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TITLE: Diagnosis and Treatment of TBI and Polytrauma during Ground and High-Altitude Evacuation using Liquid Biopsy and Extracorporeal Life Support

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14. ABSTRACT
TBI alone and in combination with polytrauma and lung injury caused up to 83% of "nonsurvivable" combat-related deaths. There is no accurate diagnosis method or viable therapeutic intervention for these casualties primarily due to the severity of injury, which can be unrecognized early on. Our proposal will address these unmet needs via utilization of a model of TBI with targeted descriptors of injury severity derived from bedside cell free DNA (cfDNA) testing, and then via addition of polytrauma and lung injury with subsequent testing of therapeutic intervention via extracorporeal life support (ECLS).

15. SUBJECT TERMS
Traumatic brain injury; extracorporeal life support; cfDNA; biomarkers

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INTRODUCTION:

TBI alone and in combination with polytrauma and lung injury caused up to 83% of "nonsurvivable" combat-related deaths. There is no accurate diagnosis method or viable therapeutic intervention for these casualties primarily due to the severity of injury, which can be unrecognized early on. Our proposal will address these unmet needs via utilization of a model of TBI with targeted descriptors of injury severity derived from bedside cell free DNA (cfDNA) testing, and then via addition of polytrauma and lung injury with subsequent testing of therapeutic intervention via extracorporeal life support (ECLS). Objectives: Base Phase 1: 1) identify and characterize biomarkers specific to TBI alone and with polytrauma; compare to control animals without injury; 2) improve understanding of risk and genetic pathophysiologic factors in TBI by determining plasma levels of various injury severity markers; Phase 2: 1) develop targeted therapeutic applications for ECLS devices and clinical guidelines to improve diagnosis, stabilization, and treatment of TBI; compare therapeutic effects of ECLS with standard of care in 72 hour studies relevant to PFC; 2) develop CPGs to treat TBI with polytrauma and hemorrhage; Phase 3: assess impact of altitude, vibration, and temperature and how they affect TBI outcomes in animals with TBI and polytrauma treated with and without ECLS at high altitude. This proposal improves characterization, diagnosis and treatment of neurotrauma resulting from TBI including precise characterization and individualized assessment of the specific TBI pathology; targeted diagnosis of TBI using liquid biopsy; use of treatment and stabilization/recovery protocols using brain sparing resuscitation and life preservation via extracorporeal life support and mechanical ventilation: all studied during ground and high altitude evacuation phases of en-route care. We test therapeutic interventions while carrying out digital data recording and development of knowledge products on utility of life-preservation approaches for surgical patients with massive combined trauma.

1. **KEYWORDS:**

   Traumatic brain injury; extracorporeal life support; cfDNA; biomarkers

2. **ACCOMPLISHMENTS:**

   What were the major goals of the project?

   Base Phase 1

   <u>Specific Aim 1:</u> Identify cfDNA, DAMPs and other injury severity markers in TBI alone and in combination with polytrauma and bleeding (months 1-12)

   <u>Major Task 1:</u> Regulatory approval (months 1-2)

   Subtask 1: Write animal use protocol and obtain IACUC and ACURO approvals for research (months 1-2)

   <u>Major Task 2:</u> Animal experiments and sample collection (months 2-12)

   Subtask 2: Conduct time control experiments (n=6, 48 hours duration) and collect samples for all planned assays (months 2-12)

   Subtask 3: Conduct Group A (TBI) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Subtask 4: Conduct Group B (ARDS from trauma [bilateral pulmonary contusion]) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Subtask 5: Conduct Group C (TBI + ARDS) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Specific Aim 2: Screen transcriptional cfDNA responses modulated by TBI alone, and in combination with polytrauma and ARDS (months 2-12)

Major Task 3: Characterize cfDNA and severity markers (months 2-12)

Subtask 6: Conduct cfDNA analysis by CGT method on samples collected in Specific Aim 1 (months 2-12):

Subtask 7: Conduct amplifiability examination of cfDNA samples (months 2-12)

Subtask 8: Characterize organ- and injury-specific markers and severity descriptors defined by analysis in Subtasks 6 & 7 in Groups A-C (months 2-12)

Subtask 9: Define dose-response relationship of cfDNA expression in TBI alone, ARDS from trauma alone, and TBI + ARDS (months 2-12)

Optional Phase 2

Specific Aim 3: ECLS as a targeted therapeutic intervention in TBI (months 13-24)

Major Task 4: Therapeutic animal studies (months 13-24)

Subtask 10: Conduct Group D (TBI+ARDS, treatment with lung-protective ventilation and judicious fluid management) animal experiments (n=12, 72 hours in duration) (months 13-24)

Subtask 11: Conduct Group E (TBI+ARDS, treatment with VV ECLS) animal experiments (n=12, 72 hours in duration) (months 13-24)

Subtask 12: Characterize organ- and injury-specific markers and severity descriptors from Groups D & E (months 13-24)

Subtask 13: Draft and publish CPGs for ECLS usage as therapeutic intervention in TBI and ARDS from polytrauma (months 22-24)

Optional Phase 3

Specific Aim 4: ECLS as a life-saving intervention for TBI during aeromedicalc evacuation: effects of altitude, vibration, and temperature changes as well as feasibility of VV ECLS in a model of PFC (months 25-36)

Major Task 5: Therapeutic animal studies with transport (months 25-36)

Subtask 14: TBI+Polytrauma group, treatment with standard of care) animal experiments (n=8, 72 hours in duration) including 7-stage ground and high-altitude evacuation (months 25-36)

Subtask 15: TBI+Polytrauma group, treatment with VV ECLS) animal experiments (n=8, 72 hours in duration) including 7-stage ground and high-altitude evacuation (months 25-36)

Subtask 16: Characterize organ- and injury-specific markers and severity descriptors from Groups F & G (months 25-36)

Subtask 17: Characterize effects of stressors of flight (altitude, vibration, temperature) on TBI management with VV ECLS (months 25-36)

Subtask 18: Draft and publish CPGs for VV ECLS management of TBI during aeromedical evacuation (months 34-36)

<u>Major Task 6:</u> Study completion (months 34-36)

Subtask 19: Preparation of manuscripts and technical reports on findings of study (months 34-36)

What was accomplished under these goals?

<u>Specific Aim 1:</u> Identify cfDNA, DAMPs and other injury severity markers in TBI alone and in combination with polytrauma and bleeding (months 1-12)

<u>Major Task 1:</u> Regulatory approval (months 1-2)

Subtask 1: Write animal use protocol and obtain IACUC and ACURO approvals for research (months 1-2)

Task Status: started Y1Q1, 100% completed Y1Q1, however unforeseen delays required re-approval. New regulatory approval completed Y1Q3, 100% completed.

<u>Major Task 2:</u> Animal experiments and sample collection (months 2-12)

Task Status: 87% animals completed. All animals in injury groups completed; only 2 out of 6 healthy controls were carried out and both had pneumonia due to a swine viral outbreak. We are planning to do the remainder 4 in the Summer months between the seasonal swine flu periods.

Subtask 2: Conduct time control experiments (n=6, 48 hours duration) and collect samples for all planned assays (months 2-12)

Subtask Status: 33% animals completed. (see above)

Subtask 3: Conduct Group A (TBI) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Subtask Status: 100% animals completed

Subtask 4: Conduct Group B (ARDS from trauma [bilateral pulmonary contusion]) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Subtask Status: 100% animals completed

Subtask 5: Conduct Group C (TBI + ARDS) experiments (n=8, 48 hours duration) and collect samples for all planned assays (months 4-12)

Subtask Status: 100% animals completed

Methods and results for Major Task 2 for the reporting period:

Animal work was started on 3 February 2020, paused in March 2020 due to COVID-19, and resumed 1 June 2020. Animal work continued through the remainder of the Y2 POP. Summary of the animal experiments completed this reporting period are below:

Animal #	Group	Date	Wt (kg)	Time to ARDS (hrs)	Survival (hrs)	Included
3	Group A	2-March-20	52.0	9	48	Yes
4	Group C	1-Jun-20	55.4	16	24	Yes
5	Group C	8-Jun-20	59.8	#N/A	48	Yes
6	Group A	15-Jun-20	57.4	17	48	Yes
7	#N/A	22-Jun-20	61.4	#N/A	#N/A	No
8	Group B	7-Jul-20	61.0	0	17	Yes
9	Group B	14-Jul-20	53.6	1	21	Yes

10	Group C	20-Jul-20	49.2	0	15	Yes
11	Group C	27-Jul-20	56.4	#N/A	48	Yes
12	Group C	3-Aug-20	56.8	0	6	Yes
13	Group A	10-Aug-20	61.0	21	48	Yes
14	Group A	17-Aug-20	52.4	22	48	Yes
15	Group A	24-Aug-20	58.2	0	48	Yes
16	Group B	31-Aug-20	55.2	1	14	Yes
17	TC	14-Sep-20	47.6	#N/A	48	Yes
18	#N/A	21-Sep-20	57.4	18	13	No
19	#N/A	28-Sep-20	58.2	0	5	No
20	Group B	5-Oct-20	61	0	48	Yes
21	Group C	13-Aug-20	61.8	#N/A	48	Yes
22	Group B	19-Oct-20	52.6	0	22	Yes
23	Group B	16-Nov-20	44.4	0	1	Yes
24	Group A	30-Nov-20	56.0	24	37	Yes
25	#N/A	7-Dec-2020	53.4	#N/A	#N/A	No
26	Group C	14-Dec-20	44.2	0	17	Yes
27	Group B	5-Jan-21	48.6	0	19	Yes
28	Group C	11-Jan-21	49.6	0	1	Yes
29	Group A	19-Jan-21	49.0	#N/A	48	Yes
30	Group B	25-Jan-21	56.2	0	30	Yes

Table 1. Large animal experiments completed during Y2 POP. Animal numbers 7, 18-19, and 25 were completed, but subsequently excluded from data analysis due to complications; they remain included here for transparency. We plan to replace them in accordance with our approved animal protocol policies. Group TC = Time Control

Key Findings or Accomplishments for Specific Aim 1:
Phase 1 injured animal experiments have been completed.

<u>Specific Aim 2:</u> Screen transcriptional cfDNA responses modulated by TBI alone, and in combination with polytrauma and ARDS (months 2-12)

<u>Major Task 3:</u> Characterize cfDNA and severity markers (months 2-12)
Task Status: started Y1Q6, 50% completed
PI Dr. Batchinsky and his co-investigator Dr. Choi held repeat (5) conference calls with Dr. Yeh. During these calls, the teams discussed in detail the COVID 19 delays and their effects on Dr. Yeh's laboratory. Dr. Batchinsky and Dr. Choi expressed concerns to Dr. Yeh that no organ specific cfDNA analysis has been performed to date by Dr. Yeh. Dr. Yeh assured the San Antonio teams that he and his lab will resume activities in the next performance period and will catch up to all performed analyses in December of 2020.

The PI received communication from Dr. Yeh on Feb 12 stating that they are still trying to work out the protocol for subtasks 6 and 7.

However, to date, Dr. Yeh has not completed the organ specific marker development citing absence of swine-specific marker reference maps and inability to adapt the human cancer reference maps as he thought would be possible at the beginning of the grant.

We are in discussions about possible alternative analyses to be performed by CIRCULOGENE (conference call planned between CEO, Dr. Yeh and PI and team).

Subtask 6: Conduct cfDNA analysis by CGT method on samples collected in Specific Aim 1 (months 2-12)
Subtask Status: Ongoing, 87% completed by CIRCULOGENE.

Subtask 7: Conduct amplifiability examination of cfDNA samples (months 2-12)
Subtask Status: Ongoing, 87% completed by CIRCULOGENE.

Subtask 8: Characterize organ- and injury-specific markers and severity descriptors defined by analysis in Subtasks 6 & 7 in Groups A-C (months 2-12)
Subtask Status:
Geneva work: systemic concentration of cfDNA, systemic DAMPs (HMGB1 and TLR4), systemic GFAP analysis, systemic plasma free hemoglobin - 87% completed. Systemic S100β analysis – 10% completed.
CIRCULOGENE work: not started, 0% completed for characterize organ- and injury-specific marker from cfDNA. Pending development and execution of new method by CIRCULOGENE

Subtask 9: Define dose-response relationship of cfDNA expression in TBI alone, ARDS from trauma alone, and TBI + ARDS (months 2-12)
Subtask Status: not started, 0% completed by CIRCULOGENE.

Methods and results for Major Task 3 for the reporting period:
In this reporting period, plasma samples from all animals completed to date (87%= *Time Control n=2, Group A n=8, Group B n= 8, Group C n=8*) were sent to CIRCULOGENE for analysis. Samples from excluded animals were not sent for cfDNA analyzing. The samples from these studies enabled CIRCULOGENE to continue their fine-tuning of cfDNA extraction by CGT method and amplification of cfDNA (*Subtask 6 and 7*). A total of 30 animal experiments were done in the current period. A total of 26 animal samples were used for laboratory base analyzing for systemic cfDNA, HMGB1, TLR4, GFAP, S100β, total plasma protein concentration (TPPC), plasma free hemoglobin (pfHb) to verify the injury severity of our model for the current project. All tissues samples were collected at end of study for histology analysis for injury severity observation, and wet-to-dry organ edema measurement.

Key Findings or Accomplishments for Specific Aim 2:
Higher cfDNA concentration was seen on post TBI, post chest contusion and post shock in animals with early mortality. The level of cfDNA may be associated with other DAMPs including kidney and brain damage biomarkers, indicating cfDNA concentration is an indicator of injury. It also assists in detecting multiorgan damage and could be useful to develop other applications in point-of-care testing to guide therapeutic interventions, aimed at reducing mortality.

Histological analysis

Hemorrhage confirmed on the frontal cortex and temporal lobe (Panel A, below), near auditory cortex, and vision cortex. Edema was observed on the brain stem by hematoxylin and eosin staining (Figure 1, yellow arrows showing hemorrhage and cell necrosis) on the Group A animal.

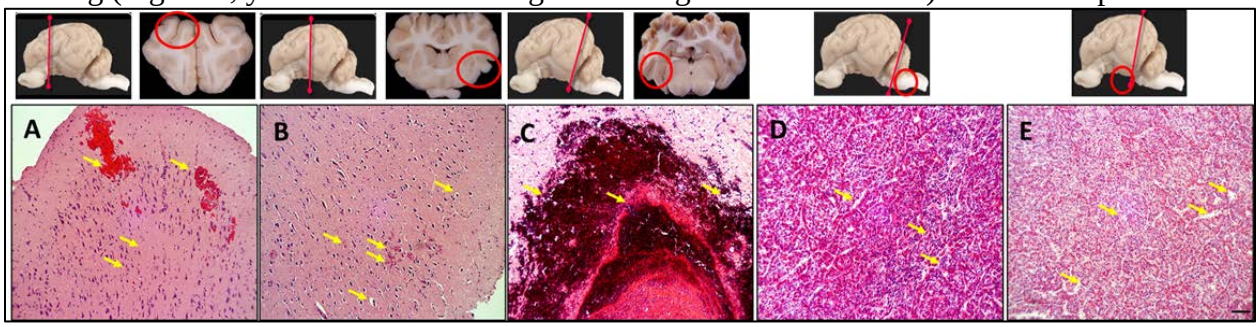

Figure 1. Histological appearance of brain damage after blunt TBI in this model

Severe edema was observed from all injured animals in Group A, B, and C. Yellow arrows indicated degeneration of acidophil over expressed basophil and hemorrhage on interstitial area. -Note that Time Control (Figure 2A) animal had pneumonia and this slide does not constitute completely normal physiology. Figure 2B shows specific pituitary injury in Group A animal with blood congestion with acidophil and basophil cell necrosis. Figure 2C shows Group B pulmonary contusion only injury and indicate severe interstitial edema with combined effects of high-volume fluid resuscitation and use of continuous rate epinephrine. Figure 2C show pituitary injury of Group C indicating similar injury pattern with Group A for cell necrosis with blood congestion.

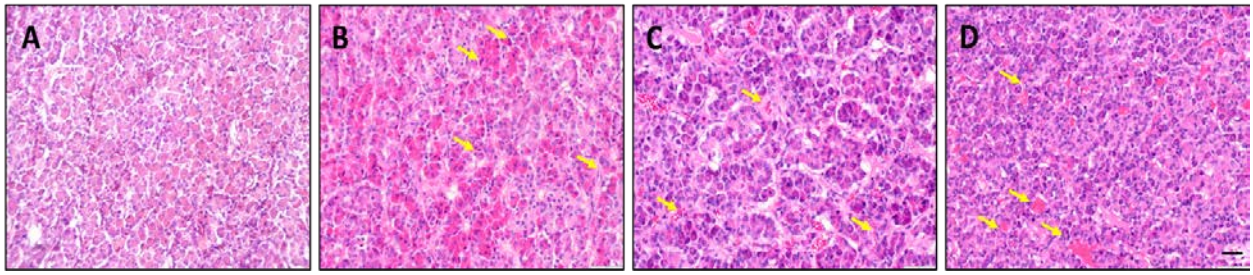

Figure 2. Histology image of traumatic brain injury for anterior pituitary (A) Time control (B)Group A (TBI) (C) Group B (PC) (D) Group C (TBI+PC). 200X magnification; scale bar 50um.

Cell free DNA, biomarkers analysis

Higher cfDNA concentration was increased after injury phase (Figure 3) on all the injury groups (time control group not included here).

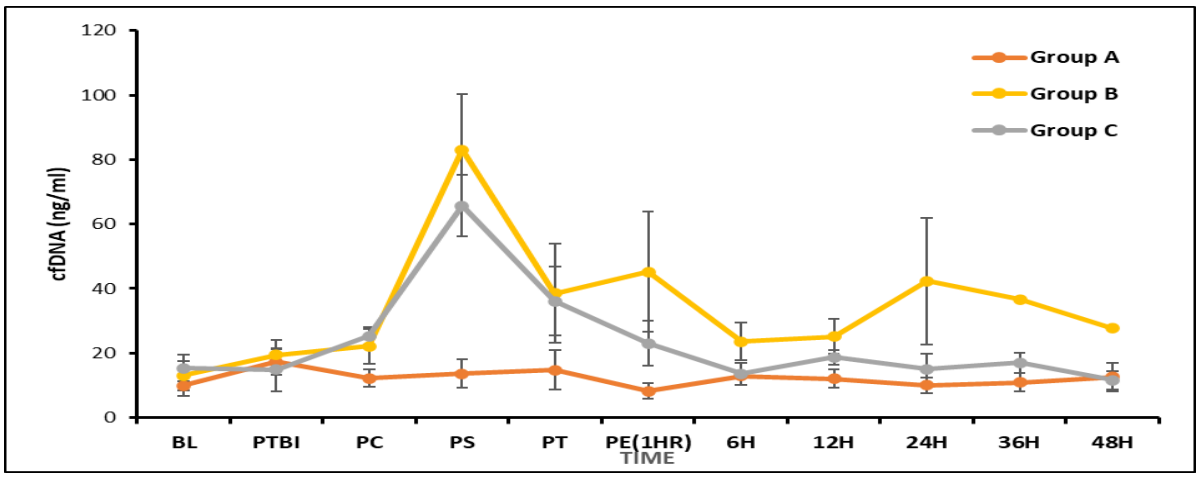

*Figure 3. Cell-free DNA levels. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury. †indicates between group difference, *indicates difference vs. baseline; $p<0.05$.*

Systemic HMGB1 was measured for injury severity assessment. Systemic HMGB1 was measured by ELISA (Tecan, Germany). Increasing level of HMGB1 was observed from PTBI, PC and PE time point of all injured animals (Figure 4).

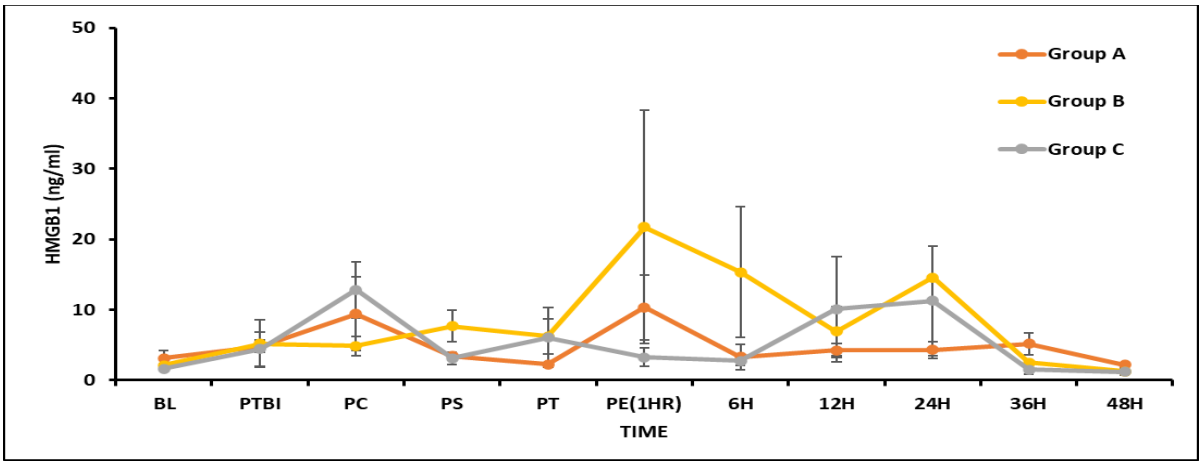

Figure 4. Systemic HMGB1 levels. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury.

Systemic TLR4 was measured for injury severity assessment. Systemic TLR4 was measured by ELISA (Abcam, USA) on samples collected from animal numbers 1 to 23. Increasing level trend of TLR 4 was observed at 12H of Group B, 36H of control and Group A (Figure 5).

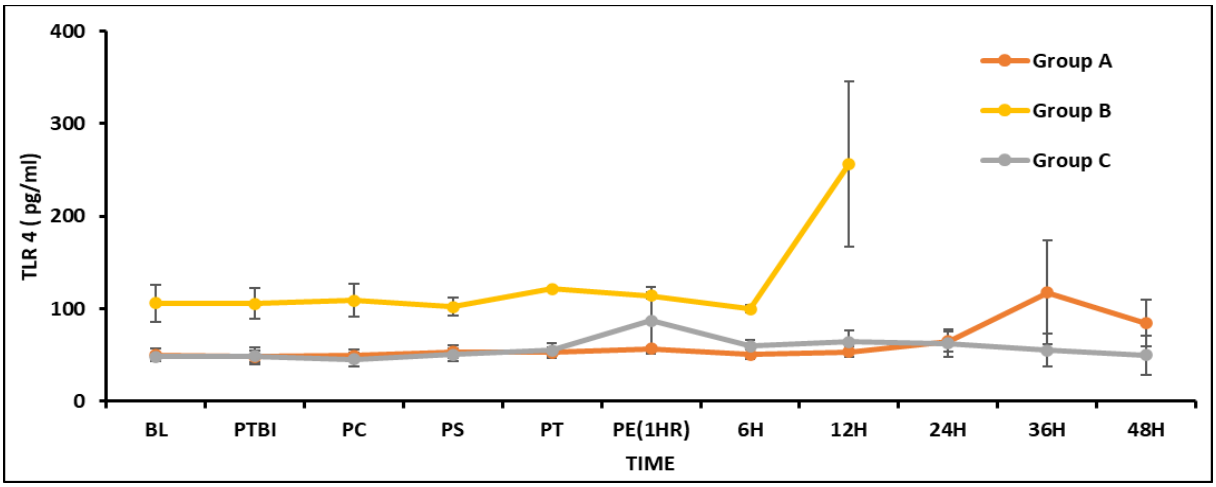

Figure 5. Systemic levels of TLR4. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury. Group B graph truncated beyond 12 hours because of loss of analyzed ELISA samples. This data will be provided later when banked samples will be re-analyzed.

Our current S100β analysis revealed a problem (lack of sensitivity) with the assay (LS Bio, USA) and we are acquiring a different assay (human-based assay) to perform the analysis on banked/frozen plasma samples.

Systemic GFAP was measured by ELISA (LS Bio, USA) for brain injury severity assessment. Higher than normal level of GFAP was detected on BL and no difference in level of GFAP was observed between groups. We are acquiring a different assay to verify the second value of systemic level of GFAP the analysis on banked/frozen plasma samples (Figure 6).

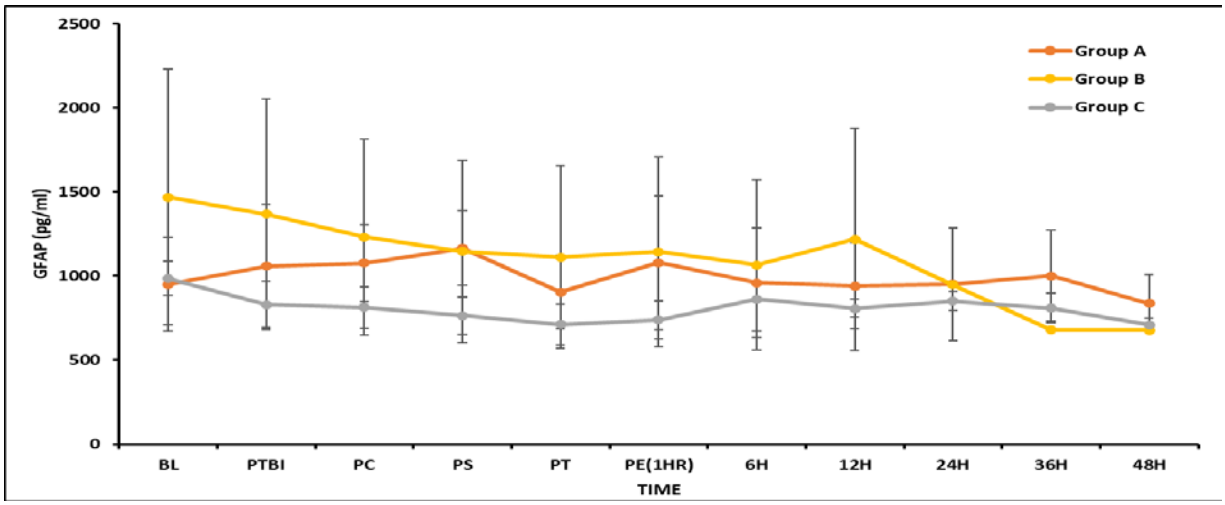

Figure 6. Systemic GFAP levels. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury.

Total plasma protein concentration was measured to verify organ permeabilization with Pierce™ BCA Protein method. No difference in level of TPPC was observed between groups (Figure 7).

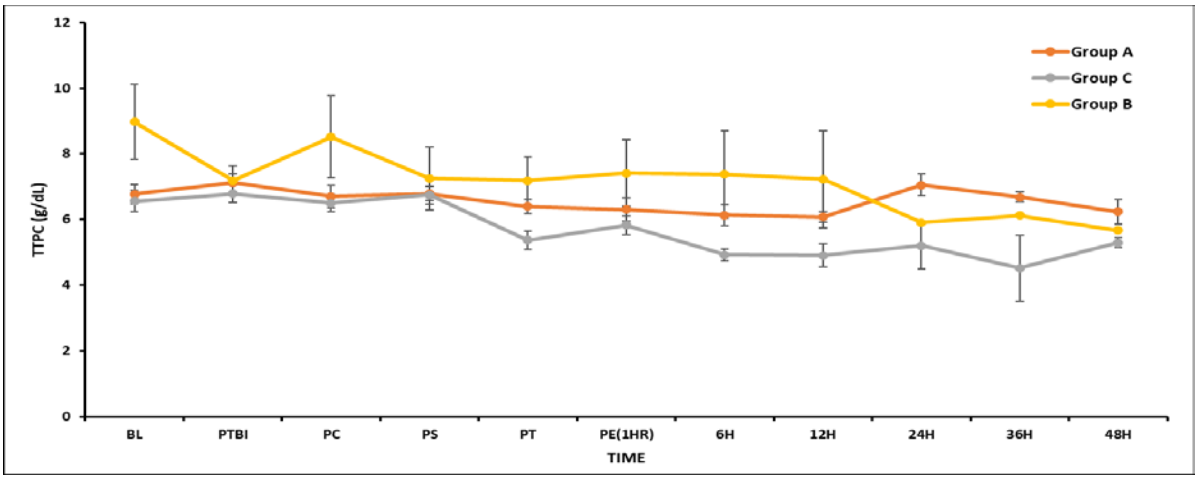

Figure 7. Total plasma protein concentration (TPPC) level. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury.

Plasma free hemoglobin (pfHb) was measured for red blood cell hemolysis injury severity assessment. The pfHb was measured by Spectramax (molecular diagnosis, USA). Increasing level of pfHb was observed from injury phase and decreased until 24h time point of all animals. No difference in level of pfHb was observed between groups (Figure 8).

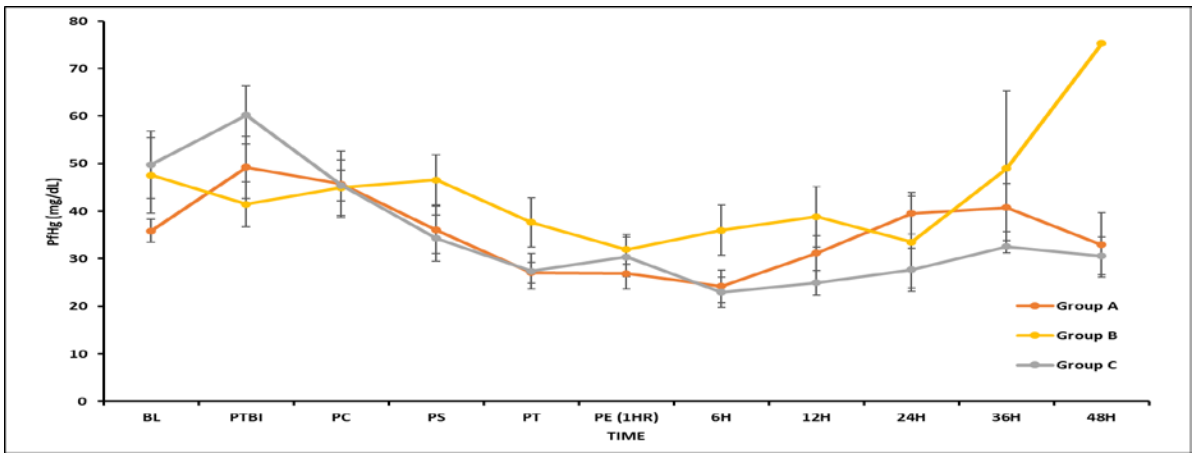

Figure 8. PfHb levels. BL: Baseline; PTBI: Post-TBI; PC: Post-Pulmonary Contusion; PS: Post-Shock; PT: Post-Transfusion; PE(1HR): Post-ECLS / 1 Hour Post-Injury.

In summary, we performed all injured animals as scheduled in Phase 1; The two planned and performed experiments with control animals developed pneumonia due to preexisting flu-like symptoms. The control animals will be carried out in year 2 (which resulted in some underspending of year 1 funds). In addition, we have not completed major equipment purchases due to vendor delays which also led to underspending.

We are formally analyzing the data collected to date; what we learned to date is that TBI leads to post-contusion hypertension and tachycardia; metabolic acidosis and autonomic dysfunction manifested in drifting heart rate and blood pressure independent of medication and fluid status.

When combined with lung injury, TBI requires careful titration of $PaCO_2$ in the post injury period, complicated by concomitant needs to remove CO_2 by the ventilator to reduce mixed metabolic acidosis. In several animals this competing priority to regulate PaCo2 for brain-specific vs lung-specific reasons caused significant management challenges, resolution of which will be more easily achieved in Phase 2 of the study when selective CO_2 removal will be regulated by the ECLS circuit rather than the ventilator alone. The model of polytrauma with TBI which we mastered here in this phase will undoubtedly relate best to the casualties which currently die from TBI with polytrauma permitting us to find new therapeutic solutions.

Work performed by CIRCULOGENE

For Subtask 6, CIRCULOGENE received plasma samples from Dr Batchinsky's team and extracted cfDNA. All samples sent from Dr Batchinsky's team have been analyzed.

For Subtask 7, CIRCULOGENE conducted amplifiability examination of extracted cfDNA samples to report as a final total concentration of data

For subtask 8 and 9 as provided for this report by Dr. Yeh: During the slow-down caused by COVID-19, CIRCULOGENE started data mining from deposited human cfDNA data to identify tissue-specific gene markers to match with currently known cfDNA information. CIRCULOGENE set out to identify tissue-specific RNA biomarkers by *in silico* data mining among several resources such as the Human Protein Atlas (HPA), Tissue-specific Gene Expression and Regulation (TiGER), Genotype-Tissue Expression (GTEx), TissGDB and TCGA databases across eight categories of annotations: TissGeneSummary, TissGeneExp, TissGene-miRNA, TissGeneMut, TissGeneNet, TissGeneProg, and TissGeneClin. They have initially identified 18 tissue-specific biomarkers for lung, brain, liver, colon, breast, pancreatic, bone marrow and prostate cancers. Two representative biomarkers are illustrated below in Figures 10 and 11.

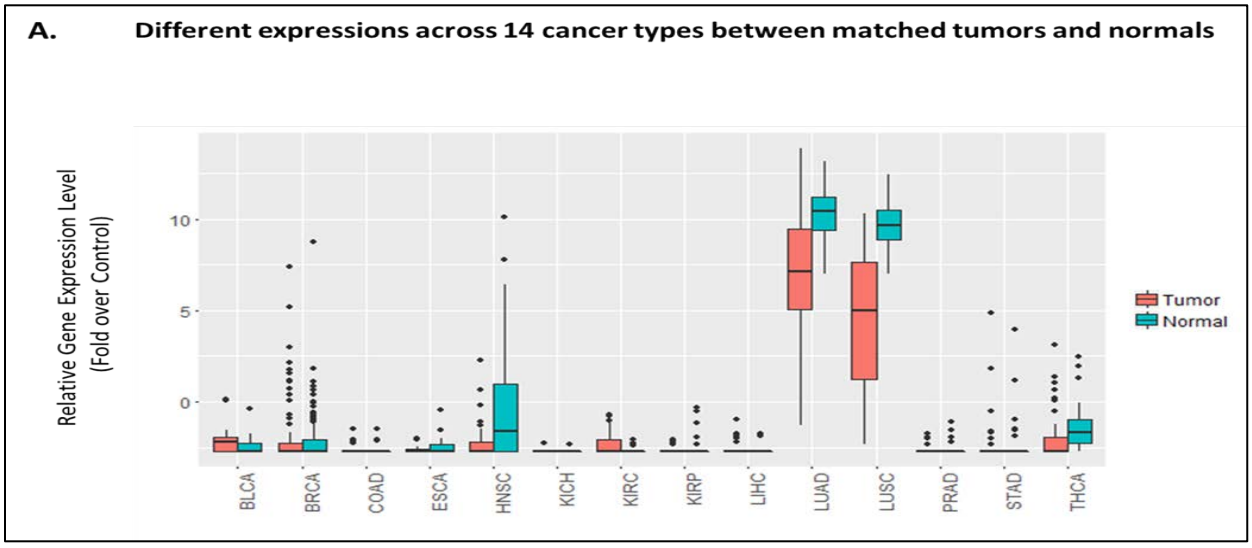

Figure 10. Expression of biomarkers across 14 cancer types compared to tumor tissue and normal tissue.

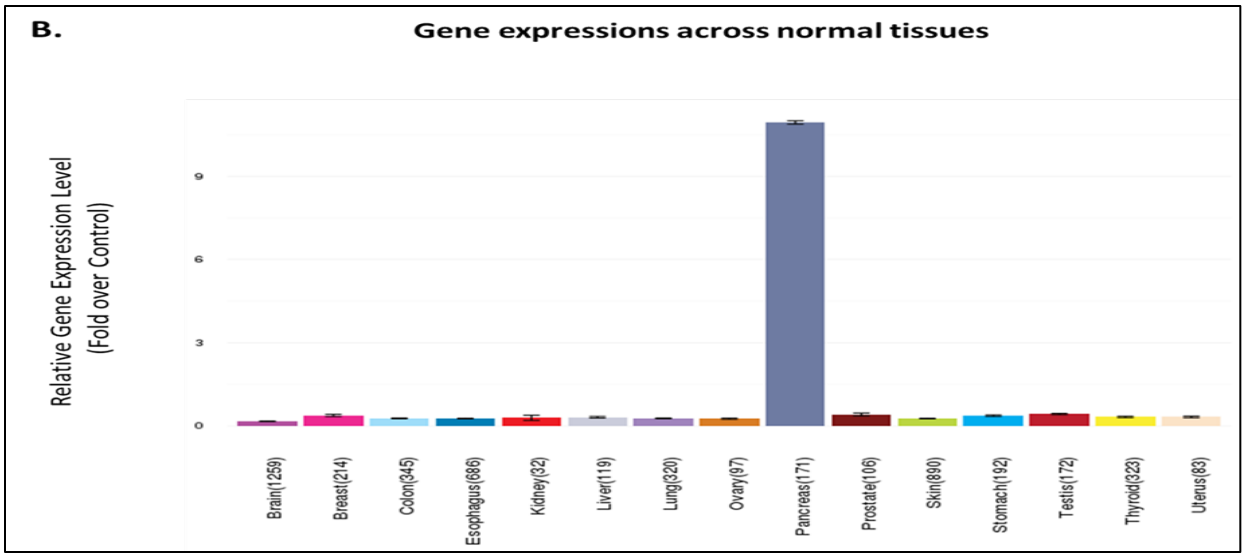

Figure 11. Gene expression across normal tissues as seen in different types of cancers.

Next, CIRCULOGENE planned to validate the tissue origin of these 18 biomarkers using plasma cfRNA or cfDNA from various archived samples housed at CIRCULOGENE. If this proof-of-concept study succeeds, we will extend our finding to the current swine study to enhance the scientific output of this effort.

Besides cfDNA/cfRNA, the most important materials in this study are reference DNA and RNA from each organ that we are targeting - Brain, Lung, Liver, kidney, Spleen, Small Intestine and Blood. These organ-specific DNAs/RNAs will serve as “genetic maps” to tell us where the elevated cfDNA/cfRNA are coming from.

Swine organ-specific DNAs/RNAs and assay reagents/buffers will be procured in the coming months. We expected and planned to start assay development in early Sept. 2020 and have preliminary data available in December 2020. However, organ specific biomarkers for cfDNA had delayed complications of the molecular biological methods.

Andriy Batchinsky PI comments with respect to collaborator performance:

> The efforts by CIRCULOGENE have been productive with respect to sample collection and overall analysis of cfDNA amounts, but has not led to a viable option to differentiate the specific injury signatures attributed to lung, kidney and heart specific cfDNA expression. More importantly, the TBI specific cfDNA work has not succeeded. At present, we are evaluating if the continued work with CIRCULOGENE will lead to a meaningful way forward.

Optional Phase 2 –*started with a partial calendar overlap with Phase 1 work and is provided here for reference. This material and all other work performed in Phase 2 will be also subject to reporting in the Phase 2 annual report.*

Specific Aim 3: **ECLS as a targeted therapeutic intervention in TBI (months 13-24)**
Methods and results for Major Task 4 for the reporting period:
Animal work was started on 1 February 2021 and a summary of the animal experiments completed this reporting period are below:

Animal #	Group	Date	Wt (kg)	Time to ARDS (hrs)	Survival (hrs)	included
1	Group D	1-Feb-21	55.4	0	21	Yes
2	Group E	8-Feb-21	61.6	24	72	Yes

Major Task 4: **Therapeutic animal studies (months 13-24)**
Task Status: Y2Q1 started, 8% complete.

Subtask 10: Conduct Group D (TBI+ARDS, treatment with lung-protective ventilation and judicious fluid management) animal experiments (n=12, 72 hours in duration) (months 13-24)
Subtask Status: started Y2Q1, 8.3% of group completed. 1 animal experiment in this group conducted 1 Feb 2021.

Subtask 11: Conduct Group E (TBI+ARDS, treatment with VV ECLS) animal experiments (n=12, 72 hours in duration) (months 13-24)
Status: started Y2Q1, 8.3% of group completed. 1 animal experiment in this group conducted 8 Feb 2021.

Subtask 12: Characterize organ- and injury-specific markers and severity descriptors from Groups D & E (months 13-24)
Status: not started

Subtask 13: Draft and publish CPGs for ECLS usage as therapeutic intervention in TBI and ARDS from polytrauma (months 22-24)
Status: not started.

Methods and results for Major Task 4 (Phase 2) for the reporting period:
In the last reporting period, Y1Q7, plasma samples from animals completed to date (Group D n=1, Group E n=1) for *Subtask 10 and 11*. *Subtask 12 has not been started due to* delayed development of the new method for organ specific biomarker for cfDNA by CIRCULOGENE.

What opportunities for training and professional development has the project provided?
Nothing to report this period.

What do you plan to do during the next reporting period to accomplish the goals?
We have scheduled animal experiments throughout the next reporting year to continue this work.

To be ready and achieve the delayed CIRCULOGENE Year 1 goals, their team is setting out a new assay development and validation plan to identify the sources of elevated cfDNA, i.e., injured organs. We plan to tackle this issue from two angles: by cfDNA epigenetic sequence pattern; and by cost- and time-efficient hybridization fluorescent assay. These two approaches

can only be made possible through CIRCULOGENE's validated Linear In Situ Amplification (LISA) technology, thanks to its high yield of cfDNA/cfRNA.

Besides cfDNA/cfRNA, the most important materials in this study are reference DNA and RNA from each organ that we're targeting - Brain, Lung, Liver, kidney, Spleen, Small Intestine and Blood. These organ-specific DNAs/RNAs will serve as "genetic maps" to tell us where the elevated cfDNA/cfRNA are coming from.

No other progress has been made to date on sample analysis by CIRCULOGENE due to delays with COVID 19 and lack of availability of swine specific injury marker reference maps.

PI comment: If CIRCULOGENE does not deliver on tasks 8 and 9 we shall replace the brain specific injury severity markers originally pursued by CIRCULOGENE in favor of a new hand-held device used for diagnosis of TBI in humans. Discussions with the company doing this work are underway and the science officer will be notified as soon as we have a definitive answer to the way forward.

3. **IMPACT: Describe 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?
   Nothing to report this period.

   What was the impact on other disciplines?
   Nothing to report this period.

   What was the impact on technology transfer?
   Nothing to report this period.

   What was the impact on society beyond science and technology?
   Nothing to report this period.

4. **CHANGES/PROBLEMS:**
   Changes in approach and reasons for change
   Nothing to report this period.

   Actual or anticipated problems or delays and actions or plans to resolve them
   Throughout this POP, the PI Dr. Batchinsky and his co-investigator Dr. Choi held repeat (5) conference calls with Dr. Yeh. During these calls, the teams discussed in detail the COVID 19 delays and their effects on Dr. Yeh's laboratory. Dr. Batchinsky and Dr. Choi expressed concerns to Dr. Yeh that no organ specific cfDNA analysis has been performed to date by Dr. Yeh. Dr. Yeh assured the PI that he and his lab will resume activities in the next performance period and will catch up to all performed analyses in December of 2020. However, Dr Yeh have not processed any organ specific biomarkers for cfDNA due to lack of laboratory resources by COVID 19 until current.

Dr Yeh proposed an alternative method for Subtask 8 - organ-specific signal expression analysis ongoing at CIRCULOGENE to apply a new type of method for developing a “bisulfite methyl-sequencing” protocol. This approach aims to remedy CIRCULOGENE’s current failed attempt to carry out organ specific methylation analyzing due to (1) inappropriate and low conversion frequencies under the conventional bisulfite-conversion protocol; (2) low yield of sequencing library; (3) too much noisy background signal; and (4) low-quality and low-coverage sequencing reads. However, the PI would like to officially notify the science officer that despite repeat requests to show and share data from the failed attempts, CIRCULOGENE refused to provide evidence of failed attempts stating that unsuccessful runs simply did not generate any data.

In addition, Dr. Yeh stated that he is unable to develop and analyze the swine organ-specific injury severity markers that CIRCULOGENE was hoping to transfer from their previous analysis of human data. Given the fact that methylation-sequencing is a labor-intensive, time-consuming and very costly assay, CIRCULOGENE thus plan to develop a higher throughput, cost- and time-efficient hybridization fluorescent assay to track and identify organ injury in Q1 2021.

The proposed alternative plan is: 1) CIRCULOGENE will buy commercially available swine tissue genomic DNA (gDNA) from brain, lung, liver, kidney, and blood as reference materials; 2) Isolate methylated DNA from these tissue DNA samples. 3) Prepare swine cfDNA from the AREVA lab animal samples using their proprietary technology and fluorescent labeling of these cfDNA as probes. 4) Hybridization of each of the 5-tissue methylated genomic DNAs with fluorescent cfDNA probes. 5) Analyze fluorescence signals to determine the source of signals according to tissue references.

Dr. Yeh, stated that his goal is to complete the above experiments by 1 March 2021. Subject to reporting in the next period.

Finally, and most importantly, the PI would like to report that the experimental and therapeutic part and objectives/tasks of this study are progressing extremely well in the AREVA lab and we have no concerns with successful completion of the overall grant as scheduled. We have identified clinically relevant mortality (Table 1) in non-control groups of animal experiments, which reinforces the clinically relevance and utility of our model.

If the event that CIRCULOGENE will prove to be a non-viable solution for execution of their assigned subtasks, the AREVA team has already began discussions with a different diagnostic company that potentially could replace and enhance diagnostics of TBI and polytrauma severity instead of CIRCULOGENE. The decision to replace sub-awardees will be made jointly with the Science officer and only with his/her approval.

Changes that had a significant impact on expenditures
Nothing to report this period.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report this period.

Significant changes in use or care of human subjects
None required to complete SOW.

Significant changes in use or care of vertebrate animals.
None.

Significant changes in use of biohazards and/or select agents
None required to complete SOW.

5. **PRODUCTS:**

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.
None this reporting period.

Books or other non-periodical, one-time publications.
None this reporting period.

Other publications, conference papers, and presentations.
Oral presentations:
None this reporting period.

Posters:
1. Garcia I, Choi J, Roberts T, et al. Increase in Systemic Levels of Damaged Associated Molecular Patterns Separates Non-Survivors from Survivors in a Model of Severe Smoke Inhalation and Burns. Presented at *36th Annual Children's National Symposium: ECMO and the Advanced Therapies for Cardiovascular and Respiratory Failure (online conference)*. Keystone, CO 2020.
2. Choi J, Roberts T, Wendorff D, Necsoiu C, Jordan B, Sieck K, Beely B, Cancio L, Batchinsky A. Local Expression of HMGB1, TLR4, AQP5 and TGFB1 in ARDS Due to Smoke Inhalation and Burns in Swine Treated with Minimally Invasive Extracorporeal Life Support. Presented at *Military Health System Research Symposium*. Kissimmee, FL; 2019.
3. Choi J, Roberts T, Wendorff D, Necsoiu C, Jordan B, Sieck K, Beely B, Cancio L, Batchinsky A. Systemic Expression of Damage Associate Molecule Pattern (DAMP)s After ARDS Due to Smoke Inhalation and Burns in Swine Treated with Extracorporeal Life Support. Presented at *Military Health System Research Symposium*. Kissimmee, FL; 2019.
4. Garcia I, Willis RP, Lee J, Roberts T, Wendorff D, Beely B, Harea G, Sieck K, Batchinsky A, Choi J. Injury Severity Validation with Bronchoalveolar Lavage Cell Analysis: Expression of HMGB1 and TLR4 After Smoke Inhalation Injury and Burns in Swine Treated with Extracorporeal Life Support. Presented at *Military Health System Research Symposium*. Kissimmee, FL; 2019.

Website(s) or other Internet site(s)
None this reporting period.

Technologies or techniques
None this reporting period.

Inventions, patent applications, and/or licenses
None this reporting period.

Other Products
None this reporting period.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Andriy I. Batchinsky, MD
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-8601-2827
Nearest person month worked:	1.7
Contribution to Project:	Overseeing and carrying out the project protocol, collecting and analyzing data, and preparing and finalizing manuscripts and reports.
Name:	Teryn Roberts
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.1
Contribution to Project:	Carrying out the project protocol, collecting and analyzing data, preparing and finalizing manuscripts and reports.
Name:	Jae Choi, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.8
Contribution to Project:	Carrying out the project protocol, carrying out pathological assessment and assays, and preparing manuscripts and reports.
Name:	Brendan Beely
Project Role:	Research Coordinator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.5
Contribution to Project:	Performing routine laboratory procedures, assisting with study protocols, and preparing reports.
Name:	Dan Wendorff
Project Role:	Laboratory Manager
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.2

Contribution to Project:	Performing routine laboratory procedures, preparation of animal protocol and oversight of IACUC review, overseeing Lab. Techs.
Name:	John Jones
Project Role:	Statistician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Assisting with data organization, statistical analyses including power analysis, and data interpretation, as well as contributing to manuscripts and reports.
Name:	George Harea
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Assisting with procurement requests, study design, and assisting with data collection and interpretation.
Name:	Isabella Garcia
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.2
Contribution to Project:	Assisting with large animal handling (anesthesia/sedation, subject monitoring, and drug administration), assisting with data collection and interpretation.
Name:	Ji Lee
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.1
Contribution to Project:	Assisting with large animal handling (anesthesia/sedation, subject monitoring, and drug administration), assisting with data collection and interpretation.
Name:	Hailee Alaniz
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.2
Contribution to Project:	Assisting with large animal handling (anesthesia/sedation, subject monitoring, and drug administration), assisting with data collection and interpretation.
Name:	Clayton Smith
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.2

Contribution to Project:	Assisting with large animal handling (anesthesia/sedation, subject monitoring, and drug administration), assisting with data collection and interpretation.
Name:	Brittney Lewis
Project Role:	Regulatory Specialist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Assisting with the drafting and review of technical documents such as manuscripts and reports and completing literature reviews.

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

None this reporting period.

What other organizations were involved as partners?

Organization Name: CIRCULOGENE Theranostics
Location of Organization: Birmingham, Alabama
Partner's contribution to the project

- Collaboration
- Sample Analysis

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: