Feasibility of pedicled vascularized inguinal lymph node transfer in a mouse model: a preliminary study

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Grant sponsor: JSPS KAKENHI; Grant number: JP16H05491

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Running title: Pedicled vascularized lymph node transfer in mouse model

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Abstract

Purpose: Vascularized lymph node transfer is becoming more common in the treatment of lymphedema, but suitable small animal models for research are lacking. Here, we evaluated the feasibility of pedicled vascularized inguinal lymph node transfer in mice.

Methods: Twenty-five mice were used in the study. An inguinal lymph node-bearing flap with a vascular pedicle containing the superficial caudal epigastric vessels was transferred into the ipsilateral popliteal fossa after excision of the popliteal lymph node. Indocyanine green (ICG) angiography was used to confirm vascularity of the flap. ICG lymphography was performed to evaluate lymphatic flow at 3 and 4 weeks postoperatively. Patent blue dye was injected into the ipsilateral hind paw to observe staining of the transferred lymph node at 4 weeks postoperatively. All transferred lymph nodes were then harvested and histologically evaluated by hematoxylin and eosin staining.

Results: In 16 of the 25 mice, ICG lymphography showed reconnection between the transferred lymph node and the afferent lymphatic vessels, as confirmed by patent blue staining. Histologically, these transferred lymph nodes with afferent lymphatic reconnection significantly regressed in size (0.37 ± 0.24 mm^2) and showed clear follicle formation, whereas those without afferent lymphatic reconnection showed less size regression (1.31 ± 1.17 mm^2); the cell population was too dense to allow identification of follicles.

Conclusions: We established a mouse model of vascularized lymph node transfer with predictable afferent lymphatic reconnection. Both the vascularization and reconnection might be necessary for functional regeneration of the transferred lymph node.

INTRODUCTION

Lymphedema is a pathologic condition resulting from lymphatic dysfunction, with tissue swelling due to localized accumulation of protein-rich fluid.[1] Surgical procedures to treat lymphedema are categorized into debulking or physiologic procedures, including lymphaticovenous anastomosis,[2] lymph vessel transplantation,[3] and lymphaticovenous implantation.[4] Vascularized lymph node transfer has emerged as a relatively new physiologic procedure that brings functional lymph nodes into the affected site and restores lymphatic flow.[5] Common donor sites for vascularized lymph node transfer include the inguinal,[5-8] axillary,[9] submental,[10,11] supraclavicular,[12,13] and lateral thoracic[14,15] regions. However, donor site morbidity remains a concern, including iatrogenic lymphedema, lymphorrhea, nerve injury, and conspicuous scar.[11,16-19] In the search for a better source of lymph node transfer, intraabdominal donor sites such as the jejunal mesentery[20] and omentum[18,21] have recently been reported to pose a lower risk for iatrogenic lymphedema.[21]

Despite its growing popularity in lymphedema treatment,[5,8,22-24] experimental evidence of the need for vascularization of transferred lymph nodes is lacking. The few small animal models of vascularized lymph node transfer that allow histological evaluation of the transferred lymph node are presently limited to rats.[25-28]

In addition to restoring lymphatic flow, lymph node transfer offers the possibility of retaining the immunologic and sentinel node functions of the affected limb.[29] A mouse model would be advantageous to investigate the immune function mechanism with numerous antibodies. We recently reported, using a mouse model inoculated with B16F10 melanoma cells, the immune-mediated antitumor effect of nonvascularized lymph node autotransplantation.[30] However, autotransplantation of lymph nodes is rarely performed in clinical situations because of lymphedema after lymphatic injury during cancer treatment.

This study evaluated the feasibility of pedicled vascularized lymph node transfer in

a mouse model. Here, we describe detailed surgical techniques and the anatomy of the vascular and lymphatic vessels in mice.

MATERIALS AND METHODS

All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University. All study procedures were performed with the approval of the Institutional Animal Care and Use Committee at Hokkaido University. Twenty-five 8-week-old male C57BL/6N mice (Sankyo Labo Service, Tokyo, Japan) were used to create the mouse model following a 2-week acclimation period. Mice were housed in cages under controlled temperature and humidity with an artificial 12-h light/dark cycle and free access to standard laboratory chow and water. Experiments were performed under general anesthesia with 2.5% isoflurane inhalation. Hair on the lower half of the body was shaved with an electric clipper and then cleared with depilatory cream 1-2 days before surgery.

Surgical Technique for Pedicled Vascularized Lymph Node Transfer

All surgical procedures were performed with the aid of a surgical microscope (OPMI pico, Carl Zeiss Meditec, Tokyo, Japan) under 4× or 10× magnification. Figure 1 shows a schematic representation of the model. Five microliters of 2% patent blue solution (Wako Pure Chemical Industries, Osaka, Japan) was injected subcutaneously into the left hind paw and left lower abdominal region adjacent to the scrotum to identify a popliteal lymph node (PLN) and an inguinal lymph node (ILN), respectively. First, the mouse was placed in the prone position and a 5-mm incision was made in the left popliteal skin across the ischial vein that was visible through the skin. A PLN was identified under the ischial vein between the biceps femoris muscle and medial hamstring muscles (Fig. 1A). After reposition to supine, a 10-mm left inguinal skin incision was made above the femoral artery. The left

inguinal region was undermined superolaterally to dissect the inguinal fat tissue from the overlying skin. An ILN receiving its blood supply from the superficial caudal epigastric artery and iliolumbar artery was identified in the middle of the inguinal fat tissue. This fat tissue was transected at the cranial border of the ILN without ligation of the iliolumbar artery to skeletonize and expose the side opposite the hilum of the ILN (Fig. 1B). The ILN-bearing flap was raised with a vascular pedicle containing the superficial caudal epigastric vessels. The femoral region between the skin incisions was undermined and the ILN-bearing flap was inserted into the popliteal region. The left inguinal skin incision was closed with 5-0 nylon sutures. The mouse was then returned to prone. The PLN was excised and the popliteal fossa was widened without injuring the ischial vein. The ILN-bearing flap was set into the vacant popliteal fossa and the fat tissue around the ILN was sutured to the biceps femoris muscle at 3 points using 10-0 nylon (Fig. 1A). The left popliteal skin incision was closed with 5-0 nylon sutures.

Intraoperative Assessment of Flap Perfusion

Vascularity of the ILN-bearing flap was confirmed using indocyanine green (ICG) angiography. After the flap was raised, a 5-mm incision was made in the left cervical skin, and the left superior vena cava was identified under the pectoralis major muscle. Five microliters of ICG solution (Diagnogreen; Daiichi Sankyo Co., Ltd., Tokyo, Japan; 2.5 mg/mL in distilled water) was injected into the superior vena cava. Fluorescence images were acquired using a near-infrared fluorescence camera (Photodynamic Eye; Hamamatsu Photonics, Shizuoka, Japan).

Postoperative Assessment of Lymphatic Flow

Lymphatic flow in the hindlimbs and body was assessed at 3 and 4 weeks postoperatively using ICG lymphography. Regrown body hair was removed using depilatory

cream before imaging. Five microliters of ICG solution was injected subcutaneously into both paws, and fluorescence images were acquired using the near-infrared fluorescence camera 15-20 min after ICG injection.

Postoperative Assessment of Afferent Lymphatic Reconnection

Four weeks postoperatively, real-time staining of each transferred lymph node was observed under a video camera-equipped surgical microscope. The left hindlimb skin was removed in the prone position. Five microliters of 2% patent blue solution was injected subcutaneously into the left paw, and a video of the hindlimb field was recorded. The transferred lymph node (n = 25) and the contralateral intact PLN and ILN (n = 8 each) were collected with the surrounding soft tissue. The transferred lymph nodes were divided into those with and those without afferent lymphatic reconnection based on patent blue staining.

Histological Assessment of Lymph Nodes

Specimens were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections (4 μm thick) were then stained with hematoxylin and eosin, and the size of each transferred lymph node with or without afferent lymphatic reconnection and the structure of surrounding soft tissue were compared with those of the contralateral intact PLN and ILN. All histologic slides were digitized using a whole slide scanner (NanoZoomer Digital Pathology; Hamamatsu Photonics), and the resulting digital images were visualized with NDP.view2 software.

Statistical Analysis

Lymph node size was reported as mean ± standard deviation. Multiple pairwise comparisons were performed using the Steel-Dwass test. Statistical analysis was performed using JMP software (version 14.1.0; SAS Institute Inc., Cary, NC). Statistical significance

was set at $P < 0.05$.

RESULTS

All mice tolerated the procedure with no postoperative complications and were sacrificed after 4 weeks. In all cases, the flap contained a single ILN in the inguinal fat tissue. The vascular pedicle varied in length from 1.5 cm to 2.0 cm (Fig. 2A) with no anatomic variations in the region manipulated. Fluorescence was detected throughout the ILN-bearing flap after ICG was injected into the superior vena cava (Fig. 2B), indicating perfusion of the ILN with blood.

ICG lymphography showed clear spotty fluorescence in the intact popliteal region (Fig. 3). At 3 and 4 weeks postoperatively, 16 of the 25 mice showed clear spotty fluorescence in the popliteal region on the manipulated side (Fig. 3A, B); the remaining mice showed no spotty fluorescence at this site but had collateral vessels and dermal backflow patterns in the hindlimb (Fig. 3C, D). Real-time patent blue staining of the transferred lymph node was observed in the 16 mice (Fig. 4) but not in the remaining 9 mice. Dissection of the stained transferred lymph node revealed a net of lymphatic vessels around it, whereas dissection of the unstained transferred lymph node showed lymphatic vessels in the fat tissue bypassing the node. ICG lymphography with clear spotty fluorescence in the popliteal region on the manipulated side after week 3 correlated with afferent lymphatic reconnection of the transferred lymph node on patent blue staining.

In all mice, the transferred lymph nodes were confirmed by hematoxylin and eosin staining. No histologic features suggestive of ischemia or necrosis were noted in any of the transferred lymph nodes. Fibrous tissue forming a capsule-like structure was observed around the transferred lymph nodes in all sections. Mean size of the transferred lymph nodes with or without afferent lymphatic reconnection (Fig. 5A, B) was 0.37 ± 0.24 (range, 0.12–1.02) mm^2 and 1.31 ± 1.17 (range, 0.30–3.81) mm^2, respectively. Mean size of contralateral intact PLNs

and ILNs (Fig. 5C, D) was 0.64 ± 0.27 (range, 0.29–1.10) mm^2 and 2.93 ± 0.82 (range, 1.89–3.93) mm^2, respectively (Table 1). Interestingly, the transferred lymph nodes with afferent lymphatic reconnection significantly regressed in size compared to those without afferent reconnection (P = 0.0307) and the intact ILNs (P = 0.0006), but clear follicle formation was observed in the cortex of each node, similar to that in the intact PLNs. Although the transferred lymph nodes without afferent lymphatic reconnection significantly regressed in size compared to the intact ILNs (P = 0.0406), the cell population was too dense to allow detection of follicles.

DISCUSSION

Vascularized lymph node transfer is still a relatively new technique. The concept was introduced in a rat model of ILN transfer by Shesol et al.[25] in 1979. Clodius et al.[6] clinically applied the technique in 1982. Since 2006 when Becker et al.[5] reported long-term outcomes in the first clinical series of patients who underwent vascularized lymph node transfer into the axilla (n = 24), the technique has become more widely used for the surgical treatment of lymphedema. Maintaining the vascular supply to the transferred lymph nodes is important for survival and preservation of function.[25,27] However, experimental studies comparing vascularized and nonvascularized lymph node transfer are still lacking in the literature.[31,32] Moreover, the immunologic activity and function of the transferred lymph nodes has not been sufficiently investigated. Developing a simple and reliable animal model would enable the biological significance of vascularized lymph node transfer to be researched further.

Several small and large animal models of lymph node transfer have been reported to date.[32] In most cases, however, lymph nodes were transferred as nonvascularized free grafts with[33,34] or without[35] fragmentation. Only a few rat models of vascularized lymph node transfer have allowed for histologic evaluation of lymph nodes transferred from the

inguinal,[25] cervical,[27] and axillary regions.[28] Rats are usually used in experimental models of vascularized lymph node transfer for several reasons, including the existence of accessible lymph node basins, the moderate size of the blood vessels, which allows microsurgical vascular anastomosis, and the relative homogeneity of rodent strains. Nevertheless, mice are preferred because they are easier to handle and relatively inexpensive. There are also numerous molecular reagents and bioassays available to investigate the mechanism of postoperative immune function in mice.

To date, no satisfactory mouse models of vascularized lymph node transfer have been developed. One study used a mouse model to evaluate drainage of lymph from vascularized ILNs transferred with an overlying skin paddle, but the experimental model was complex and the histologic evaluation of the transferred lymph nodes was not well described.[36] This prompted us to conduct the present study to establish a practical pedicled vascularized lymph node transfer in a mouse model and investigate the postoperative function of the transferred lymph nodes.

In earlier rat models, inguinal fat tissue was used as the donor site for harvesting ILNs with a vascular pedicle of the superficial epigastric vessels containing between 1 and 4 lymph nodes.[37,38] The advantages of pedicled ILN transfer include limited anatomic variation, easy flap elevation, minimal donor site morbidity, and no requirement for microvascular anastomosis. In our mouse model, a single ILN was identified, consistent with a previous study of mouse anatomy.[39] Because there is a single PLN in mice, transfer of a single ILN to the vacant popliteal fossa involves a simple one-to-one correspondence of lymph nodes at this site and the transferred lymph node can be evaluated histologically. To our knowledge, this is the first experimental study to quantify and compare changes in the size of transferred lymph nodes with or without afferent lymphatic reconnection in an animal model of vascularized lymph node transfer. Size reduction of transferred lymph nodes was significantly greater in those with afferent lymphatic reconnection than in those without it.

In mice, the PLN drains lymph from the ipsilateral hindlimb to the external sacral lymph node or ILN, and lymphatic vessels from these nodes drain proximally into the parailiac plexus. The ILN also drains lymph from the tail to the axillary lymph nodes.[40] Thus, removal of the PLN and transfer of the ILN-bearing flap to the popliteal fossa affects drainage of lymph from the tail and hindlimb. Using a mouse model, Maeda et al.[41] demonstrated that autotransplantation of a nonvascularized excised PLN into its original position restored lymphatic flow 3 weeks after transplantation. Similarly, in our mouse model, ICG lymphography showed reconnection between the transferred lymph nodes and afferent lymphatic vessels 3 weeks after transfer. These results suggest that ICG lymphography can be a noninvasive method for predicting successful afferent lymphatic reconnection to the transferred lymph node.

In a rat model of vascularized ILN transfer to the popliteal fossa with an intact vascular pedicle or with microvascular anastomosis, Rabson et al.[26] reported that lymphatic reconnection with surrounding lymphatic vessels occurred spontaneously in the transferred lymph nodes. They showed that the ideal method of dissection was to skeletonize and expose the donor node and excise the native PLN and all surrounding fat from the recipient site, which had a 57% success rate of afferent lymphatic reconnection. In our mouse model which involved skeletonizing the donor ILN and excising the PLN without the surrounding fat tissue, the success rate was slightly higher, at 64%. Given its vascular pedicle with surrounding fat tissue, skeletonization of the transferred lymph node was limited compared to nonvascularized free grafts. Other studies have reported a lower success rate of 22% for the incorporation of a nonvascularized lymph node into the existing lymphatic vasculature.[33]

In their concept of an “open” lymphatic channel, Shesol et al.[25] suggested that the number of lymphatic channels available for spontaneous lymphatic reconnection after lymphadenectomy decreases over time.[42] Moreover, lymph nodes have a high capacity for spontaneous regeneration, with lymphangiogenesis occurring rapidly after

lymphadenectomy.[22,31] Transferred lymph nodes may act as an endogenous source of vascular endothelial growth factor (VEGF)-C that facilitates lymphangiogenesis after transfer.[43,44] Endogenous VEGF-C expression in the perinodal fat and in the newly formed lymphatic vessels may play a critical role in the regulation of lymphatic regeneration and the migration of lymphatic endothelial cells from the recipient into the transferred lymph nodes.[35] To enhance integration of the lymphatic and vascular systems and the viability of transferred lymph nodes, exogenous VEGF-C has also been applied in conjunction with lymph node transfer in experimental animal models.[33,45,46]

Maintaining vascularization of the transferred lymph node ensures its survival and is essential when a lymph node-bearing block of tissue is transferred.[44] Furthermore, flow of lymph between the transferred lymph node and surrounding lymphatic vessels is required for maintenance and function of the transferred lymph node.[33,45-47] This functional reconnection is crucial because the connection regulates immune cell homeostasis.[48] Therefore, our finding that the cell population was maintained with clear follicles in the transferred lymph nodes with afferent lymphatic reconnection suggests remodeling and immunologic competence of these lymph nodes. However, our finding that the transferred lymph nodes without afferent lymphatic reconnection showed less regression in size with a dense cell population suggests that maintaining vascularization contributed to their survival but not always to their functional regeneration.

This study has several limitations. First, flap vascularity was confirmed using ICG angiography during flap elevation, but it was difficult to prove persistent vascularity postoperatively, as seen in previous studies.[27,28] Second, although the size of lymph nodes was measured in the maximum cross-section, it was not possible to make paraffin-embedded sections of lymph nodes with surrounding fat tissue in the same plane for comparison because of the flat ellipsoid shape of ILNs. Third, further research is necessary to clarify the

functional significance of the vascular element by comparing with groups of nonvascularized lymph node transfer.

This study indicated that both vascularization of the transferred lymph nodes and afferent lymphatic reconnection might be important for their functional regeneration. In the practical treatment of lymphedema, it has been established that lymph node transfer is more effective than lymphaticovenous anastomosis.[49] However, vascularized lymph node transfer as a clinical procedure mostly requires microsurgical vascular anastomoses that often result in long operating times. If vascularization of transferred lymph nodes does not affect surgical outcomes, the procedure would be omitted and nonvascularized lymph node transfer would be an alternative.[32] Additional exogenous treatment to facilitate lymphatic reconnection should also be investigated.

Moreover, a PLN could be regarded as the sentinel lymph node, which is the primary site where the immune system encounters tumor antigens in a mouse model inoculated with B16F10 melanoma cells, syngeneic and transplantable to the C57BL/6 mouse strain, into the hindlimb footpad.[30] We believe that our model will be useful for further investigating the physiologic and immunologic functions of the transferred vascularized lymph nodes as well as the biological significance of these functions in terms of tumor immunity.[26,30,50]

CONCLUSIONS

We have created a practical mouse model of pedicled vascularized ILN transfer with predictable afferent lymphatic reconnection to the transferred lymph node. Both vascularization and reconnection might be needed for functional regeneration of the transferred lymph node.

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Figure Legends

Figure 1. Schematic drawing of pedicled vascularized inguinal lymph node transfer in a mouse model. **A**, The left popliteal region in the prone position. **B**, The left inguinal region in the supine position. (PLN) popliteal lymph node, (ILN) inguinal lymph node, (IsLN) ischial lymph node, (BF) biceps femoris muscle, (MH) medial hamstring muscles, (IFT) inguinal fat tissue, (1) ischial vein, (2) superficial caudal epigastric artery, (3) femoral artery, (4) iliolumbar artery.

Figure 2. A, Length of the vascular pedicle on an inguinal lymph node-bearing flap, ranging between 1.5 and 2.0 cm. **B**, Confirmation of flap perfusion after intravenous injection of indocyanine green.

Figure 3. Fluorescence images of the hindlimbs in the prone position after injection of indocyanine green. **A**, **C**, Three weeks after surgery. **B**, **D**, Four weeks after surgery. Arrowheads indicate the intact right popliteal region. Arrows indicate the left popliteal region where the pedicled vascularized inguinal lymph node was transferred.

Figure 4. View of the left popliteal region in the prone position. Staining of the transferred lymph node (arrow) was observed after subcutaneous injection of patent blue dye into the ipsilateral paw. Arrowheads indicate a lymphatic vessel filled with patent blue dye. Asterisk denotes the ischial vein.

Figure 5. Hematoxylin and eosin staining of the lymph nodes harvested 4 weeks after pedicled vascularized inguinal lymph node transfer. Bars, 1 mm. **A**, Transferred lymph node with afferent lymphatic reconnections. **B**, Contralateral intact popliteal lymph node. **C**, Transferred lymph node without afferent lymphatic reconnections. **D**, Contralateral intact

inguinal lymph node.

Table 1. Comparison of the Size of Lymph Nodes

		Size	
Lymph nodes assessed	*n*	Mean ± SD (mm^2)	Range (mm^2)
A: Transferred lymph nodes with afferent lymphatic reconnection	16	0.37 ± 0.24	0.12–1.02
B: Transferred lymph nodes without afferent lymphatic reconnection	9	1.31 ± 1.17[a]	0.30–3.81
C: Contralateral intact popliteal lymph nodes	8	0.64 ± 0.27	0.29–1.10
D: Contralateral intact inguinal lymph nodes	8	2.93 ± 0.82[b, c, d]	1.89–3.93

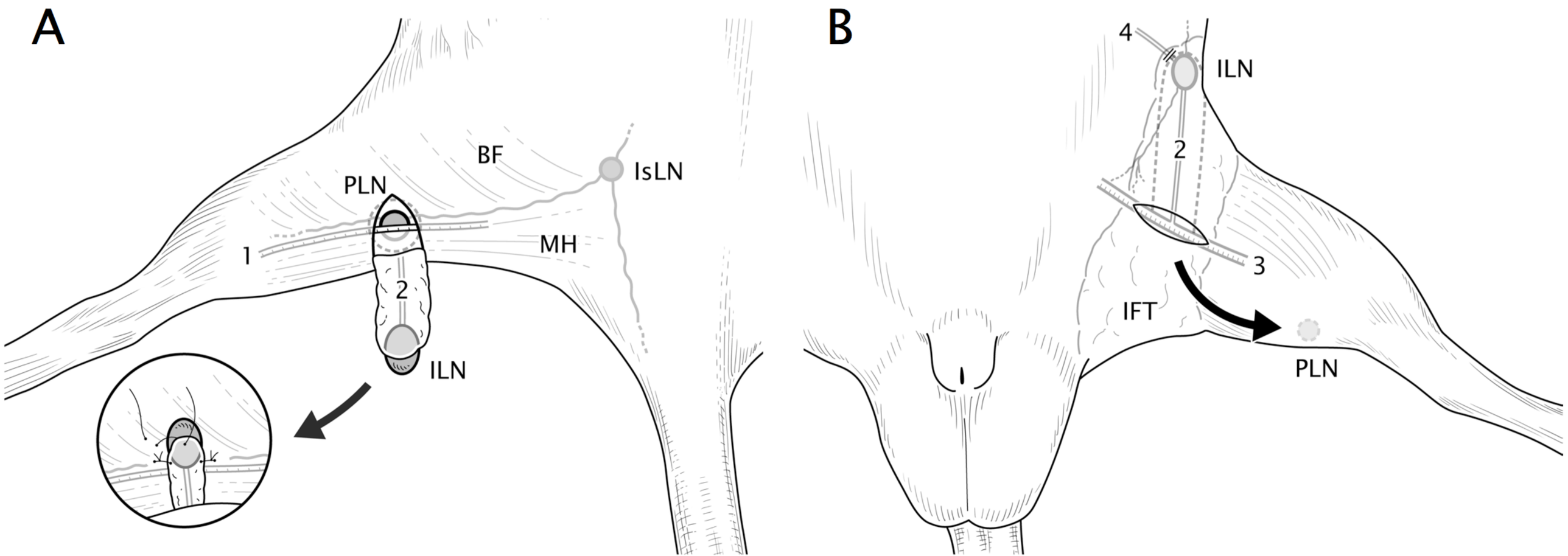
SD, standard deviation.

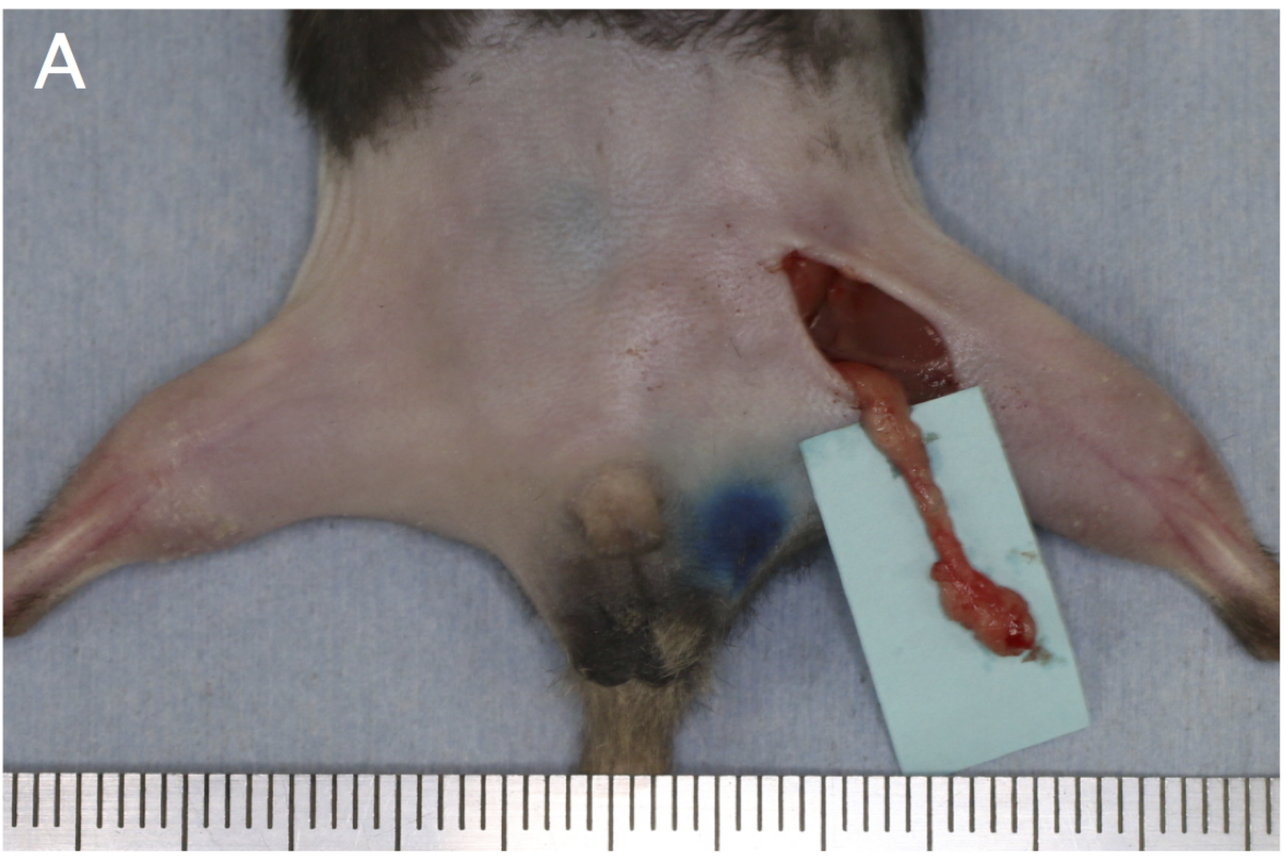
[a]*Comparison between B and A, P = 0.0307.*

[b]*Comparison between D and A, P = 0.0006.*

[c]*Comparison between D and B, P = 0.0406.*

[d]*Comparison between D and C, P = 0.0052.*


A
BF
IsLN
PLN
MH
1
2
ILN
B
4
ILN
2
3
IFT
PLN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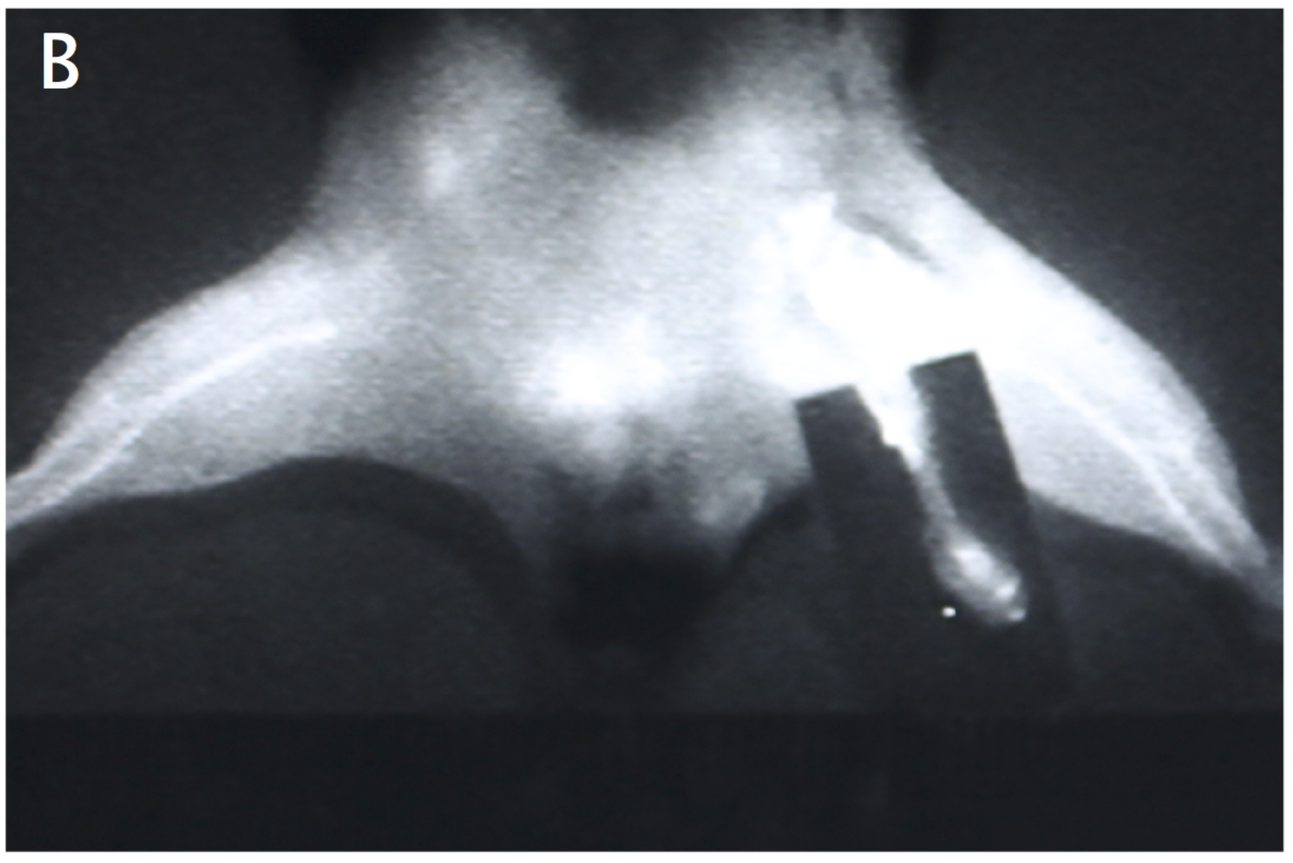
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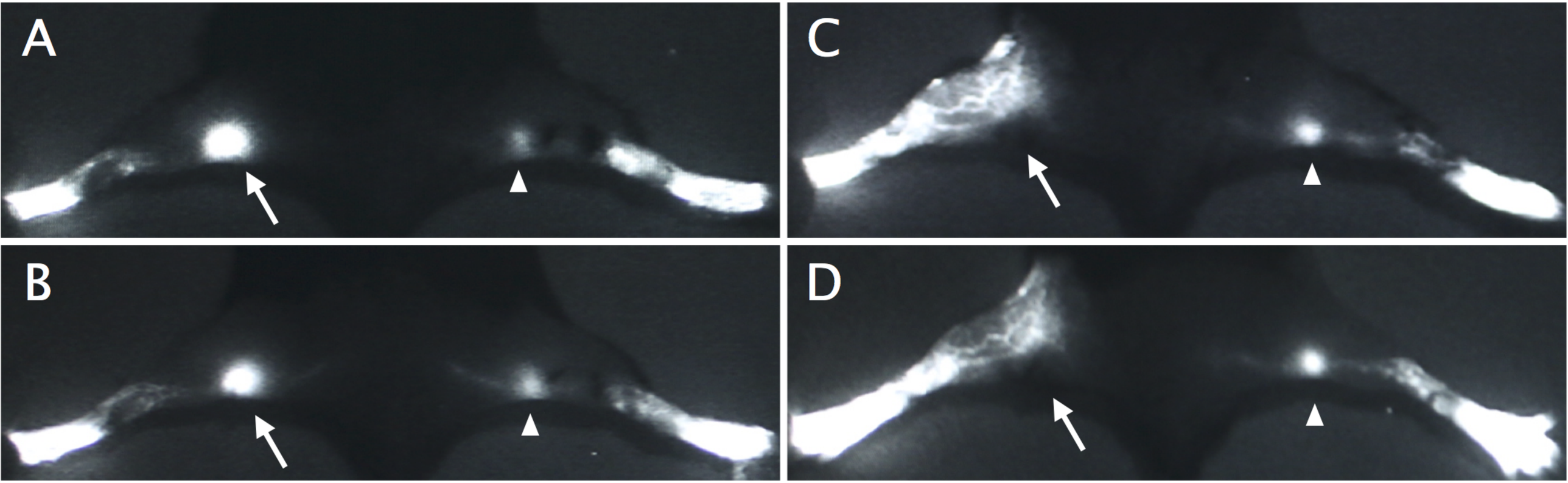
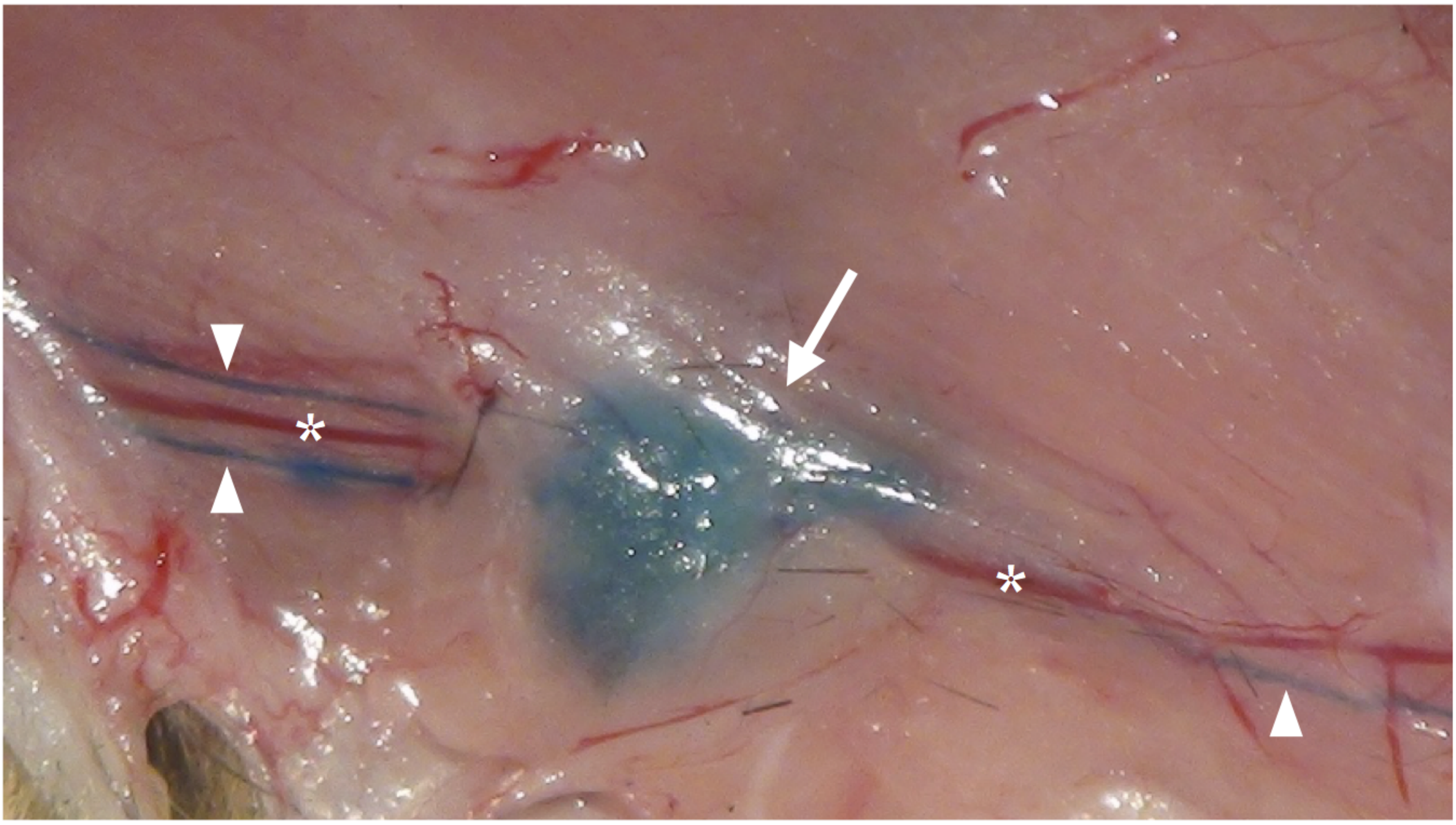
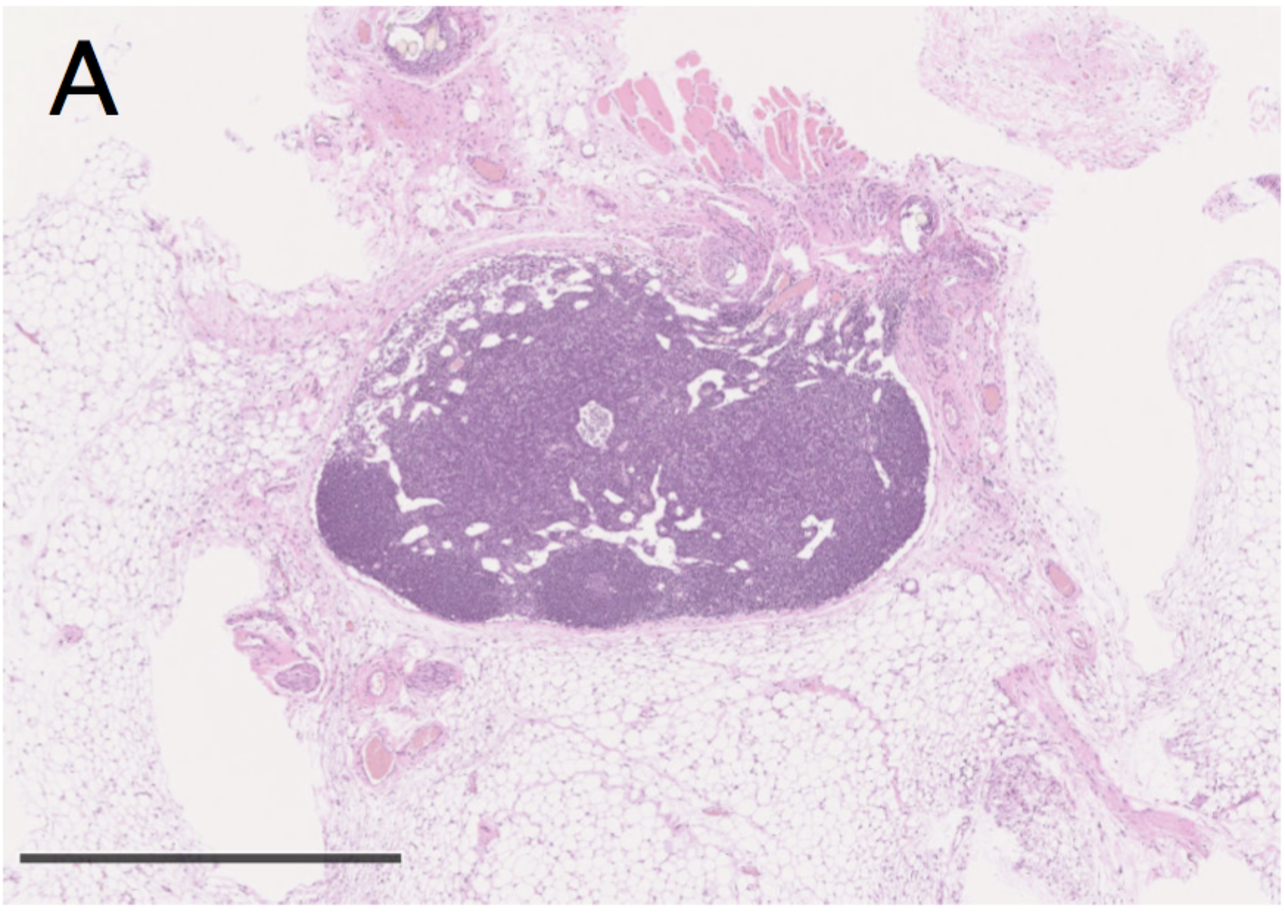

A
C
B
D

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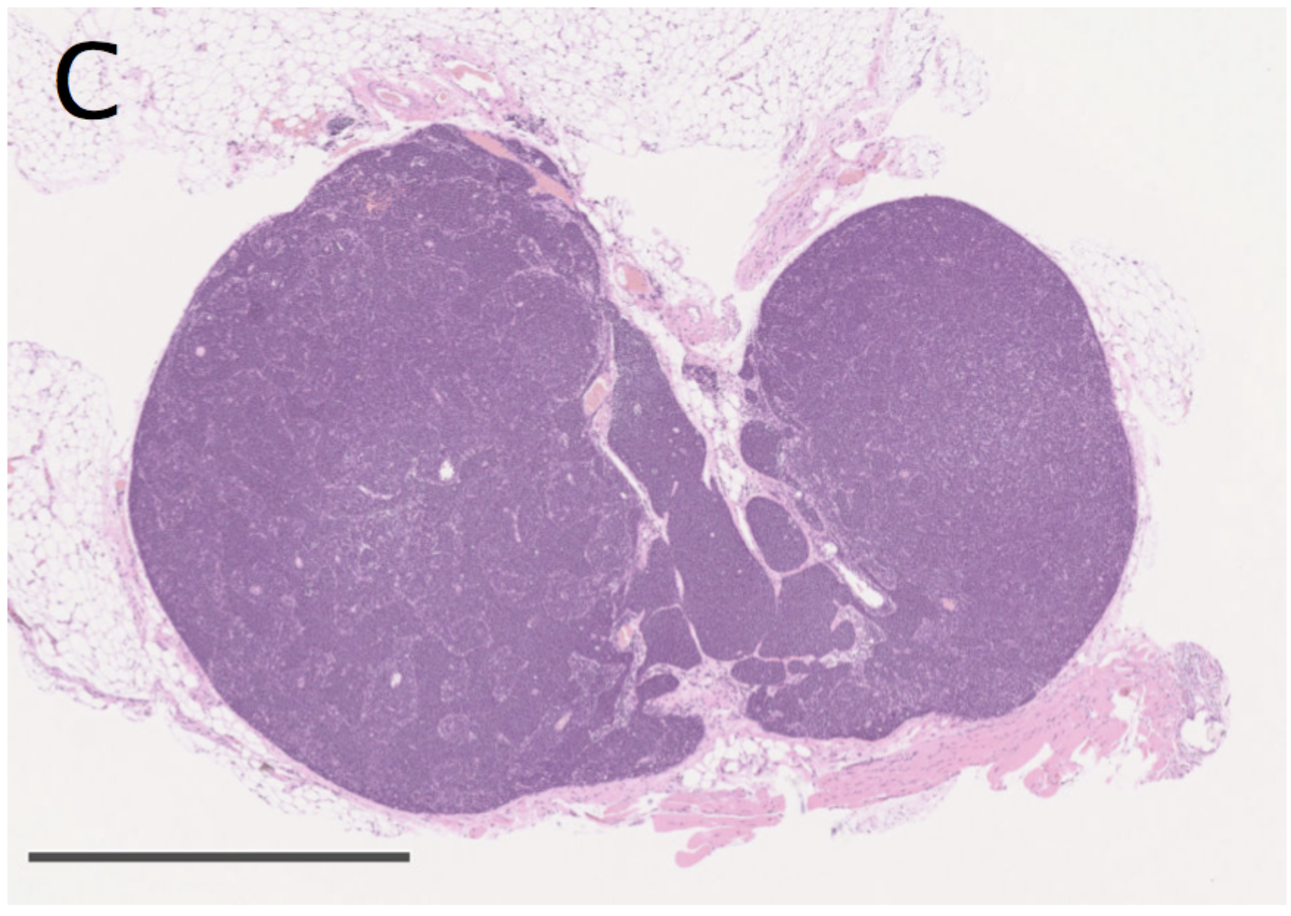
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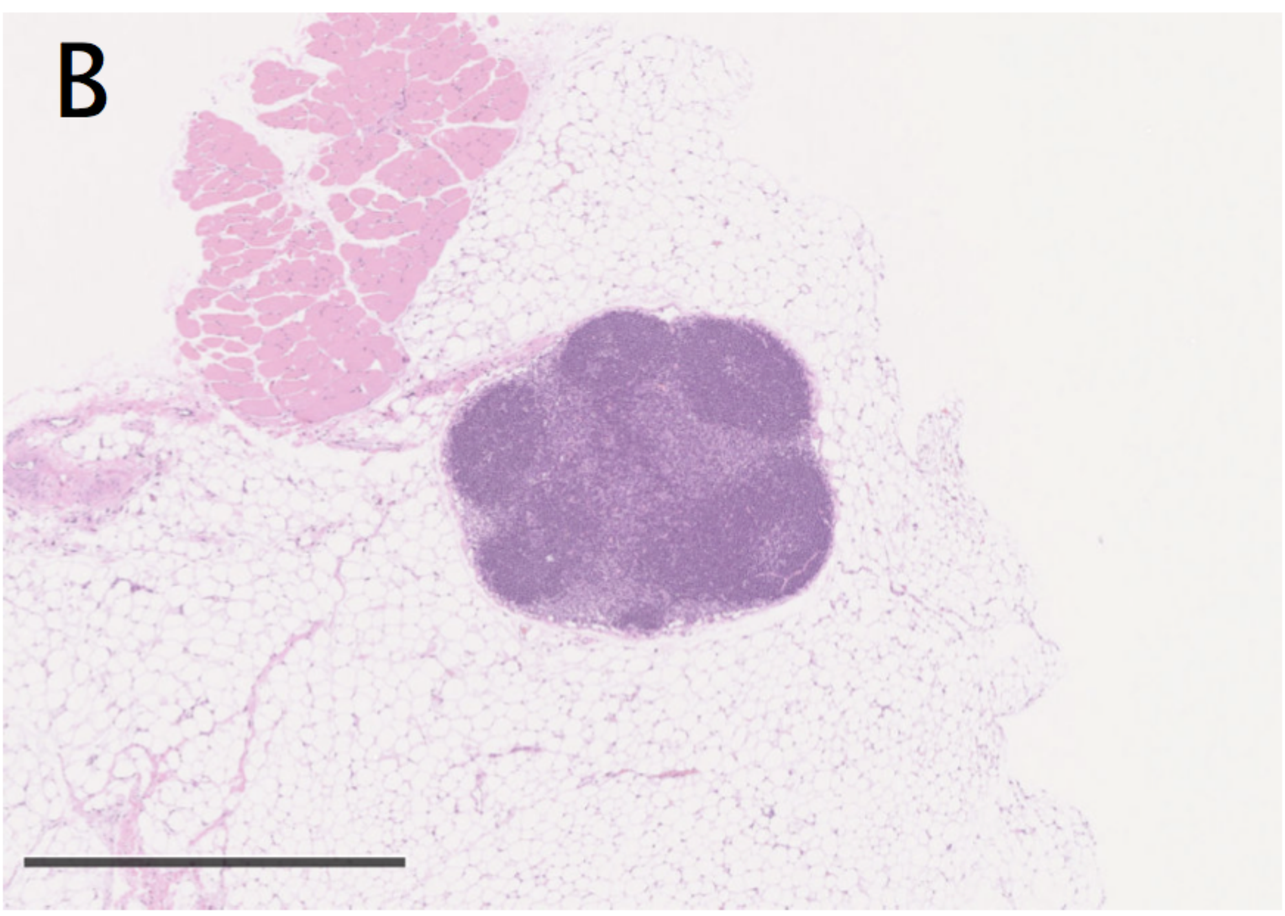
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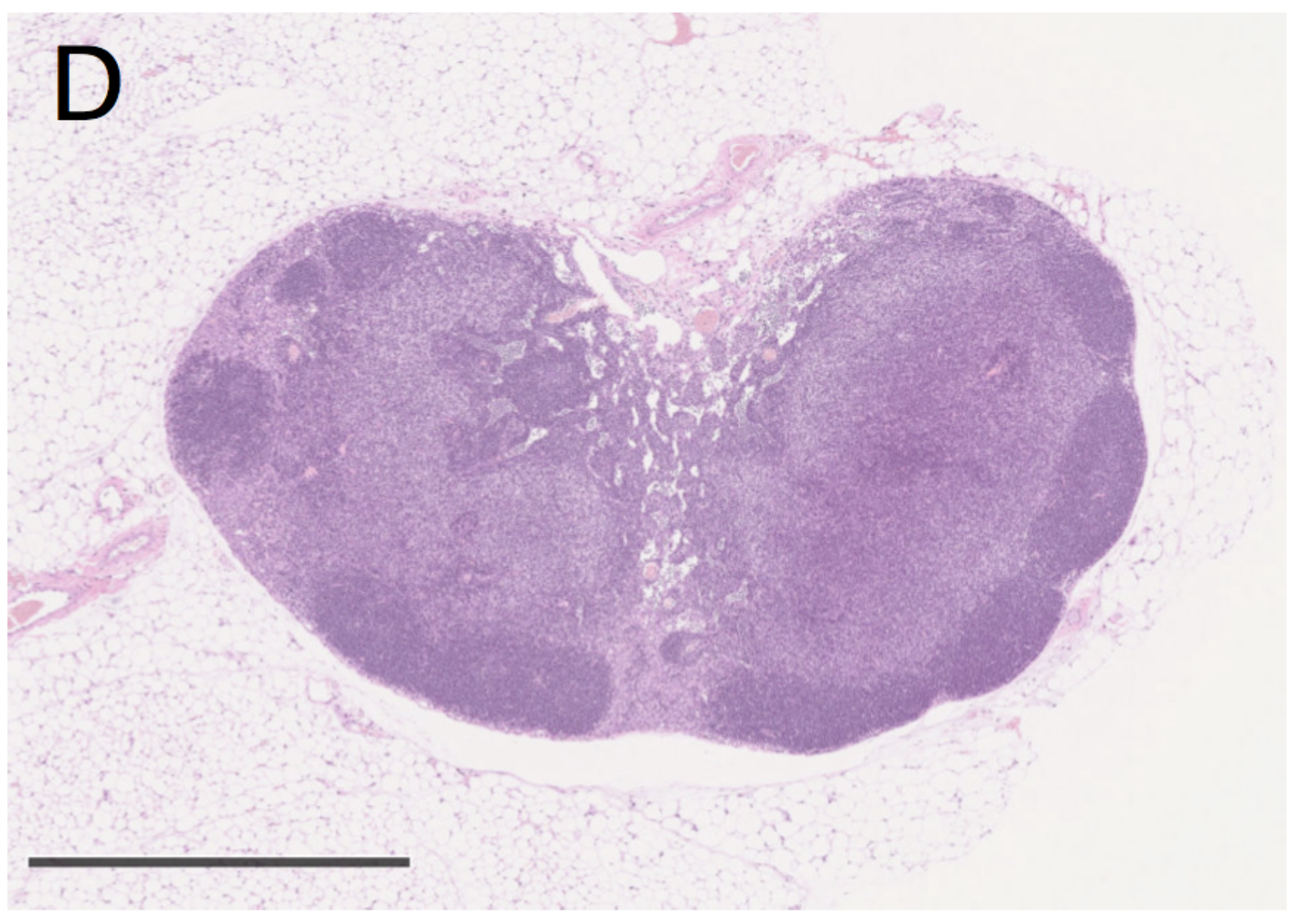
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