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# A novel pilot animal model for the surgical prevention of lymphedema: the power of optical imaging

*Bao Ngoc N. Tran, MD,*[a] *Joseph P. Angelo, PhD,*[a,b] *Jeong Heon Lee, BS,*[c] *Qing Z. Ruan, MD,*[a] *Rita G. Laurence, BS,*[d] *Hak Soo Choi, PhD,*[c] *Bernard T. Lee, MD, MBA, MPH,*[a] *and Dhruv Singhal, MD*[a,*]

[a] *Division of Plastic and Reconstructive Surgery, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts*

[b] *Department of Biomedical Engineering, Boston University, Boston, Massachusetts*

[c] *Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts*

[d] *Division of Cardiac Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts*

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ABSTRACT

*Background:* Breast cancer–related lymphedema affects more than 400,000 survivors in the United States. In 2009, lymphatic microsurgical preventive healing approach (LYMPHA) was first described as a surgical technique to prevent lymphedema by bypassing divided arm lymphatics into adjacent veins at the time of an axillary lymph node dissection. We describe the first animal model of LYMPHA.

*Methods:* In Yorkshire pigs, each distal hind limb lymphatic system was cannulated and injected with a different fluorophore (human serum albumin–conjugated indocyanine green or Evans Blue). Fluorescence-assisted resection and exploration imaging system was used to map the respective lymphangiosomes to the groin. Baseline lymphatic clearance of each hind limb lymphangiosome was obtained by measuring the fluorescence of each dye from centrally obtained blood samples. A lymphadenectomy *versus* lymphadenectomy with LYMPHA was then performed. The injections were then repeated to obtain clearance rates that were compared against baseline values.

*Results:* Human serum albumin–conjugated indocyanine green and Evans Blue allowed for precise lymphatic mapping of each respective hind limb using fluorescence-assisted resection and exploration imaging. Lymphatic clearance from the distal hind limb dropped 68% when comparing baseline clearance *versus* after a groin lymphadenectomy. In comparison, lymphatic clearance dropped only 21% when comparing baseline clearance *versus* a lymphadenectomy with LYMPHA.

*Conclusions:* We describe the first animal model for LYMPHA, which will enable future studies to further evaluate the efficacy and potential limitations of this technique. Of equal importance, we demonstrate the power of optical imaging to provide real-time lymphatic clearance rates for each hind limb.



* *Corresponding author*. Division of Plastic and Reconstructive Surgery, Department of Surgery, Beth Israel Deaconess Medical Center, 110 Francis St., Suite 5A, Boston, MA 02215. Tel.: +972 617 632 7835; fax: +972617 632 7840.
E-mail address: dsinghal@bidmc.harvard.edu (D. Singhal).

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## Introduction

Upper extremity lymphedema is one of the greatest survivorship issues amongst breast cancer survivors.[1] Patients who receive axillary node dissection and axillary radiation therapy for breast cancer are at particular risk for the development of lymphedema.[1,2] The psychosocial impact of lymphedema has been described to be as distressing as the initial diagnosis of breast cancer; patients with breast cancer–related lymphedema (BCRL) have a lower quality of life, a higher level of anxiety or depression, a higher likelihood of chronic pain, and greater difficulty functioning socially and sexually compared with breast cancer survivors without lymphedema.[3-10] Lymphedema is characterized by discomfort, functional impairment, and repeated infections.[3] Patients with BCRL incur twice the cost of medical expenses as breast cancer survivors who do not develop lymphedema. Outpatient care, especially mental health services, diagnostic imaging, and visits with moderate or high complexity, account for the difference in the economic impact between these two groups.[11]

The standard-of-care for chronic lymphedema is decongestive therapy with compression garments; however, this is palliative and not curative. Similarly, although functional lymphatic surgery, e.g. lymph node transfer and lymphovenous bypass, improve symptoms and quality of life in patients with chronic lymphedema, these procedures do not offer a definitive cure.[12-14] Therefore, focus has recently turned toward the surgical prevention of lymphedema. In 2009, our Italian colleagues introduced the lymphatic microsurgical preventative healing approach (LYMPHA).[15] For breast cancer patients undergoing an axillary lymph node dissection (ALND), which is the single greatest risk factor for the development of BCRL, divided arm lymphatics are microsurgically bypassed into adjacent veins at the time of the ALND. Four-year follow-up with this technique has demonstrated a lymphedema rate of 4.05% after ALND, and these significant reductions in lymphedema rates have been replicated in the United States.[16-19]

Various preclinical models ranging from rodents to sheep have been developed to study the physiology of lymphedema and its therapeutic intervention.[20] There is, however, an unmet need for the development of an animal model for LYMPHA, as this technique has already revealed early promising clinical results.[16-19] An animal model will allow further study to refine LYMPHA techniques and better understand indications. Moreover, an animal model that allows for real-time evaluation of lymphatic clearance would be optimal. To date, the first and only known animal model that quantifies direct lymphatic flow over time was published in 2009.[21] Using a sheep model and $I^{125}$, lymphatic flow was measured by injecting the radiotracer into afferent lymphatic channels in the hind limb of the sheep and measuring the radioactivity of blood samples from an internal jugular central line over time. The challenge of obtaining and using nuclear dyes, not only in research but also in clinical practice, greatly limits the utility of this approach.

Our laboratory previously described the use of the fluorescence-assisted resection and exploration (FLARE) imaging system to perform near-infrared (NIR) fluorescence angiography with reliable results.[22-26] We hypothesized that using the same technique with the Federal Drug Administration (FDA)–approved fluorophores, we would be able to obtain real-time lymphatic clearance rates, thereby avoiding the need for nuclear dyes altogether.

The aims of our current study were as follows: (1) to establish the first animal model for the prevention of lymphedema using an immediate lymphovenous bypass after a lymphadenectomy, and (2) to determine the ability of optical imaging agents to quantify lymphatic clearance.

## Materials and methods

### *Preparation of injectable fluorophores*

Indocyanine green (ICG, 25-mg vials) and Evans Blue (EB, 25-mg vial) were purchased from Akorn Inc (Lake Forest, IL) and Thermo Fisher Scientific (Waltham, MA), respectively, and used as received. Stock solutions were prepared with sterile water at a 2.5 mg/mL concentration, and transferred to the same volume of 5% human serum albumin (HSA, Sigma-Aldrich) to yield complexes of human serum albumin–conjugated indocyanine green (ICG-HSA) and and Evans Blue (EB-HSA), at the final concentration of 1.25 mg/mL.

### *Intraoperative NIR imaging system*

The basic design and setting of the real-time intraoperative dual-NIR channel imaging system have been described in detail previously.[23,25] In this study, 670-nm excitation light (1 mW/cm$^2$) and 760-nm excitation light (4 mW/cm$^2$) were used with white light (400nm-650 nm) at 5500 lux. Color and NIR fluorescence images were acquired simultaneously using AD-130GE camera (JAI Ltd, Yokohama, Japan) installed with custom dual bandpass prism (channel 1: 710/50, channel 2: 780lp), and custom software at rates of up to 15 Hz over a field of view that was manually adjusted by a 3CCD zoom lens (GOYO OPTICAL Inc, Saitama, Japan). In the color-NIR–merged image, 700-nm fluorescence and 800-nm fluorescence were pseudo-colored red and green, respectively. For each experiment, camera exposure time and image normalization was held constant.

### *Animal preparation*

The nonsurvival animal experiments were conducted in compliance with an approved Institutional Animal Care and Use Committee protocol (#030-2016). Yorkshire pigs were quarantined for 48 h as per the Institutional Animal Care and Use Committee guidelines and were given free access to food and water. Animals were fasted 24 h before anesthesia. Female Yorkshire pigs (Parsons EM & Sons Inc, Hadley, MA.) with a mean body weight of 37.2 kg (range, 35kg-39 kg) were induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, Iowa), intubated, and maintained on 2% isoflurane (Baxter International, Deerfield, IL). Electrocardiograph, heart rate, oxygen saturation, and body temperature were monitored during the procedure. Bilateral groins to hind feet were shaved and sterilized with povidone-iodine, and a central venous catheter was inserted into the internal jugular vein. Animals were euthanized with 86 mg/kg intravenous

pentobarbital sodium and phenytoin sodium (VIRBAC CORPORATION, Fort Worth, TX) at completion of the study.

A total of four pigs were included in this pilot study. In the first pig, we obtained our baseline clearance curves. In the second pig, we maintained a control side (no intervention) and performed a lymphadenectomy on the study side. In the third pig, we performed a sham operation on one side and a lymphadenectomy in the contralateral groin. In the fourth pig, we performed a lymphadenectomy on one side and a lymphadenectomy with lymphovenous bypass on the other side (Fig. 1).

### *Lymphatic mapping and obtaining baseline lymphatic clearance*

Following induction of anesthesia, 5 mL of 0.1% hematoxylin and eosin dye was injected subdermally at the medial aspect of each distal hind limb, allowing identification of afferent lymphatic channels. One afferent lymphatic channel was cannulated in each hind limb with a 26-gauge angiocatheter using the Seldinger technique. After securing the catheters, 6 mL of ICG-HSA and 10 mL of EB-HSA were injected, over 10 min, simultaneously into separate afferent limbs. The real-time mapping of lymphatic channel and lymph nodes were obtained by tracing NIR fluorescence and were recorded simultaneously at each emission wavelength.

Lymphatic clearance was evaluated by injecting preset amounts of each fluorophore into the peripheral afferent lymphatic channels followed by measurement of systemic fluorophore concentrations in sera, using fiber optic HR2000 spectrometers (200nm-1100 nm) (Ocean Optics Inc, Dunedin, FL). Blood samples were drawn from a right internal jugular vein at time 0, 15, 30, 45, 60, 90, 120, 150, and 180 min after the peripheral injection. Absorbance of each blood sample was obtained at 805 nm for ICG-HSA and 610 nm for EB-HSA and calculated the molar concentration based on the Beer's Law Equation.[27] Results were presented curve fitting, which was performed using Prism, version 6.0 software (GraphPad, San Diego, CA).

### *Lymphadenectomy and lymphatic clearance*

In the second model, we again plotted baseline lymphatic clearance curves for both ICG-HSA and EB-HSA to ensure reproducibility. After washout of both dyes at 3 h, we performed a lymphadenectomy on the study side. ICG-HSA and EB-HSA were then reinjected into their respective hind limbs to obtain new lymphatic clearance values. Lymphatic clearance rates after lymphadenectomy (study side) were compared against the contralateral side that underwent no intervention (control side).

### *Lymphovenous bypass and lymphatic clearance*

In the third model, we again measured baseline lymphatic clearance to ensure reproducibility. After washout of both dyes at 3 h, we performed a lymphadenectomy alone on the

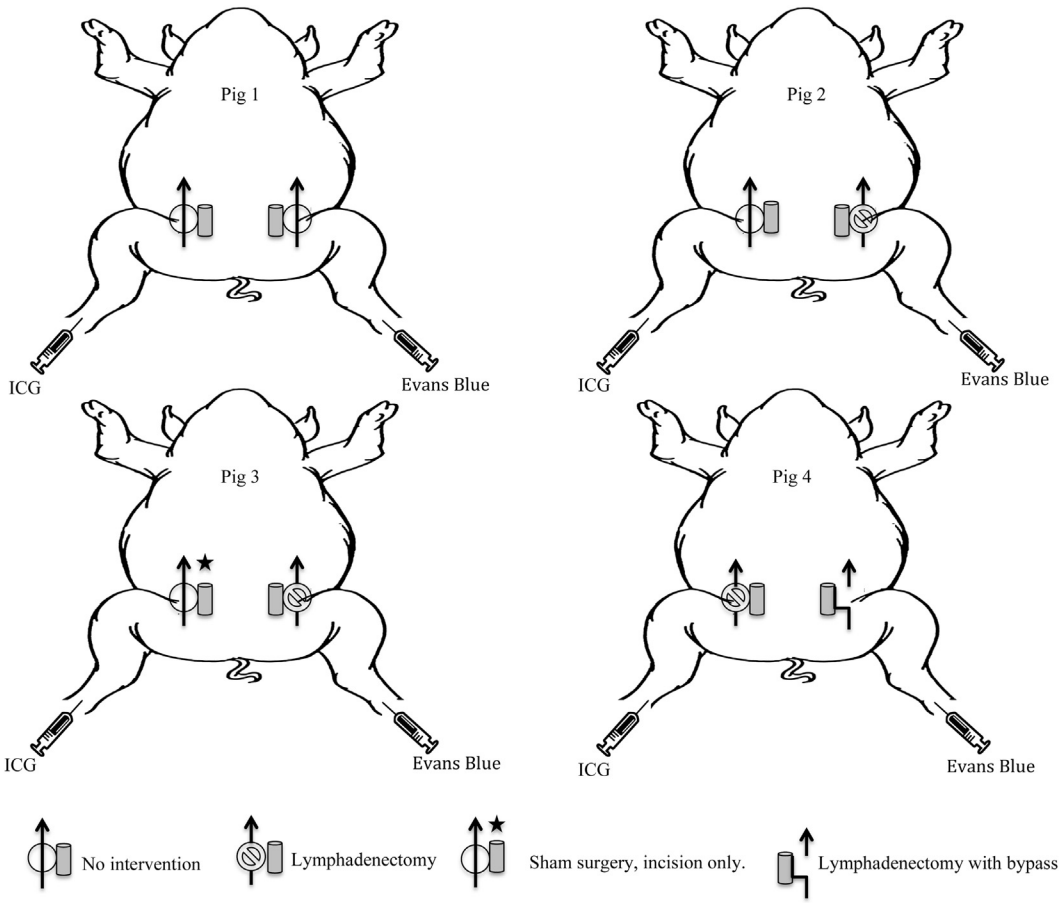


**Fig. 1 – Schematic of procedures performed in this pilot study.**

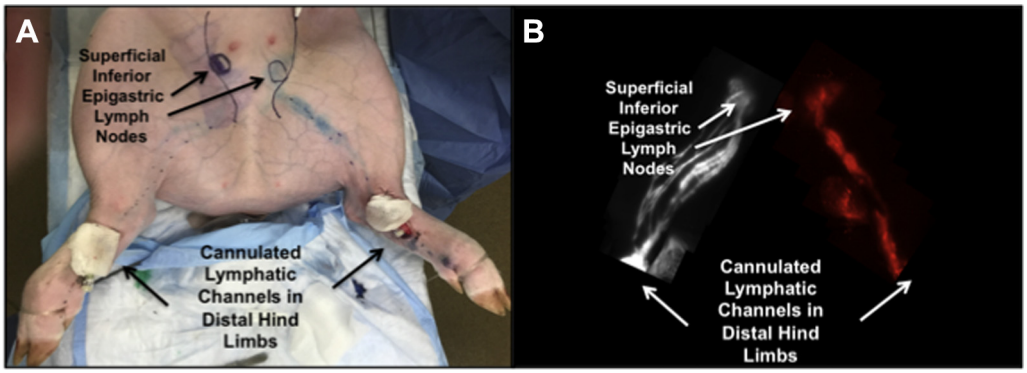


**Fig. 2 – (A) Lymphatic channels cannulated in the distal hind limbs bilaterally. (B) FLARE video capture immediately after bilateral distal hind limb injection of fluorophores. Stitching was done with Image J (NIH Image, WI) Right, ICG-HSA (white signal) at 250-ms exposure time. Left, EB-HSA (red signal) at 200-ms exposure time. (Color version of figure is available online.)**

control side and lymphadenectomy with lymphovenous bypass on the study side. A lymphovenous bypass was performed by parachuting one of two lymphatic vessels into the vein using a 9-0 nylon sutures. 10-0 suture was then used to stabilize the vein to the perilymphatic tissue. Approximately, 6-8 sutures were placed. The initial parachuting suture was then removed.[28] ICG-HSA and EB-HSA were then reinjected into their respective hind limbs to obtain new lymphatic clearance values. Lymphatic clearance rates after lymphadenectomy (control side) were compared against the contralateral side that underwent a lymphadenectomy with immediate lymphovenous bypass (study side). The restoration of flow was confirmed by NIR fluorescence tracing.

## Results

### *Lymphatic mapping*

Using the FLARE imaging system, the inguinal lymphosomes of the Yorkshire pig's hind limbs were successfully mapped to their respective target groin lymph nodes (Fig. 2). There was no evidence of fluorophore leakage from the injection site.

Before any surgical intervention in the groin, fluorophore concentrations were obtained for ICG-HSA and EB-HSA via centrally obtained blood samples over 3 h after injection into their respective peripheral afferent lymphatic channels. Baseline lymphatic clearance graphs demonstrated washout of ICG-HSA at approximately 45 min and EB-HSA at 180 min (Figs. 3 and 4).

### *Lymphadenectomy and lymphatic clearance*

The target nodes of this study were the superficial inguinal lymph nodes, which were identified and marked using our previously obtained FLARE lymphatic mapping. There was minimal anatomic variation during our lymphadenectomy dissections. All nodes were found to be solitary, close to midline, superficial and medial to the inguinal ligament, and measured 3 cm-4 cm (Fig. 5A). After lymphadenectomy, lymphatic clearance from the affected limb was decreased by 68% from baseline (Fig. 5B).

### *Lymphovenous bypass and lymphatic clearance*

An average of two afferent lymphatic channels were identified in the lymphadenectomy bed. Both identified channels were

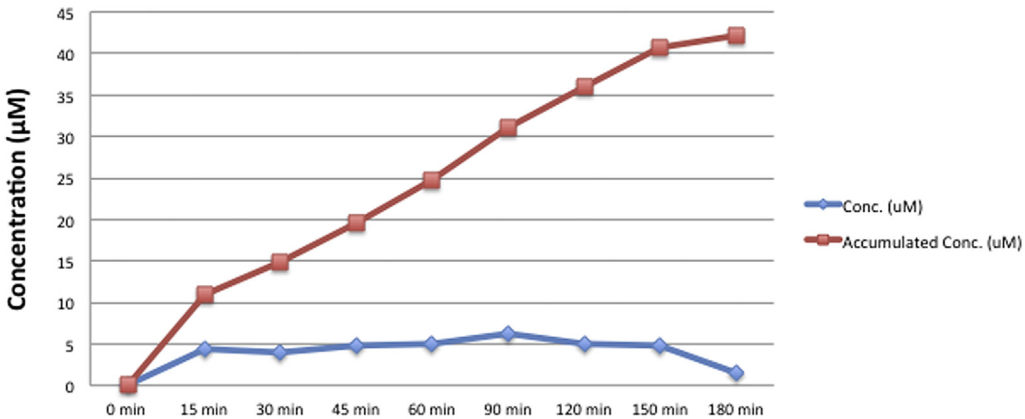


**Fig. 3 – Baseline lymphatic clearance for EB-HSA. (Color version of figure is available online.)**

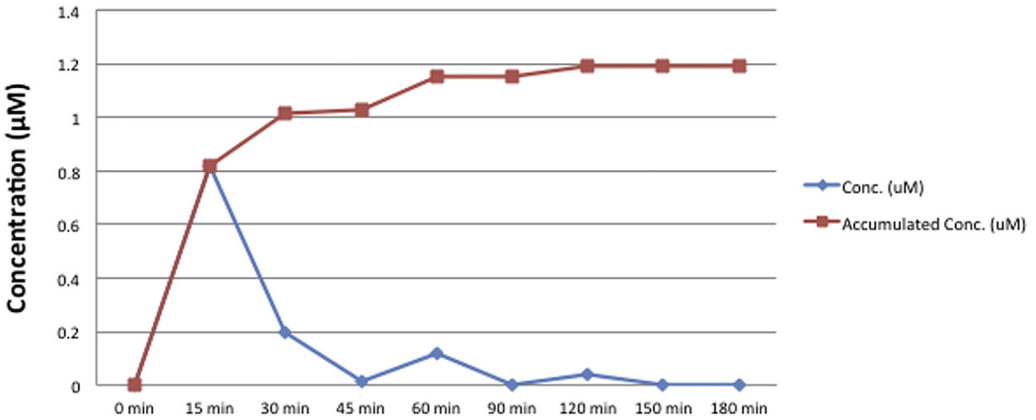


**Fig. 4 – Baseline lymphatic clearance for ICG-HSA. (Color version of figure is available online.)**

bypassed into the superficial inferior epigastric veins (Fig. 6A and B). Following lymphovenous bypass after lymphadenectomy, lymphatic clearance was only diminished 21% from baseline (Fig. 6C). Successful diversion of lymphatic flow was grossly confirmed by free passage of dye from afferent lymphatic channels into the venous pedicle. (Fig. 7).

## Discussion

We report the first successful animal model studying the physiology of the LYMPHA technique, the only known surgical prevention option for extremity lymphedema. Of significant note, we were able to quantify lymphatic flow using FDA approved optical imaging agents and report real-time effects of lymphadenectomy and subsequent lymphovenous bypass on lymphatic clearance.

There have been 24 animal models studying the physiology and therapeutic treatments of chronic lymphedema.[20] The first canine model evaluated the effects of lymphadenectomy on lymphatic physiology and consequent development of lymphedema in 1968.[29] Lymphovenous anastomosis was first explored as a potential treatment for chronic lymphedema in 1974.[30] Subsequently, this technique was replicated in both dogs and rabbits that developed secondary lymphedema after surgery and radiation.[31,32] Applications of growth factors such as vascular endothelial growth factor-C and -D in pig and mice also showed great potential for lymphatic regeneration.[33-36]

Since its conception in 2009, LYMPHA has demonstrated promising results in lymphedema prevention.[16-19] Despite these promising results, many questions remain. The surgical technique does not prevent lymphedema in all patients, and the patency of the lymphovenous bypass after regional radiation therapy and chemotherapy remains unknown. Therefore, developing an animal model for further study and

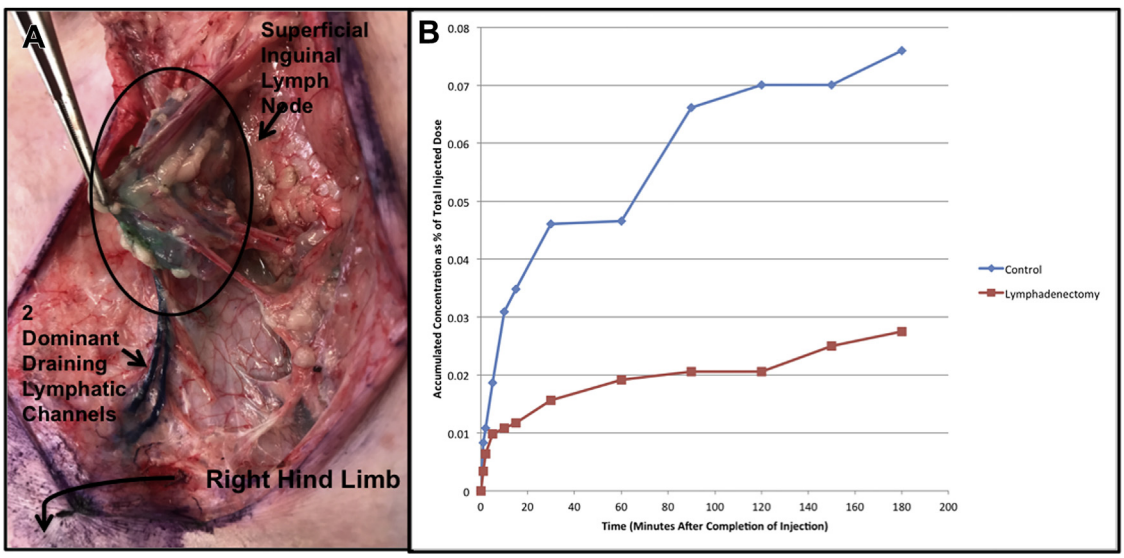


**Fig. 5 – (A) Superficial inguinal lymph node and two dominant draining lymphatic channels from right hind limb. (B) Changes of serum fluorophore concentration, control (blue curve) *versus* lymphadenectomy (red curve) using ICG-HSA for evaluation. (Color version of figure is available online.)**

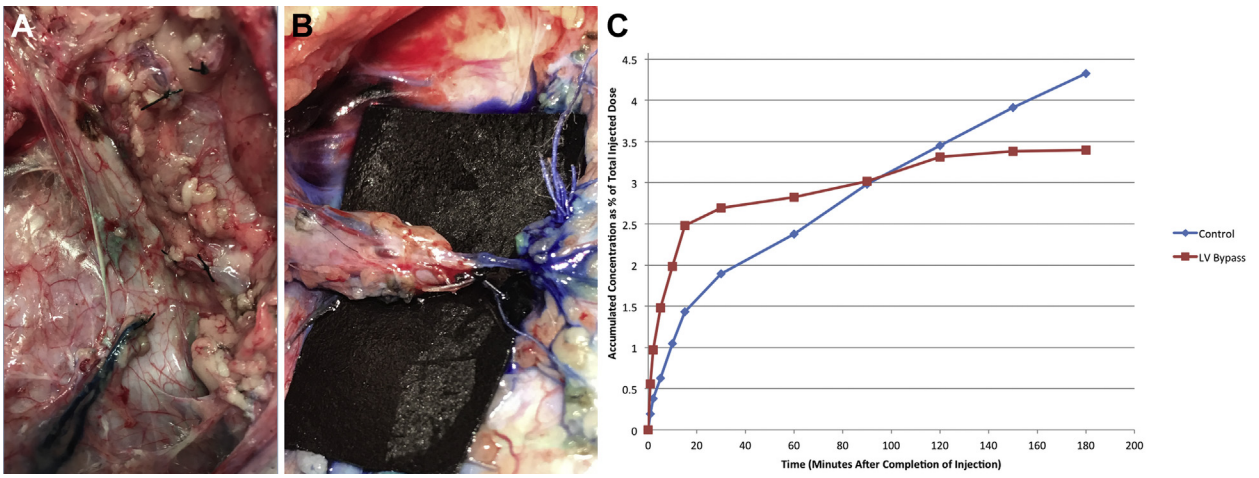


**Fig. 6 – (A) Radical lymphadenectomy. (B) Two lymphatic channels from the right hind limb are bypassed into the superficial inferior epigastric vein. Passage of blue dye is visible in the pedicle vein. (C) Changes of serum fluorophore concentration, control (blue curve) *versus* two lymphovenous bypasses following lymphadenectomy (red curve) using EB-HSA for evaluation. (Color version of figure is available online.)**

optimization of this promising approach to lymphedema is critical. Broadly speaking, this model will serve as the stepping-stone for further research in the emerging field of functional lymphatic surgery for the prevention of lymphedema in plastic surgery. We believe the Yorkshire pig to be the ideal model to study LYMPHA. Swine has been previously advocated as an ideal model for lymphedema, given the close resemblance of tissue structures to humans.[37] Moreover, the entire swine lymphatic system has been previously mapped, and the swine hind limb has been suggested as an ideal model for the training in lymphovenous anastomosis.[37,38]

Immediately after a groin lymphadenectomy, we were able to quantify a 68% reduction in lymphatic clearance of the affected limb. As our current study was nonsurvival, we were unable to translate our objective findings with the incidence of clinical lymphedema. However, we noted a significant restoration of lymphatic clearance when the lymphadenectomy was performed with an immediate lymphovenous bypass. The clinical and histological significance of this offloading effect on the affected extremity's lymphatic system deserves further study, given the early clinical promise of LYMPHA in humans.

A second important finding from our current study was the ability to use fluorophores to study real-time lymphatic clearance. The current gold standard technique to measure lymphatic flow rates uses radiotracers that have significant limitations. Aside from strict regulation limiting their clinical application in the operating room, they are not readily cleared and therefore accumulate in tissues complicating evaluation of clearance rates, and have limited utility because only one

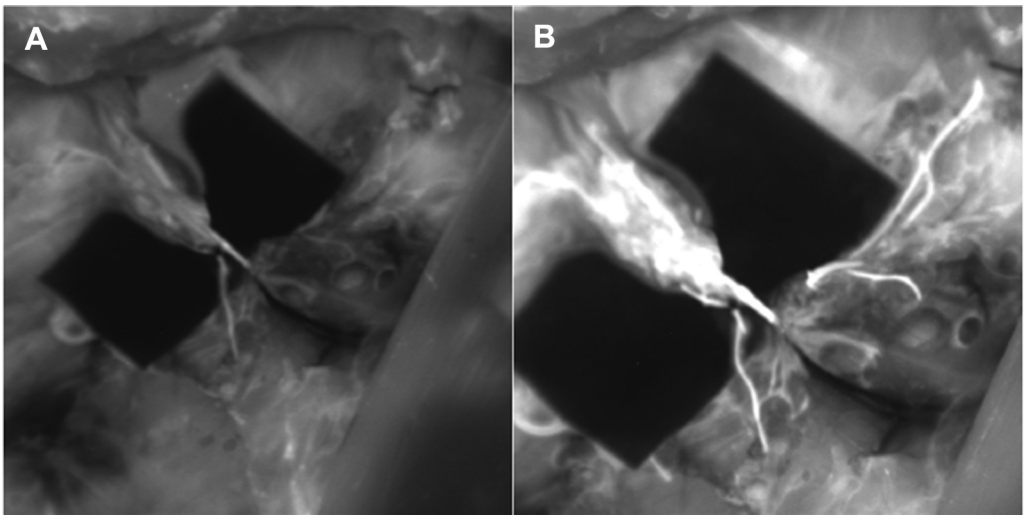


**Fig. 7 – (A) FLARE image of the lymphovenous bypass before right hind limb lymphatic injection with fluorophore. (B) Post injection image demonstrating free passage of dye from lymphatic channels into the venous system. Note that the exposure time (400 ms) was the same in images (A) and (B).**

agent can be injected and evaluated at any given time. Optical imaging agents, on the other hand, are becoming increasingly popular and readily available intraoperatively and are readily excreted allowing for accurate measures of clearance, and multiple agents can be injected simultaneously and evaluated independently.[12] Finally, there is potential for rapid translation of these agents to clinical use, as these fluorophores are currently FDA approved.

A significant limitation of our current study is that our protocol is nonsurvival, limiting our ability to correlate real-time lymphatic clearance rates to clinical lymphedema. Second, any leakage of dye from the distal hind limb injection can skew our central lymphatic clearance rates. While we were able to offset this issue by securing our angiocatheters under a microscope, we are working to develop more optically enriched fluorophores, which will allow us to use a smaller amount of injection and still be able to detect them on photospectrometry.

## Conclusion

We described the first animal model for LYMPHA, and future studies will be directed at evaluating the efficacy of this technique. Of equal importance, we demonstrate the power of optical imaging to provide real-time lymphatic clearance rates. This model will serve as the stepping-stone for further research in the emerging field of functional lymphatic surgery for the prevention of lymphedema in plastic surgery.

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Authors' contributions: B.N.N.T. conceived and designed the study; analyzed and interpreted the data; wrote the manuscript; and approved the final version of the manuscript. J.P.A. conceived and designed the study; and collected the data; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. J.H.L. conceived and designed the study; and collected the data; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. Q.Z.R. conceived and designed the study; and collected the data; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. R.L. conceived and designed the study; and collected the data; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. H.S.C. conceived and designed the study; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. B.T.L. conceived and designed the study; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript. D.S. conceived and designed the study; and analyzed and interpreted the data; provided critical revisions that are important for the intellectual content; and approved the final version of the manuscript.

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