincy, Mass) with 10 one per second applications of a 50 Hz, 100v, 200 millisecond stimulus via electrodes inserted into the gracilis and biceps femoris muscles of the thigh to simulate ambulation. With this method they were able to demonstrate similar patterns of pressure variation seen in humans, though obviously measured in the thigh not the calf. The second method is to use a Mikro-Tip® (Millar Instruments, Inc) implantable pressure sensor. This would allow pressure readings to be taken while the animal ambulates naturally and subsequently document the absence or presence of ambulatory venous hypertension. However the longer length of the implantable catheters may make placement in the tortuous superficial veins difficult and increase the risk of thrombosis or alter the flow patterns, thereby possibly altering the model.
4.8 Applications

In the absence of an ideal experimental animal model of varicose veins and venous insufficiency, the ongoing challenge for investigators is to identify effective therapies within the constraints of the available models such as Bergan’s rat AVF model. Our porcine model has scope for further development and sophistication, which may ultimately lead to an increase in our understanding of the progression of the varicose vein process and possibly the formulation of preventative and treatment strategies that will translate into patient benefits in clinical trials of pharmacological interventions for varicose veins.

4.8.1 Matrix Metalloproteinases

As has been discussed the vessel wall changes seen in this model and human varicose veins provide evidence of venous wall remodelling. A disturbed smooth muscle cell/ extracellular matrix (ECM) balance and loss of mechanical properties have been well described in varicose veins. Degradation of the ECM is regulated by a variety of proteinases but the major enzymes are considered to be matrix metalloproteinase (MMP’s), their activators and tissue inhibitors of metalloproteinase (TIMP’s). In humans there are 23 different MMPs so far identified and taken together activated MMP’s can degrade all ECM components. Thus the balance between MMP’s and TIMP’s is critical for ECM remodelling in the tissue. Studies have supported the implication that changes in the ECM of varicose veins may be associated with alterations in MMP activity. In particular studies of the MMP/TIMP balance in human varicose veins have demonstrated decreased amounts of MMP-2 and increased TIMP-1 in varicose veins compared to those of controls.
Investigations into the regional variation in proteolytic activity in varicose veins noted a greater amount of MMP-1 and MMP-13 in the proximal compared to distal varicose segments. There was no difference in their quantities in the control veins. In humans structural and functional studies have provided information as to how to manipulate their enzymatic activities. Based on those studies a large number of MMP inhibitors have been designed but have shown little efficacy. It has been suggested that these failures may be partially due to our limited knowledge of the function of MMP’s in pathological processes and as consequent leading to a lack of selective inhibitors. It has also been suggested that the challenge ahead in designing selective MMP inhibitors includes not only the identification of the enzyme critical to the relevant disease process, but also the fact that the MMP’s are all structurally similar and hence how to screen inhibitors for a particular enzyme.

Therefore, a possible application of this model would be to investigate and better understand the roles that MMP’s and their inhibitors play in the pathogenesis of varicose veins. Enabling the development of locally expressed inhibitors of MMP’s in the varicose veins.

4.8.2 Gender

There is a debate concerning the role gender plays in the development of varicose veins. This model may play a role in investigating this issue. The model could be generated in both male and female pigs and the extent or susceptibility of varicosity formation compared.
**4.8.2 Pregnancy**

In humans there is also an association with pregnancy.\textsuperscript{6, 53, 56} This reported association has been thought to be due to several reasons including: mass effect of the gravid uterus, hormonal levels and increased circulating volume. By forming AVF at each of the 3 stages of pregnancy and measuring hormone levels we may be able to investigate if there is a correlation between degree of varicosity formation and either uterine mass or hormone levels. AVF would have to be formed at each stage rather than just following them through.

**4.8.3 Evaluation of currently available pharmaceutical interventions**

**Daflon (Micronized purified flavonoid fraction)**

Flavonoid drugs have been widely used in the management of the symptoms of venous disease for a number of years. Currently Daflon 500mg is used in patient with varicose veins to aid with relief of from symptoms including pain, leg heaviness, and cramps, oedema, and possibly also prevents the appearance of varicose veins. The clinical efficacy of Daflon 500mg in venous ulcers has been demonstrated in a number of randomised controlled clinical trials, in which the rate of ulcer healing was significantly shortened.\textsuperscript{152, 153} It is thought that Daflon 500 acts on microcirculation, normalising the synthesis of prostagladins and free radicals and inhibiting leukocyte activation and trapping.\textsuperscript{154} The exact mechanisms by which Flavoniod drugs work are not clear and it is in this area of pharmacological testing that this model may initially be of use. Not to
investigate how it controls symptoms but whether or not it and how it may affect vessel wall remodelling in varicose veins or its impact on MMPs.
Chapter 5: Conclusion

The advantages and disadvantages of this model should be acknowledged.

5.1 Advantages

In opposition to the other animal studies concerned with venous insufficiency this present study has several advantages:

1. It is a simple model. The large size of the pig allows for relatively simple fashioning of the AVF.
2. It is the only model to generate varicose veins which share both phenotypic and histological features consistent with the human condition.
3. This porcine model demonstrates the presence of non arterial levels of venous hypertension not only in the saphenous veins but also within its superficial tributaries (veins A – C.), which is stable at least three months post formation of the AVF.
4. It utilises an animal of comparable size to the human. This in turn means this large animal model of venous insufficiency will allow investigations which can evaluate both pharmaceutical and surgical intervention including new sclerotherapy and valve replacement techniques that may have a clinical application.
5. Swine are a large animal which are readily available through both agricultural sources and commercial suppliers of laboratory animals while there is reduced availability of dogs due to restrictive legislation and public concern with the use of pet-type animals in research.155
6. Swine are animals which have been extensively used as surgical models for humans.

7. Their large size enables large volumes of tissue to be collected for further analysis.

8. The vascular wall response in pigs to atherogenic stimuli is similar to the response of human arteries\textsuperscript{156, 157} and this may suggest that the both porcine and human veins may respond in similar fashions.

9. It is a chronic and stable model which will allow both quantitative and qualitative evaluation of the underlying disease process, from the onset of the condition thus improving our understanding of the condition as a whole.

5.2 Disadvantages

It has to be acknowledged that this porcine model does have some disadvantages including:

1. It is a chronic model taking up to 8 weeks to develop varicosities.

2. It is expensive, costing approximately $5000 each animal.

3. It has a high failure rate, though this is comparable to other popular CVI models currently being utilised.

4. The size of the animals toward the end of the study makes them increasingly hard to manipulate and manage.

5. No clinical manifestations of chronic venous insufficiency developed during the study period. The study period was only 13-16 weeks, a significant portion of
patients with proven venous insufficiency do not develop cutaneous manifestation associated with venous hypertension for a number of years.

6. Require further characterisation and refinement with assessment ambulatory venous pressures in particular and implementation of suggested modifications.

In conclusion we describe a porcine AVF model of venous insufficiency, which is simple to create and closely replicates the human condition of venous insufficiency and superficial varicose veins. The formation of an AVF between the femoral vessels leads to the progressive loss of venous valve competence and the development of superficial varicosities, which demonstrated elements consistent with the human disease including venous reflux and hypertension and pathological remodelling of the vein wall. The mechanism by which these varicosities were formed appears to be a combination of retrograde flow accompanied by increased pressures within the superficial veins.
References


