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# Investigation of Lymph Node Transplantation as Therapy for Breast Cancer Related Lymphedema

by

Dalia Tobbia

A thesis submitted in conformity with the requirements  
for the degree of Doctor in Medicine  
Graduate Department of the Faculty of Health Sciences  
School of Medicine  
University of Dublin Trinity College

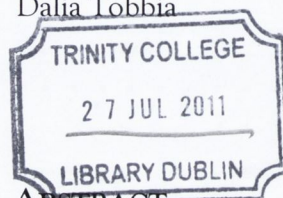
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ABSTRACT

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Humans have hundreds of lymph nodes, collections of which are found in the underarms, groin, neck, chest, and abdomen. They have long been considered 'neutral' elements in terms of lymph transport. An assumption has always been made that the impediment to lymph transport in post-surgical lymphedema is due to lymphatic vessel injury. However, these vessels are damaged routinely in surgical procedures, despite this, post-surgical edema usually resolves over time as the lymphatics regenerate and chronic edema rarely if ever occurs under these circumstances. In contrast, it is clear that the removal of a lymph node or nodes appears to be a prerequisite for lymphedema development especially if this is combined with radiotherapy. Consequently, there is reason to believe that there are other factors that require consideration. In the first instance the aim of the studies reported here was to develop an animal model of post-surgical lymphedema that would permit quantitation of lymphatic transport function after the removal of a single popliteal lymph node in sheep and correlate this with edema formation. Secondly, we used this model to test whether the transplantation of an autologous lymph node into a nodal excision site would restore lymphatic transport function and reduce the magnitude of post-surgical lymphedema.

As a measure of lymph transport,  $^{125}\text{I}$ -Human Serum Albumin was injected into pre-nodal vessels at 8, 12 or 16 weeks after surgical intervention and the plasma levels of the protein tracer were used to calculate the transport rate of the tracer to blood (% injected Radioactivity/hr). Edema was quantified from the circumferential measurement of the hind limbs. Compared with control limbs ( $17.2 \pm 0.6$ ,  $n=7$ ), lymphatic function was depressed at 8 weeks post nodectomy ( $10.6 \pm 1.5$ ,  $n=7$ ). At 12 ( $14.4 \pm 1.0$ ,  $n=7$ ) and 16 weeks ( $13.9 \pm 1.0$ ,  $n=6$ ). Lymphatic function at 8 and 12 weeks in limbs subjected to sham surgical procedures was ( $16.6 \pm 0.7$ ,  $n=6$  and  $16.1 \pm 0.7$ ,  $n=6$ ) respectively. The transplantation of avascular lymph nodes at similar times reduced lymphatic function significantly ( $12.3 \pm 0.5$ ,  $n=6$  and  $12.6 \pm 0.8$ ,  $n=6$ ). In contrast, when vascularized transplants were attempted, lymphatic function was similar to the control groups and significantly greater than that of the avascular group ( $15.8 \pm 0.9$ ,  $n=9$  and  $15.7 \pm 1.0$ ,  $n=10$ ). Lymph transport correlated significantly with the health of the transplanted nodes (scaled with histological analysis). When lymph nodes were removed, all affected limbs became edematous. The vascularized node transplants were associated with the greatest improvement in edema with the magnitude of edema in such limbs exhibiting significantly lower levels of edema than non-treated limbs. These techniques may be helpful in understanding the pathophysiology associated with cancer-related post surgical lymphedema and may facilitate the development of new strategies to treat or prevent this condition.

It is a miracle that curiosity survives formal education

---

*Albert Einstein (1879-1955)*



## DEDICATION

I dedicate this thesis to my darling husband, Dr. Thorsten Sattler, thank you for always being there, constantly encouraging me and giving me the freedom to pursue my goals. Not to mention having to endure so many unsavory dinners while I was in Canada over the past two years.

To my father Dr. Iqdam Tobbia for encouraging me to do my best and to and my mother Teresa Tobbia who has always believed in me.

## ACKNOWLEDGMENTS

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It has been a great privilege for me to spend this time in professor Johnston's lab. I could not imagine a more agreeable working environment or a more stimulating mentor. He would come to the lab several times per day to oversee the running experiments and share in the successes and frustrations of everyday lab work. His easy-going nature and flair for storytelling made even the most disheartening results seem trivial.

Special thanks to Dianna Armstrong who is undoubtedly the queen bee presiding over us, making sure the Johnston lab runs smoothly. Not to mention her touch of magic.

I was also truly lucky to have Gurjit Nagra, Lena Koh and Amy baker as my lab colleges, who helped to create a most friendly environment.

Sincere thanks to Sarah Moore for all the sheep lifting and late nights in the lab following Microsurgery.

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I would like to express my gratitude to Professor Reynolds for facilitating the submission of this thesis.

Last but not least, I would like to extend special thanks to my sister Hala Tobbia for all her support during my stay in Canada.

## DECLARATION

Apart from the exceptions noted below, all surgical procedures and experimentation contained within this thesis were performed by myself. The derivation of all mathematical equations were the work of Dr. Michael Flessner, from the University of Rochester. Computational analysis of the data was performed by M. Katic from the Department of Research Design and Biostatistics, at Sunnybrook Health Sciences Centre.

Sarah Moore performed the CD3 Antibody staining.

Technical assistance with experimental setup and animal care during these studies was provided by Dianna Armstrong and Sarah Moore.

This thesis has not been submitted as an exercise for a degree at this or any other University.

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A handwritten signature in blue ink, appearing to read "David Talar". The signature is fluid and cursive, with the first name "David" and last name "Talar" clearly distinguishable.



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## LIST OF ABBREVIATIONS

$^{125}\text{I-HSA}$	Iodinated Human Serum Albumin
$B_{\text{in}}$	Blood In
$B_{\text{in}}$ (%/hr)	Transport Rate of the Injected Radioactive Tracer to Blood
LV	Lymphatic Vessel
LN	Lymph Node
$K_{\text{exp}}$	Albumin Elimination Rate
$V_p$	Plasma Volume
$C_p(0)$	The Concentration of Tracer at Time 0
$C_p(t_i)$	The Concentration of Tracer at 4 Hours
CPM	Counts per Minute
PBS	Phosphate Buffered Saline
H&E	Hematoxylin and Eosin Staining



## CHAPTER 1:

### GENERAL INTRODUCTION

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## 1.1 Overview

The lymphatic circulation is continually responsible for transportation of extravasated fluid and protein from tissue spaces back to the blood circulation. This function is frequently disrupted when lymph nodes are removed as part of cancer diagnosis and treatment. Unfortunately, the development of post-surgical lymphedema in these patients is not uncommon.

The popular conception is that lymphatics are damaged during the excision of lymph nodes and that this causes impediment to lymph flow. In this regard, there is some scientific interest in developing molecular therapies to enhance the process of lymphangiogenesis. However, there is reason to believe that re-establishment of 'normal' fluid dynamics after tissue injury involves more than simply the regeneration of lymphatic vessels. Is it possible therefore, that the lymph nodes have important functions in maintaining fluid balance in addition to their more recognized immunological duties?

In light of this, the goal of my experimental studies over the past two years was to provide a better understanding of the pathophysiology of the lymphatic transport system. Sheep were primarily used for these in vivo experimental preparations, focusing namely on lymph clearance in the normal situation and following various surgical interventions, including the development and progression of lymphedema.



## Anatomical Design and Special Consideration of the Lymphatic System

### 1.2.1 The Interstitium

Approximately 99.9% of interstitial fluid, also known as tissue fluid exists in a gel-like state (Adair and Guyton, 1985) (Adair, 1985a). This means that in non-edematous tissues less than 1% of the tissue fluid exists as free fluid available for exchange. This gel interstitium is made up of several types of proteoglycans and collagen. Proteoglycan filaments form weak crosslinks with each other, with collagen fibers and protein molecules giving rise to the gel-like consistency of the interstitium. This means that tissue fluid does not flow freely in normal tissues. It is due to this feature of the interstitium that fluid does not collect readily in dependent tissues.

### Formation of Lymph

Lymph is an ultrafiltrate of blood containing all of the constituents of plasma. Its formation takes place at the capillary level according to Starling forces (Starling, 1894; Starling, 1896) which state that fluid transfer is determined by the difference between hydrostatic and osmotic pressures. The transcapillary hydrostatic pressure gradient causes fluid movement from the capillaries to the tissue spaces. This movement is opposed by the colloid osmotic pressure gradient which tends to transfer fluid back into the blood capillary. On the arteriolar end of the blood capillary, the hydrostatic pressure gradient is greater than the osmotic pressure gradient leading to a net movement of fluid into the interstitial spaces. This is reversed at the venular side of the capillary leading to movement of the filtered fluid back into the blood capillary. However, in most tissues of the body the hydrostatic pressure gradient is slightly greater than the colloid osmotic pressure gradient,

causing the net driving pressure directed from the capillary lumen toward the surrounding tissues to be slightly positive and thus causing a continuous leakage of plasma ultrafiltrate out of the capillaries. A small amount of protein leaks out into the tissue space as part of this process. This forms what is known as tissue fluid. The return of this extravasated fluid and protein back into the the blood circulation is an essential physiologic function of the lymphatic system.

### Structure and Function of the Initial Lymphatic Pump

Initial lymphatics possess specialized features, allowing the active removal of tissue fluid from the interstitial space. Adjacent endothelial cells form finger-like ramifications created by a series of valve-like slits in the vessel wall. Specialized filaments anchor these endothelial cells to the surrounding tissue. Due to this relationship, when the tissues expand as a result of fluid accumulation within the interstitial spaces, the lymphatic capillaries are pulled open and automatically fill with tissue fluid (Adair, 1985b). The pressure within these capillaries becomes negative when they are opened, therefore it is likely that the lymphatic pump is able to suck fluid directly from the tissue spaces and create the negative tissue interstitial fluid pressures that exist in non edematous tissues.

The valve flaps are only capable of opening inwards, directing the flow of lymph in a forward direction and preventing backflow. Moreover, endothelial cells are not tightly bound together, permitting large particles such as proteins to pass between them.



### Function of the Lymphatic Vessel Pump Beyond the Absorbing Lymphatics

The lymphatic pump consists of multiple segments separated by one-way valves referred to as lymphangions, these represent the basic subunit involved in lymph propulsion.

Forces that compress the lymph vessel causing the pumping of lymph can either result from normal movements of the body or from intrinsic contractions of the larger collecting ducts (both pre- and post-nodal). These vessels are equipped with smooth muscle cells in their walls arranged in a circular manner and vessel contractions occur in coordination with one-way valves that propel lymph flow centrally (Benoit et al., 1989; Crowe et al., 1997; Elias and Johnston, 1988; Elias et al., 1990; Hargens and Zweifach, 1977; Li et al., 1998; McHale and Roddie, 1976).

### Anatomy and Structure of the Lymph Node

Lymph nodes are small, kidney shaped organs found along the course of lymphatic vessels so that lymph draining back to the bloodstream passes through them. They tend to cluster in groups, particularly at locations where lymph vessels merge to form larger trunks, such as in the axilla. The parenchyma of the node consists of an extensive network of fine reticulin fibers which provides a loose framework for the populations of lymphocytes that inhabit the node. Lumina of the afferent and efferent vessels are continuous with a labyrinth of lymphatic sinuses through which lymph flows.

The outer cortex consists of densely packed lymphoid follicles where B lymphocytes are localized, whereas the paracortex mainly holds populations of T lymphocytes. The medulla contains primarily B lymphocytes and their derivatives. The entire node is encapsulated by a dense layer of connective tissue providing the main structural support.



Trabeculae extend from this capsule for a variable distance into the substance of the node (Young, 2001).

Blood supply to the node is derived from arteries which enter at the hilum and branch into the medulla. The number of arteries directly supplying the lymph node of mammals can range from one to three vessels (Herman et al., 1969; Herman et al., 1972; Sainte-Marie, 1968). However, in some instances there can be as many as ten to twelve arteries that penetrate the node, originating from a delicate arterial anastomotic network embedded in the surrounding fat (Heath and Brandon, 1983).

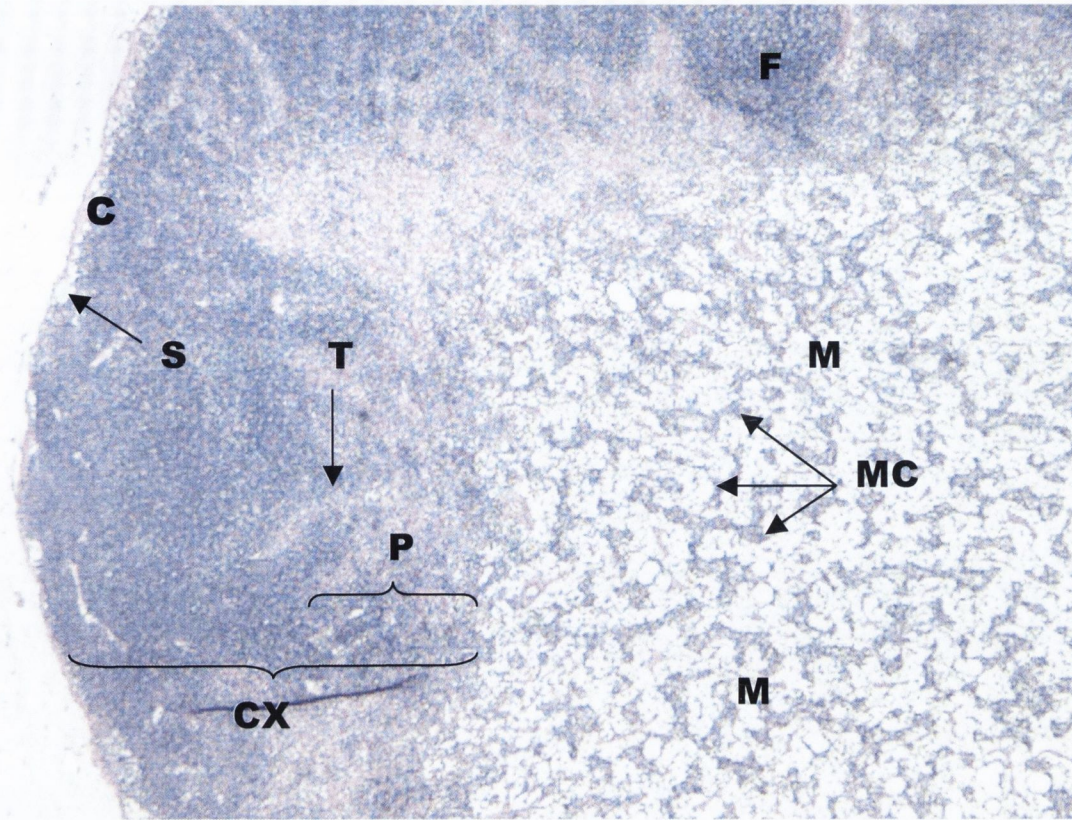


Figure 1-1 Sheep Popliteal Lymph Node Histology Stained with H&E

C - Capsule

CX - Cortex

P - Paracortical zone

S - Subcapsular sinus

F - Follicle

T - Trabeculae

M - Medulla

MC - Medullary cord



### Flow of Lymph Through Lymph Nodes

The pre-nodal (afferent) lymph vessel transporting protein and fluid absorbed from the interstitial space empties into the subcapsular sinus of lymph nodes. These lymphatic afferents branch out extensively upon reaching the surface of the node giving several terminal afferents that infiltrate into the node at multiple locations, presumably to help increase the surface area over which the lymph percolates through the node. Lymph within the node moves first through the trabecular sinuses and then into the labyrinthine medullary sinuses. Finally, it is drained from these sinuses by efferent lymphatic vessels located at the hilum of the nodes.

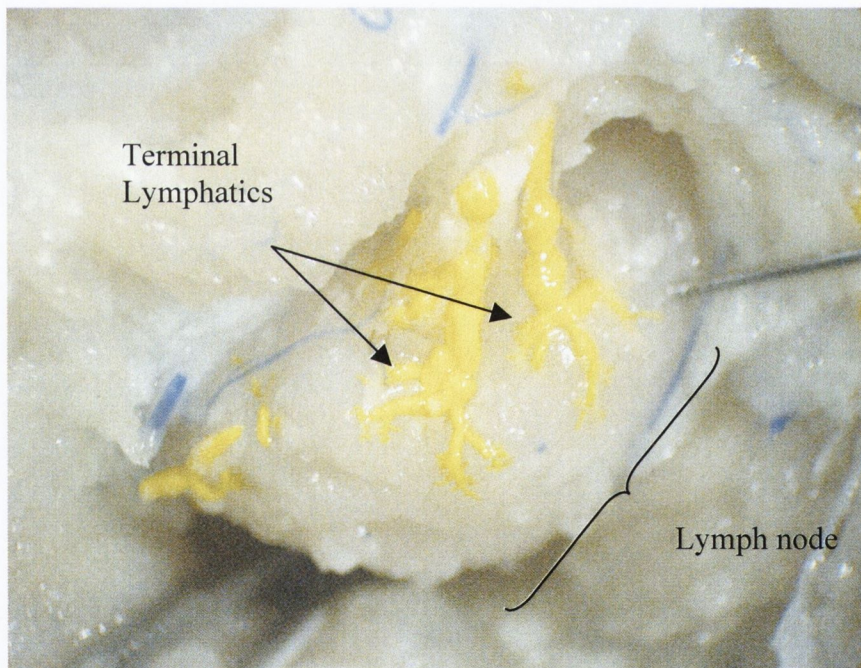


Figure 1-2 Lymphatic Vessels Highlighted with Yellow Microfil® Illustrating the Extensive Branching of the Terminal Afferents as they Pierce the Substance of the Lymph Node



### Evidence of Lymph Node Concentrating-Diluting Mechanism

Lymph is almost entirely formed at the lymphatic capillary level, however, protein concentration of lymph can be altered as it flows through the lymphatic system. Although lymph vessels have the potential to alter lymph composition (Guyton et al., 1979; Guyton., 1979), modification would appear to take place mainly within the nodes (Renkin, 1979)

In fact, one of the neglected physiological properties of lymph nodes is their ability to vent water into the local vasculature facilitating its removal from the lymphatic system, due to an extensive network of blood capillaries that are in close contact with lymph in transit through the sinuses (Belisle and Sainte-Marie, 1990; Salvador et al., 1992). Since the colloid osmotic pressure of prenodal lymph is low in most tissues of the body (i.e. the protein content of lymph is considerably lower than that of blood (Yoffey, 1970)), protein-free fluid is absorbed from the lymph into the blood capillaries in the nodes to establish an equilibrium of Starling forces across the lymph-blood barrier. Remarkably, the protein concentration of lymph can be increased as much as 300-400% after one passage through the node (Adair, 1985c; Adair and Guyton, 1983; Adair and Guyton, 1985; Adair et al., 1982). In this sense, the lymph node acts as a fluid exchange chamber.

In the studies of Jila et al., the measured intra-lymphatic pressures in upstream popliteal pre-nodal vessels using a servo-null micropipette system after obstructing flow downstream of the node, failed to produce the expected rapid rise in pressure when lymph outflow from the network was blocked downstream of the node (post-nodal). This suggests that fluid was lost from the system probably via the mechanism outlined above (Jila et al., 2007).

Although this dampening effect caused by the continuous loss of water in the nodes will have finite limits that may be overcome at higher lymph flow rates, it still represents an important 'safety mechanism' to help maintain pressure within the lymphatic system at a reasonable level when lymph flow rates are very high or when downstream flow is obstructed. This function would presumably be lost when nodes are removed in cancer patients.

### Impact of Lymph Nodes on Lymphatic Pumping Activity

Lymph nodes play a vital role within the lymphatic circulatory system; consequently their loss may have a more significant impact on tissue fluid balance than is commonly assumed.

The pressures in pre-nodal vessels can be quite high with measurements around 22mmHg achieved in rat mesenteric preparations (Zweifach and Prather, 1975) and 30mmHg in human popliteal vessels (Olszewski, 1985), facilitating lymph transport at high pressures (Eisenhoffer et al., 1994). In contrast, post-nodal pressures are considerably lower making it evident that the build-up of pressure in the pre-nodal ducts is dissipated in the lymph nodes, which appears to anatomically separate the lymphatic system into higher and lower pressure areas.

Without this dissipation of pressure within the lymph nodes, intra-lymphatic pressures in the downstream vessels are presumably higher forcing them to operate under sub-optimal conditions. This would make the lymphatic system less effective at transporting interstitial fluid and in the cancer patient, could compromise lymph transport and contribute to edema formation.



### Interaction Between Lymph Nodes and Perinodal Fat Tissue

Lymph node collections around the body are embedded in a pad of fatty tissue. Even in very lean mammals it is the last resource of fat to be depleted (Pond, 1994). The unique paracrine interplay between lymphoid cells and the adipose tissue that envelopes lymph nodes seems to play a role in the local immune response, by regulating the metabolism of lymphoid cells in the nodes, making it distinct from other fatty tissue around the body (Pond and Mattacks, 1995). Thus the local transient immune response is possibly to a significant degree dependant upon nutrients supplied by the adjacent adipose tissue (Pond, 2003). In this sense, perhaps there is an added benefit to harvesting the perinodal fat along with the lymph node in transplants.

### **Post Surgical Lymphedema Associated with Lymph Node Excision**

In a broad sense lymphedema is the development of swelling due to a functional overload of the lymphatic system exceeding its drainage capacity. Even with modern advances in research the precise pathogenesis remains elusive.

Lymphedema associated with cancer-related lymph node dissection has been termed 'the secret epidemic' (Armer, 2001). The accumulation of protein and fluid in the interstitium provides an excellent medium for the growth of organisms, leading to recurring bouts of cellulitis and lymphangitis and if left untreated it can lead to impaired limb function, psychosocial problems and in extreme cases, malignant complications and life-threatening infections. While a lymph transport deficit is recognized to be the general cause of this disorder, we are no closer to understanding why lymphedema develops in



some patients and not in others and why the edema can be unevenly distributed in the arm with some regions spared completely (Stanton et al., 2006).

What is also surprising is that this disorder does not necessarily happen acutely, but can take place many years following the initial nodectomy, and may be triggered by a minor event such as a laceration or insect bite in the affected limb. The reported incidences of lymphedema following mastectomy and axillary node removal are fairly significant ranging between 20-40% (Armer, 2001). Treatment options for these patients have been met with some success but remain controversial and are far from ideal specially with entrenched, chronic edema (Badger et al., 2004). Most individuals are offered some form of non-surgical external compression therapies. The objective of such interventions is to 'milk' the interstitial fluid and lymph through the lymphatic system in order to decongest the affected limb. This is achieved by the application of compression garments, the use of intermittent or sequential pneumatic compression devices and massage therapy. Although compression therapies do provide a degree of symptomatic improvement, they do not address the underlying problem and need to be applied continuously, possibly even on a daily basis in many patients.

Several surgical approaches for lymphedema therapy have been investigated. However they are not commonly available to patients. These include lymphatic-venous anastomoses (Campisi et al., 2001; Koshima et al., 2000; Yamamoto et al., 2003), lymphatic vessel transplantation (lymphatic to lymphatic anastomoses) (Baumeister and Frick, 2003; Weiss et al., 2003), omental grafts that contain lymphatics (Benoit et al., 2005; O'Brien et al., 1990), implantation of lymph node fragments (Fu et al., 1998; Pabst and Rothkotter, 1988; Rothkotter and Pabst, 1990), and vascularized lymph node transplantation (Becker et al., 2006; Chen et al., 1990). It is also interesting to note that

current therapeutic measures are usually offered only to patients with long-standing, established edema. Under such conditions the probability of complete reversal of symptoms seems unlikely no matter what therapeutic measures are applied. Therefore, if nodal therapy was to be applied at the time of the initial surgery or shortly thereafter and before the chronic sequelae to the limb takes place, would it have greater potential to avert or at least result in improved clinical outcomes?

#### **1.4 Lymphedema, a Problematic Complication of Breast Cancer Therapy**

Among the multitude of late side effects of breast cancer treatment, secondary upper extremity lymphedema is one of the most problematic and relatively underestimated complications. Originally described by Handley in 1908 (Handley, 1908) and first termed as “Elephantiasis Chirurgica” in 1921 by Halsted (Halstead, 1921). More than a hundred years since it’s initial description, it remains a poorly understood condition that has the potential to occur after any intervention affecting lymph node drainage mechanism. However upper extremity lymphedema occurs most commonly in association with breast cancer (Engel et al., 2003; Geller et al., 2003; Mathew et al., 2006; Petrek et al., 2000; Petrek et al., 2001; van der Veen et al., 2004; Ververs et al., 2001). It is generally viewed as an inconsequential complication of breast cancer, and when ineffectively managed leads to an increasingly debilitating condition that has no cure (Petrek et al., 2000; Petrek et al., 2001).

It is believed that the operated limb has reduced immunity due to the loss of lymph nodes, this in addition to fibrosis of tissues leading to stasis of lymph which encourages infections and secondary inflammation. If left untreated or undertreated can lead to further tissue fibrosis, hardening of skin, lobulations, blistering and skin breakdown



acting as a vicious cycle which ultimately leads to worsening of lymphedema (King MJ, 2005).

The modern approach to management of lymphedema involves patient education and promoting informed self monitoring of changes that are suggestive of early disease onset. Symptoms of heaviness, aching, skin tightness, pain, numbness, weakness or impaired arm function possibly indicate a pre-clinical phase of lymphedema onset and warrant prompt medical evaluation (Mayrovitz, 2009; Meneses and McNees, 2007; Nielsen et al., 2008).

The onset of lymphedema evolves at various rates in different individuals, there can be a significant delay from the initial surgical insult until swelling develops. It does not necessarily occur immediately post operatively, but can happen up to several years following the initial axillary trauma and often reported to be triggered by a minor event (Kosir MA, 2001; Petrek et al., 2000). Interestingly 75% of women who do develop breast cancer related lymphedema will have it during the first year post operatively and 90% by the third year (P.S. Mortimer, 1996).

## **1.5 The Psychologic Morbidity of Breast Cancer Related Arm Swelling**

Lymphedema is a chronic condition involving abnormal accumulation of protein rich fluid in the interstitial space of the affected limb after lymph node excision and radiation therapy. Breast cancer related lymphedema is a distressing condition affecting one in five breast cancer survivors. It is increasingly curable and as life expectancy improves for breast cancer survivors, more women will be living with symptoms of lymphedema.

The impact of lymphedema on quality of life is reported as being negative (Engel et al., 2003; Morgan et al., 2005) and its onset is followed by surprise, emotional distress,



sorrow and depression (Armer et al., 2004; Morgan et al., 2005; Petrek et al., 2001). For many patients developing lymphedema post cancer therapy is a constant reminder of their underlying illness and can pose a greater challenge to deal with than the cancer itself.

The stigma of this condition and the reaction of others, often causes patients to develop a poor self-image, become isolated and refrain from their normal activities. Which often results in social withdrawal and dramatic life changes (Ahmed et al., 2008; Carter, 1997; Johansson et al., 2003; Passik and McDonald, 1998).

### **The Concept of Sentinel Lymph Node Sampling**

The general tendency in breast cancer surgery over the years has been a “less is more” philosophy. Smaller portions of the breast are often removed in current practice and sentinel node excision is applied routinely as opposed to taking out many nodes in the drainage basin of the tumor.

Axillary lymph node surgery remains an essential aspect of breast cancer diagnosis and treatment. Assessment of the axilla is required in all cases of invasive breast cancer. Lymph node status assists in staging the cancer as it provides essential prognostic information which helps to guide further adjuvant treatment such as chemotherapy, hormonal therapy or radiotherapy and remove diseased nodes (Luini A, 2005; Morrell et al., 2005). Metastatic spread to the axilla occurs in approximately 30% of patients with breast cancer (Chang JC, 2004).

Until recently full axillary lymph node dissection was required for management of the axilla. However this surgery carries a significant risk of permanent lymphedema and increasingly less invasive surgical options namely axillary node sampling and sentinel lymph node biopsy are increasingly employed.

The first lymph node(s) standing guard in the axilla, that receives lymphatic drainage from breast parenchyma and the tumour is called the sentinel node. The main advantage of sentinel lymph node biopsy is that it reduces the likelihood of developing lymphedema by reducing the number of unnecessary axillary lymph node dissections. There is also a proven correlation between the number of lymph nodes removed at surgery and the development of lymphedema (Engel et al., 2003; Goffman et al., 2004; Querci della Rovere et al., 2003). Early studies proved that the sentinel node was positive in all but few cases that had any nodal involvement on subsequent full axillary lymph node resection. (Boolbol et al., 2001; Krag et al., 1998). Lymphatic mapping with sentinel node biopsy has gained wide clinical acceptance and has become the preferred method over axillary lymph node dissection, increasingly it is used to evaluate axillary status in patients with early stage disease with clinically negative nodes (Geller et al., 2003; Kwan et al., 2002; Mansel et al., 2006; Temple et al., 2002; Wilke et al., 2006). Recent literature advocates that it accurately stages the axilla while reducing arm morbidity commonly associated with traditional axillary lymph node staging (Blanchard et al., 2003; Del Bianco et al., 2008; Kim et al., 2006; Schrenk et al., 2000; Veronesi et al., 2009). However the advent of sentinel lymph node biopsy will not completely eliminate the problem of postoperative lymphedema.

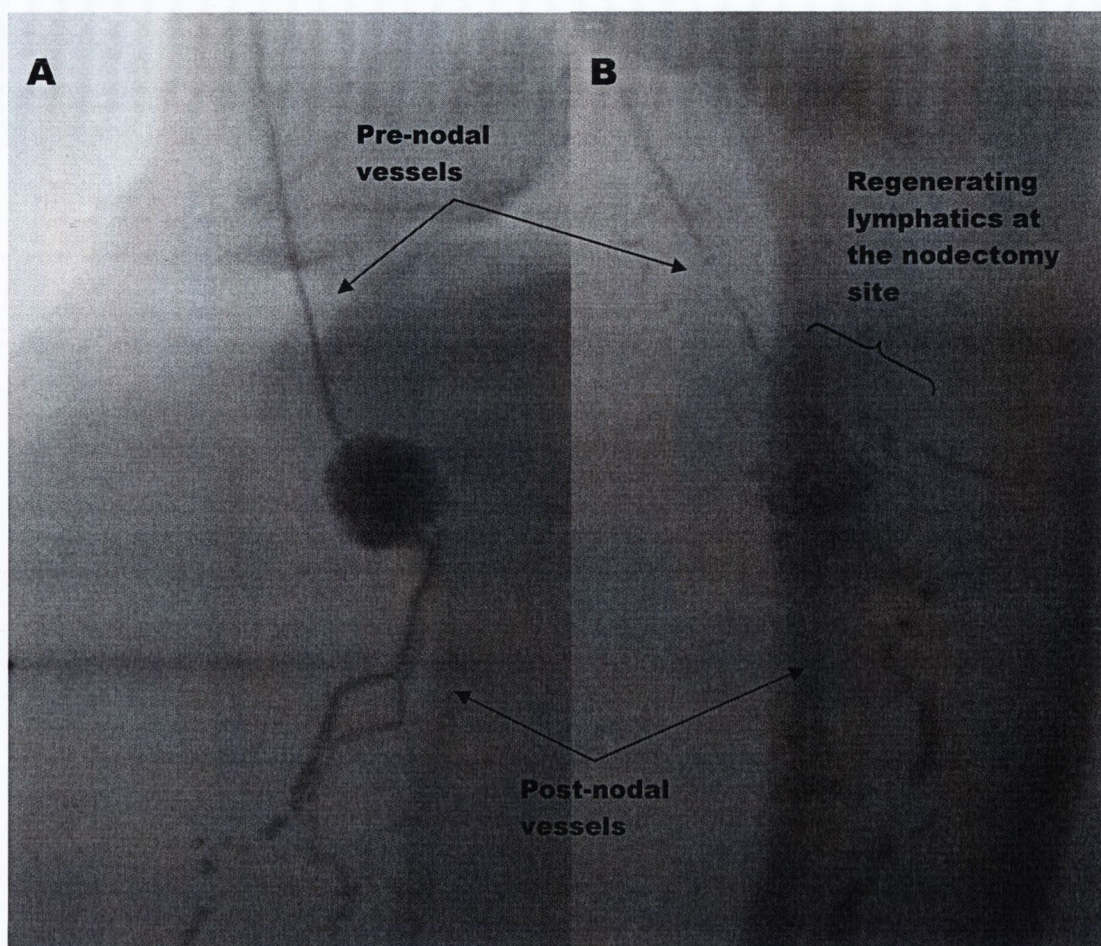
Morbidity following sentinel lymph node biopsy alone is not negligible, but still significantly less frequent as compared with level I, II and complete axillary lymph node dissection (Blanchard et al., 2003; Langer et al., 2007; Wilke et al., 2006). Patients with positive sentinel nodes will require full axillary lymph node dissection or radiotherapy (Lyman et al., 2005; Purushotham et al., 2005; Veronesi et al., 2003).



## 1.7 The Role of Lymphangiogenesis in the Restoration of Lymphatic Continuity

It is generally accepted that damage to the lymphatics is involved in the pathogenesis of lymphedema. This has led to the assumption that stimulating new vessel growth is an appropriate therapeutic measure. Several groups are trying to achieve this by applying selected agents directly to affected tissues (Szuba et al., 2002) or by introducing the molecules through gene therapy approaches (Saaristo et al., 2002; Yoon et al., 2003). While lymphangiogenesis has been researched for many years what is generally not appreciated is the fact that it is a very vigorous process (Witte, 2001; Yoffey, 1970). Indeed, once damaged, the remarkable regenerative capacity of lymphatics and their ability to develop collateral pathways around the site of obstruction soon bridge the gap (Hadamitzky and Pabst, 2008; Piller, 1985). Also an important component of the lymph transport system is the ability of pre- and post-nodal lymphatics to contract and pump lymph. From the studies of Ikomi (Ikomi et al., 2006), it would seem that the regenerating lymphatic vessels re-acquire a contractile phenotype as early as four weeks after nodal excision.





**Figure 1.3** Fluoroscopic Images of the Popliteal Lymphatic System in Sheep

A - Sheep popliteal lymphatic region in the normal/control situation

B - Illustration of regenerating lymphatics at 16 weeks post nodectomy

## **Implications of Vascularized Lymph Node Transplantation**

In the context of secondary lymphedema, the physiological role of lymph nodes in tissue fluid balance has generally been ignored. It is commonly assumed that removal of the node merely interrupts the flow of lymph between the cut ends of the pre- and post-nodal vessels.

However, there are several reasons to suspect that the re-introduction of an autologous vascularized lymph node at the excision site would have a beneficial effect. First, the transport of protein, solutes and water from the interstitium to the bloodstream involves an integrated network of lymphatic vessels and lymph nodes. The replacement of a node could help to restore the correct physiological relationships between pre- and post-nodal lymphatic vessels. Second, in combination with the appropriate cellular and molecular cues, the lymph node may provide structural orientation during lymphangiogenesis and facilitate the re-alignment of pre- and post-nodal vessels. Third, one cannot discount the possibility of immunological benefits as well. In addition, lymph nodes also act as filters and presumably provide some impediment to the metastatic distribution of cancer cells.

## **Why Address Issues Related to the Role of Lymph Nodes Within the Lymphatic System and Advance the Field?**

Lymphedema has puzzled the biomedical community for many years. There are still many unaddressed issues regarding the pathogenic mechanisms responsible for this disorder and its treatment continues to pose a challenge for health care professionals. Indeed, the exact nature of the surgically induced deficit remains to be defined. While various mouse models have proven valuable in elucidating the molecular factors that



control lymphatic vessel growth, due to their small size they are less suitable for analysis and quantitation of the physiological parameters that govern lymph flow. In this regard, there would seem to be a need for the development of animal models of lymphedema that permit the evaluation of lymphatic function and allow assessment of the practical effectiveness of surgical or other forms of therapy.

With this in mind, lymphatic vessels in sheep may provide unique opportunities since the collecting ducts are relatively large and can be manipulated individually.

In this respect, the establishment of a new approach to treatment lies in the bounds of further understanding the pathophysiology of post-surgical lymphedema.



## **General Hypothesis**

It is the absence of lymph nodes after excision for cancer that shifts the balance towards a predisposition for lymphedema and not necessarily an inability to regenerate new lymphatic vessels. Therefore, transplantation of a lymph node into the excision site will have the potential to improve tissue drainage compared with no treatment.

## **Objectives of the Experimental Studies Outlined in this Thesis**

To further expand our understanding of lymphatic system function in the normal situation and following surgical manipulation, the main goal of this thesis is to deal with the following experimental objectives:

- 1) To develop a model of lymphedema based on the removal of a single lymph node in sheep.
- 2) To quantify lymphatic transport over time and correlate this with edema formation in the lymphedema model.
- 3) Assess whether the transplantation of an autologous lymph node will reduce or prevent the development of lymphedema.

CHAPTER 2:

LYMPHEDEMA DEVELOPMENT AND LYMPHATIC  
FUNCTION FOLLOWING LYMPH NODE EXCISION IN  
SHEEP



CHAPTER 2: LYMPHEDEMA DEVELOPMENT AND LYMPHATIC FUNCTION

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## **Introduction**

Lymphatic vessels rapidly regenerate following lymph node excision in an attempt to bridge the resultant gap and re-establish continuity. However, very little is known about the physiologic properties of these newly formed vessels and how the transport capabilities of regenerating ducts are integrated functionally into a lymphatic network that includes lymph nodes as well as pre- and post-nodal collectors.

The studies contained within this chapter were designed to achieve the first two experimental objectives of developing an animal model that would permit quantitation of lymphedema and lymphatic function after lymph node excision.



## Development of the Experimental Model Used in Our Investigations

In the first instance a study using 40 sheep was performed to examine lymphangiogenesis at 6, 12 and 16 weeks following surgical obstruction of postnodal lymphatic vessels in the popliteal, prescapular, and mesenteric lymph systems (Jila et al., 2007). It was observed in this instance that the interruption to lymph flow through the popliteal lymphatic vessels resulted in the generation of a fine network of lymphatic vessels that helped to re-establish lymph flow across the site of obstruction, despite this fact a significant resistance to lymph flow was detected at all time points. Lymph nodes were not excised in these experiments and their presence appeared to lessen the impact of post nodal lymphatic obstruction, presumably by facilitating extraction of water through the nodal vasculature. The conclusions of this study inspired further investigation of the physiologic properties of newly regenerated lymphatic vessels and how they are integrated to function as channels of lymph transport over time in the presence or absence of lymph nodes or node transplants. Several dissections were performed initially to ascertain the ideal lymph node system for this study. We found the popliteal lymph node to be easily accessible and micro-dissections revealed reasonably sized vessels piercing the peri-nodal fat pad to supply blood to this node. The main pedicle was derived from a branch of either the medial circumflex femoral artery or the caudal femoral artery, the former was more consistent (arteries between 1.0-3.0mm in diameter). The main vein draining the node was a branch of the lateral saphenous vein (between 3.0-5.0mm in diameter). The laparotomies we performed on sheep revealed quite large mesenteric lymph nodes with reasonably sized vascular pedicles, and we considered harvesting these nodes for use as free transplants, however this idea was abandoned as we felt that in addition to the intra-operative risk of

bowel perforation, the recovery period after a successful laparotomy would be longer and more complicated for the animals.

Quantitative assessment of lymphatic function; the ability to transport a known mass of radiolabeled Albumin to plasma provides a quantitative measure of the lymph transport effectiveness of a given lymphatic network. Under normal conditions, molecules with molecular weights >6000 KDa injected into the limb lymphatic vessel are almost completely recovered in the thoracic duct lymph (Aukland and Reed, 1993). Thus we planned to inject radioactive protein tracer into one of the pre-nodal popliteal vessels and measure it's rate of transport to plasma. This method or variations thereof has been used by the Johnston lab to assess lymphatic drainage from the subarachnoid compartment of the brain (Boulton et al., 1997; Boulton et al., 1999) and from the pericardial space (Boulanger et al., 1999). Initially the above method of assessing lymph transport rate was tested on eleven control sheep to assess if consistent transport rates could be obtained and if this was a reproducible model for our experimental purpose. In three of the eleven animals tested, the experiments failed due to technical difficulty related to cannulation of the pre-nodal lymphatic vessels. The other eight animals showed fairly consistent lymph mass transport readings. Then we applied the same concept after lymph node excision to compare with the controls.



## Materials and Methods

### Animals

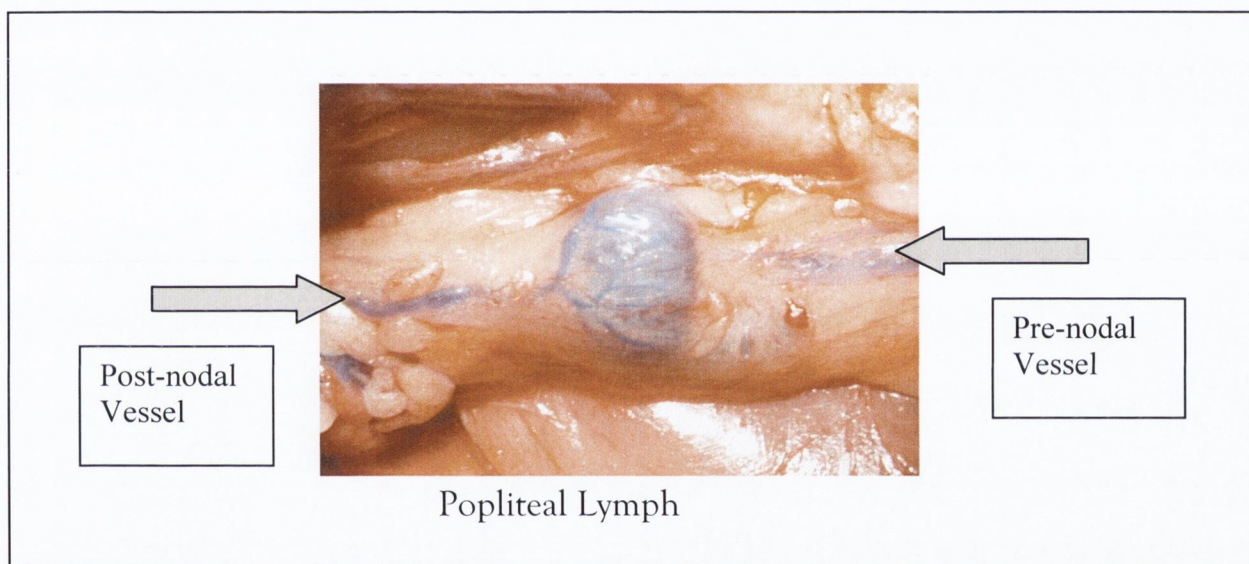
A total of 41 randomly bred male and female Dorset sheep ( $30.3 \pm 1.1$  kg) were used in this investigation. Animals were given free access to food and water for an observation period of one week preceding surgery. All experiments outlined in this paper were approved by the ethics committee at Sunnybrook Health Sciences Centre and conformed to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

### Surgery

Sheep are fasted 24 hours prior to anesthesia. The animals are anesthetized initially with 20ml I.V. injection of sodium pentothal. Subsequently, 2.0-3.5% Isoflurane is delivered through an endotracheal tube via a Moduflex, Dispomed machine with Hallowell respirator for surgical maintenance. The surgical site is shaved and washed with alcohol and betadine then draped with sterile sheets. Sterile Evans blue dye (1% in saline) is injected subcutaneously just proximal to the hoof to enhance visualization of the lymph node and the pre- and post-nodal lymphatic vessels.

A vertical skin incision (approximately 8-10cm long) is made over the lateral aspect of the popliteal region. The popliteal fossa is triangular in shape and can be found by retracting the biceps femoris muscle caudally at the level of the stifle joint. At its distal angle, a single popliteal lymph node lies embedded in a pad of fat. Once the node is exposed as illustrated in figure 2.1 the pre- and post-nodal lymphatic vessels are tied off with a silk suture and the node is excised. Hemostasis is ensured prior to closure of the

surgical site. The animals are returned to their holding pens after recovery from the anesthetic. Subcutaneous opioid analgesic is given post operatively for pain management. Antibiotic (Duplocillin) is given intramuscularly one day before surgery and again two days post operatively.



**Figure 2.1** Popliteal Node Highlighted With Evans Blue Dye Intra-operatively and Partially Dissected Free From its Surrounding Fat Pad



### Assessment of Edema

The hind legs are shaved closely and leg circumference measurements are taken at a point 10cm distal from the hock (tarsus). This landmark is highlighted pre-operatively with a skin marker. Using a blank piece of umbilical tape, circumference measurements are taken daily in the first week post-surgery and once a week after that until the animals are sacrificed. The limb circumferences are divided by the original (pre-surgical value) and expressed as percentage change over time.

To compare the edema outcomes in the various groups, the percentage change in limb circumference is plotted against time. Between the hoof and hock joints the sheep limb is fairly uniform in shape and diameter, eliminating the need for a circumference measurement at more than one point. Similarly, the forelimbs of 21 animals were shaved and measured throughout the experiments as controls at a point 10cm distal to the hock joint.

### Fluoroscopy

Imaging was performed on four sheep at eight weeks (one animal, two limbs), 12 weeks (one animal, two limbs) and 16 weeks post surgery (one animal, two limbs), in addition to one control animal (two limbs).

Evans blue dye (1% in saline) is injected subcutaneously to highlight the lymphatics prior to cannulation of an upstream popliteal pre-nodal vessel with a 26G angiocatheter. An x-ray contrast medium (1-3ml, Lipiodol, EZ-EM Canada, Therepex) is injected through the cannula and a mobile fluoroscopy system is used (BV Pulsera, Philips) to visualize the lymphatic vessels around the popliteal region.

### Immunohistochemistry

In ten animals the growth of new lymphatic vessels was assessed at the nodal excision site (lymphangiogenesis). Briefly, two methods were used to identify the lymphatic vessels. First, CFDA-SE (5(6)-CFDA SE [5-(and-6)-carboxyfluorescein diacetate succinimidyl ester] Molecular Probes # C-1157) is injected into an upstream pre-nodal lymphatic. This molecule diffuses passively into cells and remains non-fluorescent until its acetate groups are cleaved by intracellular esterases (it fluoresces green). This provided unequivocal identification of the lymphatics since the dye is introduced directly into the vascular lumen. As well, the sections are co-stained with antibodies to the lymphatic endothelial receptor for hyaluronan (LYVE-1) (tagged with Cy3-red).

Tissue blocks from the popliteal region are removed surgically. The samples are then embedded in Tissue-Tek O.C.T compound, placed in a base mold and frozen immediately at

- 80°C. Sections (7µm thick) are cut using a Leica cryostat (model Leica CM 3050S-3-1-1) and placed on glass microscope slides. The slides are washed 5 times in phosphate buffered saline (PBS-0.1 M) and blocked for one hour at room temperature with 10% goat serum in phosphate buffered saline (PBS). After washing with PBS, the sections are incubated overnight at 4°C with 1:50 or 1:100 dilutions of rabbit, anti-human LYVE-1 primary antibody (Research Diagnostics Inc.). The next day, the sections are washed with PBS and incubated with 1:100 dilutions of goat, anti-rabbit IgG antibody tagged with Cy3 (Jackson Immuno Research). In controls the primary antibody to LYVE-1 was omitted. Finally, the slides are mounted in aquapolymount and cover slipped.

Immunofluorescence microscopy is performed with a Zeiss Axiovert 100M laser scanning confocal microscope. The argon and helium/neon lasers are set to wavelengths of



488 and 543nm for excitation of CFDA-SE and Cy3 respectively. When both approaches are combined in the same tissue sample, the lymphatic vessels appear yellow-orange.

#### 2.2.6 Quantitative Assessment of Protein Transport to Plasma

The main role of the lymphatic vessel is to absorb extravasated vascular derived protein from the interstitial spaces and return it to the venous circulation. Therefore, the ability to transport a known mass of radiolabeled albumin to plasma provides a quantitative measure of the lymph transport effectiveness of a given lymphatic network. A schematic illustrating the features of the experimental design is provided in Figure 2.2. Quantitative studies were performed at 8, 12 and 16 weeks following popliteal nodectomy as well as in a node-intact group.

The animals are fasted and anesthesia is induced as previously described. The hind limbs are shaved and a final circumference measurement is made at the formerly explained landmark. A heparinized neckline is inserted into the jugular vein and secured for collecting blood samples throughout the experiment. Evans blue dye (1% in saline) is injected under the skin to highlight the lymphatics. When injected subcutaneously, the dye binds to protein and readily enters the absorbing lymphatics. It is distributed rapidly through the lymphatic network and outlines the collecting vessels clearly. An incision is made through the skin and subcutaneous tissues over the lower lateral aspect of the hind limb, extending distally from the hoof to expose several pre-nodal lymphatic vessels in close proximity to the lateral saphenous vein. With the aid of magnifying Carl Zeiss loupes (3,5x400) a single vessel is dissected free from the surrounding tissues and cannulated with a 26G angiocatheter. This is secured with 4.0 silk ties and a clamp applied distally to prevent backflow.

Saline (100μL) is then injected slowly over 30 seconds to check for any leaks at the cannulation site and to observe the flushing of the Evans blue dye in the lymphatic vessel. This process ensures the correct placement of the cannula. Radiolabeled human serum albumin (<sup>125</sup>I-HSA, 2mg in a 200μL volume) is injected into a pre-nodal lymphatic vessel over a 60 second period with a 250μL Hamilton syringe and flushed with 100μL of saline over 30 seconds. The cannula is then capped to prevent backflow. Swabs are taken from around the tip of the cannula as well as the injection site and later analyzed in the gamma counter to indicate whether or not there was a leak or spill of the radioactive tracer.

Blood samples are taken from the neckline to monitor the recovery of radioactivity in the blood over a period of four hours. Samples are taken at time zero, 15min, 30min and then every 30min up to four hours. The animal is sacrificed at the end of the experiment with 20ml of Euthanyl administered intravenously.

The concentrations of the radioactive protein tracer in plasma (CPM/ml) are divided by the amount injected to arrive at percent injected/ml, which is plotted over time. However, once a protein tracer enters blood, it re-filters back into the various tissues of the body and hence, tracer recoveries are inherently underestimated. An albumin elimination rate ( $K_{exp}$ ) has been defined in previous experiments (Boulton et al., 1997) and is used in a mass balance equation (equation 1) to reflect more accurately the ability of the lymphatic system to return protein to blood. The mass balance equation outlined below is used to estimate a single averaged mass transport rate:

$$B_{in} = \frac{[C_P(t_f) \exp(K_{exp} t_f) - C_P(0)] (K_{exp} V_P)}{\exp(K_{exp} t_f) - 1} \quad (1)$$



$B_{in}$  (blood in) = the mass transport rate (CPM/hour) is averaged from time zero to time  $t_f$  (duration of experiment) which in our case was 4 hours. The values for  $B_{in}$  derived from equation (1) are divided by the total radioactivity injected to give % injected/hr.  $C_p(t_f)$  = the concentration of the tracer at 4 hours;  $C_p(0)$  = the concentration of tracer at time zero;  $K_{exp}$  = is the coefficient of elimination of tracer from plasma. Since our previous experience indicated that this coefficient did not differ significantly between animals of various ages and weights, an average value derived from 41 animals used in previous studies (Boulanger et al., 1999; Boulton M, 1998a; Boulton et al., 1997; Boulton M, 1998b). Since the volume of distribution of the tracer (plasma volume,  $V_p$ ) would differ between animals, we adjusted the plasma recoveries to reflect this. Based on data derived in previous studies from our group (Boulanger et al., 1999; Boulton M, 1998a; Boulton et al., 1997; Boulton M, 1998b), we plotted the plasma volumes derived from 41 animals against their weights. We used a regression analysis of these data (equation 2) to calculate a plasma volume in each sheep based on the following equation:

$$y = 21.77x + 649.68 \quad (2)$$

### 2.2.7 Statistical Analysis

All data is expressed as the mean  $\pm$  SEM. The data was analyzed with a one-way analysis of variance ANOVA followed by contrasts back to baseline using a one-sided Dunnett's t-test. We interpreted  $P < 0.05$  as significant.



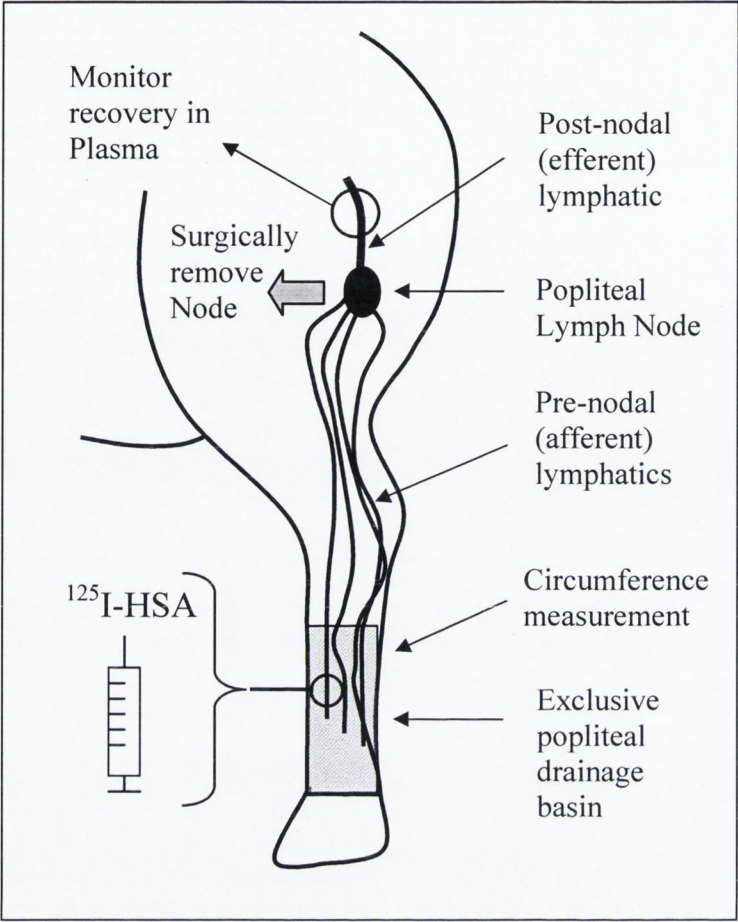


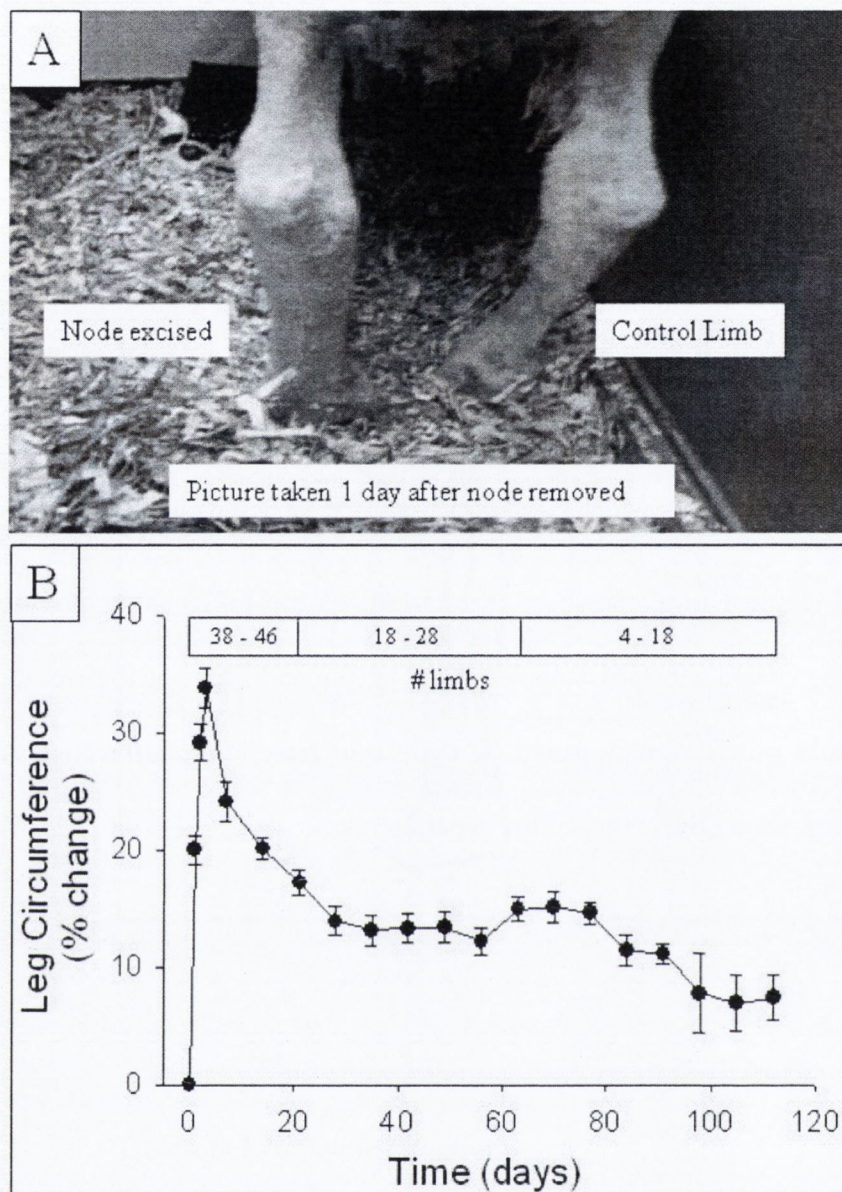
Figure 2.2      Schematic Illustrating Essential Features of the Experimental Model to Quantify Lymphatic Function

## Results

### Development of Lymphedema

The removal of a single popliteal lymph node resulted in edema formation in the lower hind limbs of all animals. An example is illustrated in Figure 2.3A. Figure 2.3B displays the averaged data. At day one after surgery the average percent increase in leg circumference was 19.5%, at day two 29.1% and at day three 33.8% (the peak of the response). The edema declined over time but in most animals did not subside completely and remained elevated until the sheep were sacrificed (up to 16 weeks post surgery). In three sheep, the edema subsided completely before the end point of the study. All of the control forelimbs which were measured showed no increase in circumference. All animals were ambulatory during the course of the experiments.





**Figure 2.3** Lymphedema Produced After the Removal of the Popliteal Lymph Node.

(A) Example of edema development following lymph node excision in comparison with a control limb.

(B) Quantification of edema after lymph node excision expressed as percentage change from pre-surgical levels. The numerals at the top of the figure illustrate the number of limbs that were assessed at various times following nodectomy.

### 2.3.2 Assessment of Lymphatic Function

When  $^{125}\text{I}$ -HSA was injected into an upstream pre-nodal popliteal lymphatic vessel in control limbs, it entered plasma with peak concentrations being achieved approximately one hour after injection (Figure 2.4). Measurement of plasma levels at 8, 12 and 16 weeks following lymph node excision revealed lower blood concentrations.

Figure 2.5 illustrates the averaged tracer mass transport rates over four hours calculated from equation one ( $B_{in}$ ). A one-way ANOVA revealed that the groups were significantly different. The values obtained at 8 ( $10.6 \pm 1.5$ ), 12 ( $14.4 \pm 1.0$ ) and 16 weeks post-surgery ( $13.9 \pm 1.0$ ) were less than that noted for the intact limbs ( $17.2 \pm 0.6$ ) but only the data at 8 weeks reached statistical significance with the Dunnett's t-test.

These data suggested that pre- and post-nodal lymphatic vessels had regenerated to a considerable extent, since the entry of tracer into plasma showed that some lymph continuity had been re-established. Indeed, at 12 and 16 weeks after surgery, lymphatic function had returned to approximately 80% of the level observed in control limbs. At 8 weeks post-surgery, only about 60% of lymph transport had been restored.



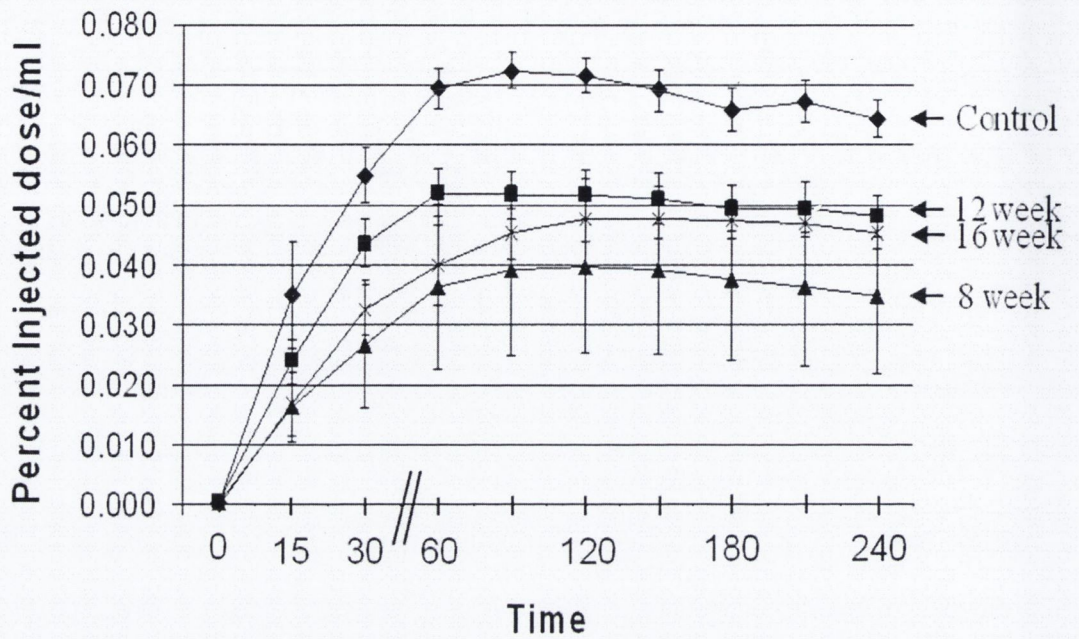


Figure 2.4 Appearance of Radioactive Albumin in Plasma Over Time  
(Quantification of Lymphatic Function)

Radioactive albumin was injected into a pre-nodal lymphatic vessel and the concentration of the tracer monitored in plasma for four hours after injection in the four groups of animals; control group (n=7) and 8 (n=7), 12 (n=7) and 16 weeks (n=6) post-surgery.

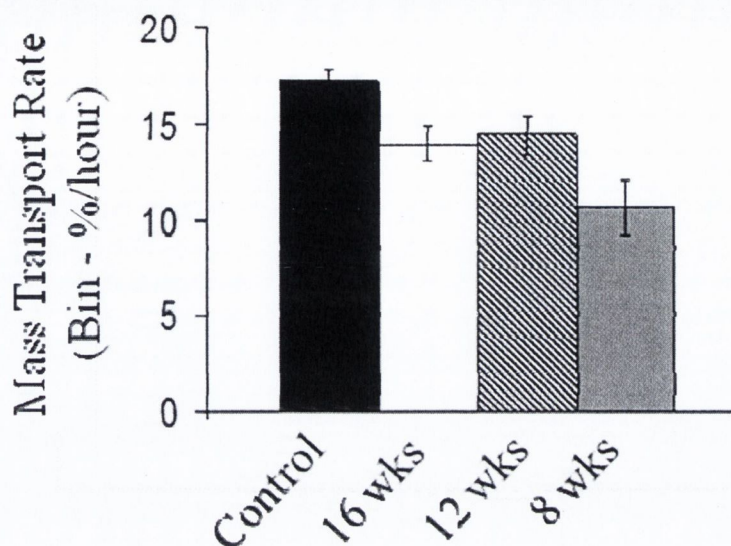


Figure 2.5 Averaged Lymphatic Mass Transport Rates (Quantification of Lymphatic Function)

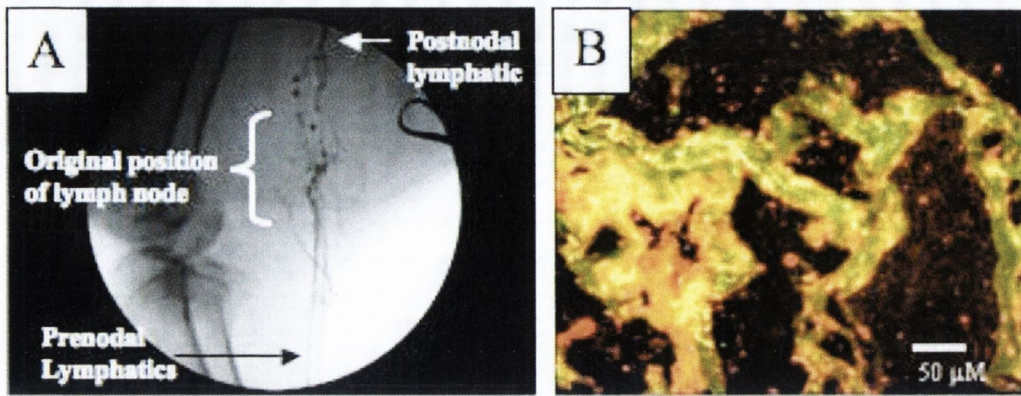
Equations 1 and 2 were used to calculate an average mass transport rate for each group of animals. Controls (n=7), 8 week (n=7), 12 week (n=7) and 16 weeks (n=6). A one-way ANOVA revealed that the groups were significantly different. The values obtained at 8, 12 and 16 weeks post-surgery were less than that noted for the intact limbs but only the data at 8 weeks reached statistical significance with the Dunnett's t-test (marked with asterisk in Figure).



## Fluoroscopy and Immunohistochemistry

Fluoroscopic analysis at 8, 12 and 16 weeks after nodal excision confirmed that some degree of fluid continuity had been established between the pre-nodal and post-nodal lymphatic vessels in the absence of the lymph node. The contrast agent injected into one of the pre-nodal lymphatics in the lower limb could be observed in the relatively large duct that would normally collect lymph from the popliteal node (post-nodal vessel) (example illustrated in Figure 2.6A). Small lymphatic vessels were observed in the nodal excision area. The possibility that these vessels were newly formed lymphatics attempting to bridge the gap left by the absent node was supported by immunohistochemistry.

We observed co-localization of LYVE-1 (red) and CFDA-SE (green) indicating that lymphatic vessels were present at the area vacated by the popliteal lymph node. An example is provided in Figure 2.6B. The newly formed lymphatics observed at 12 weeks post surgery were variable in size but at the upper end, were about 40µm in diameter. These vessels were the conduits that connected the originally placed pre- and post-nodal lymphatics and contributed to the restoration of lymph transport that was observed in the tracer and fluoroscopic studies.



**Figure 2.6** Morphological Evidence that Some Regeneration of Lymphatic Vessels has Occurred Following Popliteal Lymph Node Excision

(A) Fluoroscopy performed at 12 weeks after lymph node removal illustrating some degree of continuity between the lymphatic vessels that were originally pre- and post-nodal to the popliteal lymph node.

(B) Confocal microscopy image of regenerated post-nodal lymphatics in the nodal excision site. Tissue sections were prepared as described in the Materials and Methods. For unequivocal identification of these vessels, a non-specific cell dye CFDA-SE (green) was infused into an upstream pre-nodal lymphatic and at the same time, the tissues were stained with antibodies to the lymphatic endothelial marker LYVE-1 (red). The stained sections appeared yellow-orange indicating co-existence of the two stains. At 12 weeks, an irregular network of small interconnecting lymphatics could be observed in the area originally occupied by the popliteal lymph node. We presume that all vessels in this image are newly formed in response to the removal of the node.



## 2.4 Conclusion

Lymphedema was initiated by the removal of a single lymph node and a quantitative method was developed to assess lymphatic function over time. These techniques may be helpful in understanding the pathophysiology associated with lymphedema and may facilitate the development of new strategies to treat or prevent this condition.

CHAPTER 3:

EXPERIMENTAL ASSESSMENT OF AUTOLOGOUS LYMPH  
NODE TRANSPLANTATION AS TREATMENT OF POST-  
SURGICAL LYMPHEDEMA



<b>CHAPTER 3: EXPERIMENTAL ASSESMENT OF AUTOLOGUS LYMPH NODE</b>	
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## **Introduction**

Lymphedema in breast cancer patients represents a distressing consequence of axillary node resection. Since the actual cause of lymphedema is still being debated, the most appropriate target for new therapeutic approaches is not entirely apparent.

Following the development of a sheep model that permits quantitation of edema and lymphatic function after the removal of a single popliteal node in sheep, the objective of this chapter is to examine the impact of autologous vascularized and non-vascularized lymph node transplantation on lymphatic function and edema formation immediately following the removal of the popliteal node.



## **Materials and Methods**

### **Animals**

A total of 50 randomly bred male and female Dorset sheep ( $30.6 \pm 0.7$  kg) were used in the experiments presented in this chapter. An additional 10 sheep were used solely for anatomy dissections. All experiments outlined in this paper were approved by the ethics committee at Sunnybrook Health Sciences Centre and conformed to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

### **Anatomy Dissections**

A total of ten sheep were used for anatomy studies of the popliteal lymph node, particularly focusing on its blood supply. Sheep are fasted and anesthetized as described in earlier sections. Evans blue dye (1% in saline) is injected under the skin close to the hoof. This colors the draining lymphatics of the limb up to the popliteal node. Also in several sheep, yellow Microfil® was used. The best results were obtained using a preparation that was more dilute than that recommended in the product literature. For every 2.5ml of yellow Microfil® 2ml of diluent is added and the material catalyzed with 10% (of total volume) of the curing agent. The femoral artery is catheterized and 10 or 20ml of the Microfil® preparation is infused into the vasculature.

Polymerized Microfil highlights even the tiniest vessels penetrating the node. The popliteal lymph node with its surrounding fat was carefully dissected with the aid of either Carl Zeiss Loupes (3,5x400) or a larger Surgical Microscope Carl Zeiss (OPMI 1-FC). The arterial supply to the node seems to come mainly from the medial circumflex femoral artery (0.1-0.3mm) and to a lesser degree from the caudal femoral artery (0.1-0.2mm). Upon entering the fat pad, these vessels branch out to form an extensive arterial network, which in turn gives rise to multiple smaller vessels responsible for nurturing the node. In only one of the dissections, the main arterial branch was found to enter the node directly. The main draining vein consistently is the lateral saphenous vein (0.3-0.5mm).

It was thus decided that the node needed be harvested with the popliteal fat intact to better the chances of preserving the delicate blood supply to the node.

## **Surgical Procedures**

### *Nodectomy Followed by Avascular Node Graft Insertion*

Sheep are fasted 24 hours prior to anesthesia. The animals are anesthetized initially by I.V. injection of sodium pentothal. Subsequently, 2.0-3.5% isoflurane is delivered through an endotracheal tube via a Moduflex, Dispomed machine with Hallowell respirator for maintenance. The surgical site is shaved and washed with alcohol and betadine then draped with sterile sheets.

A vertical skin incision (approximately 8-10cm long) is made over the lateral aspect of the popliteal region. The popliteal fossa is triangular in shape and is found by retracting the biceps femoris muscle caudally at the level of the stifle joint. At it's distal angle, a single popliteal lymph node lies embedded in a pad of fat.



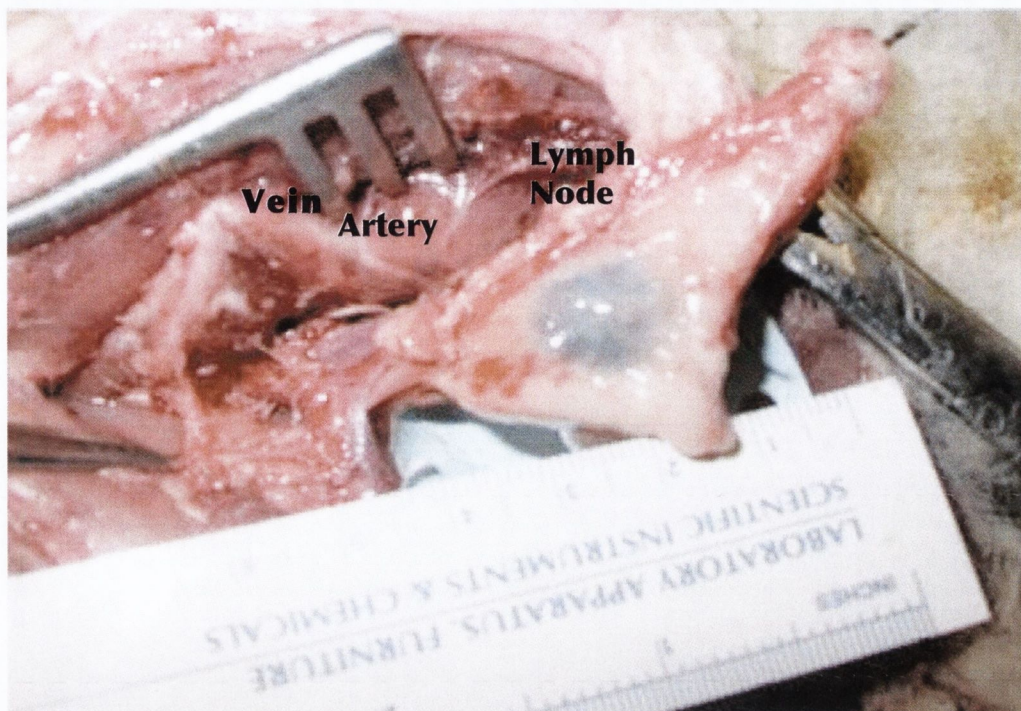
A small opening is made in the popliteal fat and the node is carefully dissected out. The pre- and post-nodal lymphatic vessels are tied off with a silk suture and the node is carefully excised. Once the nodes from both hind limbs are removed, each is then placed into the contra lateral popliteal fossa through the window opening created in the fat as a free graft without vascular reconnection. This opening is then sutured to ensure the node is not displaced. Hemostasis is performed as needed and the skin is closed.

#### *Nodectomy Followed by Lymph Node Transplant by Microvascular Anastomosis*

Sheep are fasted, anesthesia is induced and the popliteal fossa is exposed as described above. The lymph node is harvested together with the popliteal fat pad intact, to preserve the delicate nutrient vessels supplying the lymph node. The popliteal fossa is exposed by retracting the biceps femoris muscle caudally at the level of the stifle joint. The transplant tissue is carefully dissected off the surrounding muscles with the use of Carl Zeiss Loupes (3,5x 400) in a distal to proximal direction (starting distal to and dissecting towards the vascular pedicle). Care is taken not to harm the tibial and common peroneal nerves which lie just medial to the popliteal fat. The vascular pedicle formed by the lateral saphenous vein and the medial circumflex femoral artery which lie in the superior-medial angle of the popliteal fossa are identified. Great care is taken to dissect the pedicle free from the surrounding tissues before ligation and clipping of the vessels. Once the free transplant is harvested it is covered with saline soaked gauze and placed inside a sterile plastic bag, which is then placed into a dish of saline and ice. Hemostasis is ensured prior to closure of the surgical site.

The animal is then turned over and the contra-lateral popliteal lymph node is identified and excised. Recipient vessels of a suitable size and location are exposed and the surrounding tissues are cleared away, the transplant vessels are then lined up and approximated to the recipient vessels with bridging micro clamps. The vessels are sutured together microsurgically (end to end) with the use of a Carl Zeiss Microscope (OPMI 1-FC). The animals are returned to their holding pens after recovery from the anesthetic. Postoperative care and antibiotic treatment is provided as previously described.





**Figure 3.1** Popliteal Node Transplant

This Image Illustrates the Harvesting of a Free Popliteal Node Transplant With the Surrounding Fat on a Vascular Pedicle. The Node Was Highlighted With Evans Blue for Clarity.

### Sham Control Group

In this group of animals, the popliteal fat pad is exposed but left completely undisturbed. A self-retaining retractor is applied and the wound left open for one hour to duplicate the average time needed for harvest of the popliteal node transplant. Tissue hydration is maintained with a saline soaked piece of gauze, afterwards the wound is closed. The exact same is performed on the contra-lateral popliteal fossa, but this time the wound remains open for a period of three hours, which is the approximate time needed to perform the microsurgical anastomosis in the recipient limb. At the end point of the study quantitative analysis is performed on the limb that was left open for three hours i.e. the mock recipient limb of the node transplant.

### **3.2.4 Assessment of Edema**

Measurements are performed in exactly the same manner, location and frequency described previously. The hind legs are closely shaved and leg circumference measurements are taken at a point 10cm distal from the hock (tarsus). This landmark is highlighted with a skin marker for circumference measurements post operatively.

The limb circumferences are divided by the original (pre-surgical value) and expressed as percentage change over time. To compare the edema outcomes in the various groups, the percentage change in limb circumference was plotted against time and graphical integration of the area under the curves was calculated using the trapezoidal rule. To allow comparisons of experiments conducted over different times (8 and 12 weeks) an edema coefficient was calculated by dividing the areas under the curves with the duration of the experiment in days.



### 3.2.5 Fluoroscopy

In 17 sheep (19 limbs) at 8 or 12 weeks post surgery, an x-ray contrast medium (1-3ml, Lipiodol, EZ-EM Canada, Therepex) is injected into an upstream popliteal pre-nodal vessel and the lymphatics/nodes visualized at various times after surgery using a mobile fluoroscopy system (BV Pulsera, Philips).

### 3.2.6 Histological Assessment of Lymph Nodes

Popliteal nodes are fixed in 10% formalin, cut into 5mm sections and paraffin embedded. Sections (6µm) are placed on slides and then stained with Hematoxylin and Eosin stain (H&E).

In addition, one representative node slide per sheep is stained for the t-cell marker CD3 using a 1 in 200 dilution of a rabbit anti-human CD3 antibody (Dako Canada, Inc.). This is followed by sequential incubation with biotinylated link antibody, peroxidase-conjugated strepavidin and then visualized with diaminobenzadine (Dako Canada, Inc.). The sections are then counterstained with Harris's Hematoxylin, then treated with acid alcohol and ammonia water.

Lymph nodes are scaled qualitatively after transplantation on a scale of 0 to 3:

3- Normal looking nodes.

2- Nodes with some pathology (evidence of ischemic damage and loss of cellularity).

1- Partial nodes or nodes with severe damage (fibrosis).

0- Lymph nodes absent (tissue resorbed).

### 3.2.7 Quantitative Assessment of Lymphatic Function

This is performed for each animal in all of the surgical groups at either 8 or 12 weeks post operatively, exactly as described in the previous chapter.

### 3.2.8 Statistical Analysis

All data is expressed as the mean  $\pm$  SEM. The data is assessed with regression analysis, Kruskal-Wallis one-way ANOVA or t-test (unpaired) as appropriate. We interpreted

P < 0.05 as significant.

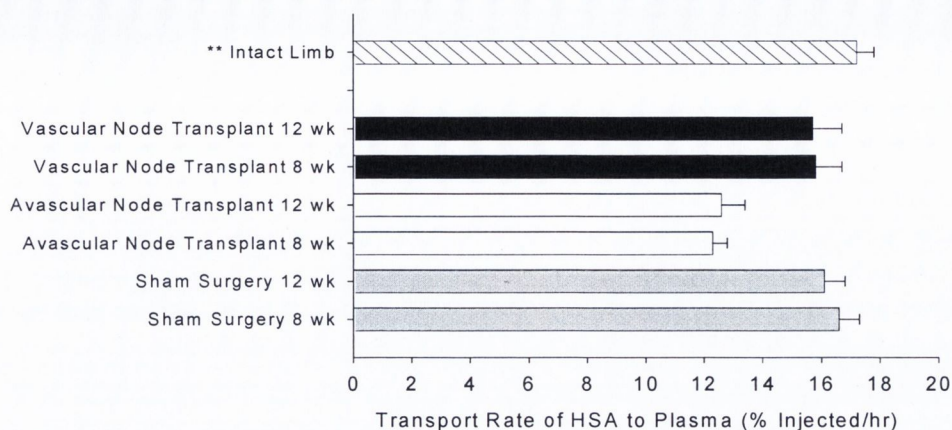


## Results

### 3.3.1 Lymphatic Function

Measurements of lymphatic function for all groups are illustrated in Figure 3.2. The transport rates of the protein tracer for the 'sham' group averaged ( $16.6 \pm 0.7\%/hr$ ) at 8 weeks and ( $16.1 \pm 0.7\%/hr$ ) at 12 weeks. These values were similar to those observed in limbs that had not been subjected to any surgical procedures ( $17.2 \pm 0.6\%/hr$ ), taken from the previous chapter and indicated that all of the surgical procedures excluding the actual removal of the nodes had little impact on lymphatic function.

The removal of a lymph node and replacement with an avascular node from the contra-lateral side gave lymphatic function values at 8 ( $12.3 \pm 0.5\%/hr$ ) and 12 weeks ( $12.6 \pm 0.8\%/hr$ ) after surgery that were significantly lower than those of the sham control group. In contrast, the replacement of the excised node with a vascularized node transplant resulted in lymph transport that was significantly greater than that of the avascular group. Indeed, lymphatic function approached sham levels ( $15.8 \pm 0.9\%/hr$ ) at 8 weeks and ( $15.7 \pm 1.0\%/hr$ ) at 12 weeks.



**Figure 3.2** Impact of Node Transplantation on Lymphatic Function

The transplantation of autologous avascular popliteal lymph nodes into the nodal excision site (white bars) resulted in lymphatic function at 8 and 12 weeks that was significantly less than that observed in the sham group at similar times (grey bars). In contrast, the transplantation of vascularized nodes (black bars) produced lymph transport values that were similar to sham controls but significantly greater than those in the avascular transplant series. The cross-hatched bar illustrates data from limbs that had no surgery, i.e. with lymph nodes intact (taken from the previous chapter). For statistical analysis, the data from 8 and 12 weeks was averaged together for each experimental group since there were no time-dependent differences in any of the experimental series. Numbers in parentheses represent the number of animals in each group.

\*\*  $p < 0.001$ .



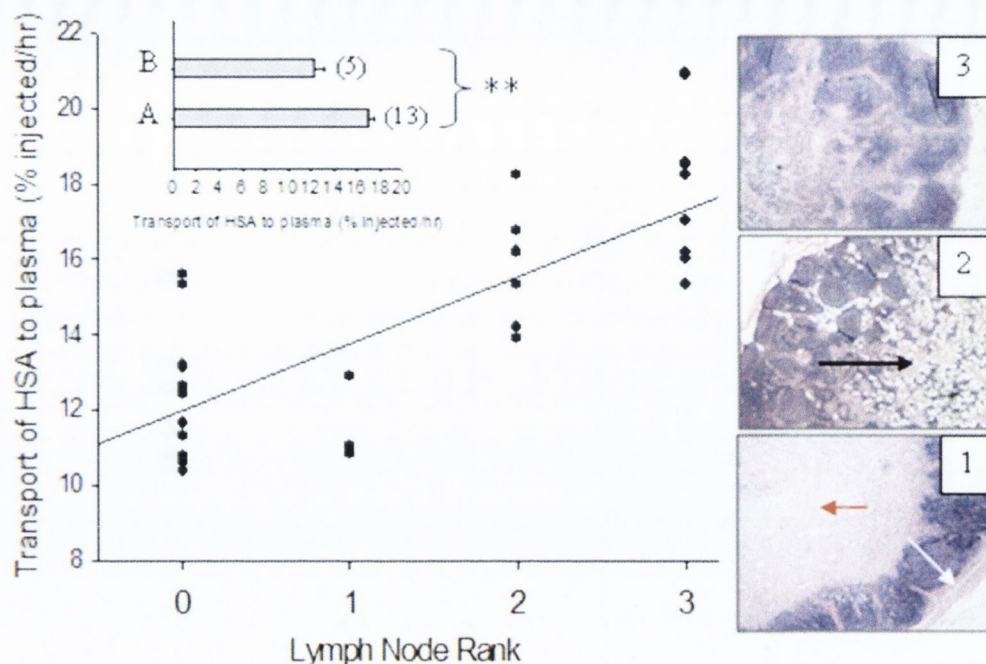
### 3.3.2 Correlation of Lymphatic Function with Health of Lymph Nodes

In the sham group, all nodes rated 2 or 3 on histological assessment indicating that the nodes were generally healthy looking. Examples are illustrated in Figure 3.3 (right upper and middle panel).

Those in the avascular transplant group fared poorly; with 7/12 classified as 0, 3/12 classified as 1 and only 2/12 classified as 2. An example of a node ranking 1 is illustrated in Figure 3.3, bottom right panel.

The vascularized node transplants were generally more successful. At 8 weeks the ratings were; 4/8 classified as 3, 3/8 classified as 2 and 1/8 classified as 0. At 12 weeks the results were 4/10 classified as 3, 2/10 classified as 2 and 4/10 classified as 0.

Figure 3.3 also shows a significant correlation between the health of the lymph nodes and lymphatic transport function. This data includes all values from the avascular and vascularized transplant groups at both 8 and 12 weeks. It is evident that lymphatic function was highest in the limbs that contained the healthiest looking lymph nodes. In the inset to Figure 3.3, we separated the vascularized node transplant data into two groups; those with lymph nodes scaled 2 or 3 (A in inset) and those scaled 0 or 1 (B). Those limbs with the healthiest nodes had significantly greater lymphatic function than those with absent nodes or with nodes exhibiting major pathological changes. The preparations with nodes scaled 2 or 3 had lymph transport averaging ( $17.1 \pm 0.4\%/hr$ ), a value that was essentially identical to limbs in which no surgery had taken place (Figure 3.2). When the vascularized node transplants were unsuccessful (nodes scaled 0 or 1), lymphatic function was significantly lower averaging ( $12.3 \pm 0.9\%/hr$ ) which was very similar to that obtained in the avascular transplants.



**Figure 3.3 Relationship Between Lymphatic Function and Lymph Node Health After Transplantation**

The data demonstrates that the subjective node rankings have different levels of lymphatic function ( $p=0.0002$ ). This association was monotonic in that increasing lymph transport was associated with increasing node rank scores ( $p<0.0001$ ). Right upper panel: example of normal node ranked 3 (H&E stain). Upper middle panel: example of node ranked 2 (H&E stain). There is evidence of ischemic injury with some loss of cellularity and lipid accumulation in the medulla (black arrow). Right lower panel: example of node ranked 1 (H&E stain). In this case, there is evidence of significant injury with major portions of the node replaced by fibrous tissue (red arrow). Additionally, the capsule is much thicker than normal (white arrow).



In the inset, the vascularized data has been subdivided into 2 groups; those preparations in which the nodes were scaled 2 or 3 (A) and those in which the nodes were scaled 0 or 1 (B). Lymphatic function was significantly greater in the transplant group with the healthiest looking lymph nodes ( $p < 0.0001$ ; unpaired t-test). Numbers in parentheses represent the number of animals in each series.

### 3.3.3 Edema

In the sham group, we noticed no change in the limb circumference over time. In all cases in which a popliteal node was placed into the nodal excision site without vascular connections, the limb became edematous. The average percent increase in leg circumference at day one was 12.7% with maximum values attained within the first week after surgery. By 12 weeks on average, the edema was still higher than the pre-surgical levels.

Figure 3.4A illustrates the averaged edema data from the vascularized node transplant series. In these experiments, lymph nodes from both hind limbs were excised and one of the limbs received a vascularized node transplant while the other limb did not. In the limbs that received a vascularized transplant, the average percent increase in leg circumference at post operative day 1 was 12.1% with peak edema also occurring during the first week. Edema in the contralateral nodectomy limbs peaked at the same time. Thereafter, limb circumference declined steadily in both limb groups with values from the vascularized transplant limbs consistently less although there was considerable variation in the data and the two groups were not significantly different. This was no doubt due to the fact that the averaged data contained both successful and unsuccessful vascularized transplants. For example, Figure 3.4B illustrates a case in which lymphatic transport in the transplant side was (17.0% injected/hr) and the node was classified as 3. The edema in the transplant limb recovered completely whereas the contralateral side (with node removed) was still edematous by the end of the experiment. In the example in Figure 3.4C, the transplant was not successful and the lymphatic function was (12.6% injected/hr) and the node classed as 0. In this case, the edema (limb circumference) in the transplant side was similar to that of the contralateral limb and did not resolve over the course of the study.



Figure 3.5 illustrates the relationship between the coefficient of edema and lymphatic function combining all of the data from the vascularized and avascular node transport limbs. While there is a tendency for the limbs with the highest lymphatic function to be associated with the lowest levels of edema, the variability in the edema data prevented this relationship from being significant (regression analysis). The insert illustrates averaged edema coefficients for these groups in addition to the limbs from which the nodes were harvested for transplant (labeled node excision). These comparisons indicate that the vascularized node transplants were associated with the lowest levels of edema. The edema coefficients in this group were significantly lower than those in the non-treated limbs (node excision only).

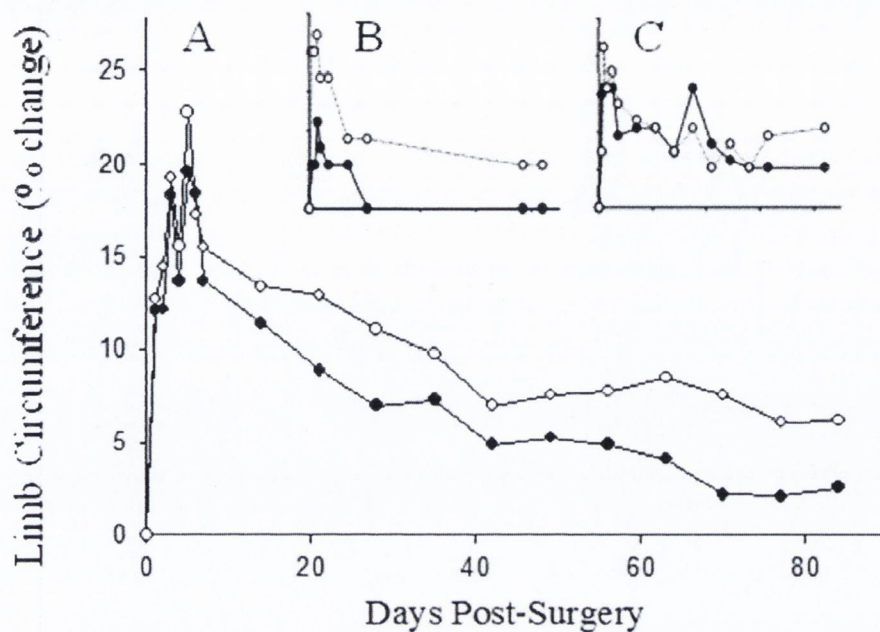


Figure 3.4 Development and Progress of Edema in the Animal Groups

Development of edema following excision of popliteal lymph node without nodal replacement (open circles) or after the transplantation of an autologous vascularized node (closed circles) (A). SE bars have been omitted from the averaged data for clarity. The edema peaks during the first week following surgery and then declines slowly over time. While there is a tendency for the averaged data to suggest that the vascularized transplant procedure lowers edema levels, this was not statistically significant (ANOVA). Inset B illustrates an example in which the nodal transplant procedure reduced edema generation over the course of the experiment



compared to the non-treated limb and at 21 days no edema could be measured. In this case, lymphatic function was (17.0% injected/hr) and the node was scaled as 3. Inset C shows an example in which the transplant was largely unsuccessful at ameliorating edema although by the end of the experiment the limb circumference was less than the contralateral untreated side. In this animal, lymphatic function was (12.6% injected/hr) and the node was scaled as 0.

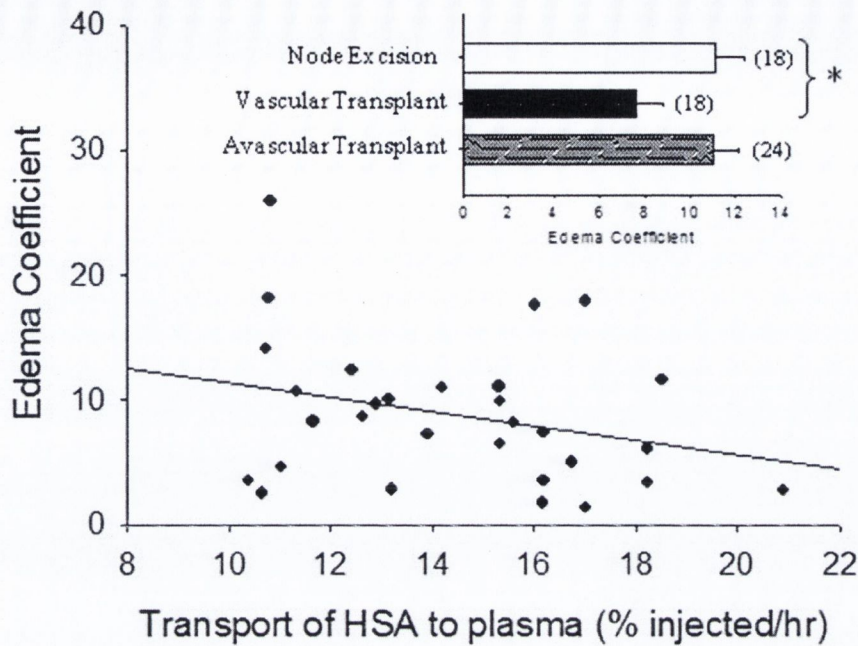


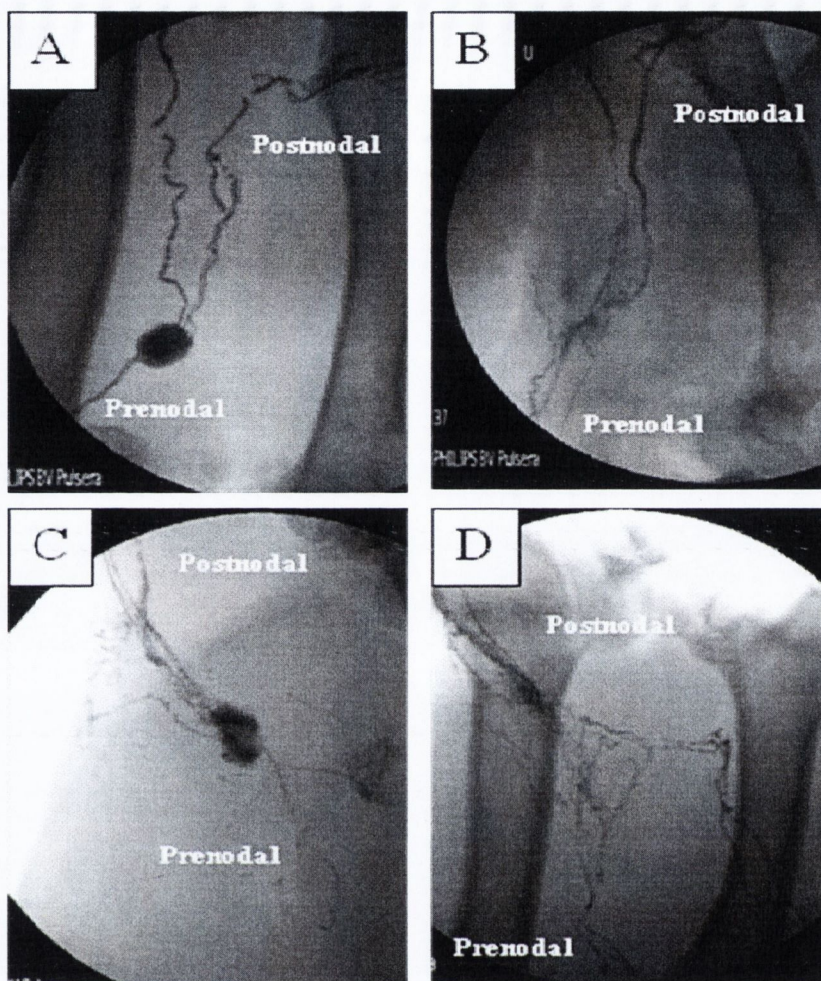
Figure 3.5 Relationship Between Edema and Lymphatic Function

In the main graph, all of the data from the avascular and vascularized transplant groups have been plotted. The trend line suggests that the level of edema declines with increasing lymphatic function but regression analysis indicates that this effect is not significant. However, the average edema coefficient for the vascularized transplant group was lower than that in the untreated node excision series ( $p=0.039$ ). On average, the vascularized group had lower edema coefficients than the avascular series but these effects were not significantly different.



### 3.3.4 Fluoroscopy Studies

In the surgical sham series, fluoroscopic analysis revealed nodal tissue, well-defined pre-nodal vessels and two or more post-nodal ducts. An example is illustrated in Figure 3.6A. In all other groups (vascular and avascular node transplants and following node excision with no transplant), fluoroscopic analysis revealed that fluid continuity had been re-established by 8 weeks. This indicated robust regeneration of lymphatic vessels. In those preparations in which an avascular node was implanted into the excision site, a distinct lymph node was not observed in most cases (example in Figure 3.6B). With vascularized nodal transplants, a node was visualized in some (Figure 3.6C) but not all limbs (Figure 3.6D). There were also no obvious differences between the 8 and 12-week time points in any of the groups.



**Figure 3.6** Fluoroscopic Images of the Popliteal Fossa Region

In Figure 3.6 (A) Image of sham preparation at 12 weeks illustrating popliteal lymph node, pre-nodal and post-nodal vessels. (B) Image taken 8 weeks after an avascular node transplant. In this example, no lymph node is present due to tissue resorption. Regenerating lymphatics have provided some fluid continuity between the pre- and post-nodal ducts. (C) Image showing successful vascularized transplant 8 weeks following surgery. The lymph node can be clearly observed and pre- and post-nodal ducts have



connected to the transplant and provided fluid continuity. (D) Image illustrating an unsuccessful vascularized transplant 12 weeks after surgery. The lymph node has been resorbed but some fluid continuity has been established by the regenerating vessels. In all examples, the pre- and post-nodal lymphatic is labeled and the area around the lymph nodes (or where the node was originally located) is circled in white.



## Conclusion

The successful re-implantation of a lymph node into a nodal excision site has the potential to restore lymphatic function and facilitate edema resolution. This result has important conceptual implications in the treatment of post-surgical lymphedema.

## CHAPTER 4:

## DISCUSSION

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## Summary of Conclusions in this Thesis

The data in this thesis, presents the development of a sheep lymphedema model that permits the integration of clinical edema with quantifiable lymphatic transport function. The data suggests that the removal of a single lymph node produces a lymph drainage deficit in the affected limb. We obtained evidence of lymphatic vessel regeneration in the area originally occupied by the lymph node. At the end point of our study, clinical edema was still evident in most animals. Utilizing this model, the replacement of an excised lymph node with a vascularized node transplant resulted in the restoration of lymphatic function to control levels in many cases and in about a half of these preparations, edema completely resolved. In contrast, when a node from the contralateral limb was inserted into the popliteal fossa without connecting the blood supply, lymphatic function was significantly less than that of the sham controls and indeed, most of these nodes did not survive the 8 or 12-week monitoring period. The average lymphatic function measured after removal of the nodes with no transplants attempted at 8 and 12 weeks was  $(10.6 \pm 1.5\%/hr)$  and  $(14.4 \pm 1.0\%/hr)$  respectively. The avascular node transplants did not improve on these values. These data raise a number of questions related to the role of the lymph node in lymph transport and suggest therapeutic potential for nodal transplantation in lymphedema patients.

## **Animal Models of Lymphedema**

The development of animal models to simulate human lymphedema has been challenging due to the remarkable regenerative capacity of the lymphatics and the formation of collateral pathways around the site of obstruction. In some cases, extensive surgical manipulation is required to elicit edema (Piller, 1985). As an example, in the studies of Olszewski (Olszewski, 1967) lymphedema was achieved in dogs by excising a circumferential strip of skin, subcutaneous tissue and fascia from the upper region of the thigh and the periosteum of the femur was stripped off. This was followed by a second procedure involving the removal of the popliteal lymph node. With varying degrees of surgical manipulation, lymphedema models have been applied to several anatomical locations and species. Commonly, a significant tissue mass is removed with attendant dissection of lymphatic vessels and nodes. Popular models include the hind limb models in dogs (Olszewski, 1967) , and rats (Liu et al., 2008; Piller, 1975), rabbit ear models (Huang and Hsin, 1983; Szuba et al., 2002) and rodent tail approaches (Boardman, 2003; Slavin et al., 1999; Swartz and Boardman, 2002). In some animal models, radiation is applied in addition to surgical tissue resection (Chen et al., 1990; Das et al., 1981).

One of the limitations of many of the aforementioned models is the difficulty in quantifying lymphatic function. Histological assessment of molecular markers is used commonly to assess the presence or absence of lymphatic vessels with attempts to correlate these findings with the clinical status of the animal (Cunnick, 2001). This approach has been popular in murine models but the functionality of the newly formed vessels is difficult to determine with immunohistochemistry. The tissue injection of various contrast agents has been used to identify newly developing lymphatic networks. For example, the intradermal injection of FITC-dextran combined with fluorescence microlymphangiography has



been used to assess the formation of new lymphatic vessels and provide some opportunity to quantify lymph transport parameters (Boardman, 2003; Swartz and Boardman, 2002). In general terms however, the functional impact of lymphangiogenesis on fluid transport remains a significant challenge in most lymphedema models, especially those that employ small species. In this regard, studies in sheep have several advantages.

### **Advantages of a Sheep Lymphedema Model**

The anatomical dimensions of the sheep lymphatic system provides an opportunity to quantify lymphatic function and offer a realistic framework for the development of therapeutic measures and methods of administration that are 'human-sized' in perspective.

Especially important is the ability to monitor the transport of a known mass of radiolabeled albumin to plasma as this provides a useful measure of the lymph transport effectiveness of a given lymphatic network. Under normal conditions, molecules with molecular weights >6000 injected into a limb lymphatic are almost completely recovered in thoracic duct lymph (Aukland and Reed, 1993). Therefore, following the injection of  $^{125}\text{I}$  HSA into a pre-nodal lymphatic vessels, one would expect that mass transport rate of this protein tracer to plasma would reflect the integrity of the lymphatic network in question. Indeed, we were able to observe measurable differences in the plasma recoveries after lymph node excision compared to control limbs.

A significant benefit to studies of the popliteal lymphatic system is that the pre- and post-nodal popliteal ducts are easy to identify and some of these are of sufficient size for cannulation. In sheep, usually only one vessel drains the node (in rare occasions there are two). Multiple pre-nodal ducts (6-12), enter the popliteal lymph node at various locations along the convex portion of the node (Heath and Brandon, 1983). Since there is no known



collateral lymphatic circulation in this area, we expected that removal of the popliteal lymph node would affect all lymph flow from the lower hind limb and induce edema, a prediction borne out by the experiments in this thesis. The ability to disrupt the regional tissue drainage in a limb by removing a lymph node makes this model especially relevant to post surgical lymphedema such as that which occurs in breast cancer patients.

In addition to the aforementioned issues, sheep lymphatics are known to regenerate after injury and in response to surgical node removal (Andrade et al., 1998; Jila et al., 2007; Kim et al., 2003). Recent data indicate that sheep lymphatic endothelial cells express the requisite molecular markers for identification and vessel regeneration is characterized by the up-regulation of factors known to be associated with lymphangiogenesis in other animal species (Jila et al., 2007).

In cancer patients, the removal of one or more lymph nodes often gives rise to acute edema, which resolves successfully. It is of course, the chronic form of edema that occurs in a subgroup of patients that is most problematic. In this regard, there is no agreed upon time beyond which one considers the edema to be chronic. For practical reasons, we did not follow the animals beyond a 16-week period. It is possible therefore, that the average edema in our animals would have resolved if the sheep were permitted to survive for longer periods. Nonetheless, the model is suitable for the measurement of lymphatic-related physiological changes over a reasonable period of time.

## **Questions Related to Lymphedema Development**

There are many questions in the lymphedema field that have to be addressed before new, rationale therapies for this condition can be developed. As one example, an assumption has always been made that the major impediment to lymph transport in post-

surgical lymphedema patients is due to lymphatic vessel injury and the inability of the regenerating vessels to remove tissue fluid adequately. However, lymphatic vessels are damaged routinely in surgical procedures and the acute, post-surgical edema usually resolves rapidly as the lymphatics regenerate.

Certainly, we observed new lymphatic vessel growth at the nodal excision site, which would agree with other accounts in the literature. In rabbits, the removal of a popliteal lymph node results in lymphangiogenesis and the formation of new ducts and collateral vessels as early as 4 weeks after node removal (Ikomi et al., 2006). Given the robust lymphatic response to injury, one might speculate that the development of lymphedema may also involve other anatomical elements and mechanisms. In this regard, it would seem that there might be something special about the removal of a lymph node.

Another issue may relate to lymph flow resistance. The resistance of large lymphatic vessels is generally believed to be low and of little physiological significance (Aukland and Reed, 1993). In contrast, lymph nodes are known to provide resistance to lymph transport and this has been estimated to be 50 to 200 times greater than that provided by the lymph trunks (Papp et al., 1971). One might expect therefore, that the removal of a node would reduce flow resistance. However, there is some evidence that the resistance to flow increases after the removal of a lymph node, at least over a relatively short period (Kim et al., 2003). The newly formed lymphatics that form in response to nodal excision exist as an irregular network of small vessels and it is possible that this arrangement provides an impediment to flow. It is of course possible that resistance will change over time as the new lymphatic network matures and remodels in an attempt to produce a configuration that may be more amenable to effective lymph transport.



Taken together, these changes would make the lymphatic system less effective at transporting interstitial fluid and, could lead to edema formation in the cancer patient.

#### **4.5 Importance of Lymph Nodes in Tissue Fluid Balance**

Tissue fluid homeostasis results from a complex interplay between the microvasculature, the interstitial matrix and the lymphatic circulation. From the interstitial fluid pressure-tissue volume relationship in a limb 'compliance curve', we learn that the normal negative limb interstitial fluid pressure provides some hydrostatic buffering effect 'safety factor' (described by Guyton and colleagues), as interstitial fluid pressure must rise before edema develops (Guyton, 1971). Negative interstitial pressures exist as long as transcapillary fluid flux and lymph transport are at appropriate levels to maintain the interstitium in a relatively dehydrated state. In the data relating to our model development (chapter two), regeneration of the lymphatic vessels over a 12-16 week period following the removal of a lymph node helped to restore a sizeable portion (about 80%) of the lost lymph transport capacity and no doubt, facilitated the resolution of a significant portion of the edema. This attests to the remarkable regenerative capacity of this circulatory system. In addition, the lymph node appears to separate the lymphatic system into higher (pre-nodal) and lower (post-nodal) pressure areas (Aukland and Reed, 1993). This is due to the fact that protein-free fluid is absorbed from the lymph into the blood capillaries in the nodes to establish an equilibrium of Starling forces across the lymph-blood barrier (Adair, 1985c; Adair and Guyton, 1983; Adair and Guyton, 1985; Adair et al., 1982). As part of this issue, lymphatic vessels contract and provide much of the energy required for lymph propulsion (Li et al., 1998). In this regard, the pressure range over which the pre- and post-nodal ducts appear to operate, is different for these vessel types (Eisenhoffer et al., 1994). Without the



dissipation of lymphatic pressure within the lymph nodes, intra-lymphatic pressures in the downstream vessels would presumably be higher and this would force these vessels to 'pump lymph' under non-optimal conditions. Therefore, the ability of the limb to remove tissue fluid may still be compromised (even at a sub-clinical level) due to the removal of the nodes and the loss of their ability to 'depressurize' the lymphatic system.

Presumably then, the damage to the lymphatics and loss of lymph nodes consumes a significant portion of the 'safety-factor' discussed above and pushes the tissue compliance curve closer to the threshold at which clinical edema may occur. An additional insult such as trauma (minor laceration, insect bite) or radiotherapy may tax the functional reserve of this limb leading to the onset of irreversible lymphedema.

## **Treatment of Breast Cancer Related Lymphedema**

### *Risk Reduction*

The initial management of lymphedema should start with efforts at preventing the disorder in the at risk population. Risk reduction advice including certain precautions and lifestyle modifications are aimed at reducing the life-long risk of post-surgical lymphedema in breast cancer survivors.

Following axillary surgery there are a multitude of precautions available for patients. Advise on practicing meticulous skin care and hygiene and avoidance of skin trauma such as cuts and injections in order to minimize the risk of infection (Mozes et al., 1982; Petrek et al., 2001; Soran et al., 2006), to avoid weight gain and increasing BMI, which are commonly recognized as risk factors for lymphedema (Clark et al., 2005; Petrek et al., 2001; Werner et al., 1991; Wilke et al., 2006). Some of the information however is controversial and largely lacks sound evidence base, such as avoiding limb constriction,

exposure to extreme temperatures, limiting of strenuous exercise and level of hand use, lifting of heavy objects or repetitive activity (Nielsen et al., 2008; Soran et al., 2006).

Precautions against air travel vary between organizations, despite the lack of convincing studies (Casley-Smith, 1996; Nielsen et al., 2008; Swenson et al., 2009).

### Conservative Management of Lymphedema

Once lymphedema is detected, management should be initiated as soon as possible. If treatment is not offered, the condition will continue to evolve over time, causing progressive tissue damage. When intervention is delayed until lymphedema is advanced, the outcome is less favorable because of chronic adipose and fibrotic changes within the tissues.

A variety of health care professional and patient inspired conservative therapies have been established to help manage lymphedema. These treatment modalities comprise a combination of techniques including lymphatic drainage, compression therapy and limb exercises. The aim of treatment is to reduce interstitial fluid volume and maintain limb circumference as small as possible.

**Complex decongestive physiotherapy:** This popular multimodality treatment was first devised by Dr. Foeldi in the mid twentieth century (Didem et al., 2005; Liao et al., 2004; Sato, 2005) and involves a combination of manual lymphatic drainage, external compression devices, skin care, fitted compression garment and limb exercises under guidance of a trained professional. If employed correctly this treatment can produce volume reductions greater than 50% in the affected limb (Casley-Smith, 1992; Erickson et al., 2001; Szuba et al., 2000).



**Manual lymphatic drainage:** This method uses light massage strokes that stimulates contractility of the lymphatic system, which ultimately encourages removal of excess interstitial fluid (Földi M, 2003; Kasseroller, 1998).

**Compression Therapy:** Includes pneumatic pumps, which envelope the limb and cyclically inflate and deflate to encourage lymphatic drainage from distal to proximal end of the limb (Brennan and Miller, 1998). Compression bandaging and compression garments both have a similar mode of action, as they are designed to provide graded compression, the greatest being at the distal end of the limb and gradually becomes less towards the proximal end thereby promoting interstitial fluid return (Yasuhara et al., 1996). Not only can significant volume reductions be achieved but also improvements in sensations of heaviness and tension. Best results can be had in combination with exercise and limb massage (Andersen et al., 2000).

**Therapeutic limb exercises:** These are mildly effective for facilitation of lymphatic drainage by varying total tissue pressure through repeated contraction and relaxation of muscles and also improving limb strength and range of motion (Johansson K, 2004). But are more effective in combination with other treatment modalities.



### Evidence for Surgical Treatment of Lymphedema

In addition to conservative therapies, several surgical techniques have been used clinically and in experimental studies in an attempt to restore lymphatic fluid drainage after tissue injury.

Lymphatico-venous anastomoses were first reported in dogs (Gilbert et al., 1976) and then in humans (O'Brien et al., 1977) for the treatment of obstructive lymphedema. In 1978 Degni proposed a new technique to bury the anastomosis (Degni, 1978). It has since become an accepted method for restoration of lymphatic drainage in both experimental and clinical settings (Chang et al., 1985; Fox et al., 1981) particularly when combined with physical rehabilitation methods (Campisi and Boccardo, 2004; Koshima et al., 2000; Yamamoto et al., 2003). The studies of Campisi et al. advocated the use of autologous interposition vein graft as an alternative technique, this consisted of inseting the vein graft between lymphatic collectors above and below the site of obstruction (Campisi C, 1991). Long term outcomes of this method showed promising results (Campisi and Boccardo, 2003; Campisi et al., 1995). Baumeister et al. first described microsurgical lymphatic grafting (lymphatic to lymphatic anastomoses) for the treatment of lymphedema in experimental dogs. This was done by means of an end-to-end anastomosis with harvested autologous lymph vessels bypassing the proximal and distal sites of the blockade (Baumeister et al., 1981). Later the same principles were applied in patients with both primary and secondary lymphedema, the latter comprising the great majority of patients with reported long term relief of lymphedema symptoms (Baumeister and Frick, 2003; Baumeister et al., 1986).

## Lymph Node Transplantation as Therapy for Lymphedema

Provided the vasculature is reconnected, data in the literature suggests that autotransplanted lymph nodes seem to survive well and they re-establish appropriate lymphatic connections (Can et al., 1998; Shesol et al., 1979). It would seem also, that avascular autologous lymph node fragments regenerate to some extent when transplanted into various tissue compartments (Pabst and Rothkotter, 1988; Rothkotter and Pabst, 1990). Node slices developed many of the lymphoid characteristics of their intact counterparts and lymphatic vessels could be observed connecting to the node structure. A rabbit ear lymphedema model even suggested that such node fragment transplants were even capable of facilitating fluid drainage (Fu et al., 1998). Addition of VEGF-C therapy has the potential to enhance the outcome of autologous lymph node transplantation and fluid drainage as compared with control transplants in nude mice (Tammela et al., 2007).

While the re-implantation of nodes as a therapeutic measure for lymphedema has not been studied extensively, it would appear from limited accounts in the literature that this procedure holds some promise. In the studies of Chen et al. (Chen et al., 1990), hindlimb lymphedema was established in dogs by surgical ablation and irradiation. After at least six months when stable lymphedema had been established, inguinal lymph nodes were transplanted into the popliteal fossa and microvascular anastomosis carried out to connect the vasculature to the node. The animals were followed for a further three or six months. In the majority of animals the transplanted nodal tissues were viable and lymphangiography demonstrated improvement of lymph drainage and a reduction of edema. However, the edema did not subside completely over the period of study. There is also human data to support this concept. Becker et al. (Becker et al., 2006), transplanted femoral nodes into the axillary region of postmastectomy lymphedema patients using



microsurgical procedures. Despite having the disease for more than five years, the authors noted that the majority of patients demonstrated marked improvement. However for the most part, lymph node transplantation remains an experimental procedure and is not commonly offered to patients. Of course, one would expect that improved clinical outcomes are more likely if the nodal therapy is applied shortly after the tissue injury and before the chronic changes to the limb make the system more refractory to treatment.

### **Does the Transplantation of a Lymph Node Facilitate the Development of Afferent and Efferent Vessels?**

Our hypothesis is that the replacement of a node could help to restore the correct physiological relationships between pre- and post-nodal lymphatic vessels and provide structural orientation during lymphangiogenesis, facilitating re-alignment of pre- and post-nodal vessels. It is well established that lymphangiogenesis is both a vigorous and highly complex process, once damaged or cut lymphatics show a remarkable regenerative capacity.

The involvement of multiple lymphangiogenic growth factors has been extensively reported. VEGF-C and VEGFR-3 are examples of lymphangiogenic factors of interest in the literature and VEGF-C has already received some attention as a therapeutic agent (Karkkainen et al., 2001; Szuba et al., 2002; Yoon et al., 2003). VEGFR-3 was one of the first molecular markers shown to be specifically expressed on the lymphatic endothelium during the progresses of lymphatic vessel development, its pattern of expression mainly influencing new lymphatic budding (Kaipainen et al., 1995; Kukk et al., 1996). The angiopoietins have also been found to influence lymphatic development. The role of this

ligand family was found to be crucial in modulating the lymphatic vasculature (Gale et al., 2002; Veikkola and Alitalo, 2002).

However in our studies we do not know exactly what guides the cut ends of the pre- and post-nodal lymphatics to find their way and assume continuity with the vascularized node transplant. Are there are specific cellular or molecular cues or any specific growth factors released by the node in situ facilitating this. Moreover there are no studies in the literature to shed light on this particular phenomena.

### **Conceptual Basis for Understanding Lymphedema Development**

The re-implantation of a lymph node into a nodal excision site appears to offer significant benefits with regard to the development of lymphedema. However, in clinical practice, the surgical complexity and potential donor site morbidity issues may limit the usefulness of this approach. Nonetheless, the lymph node may have important conceptual implications for lymphedema development and therapy. In the data relating to our model development, following the removal of a lymph node (without further intervention), the process of lymphangiogenesis was capable of restoring a sizeable portion of the lost lymph transport capacity and no doubt, facilitated the resolution of a significant portion of the edema.

This attests to the remarkable regenerative capacity of this circulatory system. However, in terms of risk to the patient, one might speculate that the functional properties of the newly formed lymphatic vessels alone may not be able to restore the full reserve capacity of the system, and a relatively subtle sub-clinical lymph transport deficit could 'prime' the limb for subsequent edema formation.



## General Conclusion

To the best of our knowledge, the experiments presented here represent the first systematic investigation of the possible therapeutic value of autologous node transplantation and the results are promising. The most unique aspect of the experimental model presented here is the consistent manner in which the transport capacity of the lymphatic system was quantified across the popliteal region. Thereby providing a deeper understanding of the inner workings of the lymphatic system. While other large animal models mainly observed for changes or reduction of clinical edema.

Traditionally, it has always been assumed that removal of the lymph node has little impact on tissue fluid dynamics other than to damage the adjacent lymphatic vessels and the most popular views on the pathophysiology of lymphedema formation tend to attribute the injury to the lymphatic vessels. However, this concept needs to be reconsidered, particularly since the association between development of chronic secondary edema and lymph node excision has been known for some time.

First, the data in this thesis describes an experimental model in sheep that permits the quantitation of edema and lymphatic function over time. This lends an important role for the investigation of the physiologic parameters that cause lymphedema. Data in this thesis also sheds some light on novel surgical treatment possibilities by attempting to replace the previously excised node. The results were very positive. From the perspective of lymphatic function, the vascularized node transplants had a clear edge over the non-vascularized transplants. Additionally, the vascularized transplants were generally associated with the lowest levels of edema.

## Future Directions

There still remain many unanswered issues regarding effective treatment possibilities for post surgical lymphedema that merit further investigation. We believe that development of therapeutic strategies focusing on the lymph node, warrant further examination.

The recognition that lymph nodes play a vital role in tissue fluid balance, leads inevitably to further assessing the possibility of re-introducing a lymph node back into the surgical excision site. This concept has a certain appeal especially as this procedure may actually prevent the development of edema if applied early. This is a key issue since most other therapeutic approaches would presumably be applied after the edema was entrenched and are therefore likely to be more resistant to intervention. Obviously there are practical limitations to nodal transplantation due to the complexity of the microsurgical procedure and the risk of donor site morbidity, therefore I believe the initial step towards possibly introducing this type of intervention in clinical practice first requires detailed anatomical studies to ascertain feasible nodes for harvest on a vascular pedicle with minimal donor site morbidity and a reproducible method of successful transplant into the surgical excision site.

It may also be possible to implant cadaveric or animal tissues as a framework for nodal regeneration, but all cellular material has to be removed and there is always a risk of introducing unwanted infectious agents. However from the experience acquired during the course of this experimental project, it would appear that lymph node fragments or grafts do not survive and become resorbed when transplanted into donor tissue compartments and therefore are probably not the most promising choice for further detailed studies.



Another option could be the development of artificial nodal tissue for this purpose. It might be possible for example to engineer some type of scaffold impregnated with appropriate host cells and/or molecular factors to provide the anatomical and molecular cues necessary for the development of a nodal structure in vivo. Although this concept of producing a viable artificial lymph node is a promising one, but in practical terms can represent a challenge to implement in the foreseeable future.

Lastly, it is important to consider that most experimental studies that investigate clinical matters are usually designed to address a single problem in isolation of all other factors that accompany the disorder. For example, in addition to lymph node excision cancer patients often receive radiation therapy to the surgical site, and it would be interesting to investigate the effects of radiotherapy on transplanted nodal tissue and the impact this would have on edema development. In this regard it is appropriate that future models for investigating novel treatments of lymphedema take such factors into account.

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