Title: Defining lymphatic microstructure and the genetic basis of lipo-lymphedema

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Statement: This thesis is being submitted in total fulfillment of the degree. This is not a jointly awarded degree.
Abstract

The lymphatic system regulates tissue fluid homeostasis, intestinal fat absorption, and immune cell trafficking. Lymphedema is soft tissue swelling secondary to lymphatic dysfunction, which results in the accumulation of tissue fluid in the interstitial space. This might occur as a primary disorder of the developing lymphatic system, or alternatively lymphedema might be an acquired disorder secondary to lymphatic injury. For example, secondary lymphedema is a common problem following cancer and cancer treatments such as lymph node surgery and radiotherapy, resulting in significant morbidity. Radiotherapy is an established risk factor for lymphedema, and in addition to causing direct injury to the lymphatic vessel, it is possible that alternative mechanisms might also contribute to radiation-induced lymphatic dysfunction, such as localized ischemia of the lymphatic wall. It is also likely that predisposing genetic risk factors are at play, as not all individuals exposed to the same risk factors will develop secondary lymphedema. Lipoedema is a different form of soft tissue swelling due to the abnormal accumulation of adipose tissue. Lipoedema and lymphatic dysfunction appear to be linked, as individuals frequently develop a degree of lymphedema, particularly as the condition progresses in severity, where it may be described as lipo-lymphedema. The cause of lipoedema and the genetic basis of the condition are currently unknown. This thesis aims to discover and define alternative mechanisms for lymphatic dysfunction in the context of secondary lymphedema, particularly focusing on the supply of oxygenated blood to the lymphatic vessel wall. We also aim to describe inheritance patterns and the genetic factors involved in lipoedema and lipo-lymphedema. Such knowledge might uncover therapeutic targets and facilitate the development of treatments for lymphedema and lipoedema, including gene therapy.
Declaration page

This thesis comprises only my original work towards the Master of Surgery degree, except where indicated in the preface. Due acknowledgement has been made in the text to all other material used. The thesis is fewer than the maximum word limit in length, exclusive of tables, maps, bibliographies, and appendices.

Preface:

i) The following work towards the thesis was carried out in collaboration with others:

- Electron microscopy sample preparation and imaging was performed by Dr Eric Hanssen and his colleagues at the Advanced Microscopy Facility, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, VIC, as a collaboration (50%). Dr Hanssen performed the electron microscopy and provided assistance in interpreting the data. The candidate planned the experiments, collected the lymphatic specimens and interpreted the data.

- Confocal microscopy imaging was performed with Mr Cameron Nowell at the Monash Institute of Pharmaceutical Sciences (MIPS), Parkville, VIC, as a collaboration. (50%) The candidate planned the experiments, collected the tissue samples, and reviewed and interpreted the data.

- Bioinformatic analysis was performed by Dr Lavinia Gordon and interpreted in conjunction with Dr Charlotte Slade at AGRF and the Walter and Eliza Hall Institute (WEHI), Parkville, VIC, respectively, as a collaboration. (50%) The candidate planned the study and interpreted the clinical significance of the candidate variants.

ii) None of the work towards the thesis has been submitted for other qualifications

iii) None of the work towards the thesis was carried out prior to enrolment in the degree

iv) No third party editorial assistance was provided in preparation of the thesis.

v) No multi-authored publications are included in the thesis

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Theatre teams
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Chapter 1:

BACKGROUND AND LITERATURE REVIEW
1.1 The lymphatic vasculature in health and disease

The lymphatic system is a network of thin-walled vessels, which form the principal conduits for lymphatic drainage in the limb (Oliver & Detmar, 2002; Shayan et al., 2006). Originally called the “white veins”, (Lord, 1968), it has now transpired that lymphatics are more than purely passive pathways for tissue fluid and chyle. They have a diverse range of functions from immune cell trafficking (Johnson & Jackson, 2014) to the regulation of intestinal fat absorption and adipose tissue metabolism (Harvey, 2005).

Lymphedema is soft tissue swelling caused by accumulation of protein rich tissue fluid in the interstitial space (Mortimer, 1998). Lymphedema can be an inherited, primary disorder, such as in Milroy disease (Oliver & Detmar, 2002; Spiegel et al., 2006), but more commonly it is an acquired phenomenon secondary to lymphatic injury (Ugur et al., 2013). Conceivably this too might have a genetic predisposition, perhaps due to inherited variation in the genes governing lymphatic development, as described for the primary lymphedema syndromes (Karkkainen et al., 2000; Francois et al., 2008; Fang et al., 2000).

1.1.1 Lymphatic Development

The most widely-accepted theory of lymphatic development describes the formation of lymphatic sacs via a process of budding from the primitive venous system, with subsequent sprouting into localized lymphatic networks (Oliver & Detmar 2002; Wigle et al., 2002; Scavelli et al., 2004), however it is possible that lymphangioblasts of mesenchymal cell origin also contribute to the process (Scavelli et al., 2004; Schneider et al., 1999). Studies in mice suggest that the “master-switch” gene sox18 induces expression of the lymphatic marker Prox1 in a subpopulation of cardinal vein vascular endothelial cells, which signals lymphatic bias at embryonic day 9 (Francois et al., 2008), followed by differentiation and budding from day 10.5 (Wigle et al., 2002; Wigle & Oliver, 1999). These latter processes appear to be Prox1 dependent, since the same venous endothelial cell subpopulation fails to express the full compliment of early lymphatic endothelial cell markers such as LYVE-1 in Prox1 null mice, and this is associated with disorganised lymphatic budding and premature arrest of lymphatic development (Wigle et al., 2002).

1.1.2 Structure, form and function of the lymphatic system.

The lymphatic network consists of three vessel subtypes within the dermis (initial and pre-collecting lymphatics) and subcutaneous plane (collecting lymphatics) (Shayan et al., 2006, Scavelli et al., 2004).
The thin-walled initial lymphatics, or lymphatic capillaries are lacking a basement membrane, smooth muscle cells and valves, and are thought to function purely to absorb tissue fluid and cells (Shayan et al., 2006; Scavelli et al., 2004). In contrast the deep collecting lymphatics comprise a basement membrane, circumferential smooth muscle layer, and valves, while the dermal pre-collectors provide a connection between the initial and collecting lymphatic subtypes, with a morphology that is intermediate between the two (Shayan et al., 2006, Scavelli et al., 2004). The deep collecting lymphatics form the principal route for lymphatic drainage, and ultimately the return of lymph to the systemic circulation (Shayan et al., 2006; Scavelli et al. 2004). Collecting lymphatics appear to achieve this flow by a combination of peristalsis driven by the mural smooth muscle pacemaker cells (Shayan et al., 2006), and extrinsic mechanical compression from adjacent arterial pulsation, and skeletal muscle contraction, while the lymphatic valves function to preserve unidirectional flow (Petrova et al., 2004; Karnezis et al., 2012). All of these processes inherently require energy, oxygen and nutrients - in short, lymphatic vessels need a blood supply.

1.1.3 Morphology and the vasa lymphaticum

It has long been established that cells within the walls of arteries and veins have their own blood supply, and this is provided by a vascular network known as “the vessels of the vessels”, or vasa vasorum (Lowenberg & Schumacker, 1948; Wolinsky & Glagov, 1967; Scotland et al., 2000). The density and depth of vessel wall penetration by the vasa vasorum appears to be determined by two factors, namely the lamellar thickness of the mural smooth muscle media of the blood vessel (Wolinsky & Glagov, 1967), and the circulating oxygen tension within its lumen (Heistad et al., 1986; Scotland et al., 2000). For this reason, despite their thinner walls, the vessel wall blood supply to veins might be more critical than the equivalent blood supply to arteries, owing to the lower circulating oxygen tension in the venous system (Heistad et al., 1986). This concept is likely to extend to the collecting lymphatics, where the circulating oxygen tension is inherently low. Agliano et al reported on the distribution of vasa vasorum in human thigh collecting lymphatics in 1997, which they described through a combination of light microscopy imaging, and models derived from 3D reconstruction software (Agliano et al., 1997). They described two plexi of lymphatic vasa vasorum, one external and the other deep to the lymphatic smooth muscle cell layer, within the subendothelial space (Agliano 1997). To our knowledge, more detailed studies on the morphology of the lymphatic vasa vasorum, or “vasa lymphaticum”, and their functional significance in the context of lymphatic injury, have not been reported. Lymphedema refers to a functional defect in the lymphatic sys-
tem, resulting from defective absorption and transport of tissue fluid from the extremities back to the systemic circulation via the peripheral collecting lymphatics and thoracic duct (Oliver & Detmar 2002; Shayan et al., 2006). Acquired lymphatic dysfunction may be the result of a variety of insults, and is particularly common following therapeutic soft tissue irradiation, and surgical disruption or excision of the draining lymph nodes of the ailla or groin during cancer therapy (Deo et al., 2004; Ugur et al. 2013). It is also likely that lymphatic injury and its clinical manifestation may be influenced by the presence or absence of an underlying genetic susceptibility (Finegold et al., 2012).
1.2 Molecular characterisation of lymphatic endothelial cells

1.2.1 Markers and development:
Although the earliest descriptions of the lymphatic system date back to Aselli in the seventeenth century (as cited in Lord, 1968), a detailed understanding of lymphatic architecture, physiology, and pathophysiology has long been limited by the lack of lymphatic-specific molecular markers to define the lymphatic endothelium, distinguishing the lymphatic vasculature from the blood vascular system (Stacker et al., 2014). The discovery of the lymphatic endothelial marker vascular endothelial growth factor receptor 3 (VEGFR3) was one of the earliest steps in defining the lymphatic vasculature, and its discovery also shed some light on the embryonic origins of the lymphatic network (Kaipainen et al., 1995). VEGFR-3 is expressed by endothelial cells of the primitive venous and lymphatic systems, while VEGFR-3 expression becomes confined to the lymphatic endothelium in the adult (Kaipainen et al., 1995).
It is a cell surface receptor, which binds the vascular endothelial growth factors VEGF-C and VEGF-D, two key drivers of lymphangiogenesis (Stacker et al., 2014). The CD44 hyaluronan receptor homologue LYVE-1 is another lymphatic endothelial cell marker (Banerji et al 1999) largely expressed by the initial and pre-collecting lymphatics of the dermis. LYVE-1 expression appears to be down-regulated in the larger subcutaneous collecting lymphatics, limiting its application as a pan-lymphatic marker (Makinen et al 2005; Shayan et al., 2006). The surface glycoprotein podoplanin is expressed by type 1 alveolar cells of the lung, osteoblasts, renal podocytes, and lymphatic endothelial cells, and is thought to be expressed by all lymphatic vessel subtypes (Evangelou et al 2005; Breiteneder-Geleff et al., 1999) making it a useful marker (Shayan et al., 2006). Podoplanin expression appears to be controlled by the homeobox transcription factor Prox-1, another widely-expressed lymphatic endothelial marker and key regulator of lymphatic development (Wigle et al., 2002).

1.2.2 Lymphatic endothelial cell isolation
Until more recently, the lymphatics were considered absent from avascular tissues including cartilage and cornea, and from the retina and brain (Oliver & Detmar, 2002), however the recent discovery of LYVE-1 and CD31 positive lymphatic vasculature lining the dural sinuses of the brain has challenged this notion, and is likely to shed light on mechanisms of immune surveillance in the central nervous system (Louveau et al., 2015). Despite our increasing knowledge of lymphatic endothelial cell markers, the isola-
tion and molecular characterization of lymphatic endothelial cells from a range of different organ and tissue types remains a significant challenge. Previous attempts at isolating and characterizing these cells have largely focused on the initial and precollecting lymphatics of human and mouse dermis (Hirakawa et al., 2003; Kriehuber et al., 2001; Podgrabinska et al., 2002) and have generally taken the approach of expanding a mixed cell population in culture prior to immunomagnetic selection for endothelial cell (CD31) and lymphatic endothelial (podoplanin or LYVE-1) markers (Hirakawa et al., 2003; Podgrabinska et al., 2002). In one case, immunomagnetic enrichment of endothelial cells from mixed dermal suspensions was followed by further expansion in culture and subsequent purification of the lymphatic endothelial cell population by flow cytometry and cell sorting for CD31+ and podoplanin+ cells (Kriehuber et al., 2001). These authors also attempted to isolate lymphatic endothelial cells directly from fresh human dermal suspensions by immunolabelling for fibrocyte (CD45), vascular endothelial (CD34) and lymphatic endothelial (podoplanin) markers and sorting by FACStar for a podoplanin+CD34+CD45- population, however the authors commented that yields of lymphatic endothelial cells isolated by this technique were low, and reverted to immunomagnetic enrichment prior to purification by flow cytometry (Kriehuber et al., 2001), highlighting some of the technical challenges encountered in isolating this rare cell type. Although undoubtedly challenging, the molecular age has facilitated the study of the lymphatic system during disease, and this thesis will focus on the lymphatics in the context of radiation injury, malignancy, and obesity.
1.3 The lymphatic system during disease

1.3.1 Radiation injury as a side effect of cancer treatment

Despite careful attempts to direct radiation specifically toward the tumor site, exposure of the surrounding tissues to ionizing radiation inevitably occurs during radiotherapy, resulting in a broad spectrum of acute and chronic tissue injury (Stone et al., 2003). At the cellular level, DNA damage, mitotic cell death and apoptosis tend to affect tissues undergoing rapid proliferation, such as the skin or the gastrointestinal tract (Stone et al., 2003), while other cell types such as fibroblasts may become senescent in the acute phase, perhaps contributing to late radiation-induced effects, such as fibrosis (Stone et al., 2003; Steel 2001). Both apoptosis and cellular senescence have been demonstrated in the dermal lymphatic endothelial cells in a murine model of acute radiation injury (Avraham et al., 2010). It is interesting to note that in this study, lymphatic endothelial cells of irradiated mice were more sensitive to the acute effects of irradiation than cultured lymphatic endothelial cells irradiated in vitro, suggesting that direct lymphatic injury might be further compounded by the associated damage to the lymphatic microenvironment (Avraham et al., 2010).

Many of the clinical manifestations of radiation injury only become apparent months or years after treatment (Rodemann & Blaese, 2007), as the so-called late effects (Stone et al., 2003). Traditionally, radiation-induced vascular injury has been considered a late event (Stone et al., 2003; Hopewell et al., 1986), however acute injury to the microvasculature has been described (Rodemann & Blaese 2007; Peña et al., 2000). For example, in a mouse model of radiation-induced gastrointestinal syndrome, blood vascular endothelial cell apoptosis was reported as early as one hour after irradiation, preceding apoptosis of cells within the villus columnar epithelium (Paris et al., 2001). This concept is supported by earlier studies suggesting that lymphatic endothelial cells of the intestine are relatively resistant to radiation-induced apoptosis, compared to blood vascular endothelium of blood vessels within the gut (Sung et al., 2006).

The collecting lymphatics boast a diameter of over 200 μm and have a structured vessel wall with distinct intima, media and adventitial layers akin to those found within the wall of an artery (Shayan et al., 2006; Scavelli et al., 2004). By logical progression, the cells within the collecting lymphatic wall would also require their own blood supply, just like arteries and veins (Agliano et al., 1997). It is conceivable that injury to this lymphatic microvasculature, the “vasa lymphaticum”, and thus localized lymphatic vessel wall
ischemia, might contribute directly to the pathophysiology of radiation-induced lymphatic injury and lymphedema.

1.3.2 Cancer metastasis: how the lymphatics are hijacked to facilitate cancer spread

The role of the lymphatic system in malignant disease is significant, indeed the lymphatics are considered integral to the process of metastatic spread of a broad range of solid tumours (Shayan et al., 2006). In these instances, tumour spread occurs via the lymphatics to the regional lymph node basins, and beyond to distant organ sites, and the lymphanagiogenic growth factors VEGF-C and VEGF-D appear to actively facilitate these processes (Stacker et al., 2014; He et al., 2005; Karnezis et al., 2012). In a mouse model of tumour metastasis it has been shown that the activation of VEGFR-3 by tumour-derived VEGF-C promotes peritumoral lymphanagiogenesis and lymphatic dilatation (He et al., 2005). Furthermore, in a xenograft model of metastatic tumour spread, the collecting lymphatics of mice bearing VEGF-D-expressing human tumour cell lines appeared dilated in comparison to non-VEGF-D-expressing tumours (Karnezis et al., 2012). These VEGF-D associated lymphatic changes appear to be mediated by prostaglandin activity, since endothelial cells of the lymphatics draining VEGF-D-expressing metastatic tumours down-regulate expression of the prostaglandin degradation enzyme 15-hydroxyprostaglandin dehydrogenase. In keeping with this, in vivo inhibition of prostaglandin synthesis by a COX-2 enzyme inhibitor has been associated with reductions in collecting lymphatic diameter and in tumour cell metastasis in mice bearing VEGF-D expressing metastatic tumours (Karnezis et al., 2012). Taken together this evidence demonstrates that the lymphatic system may actively facilitate the malignant spread of tumours with metastatic potential, through mechanisms regulated by lymphanagiogenic growth factors.

The propensity for regional lymph node spread has been recognized in the clinical setting, where the disease status of regional lymph nodes is now considered one of the key prognostic indicators in the majority of solid tumours (Bilchik et al., 2002, Morton et al., 2014). The identification and detailed histological analysis of the sentinel node, the first node or group of nodes to drain a cancer, has enabled detection of micrometastasis, or even isolated tumour cells within the node (de Boer et al., 2009). Sentinel lymph node micrometastasis predicts the disease status of the remaining nodes in a regional lymph node basin (Morton et al., 2006; Morton et al. 2014), and this forms the basis of staging by sentinel lymph node biopsy. A randomized controlled trial of 2000 patients with intermediate or thick primary melanoma con-
firmed the predictive value of sentinel node biopsy over clinical monitoring of the regional lymph nodes, and showed the value of establishing sentinel node status in terms of guiding clinical management (Morton et al., 2004; Morton et al., 2014). Rates of 10 year distant disease-free survival and melanoma-specific survival were significantly higher among sentinel node-positive patients managed by immediate surgical clearance of the remaining regional nodal basin, compared with patients undergoing observation, where surgical lymph node clearance was delayed until involvement of the regional lymph nodes was detected by clinical examination and radiological assessment (Morton et al., 2004; Morton et al., 2014). In a large cohort study of almost 3000 women undergoing evaluation and treatment for low risk primary breast neoplasms, the presence of micrometastasis or isolated tumour cells in the sentinel lymph node was associated with a reduction in 5 year disease free survival of almost 10%, while systemic adjuvant therapy conferred a 5 year disease free survival benefit of nearly 10% in node-positive patients with micrometastasis or isolated tumour cells (de Boer et al., 2009), demonstrating the clinical significance of nodal status in determining cancer survival and in guiding therapeutic regimes.

Studies in transgenic mice over-expressing VEGF-C in cutaneous tumours have shown that the sentinel nodes of tumour-bearing mice respond to VEGF-C expression by enhanced lymphangiogenesis, even before metastatic tumour cells arrive at the node (Hirakawa et al., 2007). Furthermore, metastasis from the sentinel node to distant lymph nodes and to the lung occurred more frequently in VEGF-C transgenic mice than controls, indicating the VEGF-C also promotes distant tumour metastasis beyond the sentinel node. Importantly, lung metastases were not detected in the absence of associated sentinel and distal lymph node metastasis, supporting a sequential, stepwise model for tumour metastasis via the lymphatic network (Hirakawa et al., 2007). Of the two most popular models of distant tumour metastasis, namely haematogenous spread via invading tumour blood vasculature versus lymphogenous spread to the regional nodes prior to entry into the blood vascular system (Stacker et al., 2014; Ran et al., 2010), there is clearly compelling evidence from animal models and clinical studies in support of the latter.

The “seed and soil” hypothesis originally described by Paget in 1889, attempts to explain the propensity for tumour metastases to arise at certain sites, observing that tumour cells repeatedly disseminate to specific organs such as the liver, lung and brain, implying an affinity for these locations, which cannot simply be explained in terms of relative blood supply (as cited in Fidler, 2003). In a human study on patients
with peritoneovenous shunts inserted for palliation of ascites in advanced pelvic or intraabdominal malignancy, distant metastasis occurred relatively infrequently despite a direct route of entry for tumour cells into the systemic circulation being established via the shunt (Tarin et al., 1984a and Tarin et al., 1984b). When metastatic lesions were discovered in these patients on autopsy, these almost exclusively occurred in the liver and lung - well-established sites for tumour metastasis, rather than other organs with relatively large blood supply, such as the spleen (Tarin et al., 1984a; Fidler, 2003). In a murine model of melanoma metastasis, mice receiving syngeneic B16 melanoma cell lines which were injected intravenously into the tail vein, repeatedly developed neoplastic lesions in the lungs, and in transplanted pulmonary and ovarian tissues placed heterotopically in the mouse thigh subcutaneous tissue or musculature (Hart & Fidler, 1980). In contrast, neoplastic lesions rarely developed in heterotopically transplanted renal tissue, or at sites of soft tissue trauma, indicating that specific tissue microenvironments are more receptive to circulating melanoma tumour cells than others (Hart and Fidler, 1980).

Given the significance of the lymphatic system in regional lymph node spread and beyond (Karnezis et al. 2012; Stacker et al., 2014; Hirakawa et al., 2007), it is also conceivable that lymphatics within certain tissue and organ types might actively facilitate distant tumour metastasis, perhaps via lymphangiogenesis, mechanical dilatation or chemotactic signalling. Given the propensity for tumours to metastasise to specific organs, if is highly likely that the lymphatic endothelial cells within these organs differ in their expression profiles, particularly for pathways involved in tumour metastasis.

### 1.3.3 Lymphatic dysfunction and obesity

The link between the lymphatic system and lipid metabolism has been observed for centuries. For instance Aselli described the role of gut lymphatics in the absorption and transport of lipids early in the 17th century (as cited in Harvey et al., 2008). The collecting lymphatics and lymph nodes lie within the subcutaneous adipose tissue and fat pads, respectively (Harvey et al., 2008), and perinodal adipocytes appear to play an active role in lymph node metabolism, reacting to immunological challenge by an upregulation in lipolysis, for example (Pond & Mattacks, 1998). In keeping with this, lymphatic dysfunction and defective lipid and adipose tissue metabolism also appear to occur simultaneously. In the Chy mutant mouse, a heterozygous VEGFR-3 gene mutation is associated not only with failure of lymphatic
development and defective lymphatic vasculature, but with excess subcutaneous adipose tissue deposition and chylous ascites (Karkkainen et al., 2001).

More compelling evidence for a link between defective lymphatic function and abnormal adipose tissue metabolism comes from a murine model of Prox1 haploinsufficiency, where lymphatic vascular insufficiency appears to be associated with adult onset obesity (Harvey et al., 2005). The Prox1<sup>−/−</sup> mouse becomes obese from 9 weeks of age, with evidence of adipocyte hypertrophy and abnormal hepatic lipid deposition in older obese mice (Harvey et al., 2005). In the adult mouse, lymphatic vascular abnormalities including dilated, leaking and disorganized lymphatic networks, are most striking in the intestinal and mesenteric lymphatics, and associated with chylous ascites and intestinal wall lipid deposition (Harvey et al., 2005). Furthermore the extent of adult obesity appears to correlate with the degree of lymphatic vascular dysfunction, with the accumulation of adipose tissue specifically within the vicinity of the lymph nodes and abnormal mesenteric lymphatics, implying a causal link between lymphatic leakage and abnormal fat deposition. In keeping with this, the lymph from Prox1<sup>−/−</sup> mice appears to enhance adipocyte differentiation in vitro (Harvery et al., 2005).

These findings in the mouse are supported by clinical data in patients linking lymphedema to obesity, where CT imaging has indicated that abnormal adipose tissue accumulation is a prominent feature of chronic, severe upper limb lymphedema (Brorson et al., 2006). Conversely obesity also appears to be a significant risk factor for lymphedema (Mehrara & Greene, 2014), an observation now supported by data from large-scale, prospective clinical studies in breast cancer cohorts (McLaughlin et al, 2008; Helyer et al, 2010; Mehrara & Greene, 2014), where a body mass index (BMI) in excess of 30 (clinical obesity) has been linked to a significantly enhanced risk of upper limb lymphedema compared to non-obese patients with a BMI of under 25 undergoing sentinel node sampling with or without axillary node clearance, with an odds ratio of just below 3 (Helyer et al., 2010). Even in the absence of classic clinical risk factors for lymphedema, such as malignancy, radiotherapy and lymph node surgery, extreme obesity appears to be associated with lymphedema independently (Greene et al, 2012), a finding corroborated by data from mice where a primary genetic defect in cholesterol metabolism (Apolipoprotein E mutation) has been associated with lymphatic dysfunction and abnormal lymphatic valves (Lim et al, 2009). In light of this connection between lymphedema and abnormal adipose deposition, it is perhaps not surprising that liposuction has traditionally been the mainstay of surgical treatment for lymphedema (Bagheri et al, 2005).
1.3.4 Lipoedema

Lipoedema, otherwise known as “adiposis dolorosa”, is another form of soft tissue swelling of the extremities, commonly affecting females, originally described by Allen & Hines in 1940 (as cited in Buck & Herbst, 2012). The condition is characterized by painful, symmetrical limb swelling occurring almost exclusively in females, secondary to abnormal adipose tissue deposition classically sparing the hands, feet, face, neck and trunk (Herbst, 2012). Those affected frequently report a temporal association between disease onset or exacerbations and hormonal changes such as puberty, pregnancy and the menopause, implying a hormonal basis (Child et al., 2010). The condition is typically refractive to lifestyle changes and surgical interventions targeted at obesity, with reports of the abnormal adipose tissue persisting even in the setting of extreme dieting and bariatric surgery (Bast et al., 2016). Treatment strategies currently focus on symptom management, physiotherapy, and surgical debulking (Peled et al., 2012; Baumgartner et al., 2016), however there are no cures, nor preventative measures to halt the development and progression of the disease.

There is some evidence to suggest that patients with lipoedema also have altered lymphatic structure and function. However there is currently a lack of definitive evidence to determine whether lymphatic dysfunction is a contributing factor to the pathophysiology of lipoedema, or a consequence of the disease (Harvey et al., 2008). The most advanced clinical stage of lipoedema, Stage IV disease, is characterized by non-pitting “lipo-lymphedema” (Buck and Herbst, 2016), and the condition itself is frequently misdiagnosed as lymphedema (Goodliffe et al., 2013). Further to this, in a small study of 12 patients with lipoedema, multiple microaneurysms of the dermal lymphatics were detected by fluorescence microlymphography in the affected regions of the lower limb, whereas only one microaneurysm of the thigh was reported within 12 healthy controls (Amann-Vesti et al., 2001), suggesting at least some degree of lymphatic structural disturbance in lipoedema. In contrast, in a limited case series of patients with a clinical diagnosis of lipoedema, objective measures of lymphatic function according to lymphoscintigraphy demonstrated only borderline impairment in the lower limb in seven out of ten patients, and bilateral moderate impairment in just one (Harwood et al., 1996). Clearly our understanding of the complex interplay between the lymphatic system and adipose tissue physiology remains incomplete, particularly in the setting of lipoedema.
Certain causal genetic mutations have been identified in the primary lymphedema syndromes, including mutations in the FOXC2 gene in lymphedema-distichiasis (Fang et al., 2000), SOX18 in hypotrichosis-lymphedematelangectasia (Francois et al., 2008; Irrthum et al., 2003), and VEGFR3 inactivating mutations in Milroy disease, an autosomal dominant, early onset, bilateral lower-limb oedema (Spiegel et al., 2006; Karkkainen et al., 2000). Although a positive family history is widely reported in lipoedema (Child et al., 2010), there is currently a lack of an established genetic basis for the disease. Such a discovery might lead to novel treatments for the condition, including gene therapy.
1.4 Hypotheses and aims:

Hypothesis 1
Nutrients and oxygen are supplied to the collecting lymphatic vessel wall by a blood microvascular network, the “vasa lymphaticum”. Injury to this microvasculature, and thus localized ischemia, might play a role in the pathophysiology of lymphatic injury and lymphedema.

Aims for hypothesis 1
To characterise the blood supply of the collecting lymphatics, the “vasa lymphaticum”

Hypothesis 2
Lymphatic endothelial cells can be isolated from a range of tissue and organ types. These lymphatic endothelial cells have tissue-specific molecular and functional characteristics.

Aims for hypothesis 2
To isolate lymphatic endothelial cells from different tissues and organs by flow cytometry, according to their expression of lymphatic endothelial markers, and to determine tissue- and organ-specific characteristics

Hypothesis 3
People with lipoedema have an inherited genetic predisposition, with altered expression of genes involved in adipose tissue metabolism, and lymphatic growth and regulation.

Aims for hypothesis 3
To perform genetic sequencing in patients with lipoedema and lipo-lymphedema, and their relatives
Chapter 2:

MATERIALS AND METHODS
2.1 Vasa lymphaticum in the mouse

2.1.1 Mice
Prox1-GFP BAC transgenic mice (KY221 Gsat/Mmcd), on a FVB/N-Crl:CD1(ICR) background were obtained from the institute of Molecular Biosciences Animal facility (University of Queensland). These were re-derived according to local protocols (IBC 246, AEC –AGNMBL 013/14 St Vincent’s Hospital Melbourne). Ethical approval for animal experiments was obtained from St Vincent’s Institute of Medical Research and St Vincent’s Animal Ethics Committee (AEC 015/15).

2.1.2 Murine tissue preparation

After administering a lethal dose of isofluorane, the tail was sharply detached at its base and tail tissues were harvested by making a full length, longitudinal incision on the ventral aspect in the midline, followed by circumferential, sharp dissection of the skin, subcutaneous tissues and vascular bundles en bloc, leaving the tendons and bone behind. The harvested tail tissues therefore comprised skin, subcutaneous connective tissue and fat, and two principal longitudinal vascular bundles with their associated collecting lymphatics.

Mouse flank was harvested via a longitudinal incision in the ventral midline of the trunk, extending laterally onto the ventral aspect of all four limbs. The flank tissues were reflected off the underlying deep fascia of the abdominal and thoracic wall, and limb musculature by a combination of blunt and sharp dissection, revealing the underlying subcutaneous fat, vascular bundles and lymph node basins. The inguinal fat pad and inguinal lymph node were visualized under an operating microscope and traced cranially toward the axillary lymph node basin of the forelimb. The vascular bundle comprising artery, vein and collecting lymphatic vessels running between the axillary and inguinal nodes was then harvested as a longitudinal 1 x 5 cm section from each flank comprising the full thickness of flank skin and subcutaneous fat by making sharp, longitudinal incisions either side of the vascular bundle.

For the thoracic duct and cisterna chyle harvest, following a lethal dose of isofluorane, approximately 100 μl of patent blue V dye (Guerbet, Asia Pacific Ltd) was injected intradermally and subcutaneously into the foot pads of the hind limbs bilaterally and lymphatic uptake encouraged by massage at the injection site
and repetitive passive cycling movements of the limbs. A ventral midline incision was then made over the thorax and abdomen, through the skin, subcutaneous fat. After reflecting the soft tissues off the underlying abdominal and thoracic wall, as previously described, the thoracic cavity was opened via a para-sternal approach and the abdominal wall musculature divided longitudinally to open the abdominal cavity. The intestine was displaced laterally to reveal the retroperitoneal vasculature (aorta and inferior vena cava). The cisterna chyle and thoracic duct were identified based on morphology and uptake of patent blue dye, and dissected from the adjacent blood vasculature and retroperitoneal viscera under the operating microscope. The diaphragm was divided and the thoracic duct traced proximally before dividing it at the level of the descending aorta. The specimen was fixed in a solution containing 1% (w/v) glutaraldehyde, 1.5% paraformaldehyde (PFA), 5 mM CaCl₂, in 0.1M sodium cacodylate and transported on ice to the University of Melbourne Bio21 facility, Parkville, Melbourne for embedding, processing and imaging by transmission electron microscopy.

2.1.3 Immunohistochemistry

Mouse flank tissues were fixed in 4% PFA at 4°C for 12 hours. Longitudinal sections of flank tissue were sharply divided into 1 x 0.5 cm tissue blocks before processing to the paraffin wax stage on a Thermo Ex-celsio Tissue Processor. Serial tissue sections were cut on a microtome at 5 μm and mounted on Polysine slides (Thermo Fisher), and dried overnight at 37°C. For all staining protocols, tissue sections were initially dewaxed in histolene and hydrated through graded ethanols using standard techniques, and dried overnight at room temperature prior to immunostaining.

Haematoxylin and eosin (H&E) staining was performed by immersion in Mayer’s Haematoxylin for 5 minutes, followed by washing in ammonia water, then immersion in eosin for 2 minutes at room temperature.

Sections were immunostained with the following primary and secondary antibodies:
CD31 (1:100 rat anti mouse PECA-1 (BD Biosciences)), podoplanin (1:800 hamster anti mouse podoplanin (Fitzgerald)), LYVE-1 (1:250 rabbit anti mouse (Abcam)), von Willebrand factor (1:100 rabbit anti-human von Willebrand factor (Millipore)), α-smooth muscle actin (1:300 Dako mouse anti-human α-smooth muscle actin (Agilent Technologies)), and green fluorescent protein (GFP) (1:500 rabbit anti A.
victoria GFP (Abcam), Dako rabbit anti rat 1g-biotin 1:300 (Agilent Technologies), goat anti-hamster biotinylated antibody 1:200 (Vector), goat anti rabbit-biotin 1:200 (Vector), Dako rabbit anti-mouse-biotin (Agilent Technologies),

Isotype controls: Rat IgG (Zymed), hamster 1gG (Jackson ImmunoResearch), rabbit 1gG (R&D systems), Dako mouse 1gG (Agilent Technologies)

Immunostaining was performed at room temperature, unless otherwise stated, using a tris-buffered saline (TBS) diluent and wash buffer. Antigen retrieval (if required) was performed either by incubation in 10mM tri-sodium citrate buffer at pH 6.0 at 95°C for 20 minutes, or in a Dako Proteinase K solution (Agilent Technologies) for 4 minutes. Endogenous peroxidase activity was blocked by 3% methanol in distilled water for 5 minutes, and protein blocking performed using Dako Protein Block (Agilent Technologies) for 20 minutes. Tissue sections were incubated in primary antibody at the appropriate dilution, as described, for 60 minutes, followed by a biotinylated secondary antibody, as described, for 30 minutes.

Enzyme labeling was performed by applying Dako horse-radish peroxidase (HRP)-streptavidin at 1:400 dilution (Agilent Technologies), or an avidin-biotin complex (ABC) Vector ABC Elite (Vector) for 30 minutes, and the colour developed with Dako DAB Plus (Agilent Technologies) for 5 minutes. IgG isotype controls were performed for each primary antibody at the equivalent concentration, as described. A nuclear counterstain was performed using haematoxylin, as described. Tissue sections were dehydrated through graded ethanols, cleared in histolene, and mounted in DPX (Merck).

Images were acquired by standard brightfield microscopy on an Olympus BX61 microscope with DPX71 colour camera.

2.1.4 Immunostaining of whole mounted tail tissues

Tissues were sharply divided into 0.5 x 0.5 cm segments, each encompassing a section of the vascular bundle and associated collecting lymphatics. Alternatively, in order to gain an overview of the frequency and pattern of blood microvascular branching associated with, or traversing in close proximity to the lymphatic wall, entire circumferential soft tissue specimens were obtained from the tails of Prox1-GFP mice, comprising the full tail length. These specimens comprised skin and subcutaneous tissue with two
principal collecting lymphatic vessels and associated blood vasculature running longitudinally along the full length from the tip of the tail to the base, as previously described.

Tissues were immunostained with the following primary and secondary antibodies: CD31 (1:100 rat anti mouse PECAM-1 (BD Biosciences)), von Willebrand factor (1:100 rabbit anti-human von Willebrand factor (Millipore)), or lectin (1:500 Griffonia simplicifolia lectin-biotin (Vector)), goat anti rat Cy3 1:300 (Jackson ImmunoResearch), donkey anti rat alexa fluor 568 1:100 (Thermo Fisher (Invitrogen)), goat anti-rabbit alexa fluor 568 1:100 (Thermo Fisher (Invitrogen)), or streptavidin-Dylight 549 1:800 (Jackson ImmunoResearch). Isotype controls: Rat IgG (Zymed), rabbit IgG (R&D systems).

Mouse tail tissues were fixed in 4% PFA overnight at 4°C. Staining of whole tails was performed in 15 ml Falcon tubes at room temperature, on a rotating plate, while tail segments were stained in a 24 well plate. Specimens were washed in phosphate buffered saline (PBS) (Sigma Aldrich), followed by a tissue permeabilisation step in 0.5% Triton X detergent in phosphate-buffered saline (PBS) for 60 mintues. Blocking was performed overnight in 0.1% Triton X detergent and 10% goat or donkey serum (Sigma Aldrich) in PBS. Primary antibody or Griffonia simplicifolia lectin was applied in Dako Flex Antibody Diluent (Agilent Technologies) overnight at 4°C on a rocking plate. For negative controls, rat or rabbit IgG were used at equivalent concentrations. Samples were washed in 0.1% Triton X detergent in PBS three times, and secondary antibodies applied in Dako Flex Antibody Diluent (Agilent Technologies) overnight at 4°C on a rocking plate. Samples were washed again three times as described, and a nuclear counterstain performed with 1 μg/ml DAPI (Molecular Probes) in 0.1% Triton X detergent in PBS for 15 minutes. Samples were washed twice as described and kept in 0.1% Triton X detergent in PBS until imaging.

Fluorescent microscopy imaging of tail segments was performed on an Olympus BX61 microscope with DPX71 colour camera. Confocal imaging of whole tail specimens was performed on a Leica SP8 confocal running LASX version 2.0.1. 488 nm and 561 nm lasers were used for excitation and emissions were 495-530 and 570-620 nm. All scans were performed in resonant mode (12000 Hz) with 8 times averaging using a 10 x Plan Apo objective.
2.1.5 Micro CT of mouse tail

The skin and subcutaneous tissues of the Prox1-GFP mouse tail were harvested en bloc as previously described. 1 x 2 mm specimens were taken from the proximal tail, within 1 cm of the tail base, comprising skin and subcutaneous tissue and vascular bundles, and specifically orientated about the long axis of the subcutaneous vascular bundle to incorporate blood vessels and collecting lymphatics. Specimens were orientated in preparation for electron microscopy by placing nylon microsutures at both cut ends of the vascular bundle, in the skin immediately adjacent to the vessel ends to avoid disruption to the vessel architecture.

Tissues were fixed by immersion in a solution containing 1% (w/v) glutaraldehyde, 1.5% PFA, 5 mM CaCl₂, in 0.1M sodium cacodylate. Specimens were prepared for electron microscopy using the ROTO technique (Hanssen et al., 2013) and embedded in Procure 812 resin. Micro-CT scanning was performed with a Phoenix Nanotom (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) operated using xs control and Phoenix datos|x acquisition software (both GE Sensing & Inspection Technologies) at the University of Melbourne, Parkville, VIC.

2.1.6 Scanning electron microscopy (SEM) of mouse tail

Following sample preparation (Section 2.1.5), resin blocks were trimmed to 500 x 500 x 300 um and glued to a volumescope stub. 50 nm serial sections were cut and imaged at 2 kV and 1 nA. Images were realigned using serialSM (Fremer et al., 1996).
2.1.7  Transmission electron microscopy (TEM) of mouse thoracic duct

Specimens were prepared for electron microscopy using the ROTO technique (Hanssen et al., 2013) and embedded in Procure 812 resin as described (Section 2.1.5). 70 nm tissue sections were cut in an orientation transverse to the longitudinal course of the vascular structures, and observed with a FEI Tecnai F30 transmission electron microscope (Bio21 Advanced Microscopy Facility, University of Melbourne, Parkville, VIC).
2.2 Vasa lymphaticum in human

2.2.1 Human ethics and samples

Ethical approval was granted by the Human Research Ethics Committee (HREC) of St Vincent’s Hospital, Melbourne (HREC-A 067/16) for a study on lymphatic vessel structure and function in human soft tissue biospecimens. Informed consent was obtained from all study participants for the use of surplus soft tissues removed at the time of surgery, Lymphedema lymphatic tissue specimens were obtained from the limb (leg or forearm) of patients undergoing lymphedema surgery (lymphatico-venular anastomosis). Pre-operative MR lymphangiograms were performed with the contrast agent Gadovist. Control tissues were obtained from the surplus abdominal tissue of patients undergoing a body contouring procedure (abdominoplasty) or immediate abdominal-based autologous breast reconstruction following mastectomy, in the setting of a localized primary breast malignancy.

2.2.2 Human tissue preparation

Lymphatic specimens from lymphedematous tissues were obtained intraoperatively from patients undergoing microsurgical treatment for lymphedema. Lymphatico-venular anastomosis describes the identification and dissection of the superficial, subcutaneous collecting lymphatics at the site of lymphatic malfunction, usually in the distal upper or lower limb, and microsurgical anastomosis to the subcutaneous venous system (Koshima et al., 2012). In all cases immediately prior to surgery and under general anaesthesia, 5 mg/ml indocyanine green dye (ICG) (Diagnostic Green, Germany) was injected into the dorsal webspaces of the hand or foot of the affected limb, and pre-operative lymphatic localization was achieved using an external, hand-held PDE near infrared fluorescence imager (Hamamatsu Photonics). In addition, at the same location as the ICG injection, patent blue dye (Guerbet, Asia Pacific Ltd) was injected intradermally into the dorsal webspaces of the corresponding hand or foot. At the sites of lymphatic dysfunction, 2-5 cm transverse incisions were made through the epidermis and a variable thickness of the dermis, and the residual dermis carefully divided by blunt dissection to preserve the superficial subcutaneous collecting lymphatics. Intraoperatively, lymphatic vessels were identified by their morphology and lymphatic uptake of patent blue dye in the lumen. Therefore, in summary, the collecting lymphatics were identified in the affected limb by three separate processes:

1. Pre-operative MR lymphangiogram.
2. Peri-operative percutaneous lymphatic mapping with intradermal ICG injection, as described

3. Intraoperative identification under an operating microscope, by morphology and blue dye uptake, as described.

Small (approx. 1 mm long) lymphatic biospecimens were obtained from subcutaneous collecting lymphatics within the lymphedematous tissues. Lymphatic biospecimens were bluntly dissected from the surrounding adipose tissue and orientated for analysis by the placement of 11/0 nylon microsutures (Ethicon) at both vessel ends under an operating microscope. Lymphatic biospecimens were rendered re-identifiable (coded) and transported to the O’Brien Institute, St Vincent’s Institute, Melbourne.

Control tissues were derived from the perforating vascular bundles on the deep surface of excess abdominal tissue harvested for autologous breast reconstruction in patients without lymphedema. The lymphatic vessels cannot be easily identified in these specimens in the absence of patent blue uptake, and therefore blood vessels were identified and dissected from adjacent adipose tissue and fascia under an operating microscope, and whole vascular bundles comprising lymphatic and blood vessels were sharply divided into 2-3 mm sections, and orientated en bloc by the placement of 10/0 nylon sutures (Ethicon) as described.

2.2.3 Immunostaining of human tissue sections

Tissues were fixed in 4% PFA overnight at 4°C then processed to the paraffin wax stage on a Thermo Excelsio Tissue Processor. Serial sections were cut on a microtome at 5 μm and mounted onto Polysine slides and dried as described for the mouse. Haematoxylin and eosin staining of tissue sections was performed as described in the mouse, with the reagents described (Section 2.1.3)

For immunostaining, tissue sections were dewaxed and hydrated through graded ethanols, as described. Washing steps between antigen retrieval, peroxidase quenching, primary and secondary antibody staining and enzyme labelling were performed with 0.05% tween in PBS (Sigma Aldrich). Where required, antigen retrieval was undertaken using either 10 mM tri-sodium citrate buffer at pH 6.0 in a water bath at 95°C for 20 minutes, or with Dako Proteinase K solution (Agilnet Technologies) for 4 minutes. Endogenous peroxidase activity was blocked with 3% methanol in distilled water for 5 minutes. Blocking was performed at room temperature using Thermo UltraVision Protein Block (Thermo Fisher Scientific) for CD31, D2-40 and smooth muscle actin.
immunostaining, for 5 minutes, or alternatively using 20% normal goat serum (Sigma Aldrich) in Thermo Ultra Clean diluent (Thermo Fisher Scientific) for vWF immunostaining protocols, for 60 minutes. Tissues were incubated in primary antibody diluted in Dako Envision Flex diluent (Agilent Technologies) for CD31 and D2-40 immunostaining, or 20% normal goat serum (Sigma Aldrich) in Thermo Ultra Clean diluent (Thermo Fisher Scientific) for vWF immunostaining, or Thermo Ultra Clean diluent alone for smooth muscle actin staining, at the antibody dilutions specified, at room temperature for 30-60 minutes. Negative controls were performed by substituting the primary antibody for diluent only, or in the case of D2-40 and smooth muscle actin staining, isotype controls were performed using mouse IgG at the equivalent concentration. Biotinylated secondary antibodies were applied in the same diluent as the corresponding primary antibody, at the dilutions specified. Tissues were incubated in secondary antibody at the dilutions specified for 30 minutes.

Primary and secondary antibodies were used at the following concentrations:

CD31 (1:10 rabbit anti human CD31 (Thermo Fisher Scientific)), Podoplanin (1:100 Dako mouse anti human podoplanin clone D2-40 (Agilent Technologies)), α-smooth muscle actin (1:300 Dako mouse anti human α-smooth muscle actin (Agilent Technologies)), von Willebrand Factor (1:100 von Willebrand Factor rabbit anti human (Millipore)), goat anti rabbit-biotin (1:200 (Vector)), goat anti mouse-biotin (1:200 (Vector)), Dako rabbit anti mouse-biotin (1:200 (Agilent Technologies)). Isotype controls: Dako mouse IgG (Agilent Technologies)

Enzyme labeling was performed using Vector ABC Elite (Vector), or Dako horse radish peroxidase-streptavidin (Agilent Technologies) at 1:400 dilution for 30 minutes, and the colour developed by applying Thermo DAB Plus substrate system (Thermo Fisher Scientific) or Dako DAB (Agilent technologies) for 5 minutes. Tissues were washed in distilled water and haematoxylin nuclear counterstaining performed as previously described. Tissue sections were then dehydrated through graded ethanol's and mounted in DPX (Merck).

Brightfield images were acquired on an Olympus BX61 microscope with a DPX71 colour camera.

2.2.4 Immunostaining of human whole mounted specimens

Human lymphatic specimens were fixed in 4% PFA overnight at 4°C. Samples were stained in a 24 well plate, and incubation performed on a rotating plate at room temperature unless otherwise specified. Lymphatic specimens were washed in PBS (Sigma Aldrich) for 15 minutes, and then subjected to a permeabilising step for 60 minutes in 0.5% Triton X detergent in PBS. Protein blocking was performed in 10% goat serum (Sigma Aldrich) in 0.1% Triton X detergent in PBS. Primary antibodies were diluted in 20% normal goat serum for vWF,
or Dako Envision Flex Diluent (Agilent Technologies) for CD31 and D240, and applied overnight at 4°C on a rocking plate. Specimens were washed three times in 0.1% Triton X detergent in PBS, and then secondary antibodies were applied at the dilutions specified in Dako Envision Flex Diluent (Agilent Technologies) overnight at 4°C on a rocking plate. Once again specimens were washed 3 times, as described, and nuclear counterstaining performed with 1 μg/ml DAPI (Molecular Probes) in 0.1% Triton X diluent in PBS for 15 minutes. Two further washes were performed, as described. Lymphatic specimens were transferred and stored in glycerol in preparation for imaging. Antibody co-staining of lymphatic specimens was performed as described in the following combinations: D2-40 and von Willebrand Factor, or D2-40 and CD31, both with a DAPI counterstain. Negative controls were performed by omitting the primary antibody and applying diluent only, or alternatively for D2-40, isotype controls were performed by substituting the primary antibody with the equivalent dilution of IgG, as specified.

Primary and secondary antibodies were used at the following concentration:

CD31 (1:20 rabbit anti human CD31 (Thermo Fisher Scientific)), Podoplanin (1:20 Dako mouse anti human podoplanin clone D2-40 (Agilent Technologies)), von Willebrand Factor (1:100 rabbit anti human vWF (Millipore)), goat anti rabbit Alexa Fluor 568 (1:100; Thermo Fisher (Invitrogen)), goat anti mouse alexa fluor 488 (1:100; Thermo Fisher (Invitrogen)). Isotype controls: Dako mouse IgG (Agilent Technologies)

For lymphatic wall imaging by confocal microscopy, specimens were transferred to glass slides, and immersed in glycerol to suspend the lymphatic wall and prevent vessel wall collapse. Imaging was performed on a Leica SP8 confocal running version 2.0.1 of LASX, and using a H PlanApo CS2 20x 0.75NA objective. 405 nm laser with 410-450 nm emission was used for DAPI, a 488 laser with 495-550 nm emission for Alexa Fluor 488 and a 561 nm laser with 570-650 nm emission for Alexa Fluor 568.

2.2.5 Micro CT and electron microscopy of human lymphatic specimens

For electron microscopy and micro CT, lymphatic specimens were orientated by placing 11/0 nylon microsutures (Ethicon) at either end of the longitudinal lymphatic vessel specimens. Tissues were then fixed in a solution containing 1% glutaraldehyde (w/v), 1.5% PFA, 5 mM CaCl₂ in 0.1M sodium cacodylate, and transported on ice to the University of Melbourne Bio21 facility, Parkville, Melbourne for embedding, processing and imaging by micro CT as previously described for mouse tissues (Sections 2.1.5, 2.1.6 and 2.1.7).
Resin-embedded biopsies were cut from a resin block to achieve a small sample diameter (<3 mm) allowing for resolutions of 1.221 micrometer (Case 4, 6 and control) to 1.497 micrometer (Case 5) to be achieved. Scans of samples were performed at 70 kV and 375 μA for various periods of time depending upon the number of x-ray projections collected and image averaging. Case 4 (53 minutes; 800 projections; av = 3; skip = 1; timing = 500 mS); Case 6 (66 minutes; 1000 projections; av = 3; skip = 1; timing = 500 mS); Control (57 minutes; 1500 projections; av = 2; skip = 0; timing = 500 mS); Case 5 (2 scans for 79 minutes each; 2000 projections; av = 2; skip = 0; timing = 500 mS).

Volume reconstruction of the micro-CT data was performed using Phoenix datos|x reconstruction software (GE Sensing & Inspection Technologies). The two separate volume files for Case 5 were merged using Avizo (FEI).

Transmission electron microscopy was performed as previously described in the mouse (Section 2.1.7)
2.3 Lymphatic endothelial cell isolation and characterisation in the mouse

2.3.1 Mice

6-12 week old, female Prox1-GFP BAC transgenic mice (KY221 Gsat/Mmcd), on a FVB/N-Crl:CD1(ICR) background were obtained, and ethical approval granted by St Vincent’s Animal Ethics Committee, Melbourne VIC (AEC 015/15). For control tissues, female mice on a NOD background were used.

2.3.2 Murine organ and tissue harvest

After administering a lethal dose of isofluorane, murine tissues were harvested for subsequent lymphatic endothelial cell isolation by the following techniques:

2.3.2.1 Dermis

The dorsal aspect of the ear was shaved with a blade and sharply divided at its base at the junction between the pinna and the external acoustic meatus. Ears were immediately immersed in ice-cold endothelial cell EGMV-2 media without additives (Lonza), containing 10 000 units/ml pen-strep (Life Technologies). Under an operating microscope, the ear skin was bluntly lifted off the underlying cartilage anteriorly and posteriorly, and residual adherent cartilage peeled off using forceps. Ear skin was then cut into approximately 1 x 1 mm pieces to facilitate tissue digestion.

2.3.2.2 Lymph nodes

A ventral midline incision was made over the thorax and abdomen, extending longitudinally along the flexor aspect of all limbs. The skin and underlying subcutaneous tissue were dissected off the underlying fascia of the thoracic and abdominal wall musculature and the deep fascia of the limb. The popliteal lymph nodes were identified in the hind limbs under an operating microscope by their location, colour and consistency compared to the surrounding adipose tissue. The brachial and axillary nodes were identified in the forelimbs and bluntly dissected from the surrounding adipose tissue and axillary blood vasculature. The inguinal lymph nodes were identifiable in the subcutaneous tissue of the flanks, at or adjacent to the confluence of dorsal and ventral branches of the subcutaneous blood vasculature. Lymph nodes were divided from their afferent and efferent collecting lymphatics and immediately immersed in
EGMV-2 (Lonza) containing 10,000 units/ml pen-strep (Life Technologies) on ice, as described (Section 2.3.2.1).

2.3.2.3 Collecting lymphatics

Approximately 100 μl of patent blue dye (Guerbet, Asia Pacific Ltd) was injected intradermally and subcutaneously into the footpads of the forelimbs and hindlimbs bilaterally, and lymphatic uptake encouraged by massage at the injection site and passive limb movements, as previously described (Section 2.1.2). A ventral midline incision was made over the thorax and abdomen, extending onto the limbs as described for lymph node harvest (Section 2.3.2.2). The collecting lymphatics were identified according to their location in the limb and their uptake of blue dye, under the guidance of an operating microscope. Care was taken to exclude all associated regional lymph nodes from the collecting lymphatic specimens. In the hindlimb the collecting lymphatics were located on the ventromedial aspect, afferent and efferent to the popliteal nodes, which were excluded. The collecting lymphatics were gently dissected from the surrounding loose connective and adipose tissue, and adjacent blood vasculature excluded from the specimens wherever possible. The lymphatics were sharply divided proximally and distally to the associated popliteal nodes. In the forelimb the collecting lymphatics were identified ventromedially and dorsolaterally, and freed from their associated regional nodes, adipose and connective tissues and adjacent blood vasculature. In the flanks, the identification of the long collecting lymphatic vessels running between the inguinal and axillary nodes was facilitated by the injection of 100 μl of patent blue dye directly into the inguinal node, at the confluence of dorsal and ventral branches of subcutaneous blood vasculature, which merge to form blood vessels coursing longitudinally in the flank, immediately adjacent and parallel to the collecting lymphatics. On injecting patent blue dye into the inguinal node, the flank collecting lymphatic vessels were distinguished from the surrounding adipose and connective tissues by their rapid dye uptake, and subsequently carefully dissected from the surrounding tissue and adjacent blood vasculature. Care was taken to exclude inguinal and axillary lymph nodes from the specimens. In all cases the collecting lymphatics were immediately immersed in EGMV-2 media without additives (Lonza) containing 10,000 units/ml pen-strep (Life Technologies) on ice.
2.3.2.4 Liver

Access to the abdominal cavity was achieved through a ventral midline incision. The skin and subcutaneous tissues were reflected off the underlying abdominal wall, which was then sharply divided in the midline, and the incision carefully extended cephalad to the level of the xiphisternum, and caudad to the pubis, taking care not to damage the underlying viscera. The falciform ligament was divided and the large murine liver identified in the right upper quadrant. The liver was retracted caudally and the inferior vena cava identified to the right of the midline just below the level of the diaphragm, and sharply divided. The stomach and duodenum were then retracted to locate the portal vessels and the portal vein, which was cannulated with a 21 gauge needle attached to a 20 ml syringe, and the liver flushed via the portal vein with 20 ml of ice cold Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (Lonza). The portal triad was then divided at its junction with the liver, and the gall bladder carefully removed from the liver bed. The liver was freed of its residual connective tissue attachments and immediately immersed in ice cold DMEM with L-glutamine.

2.3.2.5 Small intestine

The abdominal cavity was accessed through a longitudinal incision as described for the liver harvest. The murine small intestine was identified at its caudal end just proximal to the caecum and appendix of the large intestine. The small intestine was divided caudally and freed from its mesentery and surrounding connective tissue by blunt dissection, up to the gastro-duodenal junction where it was subsequently divided. The intestine was immediately cannulated with a 21 gauge needle attached to a 20 ml syringe and flushed repeatedly with ice cold Hank's balanced salt solution (HBSS) without calcium or magnesium (Gibco) until all visible faecal content had been removed. The intestine was immersed in ice cold HBSS without calcium or magnesium and residual Peyer's patches and mesentery carefully excised. The intestine was then cut longitudinally, and divided transversely into 0.5 cm sections in preparation for enzymatic tissue digestion.

2.3.2.6 Lung

The upper abdominal and thoracic cavities were accessed via a midline longitudinal incision as described for the mouse thoracic duct harvest (Section 2.1.2). On dividing the thoracic wall via a para-sternal approach, the sternum and ribs were carefully retracted laterally to expose the underlying thoracic viscera.
The diaphragm was accessed through an upper abdominal incision and exposed by retracting the liver and stomach caudally, and carefully split in the sagittal plane on either side of the great vessels. The right ventricle was identified in the anterior mediastinum, and cannulated with a 21 gauge needle attached to a 20 ml syringe. The lungs were flushed via the right ventricle with ice-cold PBS (Sigma Aldrich) pH 7.2, before dissecting the left and right lungs from adjacent mediastinal structures and parietal pleura of the thoracic cavity. The lungs were immediately transferred to a dish containing ice-cold PBS and residual thymus, cardiac, blood vascular and connective tissues excised. The lobes of the right lung were gently separated, and the left lung divided in two pieces in preparation for digestion.

2.3.2.7 Brain

The murine cranium was accessed via a dorsal longitudinal skin incision over the skull and cervical spine, exposing the underlying cranial vault. The skull vault was pierced and sharply divided longitudinally in the sagittal plane, just lateral to the midline to avoid disruption of the underlying sagittal sinus. The cranium was exposed by lifting the skull off the underlying dura in piecemeal fashion, and by blunt dissection, preserving the dura and sinuses. The whole brain including cerebellum was removed and rinsed in cold Dulbecco’s PBS (Sigma Aldrich) before sharply dividing into eight sections in the sagittal plane in preparation for digestion.

2.3.3 Murine tissue and organ digestion

Immediately following harvest, tissues were digested to form a single cell suspension by the following methods:

2.3.3.1 Dermis and lymph nodes

1 x 1 mm mouse ear skin sections or lymph nodes were transferred to an eppendorf containing 1 ml warmed EGMV-2 media with 10 000 units/ml pen-strep (Life Technologies), 0.7 units Liberase (Blendzyme, Roche diagnostics) and 20 μl/ml DNAse (Sigma Aldrich). This preparation was designed for the digestion of ear dermis or lymph nodes from 4-5 mice, and separate eppendorfs containing the same solution were prepared for additional Prox1-GFP and control mice. Eppendorfs containing ear dermis or lymph nodes and enzyme mix were incubated at 37 degrees for 90 or 60 minutes respectively, under con-
Tissues were disrupted mechanically by pipetting, and enzyme activity was then suspended by the addition of EGMV-2 containing 10,000 units/ml pen-strep (Life Technologies) and additives including 5% fetal calf serum (FCS) (Lonza). Cells were passed through a 100 μm filter, pelleted and resuspended in a FACS blocking buffer containing 2% FCS (Lonza), 2% BSA (Miltenyi), and 5mM EDTA (Invitrogen) in PBS (Sigma Aldrich) in preparation for antibody staining and cell sorting.

2.3.3.2 Collecting lymphatics
Tissue dissociation was achieved by enzymatic and mechanical methods as described for dermis and lymph nodes (Section 2.3.3.1).

2.3.3.3 Liver, small intestine, lung, brain
Liver dissociation was achieved through a combination of enzyme digestion of extracellular matrix and mechanical dissociation using a mouse liver dissociation enzyme mix and protocol specifically designed to generate a high yield of non-parenchymal cell types (gentleMACS, Miltenyi Biotec) and dissociator (gentleMACS dissociator, Miltenyi Biotec). Following dissociation, the liver suspension was passed through a 100 μm cell strainer, washed in DMEM with stable glutamine (Lonza), pelleted and resuspended in a FACS blocking buffer, as described (Section 2.3.3.1).

Small intestine dissociation was achieved through a combination of enzyme digestion of the extracellular matrix and mechanical dissociation using a mouse lamina propria dissociation enzyme mix (gentleMACS, Miltenyi Biotec) and dissociator (gentleMACS dissociator, Miltenyi Biotec). The lamina propria suspension was washed in a buffer containing 0.5% BSA (Miltenyi) in PBS (Sigma Aldrich) at pH 7.2, passed through a 100 μm strainer, pelleted, and resuspended in a FACS blocking buffer as described (Section 2.3.3.1).

Lung dissociation was achieved through a combination of enzyme digestion of the extracellular matrix and mechanical dissociation using a mouse lung dissociation enzyme mix (gentleMACS, Miltenyi Biotec) and dissociator (gentleMACS dissociator, Miltenyi Biotec). The lung suspension was washed in a dissociation buffer (Miltenyi Biotec), passed through a 70 μm strainer, and resuspended in FACS blocking buffer as described (Section 2.3.3.1).
Brain dissociation was achieved by enzyme digestion and mechanical dissociation using an adult brain
dissociation kit for mouse and rat (gentleMACS Miltenyi Biotec) and dissociator (gentleMACS dissociator,
Miltenyi Biotec). The brain suspension was washed in Dulbecco’s PBS (Sigma Aldrich) and passed
through a 70 μm strainer prior to debris removal and red cell removal steps (gentleMACS Miltenyi Biotec)
using a debris removal solution (Miltenyi Biotec), and red cell removal solution (Miltenyi Biotec), respec-
tively. Cells were then resuspended in FACS blocking buffer as described (Section 2.3.3.1).

2.3.4 Lymphatic endothelial cell isolation by FACS

Single cell suspensions from each tissue and organ type were resuspended in a FACS blocking buffer (Sec-
tion 2.3.3.1), and blocking performed on ice for 20 minutes. This was followed by Fc receptor blocking on
ice, with rat anti-mouse CD16/CD32 (BD Pharmingen). Cells were washed three times in a FACS washing
buffer containing 2% FCS (Lonza), and 5 mM EDTA (Invitrogen) in PBS (Sigma Aldrich) then adjusted to
10^7 cell/ml in FACS washing buffer. Immunostaining was performed in FACS blocking buffer containing
PE-conjugated Syrian hamster anti-mouse podoplanin antibody (2μg/ml, Biolegend) or PE-conjugated
Syrian hamster IgG isotype control (2 μg/ml, Biolegend). Immunostaining was performed on ice for 30
minutes, after which time cells were washed twice in FACS washing buffer and resuspended at 1 x 10^7
cells/ml in FACS washing buffer containing 0.13 μg/ml of the dead cell marker propidium iodide (Invitro-
gen).

Controls: Single fluorescence controls were used to set the gating for cell sorts, and comprised an un-
stained single cell suspension prepared from Prox1-GFP mice, and single cell suspensions from control
NOD mice stained with propidium iodide (Invitrogen) or PE-conjugated Syrian hamster anti-mouse anti-
body. Isotype controls were prepared by substituting the PE-conjugated primary antibody with PE-
conjugated Syrian hamster IgG (Biolegend).

Fluorescence-activated cell sorting was performed on an Influx Cell Sorter (BD Corp). The sort was per-
formed at 17.0 psi with a 100um nozzle. The sheath fluid was IsoFlow (Beckman Coulter), 0.9% saline.
Cells were sorted into a 96-well plate coated with 5 μg/ml mouse fibronectin (Abcam), containing 200 μl
of EGMV-2 media plus additives (Lonza), 20% FCS (Lonza), 10 000 units/ml pen-strep (Life Technolo-
gies) and 1:100 fungizone (Thermo Fischer). Media was changed at day 3-5 and replaced with EGMV-2 media plus additives (Lonza), 5% FCS (Lonza) and 10 000 units/ml pen-strep (Life Technologies). Thereafter media was changed at 48-72 hour intervals.

2.3.5 Cell Imaging

Cells in culture were imaged by brightfield and fluorescence microscopy using an Olympus 1X71 fluorescence microscope and Olympus TDP72 camera respectively, and cell morphology in culture documented and compared.

2.3.6 Immunostaining tissue sections in the mouse

Mouse tissues were fixed in 4% PFA at 4°C for 12 hours, with the exception of murine brain which was fixed for 24 hours to allow more time for the fixative to penetrate the full thickness of the specimen. Tissues were processed as described (Section 2.1.3).

Sections were immunostained with the following primary and secondary antibodies:

Podoplanin (1:400 hamster anti mouse podoplanin (Fitzgerald)), LYVE-1 (1:100 rabbit anti mouse (Abcam)), and GFP (1:500 rabbit anti A. victoria GFP; (Abcam)), goat anti-rabbit alexa fluor 488 1:100 (Jackson ImmunoResearch), goat anti-hamster biotin 1:200 (Vector) with streptavidin-Dylight 549 1:600 (Jackson ImmunoResearch). Isotype controls: Hamster IgG (Jackson ImmunoResearch), Rabbit IgG (R&D systems).

Immunostaining was performed in a similar way to the murine flank specimens (Section 2.1.3). Endogenous peroxidase blocking was not required for fluorescent labelling. Protein blocking was performed using Dako Protein Block (Agilent Technologies) for 20 minutes. Tissue sections were incubated in primary antibody at the appropriate dilution in Dako antibody diluent (Agilent Technologies) for 60 minutes, followed by a secondary antibody, for 60 minutes (goat anti-rabbit AF 488). For podoplanin immunostaining, goat anti-hamster biotin was applied for 30 minutes, followed by streptavidin-Dylight 549 for 60 minutes. For double staining, primaries were combined and fluorescent-conjugated secondaries. Isotype controls were performed by substituting the primary antibody with the equivalent dilution of IgG, as specified. To reduce autofluorescence, prior to mounting, tissues were
incubated in Sudan black dye (Neo et al., 2015). Sudan black powder (Sigma Aldrich) was dissolved in 70% ethanol to produce a 0.3% solution, and samples incubated at room temperature, then rinsed in PBS (Sigma Aldrich).

Tissue sections were dehydrated through graded ethanols, cleared in histolene, and mounted in Prolong Gold (manufacturer) with DAPI 1 μg/ml (Molecular Probes) as a nuclear counterstain. Alternatively DAPI was applied alone, in the absence of antibody staining. Images were acquired by fluorescent microscopy on an Olympus BX61 microscope with DPX71 colour camera.
2.4  **Lipoedema family pedigrees and exome sequencing**

Ethical approval for the study was granted by the HREC of St Vincent’s Hospital, Melbourne (HREC-D 117/16). Participants with a clinical diagnosis of lipoedema were recruited to the study. Clinical assessment was made by a consultant plastic surgeon running a lipoedema service, experienced in the diagnosis and management of the condition. In the absence of evidence-based diagnostic criteria, a diagnosis of lipoedema was made according to the following clinical features, as described by Wold et al in 1951 and later modified by Herbst (as cited in Herbst, 2012):

1. characteristic distribution of abnormal adipose tissue, typically on the proximal arms and lower limbs, sparing the knees, ankles and feet
2. history of onset at puberty, and/or exacerbation during pregnancy and menopause.

The diagnosis of lipoedema was further supported by the presence of the following clinical features:

1. Painful adipose tissue in the affected regions
2. History of frequent bruising, often with minor trauma in the affected regions
3. Family history of the condition

Informed consent was obtained from all study participants for the collection of peripheral venous blood samples for the purpose of genetic sequencing, and for making contact with their named first-degree relatives with the intention of inviting them to participate in the study.

2.4.1 **Lipoedema family pedigrees**

Following informed consent, a clinical history was taken via a questionnaire, asking basic information regarding past medical history and medications, as well as specific questions pertaining to the conditions lipoedema and lymphedema. These included duration of symptoms, and previous treatments. Family history was documented in the form of a family tree.

2.4.2 **Blood biospecimens**

Venous blood samples were taken from the antecubital fossa using a 23-gauge hypodermic needle and syringe. Blood biospecimens were immediately transferred to three standard 6 ml EDTA phlebotomy specimen tubes, (BD Vacutainer) with approximately 4 ml of venous blood per tube. Tubes were inverted
several times to mix the blood sample with the EDTA. Biospecimens were stored at -80 degrees prior to DNA extraction.

2.4.3 Genomic DNA extraction

Whole blood biospecimens were thawed in a water bath at 37 degrees with mild agitation. Genomic DNA was extracted using a blood and cell culture DNA midi kit (Qiagen), specifically designed to yield genomic DNA up to 150 kb in size, of suitable volume and quality for next generation sequencing. In summary, 4 ml of whole blood was subject to cell and nuclear lysis followed by protease degradation using the standardized buffer and protease preparations provided (Qiagen), heating samples to 50 degrees in a water bath for protease degradation. Samples were then applied to an anion-exchange resin within a gravity-flow operated, Qiagen Genomic tip (Qiagen) and DNA eluted using an isopropanol-based buffer provided (Qiagen). DNA was precipitated by addition of isopropanol and centrifugation, washed, and resuspended overnight in 150 μl TE Buffer (Qiagen) under gentle agitation at room temperature. The DNA extracted was subject to quality checks to determine yield, purity, length and integrity. For yield, DNA concentration of the eluate was measured by absorbance at 260 nm on a nanodrop fluorospectrometer (Thermo Scientific), and DNA purity was quantified using the same, according to the $A_{260}/A_{280}$ ratio.

DNA length and integrity were assessed by gel electrophoresis. 250 ng gDNA per sample was loaded onto a 0.8% agarose gel in 1 x TAE buffer (BioRad Laboratories) at 60V with run time of 2 hours. A GeneRuler high range DNA ladder from 10171-48502 base pairs (Life Technologies, Australia Pty Ltd) was used as a reference marker, and DNA with molecular weight >20kb and minimal “smearing” of the band, was considered suitable for next generation sequencing.

2.4.4 Exome sequencing

Exome sequencing and variant calling was performed by the Australian Genome Research Facility, Melbourne. Samples were prepared according to Agilent’s SureSelectXT Target Enrichment System for Illumina Paired-End Sequencing Library protocol. Briefly 3 μg of DNA was sheared to ~200 base pairs on a Covaris E220 system (Covaris, Woburn, MA USA). Sheared DNA underwent end-repair, A-tailing, adapter ligation and amplification. Pre-capture libraries were assessed by electrophoresis using the TapeStation
2100 DNA 1000 Tape Screen Assay (Agilent Technologies, Santa Clara, CA, USA) before hybridization with Agilent SureSelect XT Human All Exon V6 for 16 hrs, followed by capture of hybridized library using streptavidin-coated beads and amplification. The final captured libraries were assessed using the TapeStation 2100 and qPCR (Kappa Biosystems, Wilmington, MA, USA) prior to sequencing on the HiSeq 2500 sequencer (Illumina San Diego, CA USA) with 8 samples in a lane, across two sequencing runs, using the 100bp paired-end configuration.

### 2.4.5 Bioinformatics

Sequences were aligned to the human genome (hg19) using BWA mem (v0.7.12-r1039) (Li & Durbin, 2010), and duplicates were marked using Picard (version 2.6.0). The samples were then processed according to GATK (3.6) best practice guidelines (McKenna et al., 2010), with base quality score recalibration, indel realignment, duplicate removal, and SNP and INDEL discovery using HaplotypeCaller (DePristo et al., 2011, Van der Auwera et al., 2013). Annotations were added with the Ensembl Variant Effect Predictor (VEP, version 83) (McLaren et al., 2016).

Variants were called across the families, generating five Variant Call Format (VCF) files. The VCF files were subsequently filtered to target regions. These variants were further filtered according to known genes related to lipoedema, and those associated with the Human Phenotype Ontology (HPO) terms: “abnormality of adipose tissue”, “edema of the lower limbs”, “skin ulcer”, “lymphedema”, “obesity”, “cellulitis”, “lipedema”, “lipodystrophy” (Kohler et al., 2017) (Table 2.1).
Table 2.1: HPO terms (Kohler et al., 2017) applied to identify candidate variants following exome sequencing. Orphanet frequency refers to the frequency of the phenotype in the patient population according to Orphanet Inventory of Rare Disorders (http://www.orpha.net).

<table>
<thead>
<tr>
<th>Description</th>
<th>HPO Frequency</th>
<th>Orphanet Frequency</th>
<th>HPO Source Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 abnormality of adipose tissue</td>
<td>hallmark (90%)</td>
<td></td>
<td>HP:0009124</td>
</tr>
<tr>
<td>2 edema of the lower limbs</td>
<td>hallmark (90%)</td>
<td>Frequent (79-30%)</td>
<td>HP:0010741</td>
</tr>
<tr>
<td>3 skin ulcer</td>
<td>typical (50%)</td>
<td></td>
<td>HP:0200042</td>
</tr>
<tr>
<td>4 lymphedema</td>
<td>occasional (7.5%)</td>
<td></td>
<td>HP:0001004</td>
</tr>
<tr>
<td>5 obesity</td>
<td>occasional (7.5%)</td>
<td></td>
<td>HP:0001513</td>
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<tr>
<td>6 cellulitis</td>
<td>occasional (7.5%)</td>
<td></td>
<td>HP:0100658</td>
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<tr>
<td>7 lipedema</td>
<td></td>
<td></td>
<td>HP:0100695</td>
</tr>
<tr>
<td>8 lipodystrophy</td>
<td>Very frequent (99-80%)</td>
<td></td>
<td>HP:0009125</td>
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Variants were assessed by their quality score (level of confidence that the observation represents a true variant), and by their consequence (location of the variant within the gene structure), and type (impact on gene function- low, modifier, moderate, or high) according to the Ensembl variant consequences (Figure 2.1) and gene function predictions (Table 2.2). Within the Ensembl platform, predictions for the effect of an amino acid substitution on protein function were made using SIFT version 5.2.2 (Kumar et al., 2009) and PolyPhen version 2.2.2, release 405c (Adzhubei et al., 2010). Variants were described according to Human Genome Variation Society (HGVS) nomenclature (den Dunnen et al., 2016).

Results were interpreted in the context of clinical data on the family pedigrees and current literature on inheritance patterns in lipedema (Child et al., 2010).
Figure 2.1:

Location of sequence ontology consequence terms in relation to the transcript structure. Image provided by Ensembl (http://www.ensembl.org/index.html).
Table 2.2: Sequence ontology consequence terms (Eilbeck et al., 2005) listed in order of severity from most to least severe, according to estimates by Ensembl.

Table provided by Ensembl (http://www.ensembl.org/index.html).

<table>
<thead>
<tr>
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<th>Display term</th>
<th>IMPACT</th>
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<td>Transcript ablation</td>
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<td>SO:0001574</td>
<td>Splice acceptor variant</td>
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<td>Splice donor variant</td>
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<td>SO:0001587</td>
<td>Stop gained</td>
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<td>Display term</td>
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<td>SO:0001821</td>
<td>Inframe insertion</td>
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<td>SO:0001583</td>
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<td>SO:0001818</td>
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<td>SO:0001630</td>
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<td>--------------------------------------------------------------------------------</td>
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<td>SO:0001891</td>
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<td>Feature elongation</td>
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<td>MODIFIER</td>
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<td>ry_region_variant</td>
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<td>with regard to the reference sequence</td>
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<tr>
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<td>A sequence variant located in the intergenic region, between genes</td>
<td>SO:0001628</td>
<td>Intergenic variant</td>
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*Corresponding colours for Ensembl web displays*
Chapter 3:

IDENTIFYING THE ‘VASA LYMPHATICUM’ IN MOUSE AND HUMAN
Lymphedema is a common sequela of malignant disease and cancer therapies, particularly those involving surgical excision of the regional lymph nodes, and irradiation (Deo et al., 2004, Ugur et al., 2013). The presence of a microvascular network supplying the arterial and venous wall is well-documented (Lowenberg & Schumacker, 1948; Wolinsky & Glagov, 1967; Scotland et al., 2000), and these “vasa vasorum”, or “vessels of blood vessels” also appear to have a role in the pathophysiology of arterial disease, including atherosclerosis (Mulligan-Kehoe & Simons, 2014) and vasculitis (Hamaoka-Okamoto et al., 2014). Although an equivalent blood microvascular network supplying the lymphatic wall has been described in humans (Agliano et al., 1997), there are very few studies reporting on the distribution and relationship of these vessels to the lymphatic wall, and to our knowledge, there are no reports on the potential significance of this blood microvasculature in lymphatic injury and dysfunction.

There is evidence to suggest that blood microvasculature might be particularly sensitive to the deleterious effects of irradiation. For example in a mouse model of whole body irradiation resulting in the gastrointestinal syndrome, injury to the gut microvascular endothelium appeared to be the principal event (Paris et al., 2001). It is therefore plausible that injury to the lymphatic wall blood microvasculature might either cause or contribute to the process of lymphatic injury and dysfunction.

**Hypothesis:**

Nutrients and oxygen are supplied to the collecting lymphatic vessel wall by a blood microvascular network, the “vasa lymphaticum”. Injury to this microvasculature, and thus localized ischemia, might play a role in the pathophysiology of lymphatic injury and lymphedema.

**Aim:**

To characterise the blood supply of the collecting lymphatics, the “vasa lymphaticum”
3.1 Identifying the vasa lymphaticum in the mouse

3.1.1 Immunostaining serial sections of mouse flank:

To identify the collecting lymphatics, and determine their relationship to the blood vascular bundle, serial sections of mouse flank skin and subcutaneous tissues were stained for lymphatic and blood vascular markers. Collecting lymphatics in the Prox1-GFP mouse were identified according to positive GFP expression in the lymphatic endothelium and absence of LYVE-1 staining (Makinen et al., 2005), whereas dermal lymphatics within the same tissue sections stained positively for LYVE-1 (Figure 3.1). The blood vascular marker CD31 was expressed by vascular endothelial cells of the adjacent artery, and expressed more weakly by cells of the venous and lymphatic endothelium (Figure 3.1).
Figure 3.1

Light microscopy images of mouse flank tissues. Transverse serial sections of the full thickness of skin and subcutaneous tissues from the murine flank, encompassing the subcutaneous vascular bundle at x 20 magnification. 

A: H&E staining demonstrating a vein (far right), artery (middle), and two collecting lymphatic vessels (far left). 

B: GFP immunostaining showing the distribution of Prox1 expression in the lymphatic endothelium (left), but not the blood vascular endothelium of the adjacent artery and vein.

C: CD31 immunostaining demonstrating strong expression in the arterial vascular endothelium, and more weakly in the adjacent venous endothelium and collecting lymphatic endothelium.

D: Immunostaining for the lymphatic marker LYVE-1. LYVE-1 expression was absent in the blood vascular and collecting lymphatic endothelium, whereas E: LYVE-1 was expressed by the smaller calibre lymphatic vessels in the dermis.
Serial sections of the same mouse flank specimens were imaged at 40 x magnification (Figure 3.2). There was strong GFP expression in the lymphatic, but not the blood vascular endothelium, (Figure 3.2). The arterial endothelium stained strongly for CD31, while there appeared to be only minimal VWF staining in the lymphatic endothelium (Figure 3.2). No structures within or immediately adjacent to the lymphatic vascular wall stained strongly for CD31 in an equivalent pattern and intensity to the CD31 staining demonstrated in the arterial endothelium (Figure 3.2). On this basis, is possible that blood microvascular structures are not associated with the mouse collecting lymphatic wall, or alternatively they are not associated with this calibre of collecting lymphatic, in the subcutaneous tissues of the mouse flank, or finally that they are not visible in these particular flank specimens under light microscopy at 40 times magnification.
Light microscopy images of the same mouse flank subcutaneous vascular bundle as shown in Figure 3.1, but taken at x 40 magnification: A: Immunostaining for GFP, again demonstrating Prox1 expression in the lymphatic endothelium of the subcutaneous collecting lymphatics. B: CD31 immunostaining showing the strong expression of the endothelial marker CD31 in the arterial endothelium (right), and weaker expression in the endothelium of the two collecting lymphatic vessels (left). Blood vascular structures strongly positive for CD3, as seen in the arterial endothelium, were not identified within the murine collecting lymphatic wall at x 40 magnification.

To further investigate the lymphatic blood supply in the mouse, we performed whole mount immunostaining in 0.5 x 0.5 cm soft tissue specimens prepared from the tails of Prox1-GFP mice, as described (Section 2.1.4). The mouse tail contains less subcutaneous fat than the flank, thereby facilitating imaging of the subcutaneous vasculature. Critically, the tail can also be
isolated from the rest of the mouse, providing an experimental model for studying the effects of tail irradiation and lymphatic injury (Avraham et al., 2010).

CD31 immunostaining demonstrated a network of CD31-positive and Prox1-negative blood microvasculature ramifying around the collecting lymphatic, particularly at the level of the lymphatic valve (Figure 3.3). However it was difficult to determine the proximity of these blood vessels to the lymphatic wall in these specimens and under fluorescence microscopy. As such, further tail specimens were prepared with the endothelial stain Griffonia lectin, and confocal microscopy imaging performed (Figure 3.4). This demonstrated multiple Prox1-negative blood microvascular branches running in close proximity to the collecting lymphatic wall, both parallel to, and crossing, the collecting lymphatic. Although the blood microvasculature appeared to pass very closely to the collecting lymphatic, it was still difficult to determine from these confocal images whether these blood vessels were actually crossing and penetrating the lymphatic wall, or simply passing in another plane.
Fluorescence microscopy images of Prox1-GFP murine tail soft tissue specimen at x 10 magnification, following whole mount immunostaining for the vascular marker CD31. **A:** Green channel demonstrating the distribution of Prox1 expression in the collecting lymphatic endothelium. **B:** Red channel showing the distribution of CD31 expression, in the collecting lymphatic endothelium but also showing additional Prox1-negative, CD31-positive structures representing blood microvasculature (arrow). **C:** Overlay of the red and green channels demonstrating the close relationship between the blood microvasculature, ramifying around the collecting lymphatic, particularly at the level of the lymphatic valve (arrow).
Confocal microscopy images of Prox1-GFP murine tail soft tissue specimen at x 40 magnification, following whole mount staining with the endothelial stain G. Lectin. A: Green channel demonstrating the distribution of Prox1 expression in the collecting lymphatic endothelium. B: G. Lectin staining showing the Prox1-negative, Lectin-positive blood microvasculature, and weak G. Lectin staining in the collecting lymphatic endothelium. C: Overlay of the green and red channels demonstrating the spatial relationship between the blood microvasculature and collecting lymphatic wall in one plane.

3.1.2 Scanning electron microscopy of the collecting lymphatics of the mouse tail

To examine the structure of the collecting lymphatic wall in more detail, at higher resolution, we performed scanning electron microscopy on 1 x 2 mm soft tissue specimens comprising skin, subcutaneous tissue and vascular bundles harvested from the proximal tail, within 1 cm of the tail base where the collecting lymphatics are larger in calibre. Figure 3.5 is a representative image of one of the 70 nm cross-sections taken through the collecting lymphatic wall,
perpendicular to its long axis. Figure 3.5 clearly demonstrates the layers of the lymphatic vessels wall, which is approximately 100 μm in diameter, comprising lymphatic endothelium, a single smooth muscle layer, and external adventitia.

There were no visible blood vessels on or within the collecting lymphatic wall in any of the Prox1-GFP mouse-tail specimens examined in this way. This might suggest that vasa lymphaticum are not present in the collecting lymphatics of the mouse tail, or alternatively it is possible that these tiny blood vessels were simply not present within the 30 μm segment of murine collecting lymphatic specimen examined.
Representative image of scanning electron microscopy (SEM) of 70 nm transverse sections through the subcutaneous collecting lymphatic wall of the murine tail, showing the lymphatic wall ultrastructure. Structural features included a lymphatic endothelial cell (black arrow), smooth muscle cell within the lymphatic media (white arrow), and the outermost adventitia. No blood vasculature was seen on or within the lymphatic wall in the transverse sections examined by SEM.

3.1.3 Micro CT of the collecting lymphatics of the mouse tail

In order to investigate the possibility that sampling error could account for the absence of vasa lymphaticum on SEM imaging of 70 nm tail tissue sections, we prepared 1 x 2 mm murine tail soft tissue specimens as described, and imaged them by the micro CT. This imaging modality provides high-resolution with a pixel size of 1.164 um per pixel, and importantly it facilitates analysis of larger tissue sections compared to SEM. The collecting lymphatics were easily identifiable within the subcutaneous vascular bundles of the tail on the micro CT images (Figure 3.6). The sagittal view (Figure 3.6A) demonstrates the vascular bundle comprising vein, artery and collect-
ing lymphatic all running in parallel. An adjacent nerve was also identifiable within the specimen. Although detailed vessel ultrastructure could not be determined, certain morphological features of the collecting lymphatic were seen on micro CT, including the lymphatic valves in the sagittal plane (Figure 3.6A). The transverse image (Figure 3.6B) demonstrates the distances between the collecting lymphatic and its adjacent blood vasculature. In the specimen analyzed, the artery and vein were at least 100 μm away from the collecting lymphatic in the subcutaneous plane. Furthermore, there did not appear to be blood vascular branching in the vicinity of the collecting lymphatic wall. This was further supported by a 3D reconstruction model based on the same micro CT data set of the tail specimen, where no arterial branches appeared to interact with the collecting lymphatic wall (Figure 3.7).
Figure 3.6

Microscopic computed tomography imaging of murine tail soft tissues demonstrating the subcutaneous neurovascular bundle. A: Sagittal view showing a subcutaneous collecting lymphatic running parallel to the artery and vein. The leaflets of the collecting lymphatic valves are visible in this view. A nerve is also seen in the subcutaneous plane (arrows and labels). B: Transverse view demonstrating the spatial relationship between the collecting lymphatic and blood vasculature. The lymphatic appeared to lie at least 100 μm away from any blood vessels (arrows and labels), without evidence of blood microvascular branching in the region of the lymphatic wall in this view.
Figure 3.7

3-dimensional reconstruction of representative images from microscopic computed tomography of a 1 mm segment of the murine tail subcutaneous vascular bundle. **A-D:** showing 4 views at different angles, rotating about the long axis of the bundle, comprising artery and vein (white), and paired collecting lymphatics (red). **A:** multiple blood microvascular branching was demonstrated within the subcutaneous tissue plane, which appeared to lie adjacent, or in close proximity to the collecting lymphatics in this first view. **B-D:** However on further inspection at different rotational angles, the blood microvasculature can be seen passing across the collecting lymphatic in a different tissue plane, without making direct contact with the vessel wall.
3.1.4 Transmission electron microscopy of the mouse thoracic duct

To further clarify whether or not blood microvasculature is associated with the collecting lymphatic wall in the mouse, we harvested lymphatic specimens from the largest calibre of collecting lymphatic in the body - the thoracic duct, and its associated cisterna chyle. The cisterna chyli is the origin of the thoracic duct and as described in the human, the murine cisterna chilí receives the paired lumbar lymphatic trunks, which in turn receive lymph from thoracic wall, diaphragm and reproductive organs, and the iliac nodes draining the hind limb (Kawashima et al., 1964). The cisterna chilí also receives fatty chyle from the intestinal trunk draining the gut. Figure 3.8 is a montage of cross sectional TEM imaging through the murine cisterna chilí, demonstrating features of the lymphatic ultrastructure visible on TEM, including the lymphatic endothelial cell, and single layer of smooth muscle cells forming the lymphatic media, with mitochondria and myofilaments within the smooth muscle cell lumen. Despite the detailed views of lymphatic architecture of the cisterna chyli on TEM, no blood vessels were identified on or within the lymphatic wall. Based on this evidence, it is possible that a dedicated lymphatic wall blood supply does not exist in the mouse. However, we were still unable to exclude sampling error as an explanation for these negative findings.
Montage of transmission electron microscopy images of a 70 nm transverse section through the murine cisterna chili, with the additional pane demonstrating several morphological features of the collecting lymphatic wall in more detail. This includes a lymphatic endothelial cell (black arrow), and smooth muscle cell containing mitochondria and myofilaments (white arrow). Collagen fibres of the extracellular matrix are also visible (red arrow). There was no visible blood vasculature in the tissue sections examined.

3.1.5 Immunostaining of tail whole mounts

Before excluding the possibility of using the Prox1-GFP mouse tail as an experimental model for studying the vasa lymphaticum, we wanted to establish the frequency and distribution, if any, of
vasa lymphaticum in the collecting lymphatics of the tail, and to determine whether there was any consistency in this pattern across different Prox1-GFP mice. To avoid the sampling error inherently associated with imaging thin tissue cross-sections, we instead prepared whole mounts of the skin and subcutaneous tissues comprising the full lengths of the Prox1-GFP mouse tail. We envisaged that whole tail imaging could be a useful adjunct to other, more detailed imaging techniques such as micro CT and electron microscopy, by providing a means of identifying specific regions of the collecting lymphatic wall where vasa lymphaticum were present or particularly abundant, enabling a more focussed and targeted approach to further analysis.

As such, circumferential, full length soft tissue specimens were obtained from the tails of 9 Prox1-GFP mice and whole mount immunostaining performed with primary antibodies against the vascular markers CD31 (n=3) and VWF (n=3) and endothelial stain GS lectin (n=3) and imaged by confocal microscopy. There was an abundance of blood microvasculature in the full tail whole mounts, with multiple branches arising from the principal artery of the subcutaneous vascular bundle, presumed to supply the surrounding connective tissue and fat, and ramifying in the vicinity of the adjacent paired collecting lymphatics (Figure 3.9). While some of these blood vessels appeared to associate closely with the collecting lymphatic, a predictable pattern of branching toward the lymphatic wall was not observed, and certain regions of the lymphatic wall did not appear to have any adjacent blood vessels at all. It was also evident that many blood vessel branches actually crossed straight past the collecting lymphatic, rather than interacting directly with its wall (Figure 3.9). Although these findings do not exclude the presence of a microvascular blood supply to the collecting lymphatic wall in mice, they do indicate that the murine tail might not be a good model for studying the vasa lymphaticum. We therefore decided to focus on our human lymphatic specimens.
Figure 3.9

Confocal microscopy images of the full lengths of subcutaneous vascular bundles from the Prox1-GFP murine tail following whole mount immunostaining for CD31 (A), G. Lectin (B), or Von Willebrand factor (C), demonstrating the distribution of Prox1 in the endothelium of the paired collecting lymphatics in green and Prox1-negative blood vasculature in red.
3.2 Identifying the vasa lymphaticum in human lymphatics

Following ethical approval from the HREC of St Vincent’s Hospital, Melbourne, and after informed consent, six patients with lymphedema undergoing microsurgical lymphatico-venular anastomosis provided surplus soft tissue biospecimens, as described, and one control. Table 3.1 provides an overview of the six cases included in the study, their clinical histories and the sites from which lymphatic tissues were obtained. 1-2mm segments of collecting lymphatic with minimal surrounding connective tissue and fat were obtained from cases 1-6, facilitating further tissue processing, imaging and analysis.
<table>
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<th>M/F</th>
<th>Age</th>
<th>Lymphedema duration</th>
<th>Cause of lymphedema</th>
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<th>Lymphatic analysis</th>
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<tr>
<td>2</td>
<td>F</td>
<td>49</td>
<td>7 years</td>
<td>Left breast cancer, mastectomy, lymph node clearance and radiotherapy. Approx. 20 infections in the left arm</td>
<td>left forearm</td>
<td>histology – serial sections &amp; whole mounts</td>
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<td>3</td>
<td>F</td>
<td>41</td>
<td>6 years</td>
<td>Trauma left leg</td>
<td>left leg</td>
<td>histology – whole mounts</td>
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<td>4</td>
<td>F</td>
<td>50</td>
<td>4.5 years</td>
<td>Ovarian cancer - resection and right groin lymph node clearance. Identical twin has secondary lymphedema too</td>
<td>right leg</td>
<td>micro CT &amp; TEM</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>58</td>
<td>5 years</td>
<td>Surgery left thigh, complicated by seroma</td>
<td>left foot</td>
<td>micro CT &amp; TEM</td>
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<td>6</td>
<td>F</td>
<td>43</td>
<td>1 year</td>
<td>Left breast cancer. Lymph node surgery and radiotherapy</td>
<td>left forearm</td>
<td>micro CT &amp; TEM</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Control</th>
<th>M/F</th>
<th>Age</th>
<th>Procedure</th>
<th>Medical history</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>54</td>
<td>Left mastectomy and immediate DIEP flap</td>
<td>Left breast cancer. No lymphedema</td>
<td>left lower abdomen/groin from flap edge</td>
</tr>
</tbody>
</table>

DIEP = deep inferior epigastric perforator. SEM = scanning electron microscopy
3.2.1 Immunostaining in serial sections of human collecting lymphatic

Figure 3.10A-F gives representative light microscopy images of serial sections of one of the collecting lymphatic specimens from the lower limb of a male patient with a 4-year history of lymphedema, complicated by multiple infections (Table 3.1, Case 1). On light microscopy imaging at 10 x magnification, the collecting lymphatic was identified (Figure 3.10A). H&E and SMA staining demonstrated a thickened smooth muscle media (Figure 3.10A and 3.10B). The human collecting lymphatic endothelium was podoplanin and CD31 positive (Figure 3.10C and 3.10D). Light microscopy imaging at 20 x magnification also revealed several CD31-positive, podoplanin-negative blood microvasculature on and within the thickened lymphatic wall (Figure 3.10E and 3.10F), consistent with the vasa lymphaticum of the human collecting lymphatic.
Figure 3.10

Light microscopy images of transverse serial sections through the human lymphatic wall from the lower limb of a male patient with chronic lymphedema (Table 3.1 case 1). A: H&E staining at x 10 magnification. B: Smooth muscle actin staining at x 10 magnification demonstrating the thickness of the smooth muscle media in this lymphedema specimen. C: D2-40 staining at 10 x magnification showing the distribution of podoplanin expression in the lymphatic endothelium. D: CD31 immunostaining at x 10 magnification demonstrating expression of the endothelial marker within the human lymphatic endothelium. E: D2-40 staining at 20 x magnification showing in more detail the distribution of podoplanin expression within the lymphatic endothelium (black arrow). F: CD31 immunostaining at x 20 magnification showing again the expression of the pan endothelial marker within the lymphatic endothelium (black arrow), but in addition demonstrating structures within the connective tissue overlying the lymphatic and possibly within the lymphatic smooth muscle media (blue arrows) staining positively for CD31, which appear to be podoplanin-negative on D2-40 immunostaining, and may represent blood vasculature associated with the human lymphatic wall.

It was inherently more difficult to obtain isolated lymphatic specimens from control patients undergoing different procedures at different sites. However a perforating vascular bundle contain-
ing lymphatic vessels was successfully isolated from the groin of a patient undergoing abdominal-based breast reconstruction, a procedure which involves raising the full thickness of abdominal skin and fat below the umbilicus (Table 3.1, control). Figure 3.11A-F shows serial sections of the perforating vascular bundle from the control patient, without lymphedema. D2-40 immunostaining (Figure 3.11C and 3.11E) demonstrated three lymphatic vessels adjacent to the larger artery and vein, which were podoplanin positive, and more weakly positive for the pan-endothelial marker CD31 (Figure 1.11F). In contrast to the lymphedema specimen, there did not appear to be any CD31 positive, podoplanin negative blood microvasculature associated with the lymphatic wall, even on imaging at 100 x magnification (Figure 3.11E and 3.11F).
Figure 3.11

Light microscopy images of transverse serial sections through a perforating vascular bundle from the groin of a female patient without lymphedema (Table 3.1, control).  

A: H&E staining at x 10 magnification demonstrating the distribution and gross morphology of vascular structures within the specimen including an artery (arrow and label) and veins (arrows and labels) structures. Lymphatic vasculature were not clearly identifiable in the specimen at x 10 magnification.  

B: Smooth muscle actin staining at x 20 magnification, demonstrating the smooth muscle media of arterial and venous structures in brown. Lymphatic structures were not demonstrated within the specimen.  

C: D2-40 staining at 20 x magnification showing expression of the lymphatic marker podoplanin in brown, within the lumen of vascular structures not previously visible on smooth muscle actin staining, representing two thin-walled lymphatic vessels (arrows).  

D: Immunostaining for the blood vascular endothelial marker von Willebrand Factor demonstrating strongly-positive staining within the lumen of the previously-identified veins within the specimen in brown.  

E: D2-40 staining at x 100 magnification showing in more detail the structure of the thin-walled lymphatic vessels, with positive staining in the lymphatic endothelium (arrow and label) indicating expression of the lymphatic-specific marker podoplanin in brown.  

F: CD31 immunostaining at x 100 magnification demonstrating weakly-positive staining in the endothelium (arrow and label) of the thin-walled lymphatics in brown.
The difference in lymphatic structure and calibre between the two specimens analyzed (lymphedema versus control) was particularly striking. The podoplanin-positive lymphatics were thin walled and lacking a significant smooth muscle layer in the control specimen, as demonstrated by the absence of lymphatic SMA staining (Figure 3.11B). In contrast, the collecting lymphatic from the lymphedematous lower limb was larger with a much thicker wall (Figure 3.10B). It is possible that this represents a fundamental difference in lymphatic structure in lymphedema, or alternatively it might be an effect of sampling the control tissue from the groin rather than the lower limb, from a different site to the lymphedema specimens, and thus comparing two contrasting lymphatic subtypes.

### 3.2.2 Whole mount immunostaining of human collecting lymphatics

Following the identification of vasa lymphaticum in serial sections of the collecting lymphatics derived from patients with lymphedema, we wanted to gain an impression of the three dimensional distribution of these blood vessels in and around the collecting lymphatic wall. To achieve this, whole mount immunostaining was performed on lymphatic specimens from three patients with secondary lymphedema (Table 3.1). Following immunostaining, the lymphatic specimens were suspended in glycerol to prevent lymphatic wall collapse during confocal microscopy imaging. Figure 3.12 shows representative images of the lymphatic specimen taken from the right lower limb of a 41 year old, female patient with a history of right leg lymphedema secondary to trauma, undergoing LVA surgery (Table 3.1, Case 3). Immunostaining with D2-40 for the lymphatic endothelial marker podoplanin, and co-staining with the blood vascular marker von Willebrand factor, revealed a meshwork of VWF-positive, podoplanin-negative blood microvasculature on and within the lymphatic wall (Figure 3.12A-C). These small blood vessels appeared to branch and penetrate the lymphatic wall within the space immediately external to the lymphatic endothelium (Figure 3.12B). This was particularly evident in the images taken from the perspective of the lymphatic lumen outwards through the lymphatic wall (Figure 3.12C), demonstrating the overlying blood vessel network.
Figure 3.12

Representative confocal microscopy images of a human lymphatic specimen from the lower limb of a female patient with chronic lymphedema (Table 3.1, case 3), following whole mount immunostaining with both D2-40 (green) and von Wilebrand factor (red). **A:** View of the external surface of a segment of the lymphatic wall demonstrating a meshwork of VWF-positive, podoplanin-negative blood microvasculature in red overlying the collecting lymphatic wall, while D2-40 staining shows the expression of the lymphatic marker podoplanin in the lymphatic endothelium beneath in green. **B:** Further confocal microscopy imaging of the lymphatic wall segment taken from another perspective, showing the curvature of the lymphatic wall with the lymphatic endothelium in green, and, and the distribution of associated blood vasculature in red, which appear to branch and penetrate the lymphatic wall within the space immediately external to the lymphatic endothelium. **C:** Third view of the lymphatic segment, taken from within the lymphatic lumen, demonstrating the innermost lymphatic endothelium in green,
and the density of the overlying blood vessel network staining positively for the vascular endothelial marker von Willebrand factor in red.

In cross-sectional imaging, multiple VWF-positive, podoplanin-negative blood vessels were visible both on and within the collecting lymphatic wall (Figure 3.13). There also appeared to be structures within the lymphatic wall staining positively for both podoplanin and VWF, appearing yellow in colour (Figure 3.13). It is difficult to ascertain from these images alone which mural structure might be responsible for the dual staining pattern, however it is likely to represent either a lymphatic branch with weakly-positive VWF staining in the lymphatic endothelium, or a podoplanin-positive nerve surrounded by VWF-positive blood microvasculature.

**Figure 3.13**

Cross-sectional confocal microscopy image of a human lymphatic specimen from the lower limb of a female patient with chronic lymphedema (Table 3.1, case 3), following whole mount immunostaining with both D2-40 (green) and von Willebrand factor (red). This image demonstrates multiple VWF positive, podoplanin-negative blood vessels on and within with the collecting lymphatic wall (red arrows). A structure is also evident within the lymphatic wall which stains positively for both podoplanin and VWF, which is in yellow in appearance (white arrow), possibly representing a lymphatic branch or a nerve.
3.2.3 Micro computed tomography and transmission electron microscopy of human collecting lymphatics

In order to study the ultrastructure of the lymphatic wall and vasa lymphaticum in lymphedema, the lymphatic specimens from three patients undergoing LVA surgery were fixed and processed for micro CT imaging in the first instance, then transverse sections cut at the appropriate level and further imaging performed by TEM. The first specimen was obtained from the right leg of a 50 year old female patient with a 4.5 year history of right leg lymphedema secondary to a right groin lymph node clearance for ovarian cancer (Figure 3.14, Table 3.1, Case 4). The next lymphedema specimen was from the left foot of a 58 year old female with a five year history of left-sided leg lymphedema secondary to vascular surgery to the left thigh, complicated by a seroma and subsequent lymphedema (Figure 3.15, Table 3.1, Case 5). Finally, the third lymphedema specimen was obtained from the left forearm of a 43 year old female patient with a year-long history of left upper-limb lymphedema secondary to left-sided mastectomy, lymph node surgery and radiotherapy for breast cancer (Figure 3.16, Table 3.1, Case 6). Control tissue for micro CT and TEM imaging was derived from the groin region of a female patient undergoing abdominal-based breast reconstruction, as previously described (Figure 3.17, Table 13, Control). In all cases in the first instance micro CT imaging was employed to identify the vasa lymphaticum, which were then further examined by sectioning at the appropriate level of the tissue block, and imaging by TEM.

Blood microvasculature were identified within close proximity of the collecting lymphatic wall in all specimens examined (Figures 3.14-3.17), however both the blood microvasculature and the lymphatic itself appeared abnormal in the specimens from patients with lymphedema (Figures 3.14-3.16). This was particularly apparent in the lower limb lymphatic specimens from the two patients with a longer history of lymphedema of 4.5-5 years (Figures 3.14 and 3.15, Table 3.1 Case 4 and 5), compared to the specimen from the upper limb of a female patient with a relatively short history of lymphedema of 12 months (Figure 3.16, Table 3.1 Case 6). In the lower limb specimen from a female with a 4.5-year history of lymphedema (Figure 3.14, Case 4), two regions identified on micro CT contained blood microvasculature within the adventitia or surrounding
connective tissue of the collecting lymphatic, labeled regions 1 and 2 (Figure 3.14A). The collecting lymphatic itself was thickened with narrowing of the lymphatic lumen apparent on the tissue sections examined by micro CT (Figure 3.14A). TEM imaging of the first region containing blood microvasculature further demonstrated the abnormal tissue architecture of the collecting lymphatic wall (Figure 3.14B). The smooth muscle cells of the lymphatic media appeared "spiky" in morphology on electron microscopy imaging. Meanwhile, three associated blood vessels within the adjacent connective tissue also appeared grossly abnormal, with a darkened, compressed vessel wall and a very narrow lumen without circulating red blood cells in the tissue sections examined (Figure 3.14B). TEM imaging of the second region containing blood microvasculature identified on micro CT confirmed this pattern of tissue abnormalities, with "spiky" smooth muscles cells of the lymphatic wall and associated expansion of the extracellular matrix (ECM) between smooth muscle cells (Figure 3.14C). The single adjacent blood vessel within the connective tissue of the lymphatic adventitia also appeared to contain "spiky" smooth muscle cells and appeared to be lacking elastin fibres within its wall (Figure 3.14C). The blood vessel lumen was also severely narrowed without red blood cells (Figure 3.14C).
Micro CT and transmission electron microscopy images of a human lymphatic specimen from the lower limb of a female patient with a 4.5 year history of lymphedema (Table 3.1, case 4). A: Micro CT imaging in a plane running transversely through the collecting lymphatic demonstrating the gross morphology of the collecting lymphatic wall and surrounding connective and adipose tissue. The collecting lymphatic wall appears thickened with a narrow lumen (arrow). In two regions, blood microvasculature was identified within the connective tissue surrounding the collecting lymphatic (labeled 1 and 2). B: Transmission electron microscopy imaging of region 1, demonstrating the associated blood microvasculature comprising three blood vessels (bv) and demonstrating some of the ultrastructural features of the adjacent collecting lymphatic wall. The blood vessels within the connective tissue adjacent to the lymphatic adventitia appear abnormal in morphology, with a compressed, darkened vessel wall and compressed lumen without red blood cells in the sections examined. The smooth muscle later
of the lymphatic media to the right of the image also appears abnormal, with “spiky” smooth muscle cells (arrow and label). C: Transmission electron microscopy imaging demonstrating the associated blood microvasculature in region 2. This shows a single blood vessel (bv), the vascular endothelial cell nucleus (arrow and label), and narrow lumen (arrow and label). To the left of the blood vessel, once again the collecting lymphatic smooth muscle media has an abnormal morphology, with “spiky” smooth muscle cells (SMC) (arrows and label) and associated expansion of the extracellular matrix (ECM is seen labeled).

In keeping with these findings in the first lymphatic specimen, similar features were identified on micro CT and TEM imaging in the second lymphatic specimen analyzed, taken from the foot of a patient with a five year history of lymphedema (Figure 3.15, Case 5). The lymphatic wall itself appeared to contain 4-6 smooth muscle cell layers on micro CT imaging, and once again, two regions containing blood microvasculature within the adventitia/surrounding connective tissue of the collecting lymphatic were identified on micro CT, labelled regions 3 and 4 (Figure 3.15A). TEM imaging of region 3, containing blood microvasculature, demonstrated three blood vessels, all with abnormal morphology (Figure 3.15B). The lumen of these blood vessels was very narrow, and an abnormal, permeabilised red blood cell was identified in the lumen of one vessel (Figure 3.15B). TEM imaging of region 4, containing blood microvasculature, identified a single abnormal blood vessel lacking elastin fibres within the vessel wall (Figure 3.15C). Although the lumen itself did not appear significantly narrowed in this instance, there were no circulating red blood cells in the tissue sections examined.
Figure 3.15

Micro CT and transmission electron microscopy images of a human lymphatic specimen from the foot of a female patient with a five year history of leg lymphedema (Table 3.1, case 5). A: Micro CT imaging of a transverse section through the collecting lymphatic specimen demonstrating the lymphatic wall (arrow) comprising 4-6 layers of smooth muscle cells, and surrounding connective and adipose tissue containing the associated blood microvasculature (regions 3 and 4). B: Transmission electron microscopy imaging demonstrating the associated blood microvasculature in region 3, comprising three blood vessels (bv) with abnormal features including a narrow lumen (arrow and label), and an abnormal, possibly permeabilised red blood cell (rbc) within the lumen of one of the vessels (arrow and label). C: Transmission electron microscopy imaging demonstrating the associated blood microvascu-
In the lymphatic specimen from the third patient with a much shorter history of lymphedema of the upper limb of 12 months duration (Figure 3.16, Case 6), micro CT imaging demonstrated lymphatic wall thickening (region 3) and also two regions containing blood microvasculature within the adventitia/surrounding connective tissue of the collecting lymphatic wall, labeled regions 1 and 2 (Figure 3.16A). TEM imaging of region 1 demonstrated a single blood vessel with a very narrow lumen and without red blood cells (Figure 3.16B). In contrast, the blood vessel in region 2 appeared more normal in morphology, with a wider lumen containing a single red blood cell on TEM imaging (Figure 3.16C). Examination of the thickened lymphatic wall in region 3 on TEM demonstrated a more rounded appearance of the smooth muscle cells of the lymphatic media and normal ECM volume (Figure 3.16D) contrasting with the “spiky” appearance of smooth muscle cells and expanded ECM in the lymphatic specimen from the first patient, with a much longer history of lymphedema of the lower limb (Figure 3.14B and 3.14C). Although the lymphatic wall appeared thickened in the TEM imaging of region 3, the lymphatic lumen did not appear to be significantly narrowed in this specimen (Figure 3.16D). Taken together this suggests that lymphatic and blood microvascular morphology were less severely abnormal in the tissue specimen from the patient with the shortest duration of lymphedema.
Micro CT and transmission electron microscopy images of a human lymphatic specimen from the forearm of a female patient with a year-long history of upper-limb lymphedema (Table 3.1, case 6). A: Micro CT imaging of a transverse section through the collecting lymphatic specimen demonstrating the lymphatic wall and surrounding connective tissue containing associated blood microvasculature (regions 1 and 2). The smooth muscle layer of the lymphatic wall appears thickened (region 3). B: Transmission electron microscopy imaging demonstrating the associated blood microvasculature in region 1, showing a single blood vessel (bv). The lumen is very narrow (arrow and label) without any visible red blood cells (rbc). C: Transmission electron microscopy imaging of the associated microvasculature in region 2, demonstrating a single blood vessel comprising a lymphatic endothelial cell and nucleus (arrow and label) and red blood cell within the lumen (arrow and label). D: Transmission electron microscopy of the lymphatic wall in region 3, showing a normal, rounded smooth muscle cell morphology without evidence of significant narrowing of the lymphatic lumen.
Micro CT imaging of the perforating vascular bundle from the groin of the female control, without lymphedema, demonstrated contrasting morphology of the subcutaneous lymphatics and blood microvasculature compared to the lymphedema specimens (Figure 3.17). The control sample comprised perforating veins and two thin-walled lymphatics, along with blood microvasculature in the adjacent connective/adipose tissue (Figure 3.17A). Four regions identified on micro CT and labeled 1-4, were examined by TEM. Region 1 contains normal blood microvasculature, which did not appear to be associated with collecting lymphatics (Figure 3.17A and 3.17B). Blood vessel morphology appeared normal, with an open lumen containing a red blood cell (Figure 3.17B). TEM imaging of region 2 similarly demonstrated a normal blood vessel with multiple red cells in the lumen (Figure 3.17C). TEM imaging of region 3, which overlaps with region 2, demonstrated features of the nearby collecting lymphatic (Figure 3.17D), which was thin-walled, with only 1-2 smooth muscle cell layers, and had a normal lumen. In the same image, the blood vessel from region 2 was viewed again, with multiple red blood cells within its lumen (Figure 3.17D). TEM imaging of region 4 similarly demonstrated another thin-walled lymphatic and two nearby blood vessels with normal, rounded morphology and open lumens containing circulating red cells (Figure 3.17E).
Micro CT and transmission electron microscopy images of a human specimen of a perforating vascular bundle from the groin of a female patient without any history of lymphedema (Table 3.1, control). A: Micro CT imaging of a transverse section through the specimen, demonstrating two larger veins (arrows and labels), and thin-walled collecting lymphatics and blood microvasculature within the surrounding connective tissue (regions 1 to 4). B: Transmission electron microscopy imaging demonstrating blood microvasculature in region 1, which is not in the immediate vicinity of the lymphatics. This shows the features of a normal blood vessel (bv) on electron microscopy imaging, which is rounded in shape without evidence of luminal constriction, and contains a red blood cell (rbc) (arrow). C: Transmission electron microscopy imaging demonstrating normal blood microvasculature in region 2, with a blood vessel (bv) containing multiple red blood cells (rbc) (arrow). D: Transmission electron microscopy of region 3, containing the same blood vessel identified in the overlapping region 2 on the top left (arrow) and a thin-walled lymphatic comprising only 1-2 smooth muscle cell layers and a normal lumen (arrow and label). E: Transmission electron microscopy of region 4, comprising two small blood vessels (bv) on the bottom left of the image, and a thin-walled lymphatic vessel on the top right of the image (arrow and label) with normal morphology.
To gain a more global impression of the relationship between the collecting lymphatic and associated microvasculature in lymphedema, 3-dimensional reconstruction was performed on the micro CT images from the second lymphedema specimen in Figure 3.15A (Case 5). This demonstrated a network of blood vessels associated with the collecting lymphatic wall, which appeared to be more abundant in regions where the lymphatic lumen was narrower (Figure 3.18).

**Figure 3.18**

3-dimensional reconstruction of the micro CT images from a female patient with a 5 year history of lymphedema of the foot (Table 3.1, case 5), corresponding to the images in hypothesis 1 figure 15 as indicated by the superimposed orthoslice. The light blue represents the lymphatic endothelium and gives the outline of the lymphatic lumen. The green spheres give the course of the small blood vessels associated with the lymphatic wall. The blood microvasculature appears to be more abundant and closer to the lymphatic when the lymphatic lumen is narrowest or constricted (arrow).
3.3 Discussion

3.3.1 Identifying vasa lymphaticum in the mouse

Agliano et al described the vasa vasorum of collecting lymphatics of the proximal human thigh in 1997. Patients undergoing open varicose vein surgery by long saphenous vein ligation were recruited to the study and specimens were obtained from the tissues adjacent to the excised venous segments (Agliano et al., 1997). Specimens were fixed, stained with toluidine blue to define the tissue architecture, and imaged by light microscopy at 50, 100 and 160 times magnification. Based simply on vessel morphology, and with the assistance of 3D reconstruction software, the authors identified the presence of two plexi of blood microvasculature associated with the collecting lymphatic wall – one external, and one internal to the smooth muscle media (Agliano et al., 1997). The distribution of these blood vessels appeared to be linked to the organization of the lymphatic smooth muscle layer, with abundant blood microvasculature in the intervalvular regions of collecting lymphatic, where the smooth muscle media was well established. Occasionally, these vessels appeared to be associated with leaflets of lymphatic valves (Agliano et al., 1997). However in the absence of staining for lymphatic and blood vascular markers, it is difficult to conclude with any certainty that the main valved vessels described were actually lymphatics rather than smaller veins running with or branching from the long saphenous vein in the harvested specimens. In turn, it is also difficult to exclude the possibility that the so-called blood microvasculature identified within the lymphatic vessel wall were instead small lymphatic branches arising from the lumen of the main vessel, rather than blood vessels. For this reason, and in order to gain a better understanding of the vasa lymphaticum, we sought to characterize these vessels by immunohistochemistry, and detailed imaging by confocal and electron microscopy.

We initially harvested and analyzed the collecting lymphatics of the transgenic Prox1-GFP mouse (Choi et al., 2011). We failed to identify blood microvasculature associated with, or penetrating the collecting lymphatic wall in serial sections from the mouse flank under light microscopy at high magnification. Although the mouse tail is an established model for studying lymphedema and the impact of radiotherapy on the soft tissues and collecting lymphatics (Rutkowski et al., 2006, Avraham et al., 2010), the collecting lymphatic caliber is very small in the mouse tail, at
around 100 μm in diameter (Figure 3.1, Figure 3.2, Figure 3.4). Furthermore, immunostaining for three different blood vascular markers in the full lengths of nine Prox1-GFP mouse tails demonstrated multiple blood microvascular branching in the vicinity of the collecting lymphatic, occurring randomly along the length of the tail, without evidence of these blood vessels actually interacting with the collecting lymphatic wall (Figure 3.9).

There are two plausible explanations for the absence of vasa lymphaticum in the mouse specimens analyzed. Firstly, it is possible that blood microvasculature associated with the lymphatic wall is simply too small to visualize in the mouse. However, on viewing sections of tail collecting lymphatic by micro CT and electron microscopy, the so-called vasa lymphaticum was still absent from the images acquired (Figure 3.5, Figure 3.6, Figure 3.7). With detailed views down to single lymphatic endothelial cells on electron microscopy, it is much less likely that small blood vessels in the lymphatic wall would be missed purely due to their caliber. Galili et al studied the distribution of arterial vasa vasorum in porcine coronary, renal, carotid, femoral and internal mammary arteries, and noted significant variation in their density and distribution at these different sites in the body, perhaps reflecting the differing metabolic requirements or disease susceptibility of the arteries at different locations (Galili et al., 2004). We therefore moved away from the tail and flank in search of the vasa lymphaticum in the mouse cisterna clyde/thoracic duct- the largest collecting lymphatic of the body, responsible for draining lymph from the hind limbs and abdominal and pelvic viscera, and intestinal lipid absorption (Kawashima et al., 1964), where metabolic demands are likely to be high. However we still failed to identify blood microvasculature in the lymphatic wall on transmission electron microscopy of the mouse cisterna clyde (Figure 3.8).

It is possible that vasa lymphaticum do not exist at all in the mouse. For instance, there is evidence to suggest that the presence or absence of vasa vasorum and vasa venarum, supplying the wall of arteries and veins, respectively, appears to be species-specific, and vessel caliber-dependent. In 1967 Wolinsky & Glagov studied the thoracic aortas of 12 mammalian species, ranging from the mouse to the cat, dog, human, and horse. Segments of thoracic aorta were perfused with a gelatin-based mixture containing carbon ink, designed to distend and mark the
intramural vessels (Wolinsky & Glagov 1967). Segments were then fixed, allowing the gelatin to solidify, embedded in paraffin and cut for staining and examination under the microscope. Of the species examined, the mouse was the smallest in terms of gross body mass, thickness of the aortic media, at only 30 μm, and number of aortic media lamellae - only 5 in the adult mouse (Wolinsky & Glagov 1967). While some vasa vasorum were identified in the adventitial layers of the mouse aorta, no vasa vasorum were detected in the aortic media of the mouse, or indeed in any species with an aortic medial thickness below 29 lamellae (Wolinsky & Glagov 1967). This included the mouse, rat, guinea pig, rabbit and cat, whereas vasa vasorum were readily detected in the media of dog, human and sheep aortas (Wolinsky & Glagov 1967). Based on these findings, the authors concluded that below 29 lamellae, the aortic media is not dependent on vasa vasorum for oxygenation and nutrition (Wolinsky & Glagov 1967).

Caution should be exercised when extrapolating from the data on arterial wall blood supply, and directly applying these principles to the collecting lymphatics. In 1977 Brook studied the vasa venarum of human and canine veins, observing that the blood microvasculature appeared to lie much deeper within the vessel wall in veins compared to arteries, extending to the tunica intima in some specimens (as cited in Scotland et al, 2000). This suggests that, despite their thinner walls, the mural blood supply might be more critical in veins than in arteries. It is possible that this principle also extends to the collecting lymphatics, where circulating oxygen tension is even lower than the venous system (Hangai-Hoger et al., 2004).

The role of vasa vasorum in arterial pathology has been explored more extensively than the role of lymphatic wall blood microvasculature in lymphatic disease. For instance, arterial vasa vasorum have been implicated in the pathophysiology of a range of arterial disorders, including atherosclerosis (Mulligan-Kehoe & Simons, 2014), hypertension (Herrmann et al., 2005) and vasculitis (Hamaoka-Okamoto et al., 2014). Indeed, despite the fact that murine arteries do not appear to exceed the threshold thickness for the presence of vasa vasorum (Moss et al., 1968; Wolinsky & Glakov, 1967), intimal neovascularization has been demonstrated in the atherosclerotic lesions of descending aorta in apoE-deficient mice, suggesting that the distribution of arterial vasa vasorum might be significantly altered in the disease state (Moulton et al., 1999). In keeping with
this, studies on human coronary arteries have demonstrated an extensive network of mural blood microvasculature in the presence of atherosclerotic disease, which could not be demonstrated at all in healthy coronary vasculature (Barger et al., 1984; Mulligan-Kehoe & Simons, 2014).

### 3.3.2 Significance of the vasa lymphaticum in radiation injury

The impact of irradiation on the soft tissues has been widely reported (Stone et al., 2003; Rodemann & Blaese 2007), however the cellular processes responsible for the associated lymphatic dysfunction and clinical lymphedema remain poorly understood. In a mouse model of radiation-induced lymphatic dysfunction, the impact of 15 and 30 Gy on the mouse tail collecting lymphatics and surrounding tissues was explored by lymphoscintigraphy, histology and immunostaining, including TUNEL assays for apoptosis (Avraham et al., 2010). Tail lymphedema occurred only transiently, and had almost entirely resolved by 12 weeks post-irradiation in all groups. In contrast, long-term lymphatic dysfunction could be demonstrated in the tail lymphatics on lymphoscintigraphy, with reduced uptake even at 24 weeks post 30 Gy of irradiation, associated with a reduction in the number of LYVE1-positive lymphatic endothelial cells in tail tissue sections, long after clinical evidence of lymphedema had resolved (Avraham et al., 2010). In keeping with this, TUNEL assays of mouse tail sections demonstrated dose-dependent apoptosis in LYVE1-positive dermal lymphatic endothelial cells irradiated in vivo at 15 and 30 Gy, with the apoptotic response peaking at 10 hours (Avraham et al., 2010). In contrast, human dermal lymphatic endothelial cells in vitro appeared to be relatively radio-resistant, with only the higher dose of 30 Gy promoting a significant apoptotic response in culture (Avraham et al., 2010). These results therefore suggest that injury to the lymphatic endothelial cell microenvironment might contribute to the global deleterious effect of irradiation on the lymphatics.

In keeping with this, data on intestinal radiation injury in mice suggest that intestinal microvascular endothelial cells might be more susceptible to irradiation than the gut endothelium itself (Paris et al., 2001). Crypt-villus units of the murine small intestine were harvested from mice exposed to 15 Gy of whole body irradiation, and examined at 4 and 10 hours. While the apoptotic response to irradiation appeared to be maximal at 4 hours among
lamina propria cells expressing the vascular endothelial marker CD31, the onset of apoptosis was delayed to 10 hours in the columnar epithelium of the gut, suggesting that the gut microvasculature is more sensitive and more susceptible to irradiation damage (Paris et al., 2001). Furthermore, lack of the acid sphingomyelinase (asmase), an enzyme normally required by endothelial cells to mount an apoptotic response to irradiation, conferred protection from intestinal crypt damage and mortality associated with the gastrointestinal syndrome in knockout mice. Taken together this suggests that vascular endothelial, rather than gut epithelial injury, might be the principal lesion in intestinal irradiation injury (Paris et al., 2001). Whether the CD31-positive lamina propria cell population studied represents blood vessels only, or includes lymphatic endothelial cells of the lamina propria, remains to be determined, as CD31 is a pan-vascular endothelial marker expressed by both vessel types (Hirakawa et al., 2003).

Studies in mice exposed to high-dose irradiation have shown that lymphatic endothelial cells of the gut, and those within peri-tumoral microenvironments, might actually be relatively resistant to radiation-induced apoptosis compared to blood vascular endothelial cells at equivalent locations (Sung et al., 2006). Mice were exposed to whole body irradiation at 470 cGy/min, or alternatively, to study the impact of irradiation on peritumoral blood and lymphatic vasculature, cells from murine melanoma or human lung tumour lines were established and expanded in culture before injecting them into the subcutaneous tissues of the mouse flank, and mice were then irradiated with 15 Gy at 2-3 weeks post inoculation (Sung et al., 2006). Apoptosis was assessed in frozen sections of murine intestine by TUNEL assay and co-staining with primary antibodies against LYVE-1 or CD31, and images acquired by fluorescence and confocal microscopy. Whole body irradiation at a dose of 15 Gy appeared to result in minimal lymphatic endothelial cell apoptosis at 4 hours according to LYVE-1 and TUNEL co-staining, with only around 2% of the total LYVE-1-expressing lymphatic endothelial population undergoing apoptosis in the small intestinal villi (Sung et al., 2006). In contrast, 25% of CD31-positive vascular endothelial cells underwent apoptosis at the 4-hour time point. Furthermore, at all time points recorded (up to 48 hours), the proportion of apoptotic cells was significantly lower in the lymphatic endothelial cell population compared to the vascular endothelial cells (Sung et al., 2006). This resistance to irradiation-induced apoptosis in the lymphatic endothelial cell population did not appear to be confined to
lymphatics of the gut—immunostaining in peri-tumoural soft tissue specimens demonstrated a similar pattern of resistance, with virtually no lymphatic endothelial apoptosis from 4 to 48 hours post 15 Gy of whole body irradiation, although, in contrast to the findings reported in the murine small intestine, peritumoral vascular endothelial cells appeared to share this resistance to apoptosis in the face of irradiation (Sung et al., 2006). Certainly the reported findings in the murine small intestine support the concept that injury to the lymphatic microenvironment, particularly the associated blood microvasculature, might be more significant than direct lymphatic mural or endothelial cell injury in the pathophysiology of lymphatic dysfunction. Although Sung et al reported that peritumoral vascular endothelial cells were also resistant to radiation-induced apoptosis, other groups have reported contrasting data, with rates of microvascular endothelial cell apoptosis of up to 50% on irradiation in the same murine melanoma model (Garcia-Barros et al., 2003; Sung et al., 2006). If vascular endothelial cells of the vasa lymphaticum are indeed more sensitive to radiotherapy than the lymphatic vessel itself, then injury to the lymphatic wall microvasculature, and thus localized ischemia, might be responsible for lymphatic dysfunction and associated secondary lymphedema following irradiation.

3.3.3 Identifying the vasa lymphaticum in human lymphatics

We have identified vasa lymphaticum in the collecting lymphatics of the lymphadematous human limb, and characterized their morphology and ultrastructure by immunostaining and light microscopy/confocal microscopy, and furthermore by imaging at high magnification and resolution using micro CT and electron microscopy. On immunostaining and light microscopy imaging of sections of a collecting lymphatic from the leg of a patient with chronic lymphedema, we identified blood microvasculature, or vasa lymphaticum, closely associated with the adventitia of the collecting lymphatic wall and possibly extending into the lymphatic media (Figure 3.10E-F). These vessels appeared to be absent from the lymphatics in our control specimen from the groin of a patient without lymphedema (Figure 3.11E-F). Furthermore, the lymphedema specimen appeared abnormal with mural thickening, particularly of the lymphatic media (Figure 3.10), while the lymphatics within the vascular bundle of the control tissue were much smaller in caliber, thin walled and lacking a clearly-defined smooth muscle layer on light microscopy imaging (Figure 3.11).
It remains possible that these differences between lymphedematous and control tissue were a function of sampling error. The lymphatic network comprises three morphologically and functionally distinct vessel subtypes, which differ in their caliber and composition (Shayan et al., 2006). In the normal physiological state, the collecting lymphatics of the lower limb maintain the flow of lymph against gravity and the elevated interstitial pressures of the dependent limb, and their structural features reflect these functional requirements (Shayan et al., 2006). While the collecting lymphatics have a smooth muscle media, contain valves and propel lymph by smooth muscle peristalsis, the initial lymphatics are thin walled without a smooth muscle layer (Shayan et al., 2006), and inherently the energy and oxygen requirements of these two lymphatic vessel subtypes are likely to be different. The lymphatics within our control tissue specimen were thin walled and lacking of a demonstrable smooth muscle layer on SMA staining (Figure 3.11B), thereby perhaps more closely resembling the initial lymphatic subtype, and it is therefore unsurprising that the energy requirements of these vessels could be met by simple diffusion, without requiring a dedicated blood supply. In contrast, it is reasonable to assume that the energy requirements of the lower limb collecting lymphatics are much higher, and demands for an additional oxygen and energy supply to the lymphatic wall further increased in the presence of lymphatic mural thickening, and elevated interstitial pressures of the chronically lymphedematous limb (Rutkowski et al., 2010).

In keeping with this concept, on whole mount immunostaining and confocal microscopy we demonstrated a dense meshwork of blood microvasculature overlying, and penetrating the lymphatic wall in collecting lymphatic specimens from the lymphedematous limb (Figure 3.12A-C). Unfortunately due to the technical challenges encountered in isolating lymphatic vasculature from its surrounding connective tissues in control specimens, we were unable to subject control lymphatics to the same focused lymphatic wall imaging by confocal microscopy, preventing direct comparisons between the two. On micro CT and electron microscopy, further evidence for the association between blood microvasculature or vasa lymphaticum, and the collecting lymphatic wall was gathered, with blood vessels identified within the lymphatic adventitia and surrounding connective tissue. Furthermore there was evidence to suggest that adverse morpholog-
ical changes in the blood microvasculature of the vasa lymphaticum correlate with the extent of pathological lymphatic wall changes in chronic lymphedema (Figure 3.14, Figure 3.15, Figure 3.16). In particular, in the limited number of lymphedematous lymphatic specimens examined (n=3) changes in the lymphatic media smooth muscle and extracellular matrix composition and ultrastructure appeared to reflect disease severity and chronicity, and this correlation appeared to extend to the associated blood microvasculature, with mural thickening, luminal narrowing and the absence of circulating red cells (Figure 3.14, Figure 3.15, Figure 3.16). In contrast, the thin walled lymphatics of the control specimen comprised normal smooth muscles cells, and nearby blood microvasculature appeared normal in morphology with circulating red blood cells (Figure 3.17). It is conceivable that the changes in blood microvasculature observed in chronic lymphedema might contribute to the pathophysiology of the condition, with tissue and lymphatic mural hypoxia perhaps preceding lymphatic injury and dysfunction, or contributing to the process, or indeed prohibiting lymphatic recovery.

In a 3D model reconstructed from the micro CT images of a segment of lower-limb collecting lymphatic from a patient with a 5 year history of lymphedema of the foot, the associated blood microvasculature appeared to be more abundant at points where the lymphatic lumen was at its narrowest (Figure 3.18). This potentially indicates an increased requirement for a dedicated mural blood supply when lymphatic flow is reduced and circulating oxygen availability more limited, or conversely, where associated mural thickening and luminal collapse increase the energy and oxygen requirements of cells within the lymphatic vessel wall. In keeping with this concept, there is evidence to suggest that the vasa vasorum of the arterial adventitia and media respond to intimal injury and inflammation by angiogenesis and expansion, forming a more dense microvascular network (Mulligan-Kehoe & Simons, 2014; Herrmann et al., 2001). Studies in a mouse model of atherosclerosis have demonstrated that low-density-lipoprotein-receptor-deficient/ApolipoproteinB100/100 mice on a high-fat, high-cholesterol diet for 20 weeks not only develop atherosclerotic lesions in the descending aorta, but also acquire a dense plexus of vasa vasorum in the aortic adventitia (Mollmark et al., 2012). Following perfusion with a FITC-conjugated lectin, mice were sacrificed and whole mounts prepared from the descending aorta were imaged by confocal microscopy. This clearly showed that diet-induced atherosclerosis was
accompanied by significant vasa vasorum angiogenesis to form a complex, arborizing network (Mollmark et al., 2012). Further to this, in a porcine model of coronary artery atherosclerosis, pigs on a hypercholesterolaemic diet for 2-4, or 6-12 weeks developed progressive mural thickening in the left anterior descending coronary artery, accompanied by increased vasa vasorum density associated with angiogenesis and disorganized branching patterns on micro CT imaging (Herrmann et al., 2001). These changes appeared to precede functional changes in the coronary artery endothelium, as the vasoreactive response to bradykinin only became attenuated after 6-12 weeks on a hypercholesterolaemic diet (Herrmann et al., 2001).

In a murine model of Kawasaki disease, induced by intraperitoneal injection of Candida albicans water-soluble fraction, changes in the vasa vasorum were detected before microscopic changes in the coronary artery intima normally associated with Kawaskai vasculitis (Hamaoka-Okamoto et al., 2014). Mice were sacrificed at 1, 2 and 3-week time points and the inflammatory changes in the heart, coronary arteries and aorta examined by immunostaining for CD3+ and MPO+ cells. At one week, there was minimal evidence of inflammatory change, with just a few CD3+ cells detected in the aortic adventitia (Hamaoka-Okamoto et al., 2014). CD3 and MPO+ cells were only detected at the coronary arteries at 2 weeks, but by the 3 week time point the number of MPO+ cells had further increased (Hamaoka-Okamoto et al., 2014). Immunofluorescent studies with Biotin-conjugated Isolectin IB4 revealed microvasculature associated with the aortic adventitia at one week in the Kawasaki disease model, however the same vessels were not seen in control mice. SEM and Micro CT of the left coronary artery demonstratarated progressive proliferation of this mesh-like network of vessels as the inflammatory disease process progressed from 0 – 3 weeks (Hamaoka-Okamoto et al., 2014), and these changes in the vasa vasorum appeared to precede the onset of inflammatory changes within the intima of affected arteries (Hamaoka-Okamoto et al., 2014).

It is unclear what role a dedicated lymphatic blood supply might play in the pathophysiology of lymphatic disease. It is conceivable that the vasa lymphaticum might be upregulated in response to lymphatic injury, for example following trauma, irradiation and in chronic lymphedema, providing additional oxygenation to the damaged, dysfunctional collecting lymphatic wall. Equal-
ly, it is possible that injury to the vasa lymphaticum, for example by radiotherapy, and thus localized lymphatic wall hypoxia, cell death and scarring might result in lymphatic dysfunction. Further work is clearly required to delineate the precise pathophysiological processes in the collecting lymphatic wall in response to a range of insults associated with lymphatic dysfunction. Such information might also reveal potential therapeutic targets. Our studies on human collecting lymphatic vasa vasorum have been limited by a lack of well-matched control specimens. Without equivalent collecting lymphatic specimens from the lower limbs of healthy controls it is not possible at this stage to determine whether the lymphatic vasa vasorum is a normal, physiological component of the lymphatic vessel wall, or whether they arise in response to mural injury and pathology.
Chapter 4:

ISOLATION AND CHARACTERISATION OF LYMPHATIC ENDOTHELIAL CELLS FROM DIFFERENT TISSUES AND ORGANS OF THE PROX1-GFP MOUSE
The lymphatic vasculature comprises a hierarchy of branching vessels, integral to tissue fluid homeostasis, immune cell trafficking and intestinal fat absorption (Oliver & Detmar, 2002; Johnson & Jackson, 2014; Harvey et al., 2005). Increasingly the lymphatic system has been implicated in the pathophysiology of a variety of organ-specific disease states, from inflammatory bowel disease (Cromer et al., 2015) to pulmonary fibrosis (Meinecke et al., 2012). The role of the lymphatic vasculature in cancer metastasis is well described (Karnezis et al. 2012; Stacker et al., 2014; Hirakawa et al., 2007). Given the predisposition of tumours to metastasise to specific organs, it is likely that the lymphatics at these distant sites actively facilitate tumour metastasis, and is therefore conceivable that lymphatic endothelial cells within these organs differ in their expression profiles, particularly for pathways involved in tumour metastasis (Tarin et al., 1984a; Fidler, 2003). The characterisation of tissue- and organ-specific features of lymphatic structure and function has historically been hindered by a lack of lymphatic-specific markers (Stacker et al., 2014), however we now have a panel of lymphatic markers including VEGFR3 (Kaipainen et al., 1995), LYVE-1 (Banerji et al., 1999), podoplanin (Breiteneder-Geleff et al., 1999) and Prox-1 (Wigle et al., 2002), and furthermore the isolation of lymphatic endothelial cells from human dermis and lung has been described, largely through magnetic-activated cell sorting (Kriehuber et al., 2001, Lorusso et al., 2015). We aimed to isolate lymphatic endothelial cells from a range of tissues and organs in the Prox1-GFP mouse (Choi et al., 2011), and to determine their tissue-specific morphological and functional characteristics.

Hypothesis

Lymphatic endothelial cells can be isolated from a range of tissue and organ types. These lymphatic endothelial cells have tissue-specific molecular and functional characteristics.

Aims

To isolate lymphatic endothelial cells from different tissues and organs by flow cytometry, according to their expression of lymphatic endothelial markers, and to determine tissue- and organ-specific characteristics.
4.1 Isolation of lymphatic endothelial cells from the Prox1-GFP mouse by flow cytometry:

Lymphatic endothelial cells were isolated from the tissues and organs of the Prox1-GFP mice by flow cytometry according to the expression of two lymphatic markers Prox1 and podoplanin, as described (Section 2.3). Figures 4.1-4.7 show the gating applied to the cell sort for each tissue or organ studied, while Table 4.1 provides raw data on the sorts, including the proportion of live cells staining positively for the lymphatic marker podoplanin within a single cell suspension from each tissue or organ, and subsequently the proportion of these cells which expressed GFP under the control of the Prox1 promoter, indicating positive expression of the lymphatic marker Prox1 (Choi et al., 2011).
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse ear skin. **A:** FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 5.19% of recorded events. **B:** Isotype control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for only 0.64% of all events. **C:** FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate the podoplanin-positive, and Prox1 (GFP)-positive lymphatic endothelial cell population. 4.02% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse collecting lymphatics. **A:** FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 55.8% of recorded events. **B:** FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate the podoplanin-positive, and Prox1 (GFP) - positive lymphatic endothelial cell population. 0.01% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse lymph nodes. A: FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 0.88% of recorded events. B: Isotype control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for 0.20% of all events. C: FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate podoplanin-positive, and Prox1 (GFP) -positive lymphatic endothelial cell population. 3.70% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse liver. A: FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 0.49% of recorded events. B: Iso-type control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for 0.011% of all events. C: FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate podoplanin-positive, and Prox1 (GFP)-positive lymphatic endothelial cell population. 2.64% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse lung.  

A: FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 17.8% of recorded events.  

B: Iso- 

type control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for 0.019% of all events.  

C: FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate podoplanin-positive, and Prox1 (GFP) -positive lymphatic endothelial cell population.  

0.33% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse gut. **A:** FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 40.5% of recorded events. **B:** Iso-type control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for 0.62% of all events. **C:** FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate podoplanin-positive, and Prox1 (GFP)-positive lymphatic endothelial cell population. 0.59% of the live podoplanin-positive cells also expressed GFP.
Isolation of lymphatic endothelial cells by flow cytometry from a single cell suspension of Prox1-GFP mouse brain. 

A: FACS plot of PE (X-axis) and PI (Y-axis) channels showing the gating applied for the isolation of live PI-negative, podoplanin-positive cells, comprising 47.1% of recorded events. 

B: Iso-type control for the podoplanin immunostaining, with the same channels and gating applied as in plot A, showing the extent of non-specific binding of the PE-conjugated anti-mouse antibody, with PI-negative and PE-positive cells accounting for 1.46% of all events. 

C: FACS plot of GFP (X-axis) and PE (Y-axis) channels showing the gating applied to the live, podoplanin-positive cell population to further isolate podoplanin-positive, and Prox1 (GFP)-positive lymphatic endothelial cell population. 6.96E-4% of the live podoplanin-positive cells also expressed GFP.
**Table 4.1:** Isolation of organ/tissue-specific lymphatic endothelial cells from the Prox1-GFP mouse by flow cytometry

<table>
<thead>
<tr>
<th>Tissue or organ</th>
<th>Podoplanin-positive (%)</th>
<th>Percentage of podoplanin-positive events that are GFP-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermis (ear)</td>
<td>5.19</td>
<td>4.02</td>
</tr>
<tr>
<td>Collecting lymphatic*</td>
<td>55.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.88</td>
<td>3.70</td>
</tr>
<tr>
<td>Liver</td>
<td>0.49</td>
<td>2.64</td>
</tr>
<tr>
<td>Lung</td>
<td>17.8</td>
<td>0.33</td>
</tr>
<tr>
<td>Small intestine</td>
<td>40.5</td>
<td>0.59</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>47.1</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*original cell sort data, gating has since been amended.

In all cases, doublet exclusion was performed by plotting the forward scatter area against trigger pulse width, and applying gating as shown to exclude cell clumps from the sort sample. Dead cells were then gated out according to uptake of the nucleic acid dye propidium iodide, by plotting the PI channel against the PE channel (Figure 4.1A, 4.2A, 4.3A, 4.4A, 4.5A, 4.6A and 4.7A), after compensation for spectral overlap, where required. The proportion of cells which were both live and podoplanin-positive varied greatly between the different tissues and organs studied (Table 4.1), from 0.49% (liver, Figure 4.4A) to 47.1% (brain, Figure 4.7A) and even as high as 55.8% in the original gating applied to the collecting lymphatic cell suspension, although this particular result has subsequently been re-assessed and the gating reviewed (Figure 4.2). The high proportion of podoplanin-positive cells in the single cell suspension from murine brain is likely to reflect the widespread expression of podoplanin by cells of the central nervous system (Mishima et al., 2006, Tomooka et al., 2013). Isotype controls for podoplanin immunostaining were prepared from single cell suspensions from each tissue or organ type and also subjected to the same gating and analysis (Figure 4.1B, 4.2B, 4.3B, 4.4B, 4.5B, 4.6B and 4.7B). The amount of non-specific binding of the isotype control also varied between different tissues and organs from 0.011% (liver) to 1.46% (brain).
In the final stage of the cell sort, live cells positive for both PE (podoplanin) and GFP (Prox1) were selected by plotting the PE channel against the GFP channel and applying the relevant gating to select PE-positive, GFP-positive cells (Figure 4.1C, 4.2C, 4.3C, 4.4C, 4.5C, 4.6C and 4.7C). This cell population was then sorted into a 96 well plate for expansion in culture (Figures 4.8-4.14). The proportion of live podoplanin-positive cells also expressing the second lymphatic marker Prox1 varied between the tissues and organs, from 6.96E-4% in the brain to 4.02% in the mouse ear dermis. The tiny proportion of cells expressing both podoplanin and Prox1 in the brain is a significant contrast to the large podoplanin-positive cell population and reflects the widespread expression of podoplanin by other central nervous system cell types (Tomooka et al., 2013), versus the very small number of lymphatic vessels within the meninges of the murine central nervous system (Louveau et al., 2015). As such, a discrepancy in absolute yield of live podoplanin-positive, Prox1-positive cells was seen, ranging from only 50 cells isolated from the collecting lymphatics of 8 Prox1-GFP mice, to 34 000 cells isolated from the lamina propria of a single Prox1-GFP mouse small intestine.
4.2 In vitro characteristics of lymphatic endothelial cells from different organ and tissue types:

In order to compare lymphatic endothelial cells from different tissue types, cells were initially expanded in culture, and in vitro cell morphology documented by fluorescence microscopy imaging from day 0 onwards, in passage 0 and 1, facilitated by the nuclear expression of GFP under the Prox1 promoter (Choi et al., 2011), (Figures 4.8-4.14).

**Figure 4.8**

*Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the ear skin of the Prox1-GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter. A: At day 4, at x 20 magnification. The cells appear flattened in morphology and adherent to the culture plate. B: At day 4, at x 40 magnification. GFP expression is seen in the nucleus of a dividing lymphatic endothelial cell.*
**Figure 4.9**

Fluorescence microscopy image of lymphatic endothelial cells in culture, following isolation from the subcutaneous collecting lymphatics of the Prox1 GFP mouse, showing the nuclear GFP expression under the Prox1 promoter at 8 weeks, at x 10 magnification.

**Figure 4.10**

Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the lymph nodes of the Prox1 GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter.  
**A:** At day 4 at x 20 magnification.  
**B:** At day 4 at x 40 magnification demonstrating the orientation of lymphatic endothelial cells in clusters.
Figure 4.11

Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the liver of the Prox1 GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter. A: At day 1 at x 10 magnification. B: At day 1 at x 20 magnification, demonstrating the rounded morphology of liver lymphatic endothelial cells at 24 hours post-isolation and culture. C: At day 7 at x 20 magnification showing the change in cell morphology compared to day 1. Cells appear larger in size, flattened, and spread out on the plate, with an asymmetric and irregular shape. D: At day 13 at x 20 magnification showing flattening, and spreading out of the liver lymphatic endothelial cells in culture.
**Figure 4.12**

Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the lung of the Prox1 GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter. **A**: At day 0 at x 10 magnification. **B**: At day 7 at x 20 magnification, demonstrating the rounded morphology and prominent nuclei of lung lymphatic endothelial cells.

**Figure 4.13**

Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the small intestine of the Prox1 GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter. **A**: At day 6 at x 10 magnification, demonstrating the formation of cell clusters. **B**: At day 6 at x 20 magnification, showing in more detail the morphology of small intestine lymphatic endothelial cells in culture, with the formation of clusters.
**Figure 4.14**

Fluorescence microscopy images of lymphatic endothelial cells in culture, following isolation from the brain of the Prox1 GFP mouse, showing the nuclear expression of GFP under the Prox1 promoter. **A:** At day 5 at x 10 magnification. **B:** At day 5 at x 20 magnification, demonstrating the rounded appearance and prominent nuclei of central nervous system lymphatic endothelial cells in culture.

Differences in lymphatic endothelial cell size were apparent across the different tissue and organ types studied (Figures 4.8-4.14). These differences in cell size were accompanied by changes in cell morphology. In the images of liver and lung LECs at day 7, the contrast in LEC morphology between the two organ types was apparent, with liver LECs spreading out on the plate and developing cytoplasmic extensions (Figure 4.11C), while the lung LECs remained rounded with prominent nuclei (Figure 4.12B). In the images of dermal LECs in culture at day 4, this LEC subtype also appeared to flatten and spread out on the plate, with GFP expression in the dividing nucleus highlighting the cell proliferation in one of the images (Figure 4.8A and 4.8B). Once again, this contrasted with the appearance of other LEC subtypes at day 4, including lymph node LECs, which were smaller and more spherical in morphology (Figure 4.10A and 4.10B). Central nervous system LECs isolated from the brain in the Prox1-GFP mouse appeared relatively rounded with prominent nuclei at day 5 (Figure 4.14A and 4.14B), while gut LECs at day 6 had quite a distinctive morphology, with prominent cell clusters forming on the plate (Figure 4.13A and 4.13B).
4.3 Distribution of lymphatic vasculature in the tissues and organs of the Prox1-GFP mouse:

In order to study the in vivo distribution of isolated tissue or organ-specific lymphatic endothelial cells, corresponding tissues and organs were harvested from the Prox1-GFP mouse, fixed, sectioned, and immunostaining performed for the lymphatic markers podoplanin and Prox1 (GFP), as described (Section 2.3.6). This revealed tissue-specific patterns of lymphatic vascular supply, with characteristic distributions of the lymphatic network, and expression of the lymphatic markers podoplanin and Prox1 in the tissues and organs studied (Figures 4.15-4.21).

**Figure 4.15**

*Fluorescence microscopy images of a transverse section of Prox1-GFP mouse ear dermis at x 20 magnification. A: DAPI nuclear stain in blue showing the overall tissue architecture of the epidermis (arrow and label) and dermis (arrow and label) and multiple hair follicles (arrow and label). B: GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green. C: Immunostaining for the lymphatic marker podoplanin showing podoplanin-positive structures in red. D:*
GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating linear structures within the dermis staining positively for both GFP (Prox1 expression), and the lymphatic marker podoplanin, consistent with the dermal lymphatic vasculature (arrows and label).

**Figure 4.16**

Fluorescence microscopy images of a transverse section of Prox1-GFP mouse flank skin and subcutaneous tissues at x 20 magnification. **A:** DAPI nuclear stain in blue showing the overall tissue architecture with epidermis (arrow and label) and subcutaneous tissue plane containing hair follicles (arrows and label). **B:** GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green. **C:** Immunostaining for the lymphatic marker podoplanin showing podoplanin-positive structures in red. **D:** GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating GFP (Prox1) -positive, podoplanin-positive vascular structures consistent with subcutaneous collecting lymphatics in yellow (arrows and label). In the same view spherical structures with central nuclei (blue) and peripheral podoplanin-positive staining (red) are seen, possibly representing subcutaneous nerves (arrow and label).
**Figure 4.17**

Fluorescence microscopy images of a transverse section of Prox1-GFP mouse lymph node at x 20 magnification. **A:** DAPI nuclear stain in blue showing the overall tissue architecture with the outer cortex, and inner medulla. **B:** GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green, in the lymph node cortex surrounding lymph node follicles (arrows and label). **C:** Immunostaining for the lymphatic marker podoplanin showing podoplanin-positive structures within the cortex and medulla in red. **D:** GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating GFP (Prox1) -positive, podoplanin-positive vascular structures in yellow within the cortex surrounding the follicles consistent with afferent lymph node lymphatics (arrows and label), and lymphatic vasculature of the subcapsular sinuses (arrows and label).

Podoplanin-positive, GFP (Prox1)-negative structures are also present within the lymph node medulla surrounding the medullary sinuses (arrows and label).
Fluorescence microscopy images of a transverse section of the left lobe of the Prox1-GFP mouse liver at x 20 magnification. A: DAPI nuclear stain in blue showing the overall tissue architecture, with acellular areas which are likely to represent hepatic sinuses (arrows and label). B: Immunostaining for the lymphatic marker podoplanin showing podoplanin-positive structures adjacent to the hepatic sinuses in red. C: Podoplanin (red) co-staining with a DAPI nuclear counterstain (blue), further defining the distribution of podoplanin expression in the liver, which appears to be concentrated around the hepatic sinuses (arrows).
Figure 4.19

Fluorescence microscopy images of a transverse section of the Prox1-GFP mouse lung at x 20 magnification.  

**A:** DAPI nuclear stain in blue showing the overall tissue architecture of the murine lung comprising alveoli and air spaces.  

**B:** GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green.  

**C:** Immunostaining for the lymphatic marker podoplanin showing widespread podoplanin expression throughout the lung alveolar cells outlining the air spaces.  

**D:** GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating GFP (Prox1)-positive, podoplanin-positive lymphatic structures lining air spaces in yellow (arrows).
**Figure 4.20**

Fluorescence microscopy images of a transverse section of the Prox1-GFP mouse small intestine at x 20 magnification. **A:** DAPI nuclear stain in blue showing the overall tissue architecture of the small intestine, with intestinal mucosa comprising intestinal villi, lamina propria and crypts (arrows and labels), intestinal submucosa, muscularis, and serosa (arrows and labels). **B:** GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green. **C:** Immunostaining for the lymphatic marker podoplanin showing expression in the lamina propria (arrow and label) and lining the intestinal crypts (arrow and label). **D:** GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating GFP (Prox1)-positive, podoplanin-positive lymphatic vascular structures within the lamina propria and lining the intestinal crypts in yellow (arrows).
**Figure 4.21**

Fluorescence microscopy images of a coronal section of the Prox1-GFP mouse brain at the level of the frontal lobe at x 20 magnification. **A**: DAPI nuclear stain in blue showing the overall tissue architecture, comprising cerebral cortex and meninges (arrows and labels).

**B**: GFP immunostaining demonstrating the distribution of the lymphatic marker Prox1 in green. **C**: Immunostaining for the lymphatic marker podoplanin showing widespread podoplanin-positive staining in the cortex and meninges. The layers of the arachnoid and dura can be appreciated (arrows and labels), with dural vasculature and sinuses (arrows and labels). **D**: GFP (green) and podoplanin (red) co-staining with DAPI nuclear counterstain (blue), demonstrating GFP (Prox1) -positive, podoplanin-positive lymphatic vascular structures in yellow (arrows and label).

In the mouse ear skin (Figure 4.15) few vascular structures staining positively for both GFP (Prox1) and the second lymphatic marker podoplanin were identified within the dermis (Figure 4.15B-D), and a similar pattern was observed in the subcutaneous tissue of the mouse flank (Figure 4.16A-D), although GFP and podoplanin co-staining demonstrated several thin-walled vessels within the dermis and subcutaneous plane consistent with pre-collecting and collecting lymphatic vasculature (Figure 4.16D).
Within the lymph node of the Prox1-GFP mouse, areas of GFP-positive and podoplanin-positive co-staining were located within the cortex, peripheral to the lymph node follicles, and are likely to represent the afferent lymphatics of the murine lymph node.

Technical difficulties were encountered in immunostaining of the Prox1-GFP mouse liver tissue sections, with high levels of autofluorescence in the green channel, limiting any reliable interpretation of GFP-immunostaining, even after attempts at quenching the autofluorescence using Sudan black at the end of the staining procedure (Section 2.3.6). As a result, only DAPI and podoplanin immunostaining could be reliably interpreted in the liver (Figure 4.18A-C), which did demonstrate some podoplanin-positive vascular structures. Without co-staining for a second lymphatic-specific marker, the significance of these findings is difficult to determine.

In the murine lung, GFP-positive and podoplanin-positive lymphatic vasculature were identified in the lining of the ducts or sacs of the alveolar air spaces (Figure 4.19A-D). However collecting lymphatics expressing Prox1 and podoplanin appeared to be relatively few in number (Figure 4.19D). In contrast, in the small intestine of the Prox1-GFP mouse, multiple vascular structures staining positively for both GFP and podoplanin were identified within the lamina propria of the villi of the intestinal mucosa, and lining the intestinal crypts (Figure 4.20A-D). The intestinal lamina propria was the layer specifically targeted by the enzymatic and mechanical dissociation process to yield a single cell suspension from the Prox1-GFP mouse small intestine, in preparation for the isolation of a population of lymphatic endothelial cells by flow cytometry, as previously described (Section 2.3.3.3) (Figure 4.6A-C, Figure 4.13A-B). Immunostaining of tissue sections of murine brain from the Prox1-GFP mouse demonstrated the location of GFP-positive and podoplanin-positive lymphatic vasculature within the meninges, adjacent to the dural sinuses (Figure 4.21A-D), as described previously by Louveau et al (Louveau et al., 2015).

In particular, expression of the lymphatic marker podoplanin appeared to be widespread on immunostaining in certain organs and tissues, reflected by the high proportion of PE-podoplanin-positive cells isolated from single cell suspensions of the corresponding organs and tissues by flow cytometry (Figures 4.8-4.14, Table 4.1). For example, podoplanin appeared to be expressed widely in the Prox1-
GFP murine brain, with podoplanin-positive staining throughout the cerebral cortex and meninges (Figure 4.21C). Further to this, on flow cytometric analysis of a single cell suspension prepared from the Prox1-GFP mouse brain, live, podoplanin-positive cells appeared to account for 47.1% of all recorded events (Figure 4.7A-B) - observations consistent with data from other groups on the widespread expression of podoplanin in the central nervous system (Mishima et al., 2006; Tomooka et al., 2013).

Podoplanin is expressed by type 1 alveolar cells of the lung (Ramirez et al., 2003), and in keeping with this, podoplanin expression appeared particularly widespread on immunostaining of tissue sections of the Prox1-GFP mouse lung (Figure 4.19C), with podoplanin-positive staining throughout. This observation is supported by flow cytometry data from the Prox1-GFP murine lung, with live, podoplanin-positive cells accounting for 17.8% of recorded events (Figure 4.5A-B).

Although podoplanin-positive staining initially seemed to be widespread in the Prox1-GFP mouse liver, much of this appeared to be due to autofluorescence in the red channel, and was reduced by quenching with Sudan black after the staining (Figure 4.18B). Following this, a limited number of regions of the mouse liver staining positively for the lymphatic marker podoplanin were identified (Figure 4.8B-C), findings supported by flow cytometry data from the mouse liver, where live, podoplanin-positive cells appeared to represent only 0.49% of all recorded events (Figure 4.4A-B). In a similar way, podoplanin initially appeared to be expressed throughout tissue sections of the Prox1-GFP mouse lymph node, however on closer analysis much of this appeared to be background staining or autofluorescence, with the most intense podoplanin signals surrounding the lymph node follicles and co-localising with GFP (Prox1)-positive structures in the lymph node cortex (Figure 4.17B-D), and specifically outlining the lymph node sinuses of the medulla (Figure 4.17C-D). In keeping with these observations, on flow cytometric analysis of a single cell suspension of murine lymph nodes only 0.88% of all recorded events appeared to be live, podoplanin-positive cells (Figure 4.3A-B).

Relatively few podoplanin-positive structures were identified on immunostaining in the subcutaneous tissues of the Prox1-GFP mouse flank (Figure 4.16C). These structures appeared to fall into two groups – vessels with a clear lumen and positive co-staining for the lymphatic marker Prox1 (GFP) representing lymphatic vasculature, and structures with a peripheral podoplanin-positive layer and central collection of nuclei, likely to represent small branches of peripheral nerves (Figure 4.16D). In support
of this data, other groups have reported on the expression of the lymphatic marker podoplanin by Schwann cells and perineural cells of the peripheral nervous system (Jokinen et al., 2008, Schacht et al., 2005).
4.4 Discussion:

The lymphatics are considered central to the pathophysiology of tumour metastasis (Stacker et al., 2014), and it is increasingly evident that the lymphatics are involved in a wide range of inflammatory disorders, from inflammatory bowel disease (Randolph et al., 2016) to pulmonary fibrosis (Meinecke et al., 2012). However, the lack of lymphatic-specific markers, and technical difficulties in isolating lymphatic endothelial cells have long delayed progress in characterizing the lymphatic endothelium of various organ and tissue types (Stacker et al., 2014). We describe a method for the isolation of lymphatic endothelial cells from murine lymph node, dermis, subcutaneous tissue, liver, small intestine, lung, and brain by flow cytometry, based on the expression of two lymphatic-specific markers Prox1 and Podoplanin.

In 2001 Kriehuber et al. reported on the isolation of dermal lymphatic endothelial cells by flow cytometry following immunostaining of freshly-harvested mixed dermal cell suspensions with antibodies against the lymphatic marker podoplanin, CD34 and CD45. The podoplanin-positive CD34-positive and CD45—negative cell fraction, representing dermal lymphatic endothelial cells, was isolated by flow cytometry, however, low cell yields appeared to preclude further characterization of this cell population (Kriehuber et al., 2001). Instead, the authors reverted to endothelial cell enrichment of mixed dermal cell cultures by magnetic-activated cell sorting (MACS), prior to isolation of the lymphatic endothelial cell fraction by immunostaining and flow cytometry according to positive podoplanin and CD31 expression (Kriehuber et al., 2001).

Hirakawa et al. adopted a similar approach by immunomagnetic selection of lymphatic endothelial cells from human foreskin dermal cell suspensions, however they specifically differentiated between blood vascular and lymphatic endothelial cells according to CD34 expression, isolating the CD34-negative CD31-positive lymphatic endothelial cells (Hirakawa et al., 2003). They were able to demonstrate the differential expression patterns of blood and lymphatic vasculature by double immunostaining of human dermal tissue sections with antibodies against CD34 and the lymphatic markers Prox1 or LYVE-1, demonstrating the lack of CD34 expression in the Prox1+ or LYVE-1+ lymphatic vasculature (Hirakawa et al., 2003). This absence of CD34 expression by the human dermal lymphatics might account for
the low cell yields obtained by Krihuber et al in attempting to select lymphatic endothelial cells by flow cytometry based on positive CD34 expression (Krihuber et al., 2001), aside from the additional technical challenges of isolating a relatively rare cell-type by flow cytometry.

More recently, lymphatic endothelial cells have been isolated from the human lung by immunomagnetic sorting in fresh human tissue preparations (Lorusso et al., 2015). Pulmonary tissues were harvested from the clear margins of lobectomy specimens resected from 55 patients with lung cancer, and digested with collagen/dispase before plating and expansion in cell culture in an endothelial growth medium (Lorusso et al., 2015). Immunomagnetic cell sorting was performed in two stages, initially by selecting CD31-positive cells, followed by expansion of the CD31+ fraction in culture before further immunomagnetic selection for the lymphatic endothelial marker podoplanin. The purity of the isolated human lung lymphatic endothelial cell fractions was further assessed by flow cytometry after immunolabelling with an anti-human podoplanin antibody (Lorusso et al., 2015). Immunostaining of the cell cultures confirmed positive expression of the lymphatic endothelial cell markers podoplanin, LYVE-1 and Prox1. The morphology of these human lung lymphatic endothelial cells was further characterised by transmission electron microscopy (Lorusso et al, 2015).

The potential significance of the lymphatic system in pulmonary disease has been highlighted recently by data suggesting that abnormal lymphatic vasculature, particularly alterations in lymphatic mural structure, might be a contributing factor in the pathogenesis of pulmonary fibrosis (Meinecke et al., 2012). In a mouse model of pulmonary fibrosis resulting from bleomycin administration, increases in mural thickness were observed in LYVE-1-positive Prox1-positive VEGFR3-positive pulmonary lymphatic vasculature by co-staining with α-smooth muscle actin antibody – findings potentially consistent with abnormal lymphatic mural smooth muscle cell recruitment in pulmonary fibrosis (Meinecke et al., 2012). These pulmonary lymphatics also exhibited abnormal basement membrane formation, and these changes in mural structure appeared to occur early in the disease process at day 11 of bleomycin administration, prior to the onset of lung parenchymal changes consistent with pulmonary fibrosis (Meinecke et al., 2012). The structural changes were associated with impaired 488-dextran clearance in fibrotic lungs - a surrogate marker for lymphatic drainage, which was significantly reduced and associated with the build-up of fluorescent dye in the parenchyma of bleomycin-exposed mice (Meinecke
et al., 2012). In keeping with these findings, the same group also studied human lung specimens from patients with idiopathic pulmonary fibrosis, demonstrating increased pulmonary lymphatic density and lymphatic mural cell recruitment compared to control tissues from patients without pulmonary fibrosis (Meinecke et al., 2012). Together, this evidence suggests that lymphatic structural and functional changes may contribute to the pathophysiology of pulmonary fibrosis.

The isolation and characterization of lung lymphatics is key to understanding of their role in physiology and disease - particularly significant in the context of new evidence from a mouse model of neonatal pulmonary disease, suggesting that the lymphatic system is integral to the later stages of lung development, and that lymphatic dysfunction might be a contributing factor in the pathophysiology of pulmonary disease of prematurity (Jakus et al., 2014). Mice deficient in the lymphangiogenic factor CCBE1 (Alders et al., 2009) reached term gestation, however while heterozygous CCBE1+/− mice were able to oxygenate their lungs, appeared pink and remained so for over 1 hour after birth, CCBE−/− mice were cyanotic and died within 3 hours of birth, despite an intact diaphragm and apparently normal respiratory drive and gasping mechanisms (Jakus et al., 2014). The same patterns were observed in a mouse model of absent pulmonary lymphatic vasculature resulting from a mutation in the kinase domain of the lymphatic growth factor receptor VEGFR3, resulting in neonatal cyanosis and respiratory failure in VEGFR3kd/kd mice (Jakus et al., 2014). This failure of lung inflation could not be attributed to surfactant deficiency, since mice deficient in pulmonary lymphatic vasculature appeared to have normal levels of surfactant precursors and components, and normal type I and II alveolar cells on transmission electron microscopy. Instead, the lungs of neonatal CCBE−/− and VEGFR3kd/kd mice reportedly had increased alveolar interstitial thickness, reduced alveolar area and increased wet/dry ratios, in keeping with pulmonary oedema (Jakus et al., 2014).

Congenital pulmonary lymphangectasia is a rare developmental disorder characterized by abnormal dilatation of the pulmonary lymphatic vasculature in the subpleural and interlobular spaces (Bellini et al., 2003; Bellini et al., 2006). The condition is characterized by pleural effusions and pulmonary hypoplasia resulting in respiratory distress, and is usually fatal in the neonatal period (Ferreira et al., 2015). It is conceivable that defects in pulmonary lymphatic development and associated neonatal respiratory failure might account for the clinical manifestations of congenital pulmonary lymphangectasia.
in affected infants, as described by Jakus et al in lymphatic deficient mice (Jakus et al., 2014). Mice with absent pulmonary lymphatic vasculature died from respiratory failure shortly after birth. However it is important to appreciate that pulmonary expansion and establishment of respiratory function are extremely time-critical processes in neonatal adaptation, and thus pulmonary insufficiency is likely to have masked additional, perhaps fatal, organ-specific deficiencies in mice with absent lymphatic vasculature.

Other studies in mice have focused on the intestinal lymphatic system, showing that the intestinal lymphatics develop later than the blood vascular network, and are thought to originate from the intestinal mesentery (Kim et al., 2007). Mouse embryos were harvested from the amniotic cavities of pregnant mice at various embryonic stages from day 13.5 to 17.5, fixed and processed for whole mount immunostaining, or serial sections, using primary antibodies against the lymphatic markers LYVE-1, VEGFR3, Prox1 and podoplanin, and the pan-endothelial marker CD31, and imaging performed by confocal microscopy (Kim et al., 2007). While few lymphatic structures were identified in the intestinal tubes at day 13.5 or 14.5, well-developed CD31-positive blood vascular networks were visible through the intestinal wall. At the same stage, lymphatics were detected within the intestinal mesentery. By day 16.5, however, multiple LYVE1+ and VEGFR3+ lymphatics were seen branching into the intestinal wall where they formed organized lymphatic networks by day 19.5 (Kim et al., 2007), findings which suggest that, unlike the intestinal blood vascular network, intestinal lymphatics might be derived from the mesentery rather than intestinal mesoderm (Kim et al., 2007). Taking this concept further, it is plausible that the differing origins of intestinal lymphatic and blood vascular endothelial cells might account, at least in part, for the functional differences observed between the two vessel types, for example in their sensitivity to radiation injury, as previously described in chapter 3 (Paris et al., 2001, Sung et al., 2006).

The vital role of the intestinal lymphatic network in normal gut physiology is highlighted by the condition primary intestinal lymphangieactasia, or “Waldmann’s disease”, a rare congenital disorder described by Waldmann et al in 1961, characterized by dilated mucosal and submucosal lymphatics and peripheral oedema secondary to hypoproteinaemia (as cited in Vignes & Bellanger, 2008). The condition usually manifests by the age of three years in children, with a symmetrical pitting oedema of the
lower limbs and can be complicated by weight loss, GI upset, malabsorption and failure to thrive (Vignes & Bellanger, 2008). Enteroscopy and intestinal mucosal biopsy demonstrate lymphangiectasia, and yellow discoloration of the villi resulting from abnormally dilated lymphatics (Vignes & Bellanger, 2008). Studies on duodenal biopsies from patients with idiopathic intestinal lymphangiectasia have reported alterations in the expression profile of lymphangiogenic growth factors within the affected duodenal mucosal lesions (Hokari et al., 2008). They demonstrated increased expression of the lymphatic markers LYVE-1 and VEGFR3 in mucosal regions where pathological lymphatic dilatation was observed. In contrast, expression of the lymphangiogenic growth factors VEGF-C and VEGF-D appeared to be down-regulated, along with the transcription factors SOX18 and FOXC2, key regulators of lymphatic development (Hokari et al., 2008; Francois et al., 2008; Fang et al., 2000). Although the pathophysiological processes are not fully understood, it is clear from this example that a lymphatic disorder of the developing gut can result in significant functional impairment.

The central role of the lymphatic system in the pathophysiology of a range of intestinal disorders is increasingly acknowledged, particularly in the context of inflammatory bowel disease. In a rat model of trinitrobenzenesulphonic acid (TNBS) colitis, a chemically-induced colitis was generated by the administration of TNBS per rectum, and open cannulation of a draining mesenteric lymphatic enabled sampling of intestinal lymph and analysis of lymphatic diameter and flow (Cromer et al., 2015). Lymphatic flow in the TNBS-treated ileum was reportedly reduced by 40% at 24 hours, falling to 50% of the baseline at 48 hours, and these changes in lymphatic transport appeared to precede a statistically significant deterioration in histopathological scores, according to degree of epithelial and crypt loss, cellularity, vascularity, and tissue oedema, which began to deteriorate from the 48 hour time point (Cromer et al., 2015). Furthermore, increases in the number of MHC class II cells along the lymphatic vasculature were detected on mesenteric whole mounts at the 6 hour time point, with a greater that two-fold rise in the number of these immune cells (Cromer et al., 2015). This suggests that changes in the lymphatic microenvironment and associated lymphatic dysfunction might occur prior to the development of significant intestinal inflammation in inflammatory bowel disorders, and it is therefore possible that lymphatic dysfunction might contribute to the development of inflammation and tissue injury in the gut (Cromer et al., 2015).
Impaired intestinal lymphatic transport in FoxC2<sup>+/−</sup> mice, along with evidence of mesenteric and intestinal wall lymphatic valvular incompetence and reflux have been reported previously (Kriedermann et al., 2003). In humans, FoxC2 mutations reportedly cause lymphedema-distichiasis, a variable onset lymphedema of the limbs associated with a double row of eyelashes (Fang et al., 2000). The proposed central role of lymphatic dysfunction in the pathophysiology of inflammatory bowel disease is further supported by studies in a murine model of FoxC2 haploinsufficiency (Becker et al., 2015). Mice exposed to the chemical dextran sodium sulfate (DSS) developed histological signs of colitis including epithelial and crypt injury, inflammation and oedema of the submucosa. However mice heterozygous for the FoxC2 inactivating mutation developed more severe histopathological changes and clinical disease activity scores compared to DSS-treated wild type controls (Becker et al., 2015). This was associated with increased levels of inflammatory infiltrates containing neutrophils. T-cells and macrophages on immunostaining of colonic specimens (Becker et al., 2015). Although FoxC2 haploinsufficiency was not associated with gross changes in colonic lymphatic vessel size or morphology, DSS-induced colitis provoked an increase in mean lymphatic vessel area, which was significantly larger in the FoxC2<sup>+/−</sup> mice compared to wild type DSS-treated mice, along with the proportion of lymphatic vessels defined as substantially dilated (Becker et al., 2015). Taken together the data suggests that an underlying intestinal lymphatic defect characterized by lymphostasis substantially increases the susceptibility to inflammatory bowel injury associated with acute colitis (Becker et al., 2015).

Studies on human colon specimens from patients with active Crohns disease and ulcerative colitis, stained with antibodies against the lymphatic marker podoplanin have demonstrated multiple dilated lymphatics within the lamina propria and submucosa, supporting the idea that enhanced lymphangiogenesis occurs in inflammatory bowel disorders (D’Alessio et al., 2014). Other studies in humans have demonstrated evidence of lymphatic vessel wall invasion by B-cells and innate immune cells in Crohns disease, accompanied by the gathering of structures resembling tertiary lymphoid organs along the peri-nodal collecting lymphatics of the intestinal mesentery, in a manner liable to impinge on intestinal lymphatic flow (Randolph et al., 2016). In keeping with the concept of impaired lymphatic function in inflammatory bowel disorders, evidence suggests that the administration of the
lymphangiogenic growth factor VEGF-C could offer therapeutic benefit (D’Alessio et al., 2014). In human colon specimens from patients with Crohn’s disease and ulcerative colitis, VEGF-C expression is reportedly upregulated in the intestinal mucosa, along with increased VEGFR3 expression by the intestinal lymphatic vasculature (D’Alessio et al., 2014). In two separate mouse models of chronic colitis, systemic administration of an adenovirus encoding VEGF-C caused a reduction in the extent of established inflammation according to endoscopy findings and disease activity scores, along with increases in intestinal lymphatic caliber and vessel density (D’Alessio et al., 2014). Conversely, blockade of the VEGFR3 signalling cascade by administration of an anti-VEGFR3 antibody resulted in adverse clinical outcomes in these mice, and was associated with reduced lymphatic uptake of blue dye injected into the affected intestinal mucosa, indicating impaired colonic lymphatic clearance when VEGFR3 signalling is inhibited (D’Alessio et al., 2014). These data highlight the potential for therapeutic targeting of the VEGF-C/VEGFR3 lymphangiogenic signalling pathway in inflammatory bowel disease (D’Alessio et al., 2015).

Despite a historical consensus on the absence of lymphatics within the central nervous system, studies in mice have recently revealed a network of central nervous system lymphatic vessels (Louveau et al., 2015). In a study originally aimed at analysing pathways of immune cell trafficking in the central nervous system, murine meninges were carefully dissected from the overlying skull vault and immunostaining performed for antibodies against endothelial cells (anti-CD31), T-cells (anti-CD3e) and MHC class II cells (anti-MHC II) (Louveau et al., 2015). The authors discovered that most T cells appeared to lie outside the dural sinuses, and unexpectedly, that T-cells and MHC class II-positive cells seemed to be oriented linearly, along the course of CD31-positive vessels, adjacent to the dural sinuses (Louveau et al., 2015). Further analysis of these CD31-positive vascular structures by immunostaining for the lymphatic endothelial marker LYVE-1 in coronal sections of murine meninges, demonstrated positive LYVE-1 expression, while perfusion of mice with an intravenous lectin dye prior to meningeal harvest revealed a lack of dye uptake by the LYVE-1-positive vasculature, indicating a lymphatic network separate to the blood vasculature (Louveau et al., 2015). These meningeal lymphatics appeared to be responsive to the lymphangiogenic growth factor VEGF-C, with injection of recombinant VEGFR3-targeted VEGF into the murine subarachniod space resulting in vessel dilatation.
(Louveau et al., 2015). The meningeal lymphatics appeared to lack a smooth muscle layer in mice, according to α-SMA and LYVE-1 staining in meningeal specimens (Louveau et al., 2015), indicating that these meningeal lymphatics are likely to represent the initial or pre-collecting subtype, akin to lymphatics located within the dermis (Shayan et al., 2006).

To isolate meningeal lymphatic endothelial cells, the same group performed flow cytometry on a single cell suspension of the mouse meninges. Following staining with antibodies against CD45, CD31 and podoplanin they were able to successfully isolate a population of live CD45-negative CD31-positive podoplanin-positive cells, comprising less than 0.1% of all live, CD45-negative cells (Louveau et al., 2015). Unlike our study, they did not report on the appearance of these cells, or their functional characteristics in culture. It is certainly worth optimising these techniques for lymphatic endothelial cell isolation, as the central nervous system lymphatic network is currently unexplored territory, and is likely to play an important role in normal physiology, as well as pathological disorders of the central nervous system. Given the relatively well-established link between inflammation and lymphatic dysfunction, for example, in the context of inflammatory bowel disease (D’Alessio et al., 2014), it is conceivable that the central nervous system lymphatics might be involved in the pathophysiology of a range of infectious and inflammatory disorders such as meningitis and encephalitis. The lymphatic system plays an important role in immune cell trafficking - we already know that the meningeal lymphatics described by Louveau et al contained T-calls and MHC class II immune cells (Louveau et al., 2015). Given the established role of the lymphatic system in tumour spread, the meningeal lymphatics could also be implicated in central nervous system tumour growth and metastasis. Clearly there is a lot more work to be done in exploring the lymphatic network of the central nervous system.
Chapter 5:

INHERITANCE PATTERNS AND GENETIC FACTORS
IN LIPOEDEMA
Lipoedema is a progressive disorder of abnormal adipose tissue deposition associated with painful fat and a variable degree of lymphatic insufficiency (Buck & Herbst 2012; Harvey et al., 2008). The condition affects females in the majority of instances, and while little information on disease inheritance is known, it has been suggested that the disease follows an autosomal dominant pattern, with variable expression of the phenotype, particularly in males (Child et al., 2010). Beyond this, little is understood regarding the pathophysiology of lipoedema, the link between the abnormal adipose tissue and a dysfunctional lymphatic system, and the causative gene mutations (Child et al., 2010). There is a growing body of evidence supporting the concept that lymphatic dysfunction and leakage of lymph promotes the accumulation of fat, and this has been demonstrated in a murine model of Prox1 haploinsufficiency resulting in a primary lymphatic defect (Harvey et al., 2005). However the sequence of events promoting pathological adipose deposition and lymphatic dysfunction in the context of lipoedema is unclear (Harvey et al., 2008). To our knowledge a candidate gene mutation has not yet been reported in lipoedema (Child et al., 2010, OMIM number 614103).

**Hypothesis 3**

People with lipoedema have an inherited genetic predisposition, with altered expression of genes involved in adipose tissue metabolism, and lymphatic growth and regulation.

**Aims for hypothesis 3**

To perform genetic sequencing in patients with lipoedema and lipo-lymphedema, and their relatives
5.1 Familial inheritance patterns in lipoedema and/or lipo-lymphedema:

Nine patients with a clinical diagnosis of lipoedema or lipo-lymphedema consented to participate in the study, and of these, eight were able to provide details of their family tree, including family history of lipoedema and/or lymphedema (Figure 5.1). All families were Caucasian, without any history of consanguinity.

*Figure 5.1*

Family pedigrees for eight patients with lipoedema or lipo-lymphedema, describing the familial distribution of the disease.

Two of these eight affected patients had another living relative with a clinical diagnosis of lipoedema – affected twin sisters in case 2, and a mother with lipo-lymphedema and her affected 17 year-old son in case 8 (Figure 5.1). In addition to this, based on observations of the lipoedema phenotype and/or symptoms of lipoedema reported by family members, the six other affected patients (case 3, 4, 6, 7, 9 and 10) all potentially had a positive family history of the disease (Fig-
Unfortunately several of these "possibly-affected" family members were deceased, or not in regular contact, or living outside the state of Victoria, Australia, thereby precluding verification of these observations by formal clinical assessment and diagnosis.

Four families reported a possible family history of lipoedema on the paternal side – case 2, 4, 6 and 7 (Figure 5.1), with the paternal grandmother and/or great-grandmother identified as a previously-affected family member. This was based on family photographs, and/or personal observations of the characteristic body fat distribution of lipoedema, and symptom of "heavy legs". Alternatively, in case 9, a potentially-affected great-grandmother was reported on the maternal side, while in case 10 there were possibly-affected aunts on the maternal side.

On further analysis and comparison with reports in the literature (Child et al., 2010), the inheritance patterns in our eight family pedigrees appeared consistent with an autosomal dominant Mendelian condition, with variable expression of the phenotype. The lack of phenotype expression, particularly in males, potentially accounts for the condition appearing to "skip" certain generations, for example in case 4 and case 7 (Figure 5.1).
5.2 Clinical data on families with lipoedema and/or lipo-lymphedema selected for exome sequencing:

Of the eight patients providing detailed information on their family history, five affected individuals along with at least first-degree relatives, so-called “family trios”, provided a peripheral venous blood sample for the purpose of DNA sequencing (Table 5.1).
Table 5.1: Details of family “trios” providing genomic DNA for exome sequencing

<table>
<thead>
<tr>
<th>Family</th>
<th>Notes</th>
</tr>
</thead>
</table>
| **Case 2** | U1-Mother U2-Father  
A1-Daughter A2-Daughter  
| A are twins |
| **Case 6** | U3-Mother  
A1- Daughter U2-Son  
U1-Granddaughter  
| Lipoedema and lymphedema |
| **Case 8** | U2-Mother U3-Father  
A1-Daughter  
| |
| **Case 9** | U1-mother U2-father  
A1-daughter  
| |
| **Case 10** | U2-Father  
A1-daughter U1-daughter/sister  
| |

The first of these families, case 2, comprised 36 year old identical twin sisters, both with a clinical diagnosis of mild-moderate lipoedema, and their unaffected mother and father (Figure 5.1 Case 2, Figure 5.2A). The twins reported an onset of painful, heavy legs around the time of puberty at age 14 - information confirmed by their mother and father. Their legs grew in size disproportionally from this age onward, with pain and heaviness limiting their participation in sport at school. Neither the mother nor the father has a clinical history or phenotype consistent with the disease. However the father reported that the twins’ great-grandmother on their paternal side had large, heavy legs consistent with lipoedema.
Figure 5.2

Clinical images of patients with lipoedema. A: Case 2. Affected 36 year old female twins with mild-moderate lipoedema of the lower limbs. B: Case 8. Affected 46 year old female with severe lipo-lymphedema of the upper and lower limbs. Showing the clinical features of severe lower limb lipoedema, including abnormal folds of fat associated with lymphedema, and the relative sparing of the knees and feet.

The second family “trio”, Case 6, comprised a 73 year old female with both lipoedema of the legs, and secondary lymphedema of the upper limb (Figure 5.1 Case 6). Her unaffected mother, brother and daughter also consented to participate in the study and provided peripheral venous blood samples for exome sequencing. The patient gave a history of onset of lipoedema of the legs at puberty, age 14. In addition to this, she also developed secondary lymphedema of the left arm later in life, at age 54 following a left-sided mastectomy and axillary lymph node clearance for breast cancer. Of note, this patient also has a history of spontaneous chylothorax, possibly indicating a generalised, predisposing defect in the lymphatic system. While both parents were unaffected, the patient gave a history of her paternal great-grandmother having a lower limb fat distribution consistent with lipoedema.

The third family trio, case 8, comprised a 46 year old female patient with severe lipo-lymphedema of the upper and lower limbs, and her unaffected mother and father (Figure 5.1 Case 8, Figure 5.2B). She reported a transient history of lower limb lymphedema during pregnancy at age 26, followed by the
onset of lipo-lymphedema of the upper and lower limbs at age 37. Both parents were unaffected, and there was no history of lipoedema or lymphedema in past generations. However her 17 year old son has mild lipoedema of the legs, confirmed on examination by an experienced clinician.

The fourth trio, case 9, was composed of a 37 year old female with lipo-lymphedema of the legs, her mother and unaffected father (Figure 5.1 Case 9). She gave a history of onset of lipoedema at around the time of puberty, at the age of 16. This progressed in severity and was accompanied by lower limb lymphedema at the age of 31. Although neither of her parents has lipoedema, her mother, age 62, gave a history of mild, clinically-diagnosed, idiopathic lower limb lymphedema affecting her throughout adult life (Figure 5.1 Case 9). There was also a history of a possible diagnosis of lipoedema in the maternal greatgrandmother, according to observations made by family members. The offspring of the affected 37 year old patient were only 2 and 5 years of age at the time of the study, well below the age of puberty and thus making it difficult to comment on the likelihood of the condition affecting subsequent generations of that family at present.

The fifth “trio” comprised a 42 year old female with a history of lipoedema (Figure 5.1 Case 10) and her unaffected sister and father. In her case, lower limb lipoedema began at puberty at age 13. Her mother was deceased at the time of the study, precluding exome sequencing in both parents. She reported a possible history of lipoedema in her maternal aunts, albeit not confirmed by clinical assessment or diagnosis.
5.3 Genomic DNA quality assessment:

Following genomic DNA extraction as described, DNA concentration and purity (A\textsubscript{260}/A\textsubscript{280} ratio) were assessed by nanodrop fluorospectrometry (Table 5.2). DNA concentrations in excess of 100 ng/μl were achieved in all samples, and A\textsubscript{260}/A\textsubscript{280} ratios of 1.80 – 1.89 were recorded, well within the accepted range of 1.80-2.0 for genomic DNA sequencing (Table 5.2). Gel electrophoresis demonstrated good quality gDNA in all samples, with high molecular weight >20Kb and minimal “smearing” of the DNA band (Figure 5.3).

Table 5.2: Assessment of DNA concentration and purity by nanodrop fluorospectrometry.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Concentration (ng/μl)</th>
<th>A260</th>
<th>A280</th>
<th>A260/280 ratio</th>
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<tr>
<td>2 A1</td>
<td>510.7</td>
<td>10.2</td>
<td>5.62</td>
<td>1.82</td>
</tr>
<tr>
<td>2 A2</td>
<td>356.8</td>
<td>7.14</td>
<td>3.92</td>
<td>1.82</td>
</tr>
<tr>
<td>2 U1</td>
<td>408.6</td>
<td>8.17</td>
<td>4.46</td>
<td>1.83</td>
</tr>
<tr>
<td>2 U2</td>
<td>265.0</td>
<td>5.30</td>
<td>2.92</td>
<td>1.81</td>
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<tr>
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<td>767.4</td>
<td>15.3</td>
<td>8.39</td>
<td>1.83</td>
</tr>
<tr>
<td>6 U1</td>
<td>581.6</td>
<td>11.6</td>
<td>6.42</td>
<td>1.81</td>
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</table>
Figure 5.3

Gel electrophoresis of genomic DNA samples from patients and relatives submitted for exome sequencing, demonstrating the integrity and approximate molecular weight of the DNA. From left to right: **A:** high range DNA reference ladder (M1), gDNA from case 2A1, 2A2, 2U1, 2U2, case 6A1, 6U1, 6U2, 6U3, case 10A1, 10U1 and 10U2, and a second reference ladder (M2). **B:** high range DNA reference ladder (M1), gDNA from case 9A1, 9U1, 9U2, case 8A1, 8U2 and 8U3, and a second reference ladder (M2).
5.4 Exome sequencing:

Exome sequencing was performed on genomic DNA specimens from five lipoedema family trios, as described. Variants were matched to genes associated with eight selected HPO terms (Table 2.1) and three other genes known to be associated with lipodystrophy or overgrowth: Lamin A/C encoding gene (LMNA), peroxisome proliferator-activated receptor gamma (PPAR-γ) (Nolis 2014), and the nuclear receptor binding SET domain protein 1 (NSD1) (Douglas et al., 2003). We focused on variants that fulfilled an autosomal dominant inheritance model.

Of the three known genes associated with lipoedema, PPAR-γ, NSD1, and LMNA, no variants were identified for PPAR-γ and NSD1 in the five families sequenced. However in the affected individual in Case 6, with a history of lipoedema and secondary lymphedema, a homozygous GGA to G mutation in the LMNA gene on chromosome 1 was identified with a quality score of 67.5 (Table 5.3). At this location, six gene isoforms were found, all with a modifier impact on gene function (Table 5.3).
Table 5.3: Known genes – data on the LMNA mutation identified in Case 6, only found in the family member with lipoedema.

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<th>Ref</th>
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<th>Quality</th>
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<th>Consequence</th>
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<th>Feature type</th>
<th>NCBI feature</th>
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<td>Transcript</td>
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<td>MODIFIER</td>
<td>Transcript</td>
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<td>Protein coding</td>
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<td>Transcript</td>
<td>NR_047545.1</td>
<td>Misc RNA</td>
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</table>

Genotype: 1/1 = homozygous for variant allele
Further analysis of the identified variants was performed on a subset overlapping with the eight HPO terms (Table 2.1), within exonic regions only. These variants were ranked by impact on gene function according to Ensembl (Table 2.2) from highest to lowest.

There were no such overlapping hits for the individuals in Case 2. In the affected family member in Case 6, 381 of these variants were identified (unaffected family members had the reference sequence). The affected individual was heterozygous in all cases. There was relative agreement between both SIFT and Polyphen on the impact of the top 16 variants, which were classified as deleterious and probably damaging, respectively, and are listed in Table 5.4.

In the affected family member of Case 8, 33 exonic variants matched the eight HPO terms in Table 2.1. Unaffected family members had the reference sequence. The affected individual was heterozygous in all cases. The impact on gene function of the top 11 variants was classified moderate, while the rest were low or modifier (Table 5.5). Only the top two variants were also described as deleterious and probably or possibly damaging by SIFT and PolyPhen, respectively. Both were isoforms of a G for A mutation in the PRPF3 gene on chromosome 1 (Table 5.5).

Filtering of exonic variants matching the eight HPO terms previously described produced a list of 31 variants in the affected family member of Case 9 (Table 5.6). Unaffected family members had the reference sequence. The affected individual was heterozygous in all cases. Of these, the top hit was a T for A, stop gained mutation with a high impact on gene function, in the HS6ST1 gene on chromosome 2 (Table 5.6). The impact on gene function of the remaining 30 variants was classified as modifier, the top 15 of which have been included in Table 5.6.

Finally, in Case 10, filtering for exonic variants and overlap with the HPO terms described revealed 559 variants in the affected family member. Unaffected family members had the reference sequence. The affected individual was heterozygous in all cases. The top 33 with a moderate or high impact are listed in Table 5.7. The top 4 are isoforms of an A for G mutation in the BBS4 gene on chromosome
15, two resulting in a splice acceptor variant in a protein-coding region of the gene, while the other two are associated with a non-coding transcript variant (Table 5.7).
### Table 5.4: Case 6 top 16 candidate variants filtered by exonic region and overlap with HPO terms

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<th>Location</th>
<th>Gene</th>
<th>Ref</th>
<th>Alt</th>
<th>Quality</th>
<th>Genotype</th>
<th>Consequence</th>
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<th>SIFT</th>
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Table 5.5: Case 8 top 11 candidate variants filtered by exonic region and overlap with HPO terms

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Table 5.6: Case 9 top 16 candidate variants filtered by exonic region and overlap with HPO terms

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5.5 Discussion:

Lipoedema is an under-recognised, poorly understood condition of abnormal adipose tissue metabolism resulting in obesity and functional impairment. The condition, most commonly affecting females, is characterized by the bilateral, symmetrical accumulation of fat, typically affecting the lower limbs and sparing the trunk and feet, although the upper limbs may be involved in approximately 30% of cases (Buck & Herbst 2012). Wold devised a set of diagnostic criteria in 1957, which were later modified by Herbst in 2012, and include pain and easy bruising in affected regions, and resistance to dieting and weight loss (as cited in Buck & Herbst, 2012). We present clinical data on inheritance patterns in eight affected families and the preliminary results of exome sequencing in five affected family “trios”. Our data supports the findings of a previously published study on lipoedema family pedigrees (Child et al., 2010), suggesting that the condition might be autosomal dominant with variable expression of the phenotype, particularly in males.

The genetic basis of lipoedema is unknown, with few studies focused on establishing the pattern of inheritance and the underlying gene mutations. In 2010, Child et al reported on a series of 67 patients with lipoedema and their families, determining that ten of the 67 patients had at least one first-degree relative with a diagnosis of lipoedema, thus indicating a 15% familial incidence of the disease (Child et al., 2010). In one family, there were six living members with a diagnosis of lipoedema, spanning three generations – compelling evidence for an inherited predisposition to developing the disorder (Child et al., 2010). All patients and their affected family members were female, and over half gave a history of disease onset coinciding with puberty, in keeping with previous descriptions of the disease.

To explore the possibility of an X-linked dominant inheritance pattern, the group performed linkage analysis using X chromosome markers in the family with six affected members in three generations. Negative lod scores under -2 were returned for all X chromosome markers, indicating that linkage was unlikely (Child et al., 2010). In light of this, and on the basis of the inheritance
patterns observed in the ten families studied, the authors concluded that an autosomal dominant inheritance was more likely in lipoedema (Child et al., 2010). They did not however report on DNA sequencing in affected families, which would be the next step in determining the genetic basis of the condition.

Dercum’s disease is a closely-related disorder also characterized by painful fat, and may be associated with lipomatisis, (Hansson et al., 2012). Although Dercum’s disease most commonly occurs sporadically, reports on inheritance patterns in affected families are suggestive of an autosomal dominant condition with variable, gender-specific expression, such that more severe symptoms occur in affected females (Hansson et al., 2012). In a small study of three females with a clinical diagnosis of Dercum’s disease and four healthy controls, near infra-red lymphatic imaging was used to assess lymphatic structure and function. Lymphatics were visualized by intradermal injection of indocyanine green (ICG) dye at the affected sites, while equivalent regions of the body were studied in controls (Rasmussen et al., 2014). Overt evidence of lymphatic incompetence, such as dermal reflux of lymph, was not observed in patients with Dercum’s disease. However more subtle abnormalities of lymphatic structure and function were noted. These included abnormal initial lymphatic networks in and around the ICG injection sites, and dilated and tortuous collecting lymphatics with impaired flow (Rasmussen et al., 2014). Although it is difficult to differentiate between cause and effect in observational studies, these findings in Dercums disease are consistent with an association between abnormal adipose metabolism and lymphatic dysfunction.

The lipodystrophies are a group of disorders characterized by abnormal adipose tissue metabolism, resulting in fat loss, or alternatively in some cases increased deposition of subcutaneous fat, and may be subclassified into general, partial or local disorders according to their distribution and severity (Nolis 2014). The generalized and partial conditions may be further divided into inherited or acquired. For example familial partial lipodystrophy (FPL) is an acquired disorder linked to mutations in a range of genes including (PPAR-Y), a key regulator of adipocyte differentiation, and LMNA (Nolis 2014). In contrast to lipoedema, FPL is characterised by the loss of adipose tissue from the limbs, and also from the trunk, but like lipoedema the onset tends to occur at
the time of puberty, and the disordered is reportedly more readily recognised and diagnosed in females (Garg & Agarwal, 2009). Although the clinical manifestations of FPL clearly differ from the lipoedema phenotype, certain parallels can clearly be drawn between these two conditions.

Lymphatic dysfunction appears to be strongly linked to the clinical picture and/or pathophysiology of lipoedema, with the most advanced clinical stage of lipoedema being defined by the development of non-pitting limb oedema, or "lipo-lymphedema" (Buck and Herbst, 2016). Furthermore structural abnormalities of the lower limb lymphatics in lipoedema have also been reported (Goodliffe et al., 2013). The development of a mouse model of Prox1 haploinsufficiency has been critical in advancing our understanding of the complex interplay between obesity and lymphatic dysfunction (Wigle et al., 1999, Harvey et al., 2005). As previously described, the homebox gene Prox1 is expressed by a subgroup of cardinal vein endothelial cells, under the control of the transcription factor SOX18 - an early indicator of lymphatic bias at embryonic day 9 (Francois et al., 2008), which is followed by lymphatic differentiation (Wigle et al., 2002; Wigle & Oliver 1999). Prox1 expression is integral to normal lymphatic development and viable embryogenesis, with Prox1 null mice developing fatal oedema in utero, at around embryonic day 14.5 (Wigle et al., 1999), and even Prox1+/- mice appear to die at postnatal day 2-3 in the majority of genetic backgrounds tested (Wigle et al., 1999).

Unlike the majority of their counterparts lacking one copy of the Prox1 allele, Prox1 haploinsufficient mice bred on NMRI background appear to survive into adulthood, facilitating studies on the longer-term consequences of Prox1 insufficiency (Wigle et al., 1999, Harvey et al., 2005). Lymphatic function in the Prox1+/- mouse was assessed by the injection of Evans blue dye into the footpads, then tracking its uptake and transport through the hierarchy of draining lymph nodes to the thoracic duct by microscopy performed at 1-2 hours (Harvey et al., 2005). In addition, intestinal lymphatic integrity and function were assessed by the enteral administration of a fluorescent lipid, and tracing its subsequent uptake and lymphatic transport by fluorescence microscopy (Harvey et al., 2005). In the Prox1+/- mice that died in the post-natal period, abnormalities in the lymphatic vasculature were most marked within the intestine and mesentery, with evidence of chyle leak and lipid deposition within the gut wall (Harvey et al.,
However, the peripheral lymphatic vasculature was also disrupted, with impaired delivery of the Evans blue dye to the thoracic duct, and evidence of lymphatic flow occurring via aberrant pathways comprising cutaneous lymphatics or indirect networks of small and morphologically abnormal lymphatic vessels, rather than via the subcutaneous collecting lymphatics (Harvey et al., 2005). Mice surviving into adulthood developed adult-onset obesity from approximately 9 weeks of age, and subsequently circulating insulin and leptin levels became elevated compared to their wild-type littermates, hallmarks of obesity not detected in the pre-obese Prox1+/- mice at 4-8 weeks of age (Harvey et al., 2005). Importantly, it has been demonstrated that the specific deletion of Prox1 from only the lymphatic endothelial progenitor cells of the cardinal vein produces a similar phenotype with structural and functional abnormalities of the lymphatic vasculature and adult-onset obesity, suggesting that obesity might be a direct consequence of lymphatic leakage in the Prox1 deficient mouse (Harvey et al., 2005).

In a murine model of diet-induced obesity in wild-type, B- and T-cell immune deficient mice, obesity was reportedly associated with alterations in lymphatic and lymph node architecture and function, along with specific changes in immune cell content and trafficking (Weitman et al., 2013). Both wild type and immune deficient mice were given a high fat diet for 8-10 weeks and compared to mice from the same lines fed on a normal chow diet (Weitman et al., 2013). The lymphatic architecture was visualized by microlymphangiography, and FITC-conjugated dextran injections utilised to assess lymphatic transport in the tail (Weitman et al., 2013). Furthermore, dendritic cell migration assays were performed with hindpaw injections of GFP-positive dendritic cells and uptake assessed by flow cytometry of the harvested popliteal lymph nodes. Meanwhile, baseline dendritic cell migration was quantified by a FITC painting assay and flow cytometry of the draining nodes (Weitman et al., 2013). Lymphoscintigraphy was also used to assess lymphatic function in the hindlimb. The authors discovered that obesity was associated with a mild inflammatory response, with increased numbers of CD45-positive cells in the subcutaneous tissues of the affected regions in wild type mice (Weitman et al., 2013). Both lymphatic uptake and transport appeared to be impaired in obese mice, associated with abnormal dilatation of LYVE-1 positive lymphatic vessels on immunostaining (Weitman et al.,
Dendritic cell migration was also significantly impaired in obese wild type mice, with a statistically significant negative correlation between body mass and number of dendritic cells reaching the draining popliteal nodes in the hind limbs of obese wild type mice (Weitman et al., 2013). In contrast, only a mild reduction in dendritic cell migration was observed in the immune deficient mice, which did not reach statistical significance, and there was no apparent correlation with body mass, suggesting that B- and T-cell responses to obesity might play a role in the adverse effect of obesity on dendritic cell migration (Weitman et al., 2013). Additionally, the impact of obesity on lymph node size and structure was affected in the B- and T-cell deficient mice. Obesity in the wild type mice was associated with a reduction in inguinal lymph node size, reduced numbers of LYVE-1 positive peri-nodal lymphatics, and disrupted lymph node follicle organization and immune cell distribution. In contrast, obesity in the B- and T-cell deficient mice resulted in an increase in lymph node size and no change in the number of peri-nodal lymphatics (Weitman et al., 2013). Taken together these findings suggest that B- and T-cell mediated inflammatory responses to obesity are likely to play an important role in bringing about the deleterious effects of obesity on lymphatic and lymph node structure and function (Weitman et al., 2013), perhaps explaining the link between abnormal adipose tissue metabolism and lymphatic dysfunction in the late stages of lipoedema.

Taking this concept further, studies in sR3 transgenic mice have demonstrated that the lymphangiogenic growth factors VEGF-C and –D play a critical role in obesity-associated adipose tissue inflammation (Karaman et al., 2014). These sR3 mice constitutively express a VEGFR3 immunoglobulin in the epidermis, which binds to, and thus depletes systemic VEGF-C and –D (Makinen et al., 2001). Wild type and sR3 mice were fed a high fat diet for 20 weeks, and weight gain was reportedly similar in both types. In contrast, insulin sensitivity was increased in the sR3 mice, with lower fasting insulin measurements and lower insulin resistance index scores compared to wild type mice (Karaman et al., 2014). Obesity-associated hepatic steatosis was much less pronounced in the sR3 mice, while in wild type mice marked hepatic lipid accumulation was associated with a reduction in AKT phosphorylation levels on insulin stimulation - a marker of hepatic steatosis-related insulin resistance in wild type but not sR3 mice (Karaman et al., 2014). Flow cytometric analysis of macrophage subtypes within the stromal vascular fraction of subcutane-
ous fat demonstrated higher M2:M1 macrophage ratios in sR3 mice, both on normal chow and high fat diets, compared to wild type controls, suggestive of a less inflammatory milieu within the adipose tissue of sR3 mice (Lumeng et al., 2007, Karaman et al., 2014). Expression of the lymphangiogenic growth factors VEGF-C and VEGF-D appeared to be upregulated in the subcutaneous adipocytes of wild type mice after 20 weeks of high fat diet compared to chow-fed wild type controls, and furthermore macrophage migration studies demonstrated the ability to VEGF-C and VEGF-D to induce macrophage chemotaxis in vitro - a VEGFR3-dependent process which appeared to be significantly impaired in the presence of a monoclonal antibody against the VEGFR3 (Karaman et al., 2014). The same group also demonstrated that in vivo VEGFR3 blockade, achieved by the systemic administration of a VEGFR3-blocking antibody, was associated with a lesser degree of macrophage accumulation within the subcutaneous adipose tissue, and more favourable insulin sensitivity profiles in mice (Karaman et al., 2014). Overall this study suggests that VEGF-C and -D lymphangiogenic signaling pathways might provide a future therapeutic target in addressing obesity-related metabolic syndrome, and given the link between the lymphatic system and lipoedema, this information may also be relevant to the management of lipoedema.

By performing exome sequencing on genomic DNA samples from five affected lipoedema families, we have generated candidate gene mutations, which could potentially play a causative role in the pathophysiology of lipoedema. Of the exonic variants identified across five families, filtered according to eight HPO terms (Table 2.1), the four isoforms of an A for G mutation in the BBS4 gene on chromosome 15 were associated with a highly deleterious impact on gene function (Table 5.7). These were identified in the affected individual in Case 10, who was heterozygous for the variant. Unaffected family members had the reference sequence. Bardet Biedl Syndrome (BBS) is an autosomal recessive ciliopathic disorder characterized by rod-cone dystrophy resulting in blindness, cognitive impairment, genitourinary and renal abnormalities, obesity, a predisposition to the metabolic syndrome and insulin resistance (Forsythe & Beales 2003, Nahum et al., 2017, Gerdes et al., 2014). The diagnosis is clinical and the condition is very heterogenous, both in terms of clinical phenotype and genetic factors. To date 19 loci have been mapped, with a BBS gene identified at each (Eichers et al., 2006, Forsythe & Beales 2003, Nahum et al., 2017), and mutations in these BBS genes appear to result in altered ciliary function (Gerdes
et al., 2014). In a mouse model of the disease, transgenic mice lacking a transcript of one of the BBS genes, Bbs4, demonstrated gender-specific obesity, with an earlier age of onset in females at 13 weeks compared to 24 weeks in their male null counterparts, and more extreme weight gain among the female mice (Eichers et al., 2006). Critically, variable penetrance of the obese phenotype was noted, with clinical obesity in 92% of females versus only 40% of Bbs4 null males. Further to this, metabolic derrangements were noted in Bbs4 null mice, including elevated total cholesterol, total to HDL-cholesterol ratios, and elevated triglycerides, along with raised circulating insulin and leptin levels, and biochemical evidence of mild liver dysfunction, all features of metabolic syndrome (Eichers et al., 2006). In a related study, Bbs4 knockout mice were crossed with transgenic mice carrying the human BBS4 gene, generating mice with tissue-specific expression of human BBS4, for example in the testis, brain, eye, cardiac and adipose tissue (Chamling et al., 2013). This study demonstrated the ability of human BBS4 to rescue the phenotype in Bbs4 knockouts, with no significant difference in weight compared to wild-type littermates in the Bbs4 knockouts expressing the human transgene (Chamling et al., 2013). In a human Caucasian population, variants in the BBS2, BBS4, and BBS6 genes have been linked to generalized obesity, with single nucleotide polymorphisms in the BBS4 and BBS6 genes associated with childhood early-onset obesity as well as morbid obesity in the adulthood (Benzinou et al., 2006).

At the molecular level, BBS4 has been shown to regulate adipocyte proliferation and differentiation, for example, in vitro studies have demonstrated enhanced proliferation in 3T3-F442A preadipocytes transfected with a vector designed to silence BBS4 transcription (Aksanov et al., 2014). Furthermore, silencing of BBS4 gene expression appeared to result in increased adipocyte triglyceride accumulation, with multiple intracellular lipid droplets and morphological and ultrastructural changes on electron microscopy including a fibroblast-like shape, smaller lipid droplets and increased numbers of lysosomes (Aksanov et al., 2014). Taking this concept further, preadipocyte Bbs4 expression appears to be regulated by insulin and insulin growth factor (IGF), with a dose-dependent downregulation of BBS4 and BBS6 gene expression reported in 3T3-F442A preadipocytes exposed to physiological concentrations of insulin in vitro, while physiological IGF administration appeared to enhance Bbs4 transcript levels (Nahum et al., 2017). In addi-
tion, silencing of BBS4 in 3T3-F442A cells appeared to significantly impact on insulin sensitivity, with relative insulin resistance and failure to enhance glucose uptake (Nahum et al., 2017).

Taken together, the literature suggests a strong link between BBS4 gene function and adipocyte biology, and in keeping with the clinical phenotype of lipoedema, evidence from mouse models suggests that the impact of BBS4 inactivating mutations on body fat distribution might be gender-specific (Eichers et al., 2006) - compelling support for the potential role of BBS4 gene mutations in the pathophysiology of lipoedema.

A second example of an exonic variant associated with the HPO terms described (Table 2.1), and resulting in a highly deleterious impact on gene function was discovered in the affected individual in case 9 (Table 5.6). This was a T for A, stop gained mutation in the protein coding region of heparan sulfate 6-O-sulfotransferase (HS6ST1) gene on chromosome 2, and the individual was heterozygous (Table 5.6). Unaffected family members had the reference sequence. The HS6ST1 gene in chromosome 2 encodes an enzyme which catalyses sulfate transfer to the 6-O position of the N-sulfoglucosamine residue within the heparin sulfate molecule (Habuchi et al., 2000; Sedita et al., 2004). Mutations in the HS6ST1 gene have been linked to idiopathic hypogonadotrophic hypogonadism, which presents as an incomplete or absent development of sexual maturity and infertility, with reduced levels of gonadotrophins and testosterone (OMIM, MIM number 614880; Raivio et al., 2007). In the presence of anosmia or hyponosmia the condition is known as Kallmann syndrome (Raivio et al., 2007). In a large study of 338 patients with gonadotrophin releasing hormone (GnRH) deficiency, mutations in the HS6ST1 gene were discovered in 2% of individuals (Tornberg et al., 2011). Further analysis of affected individuals, their phenotype and family pedigrees suggested that idiopathic hypogonadotrophic hypogonadism in association with HS6ST1 mutations has a complex inheritance pattern, neither autosomal dominant nor recessive, and that the HS6ST1 mutation alone is unlikely to be sufficient to cause the syndrome, rather additional genetic or epigenetic mechanisms must be at work (Tornberg et al., 2011). For example, three affected males had partial rather than absent puberty, and in one male patient in the study there was reversal of hypogonadism on discontinuing testosterone replacement therapy (Tornberg et al., 2011). Although not directly linked to lipoedema, there are parallels between these
two conditions – the clinical course of lipoedema appears to be heavily influenced by the hormone environment, with a much higher reported incidence in females, and disease onset frequently linked to hormonal shifts such as puberty and pregnancy (Child et al., 2010). Furthermore there appears to be much clinical variation in the severity of the phenotype, suggesting that epigenetic mechanisms could also play a significant part in the presentation of lipoedema.

In case 8, an exonic variant was identified in the PRPF3 gene on chromosome 1, with a moderate impact on gene function – three isoforms of a missense variant in the PRPF3 gene on chromosome 1 (Table 5.5). The top two were classified deleterious by SIFT (Kumar et al., 2009) and possibly damaging by polyphen (Adzhubei et al., 2010). The PRPF3 gene encodes a protein Prp3 which interacts with small nuclear ribonucleoproteins U4 and U6, components of spliceosomes (OMIM, MIM number 601414; Wang 1997). Retinitis pigmentosa 18 is an autosomal dominant condition caused by a mutation in the PRPF3 gene (OMIM MIM number 601414; Xu et al., 1996; Chakarova et al., 2002). The condition is characterized by night blindness and loss of the peripheral visual fields due to retinal dystrophy (Chakarova et al., 2002). To our knowledge, lipoedema, lipodystrophy, and obesity are not reported as clinical features associated with the retinitis pigmentosa disease phenotype, and thus the PRPF3 identified in our lipoedema family in case 8 is more likely to be an incidental finding rather than a novel candidate gene.

In case 6, filtering of HPO-associated, exonic variants revealed a group of 16 missense variants in 5 different candidate genes in the affected family member, all classified as having moderate impact on gene function, deleterious in SIFT and possibly-damaging in Polyphen (Table 5.4). Of these, the missense variant in the FAT4 gene on chromosome 4 is particularly interesting, since mutations in the FAT4 gene have been identified in patients with Hennekam lymphangiectasia-lymphedema syndrome (OMIM, MIM number 616006; Alders et al., 2014). The condition is autosomal recessive, characterized by intestinal lymphangiectasia, generalized lymphedema, associated facial features, and cognitive impairment (Hennekam et al., 1989). The condition appears to be genetically heterogenous, with mutations in the CCBE1 gene in 23% of affected individuals (Alders et al., 2009, Alders et al., 2013), and the FAT4 gene in 20% (Alders et al., 2014) – referred to as Hennekam Lymphangiectasia-lymphedema syndrome 2 (HKLLS2) in these cases (OMIM,
MIM number 616006). Given the established link between FAT4 mutations and primary lymphedema, and the close relationship between lipoedema and lymphatic dysfunction (Harvey et al., 2008), it is clear that the FAT4 gene merits further investigation as a potential candidate gene in lipoedema.

A candidate mutation in the LMNA gene was also detected on exome sequencing in our five lipoedema families, a GGA to G downstream gene variant in the affected family member of case 6 (Table 5.3) who was homozygous for the variant. Unaffected family members had the reference sequence. As discussed previously, mutations in the LMNA gene are associated with familial partial lipodystrophy, another disorder of abnormal adipose tissue deposition and biology (Garg & Agarwal, 2009). However, on closer inspection, the identified LMNA mutation in our lipoedema families appeared to have only a modifier effect on gene function, and as such is less likely to be of clinical relevance.
Chapter 6:

SUMMARY AND FUTURE DIRECTIONS
6.1 Identifying the “vasa lymphaticum” in mouse and human:

We have identified and described the microvasculature associated with the collecting lymphatic wall, the “vasa lymphaticum” in the context of chronic lymphedema. Unfortunately the significance of these blood vessels in lymphatic physiology and disease is still difficult to determine due to the lack of equivalent human lymphatic specimens from healthy controls, without lymphedema. The technical difficulties in obtaining suitable control specimens might be overcome in the future by harvesting redundant tissue from patients undergoing lower limb amputation, perhaps after intradermal injection of patent blue dye into the distal limb prior to amputation, to identify the collecting lymphatics within the specimen. Alternatively, it might be possible to identify the collecting lymphatics of the lower limb within the excess tissues removed during varicose vein surgery, as previously described by Agliano et al (Agliano et al., 1997).

Either way, control lymphatic specimens should ideally be obtained from the same lymphatic subtype (collecting lymphatics) and the same site (upper or lower limb) in order to draw meaningful conclusions on the role of vasa lymphaticum, and to put our findings in chronic lymphedema into context. The use of the mouse tail as a model for studying the impact of irradiation on the lymphatic vasculature has been described (Rutkowski et al., 2006, Avraham et al., 2010), but unfortunately we were unable to identify the “vasa lymphaticum” in the murine flank and tail, where the lymphatics are only around 100 μm in diameter. If these vessels could be identified in the murine tail, this would provide an ideal model for assessing the impact of irradiation on the lymphatic blood supply. Future studies should also focus on characterizing the expression profiles of blood vascular endothelial cells within the microvasculature of the “vasa lymphaticum” by microdissection and microarray. Such information has the potential to uncover therapeutic targets for radiation injury and lymphedema.
6.2 Isolating lymphatic endothelial cells from different tissues and organs of the Prox1-GFP mouse.

We have described a method for the isolation of lymphatic endothelial cells from a range of tissues and organs of the Prox1-GFP mouse by flow cytometry, for two lymphatic endothelial markers. This achievement is particularly significant in the central nervous system, where lymphatic vessels have only recently been discovered (Louveau et al., 2015). In our approach to isolating lymphatic endothelial cells from the central nervous system, we harvested the entire Prox1-GFP mouse brain, including the meninges, and performed enzymatic and mechanical dissociation with a protocol specifically designed for mouse brain (Miltenyi Biotec). Although successful overall, the nervous tissue of the brain itself constituted the vast majority of the final volume of single cell suspension, with the meninges forming only a tiny fraction. It is also important to note the abundance of other cells expressing podoplanin within the central nervous system (Mishima et al., 2006; Tomooka et al., 2013), meaning that only a small proportion of isolated podoplanin-positive cells also expressed the second lymphatic endothelial marker Prox1, consistent with cells of the lymphatic endothelium. In keeping with this, our flow cytometry data from the mouse brain demonstrate that live, podoplanin positive cells accounted for 47% of events—almost half of the single cell suspension from the murine brain (Figure 4.7A, Table 4.1), whereas only 0.13% of the podoplanin-positive cell fraction was also Prox1-positive (Figure 4.7C, Table 4.1), thereby expressing both lymphatic endothelial markers. In the future it might therefore be more efficient to focus solely on the meninges when performing cell sorts for central nervous system lymphatic endothelial cells, perhaps via a skull base approach and carefully dissecting the meninges from the skull vault, as described by Louveau et al (Louveau et al., 2015). The importance of performing a cell sort for more than one lymphatic marker has been highlighted further by data on the murine lung, where type 1 alveolar cells also reportedly express the lymphatic marker podoplanin (Ramirez et al., 2003). This was reflected in our flow cytometry data where podoplanin-positive cells comprised 17.8% of events (Figure 4.5A, Table 4.1).
The isolation of subcutaneous collecting lymphatic endothelial cells by flow cytometry was extremely challenging due to the careful microscope-guided tissue dissection required in harvesting the collecting lymphatics from the mouse, and the subsequent difficulties in isolating this rare cell population within the single cell suspension rapidly enough to minimise cell death during the process. As a result, we yielded only approximately 50 collecting lymphatic endothelial cells from eight Prox1-GFP mice, and these cells were relatively sparse in culture in a 96 well plate. In order to increase cell yields, specifically in the case of the collecting lymphatics, future attempts at lymphatic endothelial cell isolation and culture will focus on the selection of podoplanin-positive cells from the collecting lymphatic single cell suspension by MACS in the first instance. This would then be followed by expansion of this subpopulation in culture prior to flow-cytometry to isolate the GFP-(Prox1)-positive cell fraction, in a similar approach to the techniques described by Kriehuber et al and Lorusso et al, as previously described (Kriehuber et al., 2001; Lorusso et al., 2015).

Following isolation and successful expansion of murine organ/tissue-specific lymphatic endothelial cells in culture, future work will focus on the characterization of these cells, including detailed assessment of cell morphology by electron microscopy, and analysis of cell function by assays for migration, proliferation, tube formation and response to growth factors. We also aim to analyse and compare the expression profiles of lymphatic endothelial cells from different organ and tissue types by RNA sequencing.
6.3 Inheritance patterns and genetic factors in lipoedema:

We have described inheritance patterns of lipoedema, and identified candidate variants which could be involved in the disease. Our data supports a previous study by Child et al in 2010 suggesting an autosomal dominant inheritance with variable phenotype expression (Child et al., 2010). The candidates identified by exome screening were filtered according to overlap with HPO terms associated with lipoedema. It is possible, however, that other important candidates might have been missed by this approach. Given that lipoedema is a disease of abnormal adipose tissue deposition and expansion, further candidates might be uncovered by focusing instead on pathways involved in tumour growth, and inflammation, for example. We also aim to recruit additional ‘family trios’ to the study for exome sequencing. In the next stage, candidates’ variants will need to be validated by Sanger sequencing, and this information will be used to generate mouse models.
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