Ceftriaxone to PRevent pneumOnia and inflammaTion aftEr Cardiac arresT (PROTECT): a randomized-controlled trial and resistome assessment

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Abbreviations

ANTHARTIC	Antibiotherapy during Therapeutic Hypothermia to Prevent Infectious Complications
BIS	Bispectral index
cEEG	Continuous electroencephalography
CPC	Cerebral Performance Category
CPR	Cardiopulmonary resuscitation
CPOT	Critical Care Pain Observational Tool
DSMB	Data Safety Monitoring Board
DSMP	Data Safety Monitoring Plan
EFIC	Exception from Informed Consent
EMS	Emergency medical services
EOP	Early-onset pneumonia
ERC	European Resuscitation Council
GCS	Glasgow Coma Scale
HR	Hazard ratio
ICU	Intensive care unit
IRB	Institutional Review Board
LAR	Legally authorized representative
LOS	Length of stay
MAP	Mean arterial pressure
MRSA	Methicillin-resistant *Staphylococcus aureus*
mRS	Modified Rankin Scale
OHCA	Out-of-hospital cardiac arrest
OR	Odds ratio
POAHC	Power of Attorney for Health Care
PROTECT	PRevent pneumOnia and inflammaTion aftEr Cardiac arrest
REDCap	Research Electronic Data Capture
ROSC	Return of spontaneous circulation
SAS	Sedation-Agitation Scale
SWFI	Sterile water for injection
TTM	Targeted temperature management
VF	Ventricular fibrillation
VRE	Vancomycin-resistant enterococcus
VT	Ventricular tachycardia

1. Trial Overview

The ceftriaxone to PRevent pneumOnia and inflammaTion aftEr Cardiac arrest (PROTECT) randomized-controlled trial and resistome assessment will evaluate the impact of prophylactic ceftriaxone on early-onset pneumonia (EOP) in patients resuscitated from out-of-hospital cardiac arrest (OHCA) and are comatose. The trial will build upon the findings from observational studies and the Antibiotherapy during Therapeutic Hypothermia to Prevent Infectious Complications (ANTHARTIC) trial.[1-3]

The PROTECT trial is a multi-center, randomized-controlled, quadruple-blind, non-commercial superiority trial in which prophylactic ceftriaxone for three days after OHCA will be compared with standard care. The trial will also study ceftriaxone's ability to suppress T-cell-mediated inflammation via increased CD73/ adenosine signaling. It will also determine the effect of prophylactic ceftriaxone on the bacterial resistome using metagenomic sequencing and resistance genotype expression.

Patients ≥18 years of age will be eligible for enrollment if they are admitted to the hospital following OHCA and are comatose. The traditional informed consent process will take place prior to any study procedures. Exception from Informed Consent (EFIC) will be invoked when a family member or legal authorized representative (LAR) is unavailable at xxxx. Bedside clinicians, study investigators, patients and outcome adjudicators will be blinded to group allocation.

All patients will receive guideline-recommended treatment. Targeted temperature management (TTM) at a patient-specific goal may be used. Neurological monitoring may include the bispectral index (BIS) and continuous electroencephalography (cEEG) until it is deemed clinically unnecessary. Seizures will be aggressively treated according to clinical practice guidelines. Sedation and analgesia will be minimized to prevent interference with neurological prognostication while still providing comfort.

Patients will be randomized 1:1 to ceftriaxone 2 gm intravenously (IV) every 12 hours for three days or matching placebo. All other aspects of care will be the same between groups. Patients will undergo shotgun metagenomics sequencing to evaluate their bacterial resistome using nucleic acids obtained from sputum samples and rectal swabs. The impact of ceftriaxone on inflammation will be examined through T-cell-mediated inflammation via CD73/ adenosine signaling.

All patients will have their neurological prognosis assessed per the European Resuscitation Council (ERC's) recommendations after OHCA. Follow-up assessment will be performed six months after cardiac arrest using the modified Rankin Scale (mRS) and Cerebral Performance Category (CPC). A good functional outcome will be defined as a mRS ≤0-3 of or a CPC 1-2. Data from this trial will be used to conduct a power calculation for multicenter study examining mortality and functional outcome.

2. Scientific Justification and Significance

OHCA occurs in ~350,000 adults each year in the United States.[4] Initial heart rhythm, as determined by emergency medical services (EMS) automated external defibrillators, is ventricular fibrillation (VF) or ventricular tachycardia (VT) in 19% of patients. Among EMS-treated patients suffering OHCA, the incidence of survival and good functional status at hospital discharge was 10.5% and 8.5% in 2019, respectively.[4] Survival to hospital discharge has remained unchanged over the past five years, suggesting an urgent need for therapeutic advancements.

2.1. Post-resuscitation Care

Resuscitation from OHCA continues after return of spontaneous circulation (ROSC) is achieved. Clinical practice guidelines published by the American Heart Association in 2020 provide an evidenced-based approach to post-resuscitation care.[5] During the initial stabilization phase, the airway is secured, respiratory parameters are optimized, and hemodynamic derangements are corrected. These processes may occur independently or concurrently during the initial stabilization phase.

Additional emergent activities are conducted concurrently once initial stabilization has been achieved. Continued management includes treating reversible etiologies of OHCA such as ST-segment elevation myocardial infarction or pulmonary embolism. Emergent activities minimize secondary hypoxic-ischemic injury to the brain and may include TTM, maintenance of normoxia and euglycemia, provision of continuous or intermittent encephalography, and lung-protective mechanical ventilation.[5]

TTM between 32-36°C (COR 1, LOE B-R) for 24 hours (COR 2a, LOE B-NR) is recommended for adults who remain comatose after OHCA (COR 1, LOE B-R).[5] Recommendations are based on two trials examining TTM (32°C to 34°C) in patients with OHCA with an initially shockable heart rhythm, one trial examining TTM (33°C to 37°C) in patients with in-hospital or OHCA with an initially non-shockable heart rhythm, one trial examining TTM (33°C to 36°C) in patients with OHCA any initial heart rhythm, and one trial examining TTM (33°C) for 24 vs. 48 hours.

Clinical practice guidelines suggest a mean arterial pressure (MAP) >65 mmHg (COR 2a, LOE B-NR), at a minimum.[5] Normoxia with an oxygen saturation of 92% to 98% should be maintained while avoiding hyperoxia (COR 2b, LOE B-R).[5] Ventilation should be adjusted to maintain an arterial partial pressure of carbon dioxide within 35-45 mmHg (COR 2b, B-R).[5] Seizures should be monitored for with intermittent or cEEG and treated aggressively (COR 1, LOE C-LD).[5]

2.2. Pneumonia after Cardiac Arrest

Pneumonia is an infection of the lungs resulting in alveolar inflammation and fluid or purulent material accumulation. It is the most common infection after cardiac arrest occurring in up to 65% of patients treated with TTM.[6] Infections are associated with increased intensive care unit (ICU) length of stay (LOS), hospital LOS, duration of mechanical ventilation, post-discharge rehabilitation need, tracheostomy, and mortality, while also reducing the incidence of a good functional outcome.[7-11]

Pneumonia results from aspiration during cardiopulmonary resuscitation (CPR), or by introduction of oropharyngeal flora into the lungs during airway management. It may also be due to gastrointestinal hypoperfusion, which leads to ischemic injury to the intestinal mucosa, bacterial translocation, and hematogenous spread. Infection might also be the result of post-resuscitation immune suppression.[12-18] The association between infection and morbidity and mortality justifies further study.

Preventing infection after OHCA may: 1) reduce exposure to broad-spectrum antibiotics and subsequent collateral damage, 2) prevent hemodynamic derangements due to local and systemic inflammation, and 3) prevent an association between infection and morbidity and mortality. These benefits must be balanced with the risk for altering bacterial resistomes in the absence of clinical infection. Accordingly, further study is warranted to understand the risk-to-benefit ratio of prophylactic antibiotics.

2.3. Antibiotic Prophylaxis in Comatose Patients after Acute Brain Injury

The first trial was a single-center, prospective, randomized-controlled, open-label study of cefuroxime 1.5 grams IV q12h for two doses in a heterogeneous cohort of patients requiring mechanical ventilation after head injury or stroke resulting in a Glasgow Coma Scale (GCS) ≤12.[19] Study drug was started <6 hours from the time of intubation. EOP occurred ≤4 days from the initiation of mechanical ventilation. A total of 100 subjects were included with 50 in each group. Incidence of EOP was 16% in the cefuroxime group and 36% in controls (p=0.02).

The second trial was a single-center, prospective, randomized-controlled, open-label study of ampicillin-sulbactam 3 gm IV q6h for 72 hours in a heterogeneous cohort of patients requiring mechanical ventilation after head injury or stroke resulting in a GCS ≤8.[20] Study drug was started <6 hours from the time of ICU admission. EOP occurred ≤4 days from the initiation of mechanical ventilation. A total of 36 subjects were included with 18 in each group. Incidence of EOP was 21% in the ampicillin-sulbactam group and 58% in controls (p=0.02).

The third study was a single-center, prospective, before-and-after study of single-dose ceftriaxone in a heterogeneous cohort of patients requiring mechanical ventilation after head injury or stroke resulting in a GCS ≤8.[21] Study drug was started <4 hours from the time of initiation of mechanical ventilation. Early-onset pneumonia occurred ≤4 days from the initiation of mechanical ventilation. A total of 129 subjects were included with 71 in the ceftriaxone group and 58 in the controls. Incidence of EOP was 2% in the ceftriaxone group and 22% in controls (p=0.001).

Although other publications have reported on the impact of antibiotic prophylaxis following acute brain injury, their methodology was less rigorous. In a single-center, retrospective, before-and-after study of ceftriaxone 2 gm IV for one dose in 172 intubated patients the incidence of EOP was reduced significantly (7.4 vs 19.8%, p=0.026).[22] A post-hoc analysis of the SPIRIT-ICU and CORTI-TC studies of traumatic brain injury determined antibiotic prophylaxis reduced EOP compared to no prophylaxis (10% vs. 32%; p<0.01).[23]

2.4. Antibiotic Prophylaxis after Cardiac Arrest

A meta-analysis, published in 2019, reported three randomized-controlled trials and eight observational studies have compared prophylactic or early antibiotics to clinically-driven or delayed antibiotics.[24] Results were hampered by the fact that two of the three randomized-controlled trials were published in abstract form only. In the overall analysis, which inappropriately combined randomized and observational studies, prophylactic or early antibiotics were not associated with a reduced incidence of combined early- and late-onset pneumonia (OR 0.58, 95% CI 0.23–1.46).

The ANTHARTIC trial, published in 2019, randomized 198 patients who suffered OHCA due to a shockable rhythm to amoxicillin-clavulanate 1 gram/ 200 mg IV every 8 hours or placebo administered for 48 hours starting within six hours of ROSC.[3] The incidence of EOP within the first seven days was reduced (19% vs. 34%; HR 0.53; 95% CI 0.31 to 0.92; p=0.03), and remained similar when the definition for EOP was changed to ≤5 days (17% vs. 31%, HR 0.53; 95% CI, 0.30 to 0.95; p=0.03).

The PROTECT trial will expand the findings of the ANTHARTIC trial in several ways: 1) inclusion of OHCA patients with all initial heart rhythms, 2) use of a lower-risk antibiotic available in the USA that has not previously been tested after OHCA, 3) study the anti-inflammatory effects of ceftriaxone to determine a

mechanism by which it may improve clinical outcomes, and 4) complete a metagenomics assessment of bacterial resistomes pre- and post-ceftriaxone prophylaxis.

2.5. Antibiotic Resistance and the Resistome

The effect of antibiotic prophylaxis on bacterial resistance after OHCA are not known, but antibiotic resistance is a global concern.[25] Emergence of resistant bacteria will be examined in treatment and control patients. Control patients may be exposed to longer courses of broad spectrum antibiotics due to higher rates of pneumonia. Drug concentrations in stool, effects of ceftriaxone on expression of resistance genes, and on richness, diversity, and relative abundance of taxa will be quantified.

Prior studies have concluded antibiotic prophylaxis in acutely brain-injured patients does not induce bacterial resistance based on limited analyses. For example, in the trial examining cefuroxime prophylaxis, the intervention did not induce bacterial resistance based similar rates in gram-negative bacilli pneumonia in both groups.[19] Advances in metagenomics allows for sample multiplexing and depth of sequence coverage (20 million reads) for the measurement of resistomes of each individual patient.

The ANTHARTIC trial evaluated intestinal acquisition of multidrug-resistant bacteria on day seven on solid selective media using stool samples, limiting their observations to conditionally cultivatable organisms.[3] No difference was found following two days of amoxicillin-clavulanate administration compared to placebo. The contemporary gold standard for evaluating bacterial resistance is resistome analysis with high-throughput sequencing, which will be utilized in the PROTECT trial.

2.6. Anti-inflammatory Effects of Ceftriaxone

Ceftriaxone reduced IFN-γ and TNF-α in the injured parietal cortex and improved learning and spatial memory function in a rat model of traumatic brain injury.[26] Ceftriaxone also reduced IFN-γ and IL-17 secretion in a mouse model of multiple sclerosis by altering antigen presentation and activation of myelin-specific T lymphocytes.[27] Other studies have shown ceftriaxone dampens excitotoxicity in by decreasing glutamate transporter function.[26,28-31] Because excitotoxicity is a possible cause of secondary brain injury following OHCA, ceftriaxone's ability to attenuate this response is promising.[32]

T cells promote neuroinflammation and neuronal cell death via IFN-γ and TNF-α.[32-34] Our preliminary data showed incubation of CD3+ T-cells from human peripheral blood with ceftriaxone for 24 hours increased expression of CD73. CD73 is an adenosine-generating enzyme, and adenosine has potent immunosuppressive and anti-inflammatory effects in T cells. Consistent with this concept, the PROTECT study will determine if CD73 level is inversely correlated with IFN-γ levels in both CD4+ and CD8+ T cells.

2.7. Study Hypothesis and Anticipated Results

Prophylactic ceftriaxone administered within six hours of ICU admission will reduce the incidence of EOP and T cell-mediated inflammation in comatose OHCA survivors. Additionally, prophylactic ceftriaxone will not negatively affect bacterial resistomes as assessed by resistance genotypes in stool or sputum.

3. Trial Design

The PROTECT trial was a single-center, randomized-controlled, quadruple-blind, non-commercial superiority trial conducted at xxxx. The inflammation analysis will be completed at xxxx. The resistome analysis will be done at xxxx and xxxx.

3.1. Inclusion Criteria

- ≥18 years of age
- Comatose (do not follow simple verbal commands)
- Have any initial heart rhythm (shockable or non-shockable)
- OHCA including the emergency department

3.2. Exclusion Criteria

- Name on EFIC opt-out list
- In-hospital cardiac arrest
- Interval >6 hours from ICU admission at an enrolling center to study drug initiation
- Hemoglobin less than 7.0 g/dL or requiring a transfusion
- Pregnant patients
- Preexisting terminal disease making 180-day survival unlikely
- Refused informed consent
- Emergent coronary artery bypass grafting
- Anaphylaxis or angioedema to beta-lactam antibiotics (i.e., cephalosporins or penicillins)
    - Beta-lactam allergies listed without a known reaction will not be an exclusion
- Under legal guardianship or prisoner
- Clinical *bacterial* infection prior to hospital admission defined as any one of the following:
    - Infectious prodrome preceding OHCA
    - Active course of antibiotics for infection prior to admission
    - Active infection documented in the electronic medical record
    - Family or surrogate endorsement of an active infection
- Active course of antibiotics for infectious or non-infectious indications
    - A single dose of an antibiotic that is NOT ceftriaxone is acceptable prior to randomization as long as the bedside treatment team does not intend to continue the antibiotic(s)
- Indication for antibiotics at the time of screening in the opinion of the bedside treatment team
- Non-English speaking
- Goals of care are not clear at the time the screening window closes and the subject is not enrolled through the EFIC pathway
- Survival through 48 hours after ROSC is unlikely to occur

3.3. Inclusion Window

The optimal time from OHCA to antibiotic initiation is unclear. Theoretically, the sooner it is administered, the more effective it may be. Prospective, controlled studies in heterogeneous cohorts of acutely brain-injured patients used four or six hours from intubation or six hours from ICU admission.[19-21] The ANTHARTIC trial, which included OHCA patients, used <6 hours from ROSC to randomization.[3] Accordingly, patients will enrolled if ≤6 hours has passed since ICU admission to study drug initiation.

3.4. Methods of Recruitment

All OHCA patients are admitted through the emergency department or directly to the ICU at XXXX. An order set or power plan for TTM within the electronic medical record is activated for all comatose OHCA patients. Order set activation sends an automated email to study investigators and research coordinators who screen patients 24 hours a day 7 days a week. LAR will be approached for informed consent, or the EFIC process will be initiated in their absence at XXXX only.

3.5. Screening

Screening will be performed in the emergency department, cardiac angiography suite, ICU or via telephone using a standard script. A screening log will be maintained for OHCA patients, whether they are enrolled or not, and the reason for exclusion. Screening logs will be only available to approved personnel.

3.6. Informed Consent

Study investigators will approach the patient's Power of Attorney for Health Care (POAHC) or LAR to provide informed consent. Patients regaining consciousness will be asked for informed consent as soon as they are able to make a decision and have the capacity to do so. Consent may be obtained in person or via remote consent process.

In the event a patient's POAHC or LAR cannot be reached for informed consent within 30 minutes, the EFIC process will be initiated at XXXX only per the Food and Drug Administration's Guidance for Institutional Review Boards, Clinical Investigators, and Sponsors on Exception from Informed Consent Requirements for Emergency Research.[36] The requirements for a clinical trial to incorporate EFIC are as follows:

- The human subjects are in a life-threatening situation that necessitates urgent intervention;
- Available treatments are unproven or unsatisfactory;
- Collection of valid scientific evidence is necessary to determine the safety and effectiveness of the intervention;
- Obtaining informed consent is not feasible because the subjects are not able to give their informed consent as a result of their medical condition;
- The intervention must be administered before consent can be obtained from the subject's legally authorized representative;
- There is no reasonable way to identify prospectively individuals likely to become eligible for participation;
- Participation in the research holds out the prospect of direct benefit to the subjects; and
- The clinical investigation could not practicably be carried out without the waiver.

After a thorough community consultation and public disclosure, the IRB at XXXX deemed it was appropriate to conduct the trial. The EFIC process will be initiated after 30 minutes of trying to contact the POAHC or LAR

Subsequent to an EFIC enrollment, efforts to contact an LAR will continue. The research team will inform LAR at earliest opportunity of the subject's inclusion in the trial, the details of the investigation, other information in the informed consent document and consent to continue in the study will be sought. The LAR will be also be notified that they may discontinue the patient's participation without penalty or loss of benefits to which the subject is otherwise entitled. Patients enrolled in the study through the EFIC

process or LAR consent will be asked for informed consent as soon as they have the capacity to do so, preferably prior to hospital discharge.

3.7. Randomization

A computer-generated allocation sequence will randomize patients in a 1:1 ratio in blocks of six. The clinical trials pharmacist at xxxx will be responsible for randomizing patients independent of study investigators. Based on the randomization, the clinical trials pharmacist will prepare the study drug blinded for delivery to the bedside nurse for administration. At non-xxxx hospitals, the investigators will contact the clinical trials pharmacist at xxxx, or their designee, to tell them of an enrollment. The xxxx clinical trials pharmacist will determine the subject number and complete the randomization.

3.8. Study Drug

Ceftriaxone for injection is a sterile, semi-synthetic, broad-spectrum cephalosporin antibiotic available for IV or intramuscular injection.[37] It's chemical formula is $C_{18}H_{16}N_8Na_2O_7S_3 \bullet 3.5H_2O$ and has a molecular weight of 661.6. It contains 83 mg (3.6 mEq) of sodium per gram of ceftriaxone activity. Study drug will be provided by xxxx.

3.8.1. Preparation

Ceftriaxone 2 gram vials will be obtained from Pfizer, which is XXXX's preferred supplier through its wholesaler. Vials will be reconstituted with 19.2 mL of Sterile Water for Injection (SWFI) resulting in a 100 mg/mL solution. The reconstituted solution is a light yellow to amber in color. After reconstitution with of SWFI, it requires further dilution with 50 mL of 0.9% sodium chloride. Overfill volume of 8 mL will not be removed from the 50 mL of 0.9% sodium chloride bag resulting in a total volume of 78 mL. This will be done to maintain blinding as well.

Matching placebo will be a 50 mL 0.9% sodium chloride bag with 20 mL of SWFI added. Overfill volume of 8 mL in the 0.9% sodium chloride bag will not be removed resulting in a total volume of 78 mL. All injections will be prepared in compliance with United States Pharmacopeia Chapter 797 Guidelines and will be classified as low risk.

3.8.2. Rationale for selection

Ceftriaxone was selected for many reasons: 1) bactericidal activity against commonly isolated bacteria in comatose OHCA patients, 2) generic availability and low cost, 3) ease of administration over 30 minutes, 4) favorable local susceptibility profile, 5) excellent safety data, and 6) potentially neuro-protective.[37]

Although informative, data from the TTM trial included isolates through 14 days of admission and ANTHARTIC through 7 days of admission.[3] Prophylactic antibiotics are intended to prevent EOP due to less resistant, community-acquired bacteria. Bacteria isolated after 72-96 hours of ICU admission tend to be more resistant and healthcare-associated.[38]

Bacteria	TTM Trial[1]	ANTHARTIC Trial[1]	xxxx Ceftriaxone Susceptibility[2]
Gram-positive			
Staphylococcus aureus	22.9%	12%	86%
Streptococcus pneumoniae	5.5%	7%	99%
Streptococcus agalactiae	1.5%	3%	not reported
Gram-negative			
Haemophilus influenzae	9.1%	22%	100%
Escherichia coli	9.1%	11%	93%
Klebsiella pneumoniae	5.1%	4%	94%
Serratia marcescens	5.1%	3%	89%
Klebsiella oxytoca	3.6%	1%	97%
Enterobacter cloacae	3.3%	3%	78%
Pseudomonas aeruginosa	2.5%	3%	not active
Enterobacter aerogenes	2.2%	2%	75%
Proteus mirabilis	2.2%	1%	98%
Moraxella catarrhalis	1.5%	1%	not reported

[1] Percentages do not equal 100 for each study as some organisms (e.g., fungi) were not included above

[2] xxxx ceftriaxone antibiogram data through December 2019

TTM = targeted temperature management

The ceftriaxone dose of 2 gm IV q12h was selected using internal antibiogram data at XXXX, as well as the cumulative fraction of response based on dose. These data appear below:

Regimen	Cumulative Fraction of Response
Ceftriaxone 1 gm IV q24h	8%
Ceftriaxone 2 gm IV q24h	45%
Ceftriaxone 2 gm IV q12h	86%

3.8.3. Labeling

In order to maintain blinding, an opaque cover will be placed over the study drug due to its light yellow to amber color.

3.8.4. Distribution and management

Study drug will be kept separate from the pharmacy supply. The clinical trials pharmacist will utilize an accountability form to document study drug inventory. Drug accountability will not be checked during

monitoring visits to maintain blinding. Study drug will be delivered by the clinical trials pharmacist or technician to the bedside nurse for administration.

3.8.5. Return and destruction

Study drug dispensed for an enrolled patient that is not administered will be returned to the pharmacy. At the end of the trial, any remaining inventory will be destroyed by the clinical trials pharmacist.

3.9. Placebo

Matching placebo of 50 mL 0.9% sodium chloride will be dispensed for patients randomized to the control arm. The 50 mL 0.9% sodium chloride bags contain 8 mL of overfill. Another 19.2 mL of SWFI will be added resulting in a total volume of 78 mL. In order to maintain blinding, an opaque cover will be placed over the bag due to ceftriaxone's light yellow to amber color. Placebo will be infused over 30 minutes.

3.10. Blinding and Unblinding

The study will be conducted in a blinded manner including: 1) patient, 2) study investigators/ coordinators, 3) pneumonia adjudication committee, 4) bedside team, and 5) outcome evaluators. The clinical trials pharmacist and members of the Data Safety Monitoring Board (DSMB) will not be blinded.

Unblinding may occur for a serious adverse event or if the bedside treatment team determines it is medically necessary. Results of unblinding will not be communicated to study investigators, members of the pneumonia adjudication committee, or the outcome evaluators. Accidental unblinding will be dealt with on a case-by-case basis and patients may continue in the study depending on the circumstance.

3.11. Outcomes

Outcomes were developed for the present study and to help with a sample size calculation for a definitive trial powered for mortality or functional outcome. They were selected by carefully reviewing prior trials and observational data.

3.11.1. Primary efficacy outcome

- Clinically-diagnosed EOP occurring <4 days after initiation of mechanical ventilation

There are no validated diagnostic criteria for pneumonia in comatose OHCA survivors. Consequently, the definition of clinical pneumonia was adapted from diagnostic criteria for nosocomial pneumonia proposed by the Centers for Disease Control and Prevention/ National Healthcare Safety Network, American Thoracic Society and the Infectious Diseases Society of America, and published definitions of pneumonia in this patient populations.[3,39,40]

During the 72-hour TTM period, clinical pneumonia will be defined as:

1. New or progressive lung infiltrate and <u>at least one of the following</u> (a or b):
    a. New purulent secretions or change in quantity or quality of sputum
    b. Worsening gas exchange defined as (any of i through iii):
        i. Oxygen desaturations or PaO_2 /FIO_2 ≤240
        ii. Increased oxygen requirements

1. Increase in daily minimum FiO2 of ≥0.20 (20 points) over the daily minimum FiO2 of the first day in the baseline period, sustained for ≥ 2 calendar days. Daily minimum defined by lowest value of FiO2 during a calendar day that is maintained for >1 hour.

iii. Increased mechanical ventilator demand

1. A sustained increase in the daily minimum PEEP of ≥ 3 cmH_2O following a period of stability or improvement on the ventilator. PEEP values from 0 to 5 cmH_2O are considered equivalent. Daily minimum defined by lowest value of PEEP during a calendar day that is maintained for >1 hour.

After the 72-hour TTM period, clinical pneumonia will be defined as:

1. New or progressive lung infiltrate and <u>at least two of the following</u> (a through e):
    a. New purulent secretions or change in quantity or quality of sputum
    b. Worsening gas exchange defined as (any of i through iii):
        i. Oxygen desaturations or PaO_2 /FIO_2 ≤240
        ii. Increased oxygen requirements
        iii. Increased ventilator demand
    c. Cough, dyspnea, tachypnea, rales or bronchial breath sounds
    d. Body temperature ≥38°C
    e. Leukopenia (<4,000 white blood cells/mm^3) or leukocytosis (>12,000 white blood cells/mm^3)

A clinical diagnosis of pneumonia will be microbiologically confirmed using flexible fiberoptic bronchoscopy with >10^4 colony forming units (CFU) per mL of pathogenic bacteria or unprotected mini-bronchoalveolar lavage with >10^4 CFU/mL of pathogenic bacteria in intubated patients. In non-intubated patients, an expectorated sputum sample will be used. Blinded, board certified pulmonologists will adjudicate any clinical or microbiological pneumonia diagnosis.

3.11.2. Secondary efficacy outcomes

- Days of antibiotic susceptibility coverage
- Microbiologically-confirmed EOP occurring <4 days after initiation of mechanical ventilation
- Microbiologically-confirmed late-onset pneumonia occurring ≥4 days after initiation of mechanical ventilation
- Clinically-diagnosed late-onset pneumonia occurring ≥4 days after initiation of mechanical ventilation
- Incidence of non-pulmonary infections
- ICU-free days in the first 28 days of admission
- Mechanical ventilator-free days in the first 28 days of admission
- ICU LOS
- Hospital LOS
- ICU mortality
- Hospital mortality
- Discharge disposition

  - Home with services
  - Home without services
  - Rehabilitation
  - Skilled nursing care
  - Hospice
  - Death
  - Transfer to another hospital
- Functional outcome at hospital discharge and six months post cardiac arrest (+/- 30 days)
  - Good functional outcome will be mRS ≤0-3 of or a CPC 1-2 (Appendices 16.1 and 16.2)
  - Functional outcome will be assessed by a research coordinator at hospital discharge and via telephone at six month follow up

3.11.3. Safety outcomes

- *Clostridioides difficile*-associated diarrhea[41]
  - Diagnosed according to the 2017 Infectious Diseases Society of American Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults:
    - Unexplained and new-onset ≥3 unformed stools in 24 hours with a positive *Clostridioides difficile* toxin gene polymerase chain reaction assay
- Type one (immediate-type) hypersensitivity reactions[42]
  - Acute symptom onset (minutes to hours) involving the skin, mucosal tissue, or both (e.g., generalized hives, pruritus or flushing, swollen lips-tongue-uvula) and at least one of the following (a through c):
    a. Respiratory compromise
    b. Reduced blood pressure
    c. Signs of end-organ dysfunction
- Gallbladder disease[37]
  - Diagnosed sonographically as an echo without acoustical shadowing suggesting sludge and the presence of ceftriaxone-calcium salt

3.12. Post-resuscitation Care

All OHCA patients are admitted to or co-managed by the Neurocritical Care or Critical Care team at xxxx. The Neurocritical Care team has 24/7 coverage 365 days a year by an attending physician and an advanced practice provider (i.e., Physician Assistant or Nurse Practitioner). Most patients are treated in the cardiac ICU, but some may be treated in another ICU per bed availability and nursing.

xxxx has managed over 1,000 patients with TTM after OHCA and it is the largest hospital in xxxx. Patients will receive early coronary catheterization, when appropriate, and will undergo multimodal delayed neurologic prognostication according to clinical practice guidelines. All care will be the same in each study group except for the study drug.

3.12.1. Targeted temperature management

Patients unable to follow simple verbal commands after ROSC will be treated with TTM.[5] If hypothermia is indicated, patients will be sedated and may receive a dose of a paralytic or chilled saline (4°C), and cooled to a target temperature. If normothermia is the targeted temperature (37.8°C) the same

interventions may be necessary, but will not be required. The Bard Medical Arctic Sun Temperature Management System®, a servo-controlled surface cooling device, will be used as needed to achieve the target temperature at xxxx.

If patients are cooled, after 24 hours, they are re-warmed over 12-16 hours, and then maintained at 36.5°C-37°C until 72 hours after ROSC. Modifications to the protocol are allowed, such as targeting 36°C in the setting of refractory shock or uncontrolled bleeding, and delayed re-warming if refractory seizures are present. The trial protocol may be amended to incorporate data from the TTM-2 trial comparing 33°C for 24 hours with normothermia (37.8°C).[43]

3.12.2. Sedation and analgesia

Propofol is preferred and is titrated by bedside nurses. Analgesia is provided with intermittent injections of fentanyl or a continuous infusion. As needed doses of midazolam may be administered. Low doses are maintained to avoid accumulation in the setting of end-organ dysfunction and temperature-mediated decreases in drug metabolism and elimination. The approach to sedation between centers may differ.

During rewarming, all sedation is interrupted. Dexmedetomidine infusion is often initiated to provide a cooperative level of sedation without respiratory depression. The Sedation-Agitation Scale is used and patients are often maintained at a score of 3-4 (Appendix 16.3). Analgesia is monitored with the Numeric Rating Scale in communicative patients and with the Critical Care Pain Observation Tool (Appendix 16.4) in those who are not communicative.

3.12.3. Shivering

Shivering is suppressed with focal counter-warming using a Bair Hugger System™. The Bedside Shivering Assessment Scale (Appendix 16.5) and BIS electromyographic activity are monitored. Vecuronium may be administered when the BSAS is ≥1. Magnesium is generally maintained ≥2.4 mg/dL. In refractory cases, dexmedetomidine or magnesium sulfate infusion may be administered. Increasing the target temperature may also be tried.

3.12.4. Seizures and epileptiform activity

Patients may be monitored with cEEG until they have awoken or it is deemed not clinically necessary. Tracings are read by epileptologists throughout the day and concerning findings are reported to the bedside treatment team. Seizures and epileptiform activity will be classified according to the International League Against Epilepsy, Neurocritical Care Society, and American Clinical Neurophysiology Society's criteria. Antiepiletpic medications may be administered. Seizure prophylaxis with an antiepileptic medication is not standard care.

3.12.5. Hemodynamics

Clinical practice guidelines suggest keeping the MAP >65 mmHg, at a minimum, with observational data supporting a MAP 80-90 mmHg.[5] MAP will be maintained >80 mmHg, unless it compromises the hemodynamics. Vasoactive medications, inotropes or mechanical circulatory support may be needed.

3.12.6. Best practices

Patients will receive stress ulcer prophylaxis. Venous thromboembolism prophylaxis will be administered unless contraindicated or there is a need for systemic doses. A bowel regimen will also be administered. Blood glucose may be maintained between 120-180 mg/dL.

3.12.7. COVID-19 considerations

Patients will be tested for, treated and monitored for COVID-19 per local hospital protocol at XXXX. Because the COVID-19 pandemic is evolving, adjustments may be necessary during the trial.

3.13. Neurological Prognostication

Patients who remain comatose will have their neurological prognosis assessed per the ERC's and the European Society for Intensive Care Medicine recommendations (Appendix 16.6).[44] The multimodal neurological prognostication algorithm will be activated >72 hours after ROSC to avoid confounding from acute metabolic disturbances, sedation, analgesia and paralysis.

3.13.1. Physical examination

Absent or extensor motor response to pain at >72 hours after ROSC in a patient who is not impacted by sedation may be indicative or a poor prognosis. Bilateral absence of pupillary and corneal reflexes at >72 hours or more after ROSC may be indicative of a poor prognosis.

3.13.2. Continuous electroencephalography

Patients may be monitored with cEEG until they have awoken or it is deemed not clinically necessary. A cEEG pattern considered malignant or without reactivity to sound or pain >72 hours after ROSC may be indicative of a poor prognosis.

3.13.3. Bispectral index

Patients may be monitored with the BIS until they have awoken or it is deemed not clinically necessary. Values <22 following the first dose of a paralytic and suppression ratio at six hours post-ROSC have been associated with poor prognosis and may be used.

3.13.4. Brain computed tomography

Brain computed tomography may be conducted for unwitnessed OHCA or if clinically warranted based on the opinion of the bedside treatment team. Evidence of diffuse hypoxemic-ischemic injury with reduced grey/white matter differentiation and sulcal effacement may be indicative of a poor prognosis.

3.13.5. Brain magnetic resonance imagining

Brain magnetic resonance imaging may be obtained >72 hours after ROSC in patients who remain comatose. Evidence of diffuse or bilateral multifocal hypoxic-ischemic lesions may be indicative of a poor prognosis.

3.13.6. Neuron specific enolase

Neuron specific enolase may be assayed at 24, 48 and 72 hours after ROSC. Values >90 mcg/L may be associated with poor outcome, while values <17 mcg/L may be associated with a favorable outcome. Consistently up-trending values may also indicate a poor outcome.

3.13.7. Somatosensory evoked potentials

Somatosensory evoked potentials may be evaluated >72 hours after ROSC in patients who remain comatose. Absent N20-responses bilaterally may be indicative of a poor prognosis.

3.14. Withdrawal of life supporting therapies

All patients will be treated for at least 72 hours unless any of the following occur: 1) it is deemed unethical to continue due to irreversible organ damage or documented medical comorbidity, 2) brain death has been established. In the event of brain death, patients will be classified as dying, and not support withdrawn. Diagnosis of brain death will follow the American Academy of Neurology guidance.[45]

3.15. Follow-up

Patients will be followed for the duration of their hospitalization and up to six months after cardiac arrest. At the time of hospital discharge, a research coordinator will determine each patient's mRS score and CPC. This will also occur at six months after cardiac arrest via telephone call.

3.16. Co-enrollment

Co-enrollment in other clinical trials, observational studies and registries was reviewed. Patients enrolled in the PROTECT trial may be co-enrolled in other interventional trials if deemed appropriate by the clinical trial steering committee.

3.16.1. ICECAP

The Influence of Cooling Duration on Efficacy in Cardiac Arrest Patients (ICECAP) trial is an adaptive study to determine if increasing durations of TTM are associated with good neurological outcome.[46] Patients may be co-enrolled in ICECAP and PROTECT.

3.16.2. PRECICECAP

PREcision Care In Cardiac ArrEst-ICECAP (PRECICECAP) uses novel, data-driven approaches to collect high resolution monitoring data from patients and will use that multi-parametric data to develop newly defined patient sub-groups with differing clinical trajectories and responses to therapies.[47] Patients may be co-enrolled in PROTECT and PRECICECAP.

3.16.3. INTCAR

The International Cardiac Arrest Registry is a worldwide repository of post-resuscitation cardiac arrest care.[48] Contributing centers input data into the INTCAR registry including cEEG, neurological imaging and neurological prognostication, amongst others. Because the registry is non-interventional, patients may be enrolled in PROTECT and contribute data to INTCAR.

3.16.4. PCAS

The PCAS study is examining cardiac arrest-associated neutrophils (CAAN) effect of CD73+ lymphocytes on neutrophil activation. Subjects enrolled the PROTECT trial will not be separately enrolled in PCAS as the analyses are the same.

4. Resistome Assessment

4.1. Specimen Handling and Transportation

Sputum sample and rectal swab will be collected prior to study drug initiation and 72-96 hours after administration of first dose of study drug (after study drug has been completed). A third rectal swab will be collected 144-168 hours after administering first dose of study drug or earlier in the case of death, comfort measures only or hospital discharge, after which no study procedures will be conducted. Missing time points do not disqualify patient for study participation and is not a protocol deviation. Total nucleic acids will be extracted and resistomes determined by shotgun metagenomic sequencing using super high-throughput methods. COVID-19 considerations are outlined in Appendix 16.7. Samples from non-XXXX hospitals may be stored and transported under various conditions not listed above but that are acceptable.

4.2. Resistome Determination

Antibiotic resistance genes were measured in material recovered from rectal swabs collected prior to study drug initiation, after study drug completion (three days), and 6-7 days after the first dose of study drug. Total nucleic acids were extracted from rectal swabs using ZymoBIOMICS DNA/RNA plus extraction kit (Zymo Research Corporation 2024). Sequencing libraries were prepared using the Kapa BioSystems HyperPlus Kit (KR1145 -v3.16). Sequencing was completed on a NovaSeq 6000 with an SP flow cell (paired-end 250 bps reads). Data was demultiplexed using bcl2fastq v2.20.0.422. FASTQ files were examined for quality using FastQC v0.11.5 and multiqc v1.11 with default settings.[49,50] Estimation of duplication rates, adapter sequence trimming and read merging was completed using fastp v0.23.2 with default settings and the flags '--include_unmerged', '--merge', '-l 50' and '-g' to remove poly-G tails produced by the NovaSeq platform.[51] Human reads were identified by mapping the data against the human reference genome (GRCh38) using bwa mem v0.7.17 and filtering the mapped reads using samtools v1.19.2 (bit flags -f 4 -F262, -f 8 -F 260, and -f 12 and -F 256).[52,53] Metagenomic assembly was completed from the unmapped reads using the SPAdes pipeline v3.15.5 with the metagenomic flag and default settings. QUAST v5.2.0 was utilized to characterize the contiguity of the genome assemblies.[54,55] Per sequence coverage statistics were calculated using bwa mem v0.7.17 and samtools v1.19.2 with default settings. BLAST v2.14.0 was utilized to compare sequences against the nt database.[56] Blobtools v1.1.1 was then used to taxonomically classify each sequence using the 'bestsum' taxonomic rule and the NCBI taxonomy database.[57] Any human contigs were removed from the assemblies based on these taxonomic assignments. PROKKA v1.14.0 was used for CDS annotation. Antimicrobial resistance genes were characterized using AMRFinderPlus using both the genome assemblies (FASTA) and the annotated genes from PROKKA (FAA).[58,59]

5. Inflammation Assessment

5.1. Specimen Handling and Transportation

Peripheral or arterial blood samples (10 mL) will be obtained at four time points for a total of up to 40 mL of blood. The four time points are: before study drug is administered (By 6 hours of ICU admission), 0-24 hours after administration of first dose of study drug, 48-72 hours after administration of first dose of study drug, and again 96-120 hours after administration of first dose of study drug . Missing time points does not disqualify patient for study participation and is not a protocol deviation. Sub-populations of

white blood cells will be measured using flow cytometry and mononuclear cells will be isolated using Ficoll-Paque™. Blood will not be collected if patient develops a hemoglobin less than 7 g/dL or requires a transfusion. COVID-19 considerations are outlined in Appendix 16.7. Blood specimens will be transported to xxxx for analysis. Samples from non-xxxx hospitals may be stored and transported under various conditions not listed above but that are acceptable.

5.2. Effect of Ceftriaxone on CD73

CD3+ lymphocytes obtained from peripheral blood before administration of study drug will be adjusted to a concentration of 106 cell/mL and incubated with 50 mcg/mL ceftriaxone or vehicle for 24 hours. Expression of CD73 in subpopulations of CD3+ T cells will be determined using multi-parametric flow cytometry after staining cells with antibodies: CD3FITC-CD73PE-CD4PeCy7-CD8APC-CD39APC/Cy7-DAPI. CD73 levels in both CD4+ and CD8+ subpopulations of CD3+ T cells will be measured.

5.3. T cell-mediated Adenosine Production

CD3+, CD4+CD3+ or CD8+CD3+ T cells will be incubated with 20 µM [8-14C] ADP (American Radiolabeled Chemicals). Radioactive [8-14C] adenosine generated by T cells will be separated from [8-14C] nucleotides on columns of acidic aluminum oxide (1.3 g/column) by elution with 4 ml 0.005 N hydrochloric acid. T lymphocyte generation of adenosine will be assayed.[60]

5.4. CD73/ Adenosine-mediated T Cell Activation

Analysis of genes involved in T cell response will be performed with total RNA isolated from CD3+ lymphocytes after their incubation in the presence of vehicle, ceftriaxone, or in combination with AMP. Total RNA will be isolated using RNeasy Mini Kit (Qiagen) and cDNA will be generated using RT² Nano PreAMP cDNA Synthesis Kit (SABiosciences). Gene expression will be analyzed using Oligo GEArray® Human T-cell and B-cell Activation Microarray (SABiosciences, OHS-053). This panel of 113 genes includes CD28, CD3E, IL10, IL12B, IL18, IL27, NCK1, and NCK2 genes involved in regulation of T cell proliferation and regulators of Th1 and Th2 development such as IFNG, IL10, IL12A, IL13, IL4, IL5, ITGAX, TLR2, TLR4, TLR7, and 11TLR9.

5.5. CD73 and Pro-inflammatory Cytokines

Multi-parametric flow cytometric analysis will be conducted on whole blood cells before study drug and on study-day 1 and study-day 3. Cells will be stained with CD4FITC-CD73PeCy7-CD8PerCP-CD3APC/Cy7 following fixation, permeabilized (BD Cytofix/Cytoperm™) for intracellular staining with anti-IFN-γ (Clone 25723) or anti-TNF-α (Clone 6402) antibodies (R&D System/Bio-techne). The percentage of CD3+ T cells, CD4+ and CD8+ subpopulations, and CD73 expression on T lymphocytes will be determined within DAPI negative (viable) cell populations using MACSQuant. Myeloid cells will be analyzed to validate ceftriaxone's effect in lymphocytes. Neutrophils and monocytes will be gated and cell surface expression of CD73 and production of TNF-α assayed as described for lymphocytes.

6. Data Collection

Data will be collected to screen, recruit, and determine eligibility of prospective OHCA subjects. Prior to consent, certain information will be gathered for screening including name, date of birth, MRN, date/time of ICU admission, details surround the cardiac arrest, pre-existing terminal disease, medical

history, infection status, allergies, legal guardianship/prisoner, and medical status. Screening logs will be maintained and reasons for exclusion will be recorded. Data collection in REDCap will be completed for patients who are screened and/or enrolled in the PROTECT Study. REDCap is a secure, web-based application for data capture.[61] Data collected may follow the International Liaison Committee on Resuscitation's Utstein criteria for OHCA.[62] Data will be collected by research coordinators or study investigators from medical records, family members, ambulance run reports, or other sources. The REDCap data collection tool has been modified to ensure demographic data for EFIC patients is captured at XXXX. In addition to the data outline below, information on serious adverse events, study drug and trial completion will be collected.

6.1. Baseline Data

- Hospital
- Age (years)
- Sex (male or female as assigned at birth or in the medical record)
    - Gender cannot be reliably collected because all patients are comatose
- Height (centimeters)
- Weight (kilograms)
- Body mass index (kg/m^2)
- Race
- Ethnicity
- Smoking Status
- Living environment prior to cardiac arrest
- Pre-arrest diseases
- Charlson Comorbidity Index (total)
- Modified Rankin Score
- Cerebral Performance Category (CPC) prior to cardiac arrest
- Immunosuppressive drugs prior to admission
    - Cyclosporine
    - Tacrolimus
    - Sirolimus
    - Everolimus
    - Mycophenolate
    - Enteric-coated mycophenolic acid
    - Mizoribine
    - Leflunomide
    - Azathioprine
    - Horse or rabbit antithymocyte globulin
    - Muromonab-CD3
    - Alemtuzumab
    - Rituximab
    - Basiliximab
    - Belatacept
    - Eculizumab
    - Bortezomib
    - Methotrexate
    - Cyclophosphamide
    - Chlorambucil
    - Tofacitinib
    - Abatacept
    - Adalimumab
    - Anakinra
    - Certolizumab
    - Etanercept
    - Golimumab
    - Infliximab
    - Ixekizumab
    - Natalizumab

- Rituximab
- Secukinumab
- Tocilizumab
- Ustekinumab
- Vedolizumab
- Daclizumab
- Hydroxychloroquine
- Sulfasalazine
- Dapsone
- Rilonacept
- Sarilumab
- Eculizumab
- Belimumab
- Brodalumab
- Guselkumab
- Mepolizumab
- Reslizumab
- Benralizumab
- Dupilumab
- Omalizumab
- Vedolizumab
- Tofacitinib
- Upadacitinib
- Baricitinib
- Corticosteroids
- Chemotherapy
- Monoclonal antibiody
- Tyrosine kinase inhibitor

6.2. Pre-hospital Data

- Witnessed arrest (yes,no, or unknown)
- OHCA location
  - Home/residence
  - Industrial/workplace
  - Sports/recreation event
  - Street/highway
  - Public building
  - Assisted living/nursing home
  - Emergency department
  - Other
  - Unspecified/Unknown/Not recorded
- Bystander CPR (yes,no, not applicable medical personnel present, or unknown)
- Bystander defibrillation (yes, no, not applicable medical personnel present, or unknown)
- Initial heart rhythm at arrival of medical personnel
  - Asystole
  - Pulseless electrical activity
  - VF
  - Non-perfusing VT
  - ROSC obtained after bystander defibrillation
  - Unknown
- Presumed cause of the arrest
  - Cardiac etiology
  - Respiratory etiology
  - Neurological etiology
  - Circulatory etiology
  - Unknown
- Date and time of OHCA
- Minutes from emergency call until ambulance/emergency team is on scene

- Time from arrest to advanced cardiac life support
- Re-arrest in the field (yes or no)
- Re-arrest in the emergency department (yes or no)

6.3. ICU Admission Data

- ICU Admission Date and Time
- Sequential Organ Failure Assessment worst (highest) score within 24 hours of admission
- Acute Physiology and Chronic Health Evaluation IV score within 24 hours of hospitalization
    - The APACHE IV score will be retrospectively calculated on all enrolled PROTECT subjects
- Acute Physiology Score
- Predicted LOS
- Glasgow Coma Scale score
- Targeted Temperature Management (yes, no, or unknown)
- Time from ROSC to target temperature
- Duration of TTM
- Target temperature
- STEMI (yes, no, or unknown)
- Cardiac angiography intervention (yes, no, or unknown)
- Shock on admission (SBP <90 mmHg for 30 minutes or need for vasoactive medications/ mechanical circulatory support)

6.4. ICU Discharge Data

- Early-onset pneumonia (yes or no)
    - Clinical (yes or no; list criteria for diagnosis)
    - Microbiologically-confirmed (yes or no; list organism and criteria for diagnosis)
- Late-onset pneumonia (yes or no)
    - Clinical (yes or no; list criteria for diagnosis)
    - Microbiologically-confirmed (yes or no; list organism and criteria for diagnosis)
- Non-pulmonary infection (yes or no)
    - List infection and organism, when available
- ICU-free days in the first 28 days of admission (days)
- Mechanical Ventilation duration (days)
- Mechanical ventilator-free days in the first 28 days of admission (days)
- Rearrest in the ICU requiring CPR
- ICU LOS (days)
- ICU mortality (yes or no)
- Date and time of first dose of study drug
- Date and time of last dose of study drug
- Total doses of study drug administered
- Total missed doses of study drug
- Date and times of missed study drug doses
- Study drug discontinuation
- Reasons for study drug discontinuation

- Days of antibiotic spectrum coverage

6.5. Hospital Discharge Data

- mRS score (0-6)
- CPC score (1-5)
- Hospital LOS (days)
- Discharge disposition (select)
    - Home
    - Inpatient Rehabilitation
    - Inpatient ventilator unit
    - Skilled Nursing
    - Hospice facility
    - Death (list cause of death including withdrawal of life support)
- Date and time of death
- Cause of death (free text field in addition to information collected above in TTM database)
- Location of patient death
    - ICU
    - Hospital
- Date and time of discharge

6.6. Six Month Data (After cardiac arrest)

- mRS score (0-6)
- CPC score (1-5)
- Did patient die post-discharge?
- Date and time of death
- Cause of death

7. Data Management

7.1. Data Handling and Storage

Documents will be retained at xxxx for 15 years, which is in compliance with the United States' Food and Drug Administration Code of Federal Regulations (21 CFR §312.62[c]).[63] Access to source documents may be permitted for trial-related monitoring and audits, when appropriate. Individual data for monitoring, carrying out quality control, and auditing biomedical research may be shared at the discretion of the study investigators. Data collection forms will be kept in a locked office (research coordinators) within a locked cabinet.

Data will be transcribed into Research Electronic Data Capture (REDCap), which is a secure, web-based application for data capture.[61] REDCap can be installed in a variety of environments for compliance with such standards as HIPAA, 21 CFR Part 11, FISMA (low, moderate, high), and international standards. The REDCap Consortium is a collaborative, international network of more than 2,400 institutional partners in over 115 countries, with >590,000 total end-users conducting 450,000 ongoing research studies.

7.2. Quality Control and Assurance

A clinical research associate appointed by xxxx IRB may visit during the study to ensure proper conduct. Items for review may include:

- Informed consent forms and EFIC documentation
- Compliance with the study protocol and procedures
- Quality of data collected in the case report form
    - Accuracy
    - Missing data
    - Consistency of the data with the source documents
- Management of the study drug
- Each visit will be recorded in a written monitoring report.

Quality control mechanisms within the REDCap data collection tool may be used to ensure the appropriateness of data.[61] For example, a birthday resulting in an age of 185 years will be flagged.

8. Adverse Events

8.1. Definitions

Adverse Event (per FDA 21CFR312.32)

Adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

Serious Adverse Event (per FDA 21CFR312.32)

An adverse event is considered "serious" if, in the view of either the investigator or sponsor (when applicable), it results in any of the following outcomes:

- Death (*see below exception)
- Life-threatening adverse event
- Inpatient hospitalization (*see below exceptions) or prolongation of existing hospitalization
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions (disability)
- Congenital anomaly/birth defect

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition (above).

*Exception to the above serious adverse event definitions:

- An adverse event that results in death is usually considered a serious adverse event. However, 40-50% of patients who survive to the hospital after OHCA will not survive their hospital stay as a result of their underlying clinical condition. Since death is a clinical outcome for this study, deaths will not be reported as serious adverse events unless the investigator deems them related to study drug or study procedures.

Unexpected Adverse Event (per FDA 21CFR312.32)

An adverse event is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended.

Ceftriaxone is an FDA approved medication and does not have an investigator brochure. Therefore, the risk information is being provided in the protocol and informed consent form.

In addition, the unexpected adverse event definition assumes a causal relationship to the study drug (ceftriaxone). If an adverse event is not considered of Reasonable or Definite Relatedness to the ceftriaxone, it would not be considered an unexpected adverse event.

Relatedness

The relatedness of an adverse event to the study drug will be defined as follows:

Relatedness	**Description**
Not	The temporal relationship between exposure and the adverse event is unreasonable or incompatible, and/or it is clearly due to extraneous causes
Unlikely	Must have both of the following 2 conditions, but may have reasonable or temporal relationship to the intervention: 1. Could readily have been produced by the subject's clinical state, or environmental or other interventions 2. Does not follow known pattern of response to intervention
Reasonable	Two of the following are true: 1. Has a reasonable temporal relationship to intervention 2. Could not possibly have been produced by the subject's clinical state or have been due to environmental or other interventions 3. Follows a known pattern of response to intervention
Definitely	All 3 of the following are true: 1. Has a reasonable temporal relationship to intervention 2. Could not possibly have been produced by the subject's clinical state or have been due to environmental or other interventions 3. Follows a known pattern of response to intervention

8.2. Reporting

Serious adverse events that meet the Xxxx IRB reporting requirements will be reported to the IRB and DSMB within five business days of principal investigator notification.

Serious adverse events resulting in death and that meet the Xxxx IRB reporting requirements will be reported to the IRB and DSMB within 48 hours of the principal investigator notification.

Unexpected adverse events deemed reasonably or definitely related to the study drug will be reported to the IRB and DSMB within five business days of the principal investigator notification.

8.3. Study Drug Discontinuation

Study drug will be discontinued if:

1. Gallbladder toxicity occurs OR;
2. Withdrawal from the trial is in the patient's best interest OR;
3. Infection develops warranting a change in the antibiotic regimen OR;
4. Subject, POAHC, or LAR declines or revokes informed consent.

In the event of stopping the study drug, patients will continue to be followed for safety and efficacy outcomes, and will collect blood, sputum, and rectal samples for the inflammation and resistome assessments.

The independent DSMB will be empowered to stop the trial for under certain circumstances:

1. Efficacy
    a. The trial will not be stopped for efficacy given the intermediate outcome of interest is pneumonia, rather than death or other concrete outcomes (e.g., stroke, myocardial infarction). Additionally, it will not allow for a complete assessment of the resistome or inflammation.
2. Harm
    a. There is a statistically significant increased risk for serious adverse events, including *Clostridium difficile*-associated diarrhea, gallbladder toxicity, or type one hypersensitivity reactions, during the interim analysis of the first n=60 patients enrolled. The p-value threshold for harm will be set at 0.05.
3. Safety
    a. Significant safety concerns emerge and the DSMB and IRB choose to pause or stop the trial.

8.4. Study Specific Adverse Events

Adverse events are common in comatose OHCA patients occurring in >90%.[64] Adverse events may be the result of the clinical context or study drug. Adverse events that meet the "serious" definition in section 8.1 will be documented and assessed using the xxxx IRB reporting criteria.

Clinical events secondary to cardiac arrest will not be reported at any grade, since these are considered symptoms of the condition being studied. These may include:

- Septic shock
- Bleeding requiring transfusion
- Bradycardia requiring pacing
- Seizure confirmed on cEEG

Study specific adverse events that are expected secondary to ceftriaxone use will be reported as required per the definitions in section 8.1 and the table below. These include:

- *Clostridium difficile*-associated diarrhea
- Gallbladder toxicity

- Type one hypersensitivity reactions

Study Specific Serious Adverse Events	Definition
Clostridium difficile-associated diarrhea	Unexplained and new-onset ≥3 unformed stools in 24 hours with a positive Clostridioides difficile toxin gene polymerase chain reaction assay
Gallbladder toxicity	Diagnosed sonographically as an echo without acoustical shadowing suggesting sludge and the presence of ceftriaxone-calcium salt
Type one hypersensitivity reactions	Acute symptom onset (minutes to hours) involving the skin, mucosal tissue, or both (e.g., generalized hives, pruritus or flushing, swollen lips-tongue-uvula) and at least one of the following (a through c): a. Respiratory compromise b. Reduced blood pressure c. Signs of end-organ dysfunction

8.5. Management

Clostridium difficile-associated diarrhea may be treated according to the Infectious Diseases Society of America and Society for Healthcare Epidemiology of America 2017 Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults and Children.[41]

Type one (immediate-type) hypersensitivity reactions may be managed as a medical emergency and managed according to the American Academy of Allergy, Asthma, and Immunology and American College of Allergy, Asthma, and Immunology 2020 Anaphylaxis practice parameter.[42]

Gallbladder disease may be treated according to severity and symptoms based on hospital standards.

9. Statistical Analysis

9.1. Sample Size

Incidence of EOP without antibiotic prophylaxis is unclear due to low-quality studies, variable definitions for EOP, varying diagnostic criteria, and inconsistent reporting of antibiotic administration. Additionally, studies examine early antibiotics, which may be symptom-driven *or* prophylactic.

In the ANTHARTIC trial, the incidence of EOP at five days was 17% in the prophylaxis group and 31% in controls (absolute difference of 14%).[3] When using their definition of EOP, the incidence at seven days was 19% in the prophylaxis group and 34% in controls (absolute difference of 15%).

In the TTM trial, pneumonia occurred in 52% of patients treated at 33°C and 46% in those treated at 36°C.[65] Pneumonia was not defined as early- or late-onset. In a post-hoc analysis, the incidence of pneumonia in patients admitted to centers administering antibiotic prophylaxis was 41% compared to 54% at centers that did not.[66] Clustering within centers was possible.

Using data from INTCAR, the incidence of pneumonia, which was not defined as early- or late-onset, was 33% with antibiotic prophylaxis. Accordingly, 60 patients will be randomized to each group (n=120 total). This provides 80% power at a two-tailed 5% level of significance to detect an absolute reduction in the incidence of EOP by 25%, from 55% to 30%.

9.2. Primary Efficacy Outcome

Success of randomization will be assessed by comparing baseline characteristics of patients in the drug and placebo groups, using standard descriptive statistics, means and standard deviations or medians and interquartile ranges, as appropriate for continuous variables and proportions for categorical variables. No p values will be presented as is typical in randomized trials. All data were analyzed on an intention-to-treat basis. We described the treatment effect of antibiotics on EOP using a risk ratio with 95% confidence intervals for treatment effect, and tested the effect using with the Chi-square or Fisher Exact test, the Mann-Whitney test for two independent samples, and or the Wilcoxon Signed-Ranks test for two paired samples, as appropriate. We considered two-tailed tests of significance with $p<0.05$ to be considered significant. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc.) or SAS version 9.4 and R version 4.0.2.

9.3. Secondary Efficacy Outcomes

The secondary efficacy endpoints will be analyzed on an intention-to-treat basis. Late-onset pneumonia and other infectious outcomes will be analyzed using the same statistical approach as the primary efficacy outcome. Mortality at day 28 will be analyzed using the Chi-square test.

9.4. Safety Outcomes

The safety endpoints will be analyzed on an intention-to-treat basis. Safety outcomes will be reported as proportions and study groups compared using the Chi-square test or Fisher exact test, as appropriate.

9.5. Resistome Assessment

Abundance of antibiotic resistance-associated genotypes in resistomes will be compared within and between treatment groups. If ceftriaxone or placebo alters resistomes, ANOVA will be performed across genotypes. The abundance of each genotype will be compared between ceftriaxone- and placebo-treated patients post-intervention by both Student's T test and $\chi 2$ to establish direct changes associated with ceftriaxone and whether those deviate from potential changes in resistomes due to ICU admission. All statistical analyses will be performed using GraphPad Prism 8 (GraphPad Software Inc). A p value <0.05 will be considered significant. To measure the acquisition of ARGs, the increase in number of ARGs relative to baseline samples were compared within each treatment group using the Wilcoxon Signed-Ranks test. In addition, the baseline number and changes in the number of ARGs targeting a priori-defined frequently administered (macrolides, beta-lactams, fluoroquinolones, sulfamethoxazole-trimethoprim, and vancomycin) and infrequently administered (tetracyclines, erythromycin, aminoglycosides, chloramphenicol, clindamycin, fosfomycin, quinupristin) antibiotics according to clinical practice guidelines were compared between treatment groups.[67,68]

9.6. Inflammation Assessment

We expect to obtain 56.3 ± 4.5 x 10^6 of total white blood cells, containing 14.7 ± 1.7 x 10^6 mononuclear cells and 6.3 ± 1.1 x 10^6 of CD3+ T cells from 10 mL of blood. We will use approximately 1 x 10^6 CD3+ T cells to determine direct effects of ceftriaxone on CD73 expression and adenosine generation. We will use approximately 3 x 10^6 of CD3+ T cells to isolate mRNA for gene expression analysis after treatment with ceftriaxone. Approximately 2-3 x 10^6 whole blood cells will be used for flow cytometric analysis. Statistical analysis will be performed using the GraphPad Prism 7.0 software (GraphPad Software Inc). Comparisons between groups will be performed using two-tailed unpaired t tests. Comparisons between several groups will be performed using one-way ANOVA followed by appropriate post-hoc tests. A p value <0.05 will be considered significant.

9.7. Missing data

Missing data will be reported at the time of publication. If further statistical analyses reveal substantial missing data, multiple imputation will be considered.

10. Committees

10.1. Data Safety Monitoring Board

An independent DSMB will be formed and include a statistician, clinical trialist and infectious diseases physician. Their separate charter has been submitted in a separate document. Composition and responsibilities of the DSMB will follow the Food and Drug Administration's Guidance for Clinical Trial Sponsors Establishment and Operation of Clinical Trial Data Monitoring (March 2006). A DSMB chair will be assigned to facilitate meaningful discussion about the trial.

The following people will be members of the DSMB:

a. Xxxx
b. Xxxx
c. Xxxx

10.2. Pneumonia Adjudication Board

A pneumonia adjudication board will be assembled prior to the trial's commencement. It will adjudicate all clinically diagnosed and microbiologically confirmed pneumonia. Their work will be done in a blinded fashion. Disputes will be reconciled through communication or with the assistance of a third adjudicator. Members of the pneumonia adjudication board will include:

a. Xxxx
b. Xxxx
c. Xxxx
d. (2018)

11. Publication

Analyses will be performed six months after cardiac arrest of the last patient. Data analysis will be performed in a blinded manner. The full-length manuscript will be submitted to a peer-reviewed international medical journal. Authorship will follow the guidelines set forth by the International Committee of Medical Journal Editors. The main publication will include the primary and secondary

efficacy outcomes, safety outcomes, inflammation data, and resistome data. Subsequent publications may be considered by the study investigators at the conclusion of the trial.

12. Funding

The trial will be funded by xxxx as part of a xxxx grant for acute care research and rural disparities. The funding source has approved the scientific merit of the trial, but will not be involved with its conduct, data analysis, manuscript development, or decision to submit for publication.

13. Timeline/Sequence of Events

1/2021 to 5/2021 – trial design, EFIC process, IRB approval

5/2021 to 6/2022 – recruitment of 60 patients, follow-up data collection, interim analysis

6/2022 to 2/2023 – recruitment of remaining 60 patients, follow-up data collection

2/2023 to 8/2023 – data analysis, preliminary data, presentation of preliminary results

8/2023 – final follow-up data collected, full data analysis, submission for publication

Sequence of Events

	By 6 hours of ICU admission	**0-24 hours after administration of first dose of study drug**	**24-48 hours after administration of first dose of study drug**	**48-72 hours after administration of first dose of study drug**	**72-96 hours after administration of first dose of study drug**	**96-120 after administration of first dose of study drug**	**144-168 hours after administration of first dose of study drug**	**Day 1 to hospital discharge**	**Hospital discharge**	**Six-month follow-up**
Screening/enrollment	X									
Study drug	X	X	X	X						
Sputum sample	X*◊				X◊					
Rectal swab	X*◊				X◊		X◊Δ			
Blood sample	X*◊	X◊		X◊		X◊				
EOP		X	X	X						
Non-pulm. infection								X		
ICU LOS								X		
Ventilator-free days								X		
Hospital LOS								X		
ICU mortality								X		
Hospital mortality								X		
Discharge disposition								X		
CDAD								X		
Type-one allergy		X	X	X						
Gallbladder disease								X		

mRS									X	X
CPC									X	X

CDAD = *Clostridioides difficile*-associated diarrhea; EOP = early-onset pneumonia; ICU = intensive care unit; LOS = length of stay; mRS = modified Rankin Scale; CPC = Cerebral Performance Category; The initiation of the study drug=time zero hours

*Blood, Sputum sample, and Rectal swab must be obtained after informed consent and prior to administration of first dose of study drug

◊ Missing time points does not disqualify patient for study participation and is not a protocol deviation.

Δ If discharge occurs before 144-168 hrs after administration of first dose of study drug, collect this rectal swab on day of discharge

14. Trial Participants

14.1. Steering Committee

14.2. Investigators

15. References

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16. Appendices

The following scales, tools and algorithms were used in the conduct of the PROTECT trial.

16.1. Cerebral Performance Category

Score	Description
1	conscious with no functional disability
2	conscious with moderate functional disability but the ability to work
3	conscious but with severe functional disability and dependence on others
4	coma or vegetative state
5	dead

16.2. Modified Rankin Scale

Score	Description
0	no symptoms
1	no significant disability despite symptoms; able to carry out all usual duties and activities
2	slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	moderate disability; requiring some help, but able to walk without assistance
4	moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs
5	severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	dead

16.3. Sedation-Agitation Scale

Score	Description	Behaviors
7	Dangerous Agitation	Pulling at the endotracheal tube, trying to remove catheters, climbing over bed rail, striking at staff, thrashing side-to-side
6	Very Agitated	Does not calm despite frequent verbal reminding of limits, requires physical restraints, biting endotracheal tube
5	Agitated	Anxious or mildly agitated, trying to sit up, calms down to verbal instructions
4	Calm and Cooperative	Calm, awakens easily, follows commands
3	Sedated	Difficult to arouse, awakens to verbal stimuli or gentle shaking but drifts off again, follows simple commands
2	Very Sedated	Arouses to physical stimuli but does not communicate or follow commands, may move spontaneously
1	Unarousable	Minimal or no response to noxious stimuli, does not communicate or follow commands

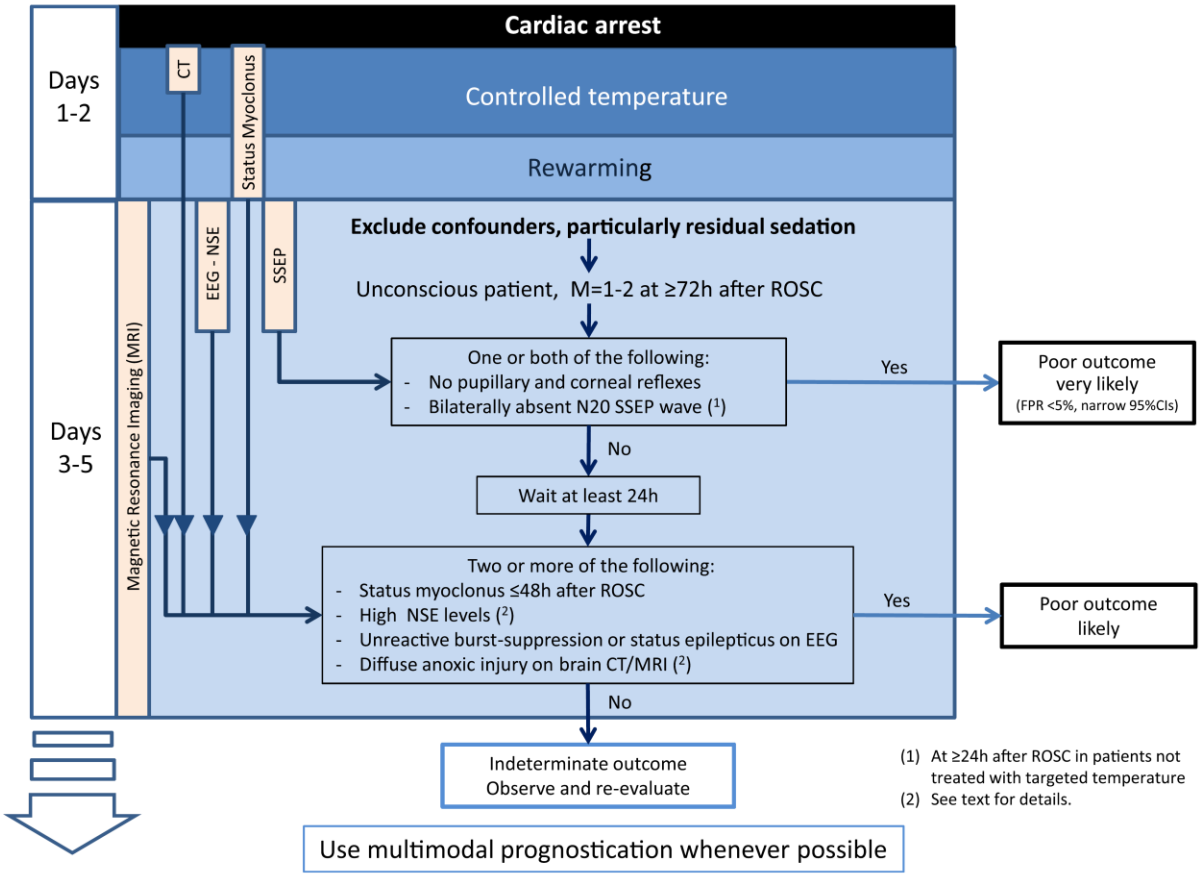
16.4. Critical Care Pain Observation Tool

Indicator	Descriptor	Score
Facial Expression	No muscular tension observed (relaxed, neutral)	0
	Presence of frowning, brow lowering, orbit tightening, and levator contraction (tense)	1
	All of the above facial movements plus eyelid tightly close (grimacing)	2
Body Movements	Does not move at all (does not necessarily mean absence of pain)	0
	Slow, cautious movements, touching or rubbing the pain site, seeking attention through movements (protection)	1
	Pulling tube, attempting to sit up, moving limbs/ thrashing, not following commands, striking at staff, trying to climb out of bed (restlessness)	2
Muscle Tension	No resistance to passive movements (relaxed)	0
	Resistance to passive movements (tense, rigid)	1
	Strong resistance to passive movements, inability to complete them (very tense or rigid)	2
Ventilator Compliance	Alarms not activated, easy ventilation	0
	Alarms stop spontaneously	1
	Asynchrony: blocking ventilation, alarms frequently activated	2
Vocalization (extubated)	Talking in normal tone or no sound	0
	Sighing, moaning	1
	Crying out, sobbing	2

16.5. Bedside Shivering Assessment Scale

Score	Description	Definition
0	None	no shivering noted on palpation of the masseter, neck, or chest wall
1	Mild	shivering localized to the neck and/or thorax only
2	Moderate	shivering involves gross movement of the upper extremities (in addition to neck and thorax)
3	Severe	shivering involves gross movements of the trunk and upper and lower extremities

16.6. European Resuscitation Council Neurological Prognostication



16.7. COVID-19 Considerations for Specimen Handling

Laboratory safety precautions against SARS-Cov2

Important, no work related to purification, isolation, or concentration of SARS-Cov2 or testing SARS-Cov2 in cell culture will be done under this protocol.

1. Working with specimens.

Two types of specimens will be used in this study (i) ten (10) ml of whole blood will be collected in the yellow top [Acid Citrate Dextrose (ACD)] BD vacutainer and (ii) five (5) ml endotracheal sputum specimens in Lukens containers. BD Vacutainer plastic tubes offer a safe method of blood collection and reduce the potential for tube breakage and specimen spillage, thereby reducing the potential for exposure to bloodborne pathogens. Lukens containers trap is securely closed with a screw-on cap to prevent specimen spillage, thereby reducing the potential for exposure to airborne pathogens.

1.1. Transport container with specimens will be opened only inside the BSL2 hood;

1.2. Specimens, the inside, and outside of transport container will be sprayed with 70% ethyl alcohol inside the BSL2 hood;

1.3. The opening and initial processing of specimens will be done only inside the BSL2 hood.

2. Personal protective equipment (PPE) and the protection of lab personnel.

2.1. Lab members will be involved in specimens handling and processing in the lab. All personnel will have completed the required CITI training and have long-term experience working with human samples using universal precautions.

2.2. Personal protective equipment that includes a disposable lab coat, glasses, gloves, and N95 mask will be worn all time during handling and processing specimens and decontamination after the work.

2.3. Biohazard caution signs designating the type of work being done and the potential risk to those in the area will be posted on doors of the lab and smaller lab room #2621

2.4. During specimens handling and specimens processing the number of people working in the smaller lab room #2621 will be limited to two lab members.

3. Major lab techniques being used in the study and safety precautions to prevent the aerosol generation and the spread of SARS-Cov2.

3.1. Initial processing of specimens.

3.1.1. Centrifugation of specimens to separate cells and fluid part of the blood or endotracheal sputum will be performed under the hood in PowerSpin LX clinical centrifuge (UNICO) placed inside the BSL2 hood.

3.1.2. After centrifugation, the fluid part of the blood or endotracheal sputum will be transferred into 1.5 eppendorf screw-capped tubes, containing information on specimens. These tubes will be placed inside the eppendorf storage boxes containing biohazards signs and information on specimens, sprayed

with 70% ethyl alcohol, and stored at -80 in a designated rack, containing biohazards signs, until further analysis.

3.1.3. Cells in the pellet will be carefully transferred into 1.5 ml eppendorf tubes with secure screw caps and washed with cold FACS buffer containing 2 mM EDTA and used for the staining of cell surface antigens and flow cytometric analysis.

3.1.4. The original specimens containers, BD vacutainer, and Lukens containers trap will be sprayed with 70% ethyl alcohol and placed in smaller biohazard bags and secured with adhesive autoclave tape. This bag will be placed into biohazards waste, located close to the BSL2 hood.

3.2. Flow cytometric analysis.

3.2.1. Cells in 1.5 ml eppendorf tubes will be stained using an array of antibodies. Unbound antibodies will be removed through centrifugation (inside the hood) and supernatant aspiration using a vacuum trap system with 1% sodium hypochlorite solution. During the entire procedure cells will be washed for five (5) times with FACS buffer containing 2 mM EDTA to remove any non-specifically bind proteins. Washout buffer will be removed using a vacuum trap system with 1% sodium hypochlorite solution.

3.2.2. Flow cytometry samples will be transferred to Flow cytometry Core using a transport container with a biohazard sign and information on samples. Cells will be analyzed using flow cytometer MACSQuant 10 located in the core facility. To prevent aerosol formation during flow cytometry, the automatic regimen of sample mixing will be disabled. Laminar fluidic system performance will be monitored using Fluorochrome vs. Time plots.

3.2.3. All flow cytometry samples will be wasted immediately after analysis into the waste system, containing 1% sodium hypochlorite solution and securely connected to the fluidic system. No flow cytometry samples will be reused for any other analysis. No flow cytometry samples will be saved after the analysis.

3.2.4. After the analysis, all remaining tubes will be placed in smaller biohazard bags and secured with adhesive autoclave tape. This bag will be placed into biohazards waste, located close to the BSL2 hood.

3.3. ELISA assays.

3.3.1. All steps, including antigen coating, incubation with specimens, incubation with detection antibody, and color developing TMB solution and all aspiration steps will be performed inside the hood. All washout buffers will be removed using a vacuum trap system with 1% sodium hypochlorite solution.

3.3.2. At the last step, 2N Sulfuric Acid will be added to all wells to kill viral particles, if any will be still retained after multiple washing steps. 96-well plate will be sprayed with 70% ethyl alcohol from outside.

3.3.3. The plates will be read using a microplate reader located close to the smaller lab room #2621.

3.3.4. After the completion of the analysis, the 96-well plate will be and placed in smaller biohazard bags and secured with adhesive autoclave tape. This bag will be placed into biohazards waste, located close to the BSL2 hood.

4. Additional lab safety precautions.

4.1. Access to the lab will be limited during the analysis of specimens from SARS-Cov2 infected patients.

4.2. Hand-washing hygiene will be performed immediately after removal of gloves or other PPE.

4.3. NO eating, drinking, smoking, applying cosmetics, or lip balm are allowed inside the lab at any time.

5. Sample transport

5.1. Samples will be transported by trained personnel according to the CDC Biosafety Guidelines for Handling and Processing Specimens Associated with COVID19, which indicates that these samples should be transported as UN3373 Biological Substances, Category B.

5.1.1. This includes transport in a triple packaged container that is leak-proof and contains enough absorbent material to soak up the entire contents of the container. The container will have a primary receptacle (i.e. sterile, leak-proof, screwcap tube with the sample inside, labeled with participant study ID and date the sample was collected), a secondary packing (i.e. styrofoam box) and a rigid outer packaging (i.e. cardboard box). The container will be able to pass a 4-feet drop test and will be labeled UN3373. The vehicle in which the samples will be transported will be equipped with a spill-proof kit.

5.1.2. The PI will ensure personnel transporting samples are appropriately trained and will document this training.

6. Prevention of aerosol formation during centrifugation

6.1. Two desktop centrifuges will be used in the study: (i) PowerSpin LX clinical centrifuge to work with specimens in original containers (specimens volume is 10 ml or less), and (ii) Eppendorf centrifuge to work with samples after initial processing (described in 3.1.). Both, PowerSpin LX centrifuge and Eppendorf centrifuge are equipped with gaskets and rotor lids.

6.2. Both centrifuges are placed inside the BSL2 hoods. One centrifuge per each of two BSL2 hoods in the smaller lab room #2621.

6.3. 1.5 ml screw-cap tubes will be used for work with post-initially processed specimens to prevent aerosol formation during opening the tubes.