AWARD NUMBER: W81XWH-19-1-0440

TITLE: Supercooled Ex-Vivo Porcine VCA Preservation to Extend the Timeline Between Procurement and Transplantation and Enable Tolerance Induction to Eliminate Immunotherapy Needs and Risks

PRINCIPAL INVESTIGATOR: Korkut Uygun PhD

CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA

REPORT DATE: November 2023

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2023	Final	01Aug2019-31Jul2023

4. TITLE AND SUBTITLE

Supercooled Ex-Vivo Porcine VCA Preservation to Extend the Timeline Between Procurement and Transplantation and Enable Tolerance Induction to Eliminate Immunotherapy Needs and Risks

5a. CONTRACT NUMBER
W81XWH-19-1-0440

5b. GRANT NUMBER
RT180063P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Korkut Uygun, PhD

E-Mail: KUYGUN@mgh.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
51 Blossom St
Boston, MA 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9 . SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project aims to develop a novel technology to preserve vascular composite allografts for extended periods. This project uses a porcine model.
Through this project, we developed a novel protocol to perfuse porcine limbs for 24h, allowing successful transplantation. Next, we aimed to extend the preservation period at 48h to match the time needed for immune tolerance protocols using mixed chimerism. We tested sub-zero non-freezing (supercooling)of porcine hindlimbs and compared it with controls. We demonstrated successful supercooling at -5°C for 48h, and assessed the recovered limbs with normothermic machine perfusion. The perfusion parameters were improved by supercooling but more optimization of the metabolic outcomes are needed before in vivo applications.

15. SUBJECT TERMS:
Organ Preservation, VCA transplantation, limb transplantation, supercooled storage

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	29	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Enabling prolonged preservation of vascularized composite allografts (VCA) is critical to enable their clinical use in a practical manner. Machine perfusion technologies have enabled dynamic organ storage for many organs, in stark contrast to the current gold standard of static cold storage. Supercooling technology, which builds on machine perfusion, has been shown to further extend preservation, allowing the increase of viable preservation time to 27 hours for human livers, 3 times the clinical average. This project aims to translate these promising results in livers to VCA, also leveraging prior studies in rats.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

VCA, preservation, supercooling, cryopreservation, transplantation, machine perfusion, Ischemia Reperfusion Injury

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. *Milestone # 1 ACURO approval obtained*. **100% complete** (February 2020)
2. *Milestone #2 Complete evaluation of Machine perfusion on VCA viability.* **100% Complete** (March 2022)
3. *Milestone #3 Develop a method to extend preservation duration for porcine limbs.* **80% Complete** (July 2023)
4. *Milestone #4 Develop a method to enable using mixed chimerism for VCA transplantation.* **10% Complete**

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Ex vivo subnormothermic machine perfusion (SNMP): Development of an Optimized protocol for 24-hr preservation of swine hindlimbs

We started the project by scaling up from our prior experience in rats to a large animal limb *ex vivo* perfusion system, and optimizing 24h acellular subnormothermic machine perfusion (SNMP).

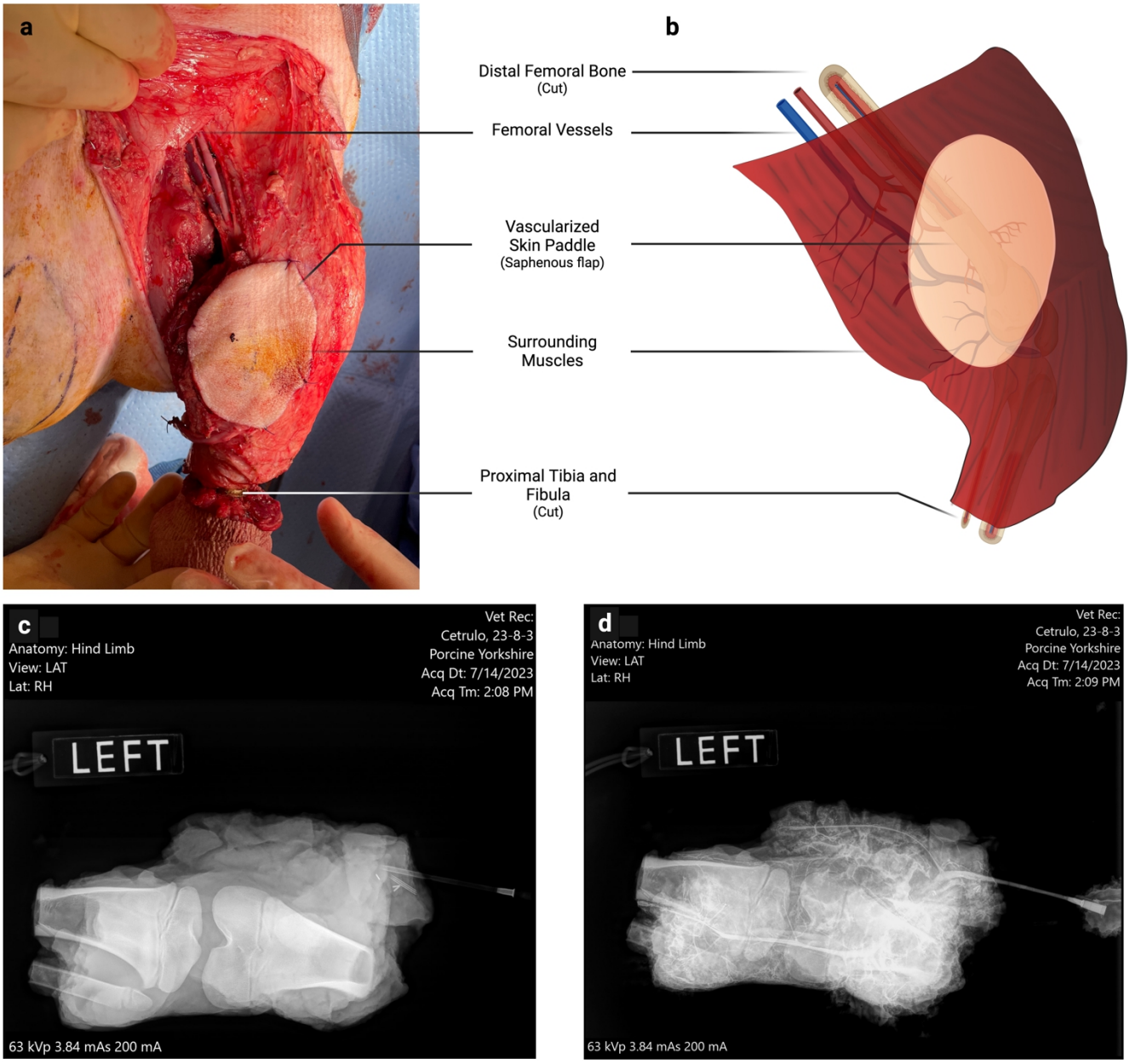

Figure 1. Surgical model of porcine VCA

a/b: intraoperative view and diagram of the partial hindlimb with dissected femoral vessels, skin apdle (saphenous flap), and muscles surrounding the femoral, tibial and fibular bones. **c**: X-Ray showing the osseous and cartilaginous content of the graft. **d:** angiography of the VCA showing adequate vascularization of all the surrounding muscles.

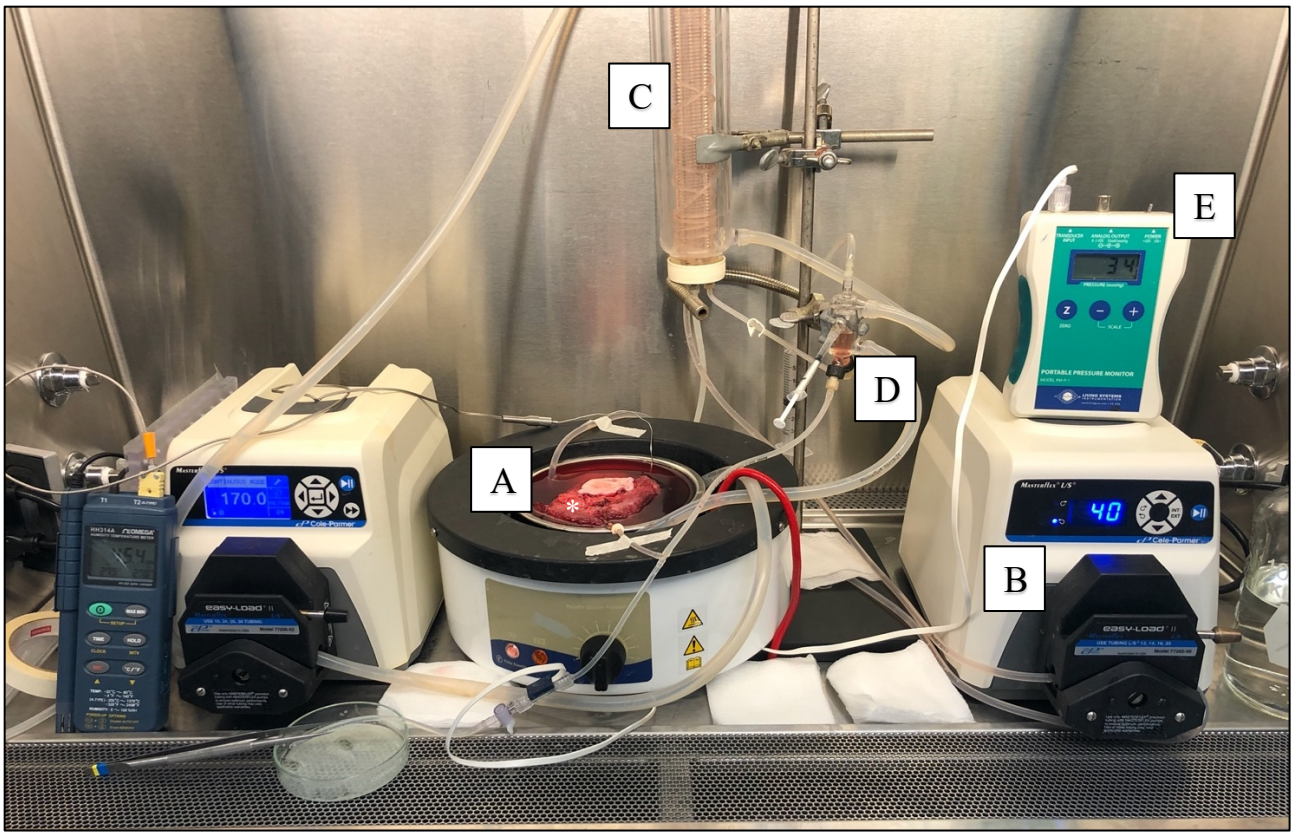

Figure 2. Continuous flow SNMP system. The perfusion system used for this experiment includes a basin (A), perfusion pump (B), an oxygenator (C), a bubble trap (D) and a pressure sensor (E). Temperature was monitored to remain at 21°C in the basin. The limb is attached to the inlet tubing through a 14G angiocatheter in the femoral artery (white *). The venous outflow drains in the basin and the perfusate recirculates in the closed circuit.

Porcine partial limbs (**Figure 1**) were initially perfused with a system of our design, including a glass oxygenator (Radnoti LLC, Covina, CA), similarly used for the perfusion of rat limbs. The initial recirculating volume was set at 500ml of Steen+ per limb (mean weight of 476g) (**Figure 2**). The massive release of potassium and lactate required us to perform two media exchanges during the 24h perfusion, each with 500ml of perfusate after 2.5 and 6 hrs of perfusion. Excessive potassium and lactate concentrations of >9mmol/L and >20mmol/L respectively after 18 hours (Fig. 1 B-C) ultimately led to discontinuation of the perfusion. For the next iteration, we therefore increased the perfusate volume to 1L per limb. Potassium and lactate concentrations were still high. To prevent further increases, we added 500ml of perfusate at 2.5hr (total circulating volume 1.5L), followed by two exchanges of 1L at 6 and 12 hrs and one of 500ml at 18 hrs. The perfusion was stopped at 21 hours due to non-viable parameters and significant edema.

Since this system did not meet the metabolic needs of a porcine limb at 21°C, we made changes: for the following experiment the addition of an oxygen carrier (HBOC-201, Hemopure®) was tested along with a hollow fiber membrane oxygenator. Simultaneous perfusion of 2 limbs was performed, with the limbs weighed 632 and 629g. Two perfusion media were tested, the first one with Steen+ as before with HBOC-201, the second one with modified concentrations of 12% BSA (vs. 15%) and 2%

PEG (vs. 0.5%). For both limbs, medias exchanges (350ml of perfusate) were performed at 6, 12, and 18hrs. In both perfusions, initial potassium concentrations were very low (Fig. 1 B), but lactate, resistances, and weight gain still indicated a limb in distress. The 12% BSA solution was worse in terms of weight gain.

Subsequentely, we made a further change in the limb perfusion system, replacing the Radnoti oxygenator with a Medtronics® Hollow fiber Membrane Oxygenator (HMO). The perfusions with this system, performed with 2L of Steen+ without need for media exchange, produced very encouraging results, with stable resistances, low potassium and lactate concentrations throughout the experiment. However, edema was still significant at >30%.

To identify the source of edema, we next performed 4 whole limb perfusions to determine whether the edema was related to the surgical design that suppresses distal limb microcirculation. The weight of the entire limbs was much greater and averaged 1200g for the first experiment. Both limbs were procured from the same pig and perfused with our custom-designed Machine Perfusion (MP) system starting with a perfusate volume of 2L. A single 1L exchange was performed after 5-hr SNMP. In this experiment, final weight gain was 18 and 33% with low resistances, but potassium and lactate concentrations were high and well above the acceptable ranges (K+ 7.5mmol/L; Lactate > 20mmol/L). Hence, for the next bilateral limb perfusion we perforemed more perfusate exchanges to counter the toxic metabolites accumulation. Using 2L of perfusate in the circuit, we did a 50% perfusate exchange at 2hrs, and 25% at 6, 12 and 18 hours. Edema was low during the first 12 hours (Figure 1D), but potassium and lactate release were still significant (Figure 1B-C). As a result, we concluded that with this model, the amount of perfusate that would have been required to maintain good parameters was too large for our system and not cost-effective.

We then studied the impact of pulsatily as a source of endothelial injury, and hence swelling, (in the Liver Assist® device) versus continuous flow (in our custom-built MP) on VCA perfusion preservation (**Figure 3**). Two partial hindlimbs procured from 20-30kg Yorkshire pigs from the same donor and placed on the liver assist or the custom device. We used our HMO oxygenated Steen+ perfusion solution. The weight of recovered limbs in each group was comparable. In each group, the limbs were placed on perfusion after approximately 16 minutes of cold ischemia (transportation to the perfusion system). Perfusion parameters are shown in Figure 4.

With this final optimization of our system, protocol and media, we were able to achieve edema limited to about 10% in both systems after 24 hours of perfusion. Edema was not different in either protocol ($p=0.71$). Resistances were also more stable in our custom system. Potassium concentrations were the same in both systems ($p > 0.05$ at each timepoint), lactates slightly higher in the custom-buit MP at 3 hour time point ($p = 0.008$) but remaining comparable at all other measurements ($p > 0.05$).

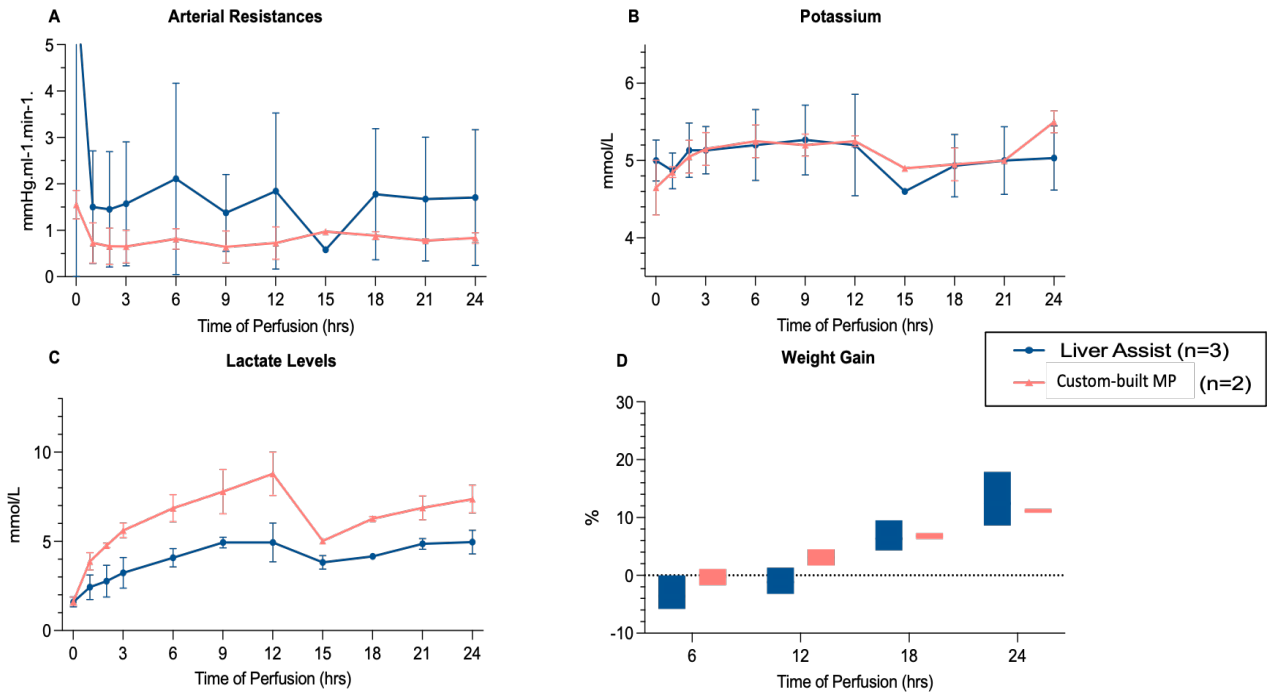

Figure 3. Optimized Continuous (Custom MP) vs. Pulsatile (Liver Assist) perfusion parameters. These graphs represent the last three experiments where the limbs were perfused simultaneously, one on the custom-built MP, and the contralateral on Liver Assist. Only two replicates are reported for the MP, since one perfusion had to be stopped after 12 hours due to a tissue embolism in the system.

With this final optimization of our system, protocol and media, we were able to achieve edema limited to about 10% in both systems after 24 hours of perfusion. Edema was not different in either protocol ($p=0.71$). Resistances were also more stable in our custom system. Potassium concentrations were the same in both systems ($p > 0.05$ at each timepoint), lactates slightly higher in the custom-buit MP at 3 hour time point ($p = 0.008$) but remaining comparable at all other measurements ($p > 0.05$).

➔ All these multiple experiments, using different surgical models, machine perfusion systems, perfusate solutions, and flow types, were compared (**Figure 4**). This important optimization work highlighted one particular setup (green curves) which was perfusing a partial hindlimb with an acellular Steen+ solution with no oxygen carrier, continuous flow, and a Medtronic HMO. Therefore, this setup was chosen to perform the next step, which consisted of transplanting porcine hindlimbs preserved for 24h using SNMP.

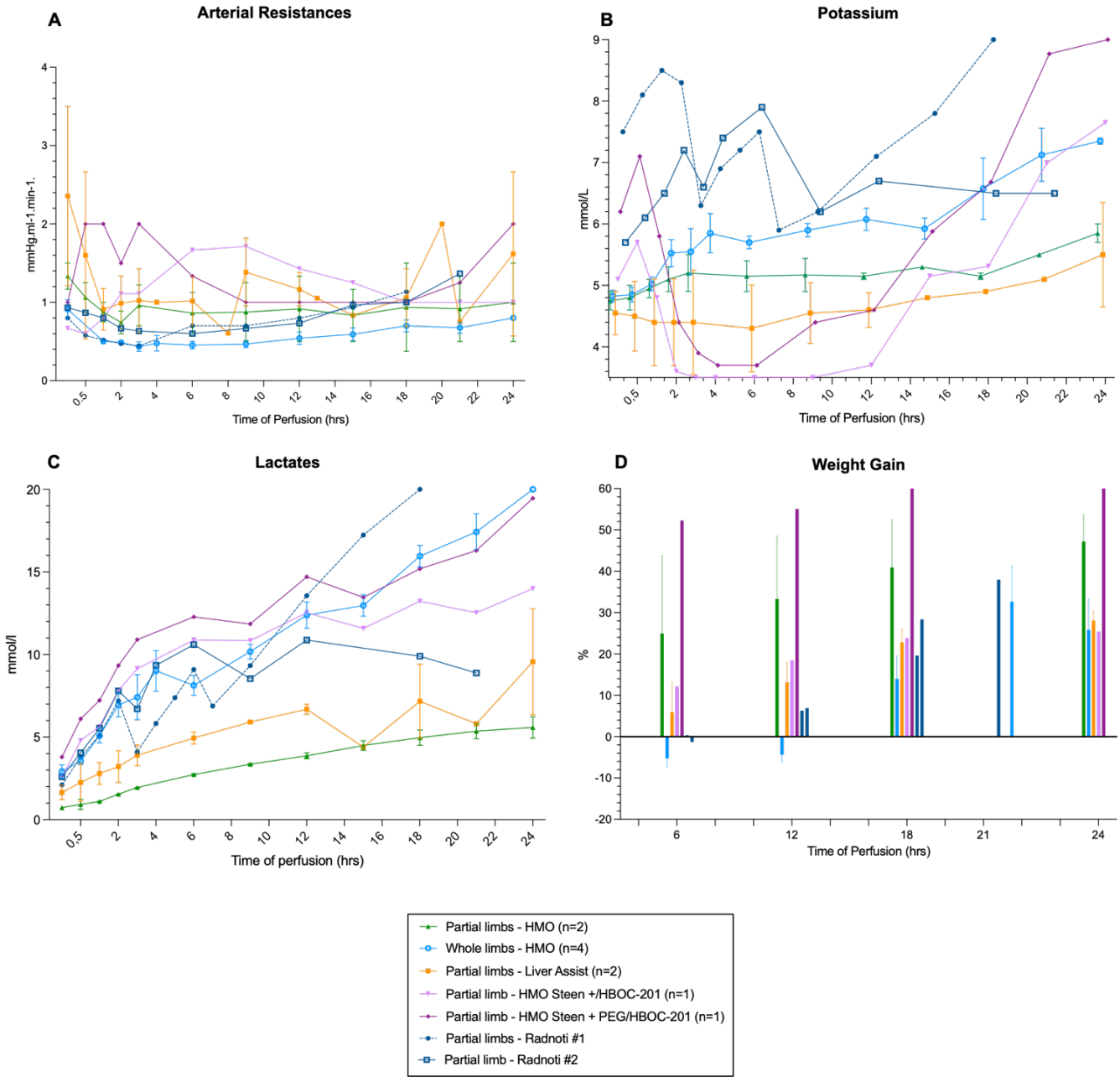

Figure 4. Perfusion parameters observed while optimizing the subnormothermic perfusion protocol to swine hindlimbs. 12 swine hind limbs were procured from Yorkshire pigs during terminal procedures in the Knight surgery operative room, with about 20minutes warm ischemia during recovery. They were then subjected to different perfusion protocols. The optimal parameters were observed with partial hindlimbs perfused with Steen+ using a HMO-based perfusion system (green curve).

2) Transplantation of porcine hindlimbs following 24h preservation with SNMP versus Static cold stored controls.

Following the optimization this perfusion protocol, we aimed to assess the viability of VCAs preserved for 24h using acellular SNMP (**Figure 5**). This part of the study consisted of procuring partial hindlimbs from Yorkshire pigs, preserving it for 24h using SNMP (n=4) or Static Cold Storage (SCS, 4°C, in HTK solution), and transplanting it in non-matched recipient pigs. The transplantation was made heterotopically (abdominal subcutaneous pocket, using the femoral vessels), and the recipient animals received Tacrolimus and Corticosteroïds to prevent rejection. The recipients and the grafts were monitored for 14 days (**Figure 6**) before euthanasia and final assessment, including repetitive blood and histology samples. One animal in the SNMP group showed early complications due to a venous coupler failure. Two animals in the SCS group were euthanized before day 14 due to poor general condition, accordingly with the IACUC guidelines and veterinarian advice.

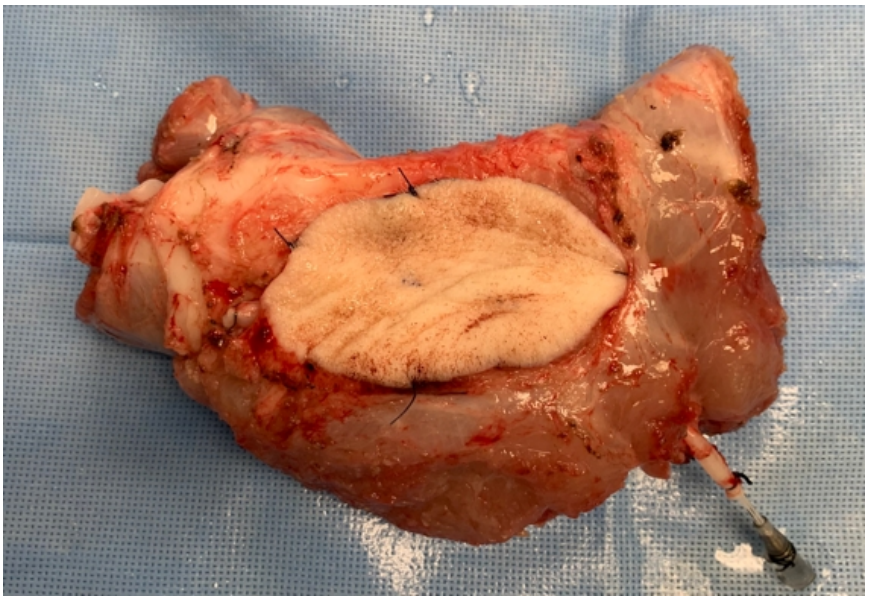

Figure 5. Partial Hindlimb at the end of the 24h SNMP preservation. The cannula is inserted in the femoral artery. The whole graft looks viable and the edema is mild (20-25%).

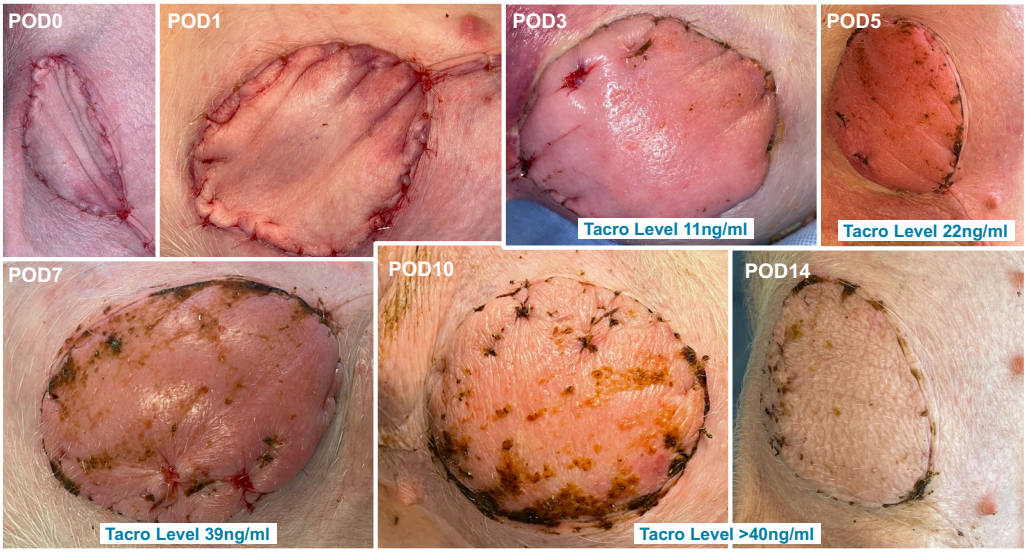

Figure 6. Follow-up of the graft after the transplantation. The skin paddle showed similar aspects in both groups (SNMP and SCS). Biopsies were procured

During follow-up of the animals, the evolution of the blood measurements revealed no differences in White blood cell count between groups. Potassium was overall higher in the SCS group but the early euthanasia of 2 animals didn't allow for reaching statistical significance. Lactate values were also higher in the SCS group.

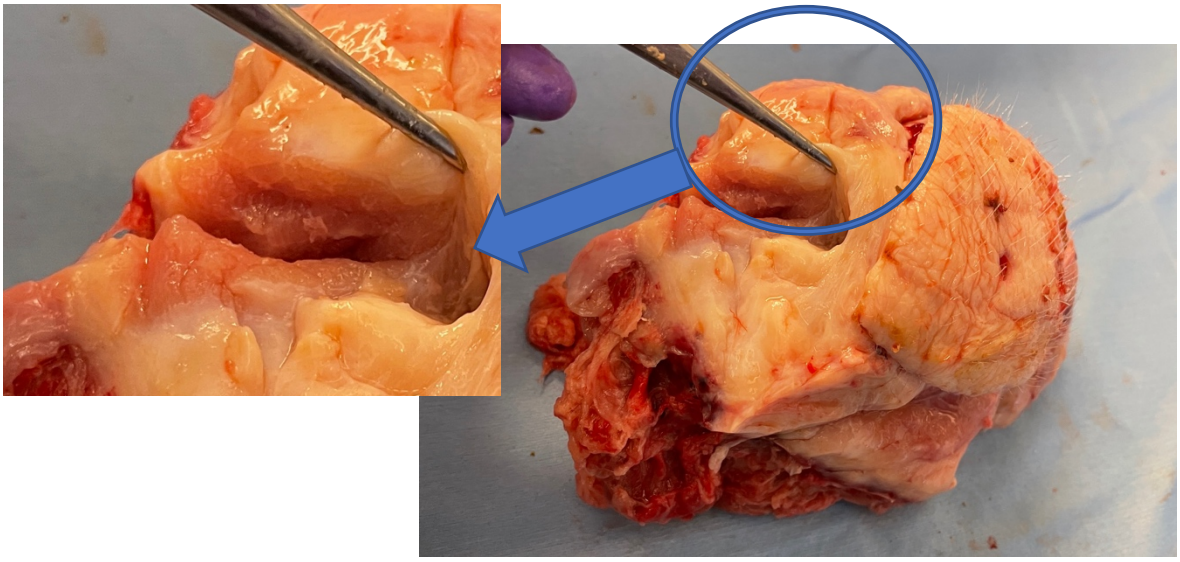

Figure 7A. Subnormothermic Machine Perfusion Group.
Macroscopic aspect of the graft at POD14 after transplantation following 24h SNMP.
The blue arrow focuses on viable muscle, which was uniform in the graft.

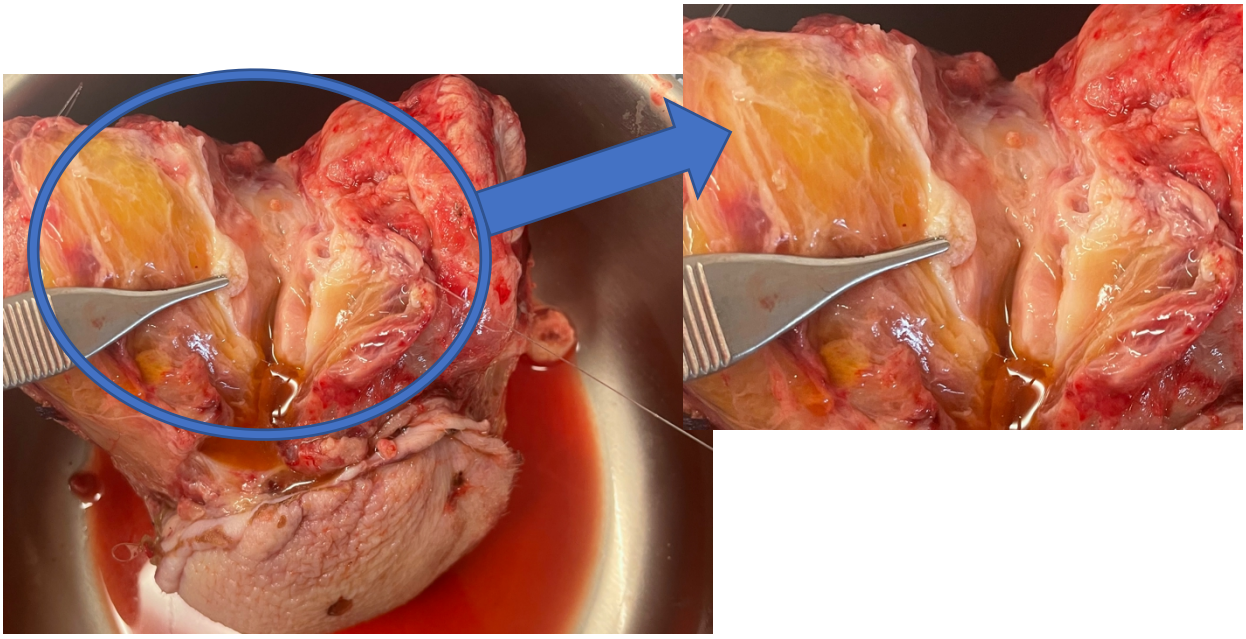

Figure 7B. Static Cold Storage Group.
Macroscopic aspect of the graft at the End of the study after transplantation following 24h SCS. The blue arrow focuses on the muscle, which was massively injured, showing a liquid degeneration for an important part of the graft.

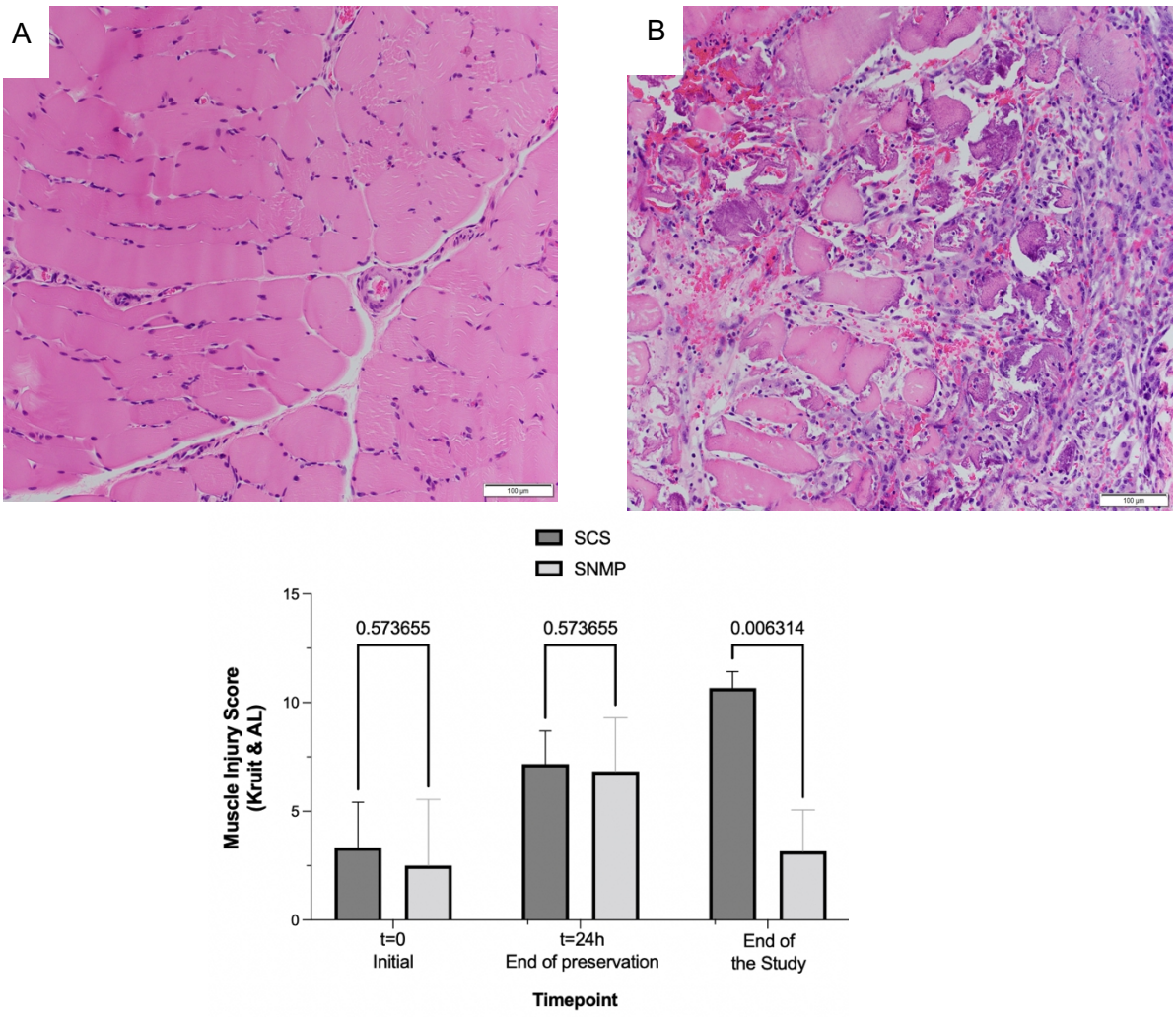

Figure 8. H&E aspect of the muscle at the end of the follow-up period on the SNMP (A) and SCS (B) groups. Figure C shows the quantification of the histology results using a muscle injury score (Kruit et al.)

The macroscopic aspect of the grafts at the end of the study (POD14 or early euthanasia) showed different aspects of the grafts, with important degeneration of the muscle fibers in the SCS group (**Figure 7).** Two pathologists analyzed the microscopic slides of the muscle in both groups (**Figure 8**), and a muscle injury score (Kruit et al.) was used to compare groups and quantify the outcomes. The score at the end of the preservation period showed no difference, but the score at the end of the study showed statistically significant differences between groups, therefore confirming the macroscopic aspect.

➔ We, therefore, have shown that SNMP allows better preservation of VCA by decreasing the ischemic injuries on the muscle. Acellular SNMP allowed 24-hour preservation prior to transplantation, and no consequences of the extended preservation were found on the recipient

animals or the grafts. To the best of our knowledge this result is the current world-best in VCA with a bone component that is validated by transplantation in a large animal model.

3) Evaluation of Supercooling in porcine VCA.

To achieve prolonged preservation of porcine VCAs over 24h, our work then focused on performing more ex vivo experiments in pig hindlimbs, consisting of 48h preservation using Supercooling (Sub-Zero Non-Freezing) techniques followed by Subnormothermic Machine Perfusion (SNMP) for the recovery. This phase was a scale-up from our previous work on rat limb supercooling (DoD RT160093). We harvested **2 partial hindlimbs**. In total, four limbs underwent supercooling (-5°C, **Figure 1**), and eight limbs underwent static cold storage (SCS, 4°C). Since supercooling involves Subnomorthermic Machine Perfusion (SNMP) Recovery using Steen+ solution (Figure 1), we separated the controls in two groups: one undergoing Static Cold Storage followed by 2h SNMP, and one undergoing SCS with no SNMP recovery. All limbs were assessed for ischemia-reperfusion injuries by a 2h of normothermic machine perfusion (NMP) with whole autologous blood, preserved for 48h in citrate bags.

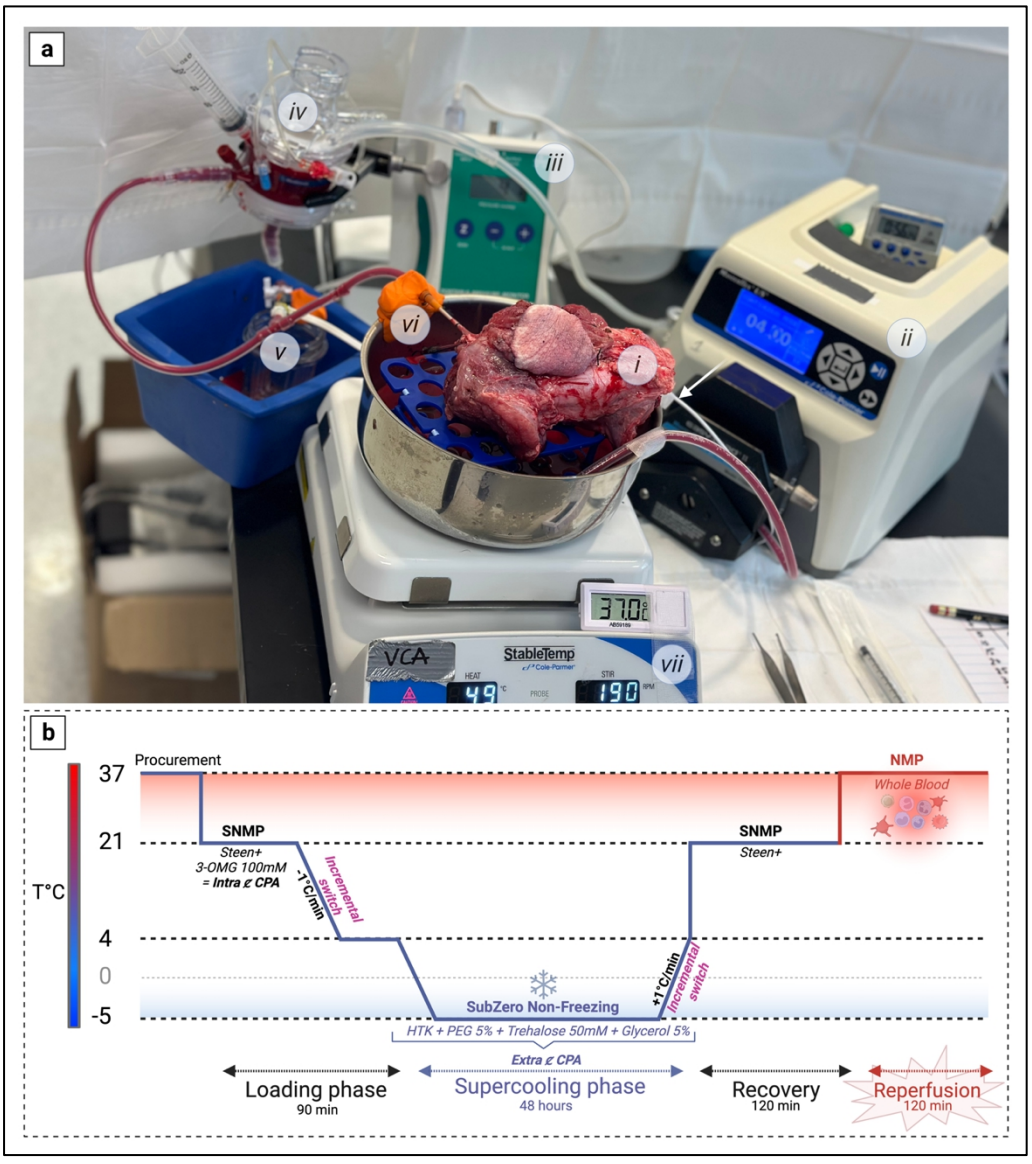

Figure 9 / **(a)** Perfusion machine used for loading and recovery of the VCAs. *i: Porcine VCA undergoing NMP; ii: Peristaltic roller pump; iii: Pressure monitor; iv: Hollow-fiber membrane oxygenator; v: Pressure sensor; vi: Arterial cannula providing the inflow in the graft; vii: Hot plate to allow warming and stirring the blood container.* **(b)** Supercooling protocol of porcine VCAs. SNMP: Subnormothermic Machine Perfusion, NMP: Normothermic machine perfusion.

The objective of the SNMP phase is to flush the metabolites (Lactate, potassium) out of the vascular tree and try to optimize the intracellular metabolic state (pH, Potassium, Sodium, Calcium). The objective of the NMP is to provoke Ischemia-Reperfusion injuries, as we would observe after transplanting the limb.

Histology samples were procured on the skin and the muscle at different timepoints (Initial, End of Preservation, End of Steen recovery (Subnormothermic Machine Perfusion), End of Blood recovery (Normothermic Machine Perfusion). Biopsy samples were fixed in buffered formalin, included in paraffin and slides were stained with H&E. Several muscle samples were procured per limb following the 2h-NMP recovery performed to provoke and reveal ischemia-reperfusion-injuries. A blinded reading from an experienced pathologist allowed scoring the injuries using a dedicated scale using the same system (Kruit et al., 2021). The analysis included inflammatory cell count, necrosis, fibrosis, edema, and overall fiber architecture. No major difference was found in the skin, accordingly with the skin's resistance to ischemia. We focused on analyzing and comparing the perfusion and metabolic parameters in the three groups. **Figure 10** displays the evolution of flow, vascular resistance, and weight gain during recovery of the VCAs, while **Figure 11** shows the metabolic parameters.

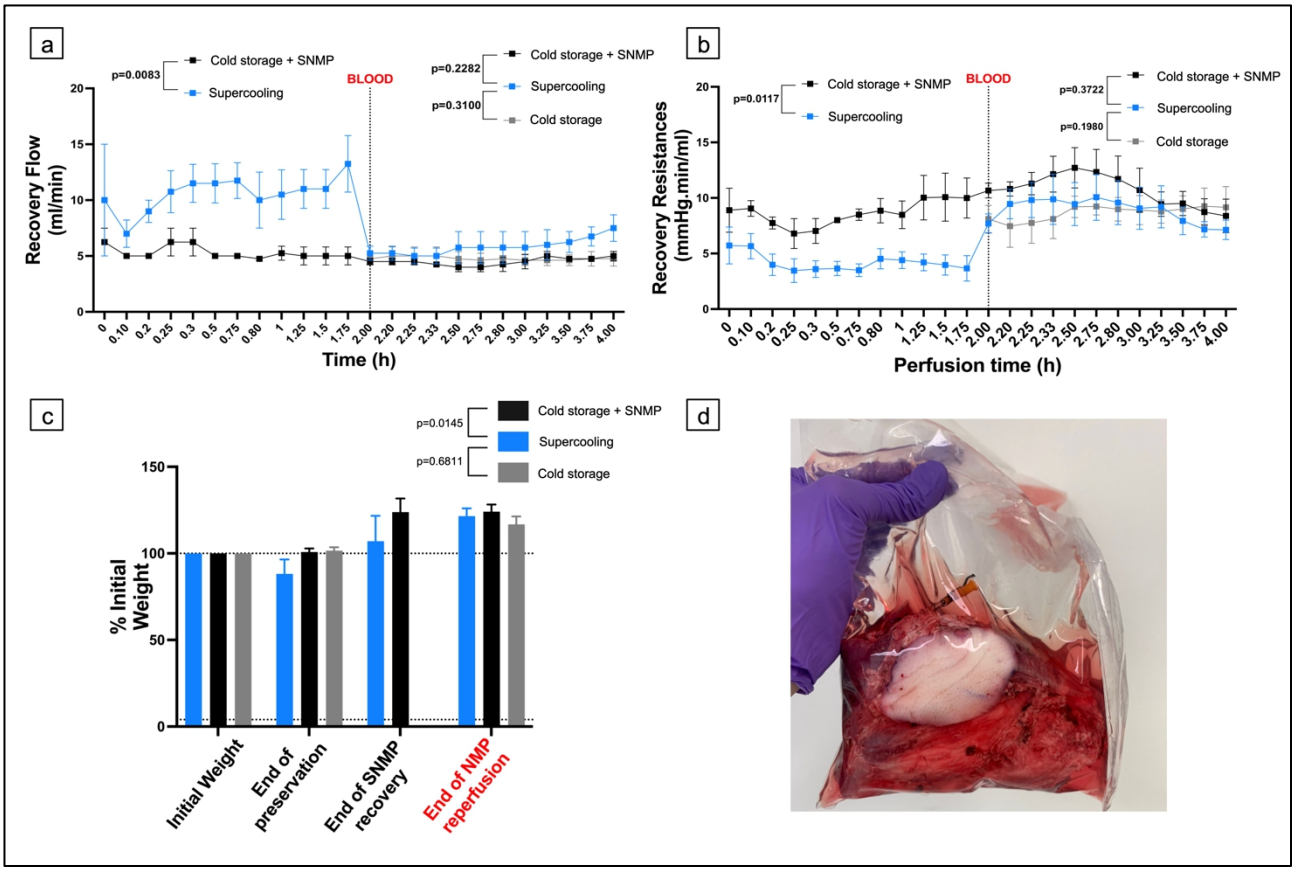

Figure 10 / Perfusion parameters of recovered porcine VCAs following 48h preservation.
At t=2h, the line marks the perfusate switch to whole blood at 37°C. **(a)** Flow rate profile during SNMP and NMP recovery showing a significantly higher flow allowed by **(b)** significantly lower vascular resistance in the SC group during the SNMP phase. No significant difference was found between three groups in the NMP phase. Note: A mixed-effects model with the Geisser-Greenhouse correction was performed to compare groups during each phase. **(c)** Weight gain following SNMP and NMP was significantly lower in the SC group when compared with CS+SNMP GROUP but not with CS GROUP. **(d)** Aspect of the stored VCA following 48h supercooling, showing the absence of ice nucleation.

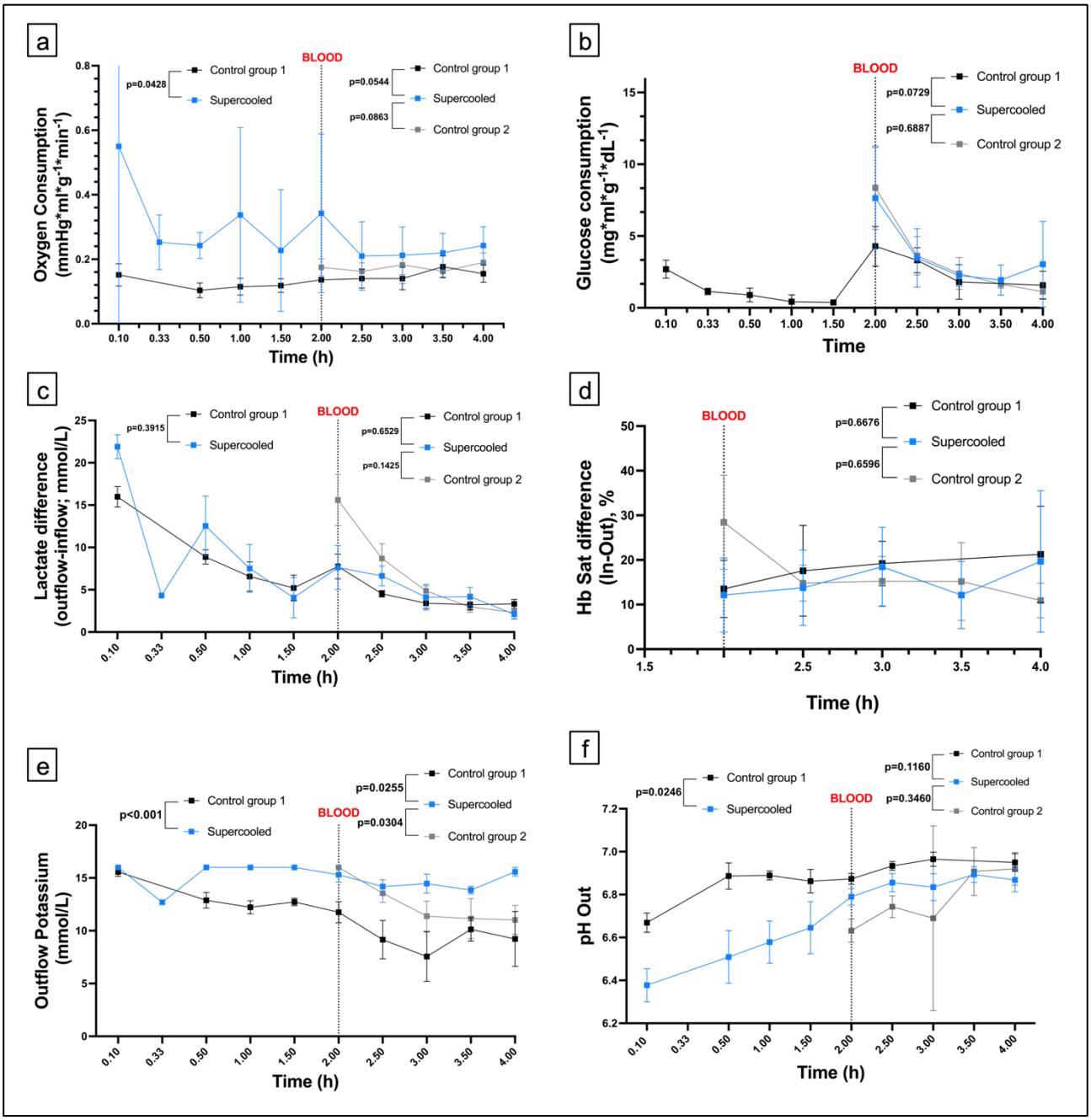

Figure 11/ Perfusate analysis during recovery. (a) Oxygen consumption was higher in the Supercooled group during recovery versus control, but the difference was only significant in the SNMP phase. **(b)** The glucose consumption was not measurable during the SNMP recovery due to interference with the 3OMG. The supercooled

limbs seemed to consume more glucose in the NMP phase versus Cold Storage+SNMP group, but the difference was not significant. **(c)** Lactate release levels were comparable between groups during both phases. **(d)** Hemoglobin arteriovenous difference was comparable between groups during the NMP phase. **(e)** Potassium release was significantly higher in the Supercooled group during the SNMP phase. **(f)** The pH was lower in the Supercooled group during the NMP phase and tended to reach similar values as control groups during the NMP phase.

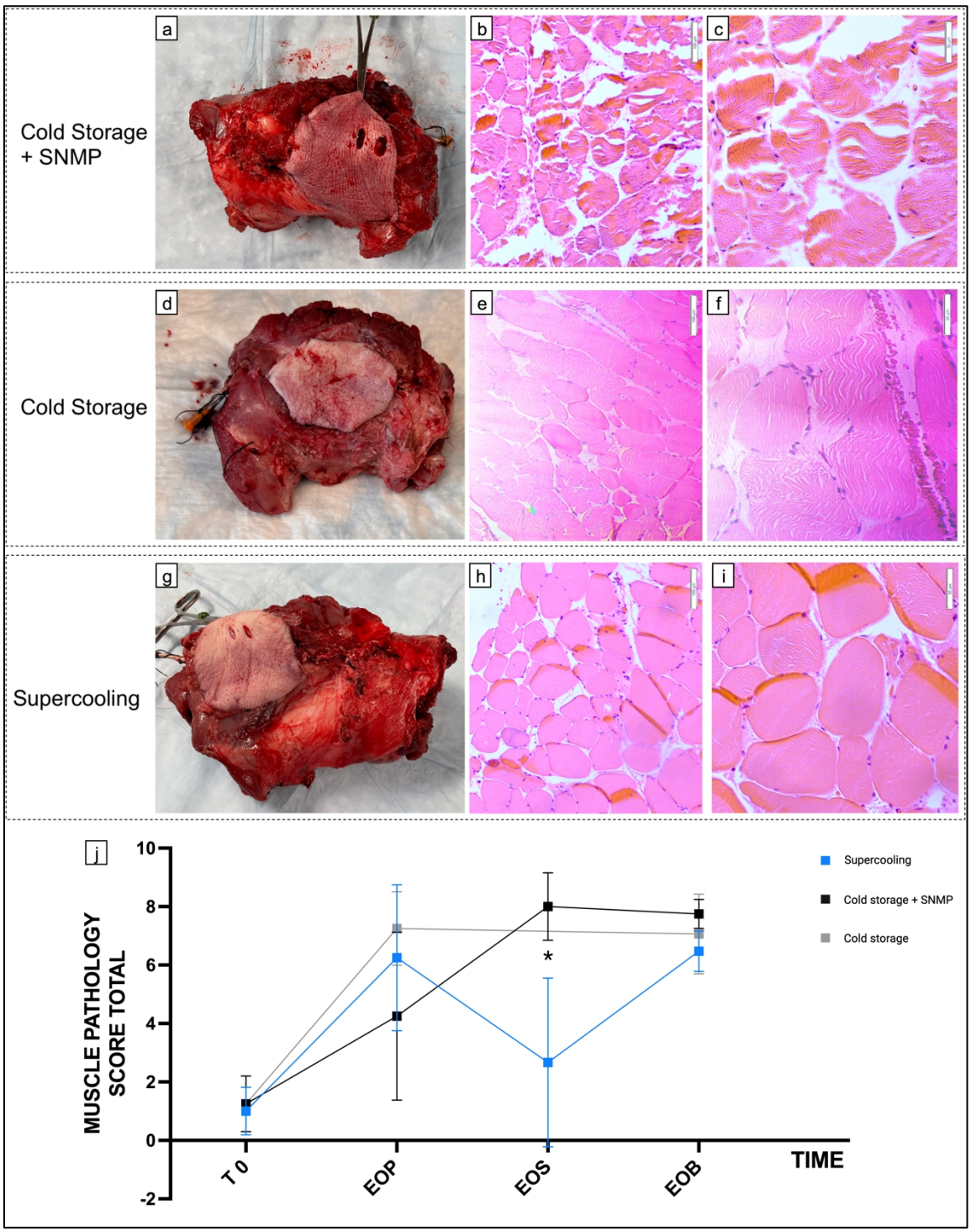

Figure 12 / Macroscopic and Microscopic results on the porcine VCAs following 48h preservation and recovery. **(a,d,g)** The macroscopic aspect of the grafts following 2h of 37°C NMP with whole blood was similar in the Cold Storage+SNMP, Cold Storage and Supercooling groups, respectively. The skin paddle presented an ecchymotic aspect which seems milder in the Supercooling group, but no significant differences were found histologically. **(b,c)** At the microscopic level, the Cold storage+SNMP group showed important interstitial edema and myocyte fiber injuries. **(e,f)** The Cold storage group showed milder edema and fiber architecture disruption. (h,i) The Supercooling group showed mild interstitial edema and minor muscle fiber injuries when compared with both control groups. **(j)** A blinded microscopic score (Kruit et al. 2021) was performed. The overall scores were compared at several timepoints for each group: T0 (initial), EOP (end of preservation), EOS (end of Steen+ SNMP recovery) and EOB (end of NMP blood recovery). A significantly lower muscle injury score was found between the Supercooling group and the Cold storage+SNMP group at the end of the Steen+ recovery. The difference between experimental and control groups was not statistically significant on the other timepoints.

This phase of the study successfully demonstrated the supercooling of porcine VCAs for 48 hours (no freezing of the limbs), restoring vascular flow, and evaluating under physiological conditions through normothermic whole blood perfusion. The MP-based recovery phases allowed for proper monitoring of the preserved grafts. Among the metrics, weight gain and vascular resistance have proven to be the earliest and most important parameters. Meyers et al. (2023) demonstrated that weight gain was correlated with microscopic muscle injury and was the earliest evidence of VCA dysfunction during ex-vivo normothermic limb perfusion in swine. In machine perfusion, vascular resistance is closely linked to endothelial cell injury: shear-stress-induced injuries cause cellular edema, which eventually increases perfusion resistance by external compression of the microvessels49. Moreover, no autonomic nerve control is possible during ex-vivo perfusion, and circulating vasoactive substances show poor action, leaving the paracrine relaxing factors as the main effector for vasodilatation. These endothelial injuries should, therefore, be estimated using flow parameters such as vascular resistance and weight gain. In this study, supercooled limbs showed significantly lower vascular resistance during SNMP recovery following 48h preservation when compared with static cold stored controls. Interestingly, this difference was not significant during the NMP phase. Moreover, the vascular resistance in the CS group was lower than in the CS+SNMP group, which could be explained by endothelial injuries during the SNMP phase that was missing in the CS GROUP. Weight gain was minor and stayed lower than 20% at the end of the NMP phase, with no significant difference between groups. However, a significant difference was found between the supercooled and static cold stored limbs at the end of the SNMP, which is consistent with the vascular resistance outcomes. Another major finding was the higher oxygen consumption of the supercooled limbs during recovery. This parameter is a direct indicator of higher metabolism, potentially linked to better cell viability permitted by supercooling. This was also suggested by a higher glucose consumption during the NMP phase without reaching statistical significance. Finally, the microscopic assessment of the muscle following normothermic blood reperfusion suggested better preservation of the myocyte structure and lower edema, with significant differences with static cold stored controls after the SNMP recovery phase.

On the other hand, the potassium release was significantly higher in the Supercooled limbs for both phases. This could be due to either rhabdomyolysis due to ischemia-reperfusion injuries or due to potassium storage and release from the CPA cocktail. This second hypothesis is more likely: the lactate release was similar in all groups, the histology showed better preservation of the muscle fiber architecture, and the CPA cocktail solution based on HTK (potassium concentration 8-9mmol/L) was loaded for 30 minutes using machine perfusion versus a simple flush in the control groups. It would

be interesting to compare the potassium release levels with a perfusate solution based on physiologic potassium concentrations, such as Steen+. We are actively working on optimizing the CPA cocktail composition.

One limitation of this work was the utilization of NMP instead of transplantation for reperfusion assessment. This 2h blood perfusion phase is obviously limiting the extrapolation to the in vivo behavior of the graft. However, this machine perfusion technique was used in several previous models as a relevant simulation for replantation. Moreover, this NMP phase seems to be more ethical regarding animal welfare since it allows for the assessment of graft quality following each preservation technique. Our experience with the previous phase of this study (see Y4 reports) showed that transplanting VCAs in a poor metabolic condition can lead to a critical decrease in the recipient animal's general condition, which needs to be avoided. Therefore, the purpose of these experiments was to act as a first phase of ex-vivo optimization to ensure adequate animal welfare according to the ACURO and Massachusetts General Hospital IACUC Guidelines.

These results show promising outcomes regarding microvascular preservation (estimated by the perfusion parameters) and histologic preservation of the myocytes and muscle fibers. More work still needs to be carried out to optimize the metabolic parameters in order to consider in vivo experimentation of VCA long storage before transplantation, opening the door to an all-in-one procedure including mixed chimerism immune tolerance. One perspective would be to combine the findings obtained in Specific Aim 1 (Optimization of a subnormothermic machine perfusion protocol, which successfully allowed 24h preservation) and Supercooling to reach 48h of total preservation. The hypothesis is that a decreased supercooling storage (24h) followed by a prolonged SNMP recovery (24h) would lead to optimized metabolic results, allowing for subsequent transplantation.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

In total, four post-doctoral research fellows and two lab technicians were trained. Training topics included surgical techniques of partial hind limb harvest in a swine model (by attending plastic surgeon Dr. Lellouch and Vice Chair of MGH IACUC Mark Randolph), machine perfusion, sub-zero non-freezing techniques, as well as scientific writing, experimental design, and various data analysis techniques supervised by Dr. Uygun and Dr. Cetrulo.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Peer reviewed scientific articles :

Tawa P, Goutard M, Andrews AR, de Vries RJ, Rosales IA, Yeh H, Uygun B, Randolph MA, Lellouch AG, Uygun K, Cetrulo CL Jr. **Continuous versus Pulsatile Flow in 24-Hour Vascularized Composite Allograft Machine Perfusion in Swine: A Pilot Study.** J Surg Res. 2023 Mar;283:1145-1153. doi: 10.1016/j.jss.2022.11.003. Epub 2022 Dec 16. PMID: 36915006.

Tawa P, Goutard M, Andrews AR, de Vries RJ, Rosales IA, Yeh H, Uygun B, Randolph MA, Lellouch AG, Uygun K, Cetrulo CL Jr. **Response Regarding: Continuous Versus Pulsatile Flow in 24-h Vascularized Composite Allograft Machine Perfusion in Swine: A Pilot Study**. J Surg Res. 2023 Nov;291:751-753. doi: 10.1016/j.jss.2023.05.031. Epub 2023 Jul 28. PMID: 37517972.

Goutard M, Tawa P, Berkane Y, Andrews AR, Pendexter CA, de Vries RJ, Pozzo V, Romano G, Lancia HH, Filz von Reiterdank I, Bertheuil N, Rosales I, How IDA, Randolph MA, Lellouch AG, Cetrulo CL Jr., Uygun K. **Machine Perfusion Enables 24-hr Preservation of Vascularized Composite Allografts in a Swine Model of Allotransplantation**. Submitted to Transplantation International, September 2023.

Berkane Y, Filz von Reiterdank I, Tawa P, Charlès L, Goutard M, Dinicu A, Mink van der Molen A, Coert HJ, Bertheuil N, Toner M, Lellouch AG, Randolph MA, Cetrulo CL Jr., Uygun K. **Sub-Zero Non-freezing Techniques for VCA preservation: Evaluating Supercooling in a Swine Model**. Submitted to Scientific Reports, September 2023.

1 article in progress : A Reliable Porcine VCA model for the Optimization of Machine-Perfusion-Based Ex-Vivo Preservation.

+ 7 abstracts presented to the American Transplant Congress (2021-23), the American Society of Reconstructive Transplantation annual meeting (2021-22), Harvard Surgery Research Day (2023), the ATP-Bio Research and Innovation Annual Meeting (2022), the French Society of Plastic Surgery (SOFCPRE) annual meeting.

DoD funding was acknowledged in all scientific articles, oral and poster presentations.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

This is the final report. Nothing to Report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The key accomplishment are ***i)*** the development of a protocol using SNMP to preserve VCA for 24h. This is to our knowledge the first description in VCA including bone, and the first time-match controlled study. This allows preservation of VCA between procurement and transplant without major injuries, potentially diminishing the risk of rejection linked to ischemia-reperfusion injuries. This is a 4x increase in viable preservation of limbs at the large animal scale based on literature.
ii) The first description of a supercooling/subzero-nonfreezing protocol in large animal VCA. This part allowed successful supercooling of a whole porcine hindlimb, followed by successful recovery with improved perfusion parameters and histology versus static cold stored controls. The metabolic outcomes still need further optimization but this acts as a robust proof of concept of extended VCA preservation using SNMP and supercooling techniques, eventually allowing enough delay to apply immune tolerance protocols.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

As an interdisciplinary project, the results are expected to have impact on the fields of plastic surgery, transplantation, biopreservation and medical systems engineering.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Licensing of patents previously developed in project W81XWH-17-1-0680, precursor to this project, are in discussion. We also expect new IP may result from this work, or alternatively the data will supporting prior patent applications.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

We are collaborating with the NSF engineering research center ATP-Bio, which includes prominent experts and members of the National Academy of Medicine. Our joint effort is focused on crafting anticipatory governance strategies for emerging preservation techniques in transplantation, including the one developed in this project. These innovative technologies are anticipated to have a substantial impact on the entire field of transplantation, including reconstructive transplant procedures.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Covid19 crisis led to major delays in large animal work. Accordingly we requested a change in project SOW to reduce transplant experiments and focus on the perfusion milestones, which was approved in May 2021. A No-Cost-Extension was also approved (Year 5) to allow testing the supercooling protocols.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing additional to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1/ Tawa P, Goutard M, Andrews AR, de Vries RJ, Rosales IA, Yeh H, Uygun B, Randolph MA, Lellouch AG, Uygun K, Cetrulo CL Jr. **Continuous versus Pulsatile Flow in 24-Hour Vascularized Composite Allograft Machine Perfusion in Swine: A Pilot Study.** J Surg Res. 2023 Mar;283:1145-1153. doi: 10.1016/j.jss.2022.11.003. Epub 2022 Dec 16. PMID: 36915006.

2/ Tawa P, Goutard M, Andrews AR, de Vries RJ, Rosales IA, Yeh H, Uygun B, Randolph MA, Lellouch AG, Uygun K, Cetrulo CL Jr. **Response Regarding: Continuous Versus Pulsatile Flow in 24-h Vascularized Composite Allograft Machine Perfusion in Swine: A Pilot Study**. J Surg Res. 2023 Nov;291:751-753. doi: 10.1016/j.jss.2023.05.031. Epub 2023 Jul 28. PMID: 37517972.

3/ Goutard M, Tawa P, Berkane Y, Andrews AR, Pendexter CA, de Vries RJ, Pozzo V, Romano G, Lancia HH, Filz von Reiterdank I, Bertheuil N, Rosales I, How IDA, Randolph MA, Lellouch AG, Cetrulo CL Jr., Uygun K. **Machine Perfusion Enables 24-hr Preservation of Vascularized Composite Allografts in a Swine Model of Allotransplantation**. Submitted to Transplantation International, September 2023.

4/ Berkane Y, Filz von Reiterdank I, Tawa P, Charlès L, Goutard M, Dinicu A, Mink van der Molen A, Coert HJ, Bertheuil N, Toner M, Lellouch AG, Randolph MA, Cetrulo CL Jr., Uygun K. **Sub-Zero Non-freezing Techniques for VCA preservation: Evaluating Supercooling in a Swine Model**. Submitted to Scientific Reports, September 2023.

5/ Berkane Y, Hayau J, Filz von Reiterdank I, Kharga A, Charlès L, Mink van der Molen A, Coert JH, Bertheuil N, Randolph MA, Cetrulo CL Jr., Longchamp A, Lellouch AG, Uygun K. **Supercooling: A Promising Technique for Prolonged Preservation in Solid Organ Transplantation, and Early Perspectives in Vascularized Composite Allografts.** Under review in Frontiers in Transplantation, August 2023.

1 article in progress : A Reliable Porcine VCA model for the Optimization of Machine-Perfusion-Based Ex-Vivo Preservation.

+ 7 abstracts presented to the American Transplant Congress (2021-23), the American Society of Reconstructive Transplantation annual meeting (2021-22), Harvard Surgery Research Day (2023), the ATP-Bio Research and Innovation Annual Meeting (2022), the French Society of Plastic Surgery (SOFCPRE) annual meeting.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

A) American Transplant Congress, San Diego CA, June 6-9th, 2023 :
Y. Berkane, I. Filz von Reiterdank, M. Goutard, P. Tawa, A. G. Lellouch, M. A. Randolph, C. L. Cetrulo, K. Uygun. Supercooling To Extend The Preservation Of VCA In A Pig Hindlimb Model.

B) ATP-Bio Research & Innovation Annual Meeting, April 13th, 2022 (Univ. of Minnesota, USA) :
Y.Berkane, M Goutard, Pierre Tawa, I Filz v. Reiterdank, H.H Lancia, E de Clermont-Tonnerre, C Guinier, N Bertheuil, M Randolph, C.L Cetrulo, A.G Lellouch, K Uygun. Successful 24 h hindlimb perfusion before transplantation in a pig model.

C) Harvard Surgery Research Day, Boston MA, March 25th, 2023:
Y. Berkane*, H. Oubari*, M. Goutard*, P. Tawa, I. FV Reiterdank, H. Lancia, AR. Andrews, MA. Randolph, AG. Lellouch, CL. Cetrulo Jr. +, K. Uygun+. Oxygenated sub-normothermic machine perfusion for extended VCA preservation. **Contributed equally*

D) Harvard Surgery Research Day, Boston MA, March 25th, 2023:
AT. Dinicu, I Filz von Reiterdank, Y. Berkane, P. Tawa, M. Goutard, M.Taggart, C. Pendexter, I. Rosales, A. Mink van der Molen, JH. Coert, M. Randolph, AG. Lellouch, CL. Cetrulo Jr*, K. Uygun*. Extended Preservation Using Supercooling Preservation of Vascularized Allografts in Rodents and Swine. **Co-senior authorship*

E) Congrès national de la SOCFPRE (French society of Plastic surgery), November 2022:
P. Tawa, M. Goutard, A. Andrews, Y. Berkane, K. Uygun, C.L Cetrulo Jr, A.G Lellouch. Influence du Flux Pulsatile sur la préservation des Allogreffes de Tissus Composites sur machine de perfusion Subnormothermique chez le porc.

F) American Transplant Congress, San Diego CA, June 6-9th, 2023:
Y. Berkane, A. G. Lellouch, A. A. Shamlou, I. Filz von Reiterdank, N. Bertheuil, J. Duisit, B. E. Uygun, M. Randolph, C. L. Cetrulo, K. Uygun. 24h Acellular Subnormothermic Machine Perfusion For VCA Preservation

G) ATP-BIO Convergent Research, September 27th, 2022:
Y.Berkane, M.Goutard, P.Tawa, I.Filz von Reiterdank, A.Shamlou, A.G. Lellouch, M. Randolph, C.L. Cetrulo Jr, K.Uygun. Subnormothermic Machine Perfusion and Supercooling to extend VCA preservation in a pig hindlimb model. Webinar presentation.

H) Pulsatile vs Continuous Flow in Swine Hindlimb Preservation using Subnormothermic Machine Perfusion: American Society for Reconstructive Transplantation Meeting – November 2021
P. Tawa, M. Goutard, R.J. de Vries, A. G. Lellouch, G. Romano, V. Pozzo, S. Maggipinto, L. Lantieri, M. A. Randolph, C. L. Cetrulo, Jr., K. Uygun.

I) American Society of Reconstructive Microsurgery Meeting – January 2022:
A Simplified Perfusion Protocol for 24-hr VCA Ex Vivo Preservation in a Swine Limb Transplantation Model M. Goutard, P. Tawa, R.J. de Vries, A. G. Lellouch, G. Romano, V. Pozzo, C. Pendexter, S. Maggipinto, L. Lantieri, S. N. Tessier, M. A. Randolph, C. L. Cetrulo, Jr., K. Uygun.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

- Nothing to report.

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

A novel protocol for swine limb ex vivo perfusion preservation was developed.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

4907701-22-0124 System and Method for Determining Perfused Tissue Viability

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged

Name: Korkut Uygun
Project Role: PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Co-led the project

Name: Mehmet Toner
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Co-led the project

Name: Shannon Tessier
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Led optimization of perfusate additives and supercooling protocol

Name: Biju Parekkadan
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Planned immunological aspects of transplant studies planned and graft histologocal and immunological assessment. Replacing the effort originally planned for Dr. Tessier as approved in the SOW change in May 2021.

Name: Casie Pendexter
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Assisted in graft recovery and machine perfusion (until Jan 2021)

Name: Sinan Ozer
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Assisted in graft recovery and perfusion and assays. Left for graduate studies (Cornell MBA program) Nov 2020

Name: Mo Mojoudi
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4
Contribution to Project: Assisted in graft recoveries, perfusion. Replaced Casie Pendexter's role in February 2021

Name:	*Ilana Reis*
Project Role:	*Dissemination Organizer*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Assisted in preparation of reports, preparation of publication materials and documentation related to IP disclosures, oversaw lab compliance and relevant reporting.*

Name:	*Lynne Stubbefield*
Project Role:	*Staff Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Assisted in preparation of reports, preparation of publication materials and documentation related to IP disclosures, oversaw lab compliance and relevant reporting.*

Name:	*Eileen Hebard*
Project Role:	*Inventory Coordinator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Responsible for ordering and coordination of supplies for perfusion devices, perfusion media components, iStat and other analytical device cartridges, and assay supplies.*

Name:	*Hannah Stowe*
Project Role:	*Research technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Performed various sample analysis including spectrometry*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding

agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing new to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

See attached quad chart.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

No additional document to report.