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2021-06-25

Mäkinen , T , Boon , L M , Vikkula , M & Alitalo , K 2021 , ' Lymphatic Malformations Genetics, Mechanisms and Therapeutic Strategies ' , Circulation Research , vol. 129 , no. 1 , pp. 136-154 . https://doi.org/10.1161/CIRCRESAHA.121.318142

http://hdl.handle.net/10138/340862
https://doi.org/10.1161/CIRCRESAHA.121.318142

unspecified
acceptedVersion

Lymphatic malformations: genetics, mechanisms and therapeutic strategies

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Short title: Lymphatic malformations

Keywords: lymphangiogenesis, sirolimus, trametinib, vascular anomaly, VEGF-C

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Total word count: 14 073

Subject terms: Vascular Disease, Vascular Biology, Animal Models of Human Disease, Genetics, Treatment

Abstract

Lymphatic vessels maintain tissue fluid homeostasis by returning to blood circulation interstitial fluid that has extravasated from the blood capillaries. They provide a trafficking route for cells of the immune system, thus critically contributing to immune surveillance. Developmental or functional defects in the lymphatic vessels, their obstruction or damage, leads to accumulation of fluid in tissues, resulting in lymphedema. Here we discuss developmental lymphatic anomalies called lymphatic malformations (LMs) and complex lymphatic anomalies (CLAs) that manifest as localized or multifocal lesions of the lymphatic vasculature, respectively. They are rare diseases that are caused mostly by somatic mutations and can present with variable symptoms based upon the size and location of the lesions composed of fluid-filled cisterns or channels. Substantial progress has been made recently in understanding the molecular basis of their pathogenesis through the identification of their genetic causes, combined with the elucidation of the underlying mechanisms in animal disease models and patient-derived lymphatic endothelial cells. Most of the solitary somatic mutations that cause LMs and CLAs occur in genes that encode components of oncogenic growth factor signal transduction pathways. This has led to successful repurposing of some targeted cancer therapeutics to the treatment of LMs and CLAs. Apart from the mutations that act as lymphatic endothelial cell-autonomous drivers of these anomalies, current evidence points to superimposed paracrine mechanisms that critically contribute to disease pathogenesis and thus provide additional targets for therapeutic intervention. Here, we review these advances and discuss new treatment strategies that are based on the recently identified molecular pathways.

Non-standard Abbreviations and Acronyms

AAV	adeno-associated virus
ADAMTS3	disintegrin and metalloproteinase with thrombospondin motifs 3
ANGPT	angiopoietin
CCBE1	collagen and calcium-binding EGF domains 1
CCLA	central conducting lymphatic anomaly
CCM	cerebral cavernous malformation
CLA	complex lymphatic anomaly
CLOVES	congenital lipomatous overgrowth, vascular malformations, epidermal nevi and scoliosis/skeletal/spinal anomalies
CLAPO	capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalized overgrowth
ERK	extracellular signal-regulated kinase
GLA	generalized lymphatic anomaly
GDS	Gorham-Stout disease
KLA	kaposiform lymphangiomatosis
KTS	Klippel-Trenaunay Syndrome
LAM	lymphangioleiomyomatosis
LM	lymphatic malformation
LEC	lymphatic endothelial cells
MRI	magnetic resonance imaging
PI3K	Phosphatidylinositol 3-kinase
PROS	PIK3CA-related overgrowth spectrum
PROX1	prospero homeobox protein 1

SMC	smooth muscle cell
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

The lymphatic system

The main function of the lymphatic vasculature in mammals is to regulate tissue fluid balance by resorbing daily 1–2 liters of fluid and solutes that have extravasated from the blood capillaries to tissue interstitium and transporting this fluid, lymph, back to the venous circulation. The lymphatic vessels also function in immune surveillance by providing a pathway for the transport of antigens and extravasated leukocytes to lymph nodes, from which antigen-primed and tissue-targeted leukocytes are dispatched to lymphatic vessels and on to the blood circulation. Lymphatic (lacteal) vessels in the intestinal villi take up dietary lipids from the gut and those in the meninges around the central nervous system participate in the outflow of cerebrospinal fluid[1–3].

The lymphatic vascular system is a unidirectional hierarchical network of vessels (**Figure 1**). Interstitial fluid from peripheral tissues is drained into the lumens of lymphatic capillaries, also called initial lymphatic vessels. Fluid enters these blind-ended lymphatic vessels via valve-like openings between flaps formed by lymphatic endothelial cells (LECs). Capillary LECs have button-like intercellular junctions and anchoring filaments that act as tissue fluid pressure mechanosensors via connecting abluminally to pericellular matrix in the discontinuous basement membrane. From the initial lymphatics, the fluid, now called lymph, flows to pre-collectors and to collecting lymphatic vessels, and further through the lymph nodes and larger collecting ducts, back to the blood circulation. The collecting vessels are surrounded by a continuous basement membrane and smooth muscle cells (SMCs) that provide rhythmic contractions to “lymphangion” segments located in between luminal valves, which prevent fluid backflow. Skeletal muscle contractions, respiration, and arterial pulsations provide further forces to lymph propulsion. Cells of the lymph nodes, tonsils, Peyer’s patches, spleen, and

thymus function in immune defense in close coordination with the lymphatic vessel network. The parenchyma in some organs, such as bone, adipose tissue, liver, kidney, muscle and endocrine glands, lacks or contains only very few lymphatic vessels[4]. Only recently, lymphatic vessels were found in meninges around the central nervous system[5,6], and a lymphatic-like hybrid vessel (Schlemm's canal) with partial LEC identity involved in ocular fluid clearance was described in the eye[7–9].

Lymphatic vasculature was long considered as a passive "drainage" for fluid and other components that have extravasated from blood circulation. Recently, however, this view has changed dramatically and lymphatic vessels are now known to actively contribute to important physiological and pathological processes[1–3]. Impaired lymphatic function leads to accumulation of fluid in between cells in tissues, resulting in lymphedema. Primary lymphedema is a disease that is not caused by obvious external reasons that damage lymphatic vessels, such as surgery, radiotherapy or filariasis infection. Several subtypes of primary lymphedema can be clinically delineated and for a number of them, mutations in genes involved in lymphatic development have been identified[10]. Furthermore, mutations that cause dysplasia or obstruction of lymphatic vessels can result in chylous ascites, chylothorax, chyluria, protein-losing enteropathy, or compromised lung function. A large proportion of the lymphedema-associated mutations involve the key lymphangiogenic growth factor VEGF-C and its receptor VEGFR3, or their downstream signaling system[11] (**Figure 2**). These include genes encoding the transcription factors FOXC2 and GATA2[12–15], which enhance VEGFR3 signaling or expression respectively[16,17], or the collagen and calcium-binding EGF domains 1 (CCBE1)[18] and the disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3)[19] proteins that regulate VEGF-C processing and activity[20–22]. Secondary lymphedemas account for most of the clinical lymphedema cases. In developed countries, the

most frequent one is upper extremity lymphedema that develops in over 20 % of breast cancer patients operated in the axilla.

In this review, we focus on lymphatic malformations (LMs) and complex lymphatic anomalies (CLAs), which are localized or multifocal lesions of the lymphatic vasculature, respectively. They can result in disruption of fluid homeostasis and immune function, accumulation of chyle, or malnutrition[23]. LMs and CLAs are thought to be caused by abnormal development of the lymphatic vascular system. LMs and most forms of CLAs are rare somatic diseases, often apparent at birth or by two years of age as soft tissue masses consisting of fluid-filled cisterns or channels. The overall prevalence is approximately 1:4000 live births. LM or CLA can cause variable symptoms, depending on the size and location of the lesions, which determine the disfigurement and functional impairment of nearby structures or organs. They can also become infected, which can lead to "natural sclerotherapy" and shrinkage of the lesion. In order to understand the lymphatic anomalies, one needs to know how lymphatic vessels develop, and the mechanisms that regulate their growth and patterning.

Mechanisms of lymphatic vessel growth, maturation and maintenance

In mice, the lymphatic vascular system starts to develop at about embryonic day (E) 10.5, when the cardiovascular system is already functioning. The first LECs emerge and migrate out of embryonic veins[24] to form primitive lymphatic structures, from which further expansion of the vasculature occurs via centrifugal sprouting towards VEGF-C that is expressed in the adjacent mesenchymal tissue[25,26]. Moreover, several additional local and/or non-venous sources of LECs contribute to vessel formation. For example, the lymphatic vasculature in the

mesentery[27], skin[28,29] and heart[30–33] form in part through lymphvasculogenic assembly of vessels from progenitors of diverse origins.

The master regulator of LEC differentiation from the venous endothelium is the prospero homeobox protein 1 (*Prox1)* gene[24]. PROX1 induces a LEC-specific transcriptional program in blood vascular endothelial cells (BECs)[34] and is required to establish and maintain LEC identity *in vivo*[24,35,36]. PROX1 is also expressed e.g. in venous and cardiac valves[37], in endothelial cells in vessels with a mixed blood-lymphatic identity in the kidney (ascending vasa recta)[38] and in the Schlemm's canal of the eye[7–9].

The VEGF-C receptor VEGFR3 is initially highly expressed in the developing blood vasculature, and its deletion in mice leads to cardiovascular defects and embryonic death at E10.5, prior to initiation of lymphatic development[39]. In the differentiating LECs, PROX1 increases VEGFR3 levels[40]. Upon activation by VEGF-C, VEGFR3 in turn regulates *Prox1*, thereby establishing a feedback loop that controls LEC identity and lymphangiogenic activity[40]. LEC progenitors that exit the veins enter into an extracellular matrix of reduced stiffness, which further increases their VEGFR3 expression through GATA2-dependent transcriptional regulation[16]. The critical role of paracrine VEGF-C for lymphangiogenesis is evidenced by the fact that deletion of the *Vegfc* gene in mice results in fluid accumulation in tissues and prenatal death due to a selective lack of lymphatic vessels[25]. Even *Vegfc* haploinsufficient mice develop cutaneous lymphatic hypoplasia and lymphedema, indicating that both *Vegfc* alleles are required for normal lymphatic development[25]. The related VEGF-D also activates VEGFR3 and can stimulate lymphangiogenesis, but it is dispensable for lymphatic vessel development in mice[41]. Before receptor activation, both factors need to be activated by proteolytic processing, which in the case of VEGF-C involves the human

lymphedema-associated genes encoding CCBE1 and ADAMTS3[20–22,42,43] (**Figure 3A**). Together with the related ADAMTS14, these proteases ensure locally restricted maturation of VEGF-C to guide directional lymphatic vessel sprouting[44]. The further expansion of the lymphatic vascular network during late embryonic and early postnatal development occurs via sprouting that is dependent on VEGF-C-VEGFR3 signaling[45,46].

After the primary lymphatic plexus has been established, it needs to mature into a hierarchical network of blind-ended capillaries, pre-collectors and collecting vessels. This occurs in late gestation and continues during early postnatal period. After birth, the lymphatic capillariy junctions transform from continuous zipper-like to button-like[47,48] in a process that is regulated by angiopoietin 2 (ANGPT2) growth factor signaling via the lymphatic endothelial TIE2 receptor[48]. An important maturation step is also the formation of lymphatic and lympho-venous valves that ensure unidirectional lymph flow and prevent blood from entering into the lymphatic system, respectively[49]. Collecting lymphatic vessels form a basement membrane and recruit SMCs concomitantly with the formation of lymphatic valves. Key regulators of collecting vessel and/or valve formation are the transcription factors FOXC2 and GATA2[17,50], as well as the EPHB4 receptor tyrosine kinase and its transmembrane ligand EphrinB2[51–53]. Establishment of unique molecular LEC identities of capillaries, valves and lymphangions further contributes to the functional maturation of the lymphatic network[54]. In addition, the lymphatic vasculature in adults acquires organ-specific features, reflecting the varying functional demands and physical conditions in each organ[4]. VEGF-C is dispensable for homeostatic maintenance of lymphatic vessels in most organs, with the exception of the intestine and meninges[55,56], but is required for lymphangiogenesis associated with wound healing[57] and upregulated in pathological conditions, for example in malignant neoplasms[58,59]. Also the ANGPT/TIE signaling system is required for the maintenance of the lymphatic-like

vessels in the eye (Schlemm's canal)[60] and kidney (ascending vasa recta)[38], but its function in the adult lymphatic vasculature is less clear.

Lymphangiogenic signaling pathways implicated in lymphatic anomalies

Signal transduction downstream of the three key receptors, VEGFR3, EPHB4 and TIE, involved in the regulation of lymphatic vessel growth and remodeling, involves a cascade of several intracellular kinases and G-proteins, some of which have been found to be mutated in lymphatic anomalies. While in lymphedemas (LEs), the genes encoding the receptors and their ligands are frequently targeted by inactivating mutations, activating mutations in genes encoding the intracellular signaling proteins dominate in LMs and CLAs (**Figure 2, Table 1**).

Table 1. Classification, genetics and clinical characteristics of LMs and CLAs.

Clinical subtype	Also known as	Causative gene(s)	Clinical features
Macrocystic LM	Cystic hygroma	Somatic activating *PIK3CA* [61,62]	Single lesion of variable size consisting of multiple large fluid-filled cysts, commonly in the neck area
Microcystic LM	Capillary and cavernous lymphangioma, Simple lymphangioma	Somatic activating *PIK3CA* [61,62]	Small fluid-filled cysts and locally diffuse infiltrative lesions
GLA	Lymphangiomatosis, Diffuse LM	Somatic activating *PIK3CA* [63]	Diffuse and multicentric proliferative lesions with multiple organ involvement
KLA	Lymphangiomatosis	Somatic activating *NRAS* [64–66] Somatic activating *CBL* [67]	A subtype of GLA, foci of spindle-shaped LECs, thrombocytopenia

GSD	Vanishing bone disease	Somatic activating *KRAS* [68]	Lymphatic vessel growth in any bone, leading to progressive bone destruction and resorption
CCLA	Lymphangiectasia, Channel type LA	Somatic activating *ARAF* [69] Germline heterozygous kinase-inactivating *EPHB4* [70]	Dilation of large lymphatic vessels

Abbreviations: LM, lymphatic malformation; GLA, generalized lymphatic anomaly; KLA, Kaposiform lymphangiomatosis; GSD, Gorham-Stout disease; CCLA, central conducting lymphatic anomaly; LA, lymphatic anomaly. Based on ISSVA classification for vascular anomalies[71]. An additional classification algorithm can be found in[72].

The receptor binding properties of VEGF-C and VEGF-D are regulated by proteolytic cleavage. **Figure 3A-C** shows schematically how VEGF-C is proteolytically processed into an active form composed of the central VEGF homology domain that can then promote the formation of both VEGFR3 homodimers and VEGFR2-VEGFR3 heterodimers[73,74], which in turn activates the receptor tyrosine kinase activity. Neuropilin 2 (NRP2) acts as a co-receptor that is required for VEGF-C-induced lymphangiogenic sprouting[75]. Subsequent intracellular signal transduction employs the RAS-ERK and PI3K-AKT pathways, which function as central downstream effectors of various receptor tyrosine kinases. Studies using cultured primary LECs showed that VEGFR2, presumably through formation of the VEGFR2-VEGFR3 complex[73], is required for VEGF-C-induced AKT activation and cell migration, while VEGFR3 homodimers promote mainly ERK activation[76]. Interestingly, the kinetics of AKT and ERK activation are different, depending on whether VEGFR2 or VEGFR3 homodimers, or whether both receptors, including VEGFR2-VEGFR3 heterodimers, are activated[76,77]. This may translate into different biological responses downstream of the different receptor complexes. Notably, however, although VEGFR3 is differentially phosphorylated in

VEGFR2/VEGFR3 heterodimers[73] and VEGFR2 can robustly stimulate signaling in cultured LECs, VEGFR2 function seems dispensable for developmental lymphangiogenesis[46]. In adult mice, a specific VEGFR2 agonist increased lymphatic vessel diameter, but not sprouting[78]. The requirement for VEGF-C processing may limit its ability to signal through VEGFR2 *in vivo*, with only the fully processed form being able to bind to VEGFR2[74], whereas *in vitro* studies have mainly utilized the mature fully processed form. Interestingly, mainly PLC-gamma/PKC-dependent activation of ERK has been reported downstream of VEGFR2 in cultured blood vascular endothelial cells[79,80].

Phosphatidylinositol 3-kinase (PI3K), particularly its catalytic subunit p110α, has emerged as a key regulator of vascular development[81]. PI3Ks are lipid kinases that regulate the plasma membrane phosphatidylinositol (3,4,5)-triphosphate (PIP_3) levels, thereby providing docking sites for cytosolic proteins to activate signaling cascades such as the PDK1-AKT-mTOR pathway (**Figure 2**). In lymphatic vasculature, PI3K signaling regulates vessel sprouting and maturation, as deletion of its *Pik3r1* gene-encoded regulatory subunits that stabilize and inhibit the catalytic p110 subunit, activated the AKT pathway and led to hyperplasia and defective valve formation in intestinal lymphatic vessels in mice[82]. Studies of genetic mouse models further showed that p110α function is essential for the formation of the lymphatic vasculature in the gut[27]. Mice lacking *Akt1*, one of the three *Akt* genes, also have atrophic lymphatic vessels, yet loss of all *Akt* genes does not block VEGF-C-induced lymphangiogenic sprouting[83].

RAS signaling, composed of the cascade of RAS-RAF-MEK-ERK kinases with multiple isoforms at each step, is equally important for lymphatic vascular development. The three RAS isoforms (H-, N- and K-RAS) co-operatively regulate LEC proliferation and lymphatic vessel growth[84]. Lymphatic vessel hyperplasia observed in mice deficient of *Rasa1* (encoding p120

RasGAP, a negative regulator of RAS) or its interacting partner Map4k4, further highlighted the important role of RAS signaling in controlling LEC proliferation vs. quiescence[85,86]. Furthermore, cross-talk between the RAS-ERK and PI3K-AKT pathways exists at several levels, as indicated in **Figure 2**. Interestingly, mice carrying a mutation in the p110α subunit of PI3K that blocks its interaction with RAS have defective lymphatic vasculature, despite normal development of blood vessels[87]. This suggests selective importance of RAS signaling through PI3K in the lymphatic vasculature. Furthermore, ERK and its target kinase RSK can inhibit TSC1-TSC2 complex activity via phosphorylation[88].

EPHB4 is a receptor tyrosine kinase that is highly expressed in venous and lymphatic endothelial cells. It binds to the transmembrane ligand EphrinB2 in neighboring cells (in *trans*), which triggers the formation of an EPH-Ephrin heterotetramer and subsequent recruitment of other ligand-bound receptors to higher-order clusters[89]. Ligand binding induces bi-directional signaling in both the receptor- and ligand-expressing cells[89]. EPHs and Ephrins can also interact in *cis*, on the surface of the same cell, which inhibits receptor clustering and signaling. EPHB4 promotes RAS-MAPK signaling in cancer cells, whereas in endothelial cells, the activated EPHB4 recruits RASA1 (p120 RasGAP) to suppress the RAS pathway[90,91] (**Figure 2**). EphrinB2-EPHB4-RASA1 signaling is required for the formation of lymphatic and lymphovenous valves[51,52,92,93] and maintenance of LEC junctions[94]. In addition, EphrinB2 regulates VEGF-C signaling by controlling VEGFR3 internalization and downstream signaling[95].

Angiopoietins also signal through the PI3K pathway, via the TIE receptors. Inactivating mutations in genes encoding the TIE1 receptor that heterodimerizes with TIE2, or its ligand ANGPT2 lead to abnormal lymphatic development in mice[96], and *ANGPT2* mutations were

recently implicated in lymphedema[97]. ANGPT2 protein has also been studied as a biomarker of lymphangioleiomyomatosis (LAM), which is a rare multisystem disease characterized by cystic destruction of the lungs and lymphatic involvement[98,99], and of some CLAs[100,101]. Interestingly, although the majority of venous malformations in humans are explained by activating mutations in *TIE2*[102], they have not been detected in lymphatic anomalies, whereas *TIE1* is currently being considered as a candidate gene in LMs with or without lymphedema[103].

Anomalies of the lymphatic system

The clinical classification of lymphatic anomalies is important for providing an accurate diagnosis and guiding treatment decisions. The classification has been largely based on clinical presentation. Molecular diagnosis has become possible with the identification of causative genes, in particular in lymphedema, for which a number of germline gene mutations have been discovered[72]. In LMs and CLAs, causative mutations have been identified only recently, and so far, in a small number of CLA patients. In this review, we have used the International Society for the Study of Vascular Anomalies (ISSVA) classification for vascular anomalies[71], which is regularly updated to incorporate increased understanding of the biology and genetics of the disease.

Cystic LMs are the most common congenital lymphatic anomalies. They occur as solitary lesions of variable size and their appearance defines their classification into macrocystic, microcystic or mixed cystic LM (**Figure 4A, Table 1**). Macrocystic LMs manifest as large fluid-filled cavities that commonly localize to the neck area, while microcystic and mixed cystic LMs contain small cysts and diffuse vessel-like lesions (**Figure 4A-B, Table 1**). LMs commonly infiltrate soft-tissues, and they can be found anywhere on the body, from extremities

to the abdominal or thoracic cavities. Sometimes the lesion contours are ill-defined, with invasive involvement of the adjacent tissues and structures, resulting in severe complications, including organ dysfunction and impairment of breathing or swallowing[104,105].

CLAs are multifocal and/or cause defects in the central collecting lymphatic channels[106,107]. They include Generalized Lymphatic Anomaly (GLA), Gorham-Stout Disease (GSD), Kaposiform lymphangiomatosis (KLA) and Central Conducting Lymphatic Anomalies (CCLA) (**Figure 4A, Table 1**). They are rare diseases with overlapping and variable clinical features, making their diagnostics and management challenging. Common characteristics among CLAs are lymphatic vascular lesions in multiple tissues and sites, including bones, as well as leakage of lymph or chyle into the abdominal or thoracic cavities (pleural, pericardial, peritoneal effusions, chylothorax, chylous ascites), or externally. CLAs can be associated with intestinal lymphangiectasias, causing protein-losing enteropathy, characterized by hypoalbuminemia, hypogammaglobulinemia, and hypoproteinemia, but also with anemia because of intralesional bleeding, and sometimes lymphocytopenia.

GLA is characterized by diffuse or multicentric invasive lesions in multiple organs, including the bones, liver, spleen, lungs, and soft tissues (**Figure 4B, Table 1**). A distinguishing feature of GLA, as well as KLA and GSD, is the invasion of lymphatic vessels into bone. However, in GLA, the bone lesions do not destroy the cortical bone, unlike in GSD, which is associated with progressive bone destruction and resorption[107,108] (**Figure 4A, Table 1**). KLA is clinically reminiscent of GLA, although the lesions are histologically characterized by presence of foci of “kaposiform” spindle-shaped LECs, and thrombocytopenia is relatively frequent[65]. The fourth subtype of CLAs is CCLA, also known as the channel type LA. It is characterized by dilation, malformation and dysfunction of the major conducting abdominal and/or thoracic

lymphatic vessels, leading to impaired lymph drainage and leaking of lymph (or chyle) into body cavities[107] (**Figure 4A, Table 1**). CCLA can cause effusions and/or protein-losing enteropathy, and it can be part of GLA, GSD or KLA. It can also cause peripheral lymphedema. CCLA, GLA, GSD and KLA can lead to respiratory failure and have poor prognosis[107].

LMs can occur in combination with blood capillary, venous or capillary and venous malformations, called e.g. capillary-lymphatic malformations (CLM). In addition, LMs are common manifestations in syndromes characterized by tissue overgrowth, such as PROS (PIK3CA-related overgrowth spectrum) that includes KTS (Klippel-Trenaunay Syndrome), CLOVES (congenital lipomatous overgrowth, vascular malformations, epidermal nevi and scoliosis/skeletal/spinal anomalies) and CLAPO (capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalized overgrowth), as well as Proteus syndrome[71].

Genetics of LMs and CLAs

LMs are sporadic diseases, and each is caused by a somatic mutation. In the majority (~80%) of common cystic LMs, a causative mutation has been identified in the *PIK3CA* gene that encodes the p110α catalytic subunit of PI3K[61,62,109] (**Table 1, Figure 2**). The LM-causative mutations in *PIK3CA* are identical to those found in venous malformations[110–112] as well as in cancer and mosaic overgrowth syndromes[113]. Interestingly, the majority of LM-associated[109] *PIK3CA* mutations, as in cancer, cluster at three 'hot spots' in the helical domain (E542, E545) and the kinase domain (H1047) of p110α, resulting in variants that potently increase the PI3K enzyme activity. Both types of hot spot mutations cause basal activation of PI3K and its downstream AKT-mTOR pathway[114], which has opened up the possibility for the therapeutic

use of inhibitors of PI3K (alpelisib) and mTOR (rapamycin) in *PIK3CA*-driven vascular malformations[115]. The mechanisms by which the hot spot mutations in the helical and kinase domains activate oncogenic potential of p110α are, however, different[114], thus they may affect LM manifestation and therapy responses. Non-hot spot mutations with weaker effects on p110α activation have been identified in a smaller proportion of LMs[116]. In addition, in the syndromic forms of LM (CLOVES and KTS), in which somatic *PIK3CA* mutations are more widely distributed in patients' tissues with a tendency towards higher mutant allele frequency in the resected tissues, the mutations tend to be non-hot spot mutations[109]. A recent study identified a correlation between the mutation type and clinical features of LMs, with non-hot spot mutations being more frequent in macrocystic lesions than microcystic lesions, accounting for a higher proportion of neck/body lesions[116]. The Proteus syndrome is caused by somatic/mosaic activating mutations in *AKT*[117], which is downstream of p110α along the signal transduction cascade.

Apart from the recurrent *PIK3CA* mutations that account for the majority of microcystic and macrocystic LM cases, a causative somatic *PIK3CA* mutation was also reported in a series of GLAs[63] (**Table 1**). In addition, mutations in genes encoding components of the RAS-MAPK pathway have recently been identified in rare cases of patients with a CLA (**Table 1, Figure 2**). These include a recurrent somatic pathogenic mutations in *NRAS* in a dozen of patients with KLA[64–66] and in *ARAF* (encoding the serine/threonine protein kinase A-RAF) in two patients with CCLA[69]. Recently, a mutation was also identified in *CBL*, which encodes a ubiquitin ligase that targets receptor tyrosine kinases for degradation, and whose loss-of-function is associated with stabilization of the kinases and subsequent activation of the RAS pathway[67]. In one GSD patient, a low-frequency G61R somatic mutation was found in *KRAS* and a G12V hot spot mutation in 12% of the alleles was detected in the affected tissue of another

patient[68,108]. All the identified mutations lead to activation of the MAPK pathway, suggesting that these lymphatic anomalies are unresponsive to rapamycin, but could be treated with MEK or ERK inhibitors. Furthermore, germline heterozygous kinase-dead mutations in the gene encoding EPHB4, which signals to suppresses MAPK signaling in endothelial cells, were found in a few patients with CCLA[70] (**Table 1, Figure 2**). The RAS-MAPK pathway (e.g. *PTPN11*, *SOS1*, *RASA1*, *BRAF*, *KRAS*) is typically mutated in Noonan and Noonan-like patients (grouped under RASopathies) who infrequently have CLA-type lesions, as recognized already a long time ago[69,118,119]. In particular, the *SOS2*-related Noonan syndromes patients were reported to have a high risk of lymphatic complications[120].

Pathogenic mechanisms of LMs and CLAs: lessons from animal models and patient-derived LECs

The recently generated animal models of microcystic and macrocystic LM[121], GLA[63,66] and GSD[108,122] have advanced our understanding of their pathogenesis and enabled the assessment of therapies. In particular, the identification of somatic *PIK3CA* mutations as drivers of LM[61,62] and GLA[63], and use of the Cre/loxP technology for inducible and cell-type-specific expression of causative mutations to mimic somatic mosaicism, have permitted development of accurate mouse models that recapitulate key aspects of human pathology. *Vegfr3-CreER^{T2}* or *Prox1-CreER^{T2}* recombinase-mediated activation of the frequent causative mutation *Pik3ca^{H1047R}* in LECs of transgenic mice was shown to promote lymphatic vessel overgrowth and formation of vascular lesions in mice[63,121] (**Figure 5A-B**). This demonstrated that oncogenic PI3K signaling drives LM pathogenesis in a LEC-autonomous manner, in agreement with the finding that *PIK3CA* mutations occur only in the LECs in human LM[61,123,124]. Whereas LEC-specific expression of *Pik3ca^{H1047R}* in the embryonic vasculature promoted formation of large cysts that

were localized predominantly to the neck region[121], late embryonic or postnatal induction caused vessel hyperbranching[63,121] characteristic of microcystic malformations (**Figure 5A-B**). Likely both LEC-autonomous and non-LEC-autonomous factors also affect the different outcomes. For example, LEC proliferation at the time of induction may influence the response to PI3K activation, which is normally inhibited in quiescent endothelium. Furthermore, developmental stage-specific biomechanical properties and composition of the tissue environment, including the extracellular matrix and immune cells may affect lesion growth and contribute to organ-specific disease manifestation.

Studies in patient-derived LECs and the mouse LM model have provided important insights into the mechanisms by which *PIK3CA* mutations alter LEC phenotype and vessel overgrowth. In agreement with the major role of the PI3K-AKT-mTOR pathway for cell growth and survival, patient-derived LECs expressing common causative *PIK3CA* hot spot mutations showed increased proliferation and resistance to cell death stimuli[123,124] (**Figure 6A**). In addition, *PIK3CA*^{H1047R}-expressing murine LECs showed increased migration *in vitro* and lymphatic sprouting *in vivo*[121]. Interestingly however, although the mutant LECs had increased basal PI3K-AKT pathway activity[121,123,124], their stimulation by VEGF-C led to AKT hyperactivation *in vitro* and, surprisingly, was required for the growth of the malformations in mice[121] (**Figure 6B**). The involvement of the VEGF-C pathway in LM pathogenesis is supported by upregulation of its receptors VEGFR3 and NRP2 in *PIK3CA*-induced lymphangiogenic sprouts in mice[121] as well as in LM biopsies and lesion-derived LECs from patients[124,125]. Notably, LM lesions in both mice[121] and humans[126] are characterized by infiltration of immune cells, which are an important source of VEGF-C. These observations suggest that paracrine mechanisms, through regulation of the immune response, critically contribute to pathological vascular growth in LMs (**Figure 6A**). However, the positive

feedback mechanism that reinforces the upstream VEGFR3-NRP2 pathway and thus promotes downstream signaling in mutant LECs remains unknown. This could require cooperation with other VEGFR3-dependent downstream components, such as the RAS-MAPK pathway or regulation of endosomal recycling of VEGFR3 by PIP3 levels[127], as has been shown for other RTKs. Interestingly, RAS signaling has also been shown to regulate VEGFR3 levels[84], potentially contributing to a feedback mechanism. It is important to consider that the mechanisms by which the causative *PIK3CA* hotspot mutations in the helical and kinase domains, and the non-hot spot mutations outside these domains, activate the oncogenic potential of p110α are different[114], which may affect their dependence on the input from other pathways.

Ectopic lymphatic vessels in bone are observed in both GLA, KLA and GSD, but they are associated with loss of cortical bone only in GSD[128]. Expression of the *PIK3CA*^{H1047R} mutation in LECs in a mouse model of GLA indeed resulted in growth of ectopic lymphatic vessels in bone[63]. Interestingly, transgenic overexpression of VEGF-C in osteoblasts also resulted in the invasion of bone by lymphatic vessels, which was additionally associated with osteoclast-mediated resorption of cortical bone resembling GSD[122]. LECs are known to cause bone destruction in mice by secretion of M-CSF, which promotes osteoclast formation and activation[129]. However, why the ectopic lymphatic vessels in bone in GLA patients do not promote ostolysis, is not clear.

The observation that only a small proportion of LECs in LM tissue carry a *PIK3CA* mutation[62,116] suggests that development of vascular lesions may not rely solely on LEC-autonomous PI3K activation. Lack of correlation between mutation burden and the severity of disease manifestation in PROS[130] indicates that this may be a common feature of *PIK3CA*-

driven mosaic disorders. Cells that acquire a *PIK3CA* mutation may trigger aberrant signaling in the adjacent normal ECs through cell-cell interactions to collectively drive pathological tissue growth (**Figure 6A**). 'Clonal co-operation', a concept that is well established in cancer[131], is supported by observations in another type of mosaic vascular overgrowth, predominantly affecting the brain vasculature – cerebral cavernous malformation (CCM). In CCM, only a small proportion of lesional ECs carry the causative mutation[132,133]. In mice, the initial clonal expansion of mutant (*Ccm3* deficient) ECs was followed by incorporation and phenotypic changes in normal ECs during the expansion of the lesions[134,135]. Analysis of low frequency genetic mosaics in combination with tracking of clones derived from a single cell are challenging when using the traditional approaches that rely on reporter and mutant alleles located in different loci[136]. Advanced genetic tools that allow generation and reliable tracing of multispectral mosaics[137,138] should allow detailed analysis of the clonality of mutant LECs and their cooperation with their normal neighbors in driving mosaic vascular overgrowth. Uncovering the microenvironmental paracrine mechanisms will be critical for the development of effective combinatorial strategies to inhibit PI3K and other (paracrine) signalling pathways (**Figure 6A**).

As discussed above, CLA-causative mutations were recently identified also in genes encoding components of the RAS-MAPK pathway. These include a somatic activating mutation in *ARAF* in CCLA[69]. Activation of the MAPK in cells expressing the mutant *ARAF* suggested that such anomalies could be treated with MEK or ERK inhibitors. Indeed, the FDA-approved MEK inhibitor trametinib rescued lymphatic phenotypes observed in a transgenic zebrafish model expressing mutant *ARAF* and allevieted remarkably symptoms in a patient with an advanced rapamycin-unresponsive CCLA caused by an *ARAF* mutation[69]. MEK inhibition was also tested in the recently developed model of *Kras*-driven GSD, where the Cre-loxP system was

used to activate a LEC-specific *Kras*^{G12D} transgene in mice[108]. This led to the formation of ectopic lymphatic vessels in the bones and, with time, large superficial lymphatic cysts[108]. The development of lymphatic valves was also inhibited, and even existing lymphatic valves regressed, causing retrograde lymph flow and chylothorax[108], similarly as in mice deficient of the negative regulator of RAS, *Rasa1*[92]. Importantly, these defects were prevented in the *Kras*^{G12D} mice using trametinib[108].

Diagnostic developments

T2-weighted magnetic resonance imaging (MRI), in addition to lymphoscintigraphy, are important tools for the visualization of abnormal lymphatic growths and lymphatic leak or obstruction[139]. Furthermore, intranodal lymphangiography is emerging as a useful technique for CLA diagnostics. It is used to determine sites of leakage for surgical ligation, and for therapeutic embolization of CCLA type lesions[140].

The increased sensitivity of mutant DNA detection in blood circulation has made it possible to diagnose mutations in amplified cell-free DNA (cfDNA) from plasma as well as cyst and pleural effusion fluid from KLA and LM patients[64,141]. Such liquid biopsies may provide opportunities to initiate targeted pharmacotherapy even prior to surgical interventions. Assessment of cytokines concentrations in plasma has identified potential biomarker proteins, such as VEGF-D in GSD and soluble VEGFR3 plus ANGPT2 in KLA[100,101]. Single-cell sequencing, proteomic, lipidomic and NMR-based metabolomics platforms for scalable, comprehensive and batch-effect free metabolic analysis should accelerate scientific breakthroughs leading to highly sensitive and accurate diagnostics. This, combined with a

better understanding of the underlying molecular mechanisms should help in stratifying patients and refining disease classification, thereby guiding the choice of treatment.

Therapeutic options

Non-pharmacological treatment strategies. Surgical resection, sclerotherapy and laser therapy can provide local control and symptomatic relief to LM and CLA patients with recurrent effusions, infections and pain. Treatment is usually done to restore or preserve function and/or for esthetic reasons[142]. Often a combined treatment is needed. The therapeutic decision depends on the location and size of the malformation, the affected tissue and the experience of the physicians. Treatment in a multidisciplinary Center secures the best therapeutic approach.

Laser ablation can be used to reduce oozing from the superficial dermal or mucosal lymphatic vesicles[143]. Most commonly, yttrium-aluminum-garnet (YAG)-laser is used[144], but re-appearance of vesicles is commonly seen. Surgical resection of LMs is challenging and requires experience, time and an accurate technical approach, as LMs often invade important anatomical structures. Surgery is best indicated for well-localized micro- and macrocystic LMs, for which complete resection, and thus cure, can be achieved. However, this is rarely feasible, and resections are commonly only partial. Sclerotherapy is frequently used for macrocystic LMs. Repeated treatments are needed, yet recurrence is common over long-term. Several sclerosing agents have been used, including 3% sodium tetradecyl sulfate, doxycycline and bleomycin, with success rates from 40 to 100%[145,146]. Eighty % of macrocystic and 50% of microcystic LMs showed significant improvement in response to bleomycin sclerotherapy during a 1-3 years of follow-up[147].

Treatment of CLAs is more complicated. Surgery is rarely possible due to the localization and extent of the lesions. Sclerotherapy can sometimes be applied for macrocystic LMs, but as the lesions are often located deep inside organs, delivery of the therapy can be difficult.

Pharmacological treatment strategies. Recent progress in the identification of germline and somatic mutations that result in activation of well-known intracellular signaling pathways in LMs and CLAs has raised interest in drug-based therapeutic interventions for lymphatic and vascular anomalies. Because the PI3K-AKT-mTOR and RAS-MAPK pathways are well known drug targets in cancer, several small molecule inhibitors have been repurposed for use in LM and CLA. The mTOR inhibitor rapamycin (also known as sirolimus) has been used for many years in the context of organ transplantation due to its immunosuppressive effect, providing its detailed pharmacological profile and mid-term and long-term adverse effects[148]. Clinical drug trials targeting vascular anomalies have increased during the past 10 years, and some of them have focused on lymphatic anomalies that will be discussed here.

mTOR inhibitor rapamycin (sirolimus). Rapamycin inhibited lymphatic vessel overgrowth in mouse models of *Pik3ca*-driven LM and GLA[63,121]. In the clinic, rapamycin was first tested on an off-label basis in patients with complex life-threatening vascular anomalies. These studies included patients with lymphatic anomalies such as KLA, capillary-lymphatic-venous malformation (CLVM) and LM patients. In retrospective analysis, rapamycin alleviated symptoms, including pain, functional limitation, and oozing in about 80% of the treated cases within 3 months[149–152], although none of the patients was completely cured.

The first prospective clinical trial included six patients with various vascular anomalies refractory to current treatments (range 14-64 years). One KTS patient with a somatic *PIK3CA* mutation[153,154], was highly symptomatic due to a large lesion, daily abdominal lymphatic oozing, chronic ulceration, recurrent infections and muscle weakness. This patient received 2 mg of rapamycin daily for 26 months before becoming eligible for a surgery of the extensive dorso-abdominal CLVM. Rapamycin stopped the oozing and infectious episodes, healed the ulceration, improved muscle tone and promoted an improved quality of life[153,154]. The patient used rapamycin for a total of six years without important adverse effects.

Other prospective trials have since confirmed the efficacy and tolerability of rapamycin. An important study on mostly pediatric patients included 22 LMs, 13 CLVMs, three LVMs and three CLAs[155]. After one year of rapamycin treatment, 89% of the patients had an improved quality of life, 80% had improved organ dysfunction, and 52% presented greater than 20% reduction in lesion size[155], yet none of the lesions disappeared completely.

Another phase II prospective trial enrolled 19 patients with extensive slow-flow vascular malformations, including 10 patients with lymphatic anomalies consisting of six LMs, two GLAs and two cases of KTS[154]. The patients were somewhat older than those in the study of Adams and co-workers[155] (median age of eight versus 15 years). They all continued to have severe symptoms and poor quality of life, even after all possible therapeutic interventions, including repeated sclerotherapies and/or surgical resections. Although none of the lesions disappeared during rapamycin treatment, functional limitations, pain, bleeding, oozing, and infections decreased in all patients. After three months of treatment, 53% of the patients had over 50% improvement in their quality of life, and an additional 32% of patients showed 20–50% improvement that persisted at the six- and 12-month evaluation timepoints[154]. Similar

results were recently observed in a single-center study using rapamycin in 56 pediatric patients (median age 24 months) with complex LMs[156].

VASE, a large ongoing prospective multicentric phase III trial, is currently evaluating rapamycin efficacy in European patients with complex slow-flow vascular malformations that are refractory to standard treatment (EudraCT2015-001703-32, NCT02638389). VASE is a collaborative network project of European centers specialized in vascular anomalies, coordinated by Prof L. M. Boon. VASE started in January 2016 and it plans to enroll 250 patients, who are treated with rapamycin for two years. Preliminary results on the first 101 patients with at least six months of follow-up were recently discussed in the ISSVA 2020 virtual meeting. Fourteen of the patients had LMs, including four GLAs, two GSDs and six cases of KTS/CLOVES. Eighty-seven percent of patients presented improvement in pain, functional limitation and/or quality of life. In 43% of the patients, more than 75% improvement in pain was recorded within three months and in 34% of the patients, greater than 75% improvement in functional limitation was obtained[157].

Rapamycin has also been tested on PROS patients, in which the relief symptoms was less impressive[158]. Rapamycin was used off-label for 39 patients prospectively, at least 5 of which were reported to have LMs. In two patients, superficial LMs decreased. A six-month treatment did not provide improvement in the quality of life and led only to a modest reduction in lesion size (mean decrease 7%), although patients with a predominant adipose hyperplasia seemed to respond more than others. An important parameter that may have contributed to the poor efficacy was the lower rapamycin plasma concentration in PROS patients (2–6 versus 10-15 ng/ml in the other studies). The authors speculated that rapamycin could be more effective in actively growing tissue than in inducing regression of prior tissue overgrowth[158].

p110α inhibitor alpelisib (BYL719). Alpelisib is a p110α specific PI3K inhibitor, which was recently approved by the US Food and Drug Administration (FDA) for treatment of *PIK3CA*-mutated breast cancer[159]. Alpelisib was first tested in a transgenic mouse model of PROS expressing a *Pik3ca* with a hot spot mutation[159]. The mice developed hypertophic lesions, mimicking those in human PROS patients[160]. Both alpelisib and rapamycin improved the survival of the transgenic mice, but unlike rapamycin, alpelisib also improved organ dysfunction. Alpesilib was also tested off label, prospectively, on 19 patients with PROS refractory to standard of care[160]. Important soft tissue overgrowth was the major sign in all patients. Eight of the patients had CLOVES, but there was no mention of LMs. Like rapamycin in lymphatic anomalies, alpelisib improved symptoms in CLOVES patients. Importantly, a 37% reduction in lesion size was observed after six months of treatment. Improvement of genital vascular malformation was observed in one patient with CLOVES syndrome treated with alpelisib[161]. Short-term adverse effects of alpelisib seem to be similar to those of rapamycin, the most frequent being mouth ulcerations. However, alpelisib also induced transient hyperglycemia in 3 patients[160], as it does in 30-60% of breast cancer patients, necessitating a careful follow-up after the initiation of treatment[159].

MEK inhibitor trametinib in the therapy of CLAs. As already mentioned, various CLAs are associated with activation of the RAS-MAPK signaling pathway (**Table 1**). The FDA-approved MEK inhibitor trametinib effectively rescued lymphatic phenotypes in a zebrafish model expressing a mutant *ARAF*[69] and in a mouse model of *Kras*-driven GSD[108]. MEK inhibitors trametinib and solumetinib could thus be used in the therapy of CLAs.

Both rapamycin and trametinib reduced the viability of *NRAS* mutant LECs, which showed abundant AKT and ERK phosphorylation and proliferation rate, cultured from a KLA patient[66]. These two drugs may have synergistic benefits, as previous studies have shown that mTORC1 activation leads to PI3K and MAPK inhibition through a negative feedback loop stemming from S6 kinase (RSK), which is downstream of mTOR[162] (**Figure 2**). Thus, treatment with mTORC1 inhibitors can increase activation of the RAS-RAF-MEK1/2-ERK pathway, whereas addition of the MEK inhibitor can abrogate the feedback, diminishing the RAS-ERK signals.

In another KLA patient with *NRAS* mutation, and in a KLA patient with a *CBL* mutation[67], low-dose trametinib induced rapid improvement of clinical symptoms[67]. Similarly, in one of the CCLA patients that had an *ARAF* mutation[69], as well as in one patient with Noonan syndrome with *SOS1* mutation and similar severe lymphatic abnormalities[163], trametinib induced remodeling of the lymphatic system and resolution of symptoms. As the PI3K-AKT and RAS-MAPK pathways have multiple interaction points (**Figure 2**), combination therapies including mTORC1 or PI3K inhibition plus RAS pathway inhibition holds promise for the management of CLAs.

Future therapeutic directions

Current treatments for lymphatic anomalies focus on targeting of LEC-autonomous activation of the mutant pathways with rapamycin or alpelisib, suggesting that other available inhibitors of these pathways could also be repurposed. Although the initial stage of lesion formation likely involves selective expansion of the mutant cells that can be targeted with these inhibitors, paracrine signaling between mutant and normal LECs and their stroma may critically

contribute to driving disease pathogenesis and thus provide additional targets for therapeutic intervention (**Figure 6A**).

As discussed above, increased paracrine VEGF-C signaling is observed in LMs. This is driven by upregulation of the VEGF-C receptors VEGFR3 and NRP2 in LECs[121,124,125], and likely further promoted by infiltration of VEGF-C-producing immune cells around the lesions in mice and human patients[121,126]. Inhibition of paracrine VEGF-C signaling using a soluble VEGF-C-trap was more effective than rapamycin in inhibiting growth of *Pik3ca*-driven LMs in mice, and when administered in combination with rapamycin, it even promoted regression of the abnormal lymphatic vessels[121]. This suggests that effective therapeutic benefit may be achieved only by targeting both the *PIK3CA*-driven signaling and the microenvironment-derived paracrine signaling, originating in part from the immune cell infiltrate. Indeed, it is possible that the beneficial effect of rapamycin is partly due to its immunosuppressive functions[148]. The importance of paracrine signaling in driving disease manifestation in lymphatic anomalies is underscored by the finding that VEGF-C-driven growth of normal LECs into the bone is sufficient to recapitulate key aspects of disease pathology, including osteolysis, in a mouse model of GSD[122]. Another interesting observation is that in LAM (lymphangioleiomyomatosis), which is characterized by cystic destruction of the lungs, core LAM cells carrying inactivating mutations in *TSC1* or *TSC2* express high levels of the other VEGFR3 ligand, VEGF-D, and thereby recruit a coating by normal LECs that induce cysts in the lungs[98,99].

Direct cell-cell interaction between mutant and normal LECs may induce a phenotypic change in the latter and thereby promote their participation in the lesions. Interestingly, mosaic inactivation of VEGFR3 in LECs in mice promoted lymphatic vessel hyperplasia by activating

proliferation of neighboring wild type LECs through cell-cell contact mediated regulation of NOTCH signaling[46], thus revealing a 'non-cell-autonomous' (i.e. not driven by cells targeted by the genetic alteration) mechanism driving pathological vessel growth. Such mechanism may be involved also in LMs and CLAs.

Taken together, emerging evidence from mechanistic studies points to paracrine mechanisms that act in synergy with oncogenic activation of LEC signaling to drive LM pathogenesis. This provides a possibility to develop new effective therapeutics. The approaches could be further explored in ongoing clinical trials, in which single small molecule inhibitors are tested using incremental dosages to avoid adverse effects without compromising efficacy. Combinatorial treatment with the VEGFR3 inhibitor to block paracrine VEGF-C signaling could allow the use of smaller doses of the small molecule inhibitors, e.g. rapamycin, resulting in less adverse effects. Such combinatorial treatments could also enhance the cellular effects of rapamycin, which, at the tolerated doses used in the clinic, effectively stops cell growth but rarely induces regression of the lesions. High drug concentration shown to promote regression of hyperplastic lymphatic vessels, but also body weight loss, in mice[164] could possibly be achieved by topical treatment locally, with minimal effects on other organs[165].

Since VEGF-C is not required for the maintenance of most lymphatic vascular beds in adults, its inhibition alone may not be sufficient for regression of hyperplastic lymphatic vessels already present in the lesions. Interestingly, whereas inflammation- or VEGF-C-induced lymphatic vessel hyperplasia persists after inflammation resolution or normalization of VEGF-C levels in the airways[164,166], inflammation-induced expansion of the lymph node lymphatic vasculature was reversible[167]. Understanding the mechanisms that prevent and promote lymphatic vessel regression in such situations could reveal additional therapeutic targets. For

example, cellular metabolic pathways in LECs expressing the oncogenic form of p110α, a key regulator of cell metabolism, could offer targetable vulnerabilities.

The revolution of immunotherapy in the treatment of cancer patients encourages testing it in other diseases; perhaps also in LMs and CLAs. However, checkpoint therapy is unlikely to be effective in the treatment of lymphatic anomalies because the LECs in these lesions should not have a high mutational burden that in cancer generates antigenic targets and predicts a favorable response to immune checkpoint inhibitors[168].

Acknowledgements

We would like to thank Dr. Sinem Karaman for help with the artwork of Figure 1, TM lab members: Dr. Simon Stritt, Milena Petkova and Dr. Ines Martinez-Corral for histological images, Dr. Veli-Matti Leppänen for Figures 3B and C, and Dr. Emmy Verschuren for comments on the manuscript.

Sources of Funding

TM lab is supported by Knut and Alice Wallenberg Foundation (2018.0218), the Swedish Research Council (2020-02692), the Swedish Cancer Society (19 0220 Pj, 19 0219 Us), Göran Gustafsson foundation and the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 814316 (V.A.Cure), and under grant agreement No 874708 (TheraLymph). MV lab is supported by the Fonds de la Recherche Scientifique - FNRS Grant T.0247.19, the Fund Generet managed by the King Baudouin Foundation (Grant 2018-J1810250-211305) and by la Région wallonne dans le cadre du financement de l'axe stratégique FRFS-WELBIO (WELBIO-CR-2019C-06). It is also funded by the Lymphatic malformation Institute (LMI), USA and the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 814316 (V.A.Cure), and under grant agreement No 874708 (TheraLymph). KA lab is supported by the Wihuri Foundation, the European Union's Horizon 2020 research and innovation programme under the grant agreement No 874708 (TheraLymph), the Novo Nordisk Foundation (NNF16OC0023554), the Sigrid Jusélius Foundation, the Hospital District of Helsinki and Uusimaa Research Grant, and the Academy of Finland (335721, 307366, 312516).

Disclosures. The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Organisation of the lymphatic vessel network. Fluid, soluble molecules and immune cells from the interstitial space enter blind-ended lymphatic capillaries that are characterized by button-like intercellular junctions, a discontinuous basement membrane, and anchoring filaments. Collecting lymphatic vessels transport the lymph via lymph nodes to venous circulation; they have zipper-like intercellular junctions, a continuous basement membrane, smooth muscle cell (SMC) coverage and intraluminal valves. Whole-mount immunofluorescence images of mouse ear skin stained for the indicated antibodies illustrate the key features of the two vessel types. DC, dendritic cell. Adapted from[169].

Figure 2. Signaling pathways for lymphatic vessel growth in normal tissues and in LM. Schematic overview of the RAS-MAPK and PI3K-AKT LEC signal transduction pathways involved in LMs and CLAs. The signaling components include protein and lipid kinases and G-proteins, and their regulatory factors. Disease-causative genes and proteins have been highlighted as indicated (Green, lymphedemas (LE); Blue, LMs; Red, CLAs), and genes with an activating mutation are underlined whereas those with an inactivation mutation are not. The RAS-MAPK and PI3K-AKT pathways regulate each other via cross-inhibition and cross-activation, with TCS1/2 and mTORC1 providing key integration points[170]. Some of these regulations have been indicated, with arrow-headed lines indicating positive regulations of substrate proteins and bar-headed lines inhibitory regulations of substrate proteins.

Figure 3. Proteolytic processing of VEGF-C. (A) Proteolytic processing of the VEGF-C prepropeptides that form an antiparallel dimer stabilized by disulphide bonds. Carboxyterminal cleavage is catalyzed by furin-like enzymes. The aminoterminal cleavage is enhanced by the

CCBE1 protein, and catalyzed by the ADAMTS3 metalloprotease. Mutations of VEGF-C, CCBE1 and ADAMTS3 cause primary lymphedemas. (**B**) Structural model of mature VEGF-C[171]. (**C**) Molecular model of VEGF-C bound to a VEGFR3 dimer[172].

Figure 4. Clinical characteristics of LMs and CLAs. (**A**) Schematic representation of the main clinical features of cystic lymphatic malformations and complex lymphatic anomalies. The body parts, organs and/or vessel types typically affected by lymphatic vessel overgrowth and malformations (blue) in the different diseases and their distinguishing features are shown. CCLA-like features can be part of GLA, GSD and KLA. (**B**) Clinical examples of lymphatic malformations, showing photos of macrocystic LM of the neck and microcystic LM of the tongue, and magnetic resonance imaging of GLA, as indicated. Yellow arrows indicate GLA pathology (chylothorax and diffuse cystic lesions). Hematoxylin and eosin (H&E) stained sections of macrocystic and microcystic LMs constituting a large cyst (asterisk) or multiple small-caliber vessels (arrows), respectively are shown below. Based on ISSVA classification for vascular anomalies[71]: LM, lymphatic malformation; GLA, generalized lymphatic anomaly; KLA, Kaposiform lymphangiomatosis; GSD, Gorham-Stout disease; CCLA, central conducting lymphatic anomaly. Scale bar: 150 µm.

Figure 5. Modelling *Pik3ca^{H1047R}*-driven LM in mice. (**A**) Schematic of the transgenes and gene induction protocols for tamoxifen-inducible LEC specific expression of the causative *Pik3ca^{H1047R}* mutation. (**B**) Immunohistochemical features of macrocystic LM after embryonic (E) induction and microcystic LM after early postnatal (P) induction in mouse skin. Whole-mount immunofluorescence staining of the LEC-specific markers NRP2 or LYVE1 reveals large cystic overgrowth in the back skin of an E17 *Pik3ca^{H1047R}*;*Vegfr3-CreER^{T2}* embryo as opposed to diffuse lesions with hyperbranched lymphatic vessel network and associated

bleeding in the ear skin of a 9-week-old *Pik3ca*^{H1047R};*Prox1-CreER*^{T2} mouse, as described in [121]. Scale bar: 200 µm (embryo skin, ear magnification), 500 µm (ear).

Figure 6. Cellular mechanisms of *PIK3CA*-driven LM pathogenesis and therapeutic opportunities. (**A**) LM development is triggered by a somatic activating *PIK3CA* mutation (*PIK3CA**) in LEC, leading to a cell-autonomous increase in PI3K-AKT signaling as well as LEC proliferation and migration, that can be targeted with PI3K pathway inhibitors, e.g. rapamycin (mTOR) or alpelisib (p110α). Paracrine signaling (black double-headed arrows) between mutant *PIK3CA** LECs (red) and normal LECs (grey) or stromal cells (green) also contribute to pathological vascular growth, offering additional therapeutic options. As an example, soluble VEGFR3 protein (VEGF-C-trap) or anti-VEGFR3 antibody can be used to block paracrine VEGF-C, likely produced by the lesion-infiltrating immune cells. This inhibits LM growth in mice[121]. Identification of additional, currently unknown ('?') paracrine mechanisms should enable further development of effective combinatorial therapeutic strategies. (**B**) Inhibition of paracrine VEGF-C-VEGFR3 signaling in the treatment of LM. Experimental outline of the LM induction and treatment of progressive microcystic *Pik3ca*^{H1047R}-driven LM by using AAV (adeno-associated viral vector)-based therapy blocking VEGF-C-VEGFR3 signaling. VEGF-C-trap that sequesters VEGF-C is shown schematically, consisting of the ligand-binding domain of VEGFR3 (blue) fused to IgG Fc-domain (black). Whole-mount VEGFR3 staining of the ears from six-week-old *LSL-Pik3ca*^{H1047R};*Vegfr3-CreER*^{T2} mice treated with 4-OHT to induce vascular overgrowth at 3 weeks of age and treated 1 week later with VEGF-C-trap (single intraperitoneal injection of AAV, as in [121]). Control mice (treated with AAV control[121]) show pronounced lymphatic hyperplasia that is effectively inhibited by VEGF-C blockade. Scale bar: 200 µm.

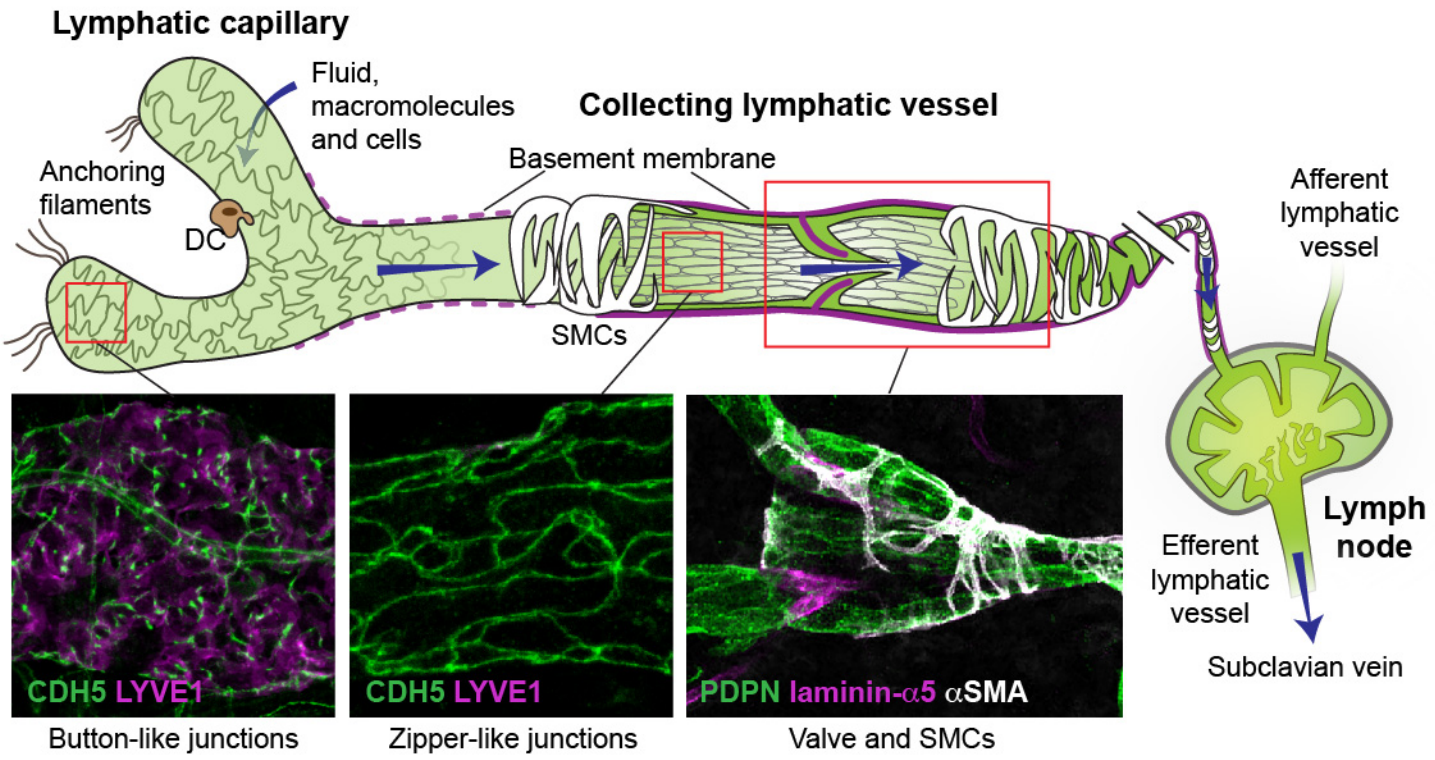
Lymphatic capillary
Fluid,
macromolecules
and cells
Collecting lymphatic vessel
Anchoring
filaments
DC
Basement membrane
SMCs
Afferent
lymphatic
vessel
Lymph
node
Efferent
lymphatic
vessel
Subclavian vein
CDH5 LYVE1
CDH5 LYVE1
PDPN laminin-α5 αSMA
Button-like junctions
Zipper-like junctions
Valve and SMCs

Figure 1

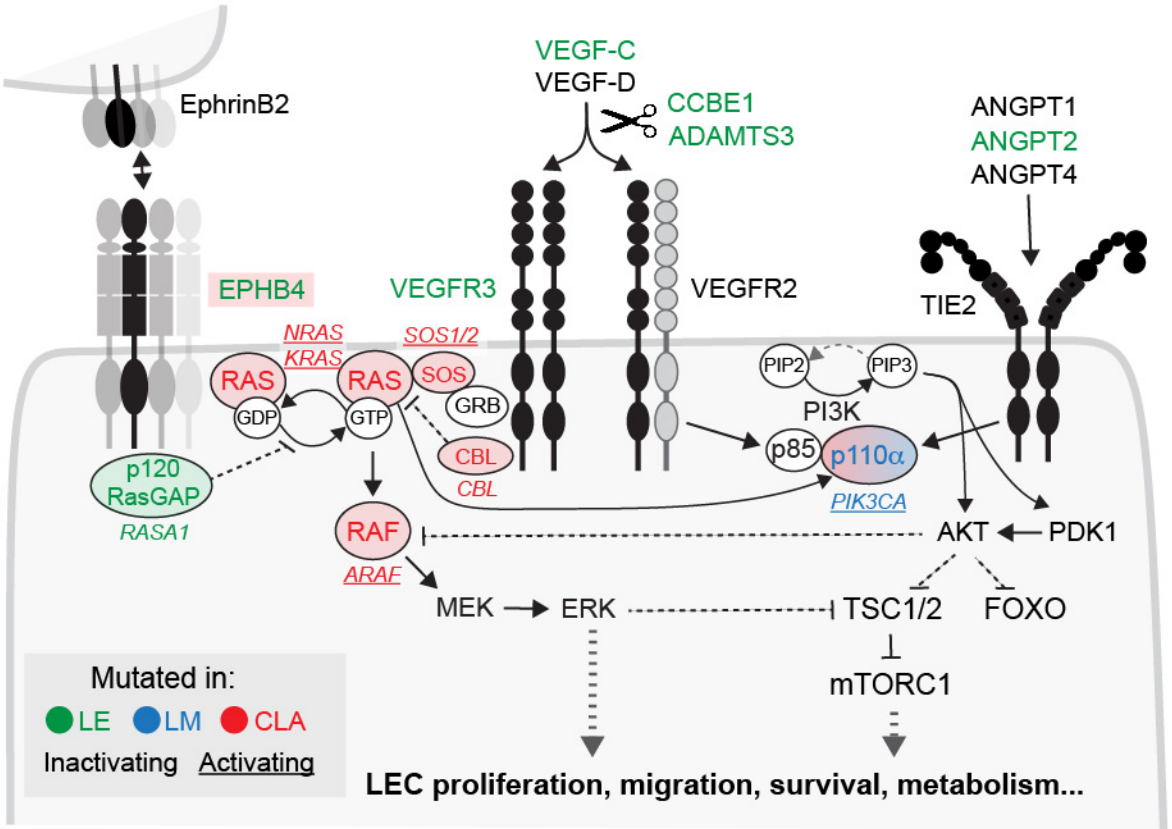
EphrinB2
VEGF-C
VEGF-D
CCBE1
ADAMTS3
ANGPT1
ANGPT2
ANGPT4
EPHB4
VEGFR3
VEGFR2
TIE2
NRAS
KRAS
SOS1/2
RAS
GDP
RAS
GTP
SOS
GRB
CBL
CBL
PIP2
PIP3
PI3K
p85
p110α
PIK3CA
p120
RasGAP
RASA1
RAF
ARAF
AKT
PDK1
MEK
ERK
TSC1/2
FOXO
mTORC1
Mutated in:
LE
LM
CLA
Inactivating
Activating
LEC proliferation, migration, survival, metabolism...

Figure 2

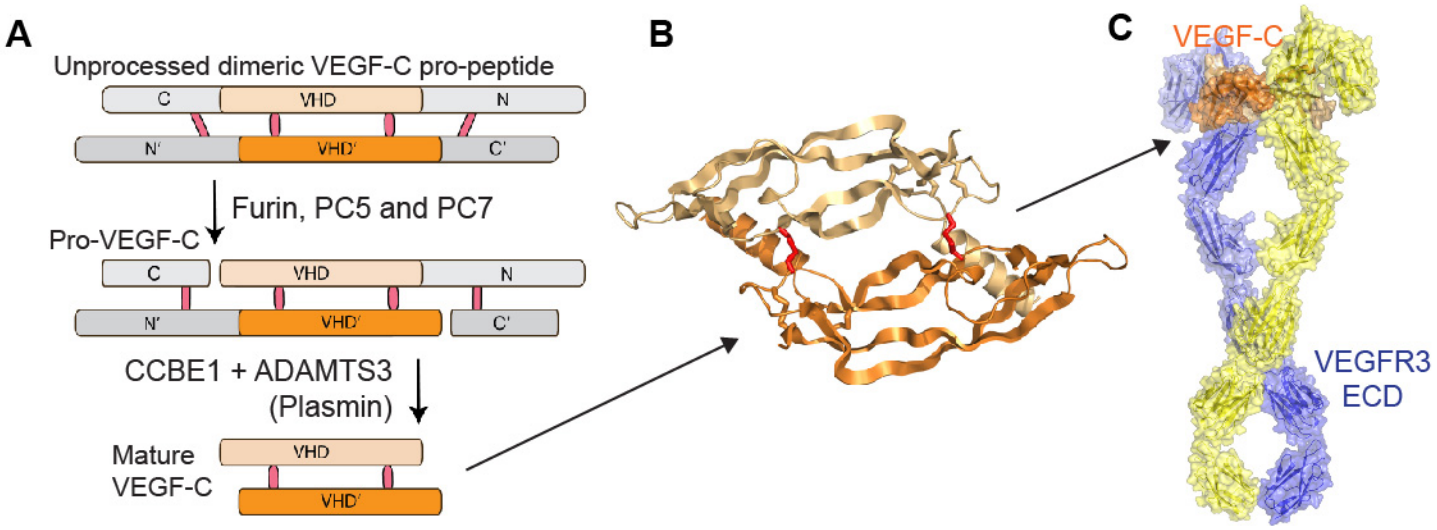
A
Unprocessed dimeric VEGF-C pro-peptide
C
VHD
N
N'
VHD'
C'
Furin, PC5 and PC7
Pro-VEGF-C
C
VHD
N
N'
VHD'
C'
CCBE1 + ADAMTS3
(Plasmin)
Mature
VEGF-C
VHD
VHD'
B
C
VEGF-C
VEGFR3
ECD

Figure 3

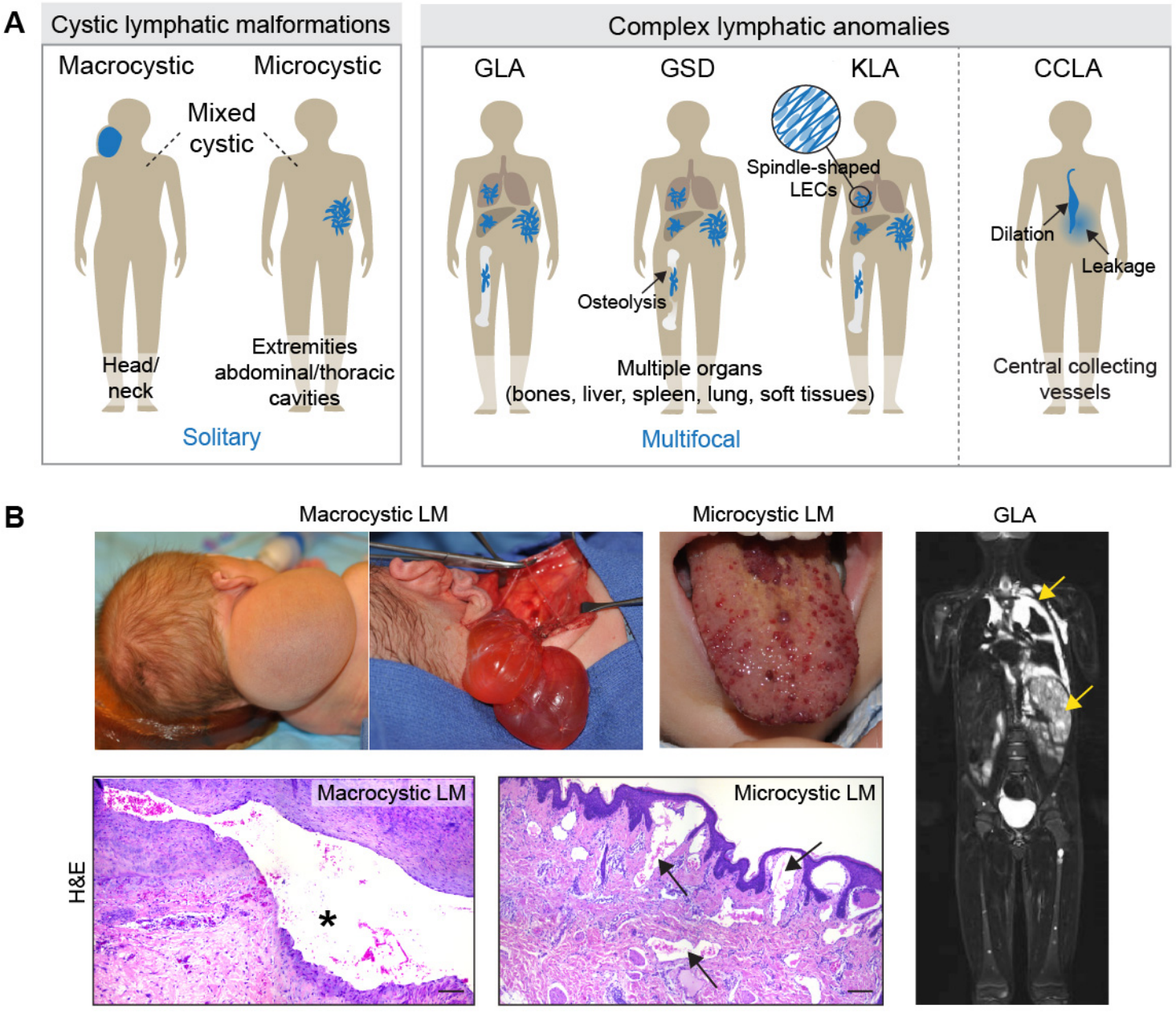
A
Cystic lymphatic malformations
Macrocystic
Microcystic
Mixed cystic
Head/ neck
Extremities abdominal/thoracic cavities
Solitary
Complex lymphatic anomalies
GLA
GSD
KLA
CCLA
Spindle-shaped LECs
Osteolysis
Dilation
Leakage
Multiple organs (bones, liver, spleen, lung, soft tissues)
Central collecting vessels
Multifocal
B
Macrocystic LM
Microcystic LM
GLA
H&E
Macrocystic LM
Microcystic LM

Figure 4

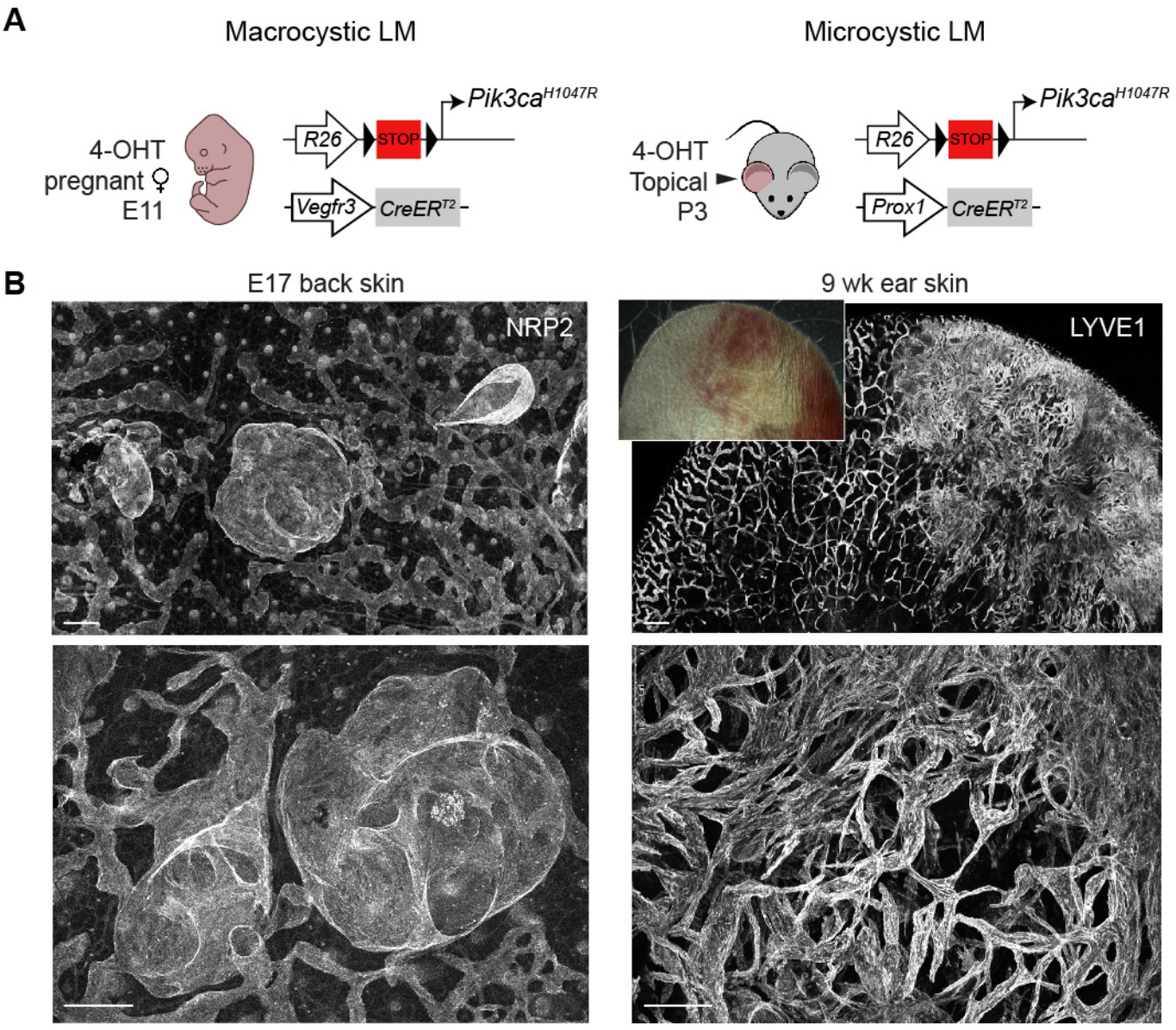
A
Macrocystic LM
Microcystic LM
4-OHT
pregnant ♀
E11
R26
STOP
Pik3ca^H1047R
Vegfr3
CreER^T2
4-OHT
Topical
P3
Prox1
B
E17 back skin
9 wk ear skin
NRP2
LYVE1

Figure 5

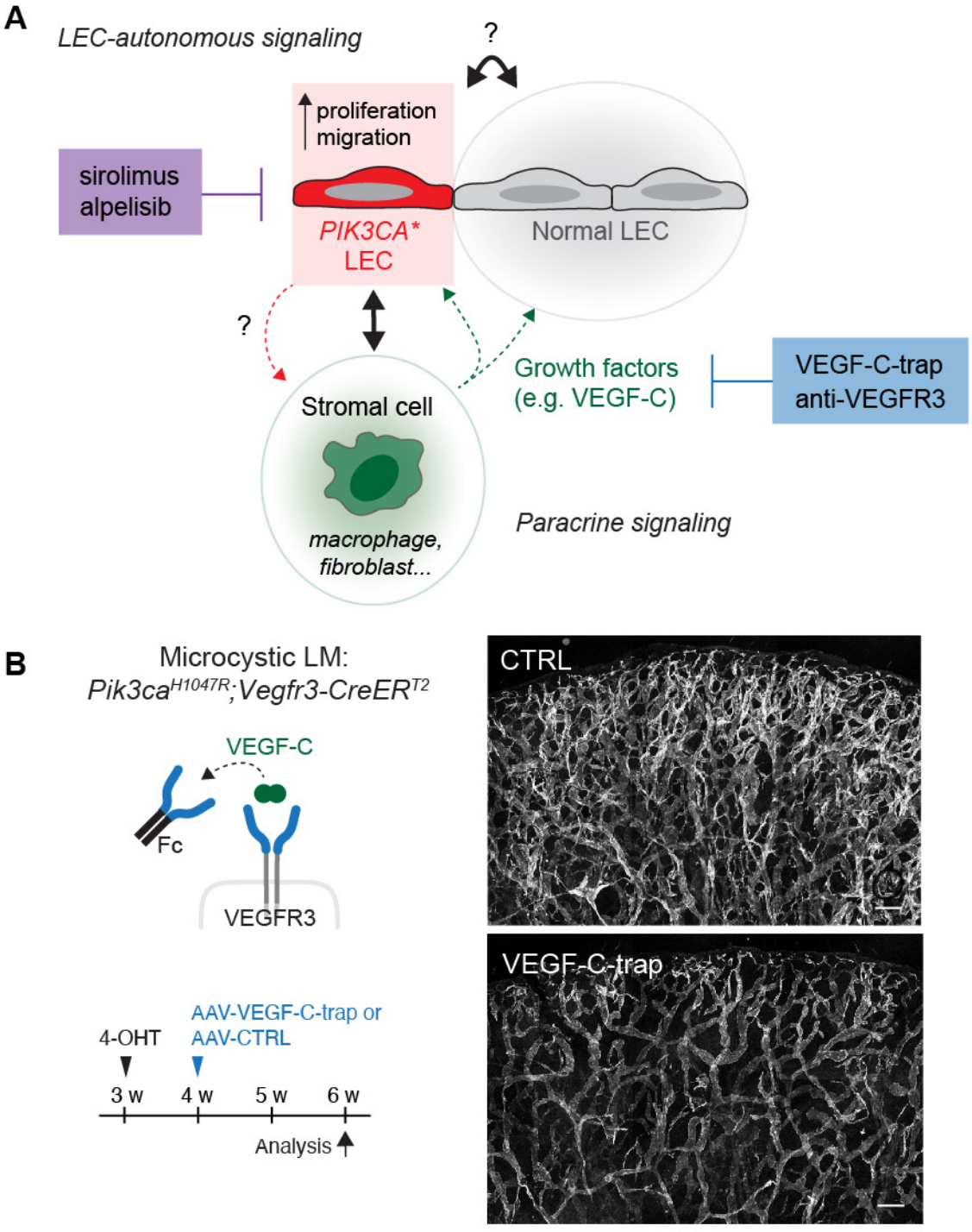
A
LEC-autonomous signaling
?
proliferation
migration
sirolimus
alpelisib
PIK3CA*
LEC
Normal LEC
?
Growth factors
(e.g. VEGF-C)
VEGF-C-trap
anti-VEGFR3
Stromal cell
macrophage,
fibroblast...
Paracrine signaling
B
Microcystic LM:
Pik3ca^H1047R;Vegfr3-CreER^T2
VEGF-C
Fc
VEGFR3
AAV-VEGF-C-trap or
AAV-CTRL
4-OHT
3 w
4 w
5 w
6 w
Analysis
CTRL
VEGF-C-trap

Figure 6