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Clinical Relevance of Animal Models of Lymphatic Dysfunction and Lymphedema

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ABSTRACT

Lymphedema is a chronic progressive condition, and treatment options are limited to physical therapy or surgical intervention, underscoring the need to develop preventative strategies. To do so, we must first understand the underlying mechanisms that contribute to the development of clinical lymphedema, which can be caused by a myriad of factors, including genetic mutations, infectious agents, and cancer treatments. Animal models are essential to study the pathogenesis of clinical lymphedema and to develop therapeutic interventions. Many animal models mimic the various aspects of lymphatic dysfunction and lymphedema seen in humans, and some species better represent different aspects or causes of lymphedema. However, no single model perfectly recapitulates human disease in a cost- and time-efficient manner; therefore, findings should be verified in multiple models and multiple species. In doing so, researchers will increase the likelihood of collecting rigorous, reliable data that could be effectively and efficiently translated into the clinic. This review explores genetic, infectious, and surgical animal models of lymphatic dysfunction and lymphedema and describes how these models can be used to understand clinical forms of lymphedema. Collectively, this information can provide valuable insight for the translational study of lymphatic diseases.

1 | Introduction

Lymphedema is the increase of fluids in the interstitial spaces due to inadequate lymphatic drainage [1–5]. Ten million people in the United States and more than 200 million patients globally suffer from lymphedema [6]. In fact, between 2012 and 2017, recurrent infections and hospitalizations due to lymphedema resulted in more than a $166 million burden on the US healthcare system [7]. The chronic, progressive nature of lymphedema, along with the limited treatment options, underscores the need to develop strategies to treat or prevent this condition. To develop preventative or therapeutic strategies, it is necessary to understand the underlying mechanisms that contribute to the development of clinical lymphedema, which can be caused by many factors, including genetic mutations, infectious agents, and cancer treatments. Thus, an array of animal models that mimic the various forms of clinical lymphedema have been developed. Earlier systematic reviews focused on surgical models of lymphedema [8]; herein, we discuss genetic, infectious, and surgical animal models of lymphatic dysfunction and lymphedema. In addition, we explore how these models can be used to understand clinical forms of lymphedema and to inform current clinical diagnostic criteria and treatment options. Together, this information can provide insight for the translational study of lymphatic diseases.

2 | Etiology of Lymphedema

2.1 | Primary Lymphedema

Primary lymphedema is an intrinsic impairment of the function and structure of the lymphatic system and is often fatal during gestation. As such, the incidence of clinical primary lymphedema is exceptionally rare, estimated at 1.15 cases per 100 000 individuals under 20 years of age [9]. Roughly 70% of primary lymphedema cases are idiopathic and have no identified genetic cause. Turner's syndrome [10–13], Meige disease [14–18], Noonan syndrome [19], Milroy's disease [20–23], and Hennekam syndrome [24–27], are just a few diseases associated with primary lymphedema out of the full classification algorithm for primary lymphatic anomaly diagnosis first developed by St. George's University Hospital (Figure 1) [28].

2.2 | Secondary Lymphedema

The most frequent cause of lymphedema is injury to the lymphatic vasculature, hence the name secondary lymphedema. Secondary lymphedema is most commonly caused by filariasis, an infectious disease, or cancer treatment, but may also arise from other surgical interventions or chronic inflammatory conditions. Here we focus on the two main causes of secondary lymphedema.

2.2.1 | Filariasis

Filariasis, a mosquito-borne disease due to an infestation of parasites like *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, is the chief reason for secondary lymphedema

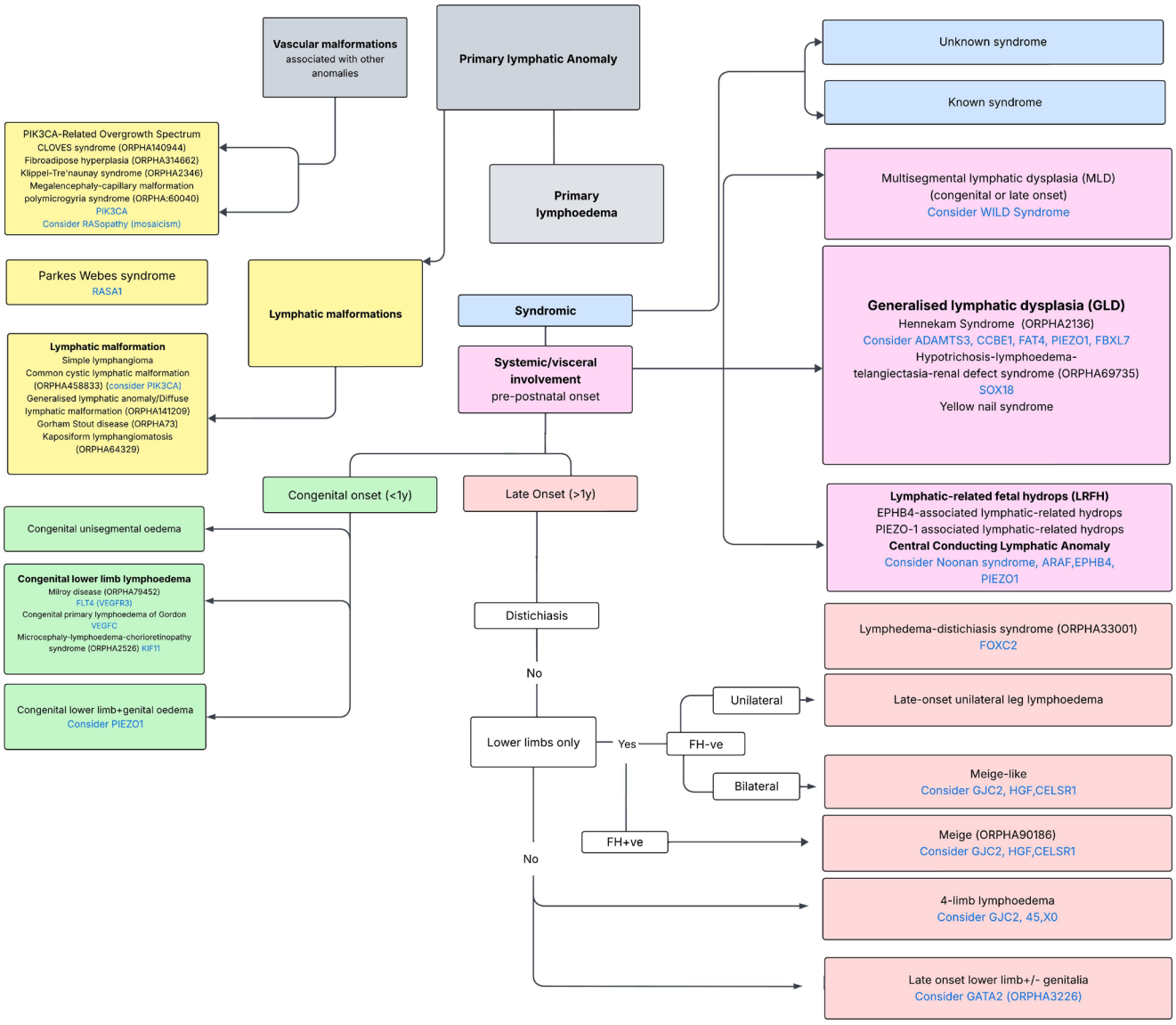

FIGURE 1 | St. George's classification algorithm for primary lymphatic anomalies diagnosis and genetic evaluation [28]. Reproduced with permission from Gordon et al. [28].

in low-income countries. According to the World Health Organization, in 2021, around 882.5 million people in 44 countries had lymphatic filariasis [29]. Live worms cause physical damage to the lymphatic valves and lymphatic vessel walls, which subsequently induces fibrosis of lymph nodes, reactive lymphoid hyperplasia, and dilatation of the collecting lymphatic vessels [30].

2.2.2 | Cancer Treatment

Cancer treatment is the leading cause of secondary lymphedema in the United States and high-income countries, with breast, gynecological, head, and neck cancers and melanoma accounting for the highest incidences [31]. Generally, resection of lymph nodes, adjuvant radiation, and chemotherapy influence lymphatic vascular impairment either independently or synergistically [32]. Trauma to the lymphatic system during surgery can result in postoperative blockage of lymphatic drainage, resulting in lymphedema. In numerous cancers, radical dissection of either inguinal or axillary lymph nodes is commonly associated with lymphedema [33]; in contrast, sentinel lymph node biopsy, or removal of only the lymph node directly draining the tumor, has a lower risk of lymphedema development as sentinel lymph node biopsy is less invasive than radical dissection to the lymphatic vasculature [34]. Adjuvant radiation therapy increases the risk of lymphedema by causing direct insult to lymphatic vessels, lowering the vessels' intrinsic pumping ability [35]; however, this injury may not be limited to cell death pathways since in vivo and in vitro irradiation of lymphatic vessels produced opposing effects [36]. The addition of anthracycline or taxane chemotherapy is also associated with clinical lymphedema development [37]. Researchers are beginning to explore the off-target mechanisms underlying the increased risk of lymphedema with these therapies [38, 39].

3 | Diagnosis

3.1 | Physical Examination

Physical examination is the most common diagnostic process for evaluating a patient for lymphedema, particularly if the lymphedema affects the patient's peripheral limbs (e.g., arms or legs). In 92% of primary lymphedema cases, the lower limbs are affected, and 50% of patients have bilateral disorders [40]. The anatomic site for secondary lymphedema generally depends on the site of infection or cancer and surgery type, with breast cancer affecting the upper extremities and gynecological cancers more often presenting with leg lymphedema, and melanoma affecting the closest limb to the primary excision site. Additionally, the more extensive the lymph node dissection surgery, the greater the incidence of lymphedema [1–3].

Pitting edema, which is detected by pressing the thumb into the dorsal region of the foot or hand of the affected limb, is a sign of early-stage lymphedema; however, patients with persistent lymphedema may no longer present with pitting edema due to fibrotic changes in the lymphedematous region. One sensitive and definite indicator of lymphedema is the Stemmer sign. To perform a Stemmer's test, the practitioner pinches the skin at the base of the fingers or toes of the affected limb. If the patient has lymphedema, the accumulation of adipose tissue, swelling, and inflammation will make it difficult to lift the pinched skin [41]. Limb circumference to measure conic geometry is often used to assess the degree of swelling [42, 43]. This is commonly achieved using a physical tape measure, but can be combined with more advanced techniques like perometry and bioimpedance analysis (BIA) to measure changes in limb volume and tissue fluid volume, respectively [44]. Some studies even suggest that perometry and BIA may be able to detect subclinical lymphedema [45–47]. Lymphedema can also affect the genitals, neck, chest wall, or other anatomical sites that are more difficult to diagnose and characterize extent with a physical examination. In these cases, diagnostic imaging is helpful.

3.2 | Imaging

Unfortunately, the diagnostic imaging approaches for lymphedema are inadequate compared to blood vessel imaging because the lymphatic vasculature is comprised of small, translucent lymphatic vessels that carry colorless lymph fluid, making it more difficult to visualize the anatomy and function of the lymphatic system compared to the blood vasculature [48]. Thus, clinically, most lymphedema cases are diagnosed with physical examination; however, during differential diagnosis, patients often undergo imaging, such as computed tomography scan, ultrasound, and magnetic resonance imaging; however, these examinations are not explicitly designed to identify lymphedema.

Lymphoscintigraphy is the gold-standard diagnostic imaging technique for detecting lymphatic dysfunction in the upper and lower extremities and can be used to confirm a lymphedema diagnosis in patients with positive signs/symptoms during the physical examination. To perform this test, the radiotracer protein 99mTc-sulfur colloid is injected into the affected limb (e.g., hand or foot) and absorbed into the lymphatic system. A gamma camera is then used to capture images. These images can be used to detect the shift of the radiotracer, thus allowing the detection of lymph nodes (inguinal or axillary lymph nodes) [49].

While lymphoscintigraphy has higher sensitivity and specificity than other imaging techniques, it fails to provide structural information due to its poor spatial and temporal resolution, and it subjects the patient to radiation. Furthermore, the process can be affected by either protocol variation or poor image resolution [50]. One of the recent techniques for visualizing lymphatic structure and increasing imaging speed is near-infrared lymphography (NIRL), in which indocyanine green, a fluorescent dye, is injected into the tissue. The dye then binds albumin and lipoproteins and is taken up by the lymphatic vessels. NIRL is more advantageous than lymphoscintigraphy in a surgical context for visualizing the structure and functional properties of lymphatic function in real time because the fluorescence can more easily be seen in vivo but also can be seen ex vivo prior to the incision of the lymphatic bed. Thus, combining lymphoscintigraphy and NIRL may

provide the most accurate picture of lymphatic function and structure [51].

4 | Staging

Lymphedema is a life-long disease that gradually worsens, regardless of etiology. Over time, the stagnant interstitial lymphatic fluid leads to chronic inflammation and the formation of subcutaneous fibroadipose tissue, further increasing the size of the affected area [52, 53]. The progression of chronic lymphedema is observed in four stages: Stage 0 is subclinical lymphedema. Patients have no visible signs of lymphedema, but impaired lymphatic transport can be monitored via lymphoscintigraphy. During Stage 1, non-pitting edema is observed, which can be attenuated by elevating the limb. As the disease progresses to Stage 2, pitting edema becomes evident and can no longer be resolved with limb elevation. In late Stage 2, edema may become non-pitting as fibrosis increases. Finally, in Stage 3, significant fibroadipose tissue accumulates in the affected area, leading to non-pitting (hard) edema, noticeable skin thickening and discoloration, and may eventually lead to elephantiasis. Unfortunately, the early stages of chronic lymphedema may go undiagnosed as patients can also experience acute postsurgical lymphedema that spontaneously resolves over time. Lymphedema can also be classified as mild, moderate, or severe. An increase in tissue fluid volume of less than 20% is considered mild lymphedema. A 20%–40% increase in tissue fluid volume is considered moderate lymphedema, and an increase in tissue fluid volume exceeding 40% is regarded as severe lymphedema [54].

5 | Current Clinical Treatments and Emerging Therapeutic Strategies

5.1 | Physical Therapy

The mainstay of treatment for lymphedema relies on manually altering fluid forces through complete decongestive therapy (CDT) comprised of compression garments, elevation, physical activity, or a specialized massage therapy called manual lymph drainage that works to gently massage lymph fluid toward working lymph vessels. CDT also includes skin care to prevent infections and diet and lifestyle modifications. Together these therapies are not curative but can effectively manage lymphedema through early Stage 2, if used regularly. As fibroadipose increases and CDT fails to relieve lymphedema, more invasive surgical approaches may be added and are detailed in the next section.

5.2 | Innovative Surgical Approaches

Several surgical approaches have been developed to either relieve established lymphedema or prevent lymphedema from developing. Liposuction to physically remove fibroadipose tissue has proven to beneficially reduce limb volume when combined with CDT. Lymphovenous bypass (LVB), where lymphatic drainage is rerouted to avoid the upstream blockage by connecting downstream lymphatic vessels to nearby veins and allowing lymph fluid to flow back into the venous circulation, has been beneficial in reducing limb volume in both upper extremity and lower extremity lymphedema compared to the CDT alone [55]. LVB also can be used in combination with vascular lymph node transplant (VLNT), a novel surgical approach where a vascularized lymph node is transplanted from a donor site (usually the patient's own neck, groin, abdomen, etc.) to the affected limb and anastomosed to the venous and arterial circulation. A prospective clinical trial demonstrated significant improvement in reduction of limb volume and cellulitis, reduced bioimpedance score, and increased quality of life in patients up to 2 years after VLNT [56]. A disadvantage of this technique is that it requires removing lymph nodes from a healthy region, potentially inducing lymphatic dysfunction in a second anatomical site.

In other efforts to prevent cancer-related lymphedema, newer techniques have been developed and incorporated into the initial tumor removal surgery to reduce the physical damage to the surrounding lymphatic vasculature [57, 58]. These include axillary reverse mapping (ARM) and preventative lymphovenous anastomosis (also referred to as LYMPHA) [59, 60]. For both of these techniques, a blue dye is injected subcutaneously to visualize the lymphatics during tumor removal surgery. For ARM, the goal is to distinguish the lymphatic vessels and lymph nodes that drain the upper limb versus those of the breast to preserve as much of the lymphatic vasculature that drains the upper limb as possible after assessing the patient's tumor burden. If necessary to remove lymphatic vessels and nodes, LYMPHA can be performed, where the cut lymphatic vessels are anastomosed to nearby veins to allow lymph fluid to drain into venous circulation. This technique is similar to LVB only that it is performed before lymphedema has developed rather than as a treatment for established lymphedema. The use of ARM and LYMPHA has reduced the incidence of cancer-related lymphedema; however, as many as 15% of breast cancer patients and 47% of gynecological cancer patients still develop lymphedema [60–70]. Thus, new research is still needed to fully understand the pathology of cancer-related lymphedema post cancer treatment.

5.3 | Pharmacological Interventions

While there are no Food and Drug Administration (FDA) approved pharmacological agents for lymphedema treatment, there are some agents in various stages of clinical trials and many that have failed clinical trials. The top candidates currently in clinical trials are actually older drugs that are being repurposed to treat lymphedema. The first is ketoprofen, a nonsteroidal anti-inflammatory drug, which showed promising results in the murine tail model of lymphedema (described later) and has made it to Phase II clinical trials for the treatment of lymphedema. In Phase I trials, ketoprofen reduced dermal thickness, improved histopathological findings, and reduced plasma granulocyte CSF (G-CSF) in patients with primary or secondary lymphedema, although no changes in limb volume or bioimpedance scores were observed [71]. Ketoprofen is thought to work to treat lymphedema by reducing leukotriene B4 (LTB4); however, the results from a Phase II, randomized, double-blind placebo-controlled clinical trial of a more specific LTB4 inhibitor, bestatin, in patients with lower extremity lymphedema were inconclusive [72]. This might be due to the study only including

patients with ≥Stage 2 lymphedema or perhaps ketoprofen's mechanism includes broader anti-inflammatory action in addition to LTB4 inhibition. While promising, ketoprofen does carry Black Box warnings regarding an increased risk of fatal heart events and stroke in patients with cardiovascular disease and is contraindicated in patients who have undergone coronary artery bypass graft surgery, thus limiting the patient population who could benefit from this therapy.

Another drug being repurposed for lymphedema treatment is tacrolimus, an immunosuppressant traditionally used to prevent organ transplant rejection and other cutaneous inflammatory or fibrotic diseases such as dermatitis, psoriasis, and scleroderma. In animal models of lymphedema (tail and hindlimb), tacrolimus improved lymphatic function by accelerating both the formation of collateral lymphatics and lymphatic pumping frequency [73]. Tacrolimus also attenuated both dermal and subcutaneous T-cell infiltration and tissue fibrosis after lymphatic injury [73]. In a Phase II, prospective, open-label single-arm trial of 18 female patients with Stage 1–2 breast cancer related lymphedema, topical tacrolimus administered daily for 6 months significantly reduced arm volume and increased quality of life; however, the changes in lymphatic function were inconclusive with only three patients showing improvement but none with worsening lymphatic function [74]. Additional trials are ongoing.

Lastly, a new monoclonal IL4/IL13 neutralizing antibody, QBX258, also recently completed Phase I trials, in which 9 female patients with Stage 1–2 breast-cancer-related lymphedema were treated once monthly with QBX258 for 4 months. QBX258 was found to significantly improve quality of life as well as skin stiffness in the lymphedematous arm by decreasing thickness, attenuating the number of proliferating keratinocytes, collagen deposition, and infiltration of mast cells and Th2 inducing cytokines [75]. However there was no change in limb volume or bioimpedance measurements with QBX258 treatment [75]. Taken together, these current and emerging therapies highlight the need for more research to develop therapeutic strategies that address the complicated pathology of lymphedema.

6 | Animal Models

In the following sections, we discuss genetic, infectious, and surgical animal models of lymphedema and how the model characteristics relate to clinical lymphedema. All animal models are summarized in Table 1, and we have attempted to match each model to the clinical lymphedema stage it most closely mimics (Figure 2), although it should be noted that models that develop into late-stage lymphedema could also be used at earlier time points to study earlier stages of lymphedema.

6.1 | Genetic Models

Genetic models allow researchers to investigate the roles of specific genes and proteins in the underlying lymphatic physiology, which may shed light on lymphedema pathophysiology. Two species have emerged that allow for genetic manipulation and provide unique insight into lymphatic pathologies: zebrafish and mice.

6.1.1 | Zebrafish

Zebrafish are an important model organism for researching lymphatic development. Although mammals are more closely related to humans in terms of evolution and physiology, zebrafish still possess about 70% of human genes, including those involved in lymphatic growth and development [76]. Their transparent embryos, along with the availability of genetically engineered transparent adult zebrafish [77], allow for detailed visualization via intravital time-lapse imaging of lymphatic development throughout the entire zebrafish life cycle—a process that is challenging in mammalian models. Additionally, zebrafish are more resilient to chemical mutagens than rodents, which allows researchers to introduce a higher density of mutations into the zebrafish genome [77]. Furthermore, zebrafish eggs are fertilized externally, facilitating in vitro fertilization and direct genetic manipulation of fertilized eggs to create transgenic or knockout lines. These procedures are more complex in rodents and other mammals [78, 79]. Consequently, genetically modified zebrafish provide a valuable opportunity for rapid study of how genetic deletions or insertions affect lymphatic anatomy and function [80]. Zebrafish readily develop lymphedema pericardially and around the eyes and intestines (Figure 3A), which can be monitored with CT and lymphangiography [81]. However, the use of zebrafish is limited by their sensitivity to environmental factors such as temperature, oxygen levels, and food availability, which can influence sex determination and complicate the control of sex-based differences or the investigation of environmental effects on lymphatic structure and function [82].

6.1.2 | Mice

Mouse models are generally regarded as the lowest mammalian model where genetic manipulation is both cost and time efficient. Genetic mouse models for the human diseases associated with primary lymphedema exist; however, only a few develop the lymphedema phenotype.

One genetic model that develops the lymphedema phenotype is the Chy mouse model, which has a missense mutation that inactivates vascular endothelial growth factor receptor-3 (VEGFR3) and has been associated with Milroy's disease [83]. These mice develop chylous ascites within the first few weeks after birth (hence the Chy name) and progressive peripheral lymphedema, measurable by limb swelling, due to hypoplastic cutaneous lymphatic vessels [83].

Mutations in connexin 47 (CX47) and connexin 43 are also linked to primary lymphedema in humans [113–115]. Connexins form gap junctions that facilitate the transport of ions and small molecules between cells and are critical to fluid movement through lymphatic vessels. Interestingly, CX47 knockout mice did not develop any lymphatic anomalies; however, in a later study, CRISPR was used to design a mouse model with a missense mutation (CX47R259C) equivalent to the human mutation (CX47R260C) associated with primary lymphedema in humans. These mutated mice have more regional lymph nodes and lymphatic channels, measurable mesenteric lymphatic reflux, and decreased lymphatic vessel contractility [84, 114]. Thus, the CX47R259C mutated mouse model can be used to study the

TABLE 1 | Overview of genetic, infectious, and surgical animal models of lymphedema.

Model	Animal	Onset of lymphedema	Duration of lymphedema	Spontaneous resolution	Characteristics	Advantages/clinical utility
Genetic						
	Zebrafish [76–82]	Various	Various	N/A	Lymphedema is developed pericardially, between the eyes and intestine	Easy to visualize the lymphatic vasculature and genetically modified Applicable for the study of developmental stages of lymphatic dysfunction
	Mouse [83, 84]	Shortly after birth	Continuous	No	Limb swelling, impaired lymph vessel contractility	Applicable for studying primary lymphedema (Milroy disease) or the role of specific proteins in lymphatic function
Infectious/Filariasis						
	Ferret [85]	3 weeks	28 weeks	No	Limb swelling, inflammation of lymph vessel wall, reduced lymph flow	Natural hosts of parasites Useful models for vaccine development
	Dog [86]	7–24 weeks	2–24 weeks	Yes	Limb swelling, lymphangitis, conjunctivitis, scrotal enlargement	
Surgical						
Tail	Mouse [87]	1–4 weeks	2–4 weeks	Yes	Increased tail volume, dermal thickening, lymphatic hyperplasia, lipid accumulation in the skin	Ability to perform non-invasive longitudinal imaging of lymph flow/function Easily reproducible Mimics volume and histopathology of clinical secondary lymphedema
	Mouse [88]		6 weeks	Yes		
	Mouse [89]		>15 weeks	No		
	Rat [90, 91]	2–3 weeks	2–4 weeks	Yes		

(Continues)

TABLE 1 | (Continued)

Model	Animal	Onset of lymphedema	Duration of lymphedema	Spontaneous resolution	Characteristics	Advantages/clinical utility
Hind Limb surgery+ radiation	Mouse [92, 93]	3–7 days	2–6 months	Yes	Hind limb swelling, fibrosis, lipid accumulation, immune cell infiltration	More sustained lymphedema Addition of radiation mimics clinical treatment regimens
	Rat [94]	Immediate	> 3 months	Yes		
	Rat [95, 96]	3 days	Varies from 10 days to 9 months	Yes		
	Rat [97]	1–2 months	4 months	Yes	Hind limb swelling with increased water content in the tissue	
	Dog [98]	3 days	2–4 weeks	Yes	Hind limb swelling	Larger mammals more closely mimic human lymphatic anatomy Can use clinical imaging methods in larger mammals
	Dog [99]	1–3 months	> 1 year	Yes	Hind limb swelling and disrupted lymphatic network	
	Pig [100–105]	N/A	N/A	Yes	No Limb swelling or difference in limb circumferences. Reduced lymph flow	
	Sheep [106]	1 week	5 weeks	yes	Hind limb swelling, impaired lymphatic transport	
Upper Limb surgery + radiation	Rhesus Monkey [107]	2 days	> 24 months	No	Increased upper limb circumference and water content, dilated lymph vessels	Anatomically most similar to humans Mimics clinical axillary lymph node dissection and radiation Lymphedema is persistent
Ear	Rabbit [108]	1–2 weeks	4 weeks	Yes	Ear volume increased	Easily reproducible Limited clinical utility
Mesenteric	Rat [109]	1 week	11 weeks	Yes	Increased tissue fluid accumulation, lymph vessel dilation	Mimics either radical lymphadenectomy or sentinel lymph node resection Can use direct cell tracking to measure lymph flow Useful to study interplay of lymphatic function and inflammation
	Rat [110]	2 days	N/A	N/A	Decreased lymph flow and reduced lymph vessel contractions	
	African Green Monkeys [111]	7 days	61 days	No	Decreased lymph flow Increased vessel dilation Increased Inflammatory cytokines and chemokines and increased specialized endogenous pro-resolving mediators	

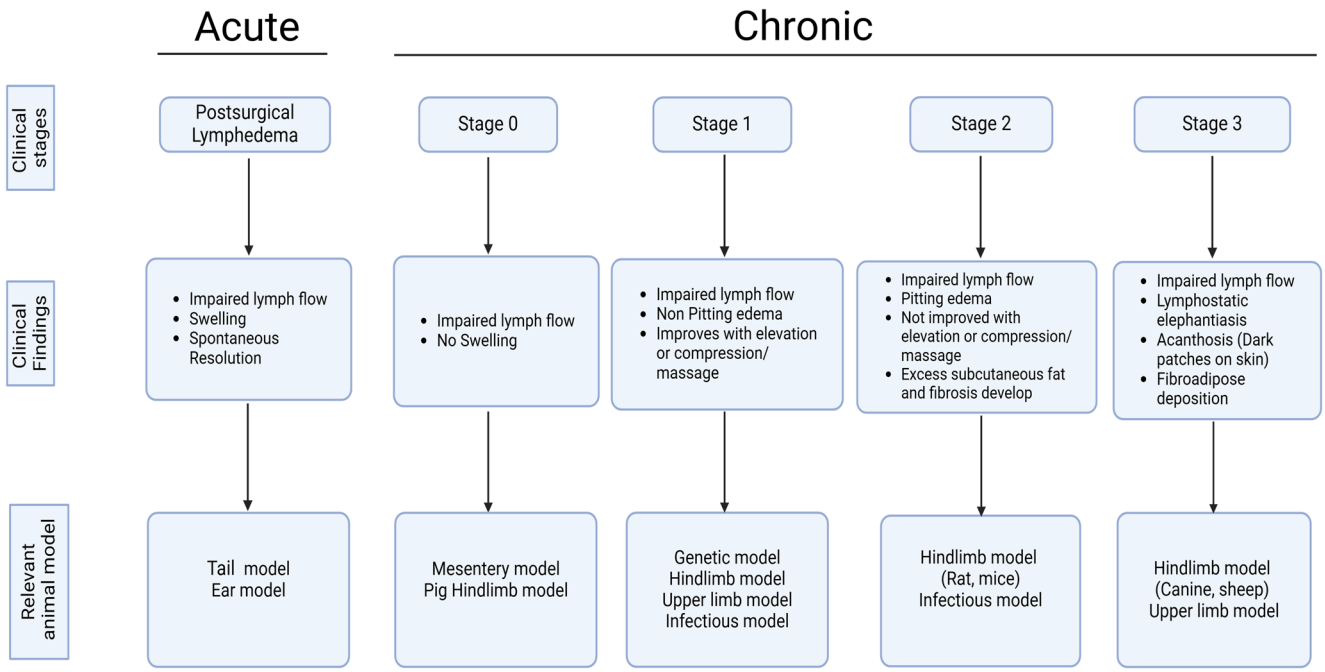

FIGURE 2 | Diagram of clinical lymphedema and corresponding relevant animal models. This figure was created using BioRender.

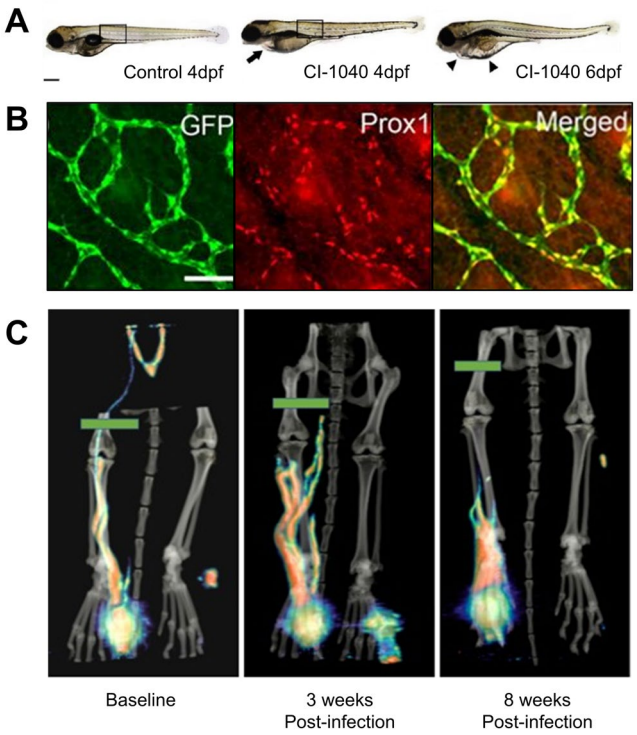

FIGURE 3 | Genetic and infectious models of lymphedema. (A) Progressive development of pericardial (black arrow) and diffuse (arrow heads) lymphedema in zebrafish after treatment with CI-1040 to prevent thoracic duct formation. Scale bar = 300 μm [80]. (B) Co-localization of GFP with Prox1 in mouse lymphatic vessels. Scale bars = 100 μm [112]. (C) Serial PET imaging of lymphatic structure and function in a ferret after subcutaneous injection of *B. malayi* in the right hind footpad. Green bars indicate the selected Region-of-Interest intended for kinetic analysis of tracer flow [85]. Reproduced with permission from Fevurly et al. [80], Choi et al. [112], and Jackson-Thompson [85].

TABLE 2 | Lymphatic reporter models.

Reporter model name	Species	Inducible/ constitutive
Prox1-GFP	Mouse [112], rat [116]	Constitutive
Prox1-tdTomato	Mouse [117]	Constitutive
Prox1-Cre-td Tomato	Mouse [118]	Inducible
Lyve1CreERT2^{tdT}	Mouse [119]	Inducible
Vegfr3-^{EGFPLuc}	Mouse [120]	Constitutive
VEGFR3-tdTomato	Mouse [121]	Constitutive

impaired lymph vessel structure and contractile activity. Still, this model may not mimic the disease progression to lymphedema seen in humans [84].

The Prox1-GFP mouse is another useful genetic mouse model where the animals do not have lymphatic pathology or lymphedema but merely GFP-tagged lymphatic vessels (Figure 3B) which makes the visualization of the translucent lymphatic vessels in solid tissue easier [112]. However, this model may be combined with infectious or surgical models to enhance the evaluation of lymphedema progression and other lymphatic anomalies that may arise. The researchers who developed this model have now developed a Prox1-GFP rat, further expanding the tools to investigate the pathogenesis of lymphedema [116]. Other lymphatic reporter mice have been developed and are listed in Table 2 [116–121]. In general, lymphatic structure and function can be studied in any other genetic mouse model; however, these

pathways may not give rise to a lymphedema phenotype and are too numerous to detail here.

6.2 | Infectious Animal Models

Mosquitos and mammals are hosts for parasites like *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* that cause lymphatic filariasis and subsequent lymphedema. In regions where these parasites are endemic, efforts have been made to control the mosquito population and generate a vaccine against lymphatic filariasis for veterinary use to curb the parasite burden in the region [86]. Mice are often used to model filariasis infection due to accessibility [122]; however, because ferrets, dogs, and cats are natural hosts of *Brugia malayi*, they make ideal models for investigating immune response and vaccine development. When infected with *Brugia malayi*, ferrets develop a lymphatic infection that mimics human disease, including inflammation of the lymphatic vessel walls that ultimately results in thickening of the intima and disorganization of collagen that extends into the perivascular and subcutaneous tissues and is accompanied by reduced lymphatic function and anatomical changes (Figure 3C) [85]. Infected dogs and cats can develop lymphedema, conjunctivitis, scrotal enlargement, and lymphangitis, but the pathology is usually transient and less severe than in humans [86]. Nevertheless, these models can be used to study how the infection causes lymphatic dysfunction and to test drugs for treating human infection.

Skin and soft tissue infections are common in patients with lymphedema; however, the mechanisms by which these infections can cause or worsen lymphedema are still under investigation. In this regard, Jones et al. [123] developed a mouse model of lymphatic dysfunction using methicillin-resistant *Staphylococcus aureus* (MRSA), a frequent cause of skin and soft tissue infections. MRSA was injected subcutaneously into the hind limb of mice, and lymphatic injury and function were measured acutely and long-term post infection. Lymphangiography of MRSA-infected mice revealed acute reductions in lymphatic function that were sustained even after animals cleared the infection and were also associated with a loss of lymphatic muscle cells within the collecting lymphatic vessels. This model provides initial evidence that skin and soft tissue infections alone may directly inhibit lymphatic function; however, this acute infection model does not result in sustained limb swelling or chronic secondary lymphedema that is seen in patients [123]. Future studies combining this acute infection with genetic or surgical models or modifying this model to a recurrent infection may provide further insight into how skin and soft tissue infections increase the risk or worsen already established lymphedema. Diphtheria is another bacterial infection that has been associated with tissue edema and lymphatic injury. In advanced diphtheria infections, lymph nodes become enlarged and surrounding tissues become edematous. A *Cre-lox* mouse model was developed using a lymphatic-specific promoter (FLT4) to express diphtheria toxin receptors on lymphatic endothelial cells and then subcutaneously injected diphtheria toxin into the ear or footpad [124]. This resulted in the ablation of the lymphatic endothelial cells and marked swelling 1 week after injection that initially resolved during the following 6- to 9-week period; however, at 9 weeks swelling again progressed along with fibrosis for up to 52 weeks, and ICG lymphoscintigraphy revealed loss of collecting lymphatic vessels accompanied by dermal lymph backflow. While globally diphtheria infections do not represent a major cause of secondary lymphedema, this model could be useful for studying lymphedema pathogenesis as well as the therapeutic potential of agents intended to enhance lymphatic endothelial cell proliferation and lymphangiogenesis, as reported in a recent study evaluating nucleoside-modified vascular endothelial growth factor C (VEGFC) mRNA lipid nanoparticles [125].

6.3 | Surgical Animal Models

Preclinical models of lymphedema were developed via surgical interventions to better understand the pathophysiology and explore novel therapeutic interventions. These surgical animal models range from big mammals (e.g., dogs, pigs, sheep, and non-human primates) to small rodents (e.g., rats and mice) and mimic lymphedema at different sites, such as the tail, hind limb, ear, and mesentery.

6.3.1 | Tail Model of Lymphedema

The tail model of lymphedema, one of the most used rodent lymphedema models, was designed to model acute disruption of lymphatic drainage caused by surgical resection of lymph nodes and lymphatic vessels. Slavin and colleagues first reported this model after ligating the lymphatics of mouse and rat samples to monitor the physiological alteration in lymphatic function through lymphoscintigraphy and fluorescence microlymphangiography [90]. The tail model of lymphedema has been used to analyze various lymphedema interventions because it is economical, easily reproducible, and, importantly, it can mimic the early volume changes in patients during the acute period after surgical cancer treatment [87–91, 126–134]. One advantage of this model is the ability to use near-infrared dyes to measure lymph flow longitudinally to assess the effectiveness of pharmacological or surgical interventions.

To induce tail lymphedema, a circumferential surgical opening is used to expose the lateral lymphatic vessels draining the tail. Then, the lymphatic vessels on one or both sides of the tail are cauterized, thus ablating the lymphatic vessels. Methylene blue or Evans Blue dye is often injected into the subcutaneous tissue at the tip of the tail to help visualize the lymphatic vessels and to prevent damage to nearby blood vasculature that could lead to tail necrosis. Lymphedema can take 21–28 days (about 3–4 weeks) to develop and is measured in real time by changes in tail volume or lymphangioscintigraphy (Figure 4A). Lymphedema is later confirmed by histology, showing characteristic dermal thickening, collagen breakdown/remodeling, lymphatic hyperplasia, and abnormal lipid accumulation in the skin (Figure 4B) [95, 128, 130, 135, 136].

However, in the standard tail model, lymphedema spontaneously resolves through unclear mechanisms after 15–20 days. Several groups modified the standard tail model to create a model with a more sustained lymphedema. For example, the ablative gap was increased to 2–3 mm (about 0.12 in) to prolong

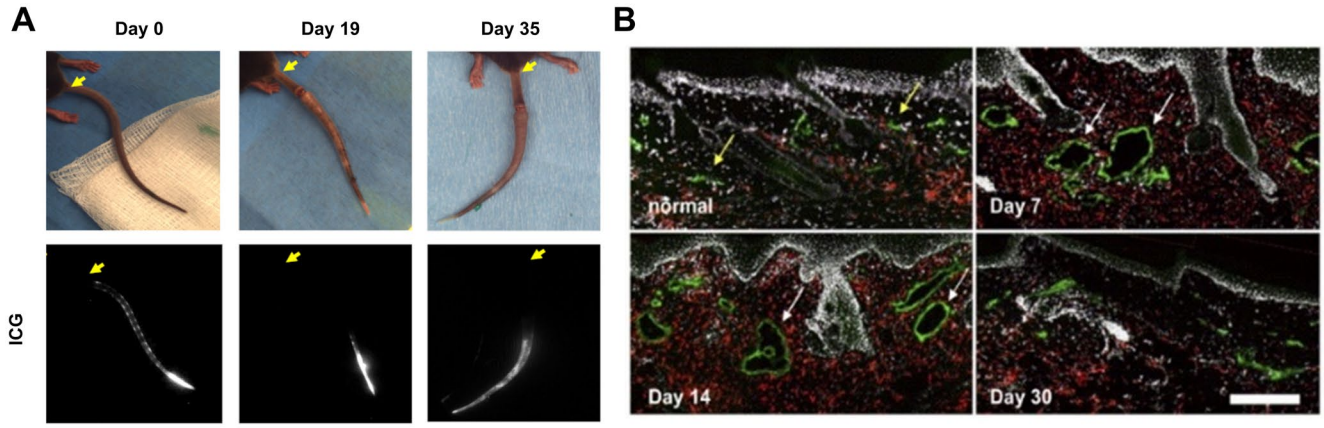

FIGURE 4 | Mouse tail model of lymphedema. (A) Assessment of lymphatic function with near-infrared laser lymphangiography in the mouse tail model. Indocyanine green (ICG) injected into the tip of the mouse tail localizes to the lymphatics and provides a measure of lymphatic function. Yellow arrow indicates the base of the tail [89]. (B) Macrophages infiltration (red) and lymphatic hyperplasia (green) during tail lymphedema. Yellow arrows indicate normal lymphatic vessels and white arrows indicate the hyperplastic lymphatic vessels. Scale bars = 200 μm [87]. Reproduced with permission from Hassanein et al. [89] and Rutkowski et al. [87]

the wound healing process, resulting in sustained lymphedema for 30 days [87]. Later, this model was modified by ligating the lymphatic vessels with the lateral veins rather than cauterizing the lymphatic vessels. Morphological changes were observed 3 weeks after surgical procedures and were more apparent after 6 weeks. After 6 weeks, the volume gradually declined from the maximum level [131]. More recently, surgical excision of the lateral lymphatic vessels, rather than cauterization, produced sustained tail swelling for 15 weeks (about 3.5 months); however, swelling peaked within the first 4 weeks and gradually declined thereafter [89]. The spontaneous resolution of swelling in the tail model of lymphedema limits the model's translatability because clinical secondary lymphedema progressively worsens rather than resolves.

6.3.2 | Hind Limb Model of Lymphedema

The hind limb model of lymphedema is frequently used to mimic limb lymphedema associated with breast or gynecological malignancies where lymph nodes may be removed for sentinel lymph node biopsy or axillary lymph node dissection. For the hind limb model in most species, a fluorescent dye or methylene blue is injected into the target paw to help visualize the lymphatic vessels [99], which may then be ligated to obstruct flow and to remove draining lymph nodes. Generally, one or more lymph nodes in the subiliac or popliteal regions are removed by surgery. The inguinal lymph nodes and surrounding fatty tissue are removed through an incision in the groin [95]. The boundaries of the dissection are superior from the margin of the external ring to the anterior superior iliac spine, laterally. The groin nodes, which are inferior to this line, are removed. Popliteal fossa dissection is an operative technique that commences from the superior aspect of the fossa and proceeds down the neural structures to the inferior aspect. The dissection entails the removal of lymph nodes and fibrofatty tissue surrounding the sciatic, tibial, and peroneal nerves. The lymph node removal is coupled with radiation to disrupt lymphatic drainage in the hind limb. Lymphedema becomes evident in 7–14 days and lasts 4–9 months. Early models that used surgery alone resulted in minimal lymphedema development or spontaneous resolution of swelling near the operation site [137]. Later models introduced a single radiation dose at the surgical site before or after surgery. The inclusion of radiation mimicked clinical treatment regimens and led to significant sustained lymphedema [99].

In general, lymphedema development can be assessed using water displacement, microcomputed tomography, magnetic resonance imaging, electronic/digital calipers, or by directly measuring lymphatic function and flow via indocyanine green lymphoscintigraphy or positron emission tomography/computed tomography. The hind limb model has been used in multiple species, including rodents and higher-order mammals, such as dogs, pigs, and sheep.

The mouse model is advantageous for studying hind limb lymphedema for several reasons. First, mice are relatively inexpensive and easy to handle, and genetic mouse models (discussed in the prior section) can easily be combined with surgical models. Also, mice develop similar anatomical swelling caused by lymph node removal and radiation as seen in humans (Figure 5A) [92, 93]. Similarly, clinical histological findings, such as fibrosis, fatty tissue deposition, and immune cell infiltration, are commonly found in the mouse hind limb lymphedema model.

Rat hind limb lymphedema models are similar to mouse models with one key exception: the lymphedema in the rat model is more persistent [94, 95]. Thus, hind limb lymphedema in rats mimics the main characteristic of clinical lymphedema but is still not as permanent as lymphedema in humans. Moreover, rats are larger in anatomy than mice, which makes it easier to see the cervical, inguinal, and popliteal lymphatics at the macroscopic level [96, 138]. However, because rats have a superficial lymphatic network that aids collateral drainage, viable lymphedema induction is more difficult in rats.

The rodent hind limb model does have limitations. Because humans have both deep and superficial inguinal lymphatics, the

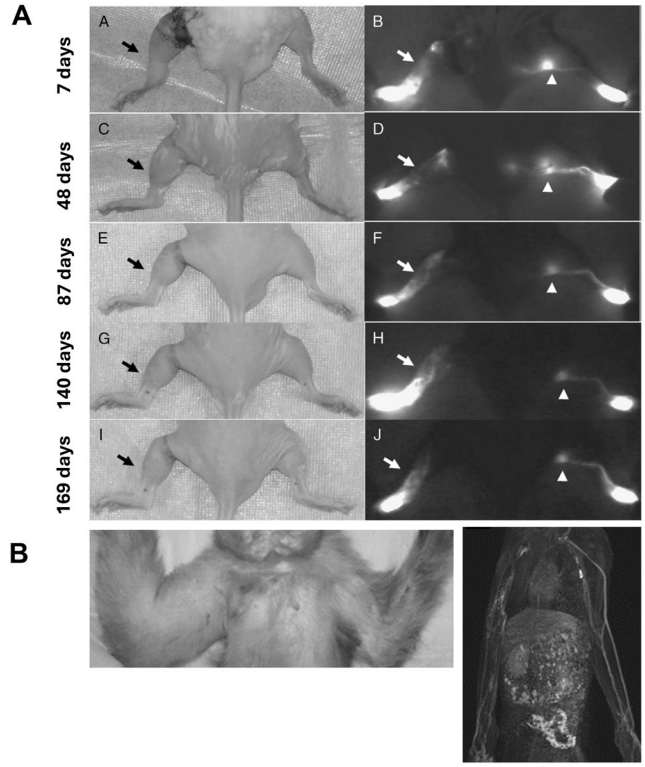

FIGURE 5 | Limb models of lymphedema. (A) Time course for leg swelling (left) and lymphatic function via indocyanine green lymphangiography (right) in a mouse hind limb model of lymphedema [93]. (B) Upper limb lymphedema in a rhesus monkey 12 months after surgery (left). The monkey also displayed delayed lymphatic flow with reticular pattern of dilated lymphatic vessels visualized with gradient-echo high-resolution MRI and digital subtraction lymphangiography (right) [107]. Reproduced with permission from Oashi et al. [93] and Wu et al. [107]

rodent hind limb model does not completely mimic the etiology of human lymphedema [97]. Still, the rodent hind limb model may be more appropriate for exploring the pathogenesis of clinical surgical lymphedema, often seen in patients' arms or legs, than in the tail model because it better aligns with human anatomy and physiology.

The lymphatic networks of larger mammals (e.g., dogs, pigs, and sheep) closely resemble the human lymphatic system, increasing the ability to translate findings from these hind limb models to the clinic. Additionally, due to the significant homology with human lymphatics, the same visualization techniques used in humans can be used in large mammals, making these animal models ideal for developing new diagnostic imaging approaches.

Dogs are thought to most closely mimic human lymphatics based on body composition, capillary permeability, decreased capacity to regenerate the lymphatic system, and lymphosome mapping; however, this model has limited use because of the cost, high mortality rate, and low availability [98, 139, 140].

Pigs are advantageous because the organization of blood and lymphatic vessels in the skin resembles that of humans. Still, pigs lack axillary lymph nodes, restricting the scope of translation because axillary lymph node dissections are commonly associated with clinical lymphedema. Pigs also fail to develop limb swelling or changes in limb circumference even months after surgery, distinguishing this model from human lymphedema disease progression [100–105].

In contrast, the sheep hind limb model produces dramatic swelling with high reliability when just a single popliteal lymph node and correlated lymphatic vessels are removed [106]. Ultimately, regardless of species, the hind limb model is useful for preclinical studies designed to develop new surgical techniques or therapeutic interventions for persistent lymphedema.

6.3.3 | Forelimb or Upper Limb Model of Lymphedema

Arm lymphedema is more common in humans subjected to breast cancer surgery. A rodent forelimb model and a non-human primate upper limb model were developed to mirror the surgical method of mammary and axillary dissection and radiation used clinically. Although rodent models exist [141, 142], the non-human primate model in rhesus monkeys is anatomically and physiologically relevant to lymphedema in the human arm [107]. To induce lymphedema in this model, mammary glands were injected with methylene blue dye to visualize draining lymphatics, and then axillary lymph nodes along with fatty tissues were dissected. The axillary regions were irradiated 2 weeks prior to and 4 weeks following surgery (30 Gy, single dose). High-resolution lymphangiography was used to detect lymphatic dysfunction (Figure 5B), and bioelectric impedance analysis was used to measure increased water content in the tissues of the affected limb. Immunohistochemistry also revealed typical lymphedema pathology. Thus, the rhesus upper limb model offers a promising avenue for exploring new therapeutic strategies for the effective management of lymphedema.

6.3.4 | Ear Model of Acute Lymphedema

The rabbit ear lymphedema model lymphedema develops 7–15 days after surgery [108]. To induce lymphedema, the skin, subcutaneous tissue, and perichondrium at the base of the ear are circumferentially excised to facilitate microsurgical dissection of lymphatics and draining lymph nodes. The main risk in the ear model is cartilage necrosis due to the removal of the perichondrium, but this was overcome by leaving a small section of the dorsal skin intact [108]. The rabbit ear also rapidly recovers within 3–4 weeks following the surgery [108, 143], thus it serves as another acute model of lymphedema likely mimicking the early volume changes observed in the acute period after surgical damage and not true chronic lymphedema as diagnosed in the clinic.

6.3.5 | Mesentery Model of Lymphedema

Mesenteric models of lymphedema were developed to enhance optical imaging to directly monitor lymph flow. Mesenteric lymphedema was first induced in rats by microsurgical removal of the regional mesenteric lymph nodes [109]. That rat model was intended to mimic radical lymphadenectomy

surgeries that remove all lymph nodes that drain a specific region. The chain of mesenteric lymph nodes was carefully resected by microdissection along the length of the superior mesenteric artery to the aortic root, leaving the distal lymphatic vessels intact and available for later studies. Lymphatic vessels dilated, and fluid accumulated in the mesenteric tissue 1 week after lymphadenectomy and gradually resolved by 11 weeks. Fluid accumulation was assessed by comparing wet and dry weights of the tissue, and vessel dilation was measured with edge detection [109].

Our group modified the rat model of mesenteric lymphedema to better recapitulate sentinel lymph node removal, which only compromises but does not fully interrupt lymph flow. In this model, a proximal lymphatic vessel draining a single mesenteric arcade is ligated to cause regional lymphatic insufficiency (Figure 6A) [110]. This model also results in less surgical trauma compared to lymphadenectomy, and lymph flow is slowed but not stopped in the mesenteric lymphatic vessels distal to the ligation. Lymphatic insufficiency is evident as reduced lymph flow in the ligated region by postoperative day 2; however, longer-term characterization of this model is still needed.

More recently, a model of intestinal lymphatic obstruction has been developed in non-human primates by ligating the central lymphatic vessels at the base of the mesentery with non-reabsorbable sutures and subsequent injection of 4% formalin into the lymph nodes draining the ligated region [111]. Similar to the rat mesenteric model, lymphatic obstruction resulted in sustained lymphostasis for up to 61 days post-surgery, characterized by pooling of injected contrast agent and no lymphatic outflow into the cisterna chyli on conventional lymphography along with increased lacteal dilation confirmed by histopathology. Surgically induced lymphatic obstruction also led to necrosis of lymph nodes and immune cell infiltration, which was most prominent 7 days after surgery and abated at later time points [111]. While originally intended to model inflammatory bowel disease, the lymphatic phenotype ranges from lymphostasis to lymphangitis and could provide insight regarding inflammation as the cause or consequence of lymphatic changes during lymphedema and other lymphatic disorders.

For mesenteric models, optical imaging can be achieved similar to other models with the use of fluorescent dyes [144, 145] before surgery and at later time points; however, the signals from dyes depend on their respective pharmacokinetics and dynamics, such as dye clearance and solubility, and injection volumes per se also may alter normal lymphatic function and lymph flow [145–147]. Therefore, an advantage of the rat mesenteric model is the ability to measure lymph flow by tracking individual cells in lymph fluid concurrent with real-time measurement of lymphatic vessel diameter to accurately quantitate flow in the absence of dye injection (Figure 6B,C) [148]. Additionally, the effects of pharmacological agents can be evaluated in the mesenteric model in real time or after long-term treatment.

With mesenteric models, the administration route of pharmacological agents should be considered. For example, intraperitoneal injection in rats generally equates to systemic dosing. Still, in these models, intraperitoneal injection may directly expose the mesenteric lymphatic vessels to much higher drug concentrations compared to systemic administration by intravenous infusion. Another significant limitation is the effect of peristalsis on mesenteric lymph flow. Aside from the intrinsic pumping of lymphatic vessels, lymph flow is also achieved by external forces, including contractions of surrounding muscles to propel lymph fluid through the lymphatic vessels. Within the mesentery, the lymphatic vessels are embedded in a sheath of connective tissue and fat rather than skeletal muscle; however, peristaltic contractions of the mesenteric wall can facilitate fluid movement through the lymphatic vessels. Fasting of animals prior to evaluation of lymph flow can minimize this effect.

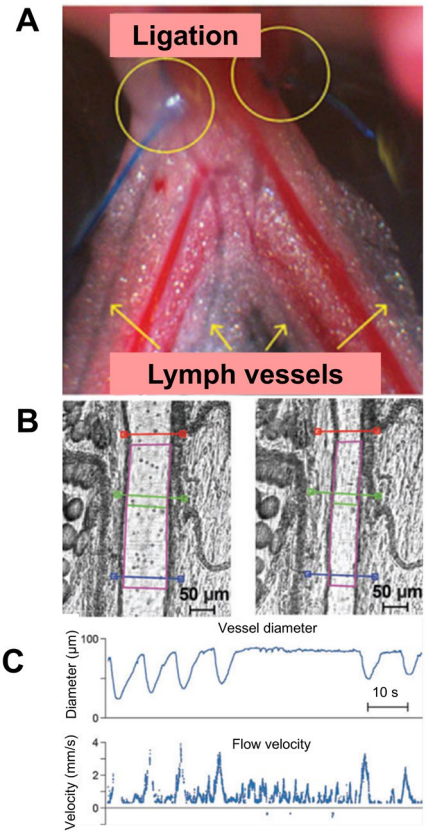

FIGURE 6 | Mesenteric models of lymphatic insufficiency. (A) Ligated lymphatic vessels (in yellow circles) and distal lymphatic vessels (yellow arrows) that were analyzed before and after ligation. (B) Edge detection and cell-tracking of in vivo mesenteric lymphatic vessel at rest (left) and during contraction (right). (C) Example diameter and cell velocity output that is ultimately used to calculate volumetric flow [110]. Reproduced with permission from Sarimollaoglu et al. [110]

While calcium-dependent contractile behavior is a shared feature of all lymphatic beds in animal models reviewed here [149–151], these models are unlikely to have the exact genotype and/or phenotype of lymphatic vessels in humans. Thus, key findings in these models should be verified in multiple models and in human lymphatic vessels when possible.

6.4 | Non-Surgical Methods

Aside from surgical ligation, there are a few non-surgical techniques that could be employed to disrupt lymph flow; however,

these methods have yet to be explored for developing animal lymphedema models. For example, combination treatment with verteporfin and photodynamic therapy is commonly used in ophthalmic surgery to treat aberrant blood vessel growth, and in animal models, it has been shown to ablate tumor-associated lymphatic vessels [152] and reduce lymph node metastasis, making it an intriguing new cancer therapy [153]. Unfortunately, these studies did not evaluate any subsequent lymphedema development. Given the profound ablation of tumor-associated lymphatics in animals and potential clinical use, this technique could be utilized as a future model of lymphedema, but a more in-depth characterization is needed. Interestingly, there are two active Phase II clinical trials investigating the ability of photodynamic therapy and verteporfin to reduce metastasis in pancreatic and breast cancer [154, 155]. It is possible that the clinical use of combined verteporfin and photodynamic therapy becomes more widespread in the future, but as of now, there is not a clear association of the clinical use of this technique and lymphedema development in patients.

7 | Conclusion and Perspectives

Although a broad array of animal models exists to study the various causes and aspects of lymphedema, each model has advantages and disadvantages. For instance, no standard definition of lymphedema exists across these models, thus preventing cross-comparison of study results from different models. In addition, most models mimic primary or cancer-related lymphedema rather than lymphedema caused by filariasis. Even though animal models exist to study lymphatic filariasis, most investigations focus on parasite control and inflammation rather than lymphatic function per se. Regardless of the disadvantages, each model has a place in lymphatic research. Ultimately, to gain FDA approval for any device or therapeutic, studies in multiple models and multiple species are needed to demonstrate effectiveness. Therefore, careful consideration should be made to select the model most appropriate to mimic the clinical lymphedema under investigation. Ultimately, there is always room for improvement, and animal models should evolve with clinical causes to keep the field progressing.

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Data Availability Statement

Data available upon reasonable request from corresponding author.

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