AWARD NUMBER: W81XWH-19-1-0437

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: Curtis L. Cetrulo, Jr., M.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA

REPORT DATE: August 2022

TYPE OF REPORT: Annual

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
August 2022	Annual	01Aug2021-31Jul2022

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-19-1-0437

5b. GRANT NUMBER
RT180063P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Curtis L. Cetrulo, Jr., M.D.

E-Mail: CCETRULO@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.

In the second year the focus was on identifying ideal perfusion parameters and initiating transplant studies.

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
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Enabling prolonged preservation of vascularized composite allografts (VCA) is critical to enable their use in a practical manner clinically. 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 exciting results in livers to VCA, also leveraging prior studies in rats.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

	Timeline	Site 1 (Cetrulo)	Site 2 (Uygun)	Anticipated number of animals
SPECIFIC AIM 1: Scale up of VCA machine perfusion protocol for swine model with transplant validation.				
Major Task 1: Scale Perfusion to Porcine Limbs	Months			
Subtask 1: Submit documents for IACUC approval	1-4	X		
Subtask 2: Submit documents for ACURO approval	3-6	X		
Milestone # 1 ACURO approval obtained	*6*			
Subtask 3: Build a scaled up Machine Perfusion protocol for pig limbs	5-24		System Development	
Subtask 4: Extend perfusion preservation of swine limbs	9-15	Limb recovery	Perfusion studies	24 (exempt protocol)
Subtask 5: Test perfusion-preservation by simulated transplantation (perfusion with reconstituted blood)	6-24	Simulated transplant studies	Ex vivo whole blood reperfusion	8
Milestone #2 Complete evaluation of Machine perfusion on VCA viability	*24*			
SPECIFIC AIM 2: Extend preservation duration in the swine hind limb transplant model				
Major Task 2: SZNF preservation of Porcine Limbs				
Subtask 1: Optimize subzero preservation of porcine VCA, including intracellular protectant loading, supercooling temperature profile, and recovery perfusion optimized in Aim 1. (Experiment: SZNF for 12, 24, 36hrs, to be determined based on final results of major task 1. Control: time matched cold storage. Endpoint: % weight gain and histology confirmation)	*12-30*	Limb recovery and assessment	Protocol Development	24 (from discarded animals, exempt protocol
Subtask 2: Test subzero non-freezing preservation in a simulated transplant model	*18-36*	Simulated transplant studies	Ex vivo whole blood reperfusion	8
Milestone #3 Develop a method to extend preservation duration for porcine limbs	*36*			
SPECIFIC AIM 3: Utilization of supercooling protocol for tolerance induction in swine				
Major Task 3: Tolerance induction via Bone Marrow + VCA transplant using extended preservation				
Subtask 1: Test inducing tolerance in a clinically practicable scenario (note that this period will likely extend into an NCE)		Transplant studies Tolerance induction	Extended preservation for transplant	8
Milestone #4 Develop a method to enable using mixed chimerism for VCA transplantation	*36 (may extend to NCE for observation)*			

What was accomplished under these goals?

We started the project by scaling up from our prior experience in rats to a large animal limb ex vivo perfusion system. We first optimized the surgical model for a 24h machine perfusion, and tested different conditions (whole limb, forelimb, partial hindlimb, pulsatile or continuous perfusion). Then, in this reporting period, we moved forward to *in vivo* transplantation after 24h preservation.

Ex vivo subnormothermic machine perfusion (SNMP): In Vivo transplantation after 24h preservation. Comparison with Static Cold Storage (control) in a time-matched fashion.

6 swine hind limbs were procured from Yorkshire pigs under general anesthesia. 3 hindlimbs were perfused using our optimized SNMP protocol, with a Steen+ perfusate. Perfusion parameters during the 24h SNMP are shown in Figure 1.

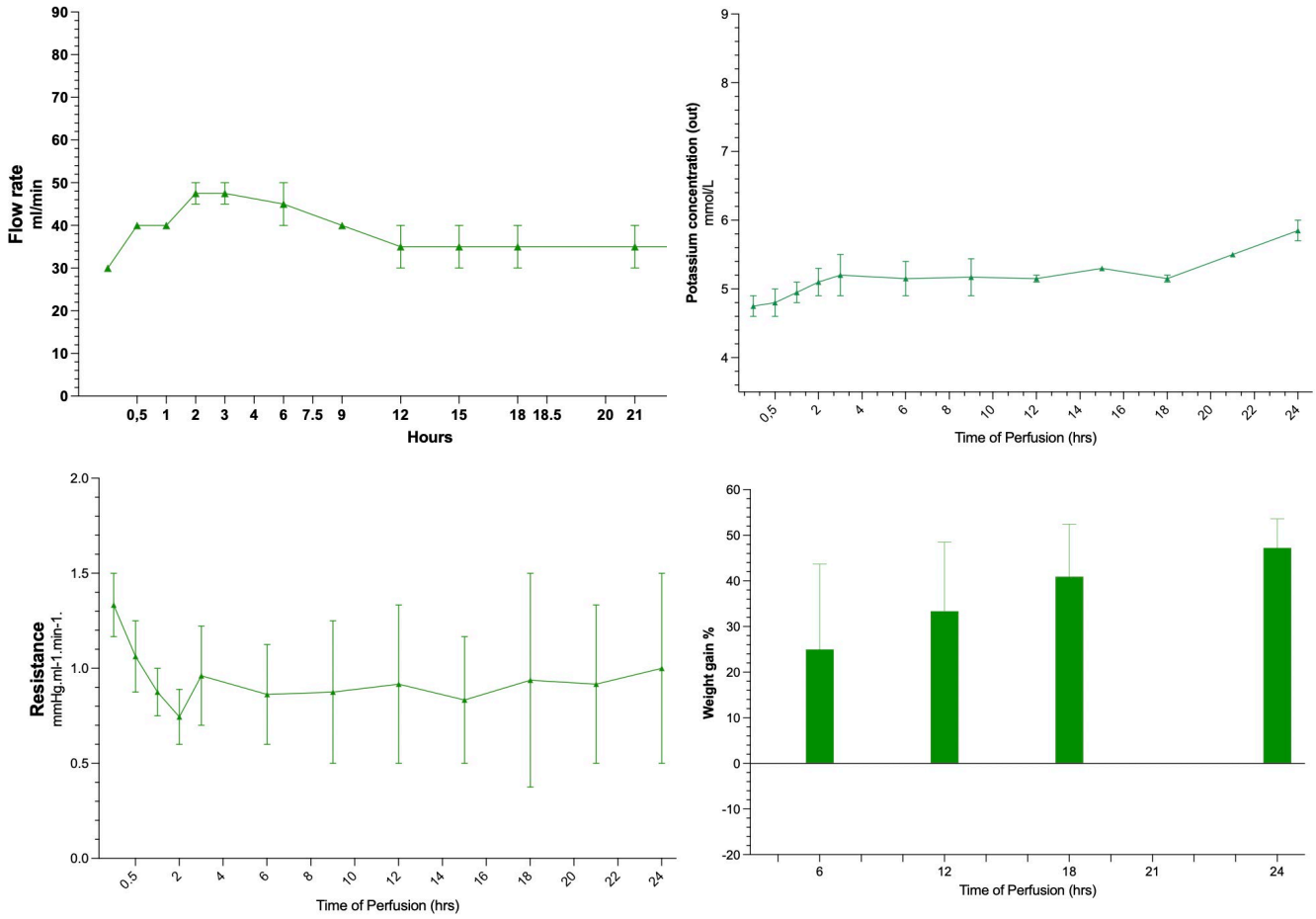

Figure 1. Perfusion parameters observed during the 24h SNMP prior to transplantation (n=3).

Three porcine partial limbs were harvested, flushed with 150ml of cold HTK before being perfused with our custom-built perfusion system including a Medtronic Affinity Pixie Hollow-fiber Oxygenator. The perfusate solution was a Steen + solution (15g/L of Bovine Serum Albumin) and a partial perfusate exchange (50%, equivalent 350ml) was performed at 12h. The 24h SNMP preservation was performed in a sterile environment.

The perfused hindlimbs were then transported back to the OR where a recipient pig has been prepared (dissection of the femoral artery and vein as recipients vessels). The hindlimb was transplanted after a flush of HTK to clear the Steen+ solution (Figure 2). The animal was then recovered, and the graft was monitored twice a day for 14 days. Immunosuppression was allowed by daily IV Tacrolimus and Corticosteroids. During this follow-up period, multiple blood draws and skin biopsies were procured. The analysis included CBC, White blood cells, Potassium, Lactate, and pro-inflammatory cytokines. At the end of the follow-up period, the animal was euthanized and a necropsy allowed macroscopic and microscopic assessment of the graft and its components: skin, muscle, and bone marrow (Figure 3).

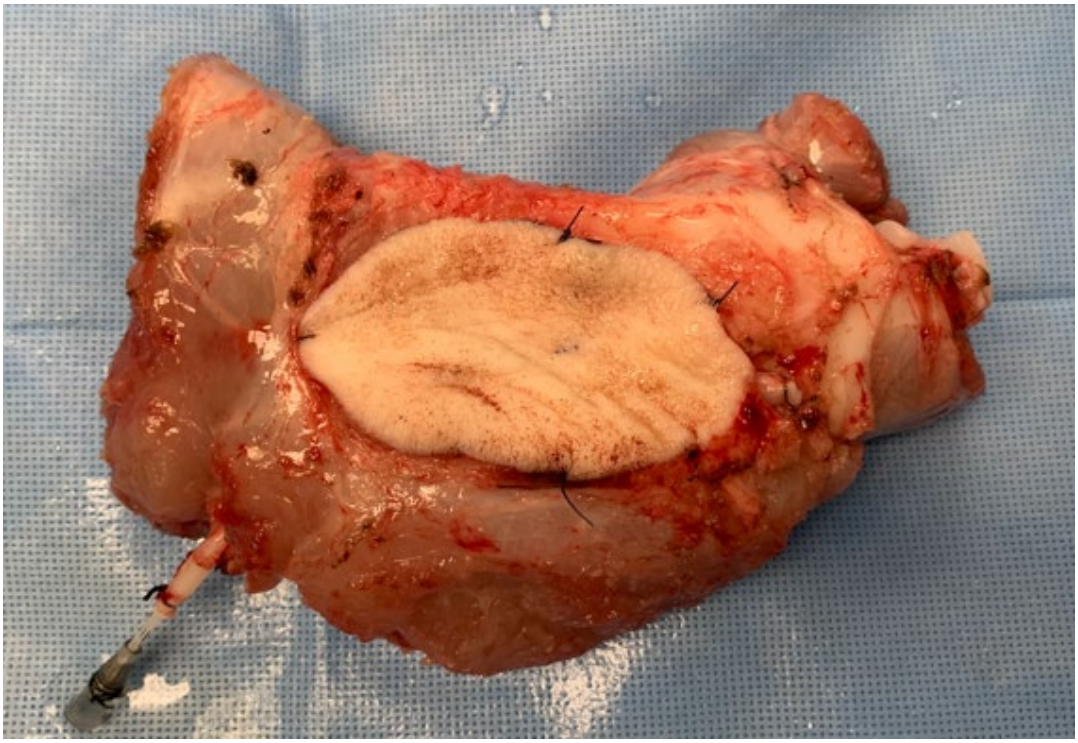

Figure 2. ***Partial Hindlimb at the end of the 24h SNMP preservation. The cannula is inserted in the femoral artery. The whole graft looks viable and the edema is mild.***

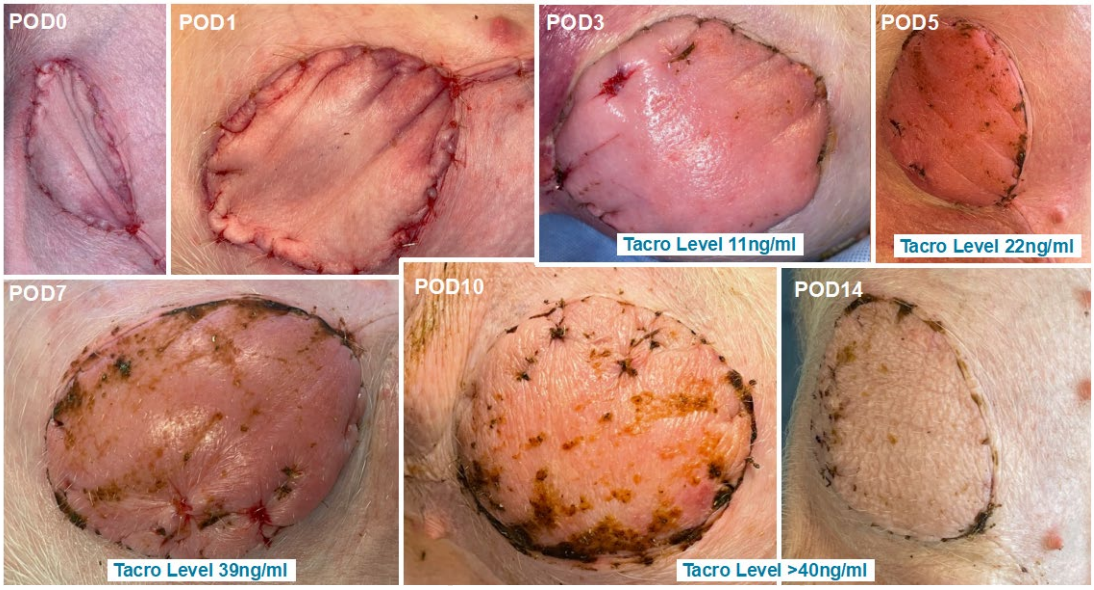

Figure 3. ***Follow-up of the graft after the transplantation.***

After n=3 successful experiments, we performed n=3 experiments consisting of the same partial hindlimb transplant after 24h static cold storage, as time-match controls. Because of the poor general conditions associated with higher potassium and lactate values, two animals in this group had to be euthanized before POD14. One animal allowed a complete follow-up period. The macroscopic and microscopic results (Figure 3) showed very important injuries in the muscle (the skin paddle was preserved in all n=3 replicates). Interestingly, the earliest euthanasia (POD6) was associated with the

highest injuries after two blinded assessments by experimented pathologists. Figure 4 shows the differences in the macroscopic appearance of the graft at the end of the follow-up period.

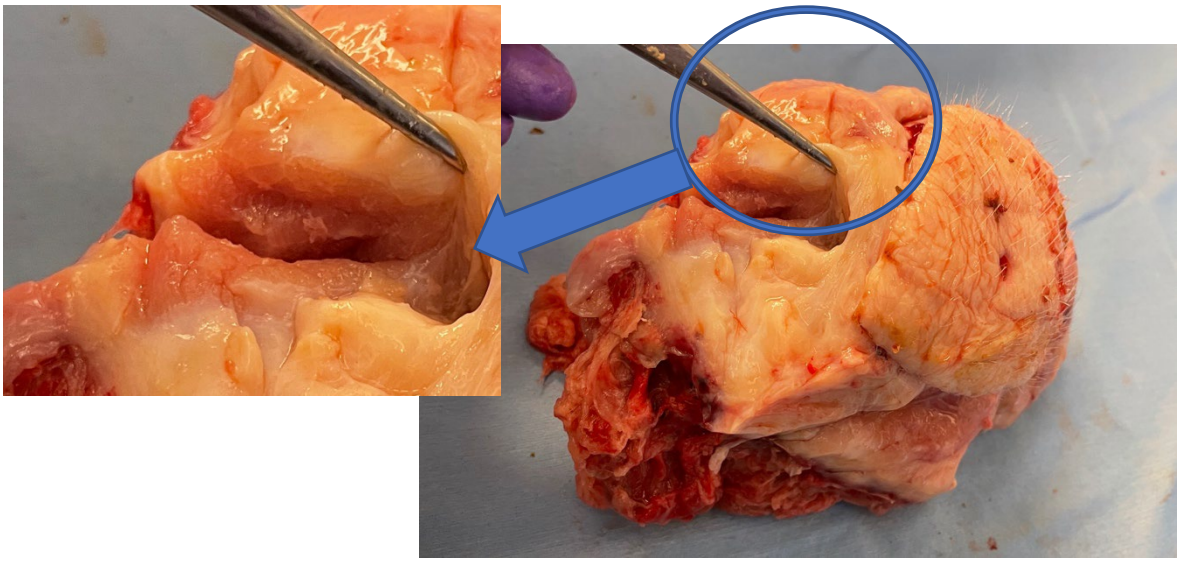

Figure 4A. ***Macroscopic aspect of the graft at POD14 after transplantation following 24h SNMP. The blue arrow focuses on viable muscle, which was uniform in the graft.***

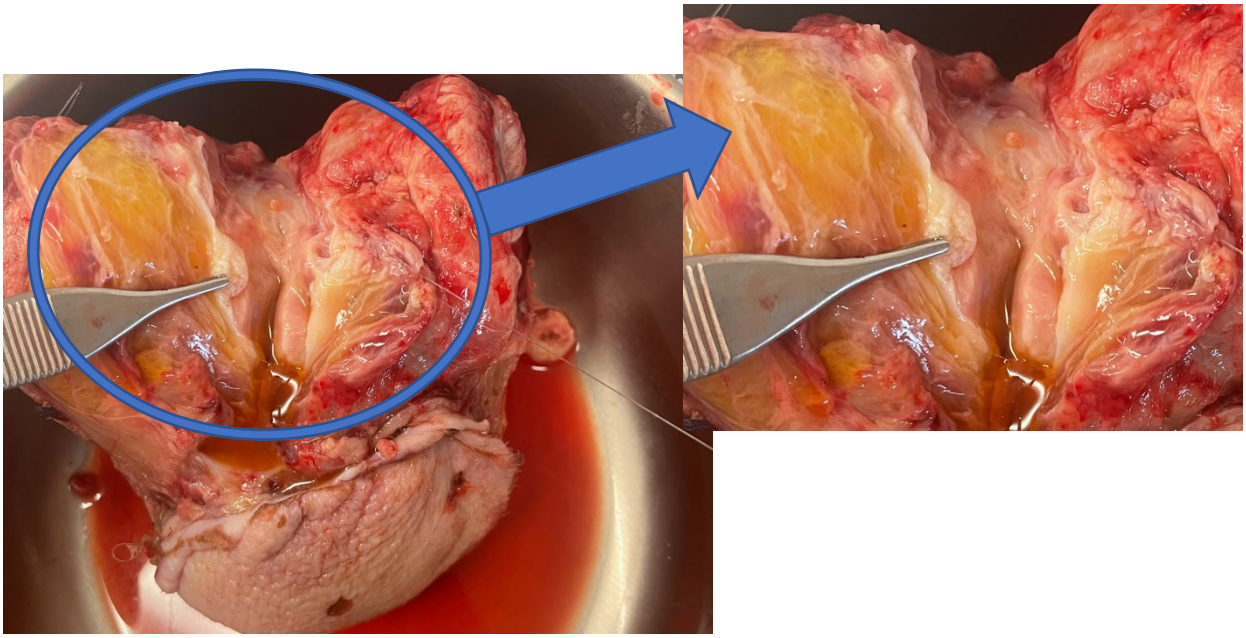

Figure 4B. ***Macroscopic aspect of the graft at the End of the study after transplantation following 24h SCS. The blue arrow focuses on the muscle, which was massively injured, showing a liquid degeneration for an important part of the graft.***

Figure 5 shows H&E slides procured in the graft's muscle. Two pathologists analyzed the slides of both groups, 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.

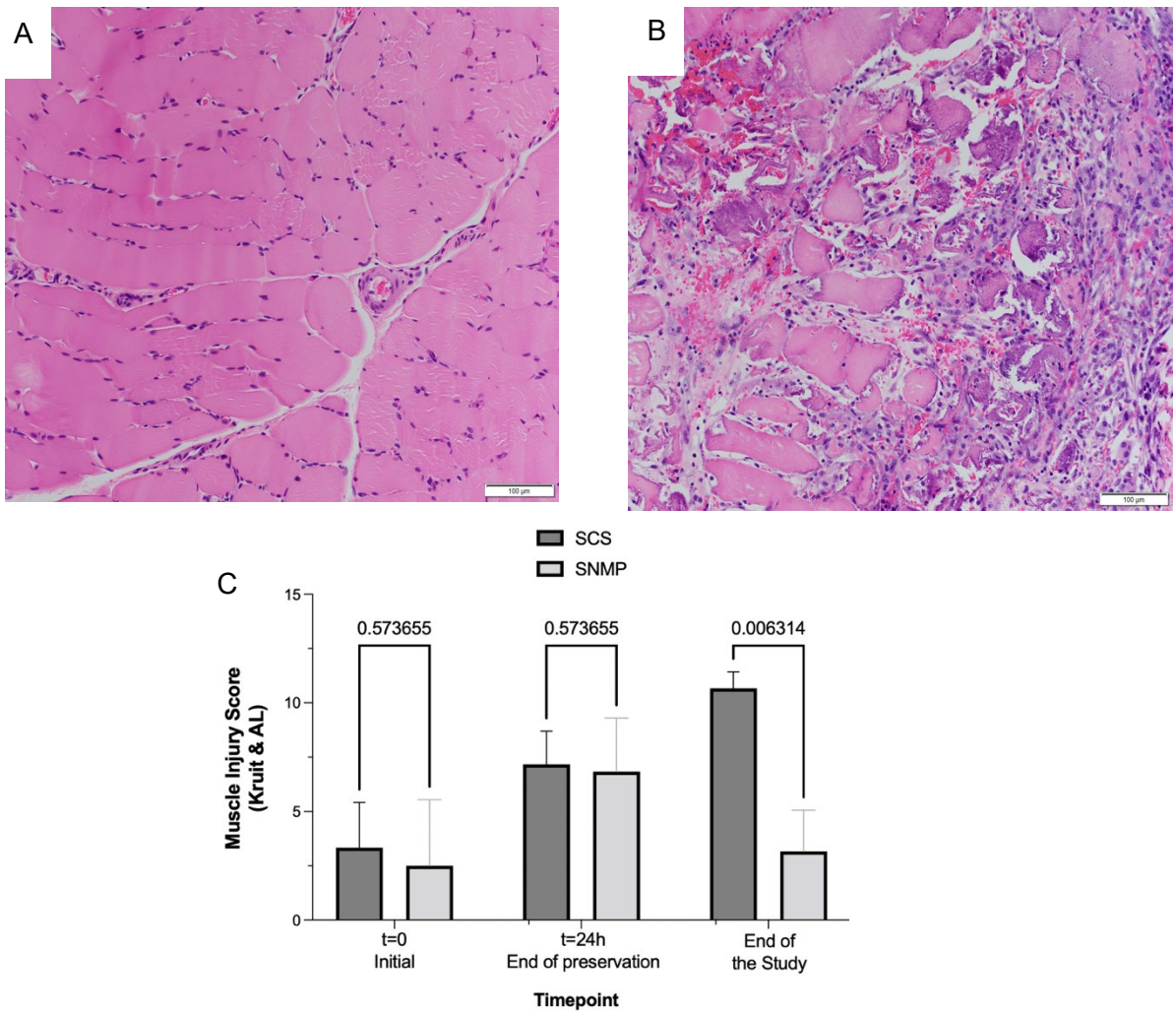

Figure 5. ***H&E aspect of the muscle at the end of the follow-up period on the SNMP (A) and SCS (B) groups. Figure C shows the quantification of the histology results using a muscle injury score (Kruit et al.)***

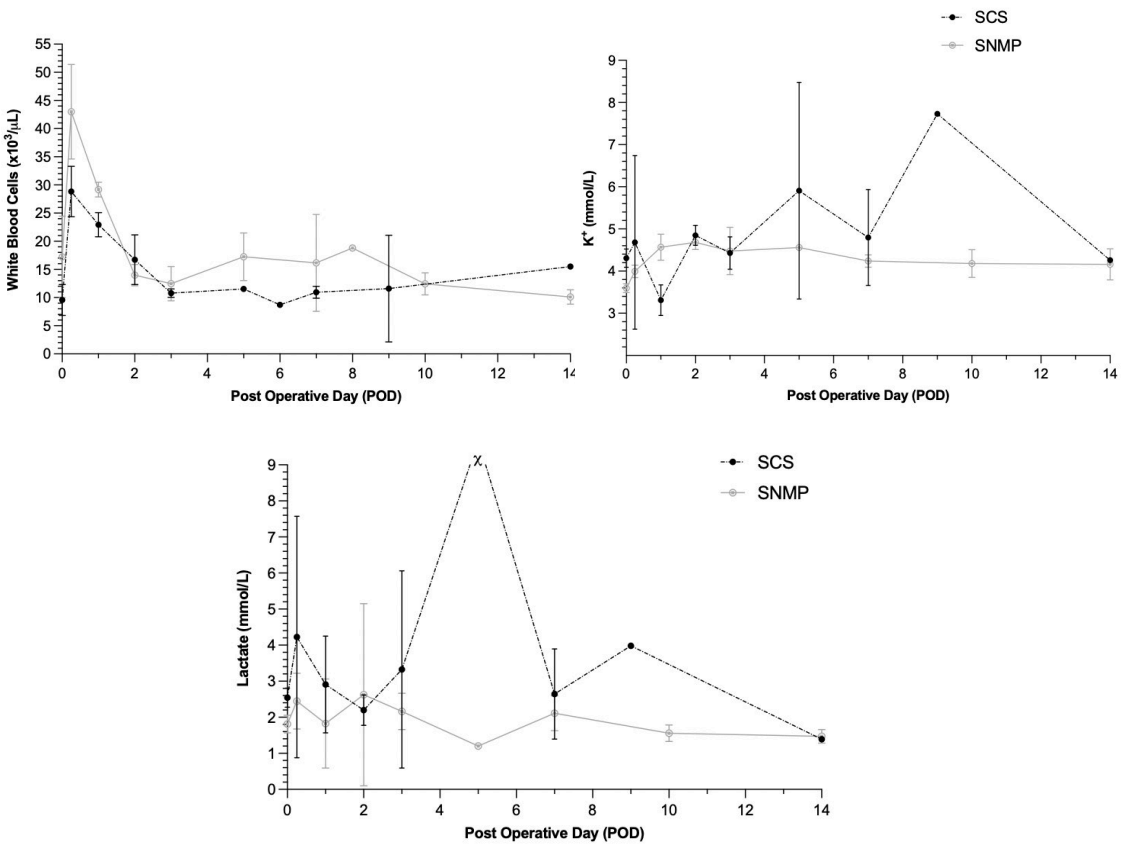

Figure 6. ***Evolution of the blood measurements during the follow-up period. WBC showed no differences between groups. Potassium was overall higher in the SCS group but the early euthanasia of 2 animals didn't allow for reaching statistical significance. Lactate values were also higher in the SCS group.***

We, therefore, have showed that SNMP allows better preservation of VCA by decreasing the ischemic injuries on the muscle. Acellular SNMP allowed 24h 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 including bone that is validated by transplantation in a large animal model.

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

Four (2 teams of 2) post-doctoral research fellows performed the transplants and the follow-up of the animals. This allowed acquiring very interesting microsurgical skills, as well as an important management capacity for the follow-up, the immunosuppressive regimen monitoring, and the basic care of the animals. They were supervised by attending plastic surgeon Dr Lellouch and Vice Chair of MGH IACUC Mark Randolph. This was also an exceptional opportunity for them to master machine perfusion techniques, as well as scientific writing, presentations, experimental design, and various data analysis techniques.

How were the results disseminated to communities of interest?

Pulsatile vs Continuous Flow in Swine Hindlimb Preservation using Subnormothermic Machine Perfusion
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.
Submitted to the American Society for Reconstructive Transplantation Meeting – November 2021

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.
Submitted to th American Society of Reconstructive Microsurgery Meeting – January 2022

Successful 24 h hindlimb perfusion before transplantation in a pig model.
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.
ATP-Bio Research & Innovation Annual Meeting, April 13th, 2022 (University of Minnesota, USA)

Influence Du Flux Pulsatile Sur La Preservation Des Allogreffes De Tissus Composites Sur Machine De Perfusion Subnormothermique Chez Le Porc
P.Tawa, M.Goutard,A.Andrews,Y.Berkane,A.Lellouch,K.Uygun,C.Cetrulo
SOFCPRE (French Society of Plastic Surgery) annual meeting, November 18th, 2022 - Paris

What do you plan to do during the next reporting period to accomplish the goals?

The immediate next step will be to peform 48h VCA preservation, with supercooling technique being our initial tool to extend preservation. Our controls will consist in the contralateral hindlimbs preserved with static cold storage. SNMP will be used to recover the limbs. Once we reach satisfactory results, we will test transplants after 48h preservation.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The key accomplishment is 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, diminishing the risk of rejection linked to IRI.

What was the 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?

No updates for the year.

What was the impact on society beyond science and technology?

We are working with the Ethics and Public Policy of the NSF engineering research center ATP-Bio, featuring many leading experts and members of the National Academy of Medicine, to develop anticipatory governance for novel preservation techniques for transplantation – such as the one we develop here. Such technologies are expected to be transformative across the field of transplantation including reconstructive transplant.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Combination of edema problems and COVID19 related issues led to revision of the SOW, which included requesting an NCE for completion of the studies.

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

Nothing significant beyond those noted in the NCE request and related SOW update.

Changes that had a significant impact on expenditures

Nothing additional to report.

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

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:

- **Publications, conference papers, and presentations**

Submitted : **24-hr Subnormothermic Machine Perfusion for Prolonged Vascularized Composite Allografts Ex Vivo Preservation - Allotransplantation in a Swine Model**
Marion Goutard MD[1-3,6]†, Pierre Tawa MD[1-3]†, Yanis Berkane, Alec R. Andrews BSc[1-3], Casie A. Pendexter BSc[2-4], Reinier J. de Vries MD PhD[2-5], Victor Pozzo MD[1-3], Golda Romano MD[1-3], Nicolas Bertheuil MD PhD, Ivy A. Rosales MD, Mark A. Randolph MAS[1-3], Alexandre G. Lellouch MD PhD[1-3,6], Curtis L. Cetrulo Jr. MD FACS FAAP[1-3]#, Korkut Uygun PhD[1-4]#

Journal publications.

Two publications being prepared for submission based on the meeting abstracts noted above in the dissemination section. A third small article on method development is under preparation.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report yet.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

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

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Curtis Cetrulo, MD
Project Role: Co-Principal Investigator
Nearest person month worked: 1
Contribution to Project: Dr. Cetrulo is responsible for overall design and direction of proposed studies, and interpretation of results.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

See attached Other support documentation. No effects on the effort in this project.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

See attached quad chart.

9. APPENDICES:

No additional document to report.