Title

Development and Physiological Functions of the Lymphatic System – Insights from Human Genetic Studies of Primary Lymphedema

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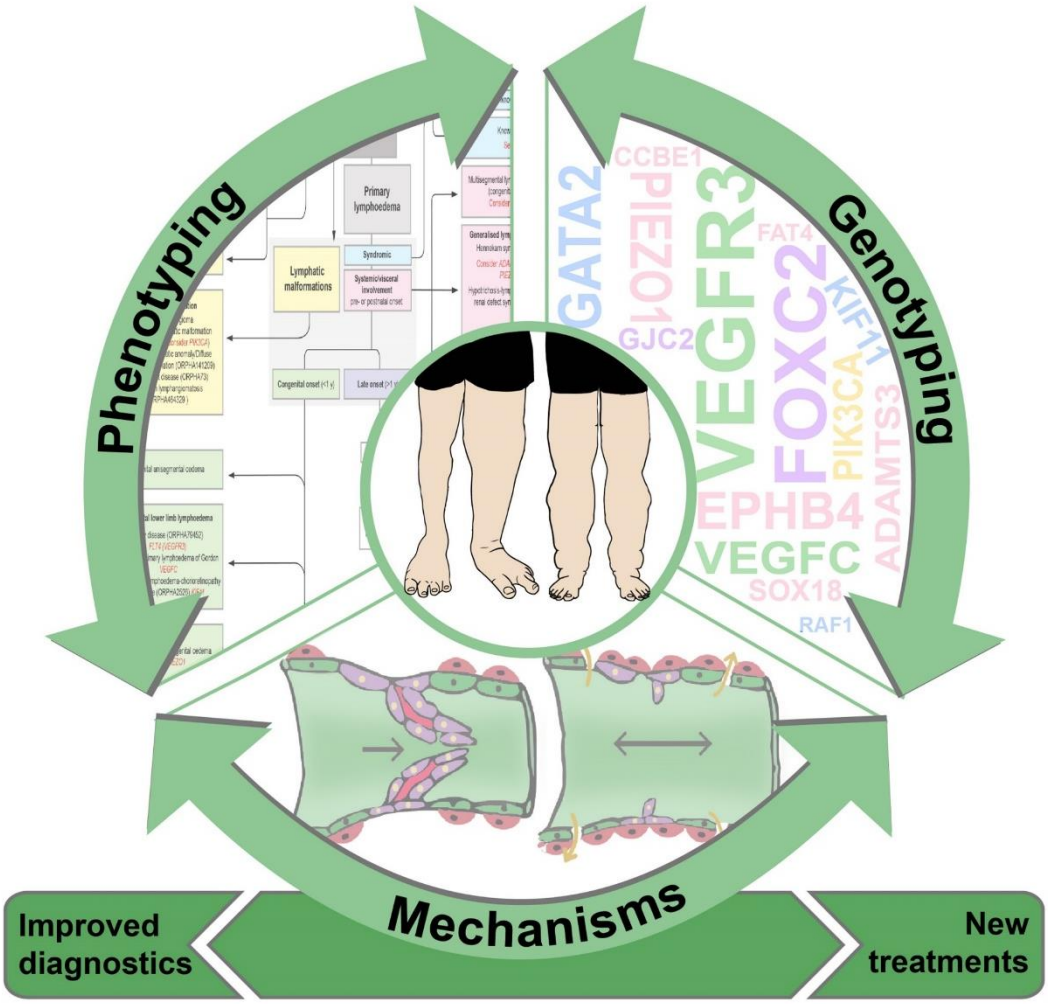
Abstract

Primary lymphedema is a long-term (chronic) condition characterized by tissue lymph retention and swelling that can affect any part of the body, although it usually develops in the arms or legs. Due to the relevant contribution of the lymphatic system to human physiology, while this review mainly focusses on the clinical and physiological aspects related to the regulation of fluid homeostasis and edema, clinicians need to know that the impact of lymphatic dysfunction with a genetic origin can be wide ranging. Lymphatic gene dysfunction can affect immune function so leading to infection; it can influence cancer development and spread; and it can determine fat transport so impacting on nutrition and obesity. Genetic studies and the development of imaging techniques for the assessment of lymphatic function have enabled the recognition of primary lymphedema as a heterogenic condition in terms of genetic causes and disease mechanisms. In this review, the known biological function of several genes crucial to the development and function of the lymphatic system are used as a basis for understanding normal lymphatic biology. The disease conditions originating from mutations in these genes are discussed together with a detailed clinical description of the phenotype and the up-to-date knowledge in terms of disease mechanisms acquired from in vitro and in vivo research models.



Clinical Highlights

Primary lymphedema is caused by an underlying genetic defect that compromises lymphatic function leading to localized lymph retention and swelling in any part of the body. The use of rigorous phenotyping, including imaging, has led to the development of a classification algorithm used to categorize lymphedema patients. Together with genotyping, through genetic studies and Next Generation Sequencing, several causal genes have been identified demonstrating that primary lymphedema is a complex heterogeneous condition. Animal and cellular models continue to be key in the discovery of pathogenic disease mechanisms.

Using genotypes hitherto identified, this review will give a collective insight into the genes critical for the development and maintenance of the lymphatic system, concentrating our focus on the genetic variants associated with lymphedema and other lymphatic abnormalities as well as the clinical phenotypes and physiological mechanisms. Multidisciplinary research efforts have led to improved diagnostics and understanding of lymphatic physiology and will hopefully soon lead to the much-needed new treatments.

1. Introduction

The lymphatic system has long been a neglected and poorly understood part of the vascular circulation, but its importance as a key contributor to human physiology and numerous diseases has now been realized (268, 288). This system, consisting mainly of lymphatic vessels and nodes, has a critical role in plasma and tissue volume homeostasis, as interstitial fluid removal in the steady state occurs via the lymphatics (225). Lymphatic vasculature transports antigens and antigen-presenting cells to lymph nodes, and therefore is a key player in immunity and tissue immunosurveillance (15, 54). The lymphatic system is also important for absorption of dietary fat in the gut, and for the regulation of peripheral tissue fat composition (407). This review makes use of what we have learned from the discovery of genes crucial for the correct development and maintenance of the lymphatic system to improve our understanding of lymphatic physiology. In particular, the review concentrates on the effect that gene mutations have in producing lymphedema and other lymphatic abnormalities as well as the physiological mechanisms whereby the clinical phenotype is produced. This review does not supersede classic reviews such as Yoffey and Courtice (411) but hopefully adds a new dimension driven by these revealing genetic advances.

1.1 Basic structure and physiology of the lymphatic system

Unlike the blood vascular system, which is a closed circuit, the lymphovascular system is predominantly a unidirectional drainage network of vessels and lymph nodes that are positioned at intervals throughout the drainage routes, consisting of vessels of ever-increasing size (Figure 1A,C). This specialized vessel network starts with blind-ended capillaries and ends in the great veins of the neck where lymph is discharged back into the bloodstream (Figure 1B). In contrast to the initial lymphatic capillaries that are composed of a single layer of endothelial cells, the larger collector vessels possess a basement membrane and smooth muscle cells (SMCs) and have intraluminal valves (Figure 1C, D).

The lymphatic endothelial cells (LECs) of the initial lymphatic capillaries overlap slightly and are connected by discontinuous adhesion junctions, also known as buttons (19). This type of architecture allows for a dynamic opening and closing of the endothelial cell junctions controlling the vascular permeability of the peripheral lymphatics. In fact, they act as a form of primary valve where interstitial fluid is permitted to enter the initial lymphatics, but cannot easily escape (Figure 1D) (343).

Once the interstitial fluid has entered the lymphatic capillaries it is called lymph. The transport of lymph through the lymphatic capillaries is thought to be dependent on cyclical changes in hydrostatic and osmotic pressures largely generated by skeletal muscle contractions, arterial pulsation, breathing, intestinal peristalsis and external body compression (42, 342, 398).

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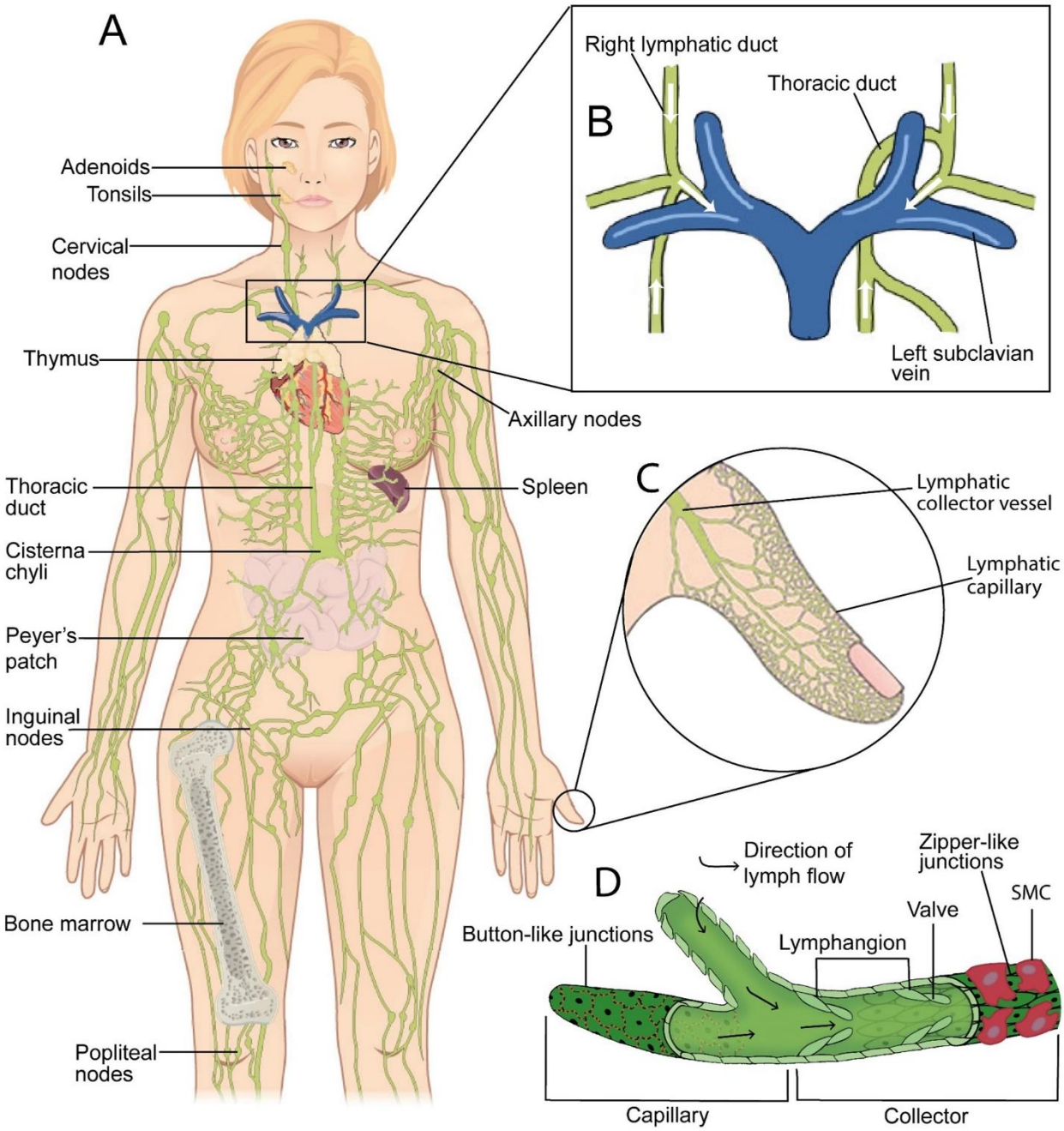

Figure 1 Anatomy of the lymphatic system. *(A) The lymphatic system includes the primary and secondary lymphoid organs and a series of lymphatic vessels, providing a one-way drainage route from all tissues back ultimately to the blood circulation via the great veins in the neck. In the primary lymphoid organs (bone marrow and thymus) immune cell production and maturation takes place, whereas secondary lymphoid organs (lymph nodes, spleen, and mucosa associated lymphoid organs such as Peyer's patch, tonsils and adenoids) are the sites for lymphocyte activation. The initial dermal lymphatic capillaries absorb interstitial material and fluid to make lymph which drains into lymphatic collectors. Lymph is pumped from the gut and lower half of the body to the cisterna chyli, a sac-like structure situated below the diaphragm, and then on to the thoracic duct. (B) The thoracic duct is responsible for the lymph drainage coming from most of the body with the exception*

of the right sides of the head and neck, the right side of the thorax and the right upper limb that drain primarily into the right lymphatic duct. Both ducts drain into the great veins of the neck. (C) The intricate dermal lymphatic capillary network drains downstream into the lymphatic collector vessels on route to the lymph nodes. (D) Oak leaf-shaped initial lymphatic capillary cells are connected via discontinuous junctions or buttons allowing the fluid to enter the system passively; the lymphatic collector endothelial cells, on the other hand, present with continuous junctions or zippers. Collectors differ from initial lymphatics by possessing intraluminal valves, smooth muscle cells (SMC) and a continuous basement membrane. Contractions of the lymphangions, the vessel segment between two valves, generate the pressure gradient ensuring the unidirectional flow of lymph. (Image in (A) modified from OpenStax College under a CC BY 3.0 license. (C) modified from OpenLearn Create under a CC BY-NC-SA 2.0 license. (D) Image 'Lymphatic capillary and collector' by St George's, University of London is licensed under CC BY-SA-4.0).

Lymph that has been passively propelled though the lumen of the capillaries eventually reaches the larger collecting vessels (Figure 1C, D). The adhesion junctions in lymphatic collector vessels are mainly continuous, zipper-like junctions, which are less permeable and thereby reduce the leakage of lymph (19, 337). The collector vessels possess SMCs surrounding their walls, which contract the vessels spontaneously and actively pump lymph onwards (383). Lymphatic collector vessel function is under the constant influence of various hydrodynamic factors as pressure or stretch, active and passive lymph pumps, intrinsic and extrinsic flow or shear stress, all of which modulate the unique contractile machinery of the collecting vessel to elicit both phasic and tonic contractions that are key to its role both as conduit and pumping system (61). Lymphatic collector vessels also have intraluminal valves (secondary valves) which ensure that lymph flows in one direction, downstream toward the lymph nodes (267, 338). Lymphatic collector vessels draining towards a lymph node are known as afferent lymphatics, and collector vessels draining beyond the lymph node are called efferent lymphatics.

The lymphatic system drains salts, proteins, cells, and fluids from the interstitial (extracellular) spaces of the body, back to the blood stream, either for ultimate disposal or recycling into the circulation (264). Put in the simplest possible terms, if the blood circulation is the supply route of the body's tissues, the lymphatic system is the cleansing and recycling service.

1.2 Physiological functions

1.2.1 The lymphatic system is essential for fluid homeostasis

The interstitial space is the extracellular space through which fluid passes on its way from blood capillaries to the initial lymphatics, thus, the source of interstitial fluid, and therefore lymph, is the transcapillary filtrate from the blood plasma (358). For years it has been taught through physiological textbooks, that according to Starling's Principles the venous capillaries

alone are responsible for the reabsorption of the bulk of filtrate generated by the arterial capillaries (Figure 2A). However, with the discovery of the glycocalyx and its role in fluid exchange in the capillary bed, there is substantial evidence (with important exceptions such as the renal cortex and medulla) that venous capillaries are not in a state of sustained fluid absorption as traditionally described. Additional processes are in action that Starling was not aware of, and a study of those including new measurements of the Starling pressures in humans, plus direct measurement of fluid exchange in blood capillaries, provides the proof that there is a continuous, but dwindling, state of filtration along the entire length of the blood capillary bed (Figure 2B) (225, 398). With the revised Starling Principle, it has been demonstrated that the most important factor in the prevention of transcapillary plasma filtrate accumulating within the body's tissues is lymph drainage (225, 261).

One element that ensures this process of blood capillary filtration in the steady state, is the glycocalyx. The glycocalyx is a jelly-like protective layer lining the luminal surface of the blood capillaries. It is produced by the blood endothelium and provides a protective barrier between the flowing blood and the vascular endothelial cells (VECs). The glycocalyx is semipermeable acting as a molecular filter and where it overlies an intercellular space, the plasma filtrate can diffuse through the glycocalyx layer into the sub-glycocalyx space and exit via the intercellular cleft into the interstitial space (Figure 2C) (394). This is thought to be the primary route for most of the filtrate.

The only time the blood capillaries reabsorb interstitial fluid, is when the Starling pressures change, such as with a fall in blood pressure. In the steady state, there is always a low protein concentration within the sub-glycocalyx space providing filtration occurs (Figure $2C_1$). If filtration declines or ceases due to a change in capillary hydrostatic pressure (such as with a decrease in blood pressure), less plasma filtrate leaves the capillaries (Figure $2C_2$). This can lead to a transient period of venous reabsorption as fluid will diffuse from the interstitium via the sub-glycocalyx space into the capillary lumen to help restore blood volume and blood pressure (Figure $2C_3$). Consequently, interstitial proteins will begin to diffuse up into the sub-glycocalyx space which will increase the protein concentration (Figure $2C_4$). Even then, and despite maintenance of low blood pressure, this protein accumulation reduces the venous reabsorption force and encourages the restoration of fluid filtration and solute washout into the interstitium (262). This way, the sub-glycocalyx acts as a regulator for filtration (162, 225).

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Fig. 2

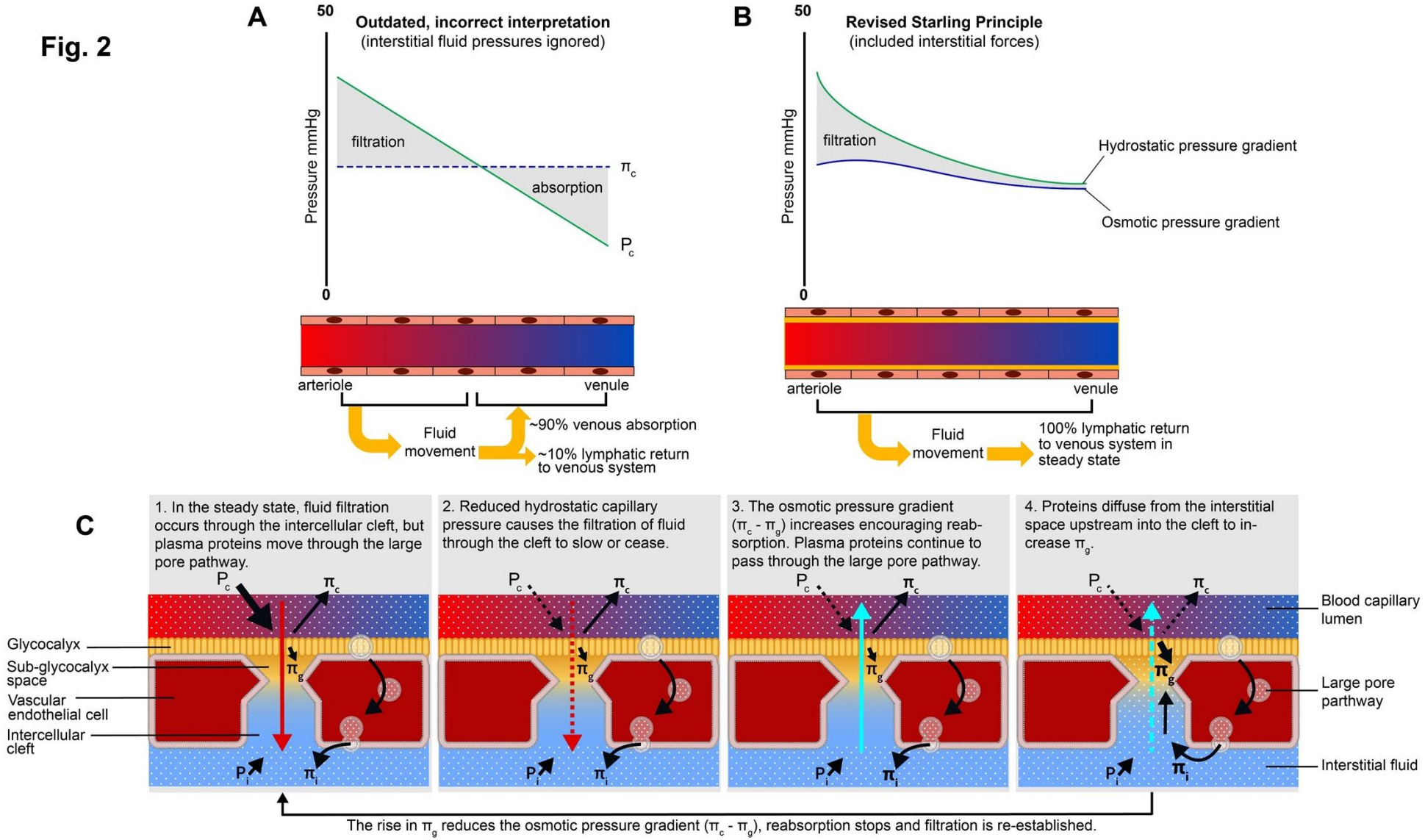

Figure 2 The revised Starling Principle and fluid homeostasis. *The Starling Principle states that fluid movement between blood and the interstitium is broadly determined by differences in hydrostatic pressure and colloid osmotic pressure between the blood plasma inside the blood capillary and the interstitial fluid in the surrounding tissues. (A) The traditional interpretation of the Starling Principle states that when blood capillary hydrostatic pressure (Pc) was larger than the plasma colloid osmotic pressure (πc) fluid would filter out of the capillaries (left yellow arrow). As the hydrostatic pressure begins to decline towards the venous end of the capillary, plasma colloid osmotic pressure was considered greater than the capillary hydrostatic fluid pressure. This would drive reabsorption and fluid would re-enter the venules by osmotic attraction (right yellow arrow). If true, then 90% of all interstitial fluid would re-enter the venous circulation while the remaining 10% would drain via the lymphatic system. This interpretation is incorrect. (B) With the direct measurement of interstitial hydrostatic fluid and colloid osmotic pressures, together with the discovery of the glycocalyx (or the "small pore pathway"), the old theory (A) has been disproven. Revision of the Starling forces has shown that, in the steady state, the forces driving filtration (green line) exceed the forces opposing filtration (blue line). Hence net, but dwindling, filtration will occur along the entire length of the capillary (grey area between the blue and green lines) with only periods of transient venous reabsorption as explained in C (when Starling forces temporarily change). This means that the bulk of interstitial fluid is drained in the lymph (yellow arrows). (C) Details of the blood vessel wall including the glycocalyx (yellow) are shown. The glycocalyx controls the local protein concentration within the intercellular cleft of the capillary wall, and hence the osmotic absorption gradient. If filtration declines or ceases, due to a drop in capillary pressure, venous absorption can occur. Due to reversed fluid movement, proteins in the interstitial fluid are drawn into the cleft where they accumulate in the sub-glycocalyx (as they cannot cross the glycocalyx). This raises local osmotic pressure of the sub-glycocalyx space to discourage reabsorption and ensures a return to a state of filtration. Therefore, according to the revised Starling model, venous absorption will only be transient. Pc = capillary hydrostatic pressure; Pi = interstitial hydrostatic pressure; πc = capillary plasma colloid osmotic pressure (COP); πg = sub-glycocalyx COP; πi = interstitial COP. (Image 'Revised Starling Principle' by St George's, University of London is licensed under CC BY-SA-4.0).*

The interstitial space is the primary source of lymph and is a major fluid compartment in the body (30). Thus, if blood capillaries, in the steady state, are in a constant state of filtration, then control of interstitial fluid volume depends critically on lymphatic function in most tissues, and interstitial fluid pressure is one of the main drivers for lymph drainage in normal skin and subcutaneous tissue. For this reason, one of the main functions of the lymphatic system is tissue fluid homeostasis, not just of the interstitium but also of body fluid generally.

1.2.2 The lymphatic system is essential for immunity and immunosurveillance

It has been known for some time that lymphatics have an important immunosurveillance function, as they represent the principal route of transport for leucocytes and soluble antigens, from peripheral tissues to their draining lymph nodes (70). Indeed, LECs are one of the first cells that come into direct contact with peripheral antigens, cytokines and immune cells during a response to infection (Figure 3A). LECs will be capable of directly determining

the adaptive immune response by influencing immune cell trafficking, promoting T-cell tolerance, and mediating T-cell homeostasis (54, 196).

Therefore, lymphatics do not act just as conduits, and as recent studies have shown, the lymphatic endothelial cells lining lymphatic vessels are known to take an active role instead in shaping both innate and adaptive immunity. While it is mostly interstitial fluid pressure that passively drives fluid into the lymphatics, immune cells are actively drawn to the lymphatic vessel through chemotaxis (Figure 3A). The best example is dendritic cells (DCs), which express the chemokine receptor CCR7 on their surface. LECs, through release of CCL21, induce directed chemotaxis of dendritic and other immune cells, which then migrate to, and into, the lymphatic vessel (54, 314). Further intraluminal gradients of chemokine control the migration of DCs through the lymphatic capillaries through crawling, and once in the lymphatic collector vessels, the DCs will be passively transported with the lymph flow to the lymph nodes. When afferent lymphatic vessels bring antigen and antigen presenting cells to the lymph nodes, dendritic cells accumulate in the subcapsular sinus, and then migrate through the floor of the sinus into the T-cell zone to present the foreign antigen to T-cells (Figure 3A).

It is evident that lymph nodes provide a specialized microenvironment for the confluence of migratory immune cells (314) and the efficient filtering of foreign matter before entering the blood circulation. They are primarily responsible for screening of pathogens such as viruses and bacteria, but also respond to cancer cells and other antigens deemed foreign e.g. allergic reaction to a drug. The lymphatic system also relies on the lymph nodes to filter and remove cellular waste, for example, debris from apoptotic cells.

Lymph nodes and other secondary lymphoid organs (e.g. the spleen) are formed during embryogenesis. Primary lymphoid organs, bone marrow and thymus, are the sites for immune cell production, whereas secondary lymphoid organs are sites where lymphocytes are activated (Figure 1A) (377). The term Tertiary Lymphoid Organs (TLOs) has been introduced to describe inducible ectopic lymphoid aggregates in tissues as a result of chronic inflammation (326). Lymph node formation is not yet well understood, but recent work using mouse models suggests that at strategic sites, usually in areas with lymphatic vessel bifurcations, extravascular mesenchymal cells start producing the chemokine CXCL13. This attracts hematopoietic-derived lymphoid tissue inducer (LTi) cells which migrate from nearby veins, and on interaction with the CXCL13-expressing cells, these become lymphoid tissue organizer (LTo) cells (29, 377). The crosstalk between the LTo and LTi cells promotes further signaling and lymph node formation (38). Lymphatics are indispensable for the initiation of the lymphatic node formation and contribute to the expansion and maturation of the developing node. Interstitial fluid from the nearby venules circulates through the developing node, and its flow is believed to potentiate the CXCL13 signal. At later stages, lymphatic collecting vessels ensure the efficient transport of LTi cells, and the formation of the lymph node capsule and subcapsular sinus. If lymphatic collecting vessel maturation is

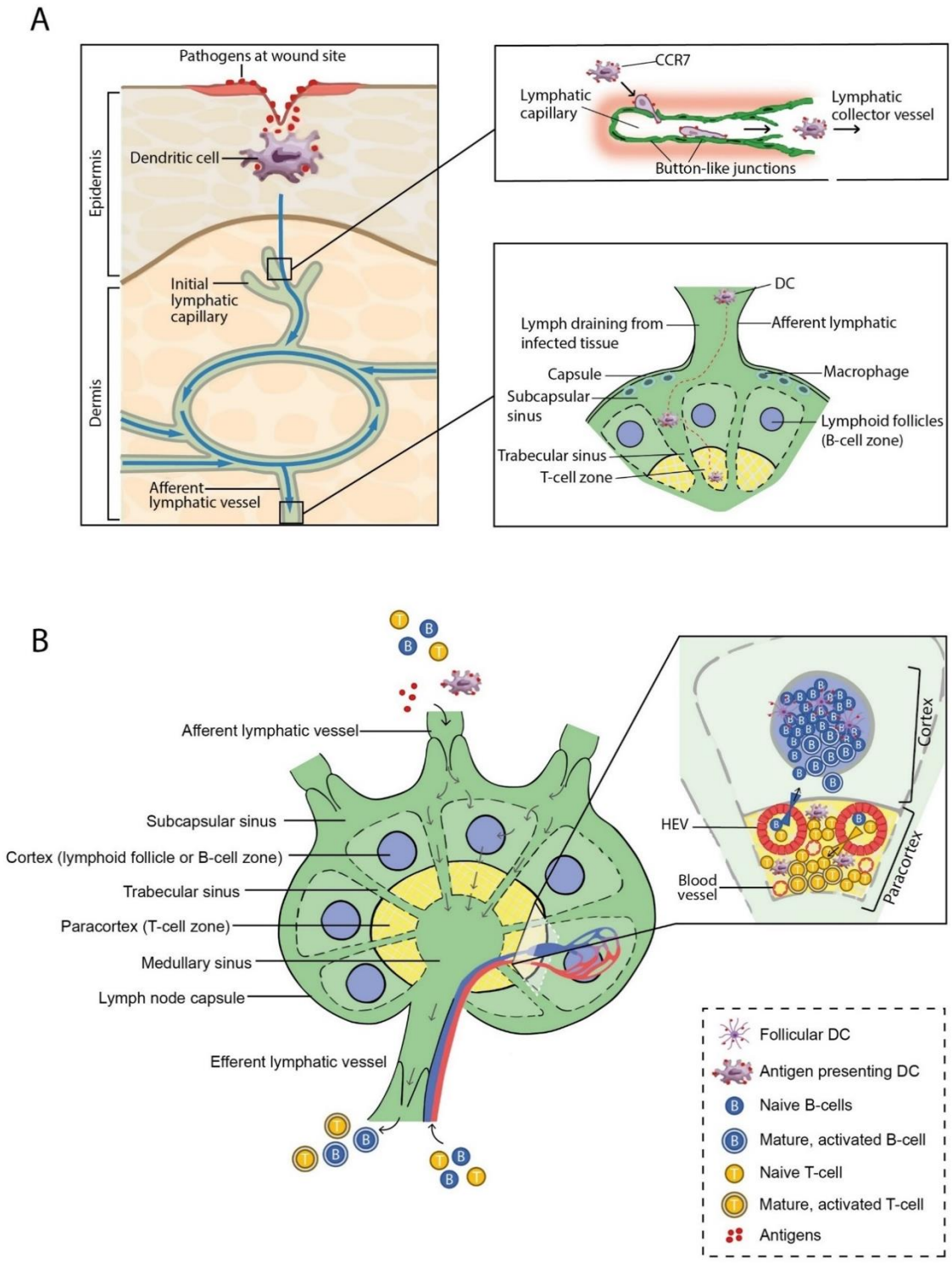

Figure 3: The lymphatic system facilitates immune cell trafficking to the lymph node and adaptive immunity*. (A) The lymphatic system does not just act as passive conduits for lymph but also plays an active role in innate and adaptive immune responses. For example, during wound healing an inflammatory response is initiated, and skin resident macrophages phagocytose invading microorganisms to clean the wound. Dendritic cells (DCs) are central to the initiation of primary immune responses by*

presenting the pathogenic antigens (red dots) on their surface. Chemokines released by LECs attract and direct the DCs towards the initial lymphatics (lymphatic capillaries). The DCs actively squeeze through the endothelial flaps (primary valves) created by the discontinuous button-like endothelial junctions. When inside the initial lymphatic, the DCs crawl along the lymphatic endothelial surface also guided by a chemokine gradient. On entering the lymphatic collector vessels, the DCs are transported passively in the direction of lymph flow to the lymph nodes where they initiate adaptive immunity. Thus, the positioning of lymph nodes along lymph drainage pathways (Figure 1), allows particles to be filtered and the lymph fluid to be screened for foreign material, particularly antigen from microbes, to induce specific immune reactions. (B) In a mature lymph node, we can distinguish three main regions: the cortex, the paracortex and the medulla. Naive lymphocytes enter lymph nodes mainly via the blood (High Endothelial Venules, HEVs) but also via afferent lymphatics. HEVs are specialized blood vessels responsible for the supply of naive lymphocytes to the lymph node. After crossing the endothelium of the HEVs into the lymph node (see insert), lymphocytes migrate to distinct areas, the B- and T-cell zones; CXCR5-expressing B-cells migrate towards CXCL13 secreted by follicular DCs, and CCL19 expressed by DCs attract T-cells via CCR7 receptors. A highly orchestrated process involving antigenic presentation by the DCs then turns naive lymphocytes into activated lymphocytes. Activated immune cells (effector cells) will leave the lymph node through the efferent lymphatics towards the next lymph node for further immune refinement until eventual discharge back into the blood circulation. Arrows indicate direction of flow. (Image 'Lymphatic system and immune function' by St George's, University of London is licensed under CC BY-SA-4.0).

prevented, for example due to genetic loss of the transcription factor FOXC2, the maturation and growth of the lymph node is stunted, with evidence of scattered LTi cells that never reach the growing lymph node (38).

As briefly mentioned, the interaction between the venous and lymphatic vascular beds is crucial to initiate lymph node morphogenesis. This lymph-venous crosstalk continues in the mature lymph node via the specialized 'high endothelial venules' (HEVs), which are found in secondary and tertiary lymphoid tissue (Figure 3B). These venules absorb fluid which leads to a more concentrated efferent lymph (2) and they actively control naïve lymphocyte trafficking from the blood circulation into the paracortex of the lymph node (5). Thus, the HEVs are an important component of the adaptive immune system. Once the naïve lymphocytes have entered the lymphoid tissue, T-cells survey dendritic cells present in the paracortex, whilst B-cells continue to move to lymphoid follicles in the adjacent cortex where they survey follicular dendritic cells (137). When the immune cells have been activated, they leave the lymph node through the efferent lymphatic vessel and then travel through afferent vessels to the next lymph node in the series (Figure 3B). Like lymph nodes, other secondary lymphoid organs such as Peyer's patches, spleen and tonsils, also provide optimal interaction of immune cells with invading pathogens (327).

The efficiency of the lymph nodes is usually very high. However, if they are overwhelmed by high concentrations of foreign matter, they become swollen and the cleansing efficiency may reduce. If lymph-borne pathogens manage to bypass the strict surveillance of the

draining lymph nodes, they can take advantage of the lymphatic system to gain access to the systemic circulation where they can disperse and cause illness (195).

An intact lymphatic system is also necessary for immunization. Using transgenic mice, which lacked dermal lymphatic capillaries but maintained intact lymph nodes and otherwise normal lymphatic vasculature, it was shown that intradermal vaccination of these mice led to a drastically reduced antibody response, indicating the important role dermal lymphatics play in mounting a competent adaptive immune response (368).

Furthermore, dermal lymphatics appear to be a determinant for boosting immune self-tolerance. In these same studies the lack of lymph drainage led to autoimmunity in aged mice. During the constant screening for foreign antigen ('non-self'), the lymph nodes must be accepting and tolerant of self-antigen ('self-tolerance') released from peripheral tissues, and the ceaseless flow of lymph through the system assures that this tolerance is maintained. If this process fails, then immune responses to self-antigen can occur, and autoimmune disease development is triggered (54).

1.2.3 The lymphatic system is essential for fat absorption and transport

Lymphatic vessels also provide lipid transport. In the gut they are directly responsible for the absorption of dietary fat, but there is substantial evidence that lymphatic function is also important for the transport of peripheral fat, participating for example in the reverse cholesterol transport to the liver.

The lymphatics continuously help to deliver nutrients to tissues. Dietary lipids are packaged into chylomicrons in the small intestines. Since these fat globules are too large to move across blood capillary walls they are transported via the lacteals (the initial lymphatic capillaries of the small intestines), where they mix with lymph to become chyle (Figure 4A). The lymphatic vessels then transport the chyle via the mesenteric lymph nodes and cisterna chyli up through the thoracic duct into the venous circulation.

Defects in the lacteal lymphatics can cause problems with lipid uptake by the intestine. For example, chylomicrons were retained in the small intestine in the *Chy* mouse model, with a heterozygous germline mutation in the tyrosine kinase domain of Vegfr3, leading to the inability of fat globules to enter the lacteals (347). In patients with intestinal lymphangiectasia, the pathological enlargement and/or blockage of the lymphatic vessels or paucity of lymphatics in the small intestines result in malabsorption of nutrients. The tortuous and dilated lacteals of the mucosa and submucosa continually leak protein-rich lymph into the bowel, and obstruction due to lymphatic hypoplasia or idiopathic malformation contributes to pressure-induced rupture of cystically dilated lymphatic vessels (Figure 4B).

VEGFR2/VEGFR3 signaling through DLL4/Notch is important for lacteal maintenance and function and deletion of *Dll4* in lymphatics led to lacteal atrophy and an increase in zipper

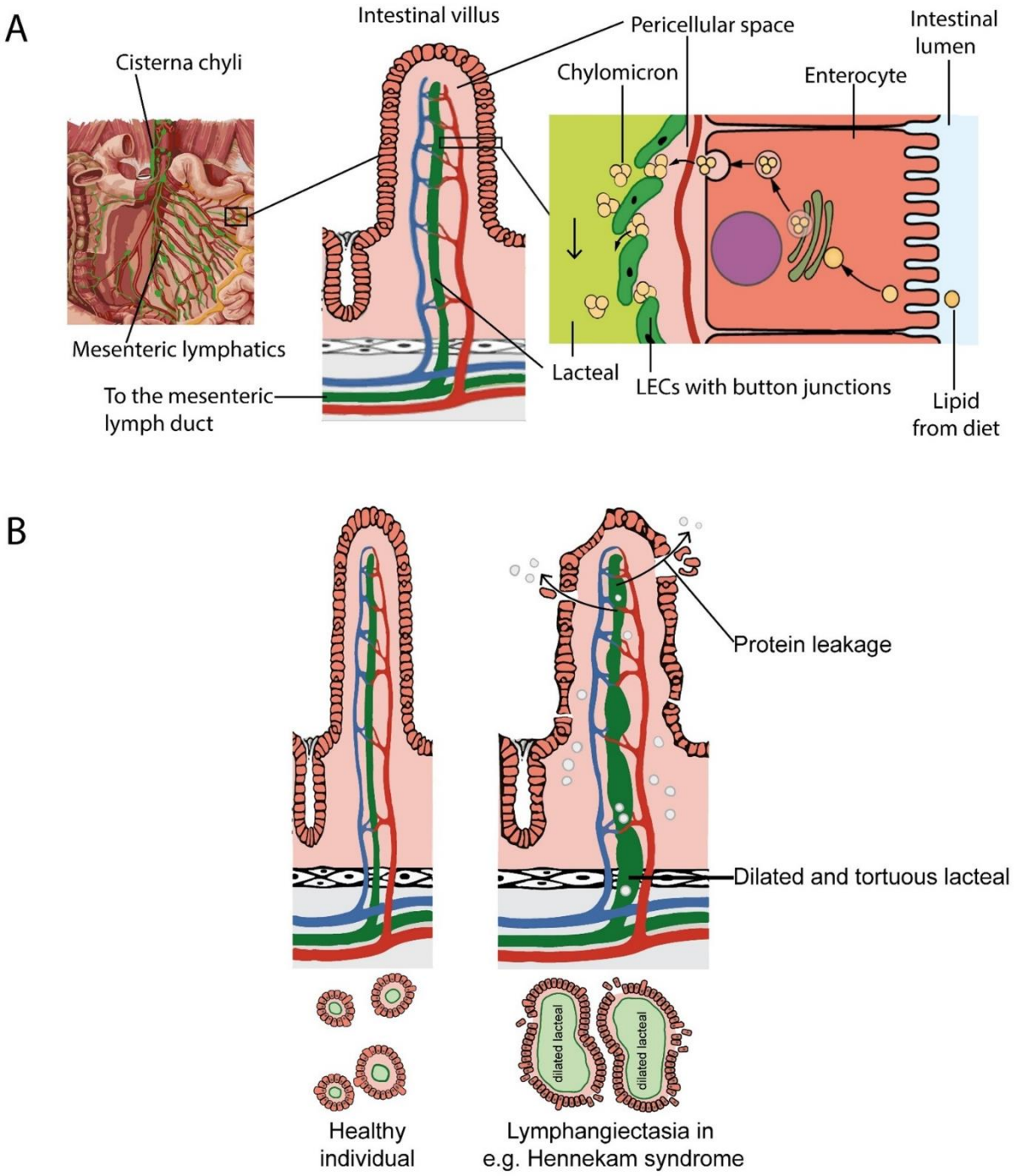

Figure 4 The lymphatic system is essential for fat absorption and its transport from the gut. *Lacteal function is compromised in some forms of primary lymphedema. (A) Lacteals, or intestinal lymphatic capillaries, are located in the intestinal villi surrounded by arterial and venous capillaries. Lacteal LECs resemble initial lymphatics but they are modified for fat absorption. Dietary lipids such as fatty acids and monoglycerides enter from the gut lumen on the apical side of the enterocytes where they combine with proteins to form chylomicrons. Discontinuous junctional openings on the lacteal wall regulate chylomicron entry into the lymphatic capillary in what is thought to be an active process involving, for example, the VEGFA/VEGFR2 or DLL4/Notch signaling pathways. Once inside the lacteals, chylomicrons are transported with the lymph via the mesenteric lymph nodes and collecting vessels into the*

cisterna chyli and through the thoracic duct up to the venous circulation at the subclavian vein. (B) Intestinal lymphangiectasia is characterized by abnormal, enlarged, and blocked lacteals in the small intestines resulting in malabsorption. Episodes of abdominal pain and diarrhea follow fat ingestion. Diagnosis is achieved by examining the intestinal wall through endoscopy and by taking a small bowel biopsy to visualize the distended lacteals. The tortuous and dilated lacteals of the mucosa and submucosa continually leak protein-rich lymph into the bowel. Mutations in CCBE1 are known to cause intestinal lymphangiectasia in Hennekam syndrome type 1. In the lacteal transverse sections, arterial and venous capillaries have been omitted for simplicity. (Image 'Lymphatic system and lipid absorption' by St George's, University of London is licensed under CC BY-SA-4.0).

junctions, resulting in the inability to take up chylomicrons (31). A high level of VEGFA availability increased signaling through VEGFR2, leading to more zipper junctions and preventing the uptake of chylomicrons into the lacteal (418). Likewise, lacteal atrophy caused by postnatal deletion of *Vegfc* impaired the absorption of lipids and led to steatorrhea (282). With this, it is becoming evident that dietary lipid absorption by the lymphatics is a dynamically controlled process, however, more research is needed to elucidate its complex molecular regulation. See Cifarelli & Eichmann (68) and Hokkanen et al. (156) for recent reviews on the topic.

In addition to dietary lipid absorption the intestinal lymphatics are still important for fluid absorption in this tissue. In humans, the liver contributes 30-50% to the thoracic lymph volume and the intestine makes the second greatest contribution to the total lymph bulk, especially after a meal. Intestinal lymph flow increases more with lipid than glucose absorption. Moreover, although the small intestine is generally considered an absorptive organ, it can be induced to secrete fluid under certain conditions (217). This presumably is what happens when intestinal lymphatics are disrupted causing diarrhea in some forms of primary lymphedema.

As mentioned at the beginning of this section, it is not only lipid transport from the gut that is controlled by the lymphatics. Peripheral tissue lipid balance also appears to be partly under lymphatic control, and several studies have shown that the lymphatics are critical for reverse cholesterol transport. When HDL transports cholesterol out of cells in the peripheral tissues, the lymphatic vessels conduct these particles into the bloodstream and back to the liver for its excretion through feces (315). Obstruction of lymphatic vessels in mice, either surgically or through genetic disruption of lymphatic function, impaired reverse cholesterol transport (230, 249) and led to increased atherosclerotic plaque formation (312). Interestingly, on the other hand, mice with hypercholesterolemia presented with edema of the paw and tail, dilated initial lymph vessels and reduced lymphatic flow (229).

Lymphedema can lead to obesity

The mechanism for the association between fat hypertrophy and lymphedema is not fully understood but it has been shown that some mouse models with impaired peripheral lymphatics present with adipose hypertrophy. For example, the *Chy* mouse (mentioned

above), which lacks dermal lymphatics and develops lymphedema, shows fat deposition in the skin (194, 328). Mice haploinsufficient for *Prox1*, a master regulator of lymphatic differentiation, develop adult onset obesity due to defective leaky lymphatic vessels particularly in the mesentery. Chyle collected from these mice promoted adipogenesis *in vitro* (102, 146). If chyle can also promote adipocyte hypertrophy *in vivo*, this would support the idea of some type of "crosstalk" between the lymphatic vasculature and adipose tissue, linking lymphatic dysfunction with obesity in humans.

In patients who have developed secondary lymphedema following breast cancer treatment, a longstanding arm swelling can be predominantly due to fat accumulation (36, 48, 153). It is thought that the chronic lymphedema is causing an inflammatory response which promotes adipose hypertrophy (416). Moreover, increased adipose tissue leads to increased collagen in the ECM (fibrosis) which in turn may exacerbate the lymphedema. Liposuction has proven successful in reducing arm volume long-term, but only through continuous use of compression garments postoperatively (153). For any of the genetic forms of primary lymphedema we are not aware of any reports indicating that lymphedema can lead to obesity.

Obesity can lead to lymphedema

We have just described that lymphatic dysfunction can lead to obesity. However, the reverse relationship also appears to be true, and it has also been suggested that obesity may be a cause of lymphedema in humans (139). Obese mice have significantly impaired lymph transport and dendritic cell migration, as well as significantly smaller peripheral lymph nodes with loss of normal T- and B-cell architecture (395). Adipose tissue accumulation caused morphological and functional changes of collecting lymphatic vessels in the extremities of diet-induced obese K14-VEGFC mice (35). More importantly, obesity increased inflammation and fibrosis leading to more severe lymphedema (336). Inflammation induced by adipose tissue caused increased lymphangiogenesis (capillary lymphatic hyperplasia), collecting lymphatic vessel dilation and loss of SMCs on collecting lymphatics, leading to impaired lymphatic contractile function and reduced lymph flow (336).

There is now convincing evidence that with an increase in adipose tissue volume, the lymphatics become dysfunctional either as a consequence of obstruction due to direct compression or indirectly due to fibrosis, which can also cause compression of the lymphatics. Others have suggested that it is the increased obesity-related inflammation that is the main reason for lymphatic dysfunction. What is evident is that there is much still to learn about this bi-directional crosstalk between the lymphatic and adipose systems.

1.3 Clinical consequences of lymphatic dysfunction

Lymphatic system dysfunction can contribute to several pathological conditions, the main one being lymphedema. Disturbances to immune cell trafficking from impeded lymph

drainage contribute to high levels of infection and sepsis. Cellulitis/erysipelas is one of the most common medical conditions presented to hospital emergency departments (66) and many of these infections are due to lymphedema (311) which in some cases may not be clinically obvious (83). Other diseases which lymphatic dysfunction may contribute to include, Crohn's disease, obesity, hypertension, atherosclerosis, recurrent infections (other than cellulitis/erysipelas), disturbed wound healing and tissue repair, asthma and chronic airways disease, glaucoma, organ rejection, and autoimmune disease (268, 306). In addition, as mentioned before, lymphatic vessels provide one of the main routes for cancer spread, contributing to the metastatic process (268, 288, 306).

1.3.1 Lymphedema

Lymphedema is caused by an insufficiency in lymph drainage, which leads to a build-up of lymph within the body's peripheral tissues. Any chronic edema arises from an imbalance between fluid input (capillary plasma filtration volume) and output (lymph drainage capacity) (150). In the steady state, there is sufficient capacity as the removal of lymph by far exceeds the production of filtrate (93, 422). If the transcapillary fluid filtration into the interstitial space exceeds the lymph drainage for an extended period of time, interstitial fluid volume increases and so edema in the interstitial space will occur (398). This is seen clinically as pitting when pressure from a digit will displace fluid away from the compressed tissue, leaving a pit or indentation of the tissue when the pressure is removed (pitting edema). The pit disappears immediately in healthy tissue, but in lymphedema patients, the redistribution of the excess fluid is slower.

Therefore, lymphedema is strictly edema occurring when a failure of lymph drainage is the dominant cause. However, all chronic edema reflects some degree of lymph drainage failure, and in many other pathological situations not related to lymphedema, lymph drainage may also be overwhelmed by high fluid filtration e.g. heart failure.

When the insufficiency in lymph drainage is intrinsic to the lymph system, then it is referred to as primary lymphedema. If extrinsic factors such as surgery or infection can be identified as responsible for the damage to the lymph drainage routes, then it is referred to as secondary lymphedema.

Lymphedema manifests with swelling usually of one or both legs, but other segments or parts of the body can be involved, such as arms, face, trunk or genitalia. Within internal organs, lymphatic dysfunction manifests with a build-up of lymph in inner body cavities, e.g. pleural and pericardial effusions and ascites, while disturbances to lymph drainage in the gut produce protein losing enteropathy and chylous reflux (Figure 4B).

Lymphedema, primary or secondary, may result in enormous swelling that can be painful and compromise mobility and function. The disfigurement from any form of lymphedema often leads to physiological and psychological morbidity. In addition, we have already

mentioned that lymphedema can be associated with severe skin changes, and predispose to recurrent infections and sepsis which can be life threatening (361). As will be seen later, some forms of primary lymphedema can present with abnormalities of systemic immunity.

1.3.2 Primary Lymphedema

Primary lymphedema, due to an intrinsic or inborn defect of the lymphatic system, is a highly heterogeneous condition in terms of age of onset, associated manifestations or underlying cause. Presentation may be *in utero*, at birth, later in childhood, or even in adulthood. Some forms of primary lymphedema manifest with life-threatening complications, e.g. fetal hydrops, pulmonary or intestinal lymphangiectasia as in Hennekam Syndrome (148), or acute myeloid leukemia as in Emberger Syndrome (245). There are many different causes of primary lymphedema and many different patterns of presentation or clinical phenotypes (136).

1.4 Methods of investigation for lymphedema in humans

Unlike veins, the lymphatic vessels cannot easily be seen because they are of a small caliber and the lymph they transport is colorless. This makes it more difficult to image the lymphatic system and measure lymph flow than it is to visualize the blood vascular system and measure blood flow. For these reasons, the lack of competent investigatory methods has historically been a limitation for the advancement of human lymphatic research until recently. With the discovery of lymphatic molecular markers such as LYVE1 (20) and Podoplanin (40), the visualization of lymphatic vessels in tissue samples has become possible. **LYVE-1**, a close relative of the leucocyte receptor CD44, is the main receptor for hyaluronic acid (HA) on the lymphatic vessel endothelium. LYVE-1 is involved in regulating the entry of e.g. dendritic cells into peripheral capillary lymphatics and possibly supports their migration toward downstream lymph nodes for immune activation (173). **Podoplanin** is a small membrane glycoprotein which belongs to the mucin-type group of proteins. It is present primarily on the endothelium of lymphatic vessels. The podoplanin antibody D2-40 has become a robust immunocytochemical marker for lymphatic endothelium, widely used in many research laboratories. The identification of these specific markers as well as PROX1 and VEGFR3 has been a determinant in the exponentially increased interest in the study of the lymphatic system.

1.4.1 Imaging techniques

The first breakthrough in visualizing lymphatic vessels in humans, other than the original observations of chyle-infused mesenteric lymphatics in the middle ages, was achieved by Hudack and McMaster using vital dyes (163).

The development of conventional radiocontrast X-Ray lymphangiography (imaging of lymphatic vessels) and X-Ray lymphography (imaging of lymph nodes and vessels) enabled the imaging of the lymphatic collector vessels for the first time, and provided valuable information on anatomical abnormalities of primary lymphedema (Figure 5A) (208, 209).

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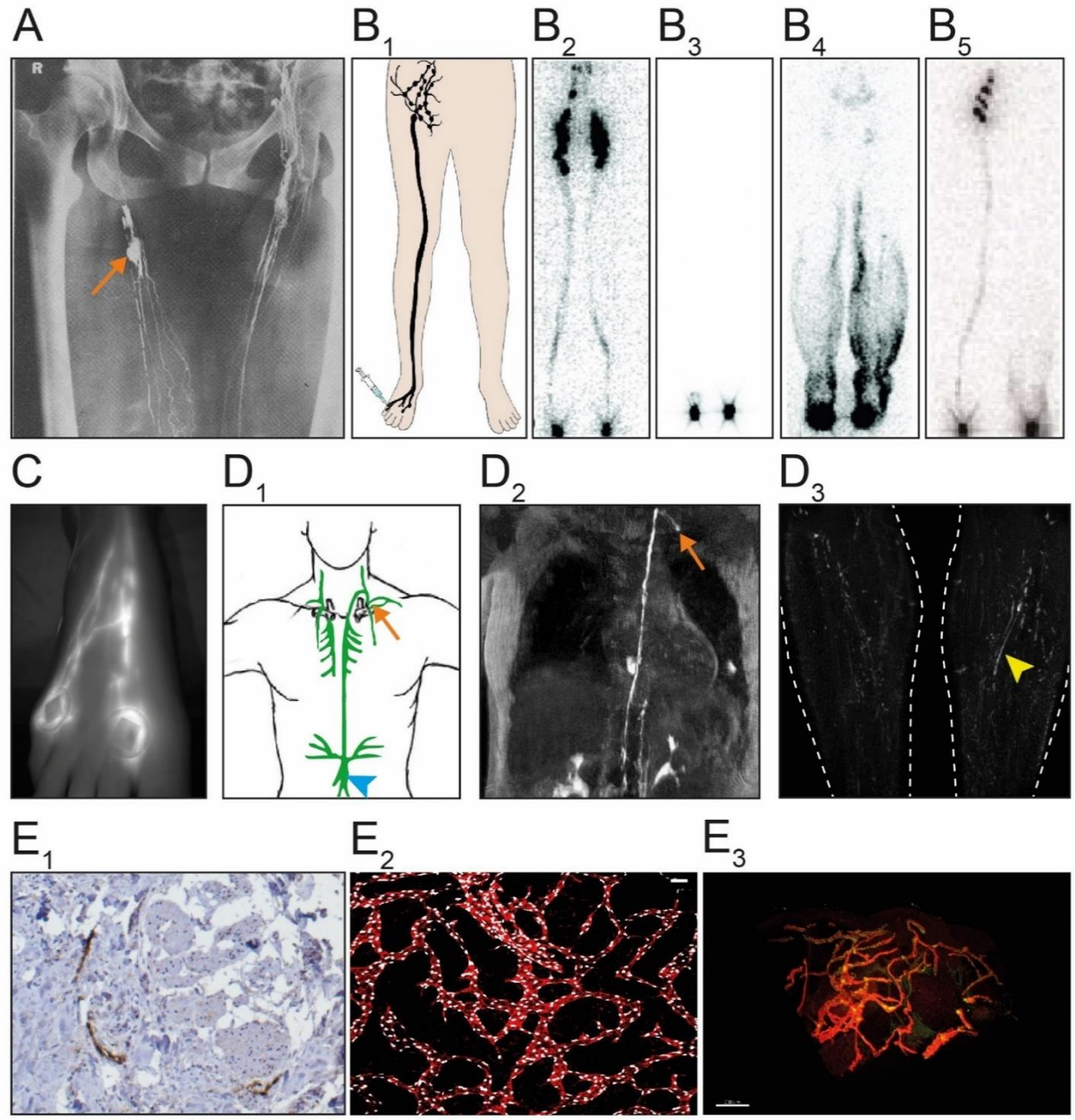

Figure 5 Methods of imaging the lymphatic system. (*A) Direct contrast X-ray lymphography involves the injection of an x-ray contrast agent e.g. Lipiodol. First, lymphatic vessels are identified by subcutaneous injection of a vital dye e.g. patent blue. Under local anesthetic, an incision is then made to expose the lymphatic vessel following the insertion of a needle into its lumen. The contrast agent can then be injected, and radiographs taken. Clear definition of lymphatic collectors and lymph nodes (arrow) can be achieved, but the procedure is invasive and rarely performed. (B1) Lymphoscintigraphy involves the injection of Technetium-99 into the web spaces between the toes (or fingers). Images using a gamma camera can be taken at specified time intervals and reveal lymph drainage channels with uptake of the contrast agent in regional lymph nodes. Removal of the contrast agent from the injection site and*

uptake in nodes reflect lymph transport (quantitative lymphoscintigraphy). (B2) Anterior view of a normal subject. (B3) Milroy patient carrying a VEGFR3 mutation showing no uptake by the initial lymphatics nor transport to the lymph nodes (functional aplasia). (B4) Lymphedema distichiasis syndrome patient carrying a FOXC2 mutation showing lymph reflux with dermal backflow seen as dark shading in the calf. (B5) Emberger patient carrying a GATA2 mutation exhibiting unilateral uptake, with the left leg showing no migration of tracer within the collecting lymphatics. (C) Indocyanine Green lymphography (ICGL) imaging of the right dorsal foot. Two indocyanine green intradermal injections are given in the toe web spaces followed by an immediate absorption of ICG into initial lymphatics. Excitation of the area of interest with laser or LED will emit fluorescence through the skin, that allows for real-time imaging with a near-infrared detector camera to visualize lymph drainage up the leg. Contractility of lymphatic collectors can be seen in real time. (D1+2) Dynamic contrast-enhanced magnetic resonance lymphangiogram (DCMRL) can image lymphatic vessels. Following bilateral injection of contrast under ultrasound guidance directly into an inguinal lymph node, central conducting vessels can be seen, like the cisterna chyli (arrowhead) and thoracic duct, highlighted in green in the drawing of the upper chest. The image is a T1 weighted MRI in which areas of contrast uptake appear bright demonstrating the thoracic duct terminating at the junction of the left subclavian and internal jugular veins, into which it drains (arrow). (D3) T2 weighted images demonstrate high signal in areas of static or slow-moving fluid. Vessel-like structures can be observed bilaterally in the legs (arrowhead). Whether these structures represent lymphatic or venous vessels remain controversial. Imaging of skin biopsies has advanced considerably with the development of modern microscopy techniques and the use of antibodies against lymphatic-specific markers. (E1) Immunohistochemistry (IHC) on paraffin-embedded 2D human skin sections showing podoplanin (PDPN) positive lymphatic vessels (brown DAB staining). Image taken at 20X magnification with a light microscope. (E2) Dermal lymphatic vasculature in the developing mouse embryo. Skin whole-mount preparation of E14.5 wildtype mouse embryos are visualized in 3D using a confocal microscope. Lymphatic endothelial cells are identified by Prox1 (white) and Vegfr3 (red) expression. Maximum intensity projections are shown. Scale bar = 100 µm. (E3). Whole-mount 3D human skin biopsy from a healthy control optically sectioned using a light sheet microscope and VIPAR analysis (164). Lymphatic endothelial cells are identified by PROX1 (green) and PDPN (red) expression. Scale bar = 100 µm. (Image credits: (C) courtesy of Dr van Zanten, St. George's University of London; (D2 and D3) Dr Ratnam, St. Georges Hospital NHS Trust, London; (E2 and E3) Dr Hägerling, Charité, Berlin. All other images are from authors' archive. Image 'Methods of imaging the lymphatic system' by St George's, University of London is licensed under CC BY-SA-4.0).

Even though it has been phased out in clinical medicine, the same principles for imaging central lymphatics following an intranodal injection of contrast are now being applied in the MRI-field (see below) (170).

Lymphoscintigraphy is currently the standard clinical investigation method and involves the interstitial injection of a radiolabeled tracer which is selectively absorbed by the lymphatic vessels, making it highly specific for investigating the lymphatic system (Figure $5B_1$). Tracking of the tracer from site of injection to the lymph nodes by gamma camera can delineate crude lymphatic vessels but only in 2D (Figure $5B_2$). This method does not give the same spatial resolution of lymphatic vessel structure as conventional lymphography. However, aspects of lymph drainage function within a whole limb and uptake in regional lymph nodes can be determined (204) and this technique can help to distinguish between different types of primary lymphedema (Figure $5B_{3-5}$) (335).

Recently, near-infrared fluorescence lymphatic imaging, otherwise known as Indocyanine Green Lymphography (ICGL), has been used to visualize the passage of dye within limb lymphatic vessels (Figure 5C), and to image relatively defined lymphatic vessels and their pumping function (316). ICGL has been primarily used for identifying lymphatic vessels suitable for lymphatic microsurgery, and in particular lymphaticovenous anastomoses (LVAs) (277, 286). Although ICGL only can detect quite superficial lymphatic vessels, it has a good spatial resolution and allows visualization of real-time movement of tracer as a surrogate for lymph. It is emerging as a promising clinical tool for imaging of lymphatic (dys)function in lymphedema patients (360).

MR lymphangiography (MRL) involves interstitial injection of a gadolinium-based MRI contrast agent. Intranodal MR lymphography has proven very useful for the visualization of central lymphatic anatomy, giving good detail of thoracic duct, cisterna chyli, and lumbar lymphatics (Figure $5D_{1-2}$) (94). This technique has become extremely useful in the visualization of thoracic lymphatic abnormalities, such as central conducting lymphatic anomalies in generalized lymphatic anomalies/generalized lymphatic dysplasia (63, 226). Protocols for peripheral limb MRL is not as well developed but could possibly allow the visualization of lymphatic collecting vessels (Figure $5D_3$). Dermal backflow in the collateral vessels and regional lymph nodes with high spatial resolution has been reported (236).

1.4.2 Tissue biopsy

Examination of affected tissue for cytopathology or histopathology has been the mainstay of diagnosis for many diseases, but not particularly lymphedema. Microscopic examination of 2D tissue sections limits the interpretation of 3D structures. Due to the nature of the technique, for example, one extended and coiled single vessel could be interpreted as multiple vessels in histological sections (Figure $5E_1$). The use of confocal microscopy on whole-mount preparations from embryonic mouse models has added an extra level of complexity to the visualization and interpretation of lymphatic networks (Figure $5E_2$). Recently, the development of light sheet microscopy has enabled digital 3D reconstruction, providing a direct visually interpretable and more comprehensive representation of the lymphatic vessels (Figure $5E_3$) (164). This new 3D imaging technique could prove a more

useful and informative tool for clinical histopathology in lymphedema patients in the near future.

1.4.3 Genotyping

In addition to improving the specificity of diagnosis for genetically derived heterogeneous diseases, and enabling a faster rate of gene discovery, Next Generation Sequencing (NGS), mainly exome sequencing, has permitted an expansion and redefinition of Primary Lymphedema disease categories (136). The ability to make a molecular diagnosis, such as the identification of germline mutations in inherited forms of primary lymphedema, or postzygotic mosaic mutations in lymphatic malformations, has transformed the management of genetically determined lymphatic disorders. Identifying the gene defect facilitates an accurate diagnosis by genetic testing, guides genetic counselling on e.g. inheritance risk, and informs on the natural history of the disease, the physiological function of that gene and the mechanism of disease. A molecular diagnosis enables the clinical features and other pathologies specific for that genotype to be defined, sometimes needed with urgency, e.g. a GATA2 mutation conveys a risk of leukemia so proactive monitoring of full blood count is required.

The use of rigorous clinical phenotyping, including imaging, led to the development of a classification algorithm of primary lymphatic anomalies (Figure 6) (74). Using this, patients were carefully categorized and DNA interrogated for gene mutations using Next Generation Sequencing, enabling the identification of several causal genes (76, 136). Together with supportive information from animal and cellular models, this has informed on the pathogenesis of primary lymphedema and genetically determined lymphatic diseases. Information about the genes and proteins that are fundamental for the development and function of the lymphatic system in health, has also been forthcoming. Using the genotypes identified to date as the basis of this review, we will attempt to give a collective insight into our current knowledge of the mechanisms causing primary lymphedema.

2. Development of the lymphatic system during embryogenesis

Lymphatic vessel development in humans starts around embryonic week six, after the onset of the blood circulation (298). During embryonic development, lymphatic vessels rapidly create a lymphovascular network through a highly stereotyped and spatially controlled lymphangiogenic program (409). In brief, the embryological development of the lymphatic system can be split into (i) LEC specification, (ii) expansion and (iii) maturation of the lymphatic tree. This developmental progression is directed by a similar, but separated, set of molecular mechanisms from the blood vasculature. Through interactions with VEGFR3, VEGFC stands as a critical regulator of this process (193, 283). The VEGFC homolog, VEGFD (the other ligand of the VEGFR3 receptor), appears to play a subtler role in regulating lymphatic vascular development compared to VEGFC (which is absolutely required for lymphatic development) (356).

The molecular control of the lymphatics does not stop here. Throughout the lifetime of the lymphatic system there is a raft of molecular signals that maintains the identity of the LECs. This allows for a healthy, functional lymphatic system, which also responds to the need for tissue remodeling and repair after an insult to the system (306).

Table 1. All genes and associated phenotypes covered in this review. *Alterations in most of the genes listed cause different types of disease. These genes have been selected because primary lymphedema is a major feature of the disease phenotype or because the role of the gene in lymphatic development is well documented. Some of the genes listed have no primary lymphedema associated with them (COUP-TFII) or no phenotype at all (PROX1), but because their role in initiating the process of lymphangiogenesis is so crucial, they have been included in this review. The genes have been color-coded according to which of the categories of primary lymphedema they are classified under in the St. George's Classification (see Figure 6). More than 20 genes are known to cause (germline) RASopathies, however, only RAF1 is covered in this review.*

Gene name	Gene MIM number	Phenotype associated with a primary lymphatic anomaly	Phenotype ID number	Inheritance
GJA1/Cx43	121014	Oculodentodigital dysplasia (ODD syndrome)	ORPHA:2710	AD
KIF11	148760	Microcephaly-lymphedema-chorioretinopathy syndrome	ORPHA:2526	AD
RAF1	164760	RASopathies (incl. Noonan syndrome)	ORPHA:536391	AD
ADAMTS3	605011	Hennekam-lymphangiectasia-lymphedema syndrome type 3	ORPHA:2136	AR
CCBE1	612753	Hennekam-lymphangiectasia-lymphedema syndrome type 1	ORPHA:2136	AR
EPHB4	600011	EPHB4-related LRFH	ORPHA:568065	AD
FAT4	612411	Hennekam-lymphangiectasia-lymphedema syndrome type 2 van Maldergem syndrome type 2	ORPHA:2136 ORPHA:314679	AR AR
PIEZO1	611184	PIEZO1-related GLD/LRFH	ORPHA:568062	AR
SOX18	601618	Hypotrichosis-lymphedema-telangiectasia-renal defect syndrome	ORPHA:69735	AD AR
FOXC2	602402	Lymphedema-distichiasis syndrome (LDS)	ORPHA:33001	AD
GATA2	137295	GATA2-deficiency syndrome (Emberger syndrome)	ORPHA:3226	AD
GJC2/Cx47	608803	Late onset 4-limb lymphedema	OMIM:613480	AD
VEGFC	601528	VEGFC-related congenital primary lymphedema of Gordon	ORPHA:79452	AD
VEGFR3/FLT4	136352	Milroy disease	ORPHA:79452	AD
BRAF *KRAS* *MAP2K1* *NRAS*	164757 190070 176872 164790	Mosaic RASopathies caused by postzygotic mutations		M
PIK3CA	171834	PIK3CA-related overgrowth spectrum (PROS)	ORPHA:530313	M
COUPTF-II	107773	Congenital heart defects, multiple types, 4 (but no primary lymphedema association reported)	OMIM:615779	AD
PROX1	601546	No reported phenotype		

Gene and phenotype MIM numbers taken from www.omim.org; ORPHA phenotype number from www.orpha.net. AD, autosomal dominant; AR, autosomal recessive; M, mosaicism due to postzygotic de novo mutations and only transmissible if the postzygotic event includes the germline (i.e. gonadal mosaicism). LRFH, lymphatic-related fetal hydrops; GLD, generalized lymphatic dysplasia.

In this review, human primary lymphedema phenotypes with mutations in genes involved in the development and maintenance of the lymphatic system will be summarized and the associated disease mechanisms caused by the distinct genotypes will be discussed. The molecules and their encoding genes selected for this review (Table 1) are either related to disorders where lymphedema is the main feature of disease (i.e. the pink, purple and green categories in the St. George's classification, Figure 6), or are related to syndromes where lymphedema is an important associated feature (i.e. linked to the blue category, Figure 6). Using insights from human genetic studies and *in vitro* and *in vivo* models, an outline of our current understanding of the role of these molecules in the developmental and physiological function of the lymphatic system will be given. The genes and molecules included can have more than one role during the development of the lymphatic system, but for simplicity the description of the human phenotype and genotype, and molecule function have been discussed in the section where they can be considered to have a first major role.

The cellular and molecular processes controlling the development of the lymphatic system are very complex and the list of genes and gene products involved is expanding each year. Not all genes with a role in the lymphatic system have an associated human clinical phenotype, therefore those genes have not been included here. For a more comprehensive overview of all the current knowledge related to the cellular and molecular processes controlling the development of the lymphatic system, the reader is directed to other reviews covering these aspects (79, 179, 373).

Furthermore, some of the molecules listed in this review also have a role(s) outside the lymphatic system. Sometimes these other functions of the molecules presented will be mentioned, but not covered in detail. For instance, PROX1 is involved in the differentiation of neuronal progenitor cells (188), VEGFC and its receptor VEGFR3 cause proliferation of the neuronal progenitor cells (159, 218), and PIEZO1 mechanosensitive channels are involved in axon regeneration (352). However, this review will only summarize the functional aspect of these molecules in relation to the lymphatic system and its development.

Genetic lineage tracing experiments in mice have shown that the majority of mammalian lymphatic endothelial cells (LECs) arise from a distinct subpopulation of endothelial cells in the cardinal vein, which become committed to a lymphatic cell fate (353) following controlled changes in the expression levels of key lymphatic specification markers. Recent investigations have shown the existence of additional sources of progenitor LECs. Through the process known as lymphovasculogenesis, lymphatic endothelial progenitor cells of a

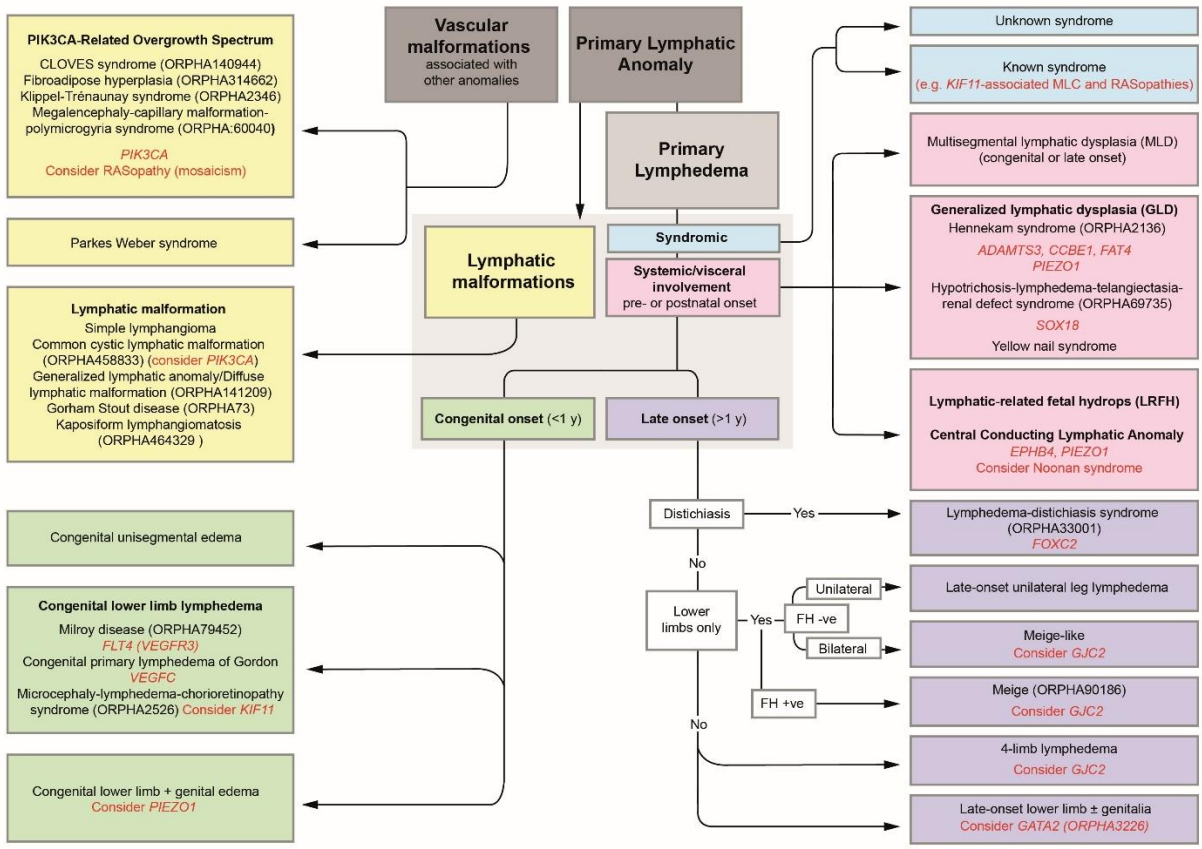

Figure 6: St. George's classification algorithm for primary lymphatic anomalies. *Clinical phenotyping together with genotyping have led to the division of patients with a primary lymphatic anomaly into five main groups, which are shown here as color-coded sections together with their various clinical sub-types of disease. This demonstrates that primary lymphedema is a complex heterogeneous condition for which several causal genes have been identified. Primary lymphedema is the major clinical feature in the green, pink and purple sections, whilst in the blue section lymphedema is not the dominant phenotypic feature. Phenotypes within the yellow section are thought to be caused by postzygotic mosaic mutations. Gene names (text in red italics) indicate the most likely molecular diagnosis for each subgroup, and in some cases, an alternative diagnosis is offered (gene names to "consider"). Only gene names included in this review are presented in this simplified version of the algorithm, for a comprehensive list of genes check the most current St. George's classification algorithm in (136). The indicated genes do not always explain the cause of disease in all patients in each group. For example, only 70% of Milroy disease patients are explained by mutations in FLT4/VEGFR3 (77). FH, family history; +ve, positive; -ve, negative; y, year. ORPHA, phenotype number from www.orpha.net. (Image 'St George's Classification Algorithm' shared by St George's Lymphovascular Research Group under the CC BY-SA-4.0 International license).*

non-venous origin contribute to the development of organ-specific lymphatic vascular beds such as the mesenteric or dermal lymphatic vasculatures (252, 357) or cardiac lymphatic vessel endothelium (210, 235, 255). This review focuses on LECs of a venous origin, and how venous blood endothelial cells become committed to a LEC cell fate, so the reader is directed to other reviews that further explore the non-venous sources of lymphatic

endothelial cells and their physiological implications (306, 344). There is also an increasing body of evidence giving insight into organ-specific lymphatic mechanisms and function. This review, however, will be biased toward dermal lymphatics, but further information on this topic can be sought in additional reviews (201, 404).

3. LEC fate specification

After morphogenesis of the dorsal aorta and the cardinal vein and the commencement of blood flow, the lymphatic system begins its development. From around embryonic day 8.5 (E8.5) in mice, Coup-TFII is expressed in all the endothelial cells of the cardinal vein and is important for promotion and maintenance of venous identity in venous endothelial cells (Figure 7A) (231, 412). At E9.0 a few cells in the jugular region of the cardinal vein, mostly scattered around the dorsolateral region, start expressing Sox18 (118, 353). The Sox18 expression is initiated after withdrawal of AKT inhibition on the RAF1/MEK/ERK pathway (89).

Around E9.5, Prox1 can be detected in this polarized group of LEC precursors (397). It has been shown that co-expression of COUP-TFII and SOX18 in venous endothelial cells activates PROX1 expression (95, 118, 158, 223, 354). This polarized expression of PROX1 in the cardinal vein indicates that the LEC specification program has been initiated (Figure 7A). It has been suggested that this polarized distribution of LEC competent cells in the cardinal vein is related to the mesodermal origins of the venous ECs. Thus, cells of paraxial mesodermal (PXM) origin, rather than lateral plate mesodermal (LPM) origin, locate to the dorsolateral part of the cardinal vein, and transdifferentiate through the COUP-TFII/SOX18/PROX1 program (Figure 7A) (359).

3.1 PROX1

Prospero-related homeobox 1 (PROX1) is a transcription factor which plays an essential role in the development of many organ systems. In the vascular system, PROX1 is specific for lymphatic vessels and is present in all nuclei of the differentiated lymphatic endothelium. It is crucial in the specification of LEC identity and maintenance of the lymphatics throughout life.

Human phenotype: Recently a human lymphedema phenotype has been suggested to be caused by mutations in *PROX1.* The report of two rare missense variants of uncertain significance (VUS) in *PROX1* in two patients with lymphedema (322) provides too few details on the phenotype, and no functional validation of the VUSs. Since the confirmation of the variants' pathogenicity awaits, this section will focus on the functional characterization of PROX1 through *in vivo* and *in vitro* studies, that feature PROX1 as one of the master regulators in lymphatic identity and development.

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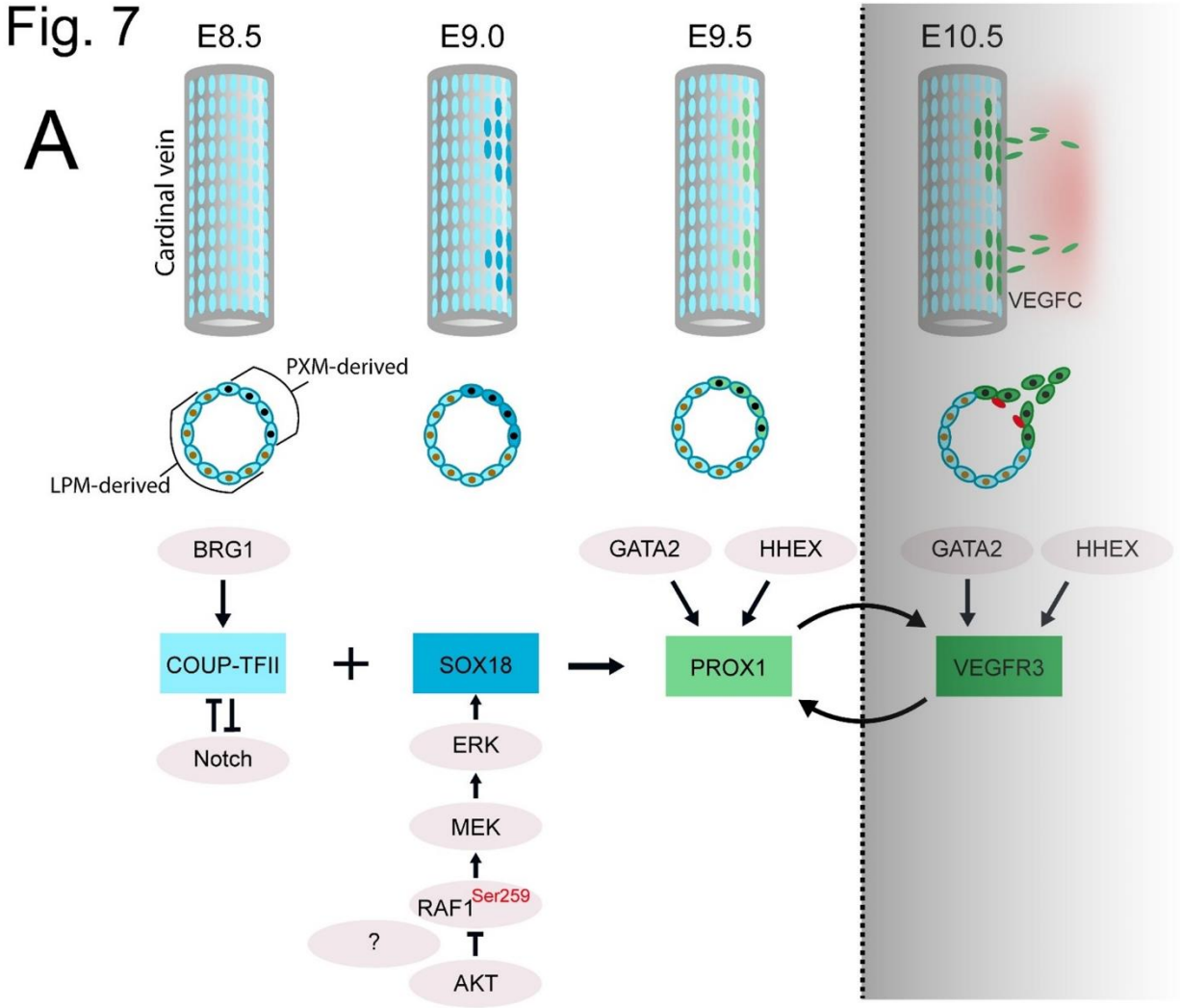
Fig. 7



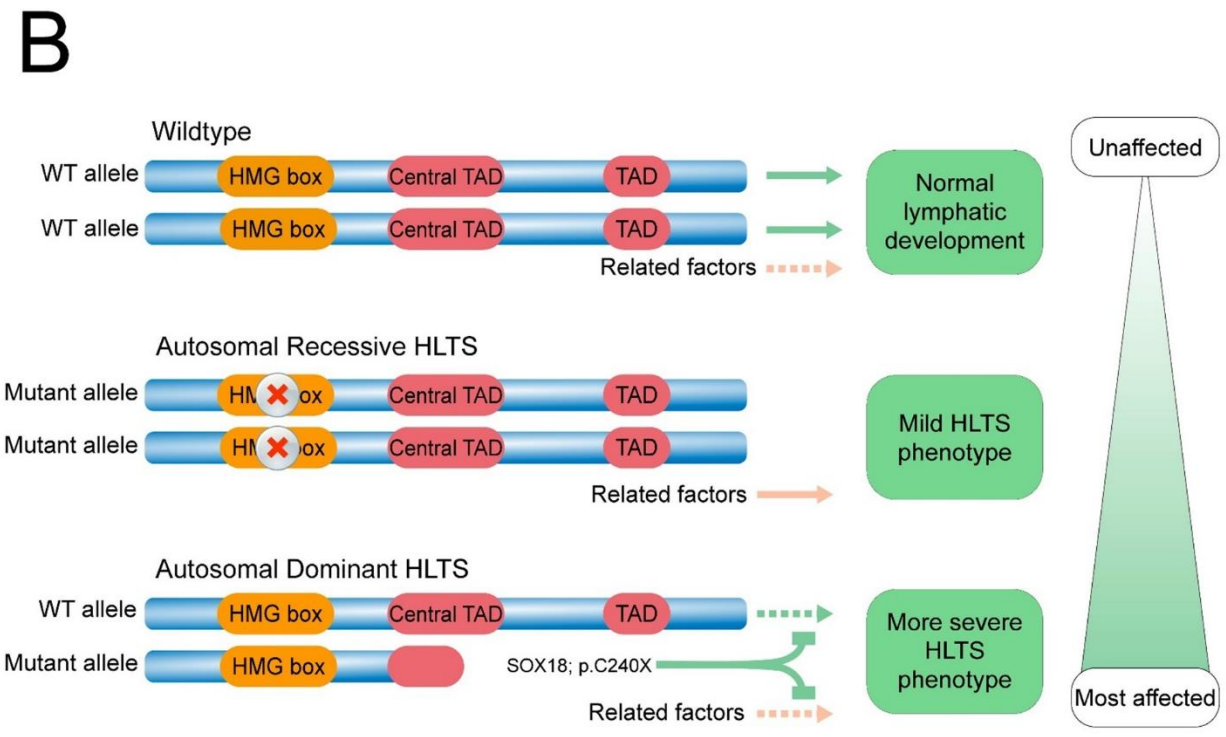


Figure 7: (A) The development of the lymphatic system during embryogenesis: LEC specification in the cardinal vein. *From around embryonic day 8.5 (E8.5) in mice, Coup-TFII, regulated by BRG1, is expressed in the nucleus of all the endothelial cells of the cardinal vein, promoting venous identity through the inhibition of the Notch pathway. At E9.0 a few cells in the jugular region of the cardinal vein start expressing Sox18. This is initiated by the reduction of an uncharacterized AKT inhibitor that releases AKT to phosphorylate RAF1 at Ser259 initiating a signaling cascade that activates SOX18. Cooperation between COUP-TFII and SOX18 induces PROX1 expression, thus around E9.5, Prox1 can be detected in polarized LEC precursors in the cardinal vein. It is thought that cells of paraxial mesodermal (PXM) origin, rather than lateral plate mesodermal (LPM) origin, locate in the dorsolateral part of the cardinal vein and are the ones able to transdifferentiate through the COUP-TFII/SOX18/PROX1 program. Other key transcription factors like GATA2 and HHEX regulate PROX1 expression. The PROX1-VEGFR3 autoregulatory feedback maintains the identity and the number of lymphatic endothelial cell progenitors.* ***(B) Predicted model for the actions of the SOX18 mutated alleles in hypotrichosis-lymphedema-telangiectasia-renal-defect syndrome.*** *Top panel: In the wildtype homozygous situation, the two SOX18 alleles are responsible for the correct lymphatic development. Each allele produces a fully active SOX18 protein which can bind to PROX1 (indicated by green arrows) triggering the expansion of the lymphatic vasculature. Middle panel: In the autosomal recessive form of hypotrichosis-lymphedema-telangiectasia-renal-defect syndrome (HLTS), missense mutations in the HMG box (red crosses) will disrupt DNA binding and therefore, mutant SOX18 will not be able to bind to PROX1 (hence no green arrows). Related factors, SOX7 and/or SOX17, can then without interference from SOX18, bind to PROX1 and compensate for some of the loss of SOX18 activity (indicated by orange arrow). The loss of SOX18 activity is not lethal, but it is not fully compensated by either SOX7 or SOX17, so patients with biallelic SOX18 mutations present with mild features of HLTS. Lower panel: In the autosomal dominant form of HLTS, patients present with truncating mutations (here the nonsense mutation p.C240X is shown). This mutant protein can bind to PROX1 via its intact HMG box and thereby antagonize the wildtype allele and the other related SOX-F group factors in a dominant negative fashion. This can interfere with the activity of the wildtype protein and lead to overall reduced SOX18 activity (indicated by broken arrows) causing a more severe HLTS phenotype. HMG box, DNA binding domain with 1st and 2nd α-helices; TAD, transactivation domain; HLTS, hypotrichosis-lymphedema-telangiectasia-renal-defect syndrome. (Image 'LEC specification in the cardinal vein' by St George's, University of London is licensed under CC BY-SA-4.0).*

Animal models: Interestingly, lymphangiogenesis in the zebrafish can proceed without *prox1* (378), so the COUP-TFII/SOX18/PROX1 specification program is not conserved in fish. We therefore need to examine mouse models and *in vitro* work performed on human cells.

Overexpression of PROX1 in cultured blood endothelial cells (BECs) reduces the expression of BEC-specific genes and induces the expression of LEC-specific markers (157, 206, 307). Venous endothelial cells, but not arterial endothelial cells, were amenable to this

reprogramming (205). If *Prox1* was deleted in mouse LECs *in vitro*, they dedifferentiated into venous BECs (184). Therefore, Prox1 acts as a binary master switch that suppresses venous BEC identity and promotes and maintains LEC identity.

Global knockout of *Prox1* led homozygous mouse embryos to develop severe edema. The mice had very few Prox1-expressing cells lining the cardinal vein and they were unpolarized (397). In this model, cells never took on a lymphatic fate but remained blood venous in origin (396). The null mice showed a complete absence of a lymphatic capillary network and died between E14.5-E15.

Conditional deletion of *Prox1* resulted in a blood vessel phenotype in homozygous animals. In addition, conditional deletion of *Prox1* postnatally or in adult mice, reprograms LECs into BECs, indicating that continuous PROX1 expression is required to maintain the LEC phenotype throughout life (184). It is suggested that LECs are one of the few differentiated cell types that require a constant expression of a certain gene such as *PROX1* to maintain their identity (184).

Disease mechanism: Thus, we have established that PROX1 is critical for the venous BEC to LEC switch, and that it needs to be constantly active to maintain lymphatic identity. What about the heterozygous mice? Is a single functional allele of *PROX1* enough to maintain normal function and preserve the wildtype phenotype? The heterozygous *Prox1* mice have fewer LEC progenitors in the cardinal vein but have a normal lymphatic capillary network at E14.5 (397). However, most heterozygous mice die within 2 to 3 days after birth, presenting with edema and chyle-filled intestines. They also lack lymphovenous valves (355). This suggests a haploinsufficient disease mechanism and that two functional copies of *Prox1* are required for survival. If the heterozygous mouse model is directly comparable to a human phenotype, one could speculate that non-immune fetal hydrops with a fatal outcome could be caused by heterozygous *PROX1* mutations. The mild, late-onset phenotype reported by Ricci et al (322) is surprising given how important this gene is in the mouse model. It is crucial that functional validation of the reported *PROX1* VUSs is carried out before any firm connection can be made to causality.

3.2 COUP-TFII

COUP-TFII (Chicken ovalbumin upstream promoter) or NR2F2 is a transcription factor which plays a critical role in controlling the development of several tissues. In the vascular system it is specific to veins and is regulated epigenetically by BRG1, a chromatin remodeling enzyme (86). COUP-TFII has many important roles in the cardinal vein endothelium. It is required to promote and maintain a venous identity and does so by inhibiting the Notch pathway (Figure 7A) (65, 412). COUP-TFII also physically interacts with PROX1, and is one of the transcription factors required for the induction of PROX1 during LEC specification (223, 354). During the late developmental stages, COUP-TFII is required for lymphangiogenic sprouting and LEC proliferation through the control of the VEGFR3/Nrp2 signaling axis (231).

Human phenotype: No human primary lymphedema phenotype has been directly linked with *COUP-TFII* mutations. A linkage study on a large Norwegian family with autosomal recessive lymphedema-cholestasis (Aagenaes) syndrome mapped the disease to a genomic region on chromosome 15q25, approximately 3 Mb from the coding region of *COUP-TFII* (52). A reliable link between Aagenaes syndrome and *COUP-TFII* has yet to be proved.

Animal models: Homozygous *Coup-TFII* mutants or endothelial specific *Coup-TFII* knockout mouse embryos die between E10.5-E11.5 from defects in vascular development. Venous fate is lost and no LEC progenitors are present (304, 412). Conditional knockout models showed that ablation of *Coup-TFII* in endothelial cells at E9.5 led to a reduced number of PROX1-expressing LEC progenitors in mutant mice (231). Deleting *Coup-TFII* later, at E11.5 after the progenitors have left the cardinal vein, led to malformed lymphatic sacs and vessels, and the mutant mice developed edema.

Disease mechanism: As Aagenaes syndrome is autosomal recessive, the affected individuals would have to be biallelic for *COUP-TFII* mutations which, according to the knockout mouse model, is most likely to result in embryonic lethality (304, 412) making a link between Aagenaes syndrome and *COUP-TFII* less likely. Furthermore, heterozygous mutations in human *COUP-TFII* have been associated with autosomal dominant congenital heart defects including atrioventricular septal defects and tetralogy of Fallot (7), but there was no mention of associated primary lymphedema. Assuming there has been no ascertainment bias of patients, and congenital heart defects are the only clinical manifestation, then it would appear that the COUP-TFII protein is expressed at sufficient levels to promote venous and lymphatic development in these heterozygous cases (haplo-sufficiency in venous ECs and LECs), but not at high enough levels to allow a complete development of the heart (haplo-insufficiency in cardiomyocytes). Such a dosage dependency has been observed in hypomorphic endothelial-specific mice where a decrease in *COUP-TFII* mRNA levels led to cardiac defects (232).

3.3 SOX18

SOX18, another transcription factor, is a member of the SOX gene family which contains a 79-amino acid HMG box, a highly conserved DNA-binding domain (39). SOX genes are important regulators of various developmental processes and SOX18 belongs to the SOX-F family which includes two other very similar genes, SOX7 and SOX17. SOX7 is important for arterio-venous identity (234, 301) and SOX17 contributes to regeneration of the endothelial barrier after injury to restore microvascular homeostasis (234). SOX18 is transiently expressed in the cardinal vein from around E9 in mice (Figure 7A, B top panel) and the HMG box domain binds to two SOX-F consensus binding sites in the *PROX1* promoter region, which then turns on PROX1 expression in the cardinal vein (95, 118, 158).

Human phenotype: Human mutations in *SOX18* have been described in patients with Hypotrichosis-lymphedema-telangiectasia-renal-defect syndrome (HLTS; ORPHA:69735).

Dependent on the type of the variant and which domain of the *SOX18* gene it resides in, HLTS can both be described as an autosomal recessive and autosomal dominant disorder. No matter what the gene mutation is, the phenotype is similar, and patients present with hypotrichosis (absence of hair including scalp, eyebrows and eyelashes) from infancy, primary non-congenital lymphedema and telangiectasia (dilatation of cutaneous blood-filled capillaries) (132). The dominant form is associated with renal defect e.g. membranoproliferative glomerulonephritis. Thin transparent skin with widespread mottling (cutis marmorata) has also been described, although the expression of the disease features can be variable.

Looking specifically at the primary lymphedema phenotype, individuals with *SOX18* mutation(s) showed variable ages of onset. The edema was usually confined to the lower limbs, but most patients also had lymphedema of the eyelids and around the eyes. Hydrocele, non-immune fetal hydrops, and progressive dilatation of the aorta have been reported (166, 375). In one case severe chylothorax and relentless pulmonary hypertension culminated in death (392).

Animal models: Unlike the homozygous *Prox1* mouse the homozygous *Sox18* mouse is viable. Complete inactivation of *Sox18* in the mouse only leads to a mild phenotype with a minor coat defect but normal growth rate and viability (302). However, this was only observed in a mouse model of mixed background. In another strain, Sox18 B6, ablation of *Sox18* led to homozygous mice with extensive subcutaneous edema, and embryonic death at E14.5 (118). These *Sox18* null mice showed a complete lack of cells expressing Prox1 and no LEC differentiation (118, 119).

Sox18 is thought to be the most important of the SOX-F members for lymphangiogenesis, but in certain strains of mice the two other Sox-F group members, Sox7 and Sox17, can functionally substitute for Sox18. It is suggested that complete deletion of Sox18 will enable Sox7 or Sox17 to bind and activate *Prox1* even though in wildtype mice and during normal lymphangiogenesis, Sox7 and Sox17 are not normally expressed. Sox7 and Sox17 have not been detected in *Sox18* mutant mice of the B6 background while *Sox18* mutant mice of the mixed background showed a clear upregulation of Sox7 and Sox17 in cells migrating from the cardinal vein, allowing these cells to express Prox1 (158). These findings indicate that in some mice strains, if Sox18 is absent, other Sox-F factors can activate *Prox1* gene expression, although they are not normally active during lymphatic development. Studies in zebrafish support this notion that other Sox-F group members can play redundant roles in vascular development (59, 149).

Disease mechanism: The heterozygous HLTS cases reported to date all carried truncating mutations, which affect the transactivation domains of SOX18 (22, 166, 265, 375, 392, 406). *In vitro* analysis of a *SOX18* truncating mutant showed that the truncated protein acted in a dominant negative manner (263). The truncated proteins were not degraded by nonsense

mediated decay mechanism and were shown to bind to the promoter of *PROX1* via its intact HMG box. Because the transactivation domain (TAD) is non-functional in the HLTS patients, as that portion of the protein is lost or partially lost, transactivation of *PROX1* does not take place. Therefore, the mutated SOX18 proteins block other SOX-F group members from binding, and transcriptional activity is suppressed in a dominant negative manner as a result (Figure 7B, bottom panel). The mutations seen in these autosomal dominant patients are very similar to the spontaneous *Sox18* mutations observed in the *Ragged* mouse. There are 4 different *Ragged* mouse lines with various frameshift mutations in or after the TAD (175, 303). The mice are very similar to the human phenotype in that they show defects of their coat, plus vascular and lymphatic dysfunction.

Two biallelic cases of HLTS have been reported (166). Both patients carried homozygous missense variants residing in the HMG box of the *SOX18* gene (Figure 7B, middle panel) The HMG box is the DNA binding domain of the transcription factor and the mutations localized in this domain are thought to destabilize the alpha-helix and thus prevent the binding of mutant SOX18 to the *PROX1* promoter. As seen in some mouse strains, SOX7 and SOX17 can then, without interference, bind to *PROX1* and compensate for some of the loss of SOX18 activity, which could explain why biallelic *SOX18* mutations in humans are not always lethal and only lead to a milder HLTS phenotype (Figure 7B, middle panel).

One of the studies analyzed human skin biopsies from an HLTS patient with a heterozygous *SOX18* mutation (406). They found that the small blood vessels were severely dilated, but the lymphatic vascular network appeared intact. Detailed histological observations of one of the *Ragged* mutants also showed hyperplasia of the endothelial cells in the microvasculature with reduced structural integrity, and it was suggested that this could explain the telangiectasia associated with HLTS (95). However, the authors proposed that the edema observed in the mice could not be explained by these vascular defects alone, and it was suggested that the lymphatic system would be impaired. The notion of lymphatic impairment is supported by the imaging result reported on one of the HLTS patients, which showed no evidence of lymphatic function as no tracer was transported from the dorsum of the foot to the groin lymph nodes on lymphoscintigraphy. This suggests a failure of initial lymphatic capillary absorption (166). However, the only lymph scan reported has only measured tracer uptake in the groin region at 32 minutes, which can be too short to truly record the activity of the lymphatic collectors as 2hr is the recommended scan time.

3.4 RAF1/MEK/ERK-MAPK

As mentioned above, RAF1/ERK signaling is important for the transcriptional activity of SOX18 (89). AKT inhibits ERK signaling via RAF1, but at around E9.0-E9.5 this inhibition is released by some unknown signal allowing ERK to become activated. This in turn activates SOX18, which binds to *PROX1* as described above (Figure 7A).

Human phenotype: Mutations in *RAF1* have been identified in a group of patients with Noonan Syndrome and LEOPARD syndrome (also known as Noonan syndrome with multiple lentigines); both under the RASopathy umbrella (297, 319). The RASopathies (ORPHA:536391) are a group of distinct genetic syndromes caused by heterozygous germline mutations in genes (>20 to date) that encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway (317). Because of dysregulation of the same pathway, the RASopathies exhibit numerous overlapping phenotypic features (69, 363).

The RASopathies may exhibit bilateral lower limb lymphedema, genital swelling with cutaneous chylous lymphangiectasia (chyle leaking from dilated skin lymphatics), and systemic involvement including intestinal lymphangiectasia and chylothoraces which may be progressive (187, 351, 402). There may be increased nuchal thickness during embryogenesis leading to persistent nuchal edema and neck webbing postnatally (211). Fetal nuchal edema is the most frequent fetal neck pathology diagnosed prenatally and is most commonly associated with chromosomal abnormalities such as Down and Turner syndromes. It is defined as fluid-filled, sometimes cystic, lesions and almost certainly results from obstructed lymph drainage from maldevelopment or delayed development of the lymph sacs.

Animal models: Although *RAF1* mutations are a relatively infrequent cause of the RASopathies, *Raf1*^{S259A} transgenic mice appear to provide a good model for understanding the mechanism of the lymphatic abnormalities in Noonan syndrome (89). Excessive Erk signaling leads to a persistent induction of Sox18 and Prox1 in venous ECs, resulting in increased transition of these venous ECs to a lymphatic fate, leading in turn, to increased outmigration of these newly specified LECs to form lymphatic sacs with subsequent development of disproportionately large, irregularly shaped, dilated lymphatics (lymphangiectasia), which wrap around arterioles and small arteries.

Disease mechanism: Gain-of-function *RAF1* mutations are observed in 3%–17% of Noonan syndrome cases (297, 319). HEK293 or COS-1 cells transfected with a *RAF1* mutation associated with Noonan syndrome had increased kinase activity and enhanced MEK and ERK activation compared with wildtype cells. If the *RAF1* mutations in the RASopathy patients also lead to excessive signaling in LECs and this leads to the same morphologic features observed in the transgenic mice, it could explain the cause of the intestinal lymphangiectasia and chylothoraces observed in RASopathy patients (308).

Lymphoscintigraphy demonstrates reflux and/or rerouting of lymphatic drainage associated with incompetent veins on the venous duplex scans. The combination of lymph and venous reflux strongly implicates a fault in lymphatic and venous valve development (187). However, the main problem in the RASopathies are probably the abnormalities in the central conducting lymphatic pathways.

3.5 Summary of LEC specification

It should be apparent from this brief introduction that transcriptional control of SOX18, COUP-TFII and PROX1 is essential to LEC specification in mammals and that disruption of the interactions between them suppresses lymphatic fate specification, which consequentially will prevent the development of a fully functional lymphatic system. An understanding of which molecules and signals activate these transcription factors is also starting to emerge as seen with the RAF1/ERK control required for the induction of SOX18 expression in venous ECs. The complexity of the molecular networks specific for commitment and differentiation of LECs is more elaborate than presented here and covered in various excellent reviews on this subject (101, 224, 240, 414).

4. Expansion of the lymphatic vascular tree

After PROX1 expression is activated in a subset of venous ECs, blood endothelial markers are down-regulated in those cells whilst vascular endothelial growth factor receptor 3 (VEGFR3) expression is up-regulated on the cell surface (179, 293). These PROX1- and VEGFR3- expressing LECs start to bud off from the cardinal vein and migrate dorsally (Figure 8A). This directed migration is guided by a molecular gradient of lymphatic specific vascular endothelial growth factor C (VEGFC). In the human embryo between weeks 6 – 9, the LECs migrate to initially form the first primitive lymphatic structures, the six primary lymph sacs (Figure 8B) (138, 330). Further sprouting of new lymphatic vessels from these primordial lymph sacs along the major veins helps create a network of lymphatic capillaries and collecting vessels that builds up the entire lymphatic vasculature (193).

Budding of endothelial cells in the initial phase of lymphatic expansion is absolutely dependent on VEGFR3-expressing LECs responding to a trail of active VEGFC molecules (142, 193). By this means, the VEGFC/VEGFR3 signaling axis plays a crucial role during this part of lymphatic development. It is now known that the matrix-interacting collagen and calcium binding EGF domains 1 (CCBE1) protein is an important player in the initial phase of lymphatic growth (37), partaking in the proteolytic cleavage of pro-VEGFC into mature VEGFC (220). The ‘A disintegrin and metalloproteinase with thrombospondin motifs 3’ (ADAMTS3) protein has more recently been identified as the co-factor that helps to cleave the VEGFC pro-peptide into an active molecule (178). In short, CCBE1 is crucial for the immobilization of pro-VEGFC, which in turn provides a substrate in the extracellular environment where ADAMTS3 proteolytically cleaves pro-VEGFC. This results in the generation of a mature VEGFC molecule, which can then initiate signaling through the VEGFR3 receptor (Figure 8A). Recent studies have given a very good insight into how VEGFC activation by ADAMTS3 and CCBE1 is regulated during lymphatic development (51, 180).

Different forms of primary lymphedema result from mutations found in genes that encode for the key proteins on the VEGFC/VEGFR3 signaling axis. They will be described independently and in depth in this section and the reader can refer to Table 2 for a

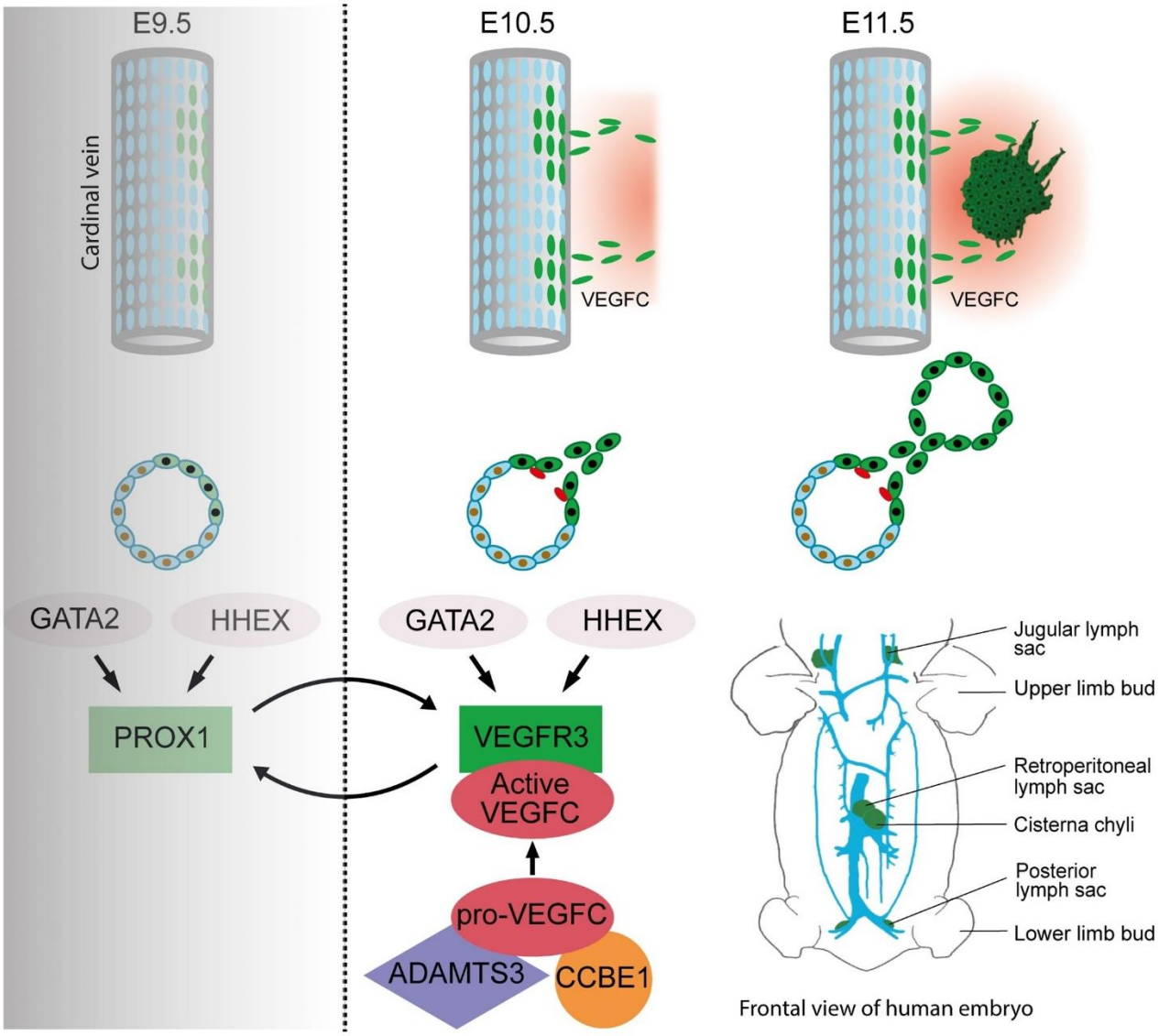


Figure 8: The development of the lymphatic system during embryogenesis. *The VEGFC/VEGFR3 signaling axis and the expansion of the lymphatic vascular tree. PROX1 expression leads to the downregulation of blood endothelial markers and the upregulation of vascular endothelial growth factor receptor 3 (VEGFR3) expression. At E10.5, these PROX1- and VEGFR3-expressing LECs (green) separate from the cardinal vein (blue) and migrate dorsally, guided by a molecular gradient of vascular endothelial growth factor C (VEGFC). Platelet aggregation at the points of LEC migration is shown (red). This initial phase of lymphatic expansion is absolutely dependent on VEGFR3-expressing LECs responding to VEGFC. Paracrine CCBE1 secretion at sites of lymphatic vessel growth promotes proteolytic cleavage of pro-VEGFC by the metalloprotease ADAMTS3. This activates and mobilizes a fully mature VEGFC molecule that on binding to VEGFR3 can activate autophosphorylation and downstream signaling. It is thought that CCBE1 binds to the extracellular matrix (ECM) while VEGFC cleavage may occur on the lymphatic endothelial cell surface. Key transcription factors like GATA2 and HHEX regulate VEGFR3 expression and the PROX1-VEGFR3 autoregulatory feedback remains important during the vascular expansion process. At E11.5 primordial lymph sacs are formed. Drawing of a human embryo (at gestational week 6 – 7,*

head not shown) displaying the first primitive lymphatic structures seen as six primary lymph sacs in green (veins in blue). The jugular lymph sacs develop around the upper jugular vein, the retroperitoneal lymph sac and cisterna chyli in the central segment of the embryo and the two posterior lymph sacs around the lower limbs near the iliac veins. (Image 'Expansion of the lymphatic vasculature' by St George's, University of London is licensed under CC BY-SA-4.0).

summary and classification of the main findings in relation to the different human genotypes and their functional consequences.

4.1 VEGFR3

Vascular endothelial growth factor receptor 3 (VEGFR3) is a tyrosine kinase receptor protein which is expressed in every endothelial cell early in development (365). At around embryonic day 12.5 (E12.5) in the mouse, Vegfr3 becomes increasingly specific for endothelial cells committed to a lymphatic lineage, i.e. the distinct venous-derived Prox1- and Lyve1-expressing endothelial cells, which are budding off and migrating away from the cardinal vein (142). Interestingly, these cells do not express any of the other vascular endothelial growth factor receptors (Vegfr1 and Vegfr2). Later in adulthood, Vegfr3 is mainly restricted to the lymphatic endothelium, where it co-expresses with VEGFR2 (329, 401). VEGFR3 can be activated by vascular growth factors VEGFC and VEGFD, though VEGFD appears not to be essential for lymphatic development (18, 356). Binding of growth factor ligands induces the dimerization of the VEGFR3 receptors, either as homodimers or heterodimers with VEGFR2, and initiates autophosphorylation of the receptors and downstream signaling cascades.

Human phenotype: Mutations in human *VEGFR3* lead to Milroy disease (ORPHA:79452) (113). This is an autosomal dominant condition and one of the most frequent forms of congenital primary lymphedema (136). Often there is a family history of lymphedema, but Milroy disease can also occur sporadically (45). There are marked inter- and intra-familial variations in severity with asymptomatic obligate carriers and the lymphedema in some patients has been reported to regress (45).

Milroy Disease is characterized by congenital (< 1 year of age), usually bilateral, lower limb lymphedema, hydroceles and wide caliber (dilated) veins. Lymphedema of the dorsum of foot is the most common manifestation in this group of patients and the pedal edema can sometimes be detected during fetal ultrasound scans in the third trimester. Non-immune fetal hydrops has also been reported in two unrelated cases with *VEGFR3* mutations (84, 131) but is not a common presentation in Milroy disease. Hydroceles (lymph accumulation in the anatomical sac around the testes) occur in one third of the males who are *VEGFR3* mutation positive (45). It can occur in some individuals even in the absence of limb lymphedema.

Animal models: Animal models have contributed notably to the understanding of the role of VEGFR3 in lymphatic development and function. *Vegfr3* heterozygous knockout mice appeared phenotypically normal at first examination (97). However, a more detailed study using ultramicroscopy demonstrated that although these mice developed peripheral longitudinal lymphatic vessels and ventral primordial thoracic duct, and peripheral LECs can be distinguished, the numbers of initial LECs outside the cardinal vein were reduced, the formation of a peripheral network was disrupted, and the ventral primordial thoracic duct was of smaller diameter (142).

Of high significance has been the establishment and investigation of the *Chy* mouse line harboring a germline heterozygous inactivating point mutation (p.Ile1053Phe) in the *Vegfr3* gene resulting in inactivation of Vegfr3 signaling (194). This mouse was originally named because of the presence of chylous ascites, since 10% of the neonatal *Chy* mice have this, although the chylous ascites resolves within 3 weeks of birth. However, it was observed that the swelling of the limbs, due to a hypoplastic subcutaneous lymphatic network, persisted.

Disease mechanism: Venous duplex ultrasound examination reveals venous valve failure in the saphenous veins of the legs in 90% of the genotyped patients (259). Venous reflux will only contribute to edema once the child is able to stand upright and the veins are subject to increased venous pressure. Therefore, the vein abnormalities would not appear to be contributory to the edema in the first year of life, which indicates that the edema is not due to venous insufficiency, but due to failure of lymphatic drainage, i.e. confirming that lymphedema is the major cause of Milroy disease.

The VEGFR3 protein has seven immunoglobulin-like repeats in its extracellular region; a transmembrane region; and an intracellular region which consists of two tyrosine kinase domains (165, 294). The majority of gene alterations associated with Milroy disease reported to date are heterozygous missense variants located in one of the two tyrosine kinase domains (135).

Functional work on the identified variants has helped in understanding the mechanisms of VEGFR3 signaling, and the consequences of these mutations on receptor activation. For example, *in vitro* studies have shown that Milroy disease mutations lead to inactivation of the tyrosine kinase domain, inhibiting trans-autophosphorylation (167, 192). It was demonstrated that the wildtype receptor was internalized and degraded at a faster rate than the mutant receptor, leading to an increased ratio of mutant to wildtype receptor on the cell surface, making dimerization with a mutant dysfunctional receptor, instead of a fully functional one, more likely. This would thereby reduce VEGFR3 signaling (192). Thus, we can suggest that the Milroy disease phenotype is caused by missense mutations antagonizing the activity of the wildtype allele in a dominant negative manner. Interestingly, *VEGFR3* mutations predicted to lead to loss of function causing haploinsufficiency have been

associated with another distinct phenotype; autosomal dominant tetralogy of Fallot with no other extracardiac malformations or primary lymphedema (183, 321).

Although there are no reports of chylous reflux and no cases of systemic involvement/intestinal lymphangiectasia in humans with *VEGFR3* mutations, the *Chy* mouse model emerged as an extremely interesting tool for researchers after a Milroy family with the same missense variant as the *Chy* mouse was reported (135). This has enabled the study of the pathophysiological consequences of missing/dysfunctional initial lymphatics. The heterozygous adult mice have swelling only in the fore and hind paws, yet lack dermal lymphatics in the fore paw, hind paw, thigh and back skin on skin biopsy (194). This is different to the human phenotype, where skin biopsy from patients with *VEGFR3* mutations reveals the presence of dermal lymphatics in foot and forearm, albeit in reduced numbers (259).

Functional studies through imaging, using lymphangiography and lymphoscintigraphy, indicated little absorption of tracer by initial lymphatics in either the *Chy* mouse or in the lower limbs of human patients (192, 259). Although there appears to be a slight reduction in dermal lymphatic vessel density in the upper limbs, there are no reported cases of upper limb lymphedema in Milroy disease, which is confirmed by lymphoscintigraphy imaging showing similar activity of tracer uptake as for healthy controls (259).

Why lymphatic function is only altered in the lower limbs and feet and not in the whole body for germline mutations in such an important gene for lymphatic function is not clear. More studies need to be undertaken to elucidate the exact mechanisms for VEGFR3 function in humans. However, taking all the knowledge acquired from the above-mentioned investigations, the mechanism of pathogenesis for Milroy disease appears to be a functional failure of the initial lymphatic capillaries rather than absent lymphatic vessels. The exact mechanisms for the uptake of fluid and molecules from the interstitium through the primary valves of the initial lymphatics into the transporting vessels remain to be determined. A study suggests that LECs can actively regulate the influx of fluids and solutes from the interstitium into the lymphatic lumen, a process partly controlled by VEGFC and VEGFR3 (41, 43). Further studies on mouse models and humans with *VEGFR3* mutations may help in better understanding this process and explain why lymphatic function fails in Milroy disease. Unlocking this mystery may guide new treatments for this group of patients.

4.2 VEGFC

VEGFC is a member of the vascular endothelial growth factor family and the main driver of lymphangiogenesis through its binding to VEGFR3. Macrophages are known to secrete VEGFC during the inflammatory process, but the sources of VEGFC during embryogenesis, particularly for lymphatic vessel development, are just starting to be discovered (373, 386). VEGFC is produced as a proprotein and the proteolytic environment is critical for controlling VEGFC bioavailability and activity (186, 350). Pro-VEGFC consists of a central VEGF homology

domain (VHD) and flanking N- and C-terminal propeptides which are proteolytically removed, with each cleavage increasing its affinity towards VEGFR3 (186). Furin mediates the cleavage of the C-terminus (349) and ADAMTS3 cleaves the N-terminus (178) generating a mature form of VEGFC that becomes the active molecule. Recent findings reveal that transcription factors of the BACH family can regulate lymphangiogenesis through modulation of VEGFC expression, demonstrating the complexity of VEGFC signaling (71), a complexity that increases as we learn more about the roles of different isoforms of VEGFC (256, 318).

Human phenotype: Mutations in human *VEGFC* have been identified as the cause of congenital primary lymphedema of Gordon (ORPHA: 79452) (134). The lymphedema is milder than in Milroy disease, but otherwise is very similar, therefore sometimes it is referred to in the literature as Milroy-like primary lymphoedema. In nearly all cases reported, swelling is confined to the lower limbs, often just the dorsum of the feet. One case has shown intermittent hand swelling (275). The lymphedema usually presents at birth and there are no other major pathological features, apart from some patients who present with prominent, varicose veins and hydrocele.

So far five families have been reported, all of whom had heterozygous loss-of-function mutations (frameshift and splicing variants) (17, 106, 134, 275). All mutations are predicted to cause truncation of the VEGFC protein, with consequential complete or partial loss of the VEGF homology domain (VHD) and C-terminal propeptide.

Animal models: Inactivation of *Vegfc* in mice, xenopus tadpoles and zebrafish allowed LEC specification in the embryonic veins, but the Prox1-expressing endothelial cells failed to bud off and migrate (193, 213, 410). Mice null for *Vegfc* are edematous, die before birth and present a complete absence of a lymphatic system in the embryo (142, 193). Imaging experiments showed that the Prox1-expressing endothelial cells remained in the wall of the cardinal vein, indicating that loss of Vegfc function halted budding off and migration of LECs (142). Heterozygous mice, on the other hand, survived to adulthood but presented with edematous paws, chylous ascites and dermal lymphatic hypoplasia.

Chy-3 mice, not to be confused with the *Chy* mice (described above in the VEGFR3 section), carry a large chromosomal deletion that includes *Vegfc* and makes them hemizygous for *Vegfc*. They were originally named because of their chylous ascites, but a further study revealed they also had lymphedema of the hind paws and approximately half of the males had lymphedema of the penis (88). *Chy-3* mice showed a hypoplastic dermal lymphatic network, whereas the blood vasculature appeared unaffected. All adult *Chy-3* mice exhibited a lateral truncal lymphatic pathway directly connecting the inguinal to the axillary lymph node. The connections between dermal superficial and deep lymphatics in the upper limbs and in all cervical regions were intact, and functionally drained the upper body. However, lymphatic tracer was not transported from the dermal to the deep truncal

lymphatic system in the lower limbs, even though the deep lymphatic vessels and nodes were present and patent (88).

Disease mechanism: VEGFC function is conserved through evolution as vegfc is also required for lymphatic vasculature development in zebrafish (213). Experimental work showed that the injection of wildtype human pro-VEGFC into zebrafish can stimulate lymphatic vessel sprouting, but when mutant human pro-VEGFC (totally or partially lacking the VHD and the C-terminus, as found in lymphedema patients) was injected, the truncated protein failed to induce lymphatic vessel sprouting (134, 275). Zebrafish mutants lacking the C-terminal domain also failed to develop lymphatics, due to the inefficient secretion of VEGFC, which indicates that the presence of the pro-VEGFC C-terminus is essential for efficient secretion of VEGFC during lymphatic development (381). The heterozygosity found in patients together with the findings from the *Vegfc* and *Chy-3* mouse models support haploinsufficiency as the disease mechanism since there is an evident need for two functional *Vegfc* alleles to maintain a healthy phenotype.

In contrast to the observations from functional imaging in mice, lymphoscintigraphy of patients with *VEGFC* mutations has shown uptake of tracer, albeit reduced, in tortuous lymphatic vessels within the lower limbs (134, 275). There is evidence of drainage rerouting, which indicates that the tracer is not following the normal tracts to the groin area, as seen in healthy controls. This contrasts with the lymphoscintigrams seen in patients with mutations in *VEGFR3*, which show no uptake within the main lymphatic vessels or nodes after 2 hours (335). Therefore, lymphoscintigraphy could prove a useful tool in differentiating between the two congenital primary lymphedema phenotypes.

It is notable that the lymphedema observed in both human and animal models associated with a partial loss of VEGFC is minimal, and reduced penetrance and asymptomatic carriers have been reported. Even a case of biallelic *VEGFC* mutations causing a Milroy-like phenotype has been reported (269). Since VEGFC appears to be so crucial for lymphangiogenesis, why a reduced level of VEGFC does not cause a more severe phenotype is unclear. There could be other factors that can activate lymphangiogenesis via VEGFR3 and so ‘rescue’ the phenotype (144, 388). Nevertheless, with a reduced availability of VEGFC, VEGFR3 activity must be affected resulting in disruption of downstream signaling pathways, possibly with similar consequences to that following from *VEGFR3* mutations.

4.3 CCBE1

Collagen and Calcium Binding EGF-domain containing protein 1 (CCBE1) is a signaling peptide secreted to the extracellular matrix by non-endothelial cells adjacent to developing lymphatic vessels (155). The protein has two domains, an N-terminal calcium-binding domain with epidermal GF-like repeats and a C-terminal domain with two collagen-like repeats.

Human phenotype: Biallelic (i.e. homozygous or compound heterozygous) mutations in *CCBE1* cause Hennekam Syndrome-1 otherwise known as Hennekam-lymphangiectasia-lymphedema syndrome (ORPHA:2136) (11), which is a rare autosomal recessive disorder. Hennekam Syndrome-1 is a disorder of the lymphatic system development that was originally reported to include lymphedema, lymphangiectasia, characteristic dysmorphism and cognitive impairment (148). There are marked inter- and intra-familial variations reported and the cognitive impairment with dysmorphic features was originally emphasized as a cardinal clinical sign, but less so in more recent publications (12, 75, 80, 172). It seems likely the dysmorphic features (round flat face, flat bridge of the nose, hypertelorism, and small mouth) are secondary to the effects of *in utero* edema when the facial structures are being formed, such as seen in the fetal hydrops cases reported (75, 346).

Extensive peripheral lymphedema and intestinal lymphangiectasia on the other hand show complete penetrance in all cases reported. The intestinal lymphangiectasia gives rise to a protein-losing enteropathy. The lymphangiectasia is usually present in the intestines (Figure 4B), but has also been found in the lung and pleura, pericardium, thyroid gland, and kidney (376). Hence, the syndrome is considered a generalized lymphatic dysplasia (136). Approximately 25% of the patients presenting with the typical phenotype described above have been found to have a mutation in *CCBE1* (12).

Animal models: ccbe1, as a regulator of lymphatic vessel formation, was first described in a zebrafish model with a coding mutation (p.Asp162Glu) in the calcium-binding EGF domain of the *ccbe1* gene (154). Fish both heterozygous and homozygous for this mutation retained a normal blood vasculature but failed to develop a functional lymphatic network. Surviving zebrafish mutants showed severe edema and the model was named accordingly *'full of fluid'* (*fof*).

Ccbe1 null mice also had normal patterning and density of blood vessels indicating that the Ccbe1 protein is not necessary for angiogenesis (37, 421). Their lymphatic system, on the other hand, lacked all lymphatic vessels and all null mice accumulated subcutaneous fluid from E13.5 leading to the development of severe edema and prenatal demise. When looking at the early development (E10.5) of *Ccbe1* null mice, Prox1 and Lyve1 were detected in endothelial cells in the dorsal part of the cardinal vein suggesting that LEC differentiation was unaffected (37, 142). However, at E11.5 a reduction of LECs expressing Prox1 and Lyve1 was seen in the cardinal vein wall and even fewer LECs were located outside the cardinal vein, which could only form abnormal sprouts, unable to coalesce into discrete lymphatic structures. Likewise, Prox1-expressing endothelial cells did not bud off from the cardinal vein in the *fof* zebrafish model (154). In contrast, the lymphatic vessel density was normal in postnatal knockout animals (51). In brief, all the *in vivo* studies demonstrated that the presence of CCBE1 is required for budding and migration of lymphatic endothelial cells, but not for the maintenance and function of adult lymphatics.

The animal studies indicated that Ccbe1 was not expressed in the LECs themselves, but spatially and temporally correlated with the migration routes of the LECs in regions of high Vegfc expression (142, 220, 325). The overlapping expression with Vegfc suggests that there might be a connection between the two proteins. *In vivo* experiments with double Vegfc/Ccbe1 knockout models showed in some aspects a more severe phenotype compared to the single gene models, with reduced numbers of initial LECs outside the cardinal vein plus short and abnormal Prox1-expressing sprouts. This supports the hypothesis of VEGFC and CCBE1 interacting synergistically (178). Indeed, Bos and colleagues showed that CCBE1 has little lymphangiogenic effect on its own, but that it can dramatically enhance the lymphangiogenic effect of VEGFC *in vivo* (37). It was speculated that CCBE1 could possibly act as a guidance molecule for Vegfc regulating budding and possibly migration. This hypothesis was supported by Jeltsch and colleagues who showed that CCBE1 mediated VEGFC cleavage through activation of ADAMTS3, concluding that CCBE1 is a permissive factor, not an instructive one (178).

Disease mechanism: Functional analysis in zebrafish of CCBE1 variants associated with Hennekam syndrome has confirmed pathogenicity (80). *In vitro* studies have shown that even though mutant CCBE1 protein can be expressed and secreted (172), its biological activity is impaired. In HEK293T cells overexpressing the Hennekam syndrome-associated CCBE1 variant p.Gly327Arg, VEGFC signaling was not activated (325). In contrast, overexpression of another Hennekam syndrome-associated CCBE1 variant, p. Arg158Cys, led to minimal loss of function. In addition, serum from patients with the p.Cys174Tyr mutation has the same VEGFC levels as healthy controls, indicating that this CCBE1 mutation has minimal functional effect (172). Taken together, this suggest heterogeneity in molecular disease mechanism depending on the mutation. This could possibly explain some of the interfamilial variation observed in Hennekam syndrome cases and why the human phenotype associated with the p.Gly327Arg variant seems to be more severe than other Hennekam Syndrome-1 mutations, with only 1 of 4 individuals surviving (11). The fact that p.Cys174Tyr has been reported in two unrelated probands with variability in expression of symptoms (11, 172) also suggests that genetic modifiers or environmental factors may be responsible for this heterogeneity.

Investigation of intestinal biopsies from a human subject showed very few lymphatic vessels, which were all distended and could explain the intestinal lymphangiectasia; one of the cardinal features of Hennekam Syndrome (11, 172). Small bowel follow-through procedure in one patient showed thickening and nodularity which was well controlled with an MCT diet (121). Ccbe1 is expressed in the developing mouse intestine (11) and would be involved in lymphatic lacteal development, therefore reduced function of CCBE1 in biallelic Hennekam patients could lead to a lack of or abnormal lymphatic vessels in the gut.

Some CCBE1-associated Hennekam patients also present with pulmonary lymphangiectasia (28, 376). *In vivo* studies have shown that lymphatic function is important in the embryonic

Table 2. Human phenotypes associated with a primary lymphatic anomaly (Part 1). *Mutations in VEGFR3, VEGFC, CCBE1, and ADAMTS3, all encoding for key proteins in the VEGFC/VEGFR3 signaling axis, cause different forms of primary lymphedema. Important findings in relation to human studies and animal models have been summarized.*

	Milroy disease	VEGFC-related congenital primary lymphedema	Hennekam syndrome Type 1	Hennekam syndrome Type 3
Human genotype and inheritance	***VEGFR3* +/-** Autosomal dominant	***VEGFC* +/-** Autosomal dominant	***CCBE1* -/-** Autosomal recessive	***ADAMTS3* -/-** Autosomal recessive
Human studies	• Reduced fluid absorption by the initial lymphatics and venous valve failure (259). • Skin biopsy from patients with *VEGFR3* mutations reveals the presence of dermal lymphatics in foot and forearm, albeit in reduced numbers (259).	• Lymphoscintigraphy of patients with *VEGFC* mutations has shown uptake of tracer in tortuous lymphatic vessels with evidence of drainage rerouting. Reduced uptake rate in the groin lymph nodes (335).	• Extensive peripheral lymphedema and intestinal lymphangiectasia (11, 148). • Investigation of intestinal biopsies from a patient with a *CCBE1* mutation showed very few lymphatic vessels (172).	• Patients with biallelic *ADAMTS3* mutations display a generalized widespread primary lymphedema, with lymphatic vessels of their bowel mesentery abnormally dilated and tortuous (340).
Animal models	• The *Chy* mouse, carrying an inactivating Vegfr3 mutation I1053F, presents defective lymphatic vessels (194). • In *Vegfr3+/-* mouse embryos, numbers of initial LECs outside the cardinal vein were reduced, the network of peripheral LECs was disrupted and the ventral primordial thoracic duct was of smaller diameter (142).	• vegfc signaling is required for the correct development of the zebrafish lymphatic system (213). • In *Vegfc-/-* mouse embryos, LECs do not sprout to form lymphatic vessels and lymphatic sacs did not form. *Vegfc+/-* mouse embryos present hypoplastic lymphatic capillaries (193). • The *Chy3* mouse (hemizygous for *Vegfc*) shows a hypoplastic dermal lymphatic network (88, 142).	• The *full of fluid (fof)* zebrafish model, carrying the mutation D162E in ccbe1, shows defective embryonic lymphangiogenesis, lacking trunk lymphatic vessels (154). • In *Ccbe1-/-* mouse embryos, there is a reduction of the number and migratory ability of initial LECs. They lack lymphatic sacs and lymphatic vessels in the skin (37, 142).	• *Adamts3-/-* mouse embryos lack lymphatic vessels in the skin (51, 177).

lung and *Ccbe1*-deficient mice with pulmonary edema often failed to inflate their lungs at birth, and it is suggested that loss of CCBE1 in human could lead to respiratory failure in (preterm) neonates (174). This could possibly explain some of the early demises observed in some of the Hennekam families (11, 75). Further studies are needed to explore in more depth the molecular and cellular basis of this disease.

4.4 ADAMTS3

'A Disintegrin and metalloproteinase with thrombospondin motifs 3' (ADAMTS3) is an extracellular matrix protein, which has been recognized for its relevance in collagen synthesis, but recent studies have shown that the substrate repertoire for ADAMTS3 is larger than initially thought (26), and it is now also recognized to play an important role in the production of mature VEGFC (Figure 8A).

Human phenotype: Biallelic mutations in *ADAMTS3* cause generalized widespread primary lymphedema, lymphangiectasia and other pathological features. Two siblings with a compound heterozygous mutation and one additional case with a homozygous nonsense mutation of *ADAMTS3* have been reported (50, 340). The two siblings from the first family presented with polyhydramnios and congenital lymphedema of the lower extremities (as well as hydroceles for the boy) (50). The girl had a protein-losing enteropathy with widespread lymphedema affecting face, abdomen, genitalia and legs. Dysmorphic features in both children were similar to those seen in patients with *CCBE1* mutations; namely, flat facies, hypertelorism, synophrys, pseudo-strabismus, upward-slanting palpebral fissures, and anteverted peaked nostrils. The homozygous case from the second family presented with an identical phenotype but the pregnancy was complicated by fetal hydrops, and the mother's first pregnancy was reported as a stillbirth due to fetal hydrops (340). The phenotype is so similar to that of *CCBE1*-associated Hennekam Syndrome 1 (see above) that the *ADAMTS3*-associated syndrome was named Hennekam Syndrome 3 (ORPHA:2136), underpinning the likelihood that the two molecules interact together. The parents from both families were unaffected with no other familial history of primary lymphedema or other lymphatic problems.

Animal models: At E12.5 all the embryos were similar in appearance, but at E13.5 embryos with a biallelic loss of *Adamts3* had skin edema and died around E15.0 (177). The embryos have normal blood vessels but completely lack lymphatic vasculature, identical to the *Ccbe1* null mouse. The *Adamts3* null mice also had remarkable liver degeneration and altered blood vessels in the placenta. Other studies of *Adamts3* knockout mice also reported lethality and lymphatic developmental defects (51, 287). In contrast, heterozygous *Adamts3* mice were viable and fertile (177). Taken together, these initial studies into the role of Adamts3 in the development of the lymphatic system clearly demonstrate its requirement for embryonic lymphangiogenesis.

Disease mechanism: Although the two compound heterozygous missense variants identified in the first family are not located in any of the domains of ADAMTS3 of known relevance, the mutated residues are fully conserved among species and also among other ADAMTS proteins, indicating the mutations could have a damaging effect on the protein. On transfection of the mutated proteins into HEK293 cells, the maturation and secretion of ADAMTS3 was altered and the protein seemed to accumulate in the intracellular space, which was confirmed by immuno-histochemistry (50). Therefore, it is very likely that the abnormal processing of ADAMTS3 is impairing the interaction with CCBE1.

Jha and colleagues investigated a heterozygous nonsynonymous variant, p.Arg565Gln, identified in a primary lymphedema patient and six unaffected family members (180). On co-transfection in HEK293T cells, the interaction between CCBE1 and the mutant ADAMTS3 protein was weak, and the extracellular localization of the proteins was altered. This suggests that the lack of ADAMTS3 extracellular availability disables or restricts the proteolytic cleavage of pro-VEGFC, with the subsequent weakening of the VEGFC/VEGFR3 downstream signaling as the molecular disease mechanism.

Heterozygous *Adamts3* mice are viable and the unaffected, heterozygous carrier parents of the ADAMTS3-associated Hennekam Syndrome children are healthy with no signs of lymphatic problems, which indicates that one functional gene copy can produce enough ADAMTS3 protein to maintain normal lymphangiogenic function in mouse and humans. Surprisingly, in contrast to the strong phenotype observed in the *Adamts3* null mouse, individuals with biallelic *ADAMTS3* mutations can survive and display generalized primary lymphedema but in comparison, with a less severe lymphatic phenotype, reduced to dilated and tortuous lymphatic vessels in the bowel mesentery (340). This indicates that the effects of the mutations in the human gene may not reflect the complete loss-of-function seen in the homozygous knockout mouse. There could be two explanations for this: (I) a species-specific compensation mechanism by other molecules present in human but not in the mouse, since for instance in zebrafish, it is only the simultaneous loss of Adamts3 and Adamts14 that produces a lymphatic phenotype (386). Moreover, during human embryonic development, activation of VEGFR3 by other growth factors is still happening (144, 388), allowing some lymphangiogenesis to take place without the need for ADAMTS3-CCBE1 activation of VEGFC. (II) Or it is possible that the reported *ADAMTS3* variants in the Hennekam Syndrome patients actually have a less severe effect on the protein than was seen *in vitro* in HEK293 cells, so that the human mutations only partially reduce the proteolytic cleavage of pro-VEGFC *in vivo* (386). This could allow enough ADAMTS3 bioavailability to drive embryonic development in general, but not enough for a completely normal development of the lymphatic network, explaining the primary lymphedema phenotype in the patients.

4.5 KIF11

EG5 or Kinesin-5, encoded by the *KIF11* gene (kinesin family member 11), is a kinesin-motor protein essential for the correct chromosome positioning, centrosome separation and establishment of the bipolar spindle during cell mitosis (110, 374). EG5 interacts with several cell cycle proteins and chromatin modifying enzymes such as histone deacetylase 1 (HDAC1) (276) or the ubiquitin ligase RNF20/40 (96) participating in that way in nuclear dynamics. EG5 overexpression is associated with poor prognosis in breast cancer (182), hepatocellular carcinoma (233) or clear cell renal cell carcinoma (181). Its crucial role in mitosis together with the association with several malignancies has made EG5 a very attractive target for anti-cancer therapies (274).

Human phenotype: Mutations in *KIF11* cause a form of primary lymphedema as part of a more complex syndrome named microcephaly-lymphedema-chorioretinopathy syndrome (MLC, ORPHA:2526) (292). MLC is characterized by microcephaly as the dominant feature, eye anomalies, intellectual disability, lymphedema and epilepsy. The microcephaly is present at birth, with subsequent slow head growth. The characteristic facial phenotype is of upslanting palpebral fissures, broad nose with rounded tip, long philtrum with thin upper lip, and prominent, large ears (185). The most common ocular abnormality is chorioretinal dysplasia, but *KIF11* mutations can also cause familial exudative vitreoretinopathy (FEVR) (323). Unlike MLC, the hallmarks of which are both central nervous system and ocular developmental defects, the primary defect in FEVR is limited to abnormal retinal vasculature (228).

The lymphedema in MLC is congenital, bilateral, and only affects the lower limbs, most often confined to the dorsa of the feet. The type of swelling is highly reminiscent of that seen in VEGFR3-associated Milroy disease, with small, dysplastic nails, deep interphalangeal creases on the toes and swelling causing doming of the dorsum of the feet. There is increasing evidence for intestinal lymphangiectasia in these patients (Personal communication). All the mutations are autosomal-dominant or *de novo*; no autosomal recessive case with a *KIF11* mutation has been described. Unaffected carriers are rare, most show some signs of disease, but with great inter- and intrafamilial variability. Family history is not a requisite for diagnosis, with *de novo* cases making up around a third (341). No genotype-phenotype correlation has been described.

Animal models: To date, lymphatic endothelial specific animal models have not been published but global heterozygous mutant mice with a genetrap insertion in Eg5 have been generated and appeared phenotypically normal (56). In contrast, homozygous embryos displayed signs of a proliferation defect already before the implantation stage. This was corroborated by Chauviere and colleagues in Eg5-deficient mice generated by gene targeting of embryonic stem cells (62). They also demonstrated that heterozygous mice are healthy, fertile, and show no detectable phenotype, whereas homozygous Eg5 embryos die during the pre-implantation stage. Thus, both studies confirm that Eg5 is crucial for cell division during

early mouse development and its function cannot be compensated by another molecular motor proteins, while other study shows that Eg5 is essential for the specification and maintenance of neural crest progenitors during Xenopus early embryogenesis (111). Up to now it is still unclear what role EG5 could play in the development and/or maintenance of the lymphatic system. Zebrafish embryos treated with an eg5 inhibitor showed high embryonic lethality and a reduction of circulating blood cells in the surviving embryos whereas knockdown of *kif11* in a transgenic fish resulted in angiogenesis defects (103). They also demonstrated that the inhibition of Eg5 represses endothelial cell migration and proliferation of both blood and lymphatic endothelial cells. When using an inducible EC-specific knockout model, loss of Eg5 postnatally led to severe growth retardation of the retinal vasculature (391). Although further study of this model could shed light on the pathophysiological role of Eg5 in the retinal phenotype observed in the MLC patients, the role of Eg5 in the lymphatic vasculature still needs elucidating.

Disease mechanism: Functional imaging of a patient with a *KIF11* gene mutation showed poor uptake of tracer by the initial lymphatics in the feet on lymphoscintigraphy, and consequently no, or much reduced, tracer uptake of the leg lymphatic vessels or groin lymph nodes (292). Although this observation is only based on one case, the result is similar to that seen in patients with *VEGFR3* mutations and could suggest a similar fault in lymphatic drainage ability. However, the precise molecular mechanisms explaining why and how mutations in *KIF11* result in primary lymphedema are still to be elucidated. The discovery of new roles of EG5 outside mitosis such as its participation in cell migration (104, 385) and intracellular transport of vesicles (384), could be the evidence for a possible role of EG5 in lymphatic endothelial cell migration and/or intracellular trafficking dynamics.

5. Maturation of the lymphatic system

At late gestation and postnatally, the lymphatic vessels in the primitive lymphatic plexus remodel to finally form a functional lymphatic vasculature (Figure 9A). This maturation process leads to the formation of a hierarchical tree composed of capillaries and collectors, making up the fully developed lymphatic system (Figure 1). The main events taking place during this phase of lymphangiogenesis include the disconnection from the venous system, ensuring the separation of the blood and lymphatic compartments, and the remodeling of the initial lymphatic plexus into initial capillaries and collecting vessels. The latter includes the development of intraluminal (secondary) lymphatic valves in the collecting vessels, ensuring the unidirectional flow of lymph through the system.

5.1 Disconnection of the primary lymphatic structures from the venous system

As described in the previous section, the LEC progenitors proliferate and migrate from the cardinal vein to assemble into primary sac like structures, which are then disconnected from the venous system (Figure 8A). One of the proposed models, the ballooning model, suggests

that the PROX1-expressing cells in the anterior wall of the cardinal vein wall undergo delamination, like the inflation of a balloon, to form the lymph sacs (Figure 9B) (120, 372). As the LEC progenitors balloon out, they take on a lymphatic phenotype and under the transcriptional control of PROX1 they begin to express podoplanin (PDPN) (295, 296). PDPN protein on LECs near the junction with the cardinal vein starts binding circulating platelets, which eventually aggregate into a firm clot. Platelet CLEC-2 receptor signaling mediates this intervascular hemostasis mechanism that is required to safeguard the lymphatic system from the high-pressure blood circulation and maintain normal lymphatic function throughout life. By E14 the lymph sac is fully disconnected from the cardinal vein and blood cells are prevented from entering the lymphatic vasculature (372).

Another proposed model, the budding model, suggests that the LEC progenitors migrate out from the venous system as strings, which then coalesce to form lymph sacs (Figure 9B) (142, 408). This way the integrity of the venous vessel wall is maintained, and vascular leakage prevented. In this model, it is suggested that the LECs and platelets may exclusively interact at the lymphovenous valves as described below.

Mice deficient in PDPN, CLEC-2, or the CLEC-2 signaling proteins SYK and SLP-76 exhibit blood-filled lymphatics during fetal life and die shortly after birth due to defective lymphatic function, and a failure to disconnect the lymphatics from the venous system (1, 33, 124, 151, 339, 362). Further study is needed to identify which model prevails in which circumstances. LEC migration and primary lymph sac formation take place at the anterior cardinal vein near the subclavian vein, the posterior cardinal vein near the iliac veins, and the primitive vena cava near the mesonephric veins (Figure 8B), and the mechanism for migration and separation could be different at each. Later, lymphatic vessels grow out from these sacs, expanding and interconnecting, and for example, the thoracic ducts ultimately connect the cisterna chyli with the lymphatic vessels of the jugular region. No human phenotype has to date been reported for any of the genes coding for these proteins. For an excellent review on the molecular and cellular mechanisms of lymphatic vascular maturation particularly in relation to the separation of lymphatics and blood vessels see Chen et al. (64).

Part of the disconnection process involves the development of lymphovenous valves in the jugular region. This is where lymph ultimately will drain into the blood circulation at the thoracic duct exit in the fully functional body (Figure 1B). However, the open, low-pressure lymphatic system cannot function properly if it is directly exposed to the high pressure of the blood-system. Therefore, junctions between the thoracic duct and the right and left subclavian veins are formed. These are the first valves to form outside of the heart and their establishment ensures the life-long separation of the blood and lymphatic systems. The process is described step-by-step in Figure 9C and it begins around E12 with the coalescence of endothelial cells at the connection sites between the primordial thoracic duct and the cardinal vein, which will form the pair of lymphovenous valves (60, 355). At around E12.5,

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Figure 9

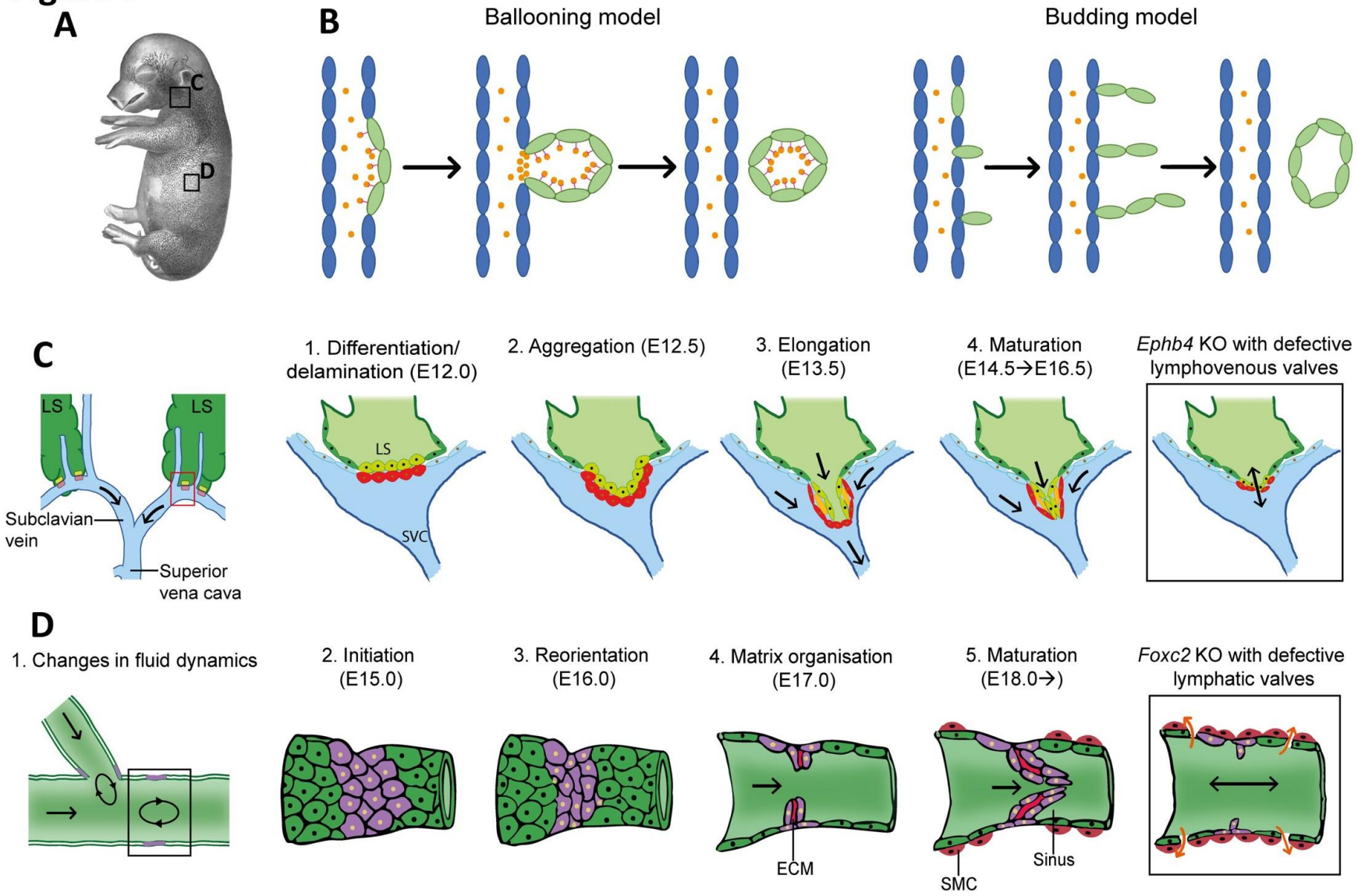


Figure 9: Maturation of the lymphatic system. *At late gestation and postnatally, the lymphatic vessels disconnect from the venous system and the primitive lymphatic plexus remodels into initial capillaries and collecting vessels, to finally form a functional lymphatic vasculature. These processes will be finely orchestrated by e.g. EPHB4, FOXC2, GATA2, PIEZO1, FAT4 and gap junctions. Although the lymphatics in the 5.5cm pig embryo (A) have not yet developed valves, it is apparent how the primary lymphatic plexus is gradually spreading over the entire body and invades the skin. (B) Proposed models for the disconnection of the primary lymph sacs from the venous system. The ballooning model suggests that the PROX1-expressing cells (green) in the anterior wall of the cardinal vein (blue) wall undergo a process known as delamination, like the inflation of a balloon, to form the primitive lymph sacs. As the LEC progenitors balloon out they begin to express podoplanin, critical for the binding and aggregation of circulating platelets (orange) to form a clot that will mark the disconnection between systems; completed by E14 in mice. The budding model suggests that the LEC progenitors migrate out from the venous system as strings before coalescing to form lymph sacs, maintaining venous wall integrity, and preventing vascular leakage. (C) Disconnection of the lymphatic system in the jugular region by the development of lymphovenous valves. At the sites where the lymph sacs (LS) interact with the jugulo-subclavian vein junctions, lymphovenous valve (LVV) development begins. 1. LECs (yellow) and venous endothelial cells (VECs, red) interact to form the LVV. Mechanistically, LECs upregulate the expression of Prox1, Vegfr3 and Cx43 whereas VECs upregulate the expression of Foxc2, Gata2 and Cx37. 2. The aggregate of endothelial cells begins to delaminate and reorientate itself, invaginating into the vein. 3. As the LVV develops, the cells elongate and align themselves to the direction of venous blood flow. 4. An opening in the middle of the aggregate develops and the valves recruit mural cells (orange) into the space between the valvular endothelial cells and the LECs. In mice deleted for Ephb4 (boxed-in example) lymphovenous valve formation does not go through its natural progression ending up with defective valves which cannot prevent retrograde flow. As a result, the Ephb4 knockout (KO) embryos have blood-filled lymphatics. Arrows indicate direction of fluid flow. (D) Development of secondary lymphatic valves in the collector vessels. The presence of intraluminal lymphatic valves in the collecting vessels, will ensure the unidirectional flow of lymph through the mature lymphatic system. 1. Prior to the initiation of secondary lymphatic valve development, bifurcations in the developing lymphatic network lead to changes in lymph flow re-circulation (curved arrows). 2. In these specific sites (purple area), valve formation initiates by activation of mechanotransduction pathways triggering a cascade of transcription factor upregulation (e.g. Prox1, Foxc2, Gata2). 3. The flow-induced changes in molecular identity lead the cells to change shape and reorientate. 4. Extra-cellular matrix (ECM, red) is deposited, promoting the formation of a ring-like constriction and some of the cells start protruding toward the vessel lumen. 5. During maturation, those protrusions elongate, and a sinus develops. Smooth muscle cells (SMC) cover the collectors except in the valve region. In Foxc2 knockout (KO) mice (boxed-in example), lymphatic collector vessel maturation is impaired with malformed valves. Arrows indicate bi-directional lymph flow due to reflux. (Image credit: (A) Adapted 'The lymphatic system in the skin of a pig 5.5cm long' by F. Sabin, Wistar Institute of Anatomy and biology. Association of American Anatomists (1904). The American Journal of Anatomy. (B-D) by St George's University of London. Image 'Maturation of the lymphatic system' by St George's, University of London is licensed under CC BY-SA-4.0).*

the cells have formed aggregates, which then begin to elongate into the lumen of the vein, forming a lymphovenous valve complex that commences to develop and mature between E14.5 and E16.5 in the mouse.

Many transcription factors and signaling pathways have been shown to regulate the development and maintenance of this complex, and current insights into these processes have been reviewed by Janardhan & Triverdi (176). The valve leaflets have two layers of endothelial cells separated by an ECM compartment; an outer layer of lymphovenous valve ECs toward the lumen of the vein and an inner layer of LECs. The expression levels of various factors vary between the two (129). The lymphovenous valves continue to develop until birth recruiting mural cells and establishing an opening between the venous and lymphatic compartments.

Platelets are also activated by PDPN signals on the LECs at the lymphovenous junctions during lymphovenous valve development and form a plug which, alongside the lymphovenous valve, is crucial for the prevention of blood entry into the lymphatic circulation (151). It is not entirely clear why two mechanisms of safeguarding the low-pressure lymphatic system from the high-pressure forces from the venous system are required, but both systems need maintaining throughout life to prevent blood mixing into the thoracic duct (151).

Lymphovenous valve defects have been reported in several murine models lacking Prox1, Foxc2, Gata2 and Cx37 (128, 189, 200, 241, 271, 331, 355) to mention just a few. Mutations in some of those genes have also been reported to cause lymphovenous valve defects in humans. These mice display defective lymphovenous valve formation and/or failure to maintain blood-lymph separation, all indicating a critical requirement for these genes to ensure unidirectional flow of lymph into blood. More recently, it has been suggested that EPHB4 could also be involved in this process (251).

5.1.1 EPHB4

EPHB4 is a tyrosine kinase receptor. The Eph receptors represent the largest subfamily of receptor tyrosine kinases (126). They are activated by interaction with specific ligands called ephrins. In the circulatory system, Ephrin–Eph signaling establishes borders between different vascular compartments (arterial, venous, and lymphatic), and helps the remodeling process by regulating the effects of vascular growth factors. The expression of ephrinB2 in arterial territories and of EphB4 in venous territories is one example. The formation of the dorsal aorta is dependent on ephrinB2 signaling, while cardinal vein formation requires EphB4, and mice deleted for either of the two have severe defects in blood vessel growth and remodeling (4, 130, 387). This interaction between the two different types of blood vascular endothelial cells (arterial versus venous) is of a repulsive nature and is essential for vascular remodeling and correct morphogenesis of the capillary beds during embryogenesis (125, 207), persisting throughout adult life.

In contrast to the repulsive signaling events that occur at the arterial-venous boundary, it is suggested that EphB4/ephrinB2 interactions can also occur between endothelial cells of the same type (4). For example, it has been proposed that both EphB4 and ephrinB2 are expressed by lymphatic vessels and that forward and reverse EphB4/ephrinB2 signaling between LECs is required for both lymphatic sprouting, and valve formation and maintenance (191, 242, 390, 419). Studies have also shown that expression levels of ephrinB2 and EphB4 in lymphatic endothelial cells dynamically change in newly formed lymphatic vessels in a cornea injury model (198).

Human Phenotype: *EPHB4* has been associated with autosomal dominant non-immune fetal hydrops and/or atrial septal defects (ORPHA: 568065) where the patients presented with a mix of lymphatic problems such as fetal hydrops, lymphedema and central conducting lymphatic anomalies (226, 251). Central conducting lymphatic anomalies represent any inborn abnormality of the visceral lymphatics, including the cisterna chyli and the thoracic duct. The abnormalities may range from dilatation giving rise to reflux, contractile failure, or obstruction of main lymphatic conducting vessels.

Some of the patients developed severe non-immune lymphatic-related fetal hydrops *in utero*, resulting in early death, and a high number of first-trimester miscarriages was observed (251). Sometimes, the hydrops improved spontaneously in those who survived the neonatal period.

Another indicator that *EPHB4* mutations lead to a lymphatic phenotype is the observation of chylothoraces in the proband described by Li et al, a condition that was corrected after a medium-chain triglyceride (MCT) diet was administered (226).

Animal models: Embryonic deletion of *EphB4* at E10.5-E14.5 led to subcutaneous edema, and analysis of the dermal skin in the mutant mice showed abnormal lymphatic vasculature with tortuous and dilated lymphatic vessels, indicating that EphB4 has an important role in lymphatic development (251). When deleting *EphB4* during either embryonic valve formation or postnatally, a complete absence of valves in the mesenteries was observed. These results suggest a critical role of EphB4 in the formation and early postnatal maintenance of lymphatic valves. This is in line with previous studies, which have shown that EphB4/ephrinB2 is expressed by lymphatic vessels and that EphB4/ephrinB2 signaling is required for both lymphatic sprouting and valve formation and maintenance (242, 390, 419).

Thus, it is clear that Ephb4 has an essential role in lymphatic valve morphogenesis. However, the lymphatic valve defects observed in the mice are unlikely to be the cause of *in utero* swelling, as their development occurs later (25, 331). Detailed analysis of mice deleted for *Ephb4* at E10.5-E12.5 demonstrated a critical requirement of Ephb4 during the early stages of lymphatic development as the lymphovenous valve-forming cells failed to extend

into valve leaflets in the majority of mutant mice when compared with wildtype (Figure 9C) (251). This led to blood in the lymphatics, and dilated vessels. It was therefore postulated that the fetal hydrops observed in the two families reported by Martin-Almedina and colleagues, was due to defective lymphovenous valve formation caused by the inadequate EPHB4 function (251). The exact mechanism by which EPHB4 controls lymphovenous valve formation is not yet known.

Loss of *ephb4* in zebrafish also led to disorganized blood and lymphatic vasculature (199, 226). Inhibition of the mTOR and MAPK pathways using mTORC1 and MEK inhibitors, showed that both types of inhibitor could independently reduce the vascular defects and restore normal vessel architecture and function, suggesting that ephb4 signals through both pathways to control lymphatic development.

Disease mechanism: Data from a human study also suggest that the dermal lymphatic vessels are dilated and on inspection of a lung biopsy from another individual from the same family, abnormally large lymphatic vessels, consistent with pulmonary lymphangiectasia, were observed (226). Furthermore, magnetic resonance lymphangiogram (MRL) of the proband showed abnormal central lymphatic anatomy in agreement with a central conducting anomaly, i.e. the thoracic duct was dilated and tortuous and with retrograde perfusion of the right lung explaining the persistent lung problems.

MRL also revealed retroperitoneal (mesenteric) masses with proliferation of lymphatic vessels around the spine, and in the lungs, which were also dysfunctional and showed abnormal flow (226).

The fact that *EPHB4* mutations do lead to a lymphatic phenotype is also evident from lymphoscintigraphic imaging. Two adults, one from each of the families described in Martin-Almedina et al showed impaired lymph drainage in the lower limb even without clinical evidence of peripheral lymphedema (251). Given its multiple roles in other cardiovascular endothelia, the impact of *EPHB4* mutations could be more widespread than just in the lymphatic endothelium. Pleiotropic effects of *EPHB4* mutations are apparent in the diverse clinical signs observed in the three reported families (226, 251). Several individuals in the three families described to date reported prominent veins and problems with early onset of varicose veins, which would be consistent with venous valve defects. In the dermal tissue biopsy mentioned above, increased numbers of venous capillaries were also observed. Telangiectasias, varicosities and venous stasis (226), all clear signs of long-term venous insufficiency, are also indicative of problems with the veins. Modeling of one of the human mutations in zebrafish did show defects in the blood vessels (226). The observation of atrial septal defects on echocardiogram in several individuals (251) also suggests that EPHB4 is essential for cardiovascular development, which correlates with the observation of cardiovascular developmental problems in mice when *EphB4* is inactivated (130).

The *EPHB4* mutations reported so far have occurred at highly conserved residues in the tyrosine kinase domain. *In vitro* functional expression studies in HEK293 cells or LECs showed that the mutant proteins are being produced but were devoid of tyrosine kinase activity, suggesting altered levels of EPHB4 signaling (226, 251).

Additional mutations in *EPHB4* have been reported recently in two other distinct clinical entities with a predominantly blood vascular phenotype: vein of Galen aneurysmal malformation (98, 382) and capillary malformation-arteriovenous malformation type 2 (CM-AVM2, MIM:618196) (13, 413). All these cases showed reduced penetrance, with considerable variance of expression, and are reported to be due to *EPHB4* loss-of-function mutations. Overexpression of CM-AVM2-associated *EPHB4* missense variants in COS7 cells showed reduced protein expression with the mutant receptors aggregating in intracellular inclusions (13). This will change the ratio of EPHB4 receptor protein to ephrinB2 in the cell membrane, which has been shown to cause dysregulation of the EPHB4/ephrinB2 signaling cascade (109). Altering the ephrinB2:EPHB4 ratio in normal VECs by either increasing ephrinB2 or blocking EPHB4 induced an AVM-like cell behavior. Whether the disease mechanism for the blood vascular predominant CM-AVM2 phenotype is the same as for the lymphatic-related phenotype needs further investigation. See Table 3 for a summary of the main findings in relation to the EPHB4 human genotype.

5.2 Remodeling and valve formation

After the establishment of the primary lymph sacs and their separation from the venous system, this primitive lymphatic plexus remodels into a hierarchical tree composed of lymphatic capillaries (initial lymphatics) and lymphatic collectors, making up the fully developed lymphatic system. As the system gains complexity, the individual LECs begin to show heterogeneity in their identity depending on their function. Some LECs within the initial lymphatic capillaries will be responsible for fluid absorption from the interstitium (lymph formation), other LECs will form the pumping collector vessels that transport lymph to the lymph nodes before eventually emptying back into the blood vascular system in the neck, while other LECs will contribute to lymphatic valve formation.

As described in the introduction, the initial lymphatics have a single layer of oak-leaf shaped endothelial cells, which partially overlap (Figure 1D). These “button-like” junctions are discontinuous giving rise to small intercellular openings (primary lymphatic valves), making the lymphatic capillaries permeable like the capillaries or microvessels of the blood circulatory system (19, 87). The endothelial cells are attached to the extracellular matrix with anchoring filaments, which allows for the opening of these intercellular junctions at high interstitial pressure (221). There are no perivascular SMCs or basement membrane in the lymphatic capillaries, and the entry of interstitial fluid and transport of lymph initially are entirely dependent on pressure gradients (342). The structure and organization of the capillary endothelium is not only highly permeable to interstitial fluid, but also

Table 3. Human phenotypes associated with a primary lymphatic anomaly (Part 2). *Mutations in EPHB4, FOXC2, GATA2, PIEZO1, GJC2, and FAT4, all encoding for proteins important for the remodeling and maturation of the lymphatic system, cause different forms of primary lymphedema. Important findings in relation to human studies and animal models have been summarized.*

	EPHB4-related LRFH	Lymphedema distichiasis syndrome	GATA2-deficiency syndrome	PIEZO1-related GLD/LRFH	Late onset 4-limb lymphedema	Hennekam syndrome Type 2
Human genotype and inheritance	***EPHB4* +/-** Autosomal dominant	***FOXC2* +/-** Autosomal dominant	***GATA2* +/-** Autosomal dominant	***PIEZO1* -/-** Autosomal recessive	***GJC2* +/-** Autosomal dominant	***FAT4* -/-** Autosomal recessive
Human studies	• Impaired lymph drainage in the lower limbs (251). • Dilated dermal lymphatic vessels (226). • Pulmonary lymphangiectasias associated with abnormal central lymphatics, suggestive of absent or dysfunctional LVs (226).	• Increased size and number of lymphatic collecting vessels and ilio-inguinal lymph nodes (82). • Tortuous vessels, lymph reflux suggestive of LV failure (46, 260, 335). • Dilated dermal lymphatic vessels and no valves on biopsy with increased investment with pericytes and SMCs (305). • Substantial venous valve loss and venous reflux (239, 258).	• No uptake into the main lymph tracts in the affected lower limbs and poor ilio-inguinal lymph node uptake suggesting failure of initial lymphatics. Some rerouting around ankle area observed in some patients suggests LV problems (245, 291).	• Poor uptake in the groin and axillae. • Superficial collector dysfunction, prominent uptake within the popliteal nodes and rerouting (117).	• Reduced uptake by the peripheral lymphatics in all 4 limbs suggesting initial lymphatic failure or peripheral lymphatic collector hypoplasia (290). • Reduced number of venous valves with shorter leaflets (239).	• No available data
Animal models	• Injection of ephb4 morpholinos in zebrafish results in lymphatic vessel misbranching (226). • Pharmacological inhibition of Ephb4 phosphorylation in WT neonatal mice causes loss of LVs (419). • *Ephb4*-/- mouse embryos show tortuous and dilated dermal lymphatic vessels, deficient LVVs and lack LVs (251).	• *Foxc2*+/- mouse models show lymphatic hyperplasia and increased size and number of lymph nodes (212, 305). • *Foxc2*-/- mouse models present with aberrant lymphatic structures and abnormal coverage with pericytes and SMCs (281, 305). • LEC-*Foxc2*-KO mouse models show increased LEC proliferation and enlarged lymphatic vessels (107) as well as impaired LV function and cell-cell junction defects (332).	• LEC-*Gata2*-KO mouse model shows enlarged jugular lymphatic sacs, irregular dermal lymphatic vessels and aberrant SMCs coverage. LVs and LVVs are disorganized (200).	• LEC-*Piezo1*-KO mouse model presents reduced number of LVs (279). • LEC-*Piezo1*-KO mouse model shows immature LVs and reduced lymphatic vessel density (67).	• *Gjc2*-/- mouse model presents normal and mature LVs. • *Gjc2*-/- *Gja1*+/- mouse model presents an initial delay in LV maturation while the *Gjc2*-/- *Gja1*-/- mouse model lacks mesenteric LVs (271).	• *Fat4* KO mice show LV abnormalities with cell polarization defects (310). • *Fat4*-/- mouse embryos present dilated dermal lymphatic vessels and a reduction in the number of lymphatic vessel branches and LVs. LEC-*Fat4*-KO dermal lymphatic vessels were wider in calibre and presented reduced number of immature LVs (34).

GLD, generalized lymphatic dysplasia; KO, knockout; LEC, lymphatic endothelial cell; LRFH, lymphatic-related fetal hydrops; LV, lymphatic valve; LVV, lymphovenous valve; SMC, smooth muscle cell.

macromolecules, pathogens and migrating cells (370), allowing easy movement of specialized cells (e.g. dendritic cells).

The organization of LECs in the lymphatic collector vessels is different from the initial lymphatic capillaries. Instead of oak-leaf shaped endothelial cells as in the capillaries, the individual LECs are spindle-shaped and the collector vessels have a basement membrane and SMCs (238, 331). They resemble more that of the endothelial cells of blood vessels which are tightly sealed to each other by continuous, “zipper-like” junctions which makes them nearly impermeable (Figure 1D). Lymphatic collector vessel permeability is regulated through ephrinB2-EphB4 signaling, which have been shown to have a critical role in the maintenance of vessel integrity in different organs of juvenile and adult mice (122).

While lymph flow into and through initial lymphatics is mainly a passive convective process dependent on changes in local hydrostatic pressure gradients, the SMCs on the lymphatic collector vessels facilitate active transport through the vessel lumen by contractile pumping (85). Collector vessels are subdivided into functional units, known as lymphangions, each separated by a distal and proximal valve. These are the pumping units controlled by extrinsic and intrinsic forces (267, 338).

The first indication of lymphatic valve development is the appearance and clustering of cells expressing elevated levels of the transcription factors, PROX1, FOXC2 and GATA2, in defined positions along the vessel, predominantly near vessel branch points subjected to complex fluid flow patterns and mechanical forces (331). This process is described step-by-step in Figure 9D and initiates at E15-E16 in mice with the spatial reorientation of these defined cell clusters. Changes in the extracellular matrix organization around E17 facilitate the development of lymphatic valve leaflets that fully mature from E18 with the encapsulation by bulbous sinus regions and the adjacent coverage by SMCs. This molecular and physiological setup is important to ensure an efficient one-way transport of lymph, as these intraluminal valves, when competent, will assure the forward propulsion of lymph through the vessels.

The action of the lymphatic intraluminal valves is important in preventing backflow of lymph, just as the venous valves are required for the efficient forward flow of blood. Interestingly, venous valves undergo comparable morphological changes during development, controlled by similar molecular programs as in the lymphatic valves (24), which could explain why both a venous phenotype (such as venous ectasia or varicose veins) and a lymphatic phenotype (lymphedema) often co-exist in the same patients.

Shear stress has been found to be an important initiator of the maturation process, as the lymph flow will provide a mechanical stimulus to which the cell reacts (331). Thus, mechanotransduction is an important regulator of vessel maturation. Endothelial cells subjected to steady laminar flow seem to promote a collector vessel phenotype, whereas

lymphatic endothelial cells subjected to disturbed flow with oscillation, which by nature occurs at branch points (bifurcations and vessel curvatures) seem to promote the development of lymphatic valves (333).

Maturation of collecting lymphatic vessels and development of the lymphatic valves happens almost simultaneously (detected at approximately E14.5–E15.5 and E16.0-E17.0 in the mouse, respectively) and are controlled by similar molecular programs (331). The most well characterized pathway is FOXC2/calcineurin/NFATC1 signaling (171, 281, 331, 332). Other molecules are also involved, e.g. GATA2 (200), connexins (189, 190), EphrinB2/EPHB4 (mentioned above) (122, 242, 390), FAT4 (310) and PIEZO1 (67, 279), all of which will be described below. The reader can refer to Table 3 for a summary and classification of the main findings in relation to the different human genotypes and their functional consequences.

5.2.1 FOXC2

Forkhead Box C2 (FOXC2) transcription factor belongs to the forkhead family. FOX transcription factors are expressed during development in a variety of tissues and are characterized by a distinct DNA-binding forkhead domain (FHD) (55, 399). They interact with chromatin and are responsible for transcriptional regulation both as activators or repressors, depending on the tissue. Stringent control of transcription by defined sets of transcription factors is essential for the correct regulation of a wide range of biological processes including cell fate determination, proliferation and differentiation events, which are necessary for the correct development of various tissue, e.g. specific forkhead transcription factors are involved in controlling the developmental steps of the circulatory system (299).

Human phenotype: The human *FOXC2* phenotype is known as lymphedema distichiasis syndrome (LDS; ORPHA:33001) and is one of the better investigated forms of primary lymphedema. Linkage studies mapped the disorder to chromosome 16q24.3 (244), and Sanger sequencing subsequently identified inactivating mutations in the *FOXC2* gene (105). LDS presents with an autosomal dominant inheritance pattern and variable expression. It is characterized by lower limb lymphedema and 94% of affected individuals present with distichiasis (which is the presentation of aberrant eyelashes arising from the meibomian glands) (46). While the distichiasis can be evident from birth, the lymphedema of the lower limbs usually develops late in childhood or later in life and sometimes not until the 5th decade, although (very rarely) congenital onset cases have been reported (115). Non-immune fetal hydrops has also been observed (100, 105, 131). The lymphedema of the lower limbs is most commonly bilateral. It is highly penetrant with 85% of individuals over 11 years of age presenting with some swelling of the lower limbs (46). Early onset varicose veins (49%), ptosis (31%), cardiac defects (7%), cleft palate (4%), spinal cysts and structural kidney abnormalities are other associated complications.

Intragenic mutations in *FOXC2* explained more than 95% of the cases with LDS (46). There is intra-familial variability in the expression of symptoms, with some individuals with a *FOXC2* mutation only presenting with distichiasis despite carrying the same mutation (46). At the molecular level, most mutations associated with LDS are frameshifts or nonsense mutations creating a premature stop codon, but missense variants have also been identified (27).

Animal models: Global *Foxc2* knockout mice die from E13.5 with skeletal abnormalities and vascular defects, while the heterozygous littermates appear normal (400). Interestingly, the FHDs of the Foxc1 and Foxc2 proteins share 99% similarity (214), and the global *Foxc1* knockout mice exhibit similar defects to the *Foxc2*-null mice (214, 400), whilst the homozygous double null mice die earlier and have more severe defects (215). This suggests that the genes have similar functions and could compensate (at least partially) for each other. Later studies have also shown how the two work in concert to control various aspects of lymphatic development (107, 280).

The initial studies on the genetically modified *Foxc2* mice did not focus on the lymphatics, but with the work of Kriederman et al., Dagenais et al. and Petrova et al., an interest in the role of Foxc2 in lymphatic development and function began to emerge (81, 212, 305). Their work showed that Foxc2 expression is not observed until E9.5 in endothelial cells of the cardinal vein and the surrounding mesenchyme in wildtype mice suggesting that Foxc2 may not be required for the initial LEC fate determination.

At around E15 there is a surge in Foxc2 expression followed by an increase in Lyve1 detected in all LECs in the entire primary plexus (281). This initiates the remodeling process. From E17.5 and into adulthood, Foxc2 expression in LECs varies depending on what identity they have taken on. (I) In capillary LECs Foxc2 expression is diminished and almost absent, whereas Vegfr3, Prox1 and Lyve1 remain highly expressed. (II) In lymphatic collector vessel LECs, Foxc2 is also downregulated, but to intermediate levels in comparison to the capillary LECs. In contrast to the capillaries, the expression of Prox1, Vegfr3 and Lyve1 is decreased. All four lymphatic markers remain at low expression levels in the lymphatic collector vessels throughout the rest of development and adulthood (332). (III) In the valve-forming regions of the lymphatic collector vessels, Foxc2 expression remains high. This high expression continues postnatally and is particularly evident in endothelial cells of the valve sinuses (332). Vegfr3 and Prox1 levels also remain high in valve leaflets (281).

The different expression profiles bring on the heterogeneity of the mature system making, for example, the LECs in the valves are molecularly distinct from those in the walls of the lymphangions (281, 305, 331). Furthermore, the high levels of Foxc2 and Vegfr3 in the valve-forming regions help suppress platelet-derived growth factor-b (Pdgf-b) to keep the vessel wall free from pericytes and SMCs, so only the vessel wall of the lymphangion includes a coating of pericytes and smooth muscle cells (Figure 9D) (332).

In homozygous mice with a LEC-specific *Foxc2* deletion, the few pups that did survive past E13.5 presented with a primordial lymphatic plexus in the skin. In the absence of the anticipated surge of Foxc2 expression at E15 to initiate the remodeling process, at E17.5 Prox1, Vegfr3 and Lyve1 remained highly expressed throughout the plexus, preventing the maturation into functional capillaries and collector vessels (281). Lack of Foxc2 expression meant that the LECs remained under the influence of Vegfc, and became highly branched as proliferation was significantly enhanced, so that in certain aspects the collector vessels of the mutant mice were similar to capillaries although they were more irregular and dilated (107). Lyve1 expression was also high in the vessels, indicating that they had not matured from capillary-like structures to collector vessels. As there was no Foxc2 to collaborate with Vegfr3 to control Pdgf-b, pericyte and SMCs patterning was further increased throughout the network also covering the lymphatic capillaries in the skin and the vessel walls in the valve regions of the collectors (Figure 9D, right panel) (281, 305). The stability of the lymphatic vasculature was also studied in this *in vivo* model, and loss of *Foxc2* lead to cell-cell junction defects (332). The loss of junctional integrity was also observed in the thoracic duct and the thick and dense basement membrane had increased permeability, which might explain the lymph leakage and chylous effusions observed in some pups. Post-natal deletion of *Foxc2* in the LEC-specific knockout mouse led to lymphatic valve agenesis, Lyve1 anomalous expression and aberrant pericytes and SMCs coverage in the vessel walls of the areas of valve degeneration (Figure 9D, right panel) (332). The Foxc2-mediated valve degeneration event can be explained by an increased ROCK activity causing a loss of adherens junctions and it has been shown that pharmacological inhibition of ROCK can restore the barrier integrity and prevented lymph leakage (280).

The heterozygous mouse phenotype with a LEC-specific deletion of *Foxc2* included ocular abnormalities such as distichiasis and a few mice (16%) exhibited hind leg swelling or periorbital swelling (212, 305). Therefore, the heterozygous *Foxc2* mice seem to recapitulate the human LDS phenotype. Histologically, the majority of pups presented with an increased number of dilated vessels with irregular lumen size. This was corroborated with lymphography, which also showed that 80% of the mice had retrograde flow (i.e. reflux) (212). The number and size of lymph nodes were generally increased throughout the body of the majority of the mice examined.

Disease mechanism: Similar to the findings from mouse studies, histological investigations of patients with *FOXC2* mutations showed dilated vessels in a skin biopsy from the affected swollen foot. The majority of the dermal vessels also had increased investment with pericytes and SMCs compared with control samples (305) suggesting that FOXC2 is also required for a pericyte/SMC-free lymphatic capillary network like observed in the mouse. Interestingly, no SMC investment was observed around the dermal lymphatic vessels in the skin biopsy from the unaffected arm of the same patient, which is consistent with the absence of lymphedema in the upper limbs of LDS patients. This also suggests that the functional dependency of LECs on FOXC2 may vary between regions of the body.

The observed enlargement of the lymphatic vessels in the patient biopsy could be explained by the hyperactivation of ERK signaling when FOXC2 expression is decreased. LECs from mice deleted for *Foxc1* or *Foxc2* presented with similarly abnormal vessels and after pharmacological inhibition of ERK this overgrowth was reversed (107). Increased proliferation of LECs was also observed *in vitro* when knocking down *FOXC2* with siRNA in human dermal LECs subjected to oscillatory shear stress (332). The cells also became more motile and the junctional integrity was lost, and this could be responsible for the impaired permeability of lymphatic collector vessels and lead to the leakage of lymph.

The lymphatic channels appear tortuous in lymphoscintigraphy images of lymphedema distichiasis patients (46, 335). Reflux of lymph within the lower limbs was also demonstrated and although there was substantial transport of tracer up the limb, there was clear accumulation of tracer activity in the lower leg and ankle suggestive of dermal backflow supporting a mechanism of reflux. Deeper popliteal nodes were also visible indicating that reflux also occurs into the deeper drainage system, another sign of abnormal lymphatic function. It was also observed that this functional failure in LDS patients was influenced by gravitational stress (dependency) (260). Abnormalities in valves caused by *FOXC2* mutations is very likely to be responsible for the reflux of lymph in the collecting vessels seen in LDS patients. In addition, all *FOXC2* mutation positive patients investigated had superficial venous reflux and approximately one third had deep venous reflux as well (258) due to reduction in size and number of venous valves (239).

Similar to mouse imaging results (212), historic imaging with direct radiocontrast X-Ray lymphography of human lower limb lymph drainage in LDS patients showed increased size and number of both lymphatic collecting vessels and ilio-inguinal lymph nodes (82). Although the anatomical resolution is not ideal in lymphoscintigraphy, the ilio-inguinal lymph nodes were clearly visualized and appeared increased in number in the patients scanned (335). This could be interpreted as hyperplasia of ilio-inguinal nodes which could be explained also by ERK overactivity in individuals with reduced FOXC2 expression levels. This aberrant formation of lymph nodes might also be associated with functional disturbances of lymph flow caused by faulty valves and vessel irregularities (348).

To understand why some of the phenotypic features are more penetrant (distichiasis in 94% of cases; lymphedema in 85%) than others (cardiac defects in 7%) it is important to get a better understanding of the molecular mechanisms of target gene interactions and transcriptional regulation. Some missense mutations located at the forkhead domain (FHD) have been found to reduce the DNA binding and transcriptional ability of the FOXC2 protein (32, 380). In contrast, four missense variants from LDS patients located outside the FHD were shown to increase transcriptional and transactivation activity in functional analysis in HeLa and COS-7 cells (380). This suggests that both gain-of-function (GOF) and loss-of-function (LOF) mutations in *FOXC2* can cause lymphedema, but a loss-of-function disease mechanism is much more common. The fact that some mutations cause LOF and others GOF

suggests that the regulation of FOXC2 is in need of fine-tuning. This is very common for many developmental processes in which forkhead proteins are involved (55). Although *FOXC2* is probably one of the most studied lymphedema genes to date, there is still much more to learn and understand. In order to be able to improve treatment for patients with *FOXC2* mutations we still need to understand more about the connections between gene mutations, protein function (or dysfunction) and the way it all contributes to the mechanisms that cause the disease. For example, correcting the incompetence of lymphatic valves in LDS patients would go a long way to correcting the lymphedema.

5.2.2 GATA2

GATA binding protein 2 (GATA2) is a member of a zinc-finger transcription factor family. The GATA2 protein has two zinc finger domains with different roles. The C-terminal zinc-finger is responsible for binding to 'GATA'-motifs of the target genes, while the N-terminal zinc finger is involved in the protein-protein interactions important for the formation of transcriptional complexes with other co-factors, which together then regulate the DNA binding activity of the C-terminus (44). On binding to the target gene, GATA2 can either stimulate or suppress the expression of that gene.

The actions of GATA2 are very complex and not yet fully understood (197), and the heterogenous clinical manifestations described below illustrate the pleiotropic functions of GATA2. It is known to play pivotal roles in embryonic development, determination of cell fate and maintenance of a wide range of biological functions throughout life. For example, GATA2 is a key regulator of hematopoiesis, where it is required for the development and maintenance of the hematopoietic stem cell pool (72), and also plays a significant role in the lymphatic system as described below.

Human phenotype: In 1979, Emberger described an autosomal dominant family with primary lymphedema associated with myelodysplasia and/or acute myeloid leukemia (ORPHA:3226) (99). Mansour and colleagues described seven new cases confirming this entity (245). In a whole exome sequencing (WES) study, heterozygous mutations were identified in the *GATA2* gene (291). Other cases have since been reported (345), including some with great intra-familial variability of expression (273). The cases described presented with primary lymphedema of the lower limbs (unilateral or bilateral), with the age of onset usually between childhood and puberty. Genital lymphatic abnormalities such as hydrocele and genital lymphedema were frequently observed. Although the primary lymphedema is usually confined to the lower limb and genital area in the individuals with *GATA2* mutations, one case of fetal hydrops and one report of possible intestinal lymphangiectasia have been reported (245).

Lymphedema often precedes the hematological abnormalities observed in Emberger patients, so it is recommended to consider this syndrome when evaluating a patient with lymphedema that fits with the described phenotype. Initially the hematological

abnormalities present as monocytopenia. The hematological transformations usually present during the 2nd decade and can develop into pancytopenia, myelodysplasia or acute myeloid leukemia with high mortality. Therefore, this is a life-threatening condition, which demands close monitoring. A high incidence of monosomy 7 or trisomy 8 in the bone marrow is frequently reported.

Other features reported include generalized cutaneous warts (presumably a result of reduced immune function), congenital sensorineural deafness and minor anomalies such as mild hypertelorism, epicanthic folds, and slender fingers.

In addition to Emberger syndrome, patients with *GATA2* mutations have been described with primary immunodeficiencies including: 'dendritic cell, monocyte, B and NK lymphocyte deficiency' or MonoMAC (91, 161), chronic myeloid leukemia (420), and 'inherited myelodysplastic syndrome/acute myeloid leukemia predisposition phenotype' (143). Reports have also shown some cases present only with primary lymphedema (160, 161, 168); therefore, it is suggested that this group of disorders represent a single spectrum of disease (152). 'GATA2-deficiency syndrome' is therefore used to include all disorders caused by sporadic or familial inactivating *GATA2* mutations (152). The disorder has a low degree of non-penetrance and the probability of being symptom free at the age of 20 is 38% but it reduces to only 8% at 40 years (92). These figures, however, could be due to ascertainment bias as asymptomatic individuals carrying a *GATA2* mutation have been reported (143, 245).

Animal models: While homozygous *Gata2* null mice die by E11.5 with severe anemia due to a failure in the formation of mature blood cells, heterozygous mice survive, but show poor expansion of the hematopoietic stem cell population (371). From the research on mice, together with human clinical studies, it can be concluded that both parental *GATA2* genes are required to produce an adequate supply of hematopoietic stem cells, otherwise there is an increased risk of developing leukemia. However, the role of GATA2 deficiency in leukemia is not yet well understood.

As the *Gata2* deleted mice die at E11.5, no lymphatic phenotype was observed. Therefore, it was not until Kazenwadel and colleagues looked at Gata2 specifically in the lymphatics of wildtype mice, that a correlation between GATA2 expression and lymphatic system development was identified (202). When comparing LECs with BECs isolated from the skin of embryonic mice, they found that *Gata2* mRNA expression was 10 times higher in LECs. Immunostaining of tissue sections showed Gata2 protein in the lymphatic vessels of embryonic (E16.5) and adult mice. Gata2 expression levels were particularly high in lymphatic valve-forming cells at E16.5 and were maintained in the valve leaflets through adulthood. It was therefore suggested that Gata2 must have a key role in valve development and maintenance.

Silencing of *Gata2* in primary LECs isolated from the dermis of E16.5 mice, led to a downregulation of other genes e.g. *Foxc2*, *Nfatc1*, *Prox1* and *Itga9*, indicating that they are partly controlled by Gata2. Expression of Gata2 thus precedes that of Prox1 and Foxc2 in lymphatic valve development (202). In a later study, the same group showed that the turbulent flow that initiates valve morphogenesis as described in the previous section, increases *GATA2* mRNA and protein expression in human LECs (200). The cells had the morphological appearance of valve-forming cells. Furthermore, the increase of GATA2 expression led to increased levels of nuclear FOXC2 expression. This led the authors to conclude that GATA2 is mechanoresponsive, and that oscillatory shear stress promotes GATA2 to activate *FOXC2* (200).

A mouse model with the selective deletion of *Gata2* in the lymphatic vasculature was generated to investigate the role of Gata2 during remodeling and maintenance. The mice had malformed lymphatic vessels that appeared tortuous and were aberrantly invested with SMCs, and had dysmorphic lymphatic valves (200). However, it is not only lymphatic valves that are regulated by Gata2, since when observing embryos from earlier developmental stages, the lymphovenous valves were also found to be malformed, and the jugular lymph sac was blood-filled. This indicated that Gata2 is necessary for the effective separation of the blood from the lymphatics through lymphovenous valve formation (200).

In addition to the oscillatory shear stress promotion of a GATA2-driven transcriptional program for lymphatic vessel maturation described above, GATA2 has also been shown to play a role earlier in lymphangiogenesis. A study has shown that as LECs migrate from a stiffer environment in the cardinal vein wall to a softer matrix outside of the cardinal vein a Gata2-driven response is induced (123). This includes upregulation of VEGFR3 in the cells and an increased responsiveness to VEGFC, thus enabling the migration and sprouting program.

The notion that GATA2 is controlling VEGFR3 expression is also supported by the findings of Coma et al. (73). Silencing of *GATA2* and *LMO2* (another transcription factor that forms a multimeric complex with GATA2) expression in cultured LECs, inhibited VEGFA/C-induced LEC sprouting in an NRP2-dependent manner. Based on these experiments, it appears that GATA2 is involved, at least partly, in the regulation of early lymphangiogenesis via the VEGFC-VEGFR3 signaling pathway.

The main conclusion from these studies is that GATA2 regulates different transcriptional responses depending on the biological context. If the LECs are exposed to a soft matrix, the upregulation of genes involved in cell migration takes place, i.e. the ‘sprouting program’ via the VEGFC-VEGFR3 signaling cascade is initiated. On the other hand, if the LECs are exposed to turbulent flow or oscillatory shear stress then the lymphovenous valve and lymphatic valve forming signaling cascades are initiated.

Disease mechanism: Skin biopsies have been obtained from two patients with GATA2-deficiency syndrome (309). Unfortunately, they were not stained for lymphatic endothelial markers, but fibrotic changes, which are a common feature in persistent peripheral lymphedema, were apparent in one of the cases. Interestingly, the skin of one of the patients (with a p.Thr354Pro mutation and primary lymphedema), showed granulomatous infiltrate in the upper and mid-dermis which cleared up after a bone marrow transplant. This suggests that the monocyte/macrophage dysfunction caused by the *GATA2* mutation is instrumental in predisposing to granulomatous disease, such as the mycobacterial infections seen in MonoMAC syndrome, and that disturbances in macrophage and lymphocyte function can also contribute to lymphedema (285).

Lymphoscintigraphy imaging of patients with GATA2-deficiency syndrome that had clinical signs of peripheral lymphedema demonstrated a lack of lymphatic uptake of intradermally injected tracer in some individuals (245, 291). This may be due to functional aplasia in the lymphatic capillaries, suggesting that the disease mechanism could be of a similar kind to that seen in Milroy disease. Therefore, the finding that GATA2 also regulates the VEGFC-VEGFR3 signaling axis is of great interest (123) and may provide an explanation for the apparent lack of tracer uptake by the initial lymphatics in some GATA2-deficient individuals. There was normal uptake of tracer in the arms of those who underwent four-limb lymphoscintigraphy (245, 291). This suggests that LEC populations in the different body regions might be heterogenous. Some rerouting around the ankle area in other patients suggests that there might also be some lymphatic valve problems.

To date more than 100 different germline mutations in *GATA2* have been identified in roughly 400 reported cases (403). Most variants reported thus far are truncating mutations (stop-gain, indel, splice site) prior to or within the zinc finger 2 domain (ZF2) and about 30% are missense mutations within the ZF2. About 10% of reported mutations are in conserved non-coding and intronic regions, which were shown to affect GATA2 transcription (160) but only a minority of the reported *GATA2* mutations have been functionally studied.

Depending on the clinical phenotype different type of mutations were found in *GATA2* and a variety of mechanistic effects observed. Variants from families with reported lymphedema, when transfected into HEK293 cells showed a significant loss of GATA2 function, including a severely reduced ability to bind the *PROX1* enhancer compared to wildtype cells (200, 291). Some *GATA2* variants found in families with no primary lymphedema retained some capacity to bind the *PROX1* enhancer, albeit not to wildtype levels (200). Yet, other variants, when expressed in HEK293T cells, have been shown to cause gain of function leading to an increase in GATA2 activity when compared to the wildtype protein (420). This differential capacity of GATA2 mutants to bind and regulate the expression of target genes was also observed by other groups (78).

In addition, depending on the GATA2 responsive element under study, the two variants investigated by Hahn et al had loss-of-function and also dominant negative effects (143).

The complexity of GATA2 function and how it responds when mutated, combined with the variable effect on the multiple responsive elements, could explain why GATA2-deficiency syndrome is a pleiotropic condition, and why some germline mutations are more likely to lead to primary lymphedema than others. To add to the complexity, analysis of five families harboring the *GATA2* p.Thr354Met mutation displayed significant intra- and inter-familial variations in myelodysplastic syndrome/acute myeloid leukemia disease latency and penetrance. So even when harboring the same mutation, the effect can vary, suggesting that individuals may require additional co-operating events for the development of the malignancy (6). What determines the variability of the other phenotypic traits of GATA2-deficiency syndromes such as lymphedema and hearing loss, also remains unclear.

5.2.3 PIEZO1

Piezos are large mechanosensitive ion channel proteins mainly expressed in non-sensory tissues (405). PIEZO1 is believed to have many different roles depending on the tissue where it is expressed, e.g. it has been shown that PIEZO1 acts as a sensor of epithelial cell crowding and stretching (140), controls blood pressure (389), and contributes to the regulation of urinary osmolarity (254) and cell volume in red blood cells (108). PIEZO1 has also been shown to have an important role in vascular biology as the protein is expressed in developing blood vessels and plays a key role in blood vessel formation (227, 313, 320).

Human phenotype: *PIEZO1* is associated with autosomal recessive generalized lymphatic dysplasia with non-immune fetal hydrops (ORPHA:568062) caused by biallelic, loss-of-function mutations (117, 237). The condition is characterized by a widespread lymphedema that can affect all segments of the body. There is a high incidence of non-immune fetal hydrops, i.e. presenting with persistent bilateral pleural effusions, ascites and subcutaneous edema, with either fetal death or a complete resolution of the hydrops postnatally. The pregnancies are frequently complicated by polyhydramnios. The babies are often hydropic at birth, with generalized edema and pleural effusions requiring ventilation for several weeks, and after introduction of milk feeds, the pleural effusions become chylous.

Sometimes lymphedema of the peripheries recurs in early childhood. This is mainly lower limb lymphedema, but can also involve the hands, arms, face and genitals. Genital edema, which may be intermittent, is frequent in males, sometimes with thickened scrotum, edema of the foreskin, and hydroceles.

Many patients have multiple episodes of cellulitis in their legs, and several of the patients have also had recurrent, severe facial cellulitis. The facial cellulitis is a striking feature and is rarely seen in other forms of primary lymphedema. Four cases with severe facial cellulitis

were reported with significant co-morbidities such as high pyrexia and respiratory distress that led to admission to intensive care with ventilation (117).

In addition to facial swelling, some of the patients had mild dysmorphic features (cupped simple ears, mild telecanthus due to epicanthic folds, and micrognathia). Some patients were also reported to have varicose veins, papillomatosis and warts. Many of the affected children had systemic involvement with chylothoraces and/or pericardial effusions. Intestinal lymphangiectasia, although rare, has been observed (PSM, personal observation).

Animal models: *In vivo* studies have shown that Piezo1 expression is high during mouse embryonic development and that homozygous disruption of the mouse *Piezo1* gene leads to embryonic lethality due to disturbed vascular remodeling and vessel organization (313). Li et al. showed similar findings in both their global and endothelial specific *Piezo1* mouse models (227). Deficiency of Piezo1 profoundly disturbed the developing vasculature and was lethal within days after the heart started beating. *Piezo1* haploinsufficiency was not lethal but abnormal endothelial cells were detected in mature blood vessels.

Furthermore, the importance of Piezo1 as a sensor of blood flow was also shown, and it was concluded that mechanistically, Piezo1 channel activity is stimulated by shear stress leading to calcium (Ca2+) entry in to the endothelial cell, increased calpain activity, and modification of the actin cytoskeleton and the focal adhesions required for cell reorganization (227). Loss of Piezo1 resulted in reduced mechanosensitivity of the endothelial cells, and failure in cell alignment in the direction of blood flow.

The role of PIEZO1 in lymphatic development has been less clear, but immunohistochemical analyses of human week-17 fetal samples showed a PIEZO1 positive signal in lymphatic vessels of the peritoneum (14), indicating a role for this protein in the lymphatics. This has recently been confirmed as Choi *et al* have demonstrated a role for PIEZO1 in lymphatic valve development (67). They showed that embryonic deletion of *Piezo1* inhibited lymphatic valve formation in newborn mice, and postnatal deletion in lymphatics caused substantial valve degeneration. They also showed that Piezo1 becomes mechanically activated when sensing oscillating shear stress, which then starts the genetic program controlling lymphatic valve development and maintenance.

In another study, deletion of *Piezo1* in two mouse lines (a BEC-specific [Tie2] and a LEC-specific [Lyve1]) resulted in pleural effusions and the pups died postnatally (279). The number of lymphatic valves was reduced as it was shown that the protrusion process in the valve leaflets (Figure 9D), which is associated with collective cell migration, actin polymerization, and remodeling of cell-cell junctions, was impaired. This study supports too that mechanical activation of Piezo1 is required for lymphatic valve formation but Foxc2 and Nfatc1 expression levels were unaffected in the *Piezo1* knockout mice. Interestingly, FOXC2 and GATA2 were upregulated *in vitro* in cultured primary LECs on upregulation of PIEZO1,

and the oscillatory shear stress-induced upregulation was abrogated by Piezo1 knockdown (67).

Piezo1 can be activated using Yoda1 (a chemical agonist). Yoda1 treatment was shown to accelerate lymphatic valve formation in animals, but also triggered upregulation of the lymphatic valve genes in cultured LECs without exposure to oscillatory shear stress (OSS) (67). More studies are required to fully understand how these interactions between shear stress signals and the protein networks controlling lymphatic valve development and maintenance are regulated.

Disease mechanism: Lymphoscintigraphy of affected individuals with generalized lymphatic dysplasia showed abnormal lymphatic drainage in both legs and arms with poor uptake of tracer in the lymph nodes in the groin and axillae (117). There are signs of failure of the superficial collector vessel function in the lower limbs, evidenced by rerouting through the skin (dermal backflow). Popliteal nodes showed prominent uptake of tracer indicating some rerouting via the deeper lymphatics, which is unusual and represents deep rerouting of the tracer.

If the cell-cell junction remodeling impairment described by Nonomura and colleagues (279) has a similar effect on the LECs in the lymphatic collector vessels in these patients as is found for Foxc2 in the mouse (280), then the vessels of the patients with generalized lymphatic dysplasia could be leaky and that could explain the superficial rerouting through the skin.

However, the imaging results suggest a reduced functioning of the initial lymphatics, which cannot be explained from the current findings in mice, which mainly relate to the role of Piezo1 in lymphatic valve development in the collectors. Nevertheless, PIEZO1 is a mechanically-gated channel and its function has been shown to be determined not only by the presence and direction of mechanical force (such as shear stress) but also by ECM proteins such as Collagen IV that modulate the channel gating sensitivity (127). It could be speculated that PIEZO1 in capillary LECs of patients with generalized lymphatic dysplasia is not being gated properly, for example due to an unresponsiveness to Collagen IV, leading to reduced functioning of the initial lymphatics. Whether Collagen IV modulates PIEZO1 by direct binding, or indirectly by signaling through other membrane bound proteins, is yet not known.

Mutations in *PIEZO1* were shown to lead to a faulty protein with reduced ion-channel activity. Through functional analysis of red blood cells isolated from patients with generalized lymphatic dysplasia a reduced abundance of PIEZO1 channels in the red blood cell plasma membrane was found (237). The absence of symptoms in the parents carrying just one PIEZO1 variant, supports the hypothesis of a loss-of-function disease mechanism. Impaired mechanical stimulation of LECs and the downstream signaling pathways, could

disrupt embryonic lymphovascular development in generalized lymphatic dysplasia patients, contributing to hydrops and childhood onset peripheral edema.

Interestingly, some variants in the *PIEZO1* gene have been shown to produce a gain of function, leading to a prolonged ion-channel activity of mutant PIEZO1 channels in red blood cells (9, 16) in autosomal dominant dehydrated hereditary stomatocytosis with or without pseudohyperkalemia and perinatal edema (DHS; ORPHA:3202; MIM:194380) (417). It is believed that the gain of PIEZO1 function in DHS may lead to a different disease mechanism, which does not affect the lymphatic system and thus the fetal hydrops reported in DHS patients may be due to a distinct underlying cause, since there are no reports of peripheral lymphedema later in life in any of the known DHS cases.

Another interesting observation is that the fetal hydrops in both conditions is able to fully resolve in the first few weeks after birth. It is unclear what causes this but unknown physiological changes may occur then, which could compensate for the defective function of PIEZO1 (250). If this enigma could be understood, the potential for treatment of this, and other lymphatic related hydrops, is clear. More work is needed to fully understand the role of PIEZO1 in lymphatic development and physiology.

5.2.4 Connexins - Gap Junctions

Gap junctions are specialized intercellular connections mediated by members of the Connexin protein family, which help in holding cells together and providing structural stability. Six connexin proteins oligomerized in the plasma membrane make up a hemichannel and when two hemichannels of adjacent cells interact, a gap junction is formed allowing direct intercellular communication.

Through these channels, small molecules (e.g. nutrients, metabolites, ions) can passively diffuse from the cytoplasm of one cell to another. However, the channels are highly selective, and the type of molecule allowed to pass through depends on which connexins constitute the channel. Thus, gap junctions can be involved in cell signaling by controlling the transfer of molecules between cells, thus regulating which pathways are activated.

Five connexins, Cx26, Cx37, Cx43, Cx45 and Cx47, have been reported to play important roles in lymphatic biology. Cx26 (*Gjb2*) has been shown to participate in dermal lymphatic vessel development in mice (90), but to date, no human lymphatic phenotype has been reported. Also, no human lymphatic phenotype is currently associated with Cx45 (*GJC1*). This connexin is expressed in the lymphatic smooth muscle layer, where pacemaker signals transmitted through these cell-cell couplings synchronize the necessary calcium rise, which leads to a coordinated contraction of the connected muscle cells in the lymphangion (58). The same authors concluded that Cx37, Cx43 and Cx47 are not involved in this process but instead these three connexins are enriched in the endothelial cells located in venous and lymphatic valves.

Connexin 37 (GJA4)

Human phenotype: The only association of Cx37 *(GJA4)* with lymphedema is a report of an increased risk for secondary lymphedema following breast cancer treatment in individuals with SNPs in the *Cx37* gene (141). However, this GWAS result still needs to be replicated in another breast cancer cohort to be confirmed.

Animal models: The expression of Cx37 in the lymphatics is very localized, mainly observed on the downstream side of the valves (189). It has been demonstrated that Cx37 expression in mice is activated by Foxc2 and oscillatory shear stress in LECs, which then promotes the nuclear translocation of NFATC1, enabling the formation of competent lymphatic valves (331). The work of Sabine et al. clearly shows that Cx37 is a crucial regulator of the molecular pathway controlling the assembly and delineation of the lymphatic valve territory in the collector vessels during development and maintenance.

Loss of Cx37 in mice prevents the organization of the valve-forming LECs, and homozygous *Cx37*-deleted mice have reduced numbers of valves, impaired lymphatic drainage (189) and the lymphovenous valves do not invaginate properly (128).

Postnatal deletion of *Cx43* in the LECs of a homozygous *Cx37* null mouse results in regression of lymphatic valve leaflets 5-7 days after induction (57). This suggest that both Connexins play a critical role together in lymphatic valve maintenance and sudden deletion of one can lead to severe valve dysfunction. Similarly, combined deletion of *Foxc2* and *Cx37* in mice disrupts lymphatic vessel growth and valve formation (190). How Cx37 relates to secondary lymphedema (for example following cancer treatment), is unclear.

Cx43 (GJA1)

Human phenotype: Heterozygous mutations in Cx43 *(GJA1)* have been associated with various forms of disease, but only autosomal dominant oculodentodigital dysplasia (ODDD; ORPHA:2710) has been reported to be associated with lymphedema (47). In addition to the characteristic features of ODDD, a patient with a missense variant in *Cx43* had bilateral lower limb edema from the age of 30 years. Initially, the lymphedema fluctuated but progressively became chronic. Two other family members with the mutation presented with lymphedema; one of them from the age of 14 years. All deep and superficial veins appeared normal on venous duplex ultrasound, only minor valve incompetence was noted in the short saphenous vein.

Animal models: The homozygous *Cx43* global mouse knockout had no lymphatic valves, and perinatal mortality was high (189). Conditional deletion of *Cx43* in the lymphatic vasculature caused altered lymphatic capillary patterning and a delay (rather than a complete block) in lymphatic valve initiation. The few valves that did develop were immature with incomplete leaflet elongation (270). The physiological consequences of these lymphatic changes were

leaky valves, insufficient lymph transport and reflux, and a high incidence of lethal chylothorax.

Disease mechanism: The p.Lys206Arg mutation reported by Brice et al. (47) sits in an important region of the protein, the SRPTEK motif, where mutations at the p.Arg202 residue have also been reported in several ODDD patients (300). This motif is believed to be important for appropriate docking of the hemichannels, and mutations in the PTEK region were shown to decrease the formation of functional gap junctions (393). The mutant Cx43 protein was localized to the cell surface of HeLa and N2A cells, where it disrupted the formation of complete and functional channels (116). Levels of normal Cx43 were also affected in those mutants, suggesting a dominant-negative molecular mechanism.

Lymphoscintigraphy imaging of lymphatic vessels in the lower limbs and arms of the ODDD patient showed no obvious anatomical abnormalities as the route of the radionuclide tracer was visible and appeared normal (47). However, quantification figures revealed reduced transport of tracer, confirming impaired lymphatic function; including the upper limbs, although there was no arm lymphedema on visual inspection of the patient. This imaging result is suggestive of an initial lymphatic vessel problem rather than a problem with the valves of the deep lymphatic collector vessels. If patients are affected by abnormal capillary patterning as found in the mouse model, it could explain an impairment in uptake of interstitial fluid. However, this is only speculation and needs further investigation.

Cx47 (GJC2)

Human phenotype: Heterozygous mutations in human Cx47 (*GJC2*) have been shown to be implicated in the development of autosomal dominant late onset four limb lymphedema (ORPHA:79452; MIM:613480) (112, 290). There was inter-familial variability of severity but the overall phenotype was one of late onset lymphedema affecting all four limbs. A significant number of these individuals also had varicose veins (290).

Cx47 mutations have also been associated with increased risk for secondary lymphedema following breast cancer treatment (114) although it could be that the breast cancer treatment has exposed an underlying primary lymphedema hitherto unrecognized. This is supported by the finding that one of the mutations identified in one of the breast cancer cases with secondary lymphedema has also been reported in an individual with late onset four limb lymphedema (114).

Animal models: Expression analysis has shown that Cx47 is widely colocalized with Cx43 on the upstream side of lymphatic and venous valves during early development (189, 271). In contrast to the homozygous *Cx43* null mice, the lymphatic valves appeared normal in the homozygous *Cx47* null mouse (271, 284). Lymphatic vessel morphology was also normal, the contractility was unaffected, and the drainage appeared normal (257). These findings suggest that Cx47 is only modestly implicated in lymphatic pathophysiology. Cx47 is also

expressed in venous valves in mouse during early development and is required for the development of peripheral venous valves, but not for the formation of central venous valves (271).

The other two Connexins, Cx37 and Cx43, are also specifically expressed in venous valves in mouse and have also been shown to be necessary for their development (239, 271, 272). The effect of *Cx37* ablation in mice is the most severe as with 100% penetrance, valves of both central and peripheral veins fail to form. Inactivation of *Cx43* in mice produces a central venous valve defect that is variable in severity. The fact that Connexins are also involved in venous valve formation indicates that venous valve and lymphatic valve morphogenesis are controlled by very similar mechanisms.

Disease mechanism: As for the Cx43 mutation positive patients, lymphoscintigraphy of Cx47 patients demonstrated significantly reduced absorption of isotope tracer from the tissue by the peripheral lymphatics, suggesting an initial lymphatic vessel problem (290) which would result in reduced absorption of fluid from the interstitium. The lymphoscintigraphy images do not suggest reflux, so lack of contractility or valve failure as the disease mechanism is less likely. This suggestion is supported by the fact that lymphatic valve development is unaffected in the mouse model. It has been borne in mind that the deep lymphatic collectors cannot be adequately imaged with currently available techniques, so these vessels could be also affected.

Like the Cx43 mutation positive patient, a number of Cx47 mutation positive patients only showed clinical signs of lymphedema in the lower limbs but had lymphoscintigraphic evidence of impairment in all four limbs (290). Whether these individuals develop clinical signs of upper limb lymphedema later in life will need to be monitored.

The patients with Cx47 (*GJC2*) gene mutations also have truncal varicose veins suggestive of venous valve failure (290), and ultrasound imaging in patients with *Cx47* mutations showed fewer venous valves with shorter leaflets (239). However, as the patients do not show any other clear signs of chronic venous insufficiency, the observed venous physiology in these individuals cannot solely explain the observed edema, thus the edema in these patients must also be a consequence of a failure in lymphatic drainage.

5.2.5 FAT4

FAT4 encodes a large cadherin-related transmembrane protein of the protocadherin family. Its ligand, DCHS1, belongs to the same protein family. FAT4 and DCHS1 are thought to exert bidirectional signaling, and thereby influence each other's gene expression (248). Together they can control cell migration, cell growth and polarized cell division through the Hippo and Planar Cell Polarity (PCP) signaling pathways. FAT4 and DCHS1 have been linked to human disease with a lymphatic phenotype reported in cases with *FAT4* gene mutations.

Human phenotype: Whole exome sequencing identified homozygous or compound heterozygous mutations in the *FAT4* gene in patients with Hennekam lymphangiectasia-lymphedema syndrome type 2 (ORPHA:2136) (10). This is an autosomal recessive primary lymphedema characterized by generalized lymphatic dysplasia both peripherally and internally. Cases of an allelic and phenotypically overlapping autosomal recessive disorder, van Maldergem syndrome (ORPHA:314679; MIM:615546), have also been reported with *FAT4* mutations (53).

Most Hennekam syndrome type 2 cases described to date have lymphedema of the limbs and lymphangiectasia affecting the gut, pericardium, lungs, kidneys and genitalia (10). The onset of lymphedema is variable and in contrast to *CCBE1*-related Hennekam syndrome, patients with *FAT4* mutations can have lymphedema onset later in childhood. All patients were described as having an unusual facial appearance with flat face, flat nasal bridge, hypertelorism, epicanthus, blepharophimosis, small ears, small mouth and irregular dentition. Cognitive impairment, hearing loss, distal limb malformations (mostly cutaneous syndactyly but also camptodactyly), osteopenia and poor overall growth were also reported.

Many of these phenotypic features are also reported for van Maldergem syndrome (246, 379), and one of the homozygous mutations (p.Glu2375Arg) associated with Hennekam syndrome type 2 (10), had already been reported in compound heterozygosity together with a truncating mutation in a patient with van Maldergem syndrome, indicating overlap also in the molecular disease mechanism for the two conditions (53). Although the phenotype is variable in both entities, with both inter- and intra-familial differences reported, it is striking how few van Maldergem syndrome cases have been reported to present with lymphedema. Whether there are different molecular mechanisms dependent on the type of *FAT4* mutation, needs further investigation. Or could the differences simply be due to ascertainment bias, with the two entities being recognized differently depending on referral pathways for recruitment? Whichever is the case, we can conclude that FAT4 plays a role in lymphatic development in humans.

Animal models: Most homozygous *Fat4* knockout mice die during the perinatal period (334) and show defects of their inner ear, kidney and neural tube indicating that Fat4 signaling affects multiple targets. The pleiotropic phenotype of both Hennekam and van Maldergem syndromes, can probably also be explained by the multiple roles of FAT4-DCHS1 signaling, but until the exact nature of this signaling pathway has been established, we will not fully understand the basis of the disease in the two syndromes. It has recently been shown that Fat4 signaling via the Hippo pathway controls neurogenesis and this could explain the central nervous system phenotype (53).

A picture is also emerging on what can be causing the lymphatic phenotype in these patients. Multiple organs, especially branching organs such as the lymphatic system, require Fat4/Dchs1 during mouse embryonic development (248) and several studies have shown

that Fat4 is also involved in the PCP signaling pathway (216, 247, 248, 334, 415). The noncanonical planar cell polarity (PCP) pathway is one of the Wnt signaling transduction pathways and regulates the cytoskeleton organization, responsible for the shape of a cell. In addition, it is known that cellular rearrangements are important for valve development (23, 25), and that these are influenced by the shear stress of the increasing flow in the developing lymphatic system (Figure 9D) (331). It has therefore been speculated that the PCP pathway could be involved in the regulation of cell orientation in lymphatic endothelial cells, and it was confirmed that the core-PCP proteins, Celsr1 and Vangl2, establish the PCP signaling required for lymphatic valve morphogenesis (367). It was later reported by the same group that it is Fat4 and Dchs1 signaling that directs the polarization during lymphatic valve morphogenesis (310). The authors showed Prox1-expressing cells are visible at E16 indicating that valve initiation was properly established, however, the valve forming cell clusters were randomly orientated, 60% of the mature valves in newborn mutant mice were abnormal and valve morphogenesis had failed. This was corroborated by Betterman and colleagues in a LEC-specific mouse model, who showed that conditional *Fat4* deletion in LECs led to mouse embryos with dilated dermal lymphatic vessels and a reduced number of immature valves (34). Thus, Fat4 has a proven role in valve formation during lymphovascular maturation, linking it to the lymphatic phenotype observed in *FAT4*-mutation carriers.

Disease mechanism: No imaging investigations have yet been undertaken for Hennekam Syndrome type 2 patients to assess their lymphatic function, so we can only speculate from animal and cell models. Genome-wide binding profiling indicates that *Fat4* is a target gene for Gata2 binding and *in vitro* studies indicate that there could be a link between FAT4 and VEGFR3 (34). The *in vitro* studies concluded that Fat4 is coordinating LEC polarity in response to flow, and that the *Fat4*-deficient human LECs that had failed to polarize remained circular and increased in size in response to laminar flow. Defective polarity could lead the *Fat4*-deficient LECs to divide in a random fashion rather than in the expected length-extending polarized manner. This could, in combination with the increased cell size, lead to the observed vessel dilation, which could be a possible explanation of the lymphangiectasia reported in some FAT4-associated Hennekam syndrome patients. However, more work needs to be carried out to fully understand the role of FAT4 in lymphatic development and physiology.

6. Other genes associated with lymphatic dysfunction

According to the current St. George's classification of primary lymphatic anomalies the genes and molecules described so far in this review are mainly associated with disorders where primary lymphedema is the main feature of the clinical presentation (Figure 6, pink, purple and green categories) or an associated feature of a syndrome (Figure 6, blue category) (136). There are additional syndromes caused by either a chromosomal abnormality (e.g. Turner syndrome with 45X0 or Phelan McDermid syndrome with 22q terminal deletion or ring chromosome 22) or by a single gene fault (e.g. Tuberous sclerosis

with *TSC1* and *TSC2* mutations, or Noonan syndrome/RASopathies) where lymphedema occurs, but since it is not the dominant phenotypic feature of those syndromes, the causative genes (if known) are not covered in this review (Figure 6, blue category) (see Gordon et al. and Brouillard et al. for lists of genes and associated syndromes) (49, 136).

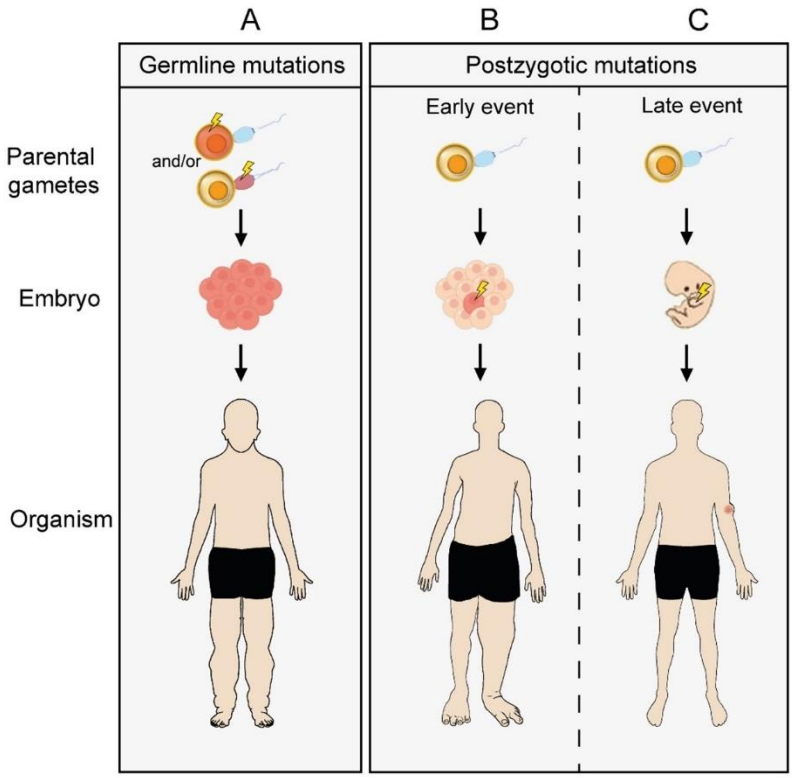
The genetic disorders covered so far in the review are all caused by germline mutations (Figure 10A), however, a further category of disorders listed by Gordon *et al.* in the St. George's classification (136) is the lymphatic malformations. Together with 'vascular malformations associated with other anomalies' they make up a group represented by isolated cases of anomalies with a somatic/post-zygotic mosaic disease presentation (Figure 6, yellow section). Most of these cases are sporadic and caused by *de novo* postzygotic mutations (Figure 10B-C).

These lymphatic malformations may be isolated, closed system anomalies, which do not connect with the main lymphatic drainage vessels (extra-truncular), and therefore do not cause lymphedema. However, other more extensive lymphatic malformations, which do interfere with main lymph drainage vessels, can cause lymphedema (truncular) (222). Sometimes the lymphatic malformations do not appear alone but as part of a combined (lymphatic-venous) vascular malformation. In this case they are usually classified as 'vascular malformations associated with other anomalies' according to the ISSVA classification (169).

The understanding of postzygotic mutations and how they can lead to disease is an emerging field. So far there have been reports of isolated cases with primary lymphatic anomalies or mixed vascular malformation caused by postzygotic mutations in the PI3K/AKT/mTOR or RAS/MAPK pathways (Figure 10D) (49, 278). As the coverage of lymphatic malformations was not the main objective of this review, the genes associated with these mosaic disorders will only be mentioned briefly.

6.1 Postzygotic PIK3CA mutations

Postzygotic mutations that activate PIK3CA have been described in a range of congenital vascular anomalies with very variable clinical manifestations, of which tissue overgrowth is the most dominant. Many were thought to be independent clinical syndromes e.g. Klippel-Trenaunay syndrome, CLOVES Syndrome and megalencephaly-capillary malformation (MCAP) syndrome, but recently the term PROS (PIK3CA-related overgrowth spectrum; ORPHA:530313) was coined to describe all these conditions due to a postzygotic mutation in *PIK3CA* (203). The phenotype of this spectrum ranges from isolated disease, such as macrodactyly, megalencephaly, or vascular malformations, to syndromes defined by tissue overgrowth, vascular malformations and epidermal nevi (e.g. CLOVES). Isolated lymphatic malformations with and without lymphedema have been reported in individuals with PROS (289, 324)..


A
B
C
Germline mutations
Postzygotic mutations
Early event
Late event
Parental gametes
and/or
Embryo
Organism

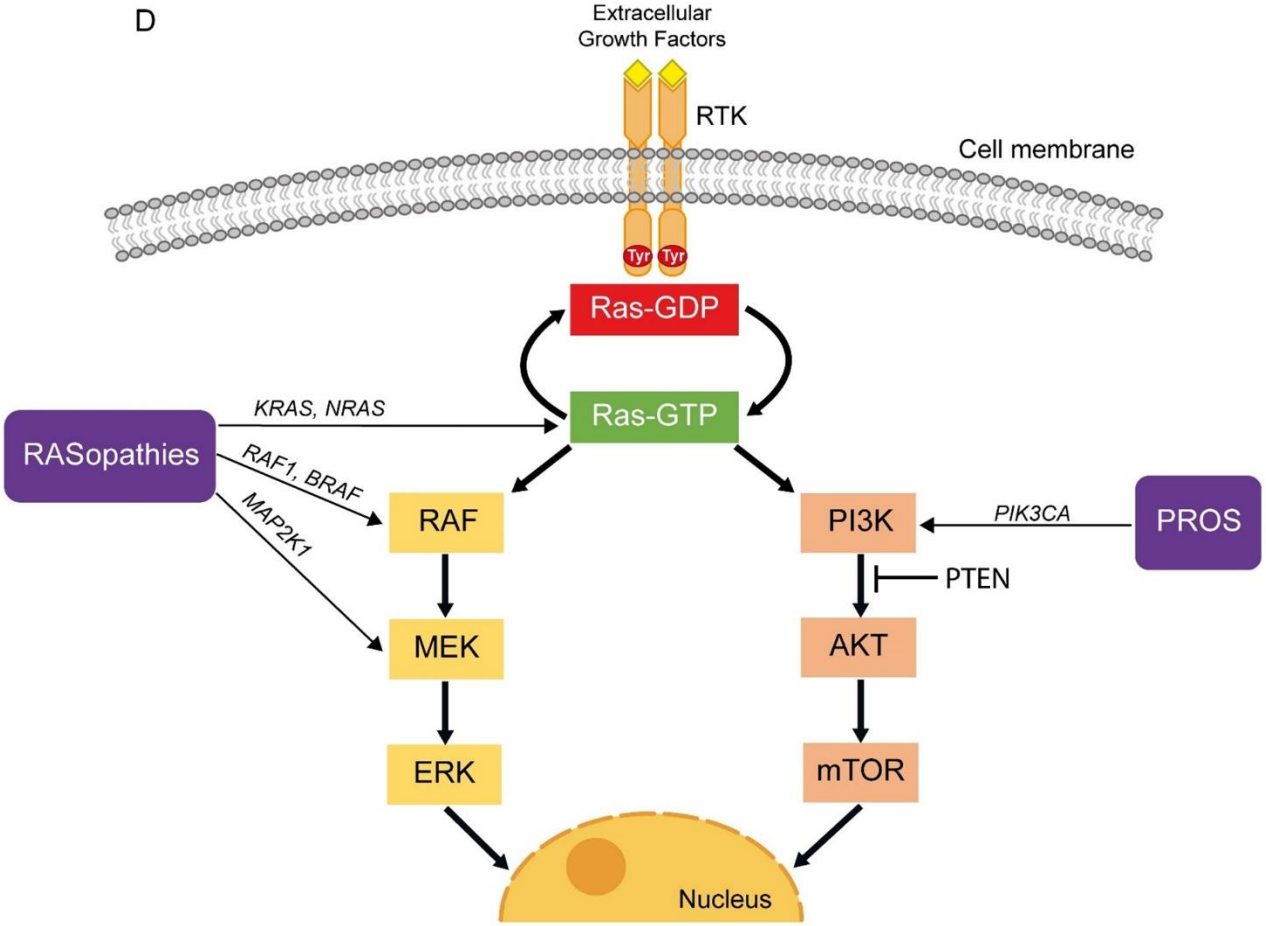

D
Extracellular Growth Factors
RTK
Cell membrane
Tyr
Tyr
Ras-GDP
Ras-GTP
KRAS, NRAS
RASopathies
RAF1, BRAF
MAP2K1
RAF
MEK
ERK
PI3K
PIK3CA
PROS
PTEN
AKT
mTOR
Nucleus

Figure 10: Impact of germline vs postzygotic mutations in the PI3K/AKT/mTOR and RAS/MAPK pathways on phenotype. *(A) Germline mutations are present in the parental gametes (sperm and/or egg) and in all cells of the future embryo. For an autosomal dominant disorder, as illustrated here with bilateral lower limb lymphedema, the mutation will have happened in either the sperm or the egg whilst the other parental gamete does not harbor the germline mutation. If the mutation happened in e.g. RAF1 this would lead to Noonan syndrome, a RASopathy, for which some of the presenting features include webbed neck, low set ears and primary lymphedema of the lower limbs. (B-C) Sometimes the parental gametes do not harbor any disease-causing mutations, but a mutation can occur during fetal development or any time after birth in any cell of the body. The postzygotic mutation will happen in a single cell, but as all its daughter cells will also carry the mutation, the severity of the phenotype depends on the timing of the postzygotic mutation and which tissue has been affected. If the postzygotic mutational event happened early during embryonic development in e.g. PIK3CA it could lead to a severe overgrowth syndrome such as PIK3CA-Related Overgrowth Syndrome (PROS), which is asymmetrical and severely affecting much of the lower body in the case illustrated in B. If the postzygotic mutation happened later or after birth it could lead to a more localized problem, which is exemplified by a localized lymphatic malformation in the left upper arm as illustrated in C. If none of the postzygotic mutations happened in cells of the germline, they are not passed on to successive generations. (D) Postzygotic mutations in the PI3K/AKT/mTOR and RAS/MAPK signaling pathways have been associated with lymphatic malformations and/or primary lymphedema. The signaling cascades activated downstream of receptor tyrosine kinases corresponding to both pathways are depicted in the simplified diagram, together with the genes associated with mosaic disorders such as PROS (PI3K/AKT/mTOR). Disorders collectively named as RASopathies are caused by germline mutations in RAS/MAPK pathway genes, and it has been shown that mosaic mutations in some of the same genes can cause a mosaic disorder including a lymphatic phenotype. (Image 'Impact of germline vs postzygotic mutations' by St George's, University of London is licensed under CC BY-SA-4.0).*

In an inducible LEC-specific mouse model carrying a *PIK3CA* mutation (p.H1047R), the timing of the postzygotic event caused by allele knock-in determined the severity of the lymphatic malformation (253). Likewise, the variable manifestation of disease observed in individuals with PROS is related to the time in development that the mutation occurred, which will determine the variant allele frequency of the *PIK3CA* mutation in the tissues (Figure 10B-C). Analysis of affected tissue from PROS patients showed that both BECs and LECs from the same patient carry the *PIK3CA* mutation, and when injecting the patient-derived cells into a xenograft mouse model, both vascular and lymphatic lesions develop (219). A low dose of mTOR pathway inhibitors can modestly reduce overgrowth and improve lymphatic malformations in these patients (3). To achieve a greater reduction, co-inhibition of VEGFC and mTOR might be necessary (253).

6.2 RASopathies caused by postzygotic mutations

The RAS/MAPK pathway is commonly activated in cancer, and dominant germline mutations in a range of the genes (>20) in this signaling pathway (like *RAF1*, which was discussed in Section 3) have been associated with Noonan syndrome and other similar disorders

collectively recognized as the RASopathies (ORPHA:536391) (363). As mentioned before, and as reported in the literature (187), patients with Noonan syndrome also present with primary lymphedema as an associated feature and are therefore placed in the blue group of the St George's classification (Figure 6). When mutations in the RASopathy genes occur postzygotically and not through the germline, a different human phenotype emerges. Postzygotic mutations in *KRAS, NRAS, BRAF* and *MAP2K1*, genes of the RAS/MAPK pathway, have been reported to cause sporadic vascular malformations, some of which were lymphatic malformations with, and without, lymphedema usually associated with high flow arterio-venous malformations (AVMs) (8). The phenotypes often included overgrowth and so resembled phenotypes seen in individuals with postzygotic *PIK3CA* mutations. Furthermore, kaposiform lymphangiomatosis, which is a proliferative lymphatic anomaly, characterized by malformed lymphatic vessels invading and damaging tissue, bones and organs, can be caused by a postzygotic mutation in *NRAS* (21, 243).

7. Concluding remarks

This review has attempted to bring together what is known about the genes causing primary lymphedema; what human phenotypes result from the causal mutations and what the disease mechanisms might be for those phenotypes. As discussed early in the introduction, the establishment of more advanced imaging techniques in the clinical settings that will allow better resolution and real-time imaging is imperative. A new promising method for the quantification of venous and lymphatic valve function is already being tested in mice and the authors are optimistic about the possibility of its translation into humans (57). 3D tissue biopsy imaging and reconstruction, together with the fast improvement in genome sequencing methodologies, will further contribute to a growing development in understanding lymphatic function and dysfunction in lymphedema patients from a clinical perspective.

Insights into the role of these disease-causing genes in lymphatic development and their possible biological function, based on the knowledge acquired from *in vitro* and *in vivo* models, are also given in this review. In some cases, the animal models were able to recapitulate meaningful aspects of the human phenotype. In other studies, distinct phenotypes were observed depending on the mouse strain used or the transgenic model generated, and the outcomes of these experiments must be interpreted with caution when making inferences from these models to human. Moreover, mice and humans have evolved to adapt to different environments and different gene compensatory mechanisms can operate in the two species. The recent developments in gene editing techniques are making it easier and faster to introduce specific point mutations for the generation of animal models. This will allow the introduction of mutations identified in lymphedema patients and might generate *in vivo* models that mimic the human phenotypes more accurately. *In vitro* models are becoming more accurate too, taking into consideration physiological flow, extracellular matrix conditions, dimensionality, chemotactic biochemical gradients, and

Call-out-box for clinicians:

- Primary lymphatic anomalies can arise from heterogeneous genetic causes reflected in a variety of physiological disease mechanisms, making the goal of an accurate clinical and molecular diagnosis for all patients a challenging task.
- Clinicians should be aware of the St George's classification pathway/algorithm, that has been designed to help with the clinical phenotyping and categorization of the patients based on age of onset, areas affected by swelling and associated clinical features.
- Both germline and somatic gene mutations have been discovered to be causative of lymphatic anomalies.
- Lymphatic malformations are mostly thought to be caused by postzygotic mutations and special considerations in terms of sample analysis are to be taken by diagnostic centers to enable a precise molecular diagnosis.
- The development of new imaging techniques for the visualization of the lymphatic system has contributed to a better understanding of the variety of physiological disease mechanism underlining lymphatic dysfunction.
- From lymphoscintigraphy to indocyanine green lymphography we are close to a better spatial resolution and imaging of real-time movement of the lymph along the limbs. Intranodal MR lymphography has proven useful for the visualization of intra-abdominal and thoracic lymphatic abnormalities and 3D-reconstruction of tissue biopsies provides a complex picture of the lymphatic dermal networks.
- The mechanistic findings observed through patient examination complement the elegant dissection of the molecular disease mechanisms obtained from the combination of in vitro and in vivo models.
- The thorough investigations of lymphatic signaling pathways at a molecular level are required for a full understanding of lymphatic biology in health and disease and the development of personalized treatments and management for patients with primary lymphedema and other lymphatic anomalies.

stiffness to better reflect *in vivo* physiology (133). In this sense 3D microfluidic (on-a-chip) devices could be promising study platforms in the near future (147).

This review focused on the venous origin of the lymphatic system, describing the processes leading to the lymphatic specification, migration and maturation of cells that arise from the cardinal vein to form the functional dermal lymphatic vasculature. However, the development of single-cell RNA sequencing is starting to bring to light the complex heterogeneity of lymphatic endothelial cell identity (364), and our knowledge on the non-

venous origin of lymphatic structures and the development of specialized lymphatic networks in several tissues in health and disease is expanding (266, 366). For a comprehensive up-to-date review on organ-specific lymphatic vasculature including the description of the meningeal lymphatic vasculature and lacteals in the small intestine, we recommend reading Petrova and Koh (306).

It becomes clear, this is a rapidly changing field and recent investigations are not only contributing to the better understanding of primary lymphedema and other lymphatic diseases but also to the role of lymphatic dysfunction in other pathologies e.g. congenital heart disease and systemic immunity. The basis for developing better treatment options for lymphedema patients is becoming more apparent and various studies are exploring such avenues (145, 369). Through this entirely new knowledge base, the lymphatic system has finally come of age, as has its representation and its involvement in human biology and disease.

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