Title:
Collateral development and arteriogenesis in hindlimbs of domestic swine after ligation of arterial inflow

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ABSTRACT

Development of collateral circulation is an important compensatory mechanism for patients with peripheral artery disease. Attempts to model and study collateral development in small animals have been hindered secondary to small subject size. Herein we describe a porcine model of hindlimb collateral development that utilizes open arterial ligation. Right hindlimb ischemia was induced in swine using two different versions of arterial ligation: Version 1 (N = 5) was ligation of the right external iliac, right profunda femoris, and right superficial femoral arteries; Version 2 (N = 5) was Version 1 plus ligation of the right internal iliac artery. Percutaneous near-infrared spectroscopy, angiography were used to determine muscle oxygen content and relative arterial diameters, respectively, at pre-ligation, post-ligation, and one-month time points. Collateral formation supplying the ischemic limb was evident at the one month time point, along with arteriogenesis of the inflow vessels. Muscle oxygen content and limb arterial indices (noninvasive cuff measurements) increased in the ischemic hindlimb at the one month time point. There was no significant difference between Version 1 and 2 with respect to arteriogenesis, arterial index, or muscle oxygen content. Histologic evaluation of ischemic hindlimb muscle at one month revealed pathologic changes analogous to those seen in patients with PAD. Two versions of hindlimb ischemia (iliofemoral interruption ± internal iliac artery ligation) resulted in collateral formation, inflow arteriogenesis, and improved perfusion pressure and oxygen content of the affected muscle. We believe that this porcine model of collateral development and arteriogenesis will be useful in the study of PAD and treatments thereof.

Abbreviations

ABI – Ankle/Brachial Index
CLI – Chronic Limb Ischemia
CIIT – Common Internal Iliac Trunk
LEIA – Left External Iliac Artery
LIIA – Left Internal Iliac Artery
LPFA – Left Profunda Femoral Artery
REIA – Right External Iliac Artery
RIIA – Right Internal Iliac Artery
RPop – Right Popliteal Artery
PAD – Peripheral Arterial Disease
Moxy – Muscle Oximetry
SmO_2 – Muscle oxygen saturation
THb – Total Hemoglobin

INTRODUCTION:

Peripheral artery disease (PAD) has a total disease prevalence of 12-14% (1-3) with an impact on functional status, quality of life, and life expectancy. In men, the incidence of PAD increases with age from 0.5% per year in the 40-55 age group to 1% per year for those older than 75 (1-3). PAD risk factors mirror those associated with coronary artery disease (CAD), including smoking, hypertension, dyslipidemia, diabetes mellitus, and family history of atherosclerosis. Up to half of PAD patients may be asymptomatic at the time of diagnosis, while other patients may present with typical claudication pain (10-35%) or atypical leg pain (40-50%)(1-3). Very few patients (1-2%) present with a threatened limb.

Treatment options for symptomatic PAD include open and percutaneous revascularization; unfortunately 20-40% of PAD patients are not candidates for revascularization secondary to unreconstructable disease, medical comorbidities, and/or other issues. For these patients, development of collateral blood flow is an important compensatory mechanism to increase oxygen delivery to ischemic limb tissue. Some studies of stem cell-based therapies on the ischemic limb in both preclinical (4-7) and clinical studies (8-13) have indicated some salutary effect on limb perfusion; but results have not been uniformly positive (14, 15).

An improved understanding of PAD has evolved with the elucidation of the role of mitochondrial dysfunction (16) and oxidative stress (17) in disease pathophysiology; however, our knowledge and ability to treat this disease is still in an early stage. Attempts at small animal modeling of PAD (typically with mice) have been hampered by reliability and utility issues secondary to size and relative ability to replicate human physiology (18-21). In many disease entities, a large animal model (such as with the pig) can replicate human anatomy, physiology, inflammatory response, genomics, and other areas more reliably than rodent models (22-26). Given these advantages of large animal modeling, we have developed and characterized a

hindlimb ischemia model in swine which could be utilized to study post-ischemia collateral development in a major arterial network.

MATERIALS AND METHODS

Animal Welfare Statement. The animals utilized to generate data for this report were maintained and treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th ed.) from the National Research Council and the National Institutes of Health (27) and also in accordance with the Animal Welfare Act of the United States (U.S. Code 7, Sections 2131 – 2159). The animal protocol pertaining to this manuscript was approved by the Institutional Animal Care and Use Committee (IACUC) of the VA Nebraska-Western Iowa Health Care System (ID number 00950) and by the IACUC of the University of Nebraska Medical Center (ID number 15-068-07-ET). All procedures were performed in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC; www.aaalac.org) and by the Office of Laboratory Animal Welfare of the Public Health Service (grants.nih.gov/grants/olaw/olaw.htm). All surgical procedures were performed under isoflurane anesthesia, and all efforts were made to minimize suffering. Euthanasia was performed in accordance with the AVMA Guidelines for the Euthanasia of Animals (28).

Experimental Subjects. Wild type domestic swine (N = 13; castrated males; age 11-13 wk) were purchased from the Animal Research and Development Center at the University of Nebraska Lincoln (ardc.unl.edu). Swine were housed individually in pens, with multiple subjects per room, and fed ad lib with standard hog feed (Purina Nature's Match Sow and Pig Complete Feed).

Experimental Design. Refer to Fig. 1. The basic experimental design included an ischemia-induction procedure (i.e., arterial ligation) on day 0, with endpoint measurement and necropsy one month later. Two methods of arterial ligation were compared in a nonrandomized fashion.

Set-Up and Anesthesia (22). On day of procedure, each swine underwent induction with ketamine (Zoetis, 2.2 mg/kg), Telazol® (Zoetis; 4.4 mg/kg) and xylazine (Zoetis; 2.2 mg/kg), given combined in single IM injection. Each subject then was weighed and endotracheally intubated. EKG, pulse oximetry, rectal temperature, and lingual endtidal CO_2 ($EtCO_2$) monitors were placed. The subject was placed on a water-circulated warming blanket that was set at 102˚F. An auricular intravenous (IV) line was placed. Anesthesia was maintained with isoflurane (0.5-1%) and supplemental oxygen (3-5 L/min) using a Matrx® ventilator (midmark.com). The ventilator rate initially was set at 12-15 breaths per minute with a tidal volume of 10 mL/kg, and subsequently adjusted to maintain the EtCO2 at 40-50 mm Hg. Cotton blankets were placed over non-surgical areas to minimize loss of core temperature. Vital signs were continuously recorded to a laptop computer via a Bionet BM5 monitor (www.bionetus.com). A single dose of cefovecin sodium (Zoetis; 8 mg/kg IM) was given before incision.

Muscle Oximetry & Arterial Indices. Extremity muscle oximetry (Moxy) measurements were measured transcutaneously in the supine position under general anesthesia using an ANT Fortiori Design device (moxymonitor.com) with Peripedal software (peripedal.com); see Fig. S3 for technical details. The Moxy device has a noninvasive probe which measures muscle oxygenation (SmO_2) and total hemoglobin concentration (THb) (Fig S4). For determination of arterial indices, a pediatric-size sphygmomanometer cuff was applied to each extremity (see Fig. S3), and systolic pressure was measured with the aid of doppler device [Model 811-B, Parks Medical Doppler (parksmed.com). The hindlimb arterial pressure index ("ABI", meant to be the porcine analog of the clinical ankle-brachial index) was determined as a ratio of hindlimb systolic blood pressure over the forelimb systolic pressure (Fig. S3).

Laparotomy, Vessel Exposure, and Angiography. With the subject under general anesthesia and in the supine position, the chest, abdomen, groins, and bilateral lower extremities were shaved with an electric clipper, washed with soap and water, and then prepped using ChloraPrep™ applicators (chlorhexidine gluconate/isopropyl alcohol; BD Medical). In the first subject, vessel exposure was performed transabdominally through a right paramedian incision (Fig. S2), which began in the midline 7-8 cm above the urethral meatus and extended caudally to the right of the meatus and midline urethra and medial to the right-sided nipple line. Once in the peritoneal cavity, the viscera were gathered in a wet towel and retracted superiorly and to the left. The peritoneum overlying the distal aorta and trifurcation was incised, and the right hindlimb arterial system was exposed with sharp dissection. To gain access to the more distal right-sided pelvic vessels, the paramedian skin incision was extended over the right inguinal ligament and onto the right medial thigh, towards but not reaching the right stifle (knee) joint. The right inguinal ligament itself was not divided. For the subsequent ten subjects, vessel exposure was obtained with a retroperitoneal approach through a similar right paramedian incision that began level with the urethral meatus and medial to the right nipple line, and then extended inferiorly across the right inguinal ligament (not divided) and onto the right inner thigh. The abdominal wall layers were incised carefully down to the peritoneum, and then the dissection was performed laterally between the peritoneum and abdominal wall to develop the retroperitoneal space. The peritoneal sac, containing the intraabdominal organs, was retracted superiorly and to the left, which exposed the distal aorta and right-sided pelvic vasculature.
In all subjects an arterial line (20-gauge angiocatheter) was placed in the infrarenal aorta, 4-5 cm above the aortic trifurcation (refer to Fig. S1), through a site controlled with a 6-0 polypropylene U-stitch. This line was used for arterial blood pressure monitoring and angiography. A baseline aortogram with runoff was obtained with injection of 10 mL of Visipaque™ 320 (Iodixanol; GE Healthcare) and C-arm fluoroscopy (GE OEC 9900 Elite).

Arterial Interruption. Once baseline angiography was obtained, the region of the aortic trifurcation was dissected (Fig S1). The right external iliac artery (REIA) then was dissected down to the bifurcation of the right superficial femoral artery (RSFA) and profunda femoris artery (RPFA). The right lateral circumflex iliac and lateral circumflex femoral artery (RLCIA and RLCFA, respectively) were suture ligated with 3-0 Vicryl. Distal dissection exposed the bifurcation of the SFA into the right popliteal and saphena arteries (RPA and RSA, respectively), and continued onto the popliteal and saphena arteries for an additional 4 cm (Fig. Fig. 2,3). Small unnamed proximal branches off the right popliteal artery were suture ligated. A continuous segment of the REIA-RSFA-RPA then was excised (Fig. 2). This constituted version 1 arterial interruption procedure. Version 2 (Fig. 3, 4) consisted of version 1 plus ligation of the right internal iliac artery (RIIA). To summarize (Fig. 1):

Version 1 Interrruption: ligation/excision of the REIA, RSFA, RPFA, RLCIA, RLCFA, RPA, RSA.

Version 2 Interruption: version 1 + RIIA ligation

A completion angiogram was performed after each arterial interruption procedure. The aortic puncture site utilized for angiographic access then was closed by tying the previously-placed U-stitch. The abdominal incision was closed anatomically in layers, with 2-0 polyglactin 910 in the peritoneum and posterior layers, 0-polydioxanone in the anterior aponeurosis, 3-0 polyglactin 910 in the panniculus carnosus, and 4-0 polyglactin 910 in the skin. Cyanoacrylate glue was applied over the skin incision; no other incisional dressing was applied. The animal's recovery from anesthesia was monitored until the subject was awake and mobile.

Terminal procedure: Subjects underwent their terminal procedure 4 weeks after arterial interruption. After induction of general endotracheal anesthesia, the right carotid artery was exposed with a cervical cut-down. Hindlimb perfusion measurements (muscle oximetry and arterial indices) were performed as described above. A catheter was then placed into the right common carotid artery and advanced into the aorta using the Seldinger technique. An abdominal aortogram with distal runoff was performed. Bilateral hindlimb dissection was performed prior to euthanasia, biopsies of the bilateral gastrocnemius (medial and lateral heads), soleus, and posterior tibialis muscles were performed (Fig. S5), with immediate methacarn fixation

Arterial Diameter Analysis: Each angiogram obtained during the procedure was saved into the Digital Imaging and Communications in Medicine (DICOM) format within the GE OEC 9900 Elite C-arm. Images subsequently were accessed using the RadiANT DICOM Viewer (Version 4.0.2). The cross-sectional diameter of the distal aorta was defined as 1. Relative diameter of each distal artery was expressed as a fraction of distal aortic diameter.

Statistical analysis: Data were analyzed using ANOVA within Microsoft Excel; the level of significance was defined as $p < 0.05$.

RESULTS

Perioperative Events. The swine mean weight was 31.9 ± sd 3.8 kg (range = 25.2 – 33.6 kg). Two of our domestic swine expired prematurely. One had to be sacrificed four days post-procedure due to the finding of a rectal prolapse with concerns of viability. A discussion was had with the veterinarian regarding the prolapse and it was stated that a prolapse can occur spontaneously and unrelated to the procedure. However, due to the requirement of prolonged post-operative observation and the difficulty of keeping the rectal prolapse reduced, the decision was made to sacrifice the animal. The second animal expired three days post-operatively due to the development of bowel ischemia. Autopsy demonstrated profuse ischemia of the bowels and the finding of an enlarged stomach; it was hypothesized that the condition of the swine developed from being overfed. These animals were replaced with subsequent animals. Two animals in the study developed ischemia of the right hoof. Their mobility was affected significantly, but each animal survived until necropsy.

Collateral Formation. ABI was measured post-ligation and at necropsy for each version. There were a total of 11 subjects but one was omitted due to missing data. The ABI was calculated pre-ligation (ABI), post-ligation (ABI_0) and at pre-necropsy (ABI_f) and the means are shown in Table 1. A paired t-test was performed for each version was performed which showed a significant change in ABI from post-ligation to pre-necropsy ($p<0.05$). An ANOVA was performed between each version and there was no significant difference between ABI with either version.

Angiogram of version 1 ligation at baseline (Fig 3a,b) demonstrated no flow through the REIA or any of the other distal vessels of the right limb. However, at necropsy (Fig 3c), it was found from

the version 1 ligation that there was development of a dominant collateral pathway of the RIIA to the RPFA and RSFA/popliteal artery of the ischemic limb. In addition, there was reconstitution of the ligated right profunda femoral artery and the superficial femoral artery as a result of the collateral vessels that derived their main inflow from the right internal iliac artery. There were two co-dominant collateral pathways appreciated in version 2 (Fig 5). The first pathway connected the common internal iliac trunk and left internal iliac artery to the reconstituted RIIA which then supplied the RPFA and RSFA/popliteal arteries. The second pathway connected the left profunda artery to the reconstituted RPFA. Table 2 lists the Moxy readings for subjects in each version expressed as mean ± standard deviation. Figure 9 the data shows the contrast between Version 1 and Version 2. Overall there was no significant difference between each version of arterial ligation.

Histology. The slides that were prepared are seen in Fig 8 and were compared to slides of human PAD subjects. The control slides show round muscle fiber bundles with minimal fibrosis. In subjects with PAD or some sort of arterial occlusion the muscle bundles become more vacuolated and significant fibrosis can be seen. The slides between the swine subjects and human subjects are comparable in showing the histological changes of PAD.

Arteriogenesis: Quantification of the arterial size changes were made (Table 3). The relative arterial diameter changes of the common internal iliac trunk, right internal iliac, right profunda, right popliteal, left external iliac, left internal iliac, and left profunda arteries were measured. For version 1, the mean luminal diameter of the RIIA at termination increased by 39.4± 5.5% ($P<0.01$) compared to baseline. The two co-dominant collateral pathways in version 2 were quantified by the increase of mean diameters of the common internal iliac trunk and left profunda artery increased at termination by 23.7± 7.6% and 24.8± 7.4%, respectively ($p < 0.05$). A paired t-test was used to calculate p-values.

DISCUSSION

The importance of developing an animal model to imitate human PAD/CLI is essential to the discovery and innovation of disease-specific treatments. Various models, typically mice, have been used in the study of vascular compensation in PAD (29). Characterization of the murine model has included variations in the level of arterial occlusion to the utilization of unique procedural methods to achieve arterial occlusion in the evaluation of hindlimb ischemia (30-32). Despite the existence of well characterized murine models of ischemia, they are hampered by issues of small size and relative ability to mimic human biology. A porcine model of hindlimb ischemia, on the other hand, should prove to be better model in terms of size and ability to model human physiology (22-26). The creation of a large animal model has great potential for the advancement and translation of angiogenic therapies for humans. The advent of stem cell therapies has opened a new frontier in therapeutic medicine; however, much more of this treatment modality needs to be studied, understood, and optimized. The animal model can help in elucidating questions such as the optimal dosing and delivery method for stem cell therapy, the cell type that provides superior therapeutic results, and the ability to quantify the salutary effects cellular therapy can have in the treatment of limb ischemia (33-37).

The premise of this investigation is to characterize a porcine hindlimb ischemia model and study the arterial network and the collaterization that develops. Previous work has been performed to understand the porcine ischemia model using an endovascular approach (38). Endovascular occlusion has the benefit of minimizing tissue damage along with the potential of creating a modular ischemia model to assist in the creation of discrete levels of ischemic severity. The difficulties of this approach is the material cost associated with the use of stent-grafts, the limitation to the extent of the occlusion achievable, and the prohibition of placement of a graft in the region of the inguinal ligaments or other areas of flexion.

Certain investigative groups have employed a variation to the open approach with the use of

ameroid constrictors to achieve their occlusion. The benefits of utilizing ameroid constrictors include a smaller incision and the ability to create a more gradual ischemia (18). However, the disadvantages of this approach include variation in the severity of the ischemia and the issues of quality-control of the ameroids themselves, as there can be discrepancies in the depth and shape of the internal slots (31).

Our traditional open model confers some unique advantages. The first benefit of this model is the adaptability of the procedure. The extent of ligations utilized in this model can be customized towards the degree of ischemia desired. Future investigators can tailor the degree of ischemia required for their study by changing the amount of ligation as well as choosing the vessel being ligated (i.e. including or excluding the internal iliac artery). The second benefit of the open model is the relative cost saving that is achieved and the scalability of this model without exponential increase in resource utilization, hence helping each respective investigator to remain within the confines of reasonable financial parameters. The third advantage of the open model is reproducibility of the model. The open model confers the ability to produce a high-fidelity model by giving investigators the capability to achieve occlusion at precise anatomical landmarks and obviates the limitation/errors inherently built-in imaging and device deployment.

Another aspect that is unique to this project is the careful characterization of arteriogenesis and collateral formation. This was observed not only in the ischemic limb but also in the control limb. We were able to demonstrate the specific locations within the porcine vascular network that experienced the greatest growth after induction of ischemia. The quantification of the specific arterial growth was used to support the subjective finding of the reproducible collateral formation we observed amongst the different versions of hindlimb ischemia. It is believed that arteriogenesis is a reactive process to the blood flow redistribution that occurs in occlusive disease (39-42). The changes in fluid dynamics of the downstream vessels causes an increase in shear forces that leads to upregulation of the cell adhesion molecules with subsequent rise in

cytokines and growth factors, and ultimately to the endothelial and smooth cell proliferation that results when the arterial lumen increases. Given the current understanding of the pathophysiology of the vessel remodeling that occurs in PAD, we can use the swine ischemia model to test angiogenic therapies in a quantitative fashion, as well as investigating/improving delivery method, dosing targets, and the effectiveness of various therapies.

The open model does harbor some limitations. The first limitation of this model is the acute nature in which limb ischemia is created. PAD results from progression of atherosclerotic disease which is a gradual process. Furthermore patients with PAD often have significant comorbidities such as hyperlipidemia, diabetes, and coronary artery disease. Our swine population did not have any of these comorbidities and it could impact the veracity of this model. The last limitation of this model is the requirement of large incisions needed for the procedure. The degree of tissue manipulation and pain associated with this procedure could potentially present as a cofounding factor when studying potential aspects such as functional status and gait of our swine model. Only 11 subjects were used to calculate final values because there were issues with other subjects, either because of subject issues or data collection issues. Perhaps if all subjects were viable until necropsy there could be more significance seen in ABI or Moxy readings.

CONCLUSION

Our open swine hindlimb ischemia model holds great promise in the translational research of PAD. The deliberate characterization of the collateral network formation, and the objective measurement of the arteriogenesis that occurs are unique and pioneering. With a quantitated understanding of the vascular remodeling that develops with our ischemia model, we can utilize it to empirically test future therapies in our goal of optimizing existing treatment regimens or innovating novel therapies in the study of PAD.

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DISCLOSURES

The authors declare no competing interests.

Table 1. Right (ischemic) hindlimb ABI, Version 1 vs. 2.

	t = 0			
Version (N)	ABI, pre	ABI, post	ABI, 30 d	ANOVA
1 (5)	0.998 ± 0.225	0	0.624 ± 0.25	0.00013
2 (5)	1.236 ± 0.303	0	0.616 ± 0.35	0.00038
T-test	0.196	1.000	0.968	

ABI expressed as mean ± standard deviation. Terms “pre” and “post” refer to before and after arterial ligation. The 30 d ABI was determined immediately prior to necropsy.

Table 2. Percent change of relative arterial diameters, Version 1 vs. 2.

Artery	Version	RD, day 0	RD, d 30	p**
CIIT	1	6.70 ± 0.34	7.87 ± 0.44	0.004
LEIA	1	5.51 ± 0.21	5.84 ± 0.32	0.32
RIIA*	1	3.75 ± 0.31	5.19 ± 0.21	<0.001
LIIA	1	3.65 ± 0.19	3.85 ± 0.24	0.14
RFPA*	1	2.58 ± 0.29	3.02 ± 0.25	0.018
LPFA	1	3.25 ± 0.25	3.25 ± 0.25	0.99
RPop	1	3.24 ± 0.37	3.00 ± 0.32	0.26
CIIT	2	6.68 ± 0.26	8.24 ± 0.85	0.004
LEIA	2	5.84 ± 0.32	6.92 ± 0.75	0.019
RIIA*	2	3.13 ± 0.24	3.44 ± 0.74	0.4
LIIA	2	3.90 ± 0.33	4.72 ± 0.33	0.004
RFPA*	2	3.35 ± 0.39	3.67 ± 0.18	0.13
LPFA	2	3.50 ± 0.35	4.42 ± 1.02	0.09
RPop	2	3.37 ± 0.39	2.65 ± 0.40	0.02

Relative diameter (RD) of each artery was expressed as a fraction of distal aortic diameter (which was defined as 1). CIIT = common internal iliac trunk; LEIA = left external iliac artery; RIIA = right internal iliac artery; LIIA = left internal iliac artery; RPFA = right profunda femoral artery; LPFA = left profunda femoral artery; RPop = right popliteal artery. *Reconstituted vessel. **Paired t-test.

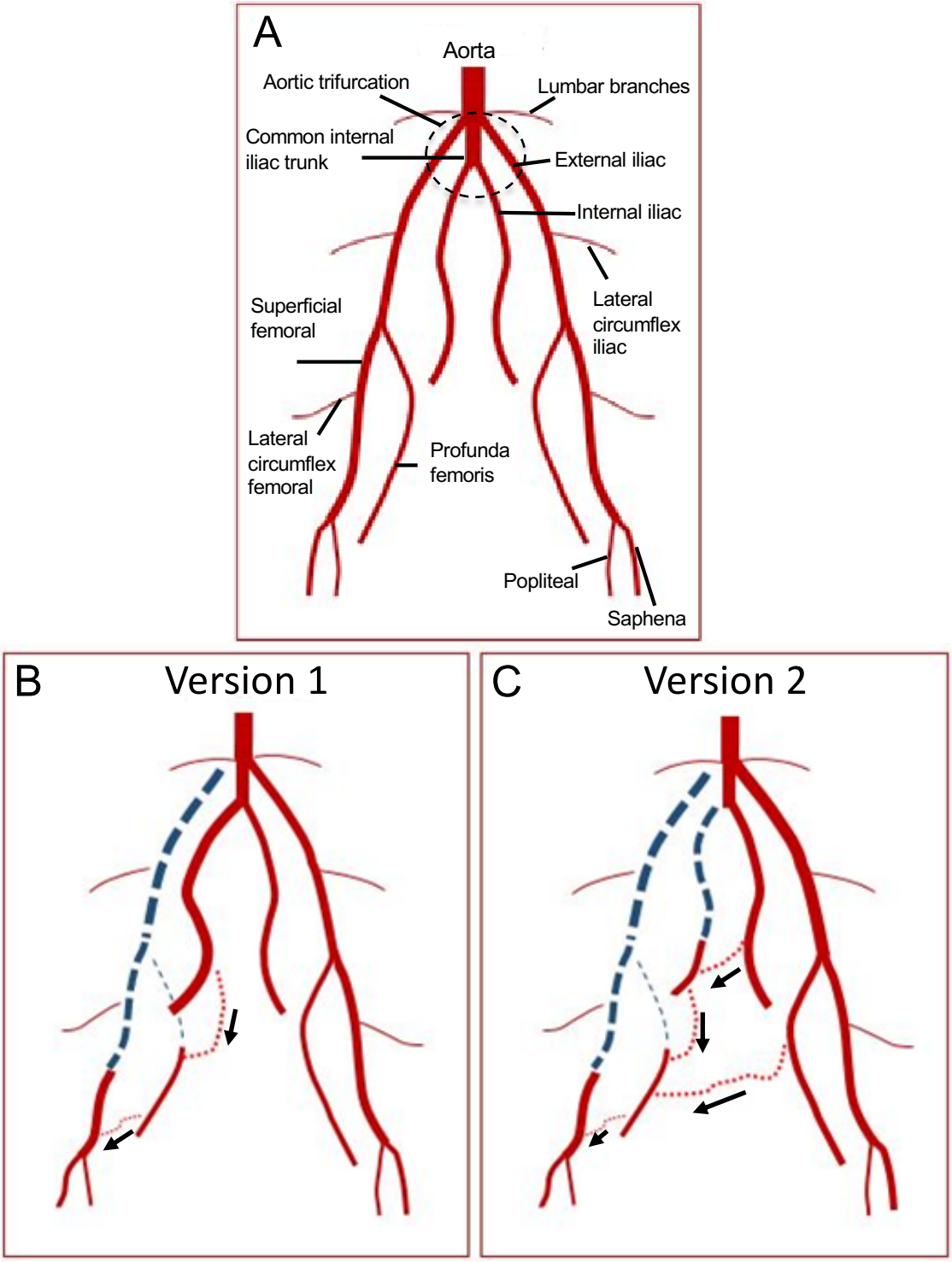


Fig. 1: Schematic of interruption of the arterial supply to the porcine hindlimbs. A: Schematic of uninterrupted blood supply to the hindlimb. B. Version 1: Ligations of the iliofemoral axis comprised of right external iliac artery, right lateral circumflex iliac & femoral, right profunda femoris, along with right popliteal and saphenous arteries. C. Version 2: ligation of version 1 along with right internal iliac artery. Dashed blue lines: Excised vessels. Dotted red lines: Collateral vessels. Arrows indicate direction of collateral flow.

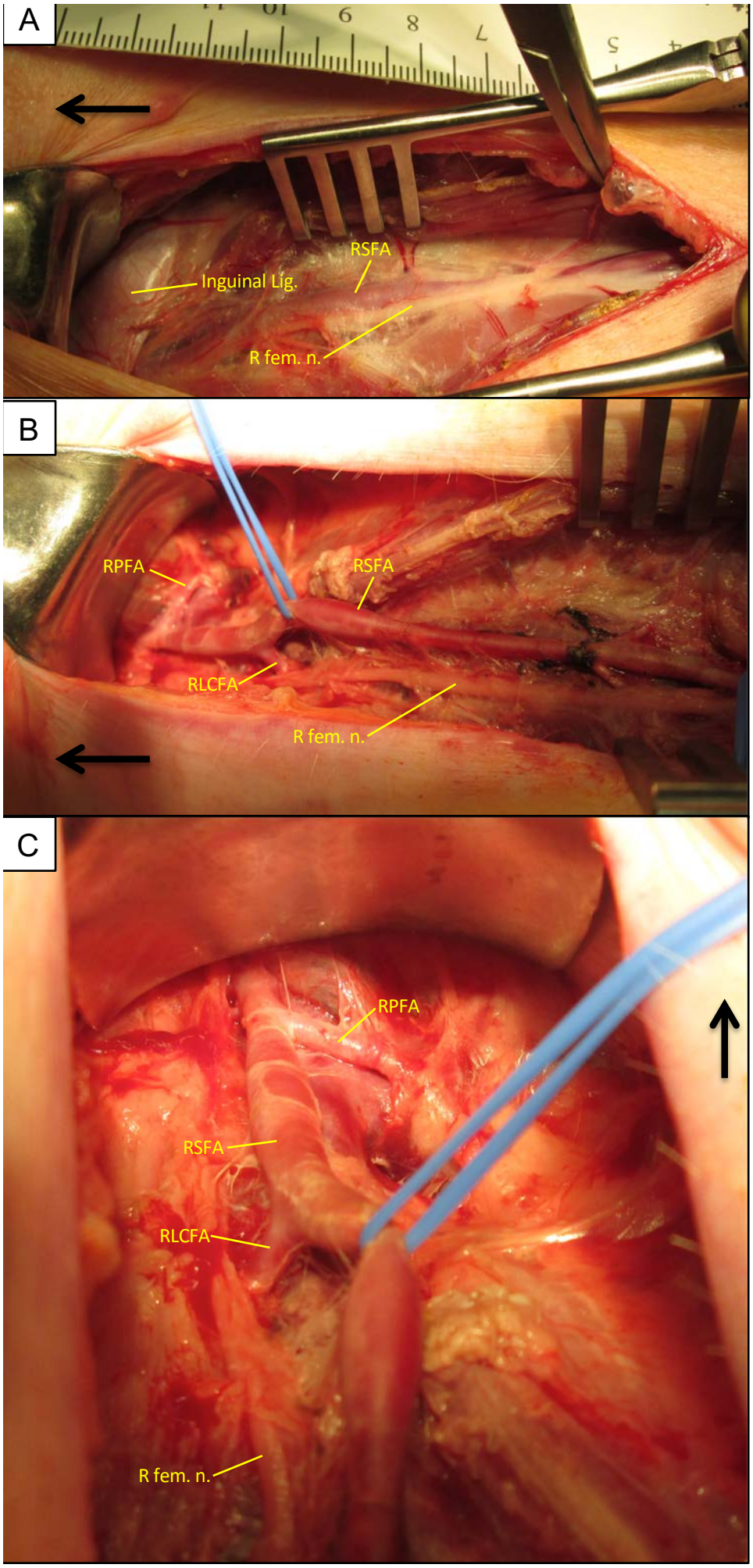


Fig. 2. Swine 383. No angios done 2˚ C-arm malfunction.

Typed Op Note:
"We dissected and controlled the right PFA, by lifting up the inguinal ligament and reaching into the right retroperitoneal space. We then dissected the right SFA all the way to its bifurcation to right popliteal and right saphenous arteries. We attempted to do an angiogram by accessing the right SFA in the mid-thigh (see hand drawn figure) but angiogram could not be completed because the fluoro machine broke. Angiogram was then abandoned and we proceeded with the arterial ligations. We ligated and divided the right PFA, in the retro-peritoneum. We left the femoral artery intact with axial patency into the right lateral circumference femoral [RLCFA] artery (a large collateral thigh branch). We ligated and divided the right SFA, distal to the right lateral circumference femoral artery. We than ligated and divided the proximal saphenous [RSaphA] and the popliteal [RPopA] arteries just distal to the right SFA bifurcation. At the end of the procedure the right foot was pale and cool."

A. Initial dissection of R groin vessels. Black arrow = cephalad.
B. Further dissection of R groin vessels, showing R Lat Circumflex Fem A, which would be preserved.
C. Rotated view of panel B.

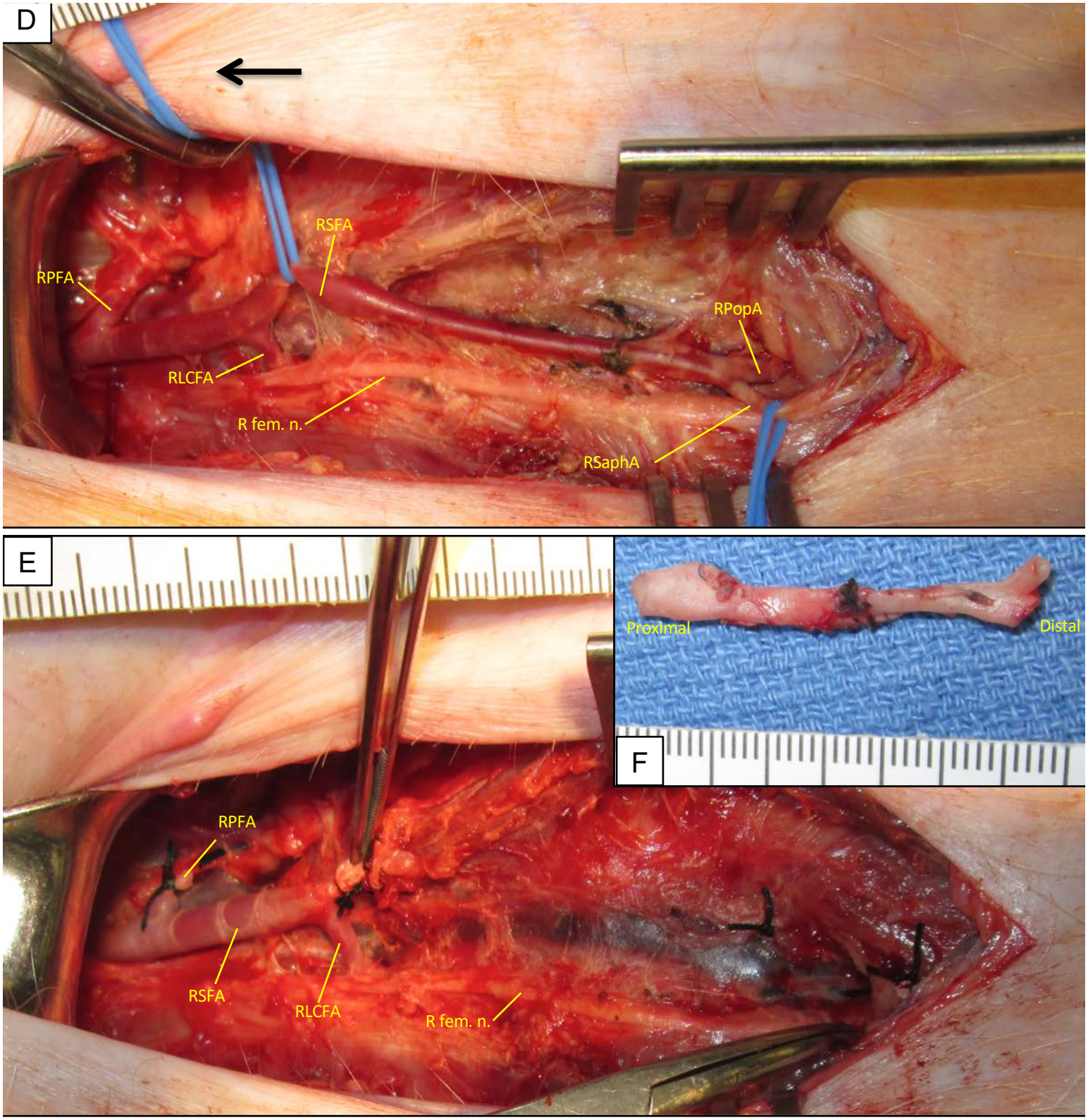


Fig. 2. Swine 383. Initial ligations performed on 09/26/2016. No angios done 2˚ C-arm malfunction.

D. Dissection of R groin vessels, showing distal bifurcation of RSFA into R saphena and R popliteal arteries. Black arrow = cephalad.
E. Planned ligations with RSFA excision have been performed. Note preservation of RLCFA.
F. Excised segment of RSFA.

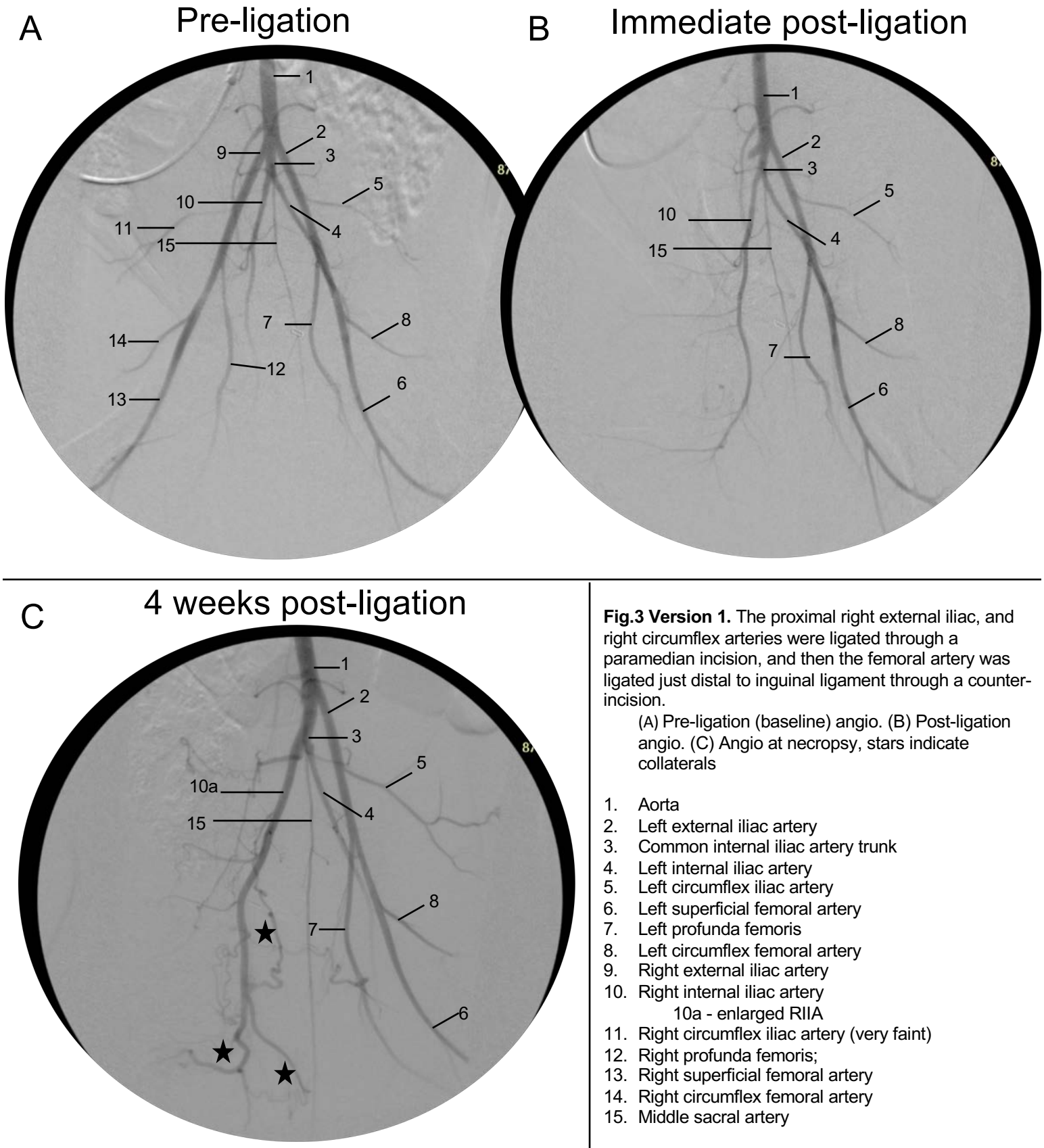


Fig.3 Version 1. The proximal right external iliac, and right circumflex arteries were ligated through a paramedian incision, and then the femoral artery was ligated just distal to inguinal ligament through a counter-incision.

(A) Pre-ligation (baseline) angio. (B) Post-ligation angio. (C) Angio at necropsy, stars indicate collaterals

1. Aorta
2. Left external iliac artery
3. Common internal iliac artery trunk
4. Left internal iliac artery
5. Left circumflex iliac artery
6. Left superficial femoral artery
7. Left profunda femoris
8. Left circumflex femoral artery
9. Right external iliac artery
10. Right internal iliac artery
    10a - enlarged RIIA
11. Right circumflex iliac artery (very faint)
12. Right profunda femoris;
13. Right superficial femoral artery
14. Right circumflex femoral artery
15. Middle sacral artery

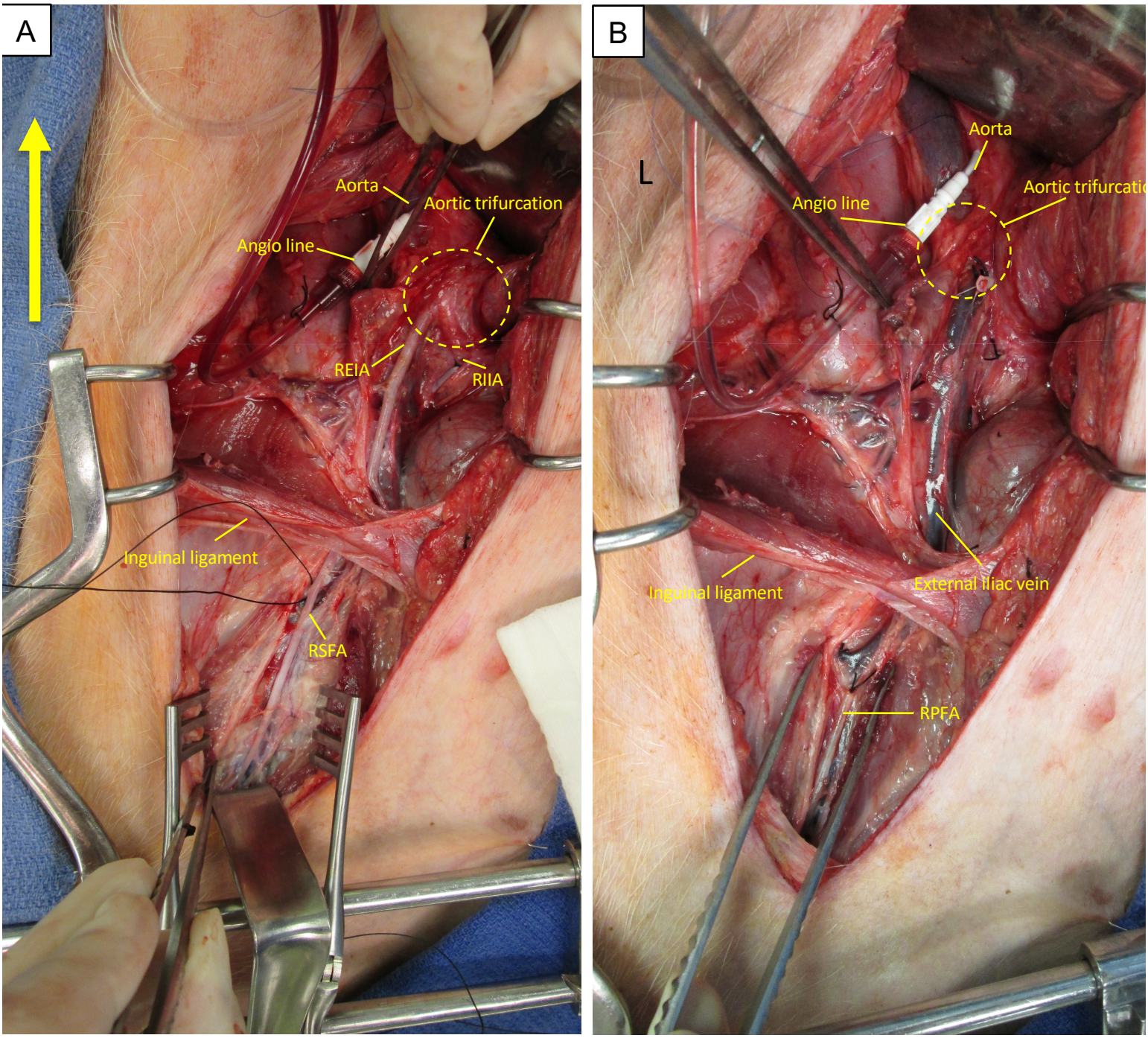


Fig 4. Version 2 dissection
A. Pre-ligation where RSFA and RIIA are exposed.
B. Post-ligation image that shows removal of REIA extending down to RSFA .
C. Resected segment of artery from B. The yellow arrow indicates cephalad direction.RIIA: right internal iliac artery; RSFA: Right superficial femoral artery; RPFA: Right profunda femoris artery

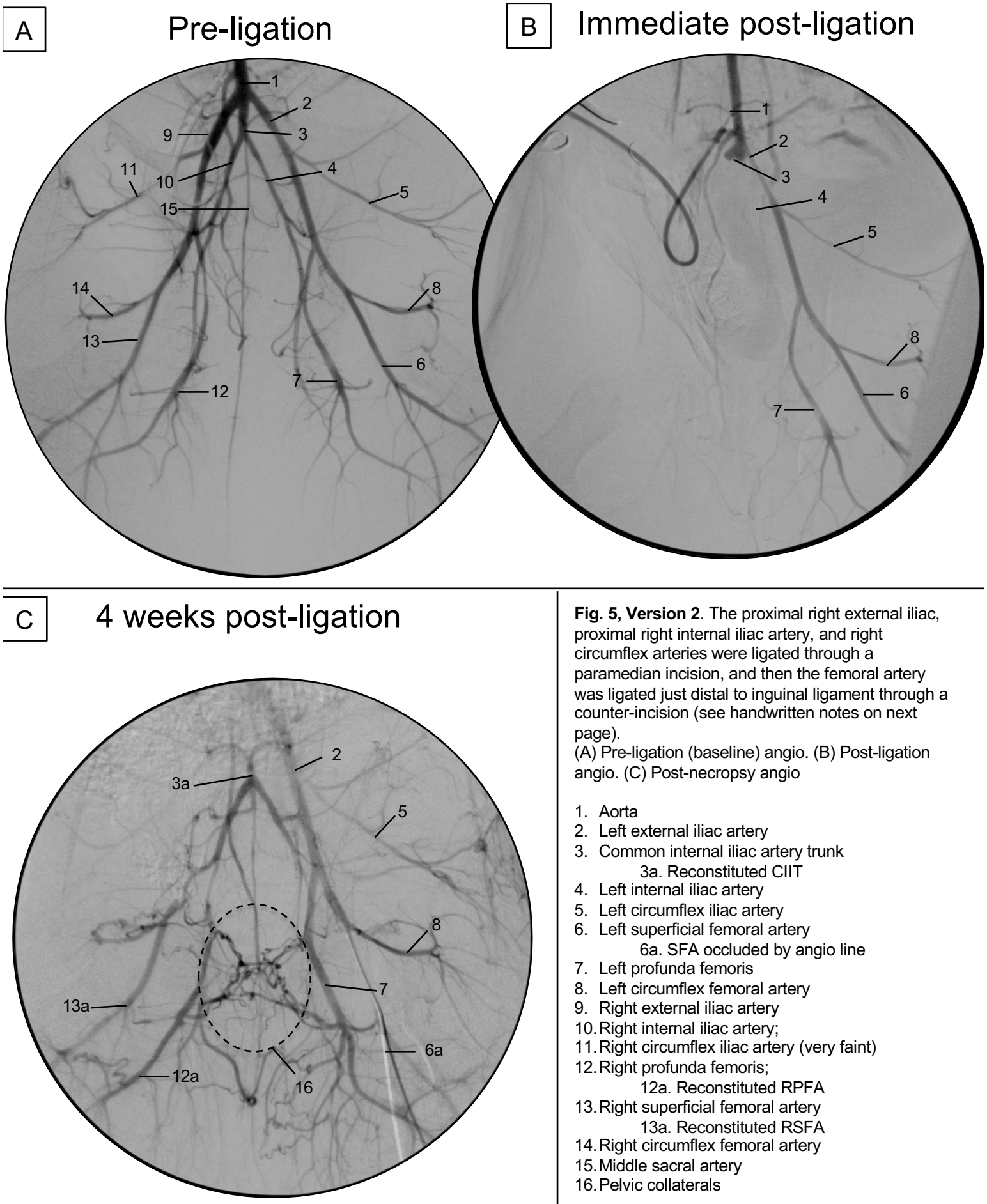
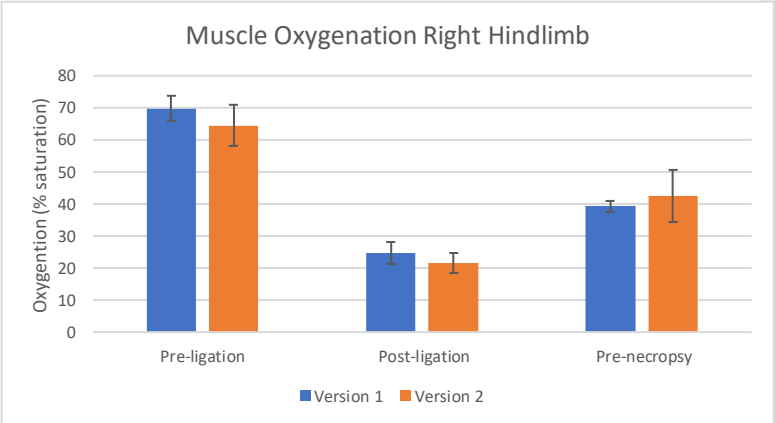
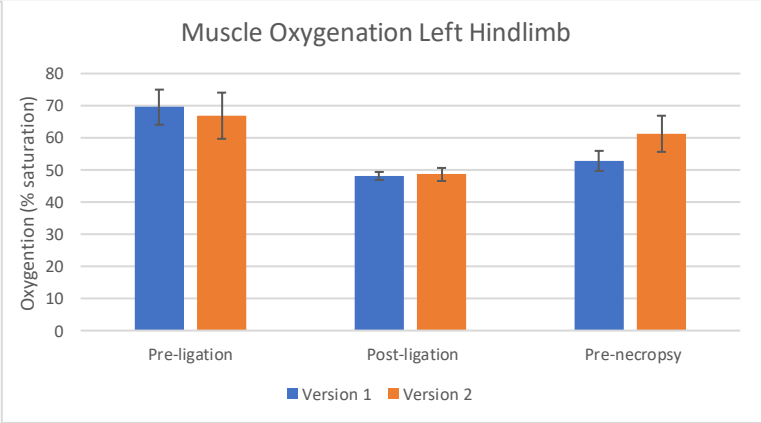
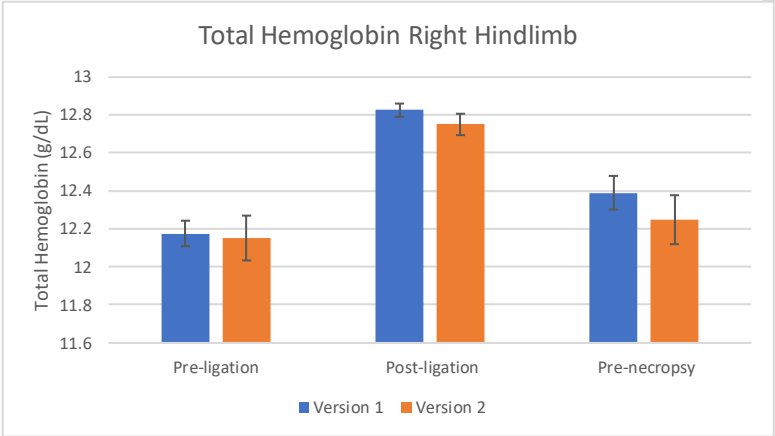


Fig. 5, Version 2. The proximal right external iliac, proximal right internal iliac artery, and right circumflex arteries were ligated through a paramedian incision, and then the femoral artery was ligated just distal to inguinal ligament through a counter-incision (see handwritten notes on next page).
(A) Pre-ligation (baseline) angio. (B) Post-ligation angio. (C) Post-necropsy angio

1. Aorta
2. Left external iliac artery
3. Common internal iliac artery trunk
   3a. Reconstituted CIIT
4. Left internal iliac artery
5. Left circumflex iliac artery
6. Left superficial femoral artery
   6a. SFA occluded by angio line
7. Left profunda femoris
8. Left circumflex femoral artery
9. Right external iliac artery
10. Right internal iliac artery;
11. Right circumflex iliac artery (very faint)
12. Right profunda femoris;
    12a. Reconstituted RPFA
13. Right superficial femoral artery
    13a. Reconstituted RSFA
14. Right circumflex femoral artery
15. Middle sacral artery
16. Pelvic collaterals







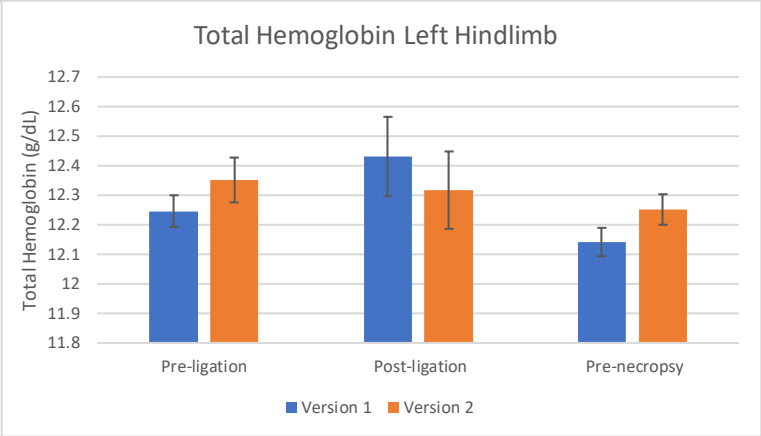


Fig 6. Comparison of Moxy data between Version 1 and Version 2. Overall these graphs show that the method of arterial ligation does not affect the amount of distal muscle oxygenation of hemoglobin carrying capacity. Either ligation version could be valid in producing a limb ischemia model.

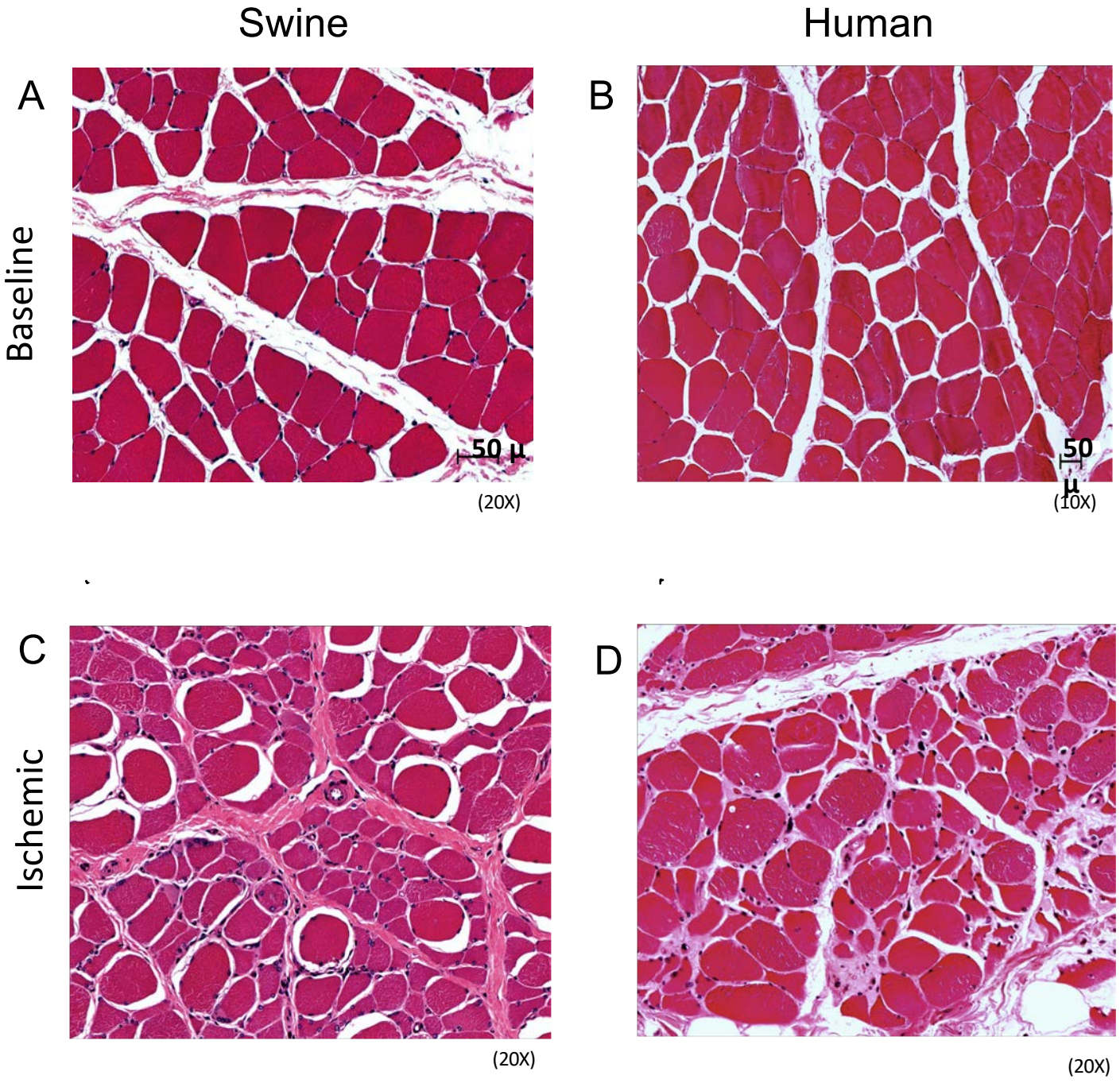


Fig 7. Histological slides demonstrating myopathy in PAD. Panels A and C are biopsies taken from swine gastrocnemius muscle. Panels B and D are biopsies taken from human gastrocnemius muscle. Panels A and B are biopsies from nonischemic muscle. Panels C and D show the histopathologic changes in subjects with chronic ischemia. .