Utilising lymphatic cell markers to visualise human lymphatic abnormalities

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Abstract

The *in vivo* visualisation of the human lymphatic system is limited by the mode of delivery of tracing agents, depth of field and size of the area examined, and specificity of the cell markers used to distinguish lymphatic endothelium from the blood vessels and the surrounding tissues. These limitations are particularly problematic when imaging human lymphatic abnormalities. Firstly, limited understanding of the lymphatic disease aetiology exists with respect to genetic causes and phenotypic presentations. Secondly, the ability of a tracer to reach the entire lymphatic network within the diseased tissue is suboptimal. Thirdly, what is known about the expression of lymphatic endothelial cell markers, such as podoplanin, LYVE-1, PROX-1 and VEGFR-3 in rodent lymphatic vessels and healthy human lymphatic endothelial cells may not necessarily apply in the human lymphatic disease settings. The aim of this review is to highlight challenges in visualising lymphatic vessels in human lymphatic abnormalities with respect to distribution patterns of the cellular markers currently employed to visualise abnormal human lymphatic vessels in experimental settings. Integrating these limitations into current technological designs aimed at improving the diagnostic visualisation of the human lymphatic vasculature *in vivo* is likely to improve the current ability to image human lymphatic diseases.

Abstract Figure

Abstract figure legend

Podoplanin labelled lymphatic malformation cysts. Note the variability in immunolabelling of podoplanin in the large cyst (#) when compared to strongly stained smaller lymphatic vessels (*) and negatively stained blood vessels (arrow).

Short title

Lokmic: Visualising lymphatic abnormalities

Keywords

Vascular, lymphatic, anomaly, imaging, cell, surface, markers

Introduction

Abnormalities of the lymphatic vascular system are classified as vascular anomalies [1]. Vascular anomalies are broadly grouped into vascular tumours (i.e. infantile haemangioma, commonly known as a strawberry birthmark) and vascular malformations (i.e. venous malformation, capillary malformation, arteriovenous malformation and lymphatic malformation). This distinction is based on *in situ* endothelial cell proliferation characteristics with tumours having a high mitotic rate, whereas the malformations have a division rate similar to that of normal endothelium, which is low [2]. Congenital or acquired deficiency in the lymphatic system leads to defective absorption of the interstitial fluid, lymph, evident either as the swelling in the interstitial tissue, such as observed in primary lymphedema, or accumulation of lymph in the localised lymphatic structure, such as observed in a lymphatic cyst formed in lymphatic malformations (LM). Since the discovery of the lymphatic system and demonstrations of its role in maintaining fluid homeostasis an avid interest has existed in

methods that would enable the clinicians and researchers to distinguish these vessels *in vivo* and *in vitro*, from their well studied counterparts, the blood vessels.

From the *in vitro* perspective, the current imaging of the lymphatic system is confined to histology sections of various thickness stained with cell markers expressed on the lymphatic endothelial cells (LECs) but not on vascular endothelium [3]. These include lymphatic vessel endothelial hyaluronan receptor (LYVE-1) [4, 5], podoplanin, a type I transmembrane glycoprotein [6-8], a homolog of the *Drosophila melanogaster* homeobox gene prospero-1 (PROX-1) [9] and Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) [10, 11] used either individually or in combination with each other. Another discriminatory factor frequently used to morphologically and immunohistochemically distinguish lymphatic vessels is that LECs express CD31 while CD34 is not detectable [3].

From the *in vivo* perspective, ideally, the interest is in imaging the lymphatic vascular tree as one entity at the highest possible resolution. Hence, technologies that would allow simultaneous visualisation equally well at the initial lymphatic capillary level and the larger calibre lymphatic vessels, such as lymphatic ducts, are needed. Currently, the *in vivo* imaging of the entire lymphatic vascular system as one entity at one time is limited by the resolution capacity, the area that can be imaged and the depth of the field viewed [12]. Examples of the clinical imaging techniques used to visualise inborn and acquired human lymphatic abnormalities include lymphangiography (rarely used nowadays), lymphoscintigraphy, contrast-enhanced magnetic resonance lymphangiography, computed tomography, positron emission tomography scans and near-infrared indocyanine green fluorescence imaging (not currently approved for human use) [12, 13]. Table 1 summarises the capacity of these techniques to visualise lymphatic system structure and function in human. Clinical imaging of human lymphatic system employed for diagnostic purposes can visualise the formation of larger collateral

lymphatics, filling defects and obstruction of lymphatic vessels and lymph nodes [12]. However, this technology does not allow *in vivo* examination of the tissue-specific LEC heterogeneity or real time visualisation of LEC responses to acute and/or chronic disease states. In the case of lymphatic abnormalities, real time visualisation of lymphatic vessel behaviour under the influence of LEC-specific germline and/or somatic mutations in experimental models throughout the lifespan is yet to be achieved.

This review will highlight several challenges in visualising lymphatic vessels in human lymphatic abnormalities from anatomical point of view with respect to distribution of the LEC surface markers currently used to visualise human lymphatic vessels [3, 14, 15].

Technique	Depth of view	Spatial Resolution	Visualisation of lymphatic structures and function
X-ray lymphangiography	Whole body	<1 mm	Demonstrates LN architecture and size, afferent and efferent lymphatic vessels, does not demonstrate normal initial lymphatics but will demonstrate dilated lymphatics

			associated with lymphedema
Lymphoscintigraphy	Whole body	H1 cm	Visualisation of LNs, LN drainage and large diameter lymphatic vessels, no evidence of visualisation of initial and collecting lymphatic vessels, detects gross abnormalities of the lymphatic system
Contrast-enhanced magnetic resonance lymphangiography	Whole body	H1 mm	Larger lymph vessels, enlarged lymph nodes, detects lymph flow and allows lymph velocity calculations, cannot show lymph flow in real-time
X-ray computed tomography (CT) lymphangiography	Whole body	1-2 mm	Visualisation of LNs and larger lymphatic vessels
Positron emission tomography (PET)	Whole body	H2 mm	Resolution can be improved if used in combination with CT, cannot visualise superficial lymphatic vessels
Near infrared indocyanine green fluorescence imaging	3-4 cm tissue depth	H200 micron at tissue surface	Superficial lymphatics (initial, collecting and conducting vessels), detects stagnant lymph and provides limited information on lymph flow in real-time, visualises draining LNs

Table 1: Summary of imaging modalities for in vivo visualisation of the human lymphatic vasculature [12, 13]

General structure and function of the human lymphatic vascular system

A balance between the formation and absorption of the interstitial tissue fluid is maintained by lymphatic system, which is comprised of lymphatic vessels and lymph nodes. The system also participates in the immune surveillance, absorption of fats and fatty acids from the small intestine and

their transport to the liver, and reabsorption of cells and protein components that have escaped from the capillary and post-capillary venules into interstitial tissue due to differences in hydrostatic and colloid pressure present in the capillary bed [15-17].

The absorption of the lymph starts at the initial collecting lymphatics, also known as initial lymphatic capillary (Figure 1A). While extensive structural variation between lymphatic capillaries in different tissues has been reported [18, 19], the general structure of the initial lymphatic capillary is that of blind-ending endothelium-lined thin-walled channel of large irregular diameter (20-70 μm) [8, 18] that contains specialised junctions and anchoring filaments. The endothelium lining the initial collecting lymphatics is oak leaf shaped where flaps overlap with each other 18 to form fluid inlets while the cell junctions joining those LECs are depicted as discontinuous buttons composed of VE-Cadherin and PECAM-1 (platelet endothelial cell adhesion molecule-1, also known as CD31) located along the sides of the flaps [20] (Figure 1B). In tracheal lymphatics, the button-like junctions are found in the first 750 μm of the lymphatic vessel [20]. The gaps located between button-like cell junctions are also sites where immune cells enter the lymphatic system [21].

The basement membrane covering the initial lymphatics is either absent or discontinuous and no pericyte-equivalent cells have been observed here [8, 22]. The discontinuous membrane is composed of collagen IV, laminins, perlecan and nidogen [21]. The initial lymphatics are anchored to the surrounding tissue via short anchoring filaments. These filaments are composed of bundles of 4-12 nm thick microfibrils which originate from the abluminal LEC surface and project into collagenous interstitial tissue [23] through their anchorage to elastin [24]. The anchoring filaments play a major role in regulating the uptake of interstitial fluid through their response to changes in interstitial fluid pressure [25].

The initial collecting lymphatics coalesce and drain into pre-collecting and collecting lymphatic vessels, which are characterised by continuous smooth muscle cell (SMC) layer, continuous basement membrane, generally paired intraluminal semilunar valves and spindle-shaped LECs [18, 19] (Figure 1A and 1C). An area in the collecting lymphatics located between two consecutive lymphatic valves is called lymphangion [17]. The structure of the lymphatic valves prevents the backward flow of lymph between lymphangions [26]. The LECs are connected to one another by zipper-like cell junctions, which appear approximately 750 μm away the start of the initial lymphatic capillary [20]. The movement of lymph is facilitated by the intrinsic contractile activity of the SMCs covering lymphatic collecting vessel [27] and adrenergic, cholinergic and peptidergic innervation [28]. The basement membrane of the collecting lymphatic vessel wall is more continuous and is composed of collagen IV, laminin, perlecan and nidogen [21] and fibronectin [29, 30]. The LECs that line the semilunar valves also make a basement-membrane-like extracellular matrix composed of collagen IV, laminin $\alpha 5$ chain and fibronectin-EIIIA/EDA splice isoform [31].

Collecting lymphatics drain the lymph into one or more lymph nodes (here they are known as afferent lymphatics) where the immune system surveys the lymph for foreign antigens. From the lymph node, the lymph enters the efferent collecting lymphatics and continues its voyage towards the cervical lymphovenous portals. These portals are sites where lymph is returned back to the venous blood circulation [32]. In particular, on the right side of the human body, three main lymph trunks (the right jugular trunk, the right subclavian trunk and the right bronchomediastinal trunk) either independently drain into the right subclavian vein or converge into right lymphatic duct, which then drains into right subclavian vein [32]. On the left side, four main lymph trunks (the left jugular trunk, the left subclavian trunk, the left bronchomediastinal trunk and thoracic duct) either independently drain into the venous system in the area of the junction of the internal jugular and subclavian veins or converge into thoracic duct, which then empties in the area of the junction of the internal jugular and subclavian

veins to return to systemic blood circulation [32]. Not all lymph passes through the lymph nodes. For example, lymph vessels of the thyroid gland, oesophagus and of the coronary and triangular ligaments of the liver drain directly into the thoracic duct [33].

Figure 1: (A) Structure of lymphatic vessels. Initial collecting lymphatic vessels or capillaries are anchored to the surrounding tissue by anchoring filaments, which facilitate lymph collection and uptake of cellular materials. The lymph is then carried through collecting lymphatic vessels, composed intraluminally of elongated LECs and abluminally of SMCs and basement membrane. (B) The oak-shaped LECs of initial capillaries are connected by button-like junctions containing VE cadherin and PECAM-1 and contain flap valves that permit the entry of the lymph and cellular materials. (C) The elongated LECs of collecting lymphatic vessels are connected with zipper-like tight junctions that do not permit entry of the lymph flow from the interstitial tissue but instead act as

conduits towards the lymph node (D). The formation of lymph is due to movement of fluid from capillaries into interstitial tissue and subsequent movement of that fluid into the initial lymphatic capillary. Subsequent unidirectional movement of the lymph through lymphatic vessels towards the systemic blood circulation is facilitated by lymphatic valve, abluminal lymphatic vessel SMCs, the surrounding skeletal muscle, active and passive motion and breathing.

Cell markers for the lymphatic endothelium

The most commonly used markers for microscopic experimental imaging of the 2-D or 3-D lymphatic networks are LYVE-1 [4, 5], podoplanin [6, 7], PROX-1 [9] and VEGFR-3 [10, 11]. None of these markers is restricted to the LEC lineage alone so when employing them to visualise the lymphatic vessels it is important to consider the type of tissue or organ where the targeted lymphatics reside and the presence of inflammation or a pathological process within that tissue. For example, healthy human liver sinusoidal endothelial cells express LYVE-1 [34], while hepatocytes express PROX-1 [35], a nuclear transcription factor termed as the master-regulator of LEC fate [16]. Thus, identifying lymphatics in the liver, when the classical liver lobular structures cannot be visualised (as it can be on a histology slide), using these markers alone to target the lymphatics would be challenging. In normal human skin, the expression of LYVE-1, VEGFR-3 and podoplanin is confined to the lymphatic endothelium. However, in the presence of inflammation, VEGFR-3 becomes upregulated on most proliferating blood vessels [36], again posing a challenge to distinguish the lymphatic vessel from blood vessels.

It was reported that LYVE-1 is generally expressed on LECs of small lymphatic vessels but not on LECs from collecting lymphatics [21]. However, in human LMs, LYVE-1 is not strongly expressed by all LM cysts [37] (Figure 3F). Similarly, podoplanin is expressed on LECs of all lymphatic vessel subtypes [6, 7, 38] but varies greatly in larger LM cyst (Figure 3B) as often regions devoid of

podoplanin expression are observed [39]. PROX-1, supposed to be present in all LECs, regardless of vessel type, is not histologically evident in all LECs lining the LM cysts *in situ* [40, 41].

Integrin $\alpha 9$, a receptor for VCAM-1 (vascular cell adhesion molecule -1) was identified as a potential marker of mouse LECs as it is highly expressed on LECs lining the lymphatic valves [30, 31]. However, this has not been validated for reliable use to label human lymphatic vessels due to issues associated with the antibody application in human tissues (Z. Lokmic, unpublished observation). **COLEC 12**, a gene that codes for Collectin-12 protein, a scavenger receptor, has also been suggested as another LEC marker [14]. COLEC 12 has also not been extensively validated as a LEC marker either in animal or human tissues. **CLEVER-1** (common lymphatic endothelial and vascular endothelial receptor-1), also known as stabilin-1 or FEEL-1) has been reported in mouse skin lymphatic vessels, macrophages and vascular endothelium responding to inflammation [42]. Like COLEC 12, CLEVER-1 also requires extensive testing before it is confirmed as a suitable human LEC marker for identification of abnormal or diseased lymphatic vessels.

The early strategies of LEC isolation relied heavily on histological identification of lymphatic vessels as CD34^{Neg} CD31^{Pos} vessels [3]. However, in pathological conditions affecting lymphatic system, focal expression of CD34 is detected within the endothelial lining of the LM cysts suggesting that this approach is not as robust in this setting [9]. This concern is further substantiated by the results observed in the multi-parameter fluorescently activated cell sorting (FACS) of LECs derived from foreskin and LM where the CD34 expression was detected at very low levels [43].

Lymphatic abnormalities: malformations or tumour?

In 1982, Mulliken and Glowacki proposed that childhood vascular lesions (anomalies) be classified into vascular tumours or vascular malformations based on the clinical presentation and presence of

endothelial cell mitotic activity [2]. Specifically, in the vascular tumours the endothelial mitotic activity was increased during tumor proliferative stage whereas in vascular malformations the endothelial mitotic activity was considered to be normal i.e. did not differ from that observed in healthy vasculature. This was irrespective of the type of vessel affected by the malformation. Low proliferative index is consistent with the observation that the vascular malformation grows with the child and is relatively stable whereas the behaviour of vascular tumors is more unpredictable in terms of growth and progression. This division of vascular anomalies into vascular tumours and vascular malformations was accepted by the International Society for Study of Vascular Anomalies (ISSVA) in 1996 [44], further refined in 2014 and is now generally known as the ISSVA Classification for Vascular Anomalies [1]. The ISSVA classification has been of enormous help to clinicians and basic scientists who often faced inconsistent terminologies when conducting systematic reviews aimed at furthering the cell-based research of these conditions. Simplification of terminology and clear guidelines on what constitutes a particular anomaly has enabled an easier and more uniform approach to researching these conditions and communicating research findings.

In addition to classification into tumours and malformations, the lymphatic disorders are also broadly grouped into acquired and congenital. Acquired lymphatic abnormalities include lymphangitis, an inflammation of lymphatic vessel arising from an infection, and secondary lymphedema, a consequence of significant damage to the lymphatic network due to extensive surgical procedure, lymph vessel obstruction, trauma or anti-cancer therapy such as radiation. Congenital lymphatic disorders are present at birth or become evident in early years of life and they grow with the child. These are caused either by the germ-line or somatic mutations although for many lymphatic anomalies, the genetic cause is not known [45, 46]. Primary lymphedema and lymphatic malformations (LMs) are most common of examples of congenital lymphatic anomalies.

Primary lymphedema

Primary lymphedema is a consequence of underdeveloped (hypoplastic) or abnormally developed (dysplastic) lymphatic vessels rather than overgrown cystic structures. Due to these defects, the interstitial fluid accumulates in the tissue causing damage to the surrounding tissues. While the onset can occur during infancy, childhood, adolescence (lymphedema praecox) and adulthood (lymphedema tarda), about 49% of cases present in infancy with approximately 92% of those cases affecting lower extremities [47]. Primary lymphedema can occur on its own or as part of a syndrome, such as Milroy's disease [48].

Germline mutations have been identified for many clinical presentations of primary lymphedema. These include VEGFR-3 gene (Milroy's disease) [49], FOXC2 (lymphedema distichiasis) [50], SOX18 (hypotrichosis-lymphedema-telangiectasia) [51] and CCBE1 (Hennekam Syndrome) [52], GJC2 [53], GATA 2 (Emberger Syndrome) [54], KIF11 (microcephaly-lymphedema syndrome) [55] and PIEZO1 (generalised lymphatic dysplasia with non-immune hydrops fetalis) [56]. Lymphatic vessels present in the tissue affected by primary lymphedema are reported to stain strongly positive for podoplanin [57]. However, careful histological studies examining cases of different types of primary lymphedema with respect to their genetic diagnosis, age of presentation and describing the patterns of podoplanin, LYVE-1, PROX-1, VEGFR3, CD34 and CD31 expression in dysplastic and hypoplastic lymphatic vessels remain to be reported.

Lymphatic malformations (LMs)

LMs (Figure 2A and 2B) are congenital vascular malformations that arise during embryonic/fetal development, or are detected within the first years of life, with prevalence estimated at 30 per 100 000 births [58]. They can be found anywhere in the body where lymphatic vasculature exists, although those in cervicofacial region appear most dramatic and are more likely to cause immediate clinical

issues in terms of airway patency, feeding difficulties, tissue deformity, infection and LM haemorrhage and thrombosis [2, 59]. LMs can appear as a singular condition or as a part of a larger syndrome such as CLOVES (congenital lipomatous overgrowth vascular malformation epidermal nevi and scoliosis/skeletal/spinal abnormalities) [60], Proteus Syndrome [61] and Klippel-Trenaunay Syndrome [46].

Figure 2: (A) Clinical presentation of macrocystic LM in chest/neck area, (B) microcystic LM on a tongue and (C) KLA of the knee.

While ISSVA classification groups LMs into slow-flow malformations [1], there is no conclusive imaging evidence that the LMs are connected to the rest of the lymphatic vascular system and therefore allow lymph flow to occur. There is also no evidence that the LM cysts have the capacity to actively pump the lymph. Instead, LMs appear to be localised overgrowth of functionally impaired collection of LEC-lined cysts arising from a somatic mutation in PIK3CA gene in the LM-LECs [62-64]. PIK3CA gene regulates pathways involved in cell growth, cell division, cell metabolism and cell survival processes [64] and is linked to tumorigenesis [65]. Microscopically the lymphatic dysmorphogenesis in LMs is characterised by the presence of cysts of various sizes, which are filled with lymph containing proteinaceous fluid, white blood cells and occasional red blood cell. The inner wall of the cyst (tunica intima) is lined with LECs, however the middle wall (tunica media) is

characterised by disorganised SMC orientation [39]. While LMs are treated with combination of sclerotherapy and surgery, the size of the LM cysts determines whether a child can be treated with sclerotherapy (microcysts can be too small to inject with the sclerosing agent) to obliterate the existing cyst [66]. Regardless of the treatment option, the LMs cannot be completely removed from the body due to their infiltrative distribution throughout the tissue structures [59]. LMs also contain lymphatic valves, albeit these are rare to see and often appear small and deformed probably due to cyst fluid pushing the vessel walls apart (Z, Lokmic, unpublished observation).

Two methods have been reported that specifically target the isolation of human LM-LECs from LM lesion. Those are magnetic bead-based isolation based on $CD34^{Neg}CD31^{Pos}$ [39, 67] or $CD31^{Pos}Podoplanin^{Pos}$ selection [64] and fluorescence-activated cell sorting (FACS) to separate $CD34^{Low}CD31^{Pos}VEGFR3^{Pos}Podoplanin^{Pos}$ LEC population [43]. Currently, neither method can distinguish between normal LECs and LM-LECs, hence the cell isolate derived from LM tissue contain both normal and abnormal LECs. This highlights the need to search for novel LEC markers to distinguish normal from diseased LECs. While proteomics might offer a solution to this issue [68], this would be an enormously challenging task, as appropriate controls are hard to define. For example, it is not know which type of a healthy lymphatic vessel is actually equivalent to the LM cyst. Intuitively, presence of the intraluminal valve and the abluminal SMCs would suggest that a collecting lymphatic vessel is the best option as a control tissue. However, if one also considers the diameter of the cyst, one must ask if the thoracic duct equivalent would be more appropriate. In addition, due to low number of LECs obtained during cell isolation procedure, the subsequent expansion of cells *in vitro* further imposes its own limitations due to LEC adaptation to the *in vitro* environment [39, 67].

Regardless of the method of cell isolation used, based on the observation of foreskin LEC behaviour, it is reasonable to expect that within the mixture of normal and abnormal LECs the normal LECs would start to senesce much earlier than LM-LECs. Normal foreskin LECs start to senesce between passage 6 and Passage 8 (assuming passage 0 at time of tissue digestion and first cell plating), whereas LM-LECs start to senesce between passages 11-13 [43, 67]. This is not to say that senescent cells are not observed in early passages of LM-LEC cell culture (remember the cell isolate contains both normal and abnormal LECs), only that beyond passage 11 the cells may no longer be useful for *in vitro* cell culture experiments. With respect to the composition of normal and abnormal LECs, it is worth keeping in mind that as LM-LECs proliferate faster *in vitro* and have reduced apoptosis when compared to foreskin LECs [39], they will outgrow the normal LECs early on. Hence, the later cell passages will predominantly contain abnormal LECs. Note that LM-LEC proliferation *in vitro* is not an endless capacity, which would otherwise be consistent with endothelioma (neoplastic cell) behaviour. Instead, LM-LECs rapid proliferation comes to a rather abrupt end for unknown reasons [67].

There are two mouse xenograft models of human LM. One model is based on implantation of a silicone chamber containing LM-LECs into a mouse groin [39], whereas the other is based on subcutaneous implantation of LM tissue pieces [69]. Both models allow easy retrieval of the xenograft and both are easily adaptable for *in vivo* visualisation of the developing xenograft for at least 2 months post-implantation. Animal model that spontaneously develops an equivalent to human LM remains to be developed.

While advances have been made in understanding the pathophysiology of LMs through studies of LM-LEC *in vitro* behaviour, identification of PIK3CA somatic mutation as a possible cause, and the *in vivo* xenograft development, there is much left to resolve. For example, it is unclear if the cervical

LM-LECs differ phenotypically from the LM-LECs located in other parts of the body such as extremities. Do both arise from the same kind of progenitor LEC that contains PIK3CA mutation? Do all LECs lining the LM cyst contain PIK3CA mutation or is the cyst lined by both mutated and non-mutated LECs? Are different sized cysts reflection of the vessel of origin i.e. initial lymphatic capillary versus collecting lymphatic vessel or simply a reflection of space and composition of the tissue and matrix in which they can grow?

The interesting fact about the LMs is that the LECs of the small calibre LM cysts are strongly positive for podoplanin (clone D2-40), whereas the cells lining the larger cysts have either focal, weak or absent positivity for this marker [39, 70] (Figure 3B). This leads to two possibilities. Either the observed focal positivity is an optical illusion resulting from overstretching of the endothelium lining the large cysts, or alternatively podoplanin expression has been downregulated or lost in the endothelium associated with the macrocysts. The LMs are lined abluminally by the supporting SMC, that are organised in an irregular fashion (Figure 3 D and 3E), suggesting that these vessels are either not equivalent to initial capillaries or alternatively that these might have started as initial collecting lymphatics but through abnormal cell communication between mutated LECs and their environment, the abnormal vessel then acquired SMCs. So far, all of LM research has focused on LM-LECs and no research has been conducted on LM SMCs to investigate their contribution to this condition.

Another interesting anatomical observation that can impact the imaging and diagnosis of LM is that LMs can be perfused with blood without necessarily showing signs of the thrombus formation [39]. This may be because LM create communications with veins leading to blood perfusion of LMs thus creating lymphatico-venous malformations, a simple merging of a LM with the normal venous system. However, it is unclear whether lymphatico-venous malformations exist as an independent and unique entity. Collectively, the information presented here clearly identifies the complexities

surrounding the LM research and highlight a need to consider the variable expression of LEC markers in aiming to better visualise these *in vivo* if researchers are to progress in understanding the pathophysiology and functional consequences of LM cyst formation.

Figure 3: Immunolabelling (brown stain) histology of the human lymphatic malformation cysts. (A) CD31 is a pan-vascular endothelial cell marker that will highlight both vascular and lymphatic vessels. (B) Podoplanin (clone D2-40) has a variable expression on larger lymphatic cysts (#) and a strong positivity on small calibre lymphatic vessels (arrow). Note that podoplanin stain will not differentiate normal from abnormal lymphatic vessels. (C) VEGFR-3 labels lymphatic malformation cysts (#) as well as inflamed vessels such as capillaries (arrow). (D) LYVE-1 is another lymphatic marker used to identify LM cysts. (E) Desmin is a marker of SMCs, note discontinuous SMC coverage of LM cysts and unusual SMC orientation. (F) Alpha smooth muscle actin (a-SMA) is also a marker of SMCs. Note that figure E and F are serial sections and that the stain highlights the heterogeneity in SMC phenotype.

Gorham-Stout disease

Gorham-Stout disease, also known as vanishing bone disease or phantom bone disease [71], is characterised the presence of intraosseous LM in which the affected bone is progressively destroyed by the developing and proliferating LM. It is unclear why bone destruction is so dominant in this disease; however, it is likely that the LM produces as yet unknown factors that precipitate acceleration of bone lysis. Bruder *et al* reported that the massive bone loss observed in these patients is seen in bone cortex and medulla with areas of osteolysis showing prominent osteoclast-mediated bone reabsorption and fibrous replacement [70]. Histologically, the lymphatic vessels in Gorham Stout disease are reported as podoplanin positive large diameter vessels [70, 72] but a careful dissection of the podoplanin staining patterns, including vessel morphology, and systemic evaluation of other LEC markers based on examination of serial tissue sections is missing.

Generalised lymphatic anomaly

Generalised lymphatic anomaly (GLA), also known as lymphangiomatosis, is a multisystem disorder as it involves diffuse or multi-centric proliferation of lymphatic vessels in several organs systems and can involve visceral (such as spleen, lung, liver and intestine) and non-visceral organs (such as extremities and mediastinum) [72]. However, in contrast to Gorham-Stout disease, GLA is not characterised by cortical loss and progressive osteolysis and is often fatal [73]. Histologically, the GLA lymphatic vessels were reported positive for all lymphatic markers discussed herein [72]. However, reports of careful examination of staining patters of LEC markers on serial sections have not been found.

Lymphangiomyomatosis (LAM)

This very rare condition of unknown origin affects only women, most frequently during reproductive years, that starts at an unknown site and spreads via lymphatics to the lungs [74]. Histologically, this tumour is composed of clusters of LAM cells and proliferating lymphatic vessels that are podoplanin positive [75].

Kaposiform lymphangiomatosis (KLA)

KLA (Figure 2C) is classified by the World Health Organisation as a rare tumour of the lymphatic vessel origin with capacity to metastasise [76]. However, the ISSVA classification places this condition into provisionally unclassified vascular anomaly [1]. This is most likely because the imaging and presenting features of KLA identify it as an invasive and aggressive condition while histologically KLA displays low mitotic rate [77]. KLA histology is characterised by abnormally dilated lymphatic vessels and clusters of ovoid to spindle-shaped cells (Figure 4) that may include slit-like lumens containing erythrocytes [70]. However, while grouped, these cell sheets do not form lobules or confluent masses. Instead, they appear to be pattern-less clusters that do not harbour lumens, which would suggest a lymphatic vascular structure either present within or arising from these clusters [70]. KLA vessels have been reported as strongly positive for VEGFR3, D2-40, PROX-1, CD31, LYVE-1 and weakly positive for CD31, CD34 and factor VIII-related antigen [78]. Example of expression of LYVE-1, Podoplanin, PROX-1, VEGFR-3 and VEGF-A in KLA is presented in Figure 4. While it mostly affects soft tissue and viscera, KLA can affect bones (i.e. ribs, clavicle, scapula, vertebra, sacrum, ilium, femur, radius, ulna and wrist bones) [70]. Haematological abnormalities such as haemorrhage and low platelet counts are associated with KLA [77].

The presentation of this tumour raises many questions. Are spindle-shaped cell clusters source of KLA stem cells that can differentiate and give rise to the abnormal lumenised lymphatic vessels that are a feature of KLA or is this cluster a result of reactive lymphangiogenesis? What is the mitogenic potential of these cells and ability to contribute to the aggressiveness of this condition? Are KLA LECs derived from the tumour vessels different to those derived from KLA cell clusters or to LECs derived from other types of lymphatic anomalies or normal tissues? From an imaging perspective, could an injection of one or more lymphatic cell markers into the diseased tissue reach the KLA cell clusters even if they are reported to be devoid of lumenised structures?

Figure 4: Immunolabelling (brown stain) histology of the clinically diagnosed KLA cell island composed of closely packed spindle-shaped cells (*) and associated fully lumenised lymphatic vessel (#). (A) Immunoglobulin stain to account for non-specific labelling (B) LYVE-1, (C) Podoplanin, (D) PROX-1, (E) VEGFR-3 and (F) VEGF-A, a proangiogenic and lymphangiogenic growth factor.

Papillary intralymphatic angioendothelioma (PILA)

Papillary intralymphatic angioendothelioma (PILA) is a very rare tumour that mainly affects infants and children [79]. It is formed in the subcutaneous tissue and less frequently, the dermis. Microscopically PILA is characterised by intercommunicating, dilated vascular spaces that contain intravascular tufts with cuboidal or columnar well-differentiated endothelial cells and hyaline cores that stain positively for Collagen IV, a basement membrane component [80]. The tumorigenic endothelial cells lining vascular spaces and forming intravascular tufts are positive for vimentin, factor VIII-related antigen, CD31, focally positive for CD34 and negative for keratin markers, epithelial membrane antigen, S-100 and desmin but interestingly, for a tumour reported to grow up to 40 cm, the mitotic activity is reported as low [80].

Lymphangiosarcoma

It is unclear whether lymphangiosarcoma is an independent entity of angiosarcoma, a tumour that arises after chronic primary or secondary lymphedema such as that associated with radical mastectomy and subsequent radiation therapy. Regardless of the classification, the pathogenesis of this rare condition is unknown. The tumour consists of both solid and vascular components that have been reported to stain strongly for podoplanin [81].

Emerging technologies

Identifying the individual LEC surface receptors *in vivo* such as LYVE-1 and podoplanin through use of fluorescently labelled antibodies presents significant technical challenges. For example, the fluorescent labels are susceptible to photo-bleaching and background auto-fluorescence often hinders clear imaging. From biological point of view, injection of these compounds can also lead to cytotoxicity and precipitation of the host immune responses associated with the foreign body

introduction [82-84]. One way to bypass these serious concerns is through the development of alternative labelling methodologies that would enable the identification of the targeted receptor without precipitating serious side effects. An example is the use of golden carbon nanotubes conjugated to LYVE-1 receptor antibody, which are then delivered to the mouse system and detected by laser-induced photoacoustic waves [82]. However, this methodology is not approved for use in humans and is limited by a passive uptake of the labelled antibody that according to the literature is not likely to label all lymphatic vessels [21]. In addition, none of the antibodies discussed herein are approved for *in vivo* use in humans.

In addition to the above discussed issues with the expression of LEC surface markers, the depth of imaging and resolution are also significant problems for both *in vitro* and *in vivo* visualisation strategies [13]. Most 3-D *in vivo* visualisation techniques rely on injecting tracers into the tissue space surrounding the lymphatic vessels (Table 1) and subsequent transport of that tracer into the initial lymphatics and ensuing lymphatic network. These tracers are either opaque (such as Patent Blue used in lymphography), radioactive (^{99m}-technetium, used in lymphoscintigraphy and single-photon emission computed tomography), ultra-small super-paramagnetic iron oxide (used in magnetic resonance lymphography) or fluorescent nature (such as indocyanine green used in near-infrared fluorescence imaging; this label is not yet approved for human use) [12,13]. Regardless of the choice, only limited lymphatic structural area will be visualised, as the tracer injected in the surrounding tissue area will only highlight the local lymphatic network, including lymph nodes, draining that area. With the advances in emerging technologies like those arising from the biophotonics field this will change and lead to improved design of optical imaging and permit simultaneous use of multiple LEC markers for *in vivo* imaging of human lymphatic vasculature. Only then will further understanding of the pathogenesis of the lymphatic abnormalities be achieved.

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Table 1: Summary of imaging modalities for in vivo visualisation of the human lymphatic vasculature

Technique	Depth of view	Spatial Resolution	Visualisation of lymphatic structures and function
X-ray lymphangiography	Whole body	<1 mm	Demonstrates LN architecture and size, afferent and efferent lymphatic vessels, does not demonstrate normal initial lymphatics but will demonstrate dilated lymphatics associated with lymphedema
Lymphoscintigraphy	Whole body	H1 cm	Visualisation of LNs, LN drainage and large diameter lymphatic vessels, no evidence of visualisation of initial and collecting lymphatic vessels, detects gross abnormalities of the lymphatic system
Contrast-enhanced magnetic resonance lymphangiography	Whole body	H1 mm	Larger lymph vessels, enlarged lymph nodes, detects lymph flow and allows lymph velocity calculations, cannot show lymph flow in real-time
X-ray computed tomography (CT) lymphangiography	Whole body	1-2 mm	Visualisation of LNs and larger lymphatic vessels
Positron emission tomography (PET)	Whole body	H2 mm	Resolution can be improved if used in combination with CT, cannot visualise superficial lymphatic vessels
Near infrared indocyanine green fluorescence imaging	3-4 cm tissue depth	H200 micron at tissue surface	Superficial lymphatics (initial, collecting and conducting vessels), detects stagnant lymph and provides limited information on lymph flow in real-time, visualises draining LNs

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