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NCT03799874: Safety and Efficacy Study of Inhaled Carbon Monoxide to Treat Acute Respiratory Distress Syndrome (ARDS), A Phase II Trial of Inhaled Carbon Monoxide for the Treatment of Acute Respiratory Distress Syndrome (ARDS)

Protocol and Statistical Analysis Plan

The therapeutic use of inhaled carbon monoxide (iCO) and carbon monoxide-releasing molecules is a dynamic field with promising pre-clinical findings of anti-inflammatory and other effects. Human trials in IPF and sepsis-induced ARDS have demonstrated safety of fixed low-dose iCO but have not adequately examined efficacy, nor sufficiently analyzed biological pathways/biomarkers relevant to iCO effect.

We therefore set out to test the safety and efficacy of fixed-dose iCO in ARDS. The study was halted due to low enrollment, but we obtained permission to combine the biospecimens from this study with our previously published Phase 1 iCO safety study (NCT04870125) to perform extensive analyses, including plasma proteomics, lipidomics, metabolomics, mitochondrial DNA levels, cytokine levels, and urine studies. We identified important findings that we feel are of significant value to the scientific community.

1. There were no attributable safety concerns from the study.
2. We noted differential plasma protein expression between placebo- and iCO-treated subjects of a novel target, CD300A, pointing to a new potential pathway of investigation in ARDS therapeutics.

We are in the process of preparing these important findings for publication. Thank you for your help in sharing the findings of our study.

Sincerely yours,

Rebecca M. Baron, M.D.

**A Phase II Trial of Inhaled Carbon Monoxide for the Treatment of Acute
Respiratory Distress Syndrome (ARDS)**

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Summary of Changes from Previous Version:

Affected Section(s)	Summary of Revisions Made	Rationale
Cover, 2, 4.5, 9.1.1, 10.1	To enhance the biologic analytic power, samples from 12 subjects enrolled in a similar, already completed study will be similarly analyzed for these biomarkers, as well as in more extensive biologic 'omics profiling that will permit more detailed analyses of these pathways in correlation with clinical data.	The sample power analyses will be greatly enhanced by adding samples from a previous, similar already completed study.

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STATEMENT OF COMPLIANCE

The trial will be conducted in accordance with International Conference on Harmonisation Good Clinical Practice (ICH GCP), applicable United States (US) Code of Federal Regulations (CFR), and the U.S. Army Department of Defense (DoD) Terms and Conditions of Award. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without prior agreement from the Investigational New Drug (IND), funding agency and documented approval from the Institutional Review Board (IRB), except where necessary to eliminate an immediate hazard(s) to the trial participants. All personnel involved in the conduct of this study have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the IRB for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. All changes to the consent form will be IRB approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title:	A Phase II Trial of Inhaled Carbon Monoxide for the Treatment of Acute Respiratory Distress Syndrome (ARDS)
Study Description:	<p>Multi-center, prospective, randomized, partially double-blind, placebo-controlled Phase II clinical trial of inhaled CO (iCO) for the treatment of ARDS.</p> <p><u>Hypothesis:</u></p> <ul style="list-style-type: none"> • Low dose iCO will be safe and well-tolerated and will lead to a reduction in mitochondrial DNA (mtDNA) levels on day 5 compared with baseline. • Low dose iCO will reduce the severity of lung injury and non-pulmonary organ failure in ARDS patients. • Low dose iCO will reduce mitochondrial dysfunction, attenuate inflammasome activation, and promote resolution of inflammation in ARDS patients. Furthermore, we hypothesize that modulation of these pathways will predict improvements in the clinical outcomes of our Phase II trial.
Objectives:	<p><u>Primary Objectives:</u></p> <ul style="list-style-type: none"> • To evaluate the safety and tolerability of inhaled carbon monoxide (iCO) in patients with ARDS. • To examine the efficacy of low dose iCO therapy in patients with ARDS, as assessed by mtDNA levels. <p><u>Secondary Objectives:</u></p> <ul style="list-style-type: none"> • To evaluate the effect of low dose iCO therapy on lung injury and non-pulmonary organ failure in ARDS patients. • To examine the biologic readouts of low dose iCO therapy in patients with ARDS.

Endpoints:	<p><u>Primary Safety Endpoint:</u> The primary safety endpoint is to evaluate the safety of inhaled CO, by determining carboxyhemoglobin (COHb) levels and the incidence of pre-specified administration-related adverse events.</p> <p><u>Primary Efficacy Endpoint:</u> The primary efficacy endpoint is to compare the effects of iCO versus placebo on mtDNA levels from baseline to day 5 (or death, whichever comes first) in patients with ARDS.</p> <p><u>Secondary Endpoints:</u></p> <p>The secondary endpoints are to examine the effects of iCO versus placebo on the following:</p> <ul style="list-style-type: none"> • Lung injury score (LIS) as measured on days 1-5, and 7 • PaO₂/FiO₂ ratio on days 1-5, and 7 • Oxygenation Index (OI) on days 1-5, and 7 • Dead Space Fraction (Vd/Vt) on days 1-3, and 7 • Sequential Organ Failure Assessment (SOFA) score on days 1-5, 7, 14, 28 • Biomarkers <ul style="list-style-type: none"> ○ Autophagy markers (eg. LC3B) ○ Inflammasome-dependent cytokines (eg. IL-18) ○ Lipid mediators (LM) and specialized pro-resolving mediators (SPMs) ○ Mitochondrial quality control biomarkers (eg. Pink1, Wipi1) ○ Biomarkers of inflammation (eg. IL-6, IL-8, IL-10, IL-1Ra)
Study Population:	All intubated patients ≥ 18 years old with ARDS according to the Berlin criteria will be eligible for inclusion. Subjects will be recruited from the medical, surgical, cardiac, and burn intensive care units (ICUs) at each center with a specific focus on patients with ARDS secondary to infection, trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
Phase:	Phase II
Description of Sites/Facilities Enrolling Participants:	The trial will be conducted at seven tertiary care medical centers including Weill Cornell Medicine/New York-Presbyterian Hospital, New York-Presbyterian Brooklyn Methodist Hospital, Brigham and Women's Hospital (BWH), Massachusetts General Hospital (MGH), Duke University Hospital, Durham Regional Hospital, and Washington University.
Description of Study Intervention:	<ul style="list-style-type: none"> • 32 intubated, adult subjects with ARDS will be randomized in a 1:1 ratio to receive either inhaled CO or inhaled air placebo for up to 90 minutes daily. • Subjects will be randomized to CO will be administered 200 ppm iCO to achieve a target COHb level of 5-8%. • Treatment will continue for 3 days, until discontinuation of mechanical ventilation, or death, whichever comes first.
Study Duration:	48 months
Participant Duration:	6 months

1.2 ABBREVIATIONS

ABG	Arterial Blood Gas
AE	Adverse Event
ALI	Acute Lung Injury
Ang-2	Angiopoietin-2
ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar Lavage
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
BWH	Brigham and Women's Hospital
CFK equation	Coburn-Foster-Kane equation
CFR	Code of Federal Regulations
CHF	Congestive Heart Failure
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
CO	Carbon Monoxide
COHb	Carboxyhemoglobin
CONSORT	Consolidated Standards of Reporting Trials
CPAP	Continuous Positive Airway Pressure
CRF	Case Report Form
CT	Computed Tomography
DBP	Diastolic Blood Pressure
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DLCO	Diffusing capacity of the Lung for Carbon Monoxide
DoD	Department of Defense
DSMB	Data and Safety Monitoring Board
eCRF	Electronic Case Report Forms
EKG	Electrocardiogram
FDA	Food and Drug Administration
FiO ₂	Fraction of Inspired Oxygen
GCP	Good Clinical Practice
GCS	Glasgow Coma Scale
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
HRPO	Human Research Protection Office
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
iCO	Inhaled Carbon Monoxide
ICU	Intensive Care Unit
IDE	Investigational Device Exemption
IL-1 β	Interleukin 1 β
IL-1Ra	IL-1 receptor antagonist
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-18	Interleukin 18
IMV	Intermittent Mechanical Ventilation
IND	Investigational New Drug Application
INR	International Normalized Ratio

IRB	Institutional Review Board
ISM	Independent Safety Monitor
ITT	Intention-To-Treat
LAR	Legally Authorized Representative
LIS	Lung Injury Score
LM	Lipid Mediator
MedDRA	Medical Dictionary for Regulatory Activities
mBW	Measured Body Weight
mtDNA	Mitochondrial DNA
MGH	Massachusetts General Hospital
MOP	Manual of Procedures
NCT	National Clinical Trial
NIH	National Institutes of Health
NO	Nitric Oxide
NYHA	New York Heart Association
OHRP	Office for Human Research Protections
OR	Odds Ratio
ORP	Office of Research Protections
PaCO ₂	Partial pressure of arterial carbon dioxide
PaO ₂	Partial pressure of arterial oxygen
PBMC	Peripheral Blood Mononuclear Cell
PEEP	Positive End-Expiratory Pressure
PI	Principal Investigator
Pplat	Plateau pressure
ppm	Parts per million
PS	Pressure Support Ventilation
RAGE	Receptor for Advanced Glycation Endproducts
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SBP	Systolic Blood Pressure
SBT	Spontaneous Breathing Trial
SOA	Schedule of Activities
SOC	System Organ Class
SOFA	Sequential Organ Failure Assessment
SOP	Standard Operating Procedure
SpCO	Non-invasive COHb by pulse oximetry
SPM	Specialized Pro-resolving Mediator
SRC	Scientific Review Committee
UAB	Unassisted Breathing
UP	Unanticipated Problem
US	United States
VBG	Venous Blood Gas
VFD	Ventilator-free Days
V _A	Alveolar ventilation
Vd/Vt	Dead space
vWF	von Willebrand factor
WBC	White Blood Cell

1.3 SCHEMA

TIME-EVENTS SCHEDULE

Measurement/Event	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 14	Day 28	Day 60	6M
Demographic/Admission Data/Medical Hx	X										
Etiology of ARDS	X										
Physical Exam	X										
Height/Body weight	X										
Time on ventilator prior to enrollment	X										
Pregnancy test	X										
Vital Signs (HR, SBP, DBP, MAP, T) ^B	X	X	X	X	X	X	X				
Central venous pressure ^B	A	A	A	A	A	A					
Electrocardiogram (EKG)	X	X	X	X	A	A	X				
Ventilator parameters ^{B, C}	X	X	X	X	X	X	X	X			
Chest X-ray (CXR) review ^D	X	A ^D	A ^D	A ^D	A ^D	A ^D	X				
Lung Injury Score (LIS)	X	X	X	X	X	X	X				
APACHE II Score	X										
SOFA Score ^E	X	X	X	X	X	X	X	X	X		
Glasgow Coma Score (GCS)	X	X	X	X	X	X	X	X	X ^F		
Richmond Agitation Sedation Scale (RASS)	X	X	X	X	X	X	X				
Arterial Blood Gas (ABG)	X										
ABG; Venous Blood Gas if ABG not available ^C		X	X	X	A	A	X	A			
SpO2	X	X	X	X	X	X	X	A			
ScvO2	A	A	A	A	A	A					
Complete blood count (CBC) ^C	X	X	X	X	A	A	A	A	A		
Basic metabolic panel ^C	X	A	A	A	A	A	A	A	A		
PT/PTT/INR ^C	A	A	A	A	A	A	A				
Serum CK, AST, ALT, Albumin, Total Protein ^C	A	A	A	A	A	A	A				
Bilirubin ^C	X	X	X	X	X	X	X	X	X		
Lactate ^C	X	X	X	X	A	A	A				
CO Administration Parameters / Treatment		X ^G	X ^G	X ^G							
Pulmonary Dead Space (Vd/Vt) Measurement		X	X	X			X				
COHb and SpCO		X ^G	X ^G	X ^G							
Vasopressors and inotropes ^B	X	X	X	X	X	X	X	X			
Fluid intake and output ^B	X	X	X	X	X	X					
Renal replacement therapy status	X	X	X	X	X	X	X	X			
Concomitant medications	X	X	X	X	X	X	X				
Adverse event monitoring ^H		X	X	X	X	X	X ^H				
Plasma isolation		X	X	X		X					

Measurement/Event	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 14	Day 28	Day 60	6M
RNA banking		X	X	X		X					
DNA banking – Cell pellet isolation		X	X	X		X					
Primary cell (PBMC) isolation		X				X					
Urine sample collection		X	X	X		X					
Discarded BAL and plasma ^I	A	A	A	A	A	A	A				
Microbiological results ^J	A	A	A	A	A	A	A	A	A		
Vital Status									X	X	X
Discharge Status									X	X	
Ventilator Status									X	X	
Vasopressor Status									X	X	
Neurocognitive Outcomes-MoCA-BLIND, Hayling											X

X - Required

A - When available

B - Data gathered at times indicated or until patient achieves 48 hours of unassisted breathing, whichever is sooner. Data will be obtained pre- and post-treatment on study drug administration days.

C - Measure during reference period (03:00-10:00), obtain at time closest to study drug administration if applicable

D - CXRs done for clinical purposes will be reviewed on days 1-5. If the subject is extubated during days 1-5, a chest X-ray will be ordered by the study team the day following extubation, if not ordered by the clinical team.

E - Record clinically available creatinine, platelets, bilirubin, SBP and vasopressor use

F - Gathered on day 28 or on discharge date

G - Daily on days of study drug administration

H - AEs will be captured until study day 7

I - Obtained from discarded biobank (site specific) if available

J - Microbiological results recorded as they become available

1.4 DEFINITIONS

Acute Kidney Injury: Acute kidney injury network Stage 3 disease, defined as a threefold increase in creatinine from baseline or the need for dialysis

Completing 48 hours of UAB (from weaning form): Defined as the date (calendar day) that the subject reaches exactly 48 hours of UAB. Example: if subject meets UAB at 1900 on 6/1/06 and does not return to AB, then the date of completing 48 hours of UAB would be 6/3/06.

Date of first UAB (from Study Termination form): Defined as the first day that the subject is on UAB from midnight to midnight. Example: if subject meets UAB at 1900 on 6/1/06, then the date of first UAB would be 6/2/06, as long as subject does not return to AB on 6/2/06.

Day zero: Defined as day of randomization. Study days are calendar days.

Drug held/hold drug: Study medication withheld for 24 hours.

Drug permanently discontinued: Study medication stopped for remainder of the trial.

Enrollment: The primary analysis population will be the modified intention-to-treat (MITT) population. All randomized subjects treated with at least one dose of the study drug will be included in the analysis. Because some patients may withdraw or have a change in clinical status precluding dosing of study drug (eg. post-randomization lactic acidosis), we may randomize additional patients to achieve the enrollment goal of 32 patients who are randomized and treated with at least one dose of the study drug. We expect this to be an uncommon occurrence. Patients who are randomized but do not undergo any study procedures or study treatment will not be included in the study analysis.

Extubation: Removal of an orotracheal, nasotracheal tube, or unassisted breathing with a tracheostomy.

Home: level of residence or health care facility where the patient was residing prior to hospital admission

Hospital Mortality to Day 60: This primary endpoint includes all deaths following randomization in any health care facility prior to discharge “home” until study day 60. Study subjects still in a health care facility at study day 61 are considered alive for this endpoint.

Interruption of Dosing During Drug Administration: Study medication prematurely stopped prior to 90 minutes.

NYHA: New York Heart Association Class IV subjects (defined as subjects who have cardiac disease resulting in inability to carry out physical activity without discomfort. Symptoms of cardiac insufficiency or an anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased).

Study Drug: Defined as inhaled carbon monoxide at 200 ppm or placebo.

Study hospital: Defined as the hospital where the patient was enrolled.

Study withdrawal: Defined as permanent withdrawal from study before completion of study activities. This does not include those subjects who have completed the protocol procedures or stopped procedures because

they have reached unassisted breathing. If a patient or surrogate requests withdrawal from the study, the investigators will seek explicit permission to continue data collection.

UAB (Unassisted Breathing): Spontaneously breathing with face mask, nasal prong oxygen, or room air, T-tube breathing, tracheostomy mask breathing, or CPAP ≤ 5 without PS or IMV assistance, or the use of noninvasive ventilation solely for sleep-disordered breathing. Assisted breathing is any level of ventilator support at pressures higher than the unassisted breathing.

2 STUDY SUMMARY

Title: A Phase II Trial of Inhaled Carbon Monoxide for the Treatment of Acute Respiratory Distress Syndrome (ARDS)

Objective: To evaluate the safety, tolerability, and efficacy of inhaled carbon monoxide (iCO), as well as to examine the biologic readouts of low dose iCO therapy in patients with ARDS.

Hypotheses:

- Low dose iCO will be safe and well-tolerated and will reduce mtDNA levels in ARDS patients.
- Low dose iCO will reduce the severity of lung injury and non-pulmonary organ failure in ARDS patients.
- Low dose iCO will reduce mitochondrial dysfunction, attenuate inflammasome activation, and promote resolution of inflammation in ARDS patients. Furthermore, we hypothesize that modulation of these pathways will predict improvements in the clinical outcomes of our Phase II trial.

Study Design:

1. Multi-center, prospective, randomized, partially double-blind, placebo-controlled Phase II clinical trial of inhaled CO for the treatment of ARDS.
2. 32 intubated, adult patients with ARDS will be randomized in a 1:1 ratio to receive either inhaled CO or inhaled air placebo for up to 90 minutes daily.
3. Subjects will be randomized to CO will be administered 200 ppm iCO to achieve a target COHb level of 5-8%.
4. Treatment will continue for 3 days, until discontinuation of mechanical ventilation, or death, whichever comes first.
5. Patients will be followed for 60 days or until discharge from the hospital to home with unassisted breathing, whichever occurs first.
6. Vital status and neurocognitive testing will be performed at 6 months via telephone interviews.

Sample Size/Interim Monitoring:

1. A total of 32 subjects will be enrolled.
2. The primary analysis will be safety and efficacy.
3. Trial progress will be monitored by an independent Data and Safety Monitoring Board (DSMB). The DSMB will monitor trial quality and feasibility approximately every 6 months and will be available as needed on *ad hoc* basis.

Study Population: All intubated patients ≥ 18 years old with ARDS according to the Berlin criteria will be eligible for inclusion. Subjects will be recruited from the medical, surgical, cardiac, and burn ICUs at each center with a *specific focus* on patients with ARDS secondary to infection, trauma, transfusion, burns, hemorrhagic shock,

inhalation, and/or oxygen exposure.

Primary Endpoint: The primary endpoint is to evaluate the safety of inhaled CO, by determining COHb levels and the incidence of pre-specified administration-related adverse events.

Primary Efficacy Endpoint: The primary efficacy endpoint is to compare the effects of iCO versus placebo on mtDNA levels from baseline to day 5 (or death, whichever comes first) in patients with ARDS.

Secondary Endpoints:

The secondary endpoints will examine the effects of iCO versus placebo the following outcomes:

- Lung Injury Score (LIS) on days 1-5, and 7
- PaO₂/FiO₂ ratio on days 1-5, and 7
- Oxygenation Index (OI) on days 1-5, and 7
- Dead Space Fraction (Vd/Vt) on days 1-3, and 7
- Sequential Organ Failure Assessment (SOFA) score on days 1-5, 7, 14, 28

Additional secondary endpoints will examine the effects of iCO versus placebo on biomarkers of mitochondrial dysfunction, inflammasome activation, and lipid mediators in patients with ARDS.

- Autophagy markers (eg. LC3B)
- Inflammasome-dependent cytokines (eg. IL-18)
- Lipid mediators (LM) and specialized pro-resolving mediators (SPMs)
- Mitochondrial quality control biomarkers (eg. Pink1, Wipi1)
- Biomarkers of inflammation (eg. IL-6, IL-8, IL-10, IL-1Ra)
- To enhance the biologic analytic power, samples from 12 subjects enrolled in a similar, already completed study will be similarly analyzed for these biomarkers, as well as in more extensive biologic 'omics profiling that will permit more detailed analyses of these pathways in correlation with clinical data.

Exploratory Endpoints:

- Ventilator-free days at day 28
- ICU-free days at day 28
- Hospital-free days at day 60
- Hospital mortality to day 28 and 60

Focused Safety Analysis: The incidence of elevation in plasma COHb $\geq 10\%$ measured on study days 1-3 and pre-specified administration-associated adverse events and serious adverse events.

Study Drug Dosing: All study drug doses will be administered via inhalation using a mechanical ventilator approved for nitric oxide (NO) delivery and the CO Delivery System. The study drug will be blinded to the study staff using identical tanks containing either CO or placebo air. The administering respiratory therapist (RT) and a physician study staff member OR a physician study staff member alone will be unblinded to the treatment assignments.

Completion of study drug administration: Study drug administration will be stopped when one of the following conditions is met, whichever comes first:

1. Completion of the third dose of study drug

2. Discontinuation of mechanical ventilation
3. Death
4. Pre-specified criteria met for permanent discontinuation of study drug (**Section 7.1.8**)

3 INTRODUCTION

3.1 STUDY RATIONALE

The purpose of this study is to assess the safety and efficacy of low dose inhaled carbon monoxide (iCO) therapy in mechanically ventilated patients with ARDS. By studying subjects with ARDS, we have targeted a disease that has been well studied in rodent and non-human primate preclinical models. Furthermore, by focusing on intubated subjects with ARDS, we have chosen a group with higher disease burden and thus likely to have both increased mortality and an increased opportunity for benefit, including a reduction in the requirement for mechanical ventilation.

Mitochondrial dysfunction is associated with increased disease severity and poor outcomes during sepsis and may be a key mechanism underlying ARDS and multiple organ dysfunction syndrome in critically ill patients¹. Furthermore, early activation of mitochondrial biogenesis has been associated with improved survival in critical illness². We and others have demonstrated that circulating mitochondrial DNA (mtDNA) levels are significantly increased in patients with sepsis and ARDS and correlate with mortality in critically ill patients^{1,3,4}. We have also shown that CO can inhibit mitochondrial reactive oxygen species (ROS) generation, inhibit translocation of mtDNA into the cytosol, and preserve mitochondrial function in macrophages⁵. CO also has been shown to activate mitochondrial biogenesis in skeletal muscle in humans⁶. Taken together, these findings suggest that preservation of mitochondrial function may be a key mechanism by which CO protects against ARDS and multiple organ failure in critical illness.

3.2 BACKGROUND

3.2.1 INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a devastating disease affecting military, veteran, and civilian populations. ARDS is a syndrome of severe acute lung inflammation and hypoxemic respiratory failure with an incidence of 180,000 cases annually in the United States^{7,8}. Despite recent advances in critical care management and lung protective ventilation strategies⁹⁻¹², ARDS morbidity and mortality remain unacceptably high¹³⁻¹⁵. Furthermore, no specific effective pharmacologic therapies currently exist. Although pneumonia and sepsis are the most common causes of ARDS¹⁴, ARDS also frequently develops after trauma, burn injury, hemorrhagic shock, massive transfusions, and inhalational injuries, all critical challenges of military medicine and combat situations.

Trauma is a significant risk factor for ARDS development^{14,16-22} in both civilian and military populations and is associated with increased morbidity, prolonged duration of mechanical ventilation, and extended intensive care unit (ICU) and hospital lengths of stay^{16,19,22}. Additionally, a recent study on combat-associated respiratory failure revealed that ARDS is independently associated with increased mortality in modern US combat casualties²³. Furthermore, burn injury^{13,24,25}, pulmonary contusion²⁶⁻²⁸, shock, blood transfusions^{21,29,30}, aspiration, pneumonia, and sepsis have all been shown to be associated with the development of ARDS in trauma patients^{14,17,18,31}.

The lack of specific effective therapies for ARDS indicates a need for new treatments that target novel pathways. Carbon monoxide (CO) represents a novel therapeutic modality in ARDS based on data obtained in experimental models of ARDS over the past decade.

3.2.2 CO IS AN ENDOGENOUSLY PRODUCED GAS WITH PLEIOTROPIC BIOLOGICAL FUNCTIONS

CO is endogenously produced in the body by heme oxygenase-1 (HO-1), one of the few inducible molecules that can protect the lung from an increased oxidant burden under circumstances of stress³². HO-1 is ubiquitously expressed, and is responsible for degradation of heme to biliverdin, free iron, and CO. While all three products of its activity have been shown to possess cytoprotective properties, CO is the product that has been most extensively studied with respect to lung disease. This colorless, odorless diatomic gas has been shown to exert biological functions as diverse as protection against oxidative injury³³⁻³⁵, inflammation³⁶, and cell death^{37,38}, inhibition of cell proliferation³⁹, suppression of matrix production⁴⁰, increased fibrinolysis⁴¹, as well as enhanced phagocytosis^{42,43} and macrophage efferocytosis⁴⁴, all of which may be important in the pathogenesis of ARDS and underlying infection. Recently, we have demonstrated several mechanisms by which CO exerts these beneficial effects including activation of mitochondrial biogenesis⁴⁵⁻⁴⁸, enhancement of autophagy^{43,49,50}, suppression of mitochondrial dysfunction and inflammasome activation⁵, as well as acceleration of resolution of inflammation via production of specialized pro-resolving lipid mediators (SPMs)^{44,48,51-53}.

3.2.3 ADMINISTRATION OF INHALED CO PROTECTS AGAINST LUNG INJURY IN ANIMAL MODELS

Our laboratory and others have demonstrated that CO at a low physiological dose confers cytoprotection via potent anti-inflammatory, anti-apoptotic, and anti-proliferative effects³³⁻³⁹. These responses are regulated by cell- or tissue-specific, and stimuli- or stress-specific signaling pathways⁵⁴. We and others worldwide have demonstrated that CO mediates cytoprotection in a variety of tissue injury models including experimental models of sepsis and acute lung injury (ALI) (**Table 1**)^{34-36,38,40,42,43,48,55-65}. These studies have shown that low dose CO confers tissue protective effects in experimental ALI models including hyperoxia and endotoxin exposure, bleomycin, ischemia/reperfusion, and ventilator-induced lung injury (VILI)^{34,35,40,55-60}.

We have also recently shown that low dose iCO accelerates resolution of ALI in a clinically-relevant baboon model of pneumococcal pneumonia^{48,51,52,66}. Administration of iCO at 200 ppm for 60 minutes at 48 hours following *S. pneumoniae* inoculation significantly attenuated histologic lung injury scores and reduced lung wet-to-dry ratios at 8 days⁴⁸. CO-treated animals had significantly increased expression of citrate synthase and increased ATP synthase staining in alveolar type 2 cells and macrophages suggesting that CO induces mitochondrial biogenesis⁴⁸. We also found that CO had systemic anti-oxidant effects with augmentation of SOD2 expression in the kidney, which co-localized with cytochrome *c* consistent with attenuated mitochondrial injury and cytochrome *c* retention in intact mitochondria⁴⁸. CO also reduced pro-inflammatory urinary cysteinyl leukotrienes⁵² and partially restored levels of circulating SPMs⁵¹.

In addition, we and others have demonstrated that CO decreases inflammation, enhances phagocytosis, and improves mortality in models of sepsis including endotoxemia^{36,61,62}, hemorrhagic shock⁶³ and cecal ligation and puncture (CLP)^{42,43}. Furthermore, CO has been shown to have beneficial therapeutic effects in pre-clinical models of other diseases including pulmonary hypertension⁶⁷⁻⁷⁰, vascular injury⁷¹⁻⁷⁵, and transplantation^{58,59,76-84} (**Table 1**).

Table 1: Pre-clinical studies of inhaled CO

Model	Year	Species	CO (ppm)	Outcome	Reference
Lung injury (VILI, acid, hyperoxia, LPS, pneumonia)	1999, 2003, 2004, 2008, 2009, 2010, 2015	mouse, rat, macaques, baboon	10-250, 500	Less inflammation & lung injury	34,35,48,55-57,64
Bleomycin lung fibrosis	2005	mouse	250	Decreased lung hydroxyproline, fibronectin, collagen	40
Ischemia-reperfusion (hind leg, lung)	2001, 2003, 2006, 2007, 2009	mouse, rat	250, 500, 1000	Less remote organ inflammation, less apoptosis, improved survival	38,41,60,85-87
Transplantation (liver, lung, kidney, heart, intestine)	2001, 2003, 2004, 2006, 2007, 2008, 2009	mouse, rat	20, 250, 400, 500	Improved survival & graft function, less inflammation & apoptosis	58,59,76-79,81-84,88
Endotoxemia	2000, 2003, 2004	rat, mouse	10-250	Improved survival, decreased inflammation	36,61,62
Hemorrhagic shock	2005	mouse	250	Decreased end organ injury/ischemia	63
Cecal ligation and puncture	2008, 2014	mouse	250	Improved survival, decreased inflammation, enhanced phagocytosis	42,43
Pulmonary arterial hypertension	2006	rat, mouse	50, 250	Reversal of established PAH & reversal of remodeling	67
Vascular injury	2003, 2005, 2006, 2007	mouse, pig	100, 250-500	less intimal hyperplasia, reduced thrombosis	71-75
Cardiopulmonary bypass	2004, 2008, 2009	pig	250	Less lung injury, decreased cardiac edema, apoptosis	89-92
Doxorubicin cardiomyopathy	2007	mouse	500	Improved cardiac function	93,94
Asthma	2003	mouse	250-1000	Reduced inflammation & bronchoconstriction	40,95,96
Cerebral malaria	2007	mouse	250	Reduced incidence of cerebral malaria	97
Hepatitis	2003, 2007	mouse	100, 250, 500	Improved survival, decreased apoptosis	98,99

Model	Year	Species	CO (ppm)	Outcome	Reference
Colitis, ileus	2005	mouse, rat, pig	250	Reduced injury & inflammation, improved motility	67,100-102
Sickle cell disease	2009	mouse	250	Reduced leukocytosis	103
Collagen-induced arthritis	2009	mouse	200	Improved arthritis score, less inflammation	104
Ureteral obstruction	2008	mouse	250	Reduced renal fibrosis	105

3.2.4 LUNG PROTECTIVE EFFECTS OF CARBON MONOXIDE

Numerous studies have examined the protective effects of low concentrations of CO on the pulmonary parenchyma and vasculature. Inhaled CO prolongs survival and prevents tissue injury and epithelial cell death in rodents subjected to high oxygen stress³⁴. CO also reduces lung cell apoptosis during lung ischemia-reperfusion injury in mice⁶⁰ and prevents tissue injury during mechanical ventilation in mice by preventing alveolar-capillary barrier dysfunction and reducing inflammation⁵⁵⁻⁵⁷. Low concentration iCO can also reverse established pulmonary hypertension in rats⁶⁷ and has been shown to protect against endothelial apoptosis³⁷. In addition, CO has been shown to de-repress fibrinolysis and to inhibit expression of plasminogen activator inhibitor-1, which could alter the progression of fibrosis⁴¹. CO has also been shown to confer protection in a number of additional disease models, including asthma, vascular injury, transplantation and fibrosis (**Table 1**). These studies collectively have provided a rationale for pursuing the clinical applications of CO, including the trials outlined below.

3.2.5 CO DELIVERY VIA AN INHALED ROUTE IS SAFE IN HUMAN SUBJECTS

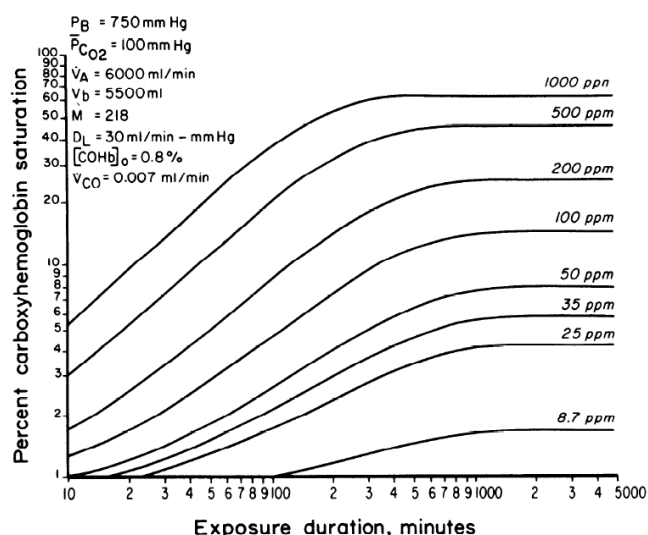


Figure 1. COHb levels as a function of exposure duration and CO concentration as determined by the CFK equation. (Peterson et al., 1975)

CO has proven to be an ideal gas for developing theoretical uptake equations. The formation of carboxyhemoglobin (COHb) on the basis of CO exposure is well described by a physiologically based pharmacokinetics model developed by Coburn in 1965 and is referred to as the Coburn-Foster-Kane equation (typically identified as the CFK or CFKE) in the literature¹⁰⁶. This model has been tested and confirmed in humans for varying inspired CO concentrations and durations of exposure¹⁰⁷⁻¹¹³ (**Figure 1**).

Extensive data is available regarding the safety and tolerability of low dose iCO in healthy volunteers^{6,111,114-118} and more recently, in subjects with chronic obstructive pulmonary disease (COPD)¹¹⁹ and idiopathic pulmonary fibrosis (IPF) (**Table 2**). Previous studies have carefully measured COHb levels in response to iCO and demonstrated that low dose iCO is safe in healthy normal

volunteers^{6,45,111,114-118}. Stewart et al. performed 25 exposures to known CO concentrations in healthy volunteers; in this study, exposure to 100 ppm CO for 8 hours resulted in COHb levels of 11-13% with no adverse effects in time estimation, steadiness, manual dexterity, EEG, and evoked potentials¹¹⁶. In a study aiming to simulate cigarette smoke inhalation, Zevin et al. exposed healthy volunteers to iCO (1200-1500 ppm) for 10 minutes, repeating every 45 minutes for 16 hours per day for 7 days¹¹⁷. In this study, mean COHb levels were $5 \pm 1\%$ and no adverse events (AEs) were reported¹¹⁷. Pecorella et al. has also demonstrated safety (COHb $5.4 \pm 0.79\%$) and activation of mitochondrial biogenesis in healthy individuals after CO inhalation at 200 ppm for 1 hour per day for 5 consecutive days⁴⁵.

Table 2: Clinical Trials of CO in Human Subjects

Study	CO Exposure	COHb	Adverse Effects
Stewart (1970) ¹¹⁶	100 ppm for 8 h	11-13%	No adverse effects
Peterson (1975) ¹¹¹	50-200 ppm, up to 5.25 h	1-20%	None noted
Hausberg (1997) ¹¹⁴	1000 ppm x 30 min, then 100 ppm x 30 min	$8.3 \pm 0.5\%$	None reported
Zevin (2001) ¹¹⁷	1500 ppm x 10 min, then every 45 min x 16 h for 7 days	$5 \pm 1\%$	None reported
Ren (2001) ¹¹⁸	4000 ppm until COHb ~ 10%, then repeated to keep COHb ~ 10% for 8 h	$9.7 \pm 0.1\%$	None reported
Mayr (2005) ¹¹⁵	500 ppm for 1 h	6.5-7.7%	Mild headache in 1 subject
Rhodes (2009) ⁶	100 ppm for 1 h for 5 days	$3.3 \pm 0.6\%$	No adverse effects
Pecorella (2015) ⁴⁵	200 ppm for 1 h for 5 days	$5.4 \pm 0.79\%$	No adverse effects
Bathorn (2007) ¹¹⁹	125 ppm for 2 h, 4 consecutive days (COPD)	2.1-3.4%	2 COPD exacerbations, judged unrelated
NCT00094406 (IND# 70,694)	100 ppm for 6 h	$6.5 \pm 1.7\%$	No adverse effects
Rosas (2017) ¹²⁰	100-200 ppm x 2 h, 2 times weekly x 12 wks (IPF)	3-4%	Well tolerated, no SAEs related to CO
NCT02425579 ¹²¹	100 or 200 ppm for 90 min for up to 5 days	$3.48 \pm 0.7\%$ (100 ppm) $4.9 \pm 0.28\%$ (200 ppm)	Well tolerated, no SAEs related to CO

In a study evaluating the effects of hypoxemia, hemodilution, and carboxyhemoglobinemia on respiratory control, Ren et al. exposed 11 normal volunteers to an iCO treatment regimen aiming to maintain a COHb level of 10% for 8 hours¹¹⁸. COHb levels ranged from 9.1 to 10.5% (mean 9.7%) and no AEs were reported¹¹⁸. Similar results have been published in a number of other studies, and none have reported AEs^{6,45,111,114,115}. In fact, baseline COHb levels of 3% have been reported in some urban areas¹¹⁶ and levels as high as 10-15% may be observed in asymptomatic chronic smokers¹²²⁻¹²⁴.

We have conducted three studies, one in normal human subjects (NCT00094406), one in subjects with IPF (NCT01214187)¹²⁰, and one recently completed Phase I trial in sepsis-induced ARDS (NCT02425579). In our placebo-controlled study of 24 healthy volunteers (11 females; mean age 26.2 ± 5.2 yrs), individuals were randomized to treatment with room air or iCO (100 ppm) for 6 hours following endotoxin instillation. CO

treatment was well tolerated and mean COHb levels were $6.5\% \pm 1.7\%$ in CO-treated subjects. There were no significant differences in vital signs, neurocognitive studies (including immediate and delayed memory, attention, language and visuospatial/constructional function), or AEs (**Table 3**).

Table 3: Vital Signs and Laboratory Parameters in CO-Treated Healthy Volunteers.

Parameter	Room Air	CO (100 ppm)	p value
Respiratory rate	17.7 ± 3.6	16.7 ± 4.0	0.5
Heart rate	84.5 ± 14	83.8 ± 15	0.9
Temperature °C	36.9 ± 0.4	37.2 ± 0.4	0.15
Mean arterial pressure (mm Hg)	92.8 ± 11	98.7 ± 11	0.21
Carboxyhemoglobin (%)	1.1 ± 0.7	6.5 ± 1.7	0.001
Oxyhemoglobin (%)	96.3 ± 2.1	92.3 ± 1.9	0.002
PaO ₂	96.5 ± 11	94 ± 7.3	0.54
Arterial pH	7.4 ± 0.02	7.41 ± 0.02	0.29
Lactate (mmol/L)	0.7 ± 0.26	0.62 ± 0.19	0.41

In addition to studies in healthy volunteers, prior work has demonstrated the feasibility of administering low dose iCO to subjects with COPD¹¹⁹. In this study, ex-smoking subjects with stable COPD were treated with iCO at 100-125 ppm for 2 hours per day on 4 consecutive days. This led to COHb levels of 2.1-3.4% with a maximal individual COHb level of 4.5%. Inhalation of CO by subjects with stable COPD led to trends in reduction of sputum eosinophils and improvement in methacholine responsiveness¹¹⁹. In our recently completed 8-center Phase I/II trial of low dose iCO in IPF^{120,125}, 58 subjects were randomized to treatment with iCO (100-200 ppm) or 21% O₂ for 2 hours, twice weekly for 12 weeks. Inhaled CO was well-tolerated with no significant difference in AEs or neurocognitive function by the Montreal Cognitive Assessment (MoCA) between iCO-treated and control subjects¹²⁰.

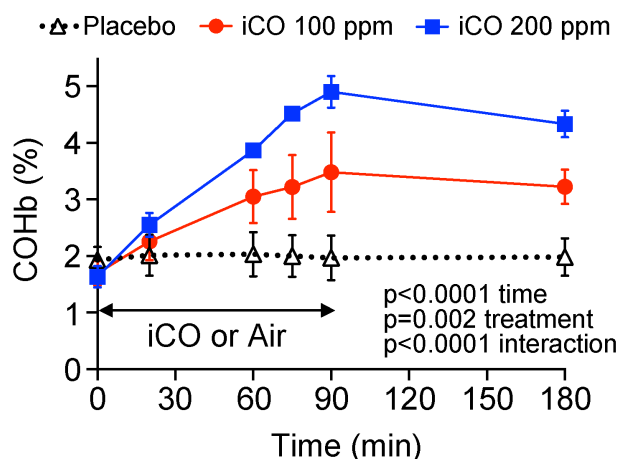


Figure 2. Low dose iCO can be safely and accurately administered in mechanically ventilated subjects with ARDS.

minutes (Δ 90 min COHb) was 2.5% in Cohort 1 and 4% in Cohort 2. There was no significant increase in COHb in placebo subjects.

Taken together, these findings demonstrate that experimental administration of several different concentrations of CO is well tolerated and that low dose iCO can be safely administered to subjects in a controlled research environment.

More recently, we completed a Phase I trial of low dose iCO in patients with sepsis-induced ARDS¹²¹. Twelve participants were randomized to iCO or placebo air 2:1 in two cohorts. Four subjects each were administered iCO (100 ppm in Cohort 1 or 200 ppm in Cohort 2) or placebo for 90 minutes for up to 5 consecutive days. CO treatment was well tolerated and no participants exceeded a COHb level of 10%. There were no administration-associated AEs or study-related serious AEs (SAEs). Baseline COHb levels were not different across treatment groups. CO-treated participants had a significant increase in COHb ($3.48 \pm 0.7\%$ [Cohort 1]; $4.9 \pm 0.28\%$ [Cohort 2]) compared with placebo-treated subjects ($1.97 \pm 0.39\%$), which did not differ from baseline levels (**Figure 2**). The maximum COHb level in Cohort 1 was 4.4% and 6.8% in Cohort 2. The maximum change in COHb from baseline to 90

3.2.6 STEADY STATE DIFFUSING CAPACITY AND THE SAFETY OF INHALED CARBON MONOXIDE

CO is also a diagnostic gas that has been used for more than a century to evaluate lung function, and in particular, in the steady state diffusing capacity test to determine the function of the alveolar-capillary membrane. The steady state method of measuring the diffusing capacity of the lung dates back more than 100 years, based on the research of Haldane and Smith. In the early 1900's, Krogh and Barcroft developed it into a standard test procedure for both understanding alveolar membrane function, as well as for diagnosing diseases of the alveolar-capillary membrane. Up until the 1970's, steady state diffusing capacity was the standard diagnostic test for pulmonary laboratories worldwide, at which point it was replaced by the single breath diffusing capacity test due to its better accuracy and being less time consuming. The steady state diffusing capacity test is still used today in some instances where the single breath procedure is not as practical, such as measurements during exercise. The procedure for steady state diffusing capacity testing entails patients inhaling 0.1% CO (1000 ppm) for seven minutes¹²⁶. As duplicate or triplicate measurements are required for most lung function tests, this suggests that hundreds of thousands of people have inhaled 1000 ppm for 14 to 21 minutes with no known reports of adverse events associated with the test. Based on the curves from the CFK equation (**Figure 1**), it is likely that this same number of people had their COHb levels raised above 3-6% following the diagnostic procedure. This longstanding diagnostic procedure reinforces that inhaling a constant concentration of low dose CO can be safely done without significant adverse events.

3.2.7 DELIVERY OF INHALED CO TO MECHANICALLY VENTILATED SUBJECTS



Figure 3. CO Delivery System (12th Man

In order to study inhaled CO in mechanically ventilated subjects with ARDS in this Phase II trial, a Carbon Monoxide Delivery System (COventDS) (**Figure 3**) developed by 12th Man Technologies will be used (**Appendix A- CO Delivery System**). The CO Delivery System is a microprocessor-based constant gas concentration delivery system that can be used to deliver operator-specified concentrations of CO to mechanically ventilated patients. The CO Delivery System delivers an operator set, constant concentration of CO gas into the inspiratory path of the patient breathing circuit independent of the patient's inspiratory flow, while the patient's respiration is supported by a ventilator. For safety reasons, the CO Delivery System has twin microprocessors such that the division of control is split between a closed loop proportional-integral-derivative (PID) controlled mixing module that is only involved with monitoring the patient flow and mixing of the gases, and an interface module that is the working face to the user for control and monitoring functions. This second microprocessor watches the delivery subsystem, monitors the inspired gas for deviations from the set concentration, and monitors inspired oxygen.

The heart of the system is comprised of three components including an inspiratory flow monitor with a ratio-metric matching CO injector module, an inhaled gas monitoring module, and a gas mixing subject interface/breathing valve for spontaneously breathing subjects. It is a variable inspiratory flow delivery system that matches the patient's inspiratory flow with the injected 5000 ppm CO to deliver the operator set CO concentration on the LCD user interface. It is a breathing-initiated delivery system and the CO is blended into the inspiratory gas stream only as long as flow is being delivered to the patient and at the exact proportions to maintain the desired concentration, independent of any change in breathing pattern, flow rate, respiratory rate, or tidal volume. The analyzer, with alarm functions, monitors the inhaled CO and O₂ concentrations.

The CO Injector is a core component for the delivery of CO. The CO Injector is constructed of CO-compatible materials and consists of a pressure regulation circuit that reduces the 40-60 psig inlet CO gas source to the optimal pressure for its proportional flow control valve. Upon sensing inspiratory flow by the patient with the flow monitoring interface, the injector module will track the flow and match the volume of CO injected to the volume of inspired gas to keep the concentration of CO constant independent of the patient's pattern of inspiratory flow. The PID controlled mixing module's sole function is to read the patient's inspiratory flow and inject CO proportional to that flow in 1 millisecond intervals. Alarms will sound on high or low CO or O₂ concentrations. Should the inspired CO concentration rise above 660 ppm, in addition to the alarms, the system will stop injecting CO into the circuit.

3.2.8 TESTING THE CO DELIVERY SYSTEM IN BABOON MODEL OF PNEUMOCOCCAL PNEUMONIA

We evaluated the safety and efficacy of the CO Delivery System developed by 12th Man Technologies in a baboon model of *S. pneumoniae* pneumonia (**Figure 4**). Five juvenile, male colony-bred baboons (*Papio cynocephalus*) were intubated, sedated, and mechanically ventilated⁴⁸. The animals underwent bronchoscopy and a baseline

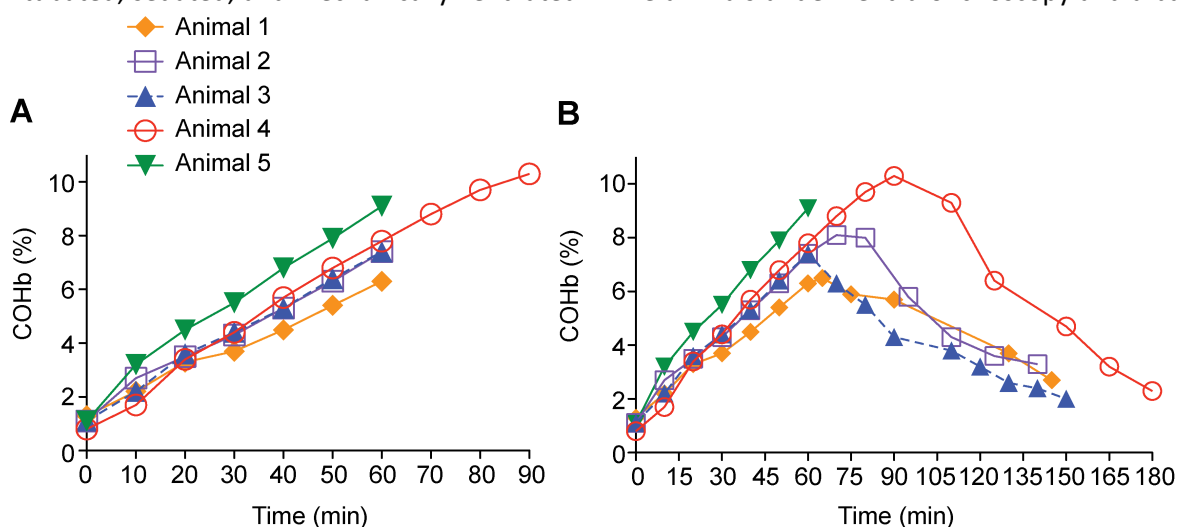


Figure 4. Delivery of inhaled CO at 200 ppm and COHb levels in baboon pneumonia model.

bronchoalveolar lavage (BAL) was performed followed by instillation of *Streptococcus pneumoniae* (10^8 - 10^9 CFU) in the right and left lower lung zones. At 24 or 48 hours post-inoculation, animals were sedated, intubated, ventilated, and underwent a repeat bronchoscopy and BAL.

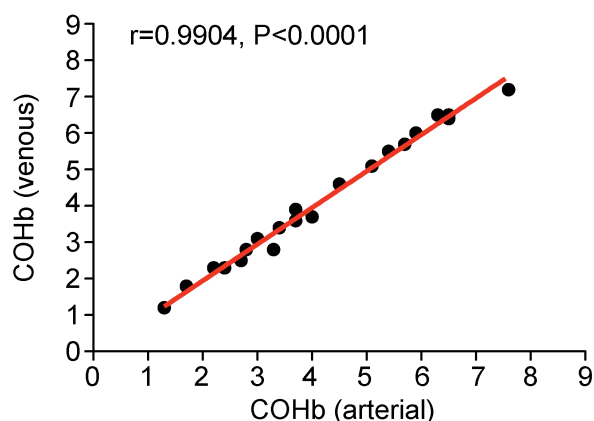


Figure 5. Correlation between arterial and venous COHb levels.

The CO Delivery System was calibrated using 100 ppm and 400 ppm CO tanks and readied for use with a 5000 ppm CO source cylinder. All CO tanks contained CO gas at the specified concentration in air. Ambient CO levels were monitored during the CO delivery device assembly, calibration, and continuously throughout the experiment using a CO detector.

Following bronchoscopy, animals were administered iCO at 200 ppm through the ventilator via the CO Delivery System for 60-90 minutes. After iCO treatment was completed, animals were administered supplemental FiO₂ for 60-90 minutes until COHb levels returned to near baseline levels.

Arterial blood was drawn before, during, and after CO delivery at 10-15 minute intervals and arterial blood gas (ABG) and COHb measurements were performed. In certain experiments, both venous and arterial blood samples were drawn simultaneously for measurement of venous and arterial COHb levels respectively (**Figure 5**). COHb levels were measured using the IL 682 Co-oximeter and, in certain experiments, using the AVOXimeter 4000 Co-oximeter. After CO exposure, animals were administered ceftriaxone daily for a total of 3 days.

After one hour of iCO administration at 200 ppm, animals achieved the pre-specified goal COHb level of 6-8%. At baseline, arterial COHb levels were $1.1 \pm 0.2\%$ and increased linearly to $2.4 \pm 0.6\%$, $3.7 \pm 0.5\%$, $4.5 \pm 0.7\%$, $5.5 \pm 0.8\%$, $6.6 \pm 0.9\%$, and $7.6 \pm 1\%$ at 10, 20, 30, 40, 50 and 60 minutes of iCO administration⁴⁸, respectively ($p < 0.0001$) (**Figure 4A**). One animal was intentionally given a prolonged (90 minute) exposure which similarly demonstrated a linear rise in COHb to 7.8%, 8.8%, 9.7%, and 10.3% at 60, 70, 80, and 90 minutes, respectively. Peak COHb levels decreased following administration of FiO_2 1.0, returning to near baseline levels after 82 ± 9.5 minutes⁴⁸ (**Figure 4B**). Throughout the exposure, ambient CO levels remained ≤ 1 ppm. Low dose iCO treatment was well-tolerated with no significant differences in pre- and post-CO heart rate, blood pressure, temperature, PaO_2 , or minute ventilation.

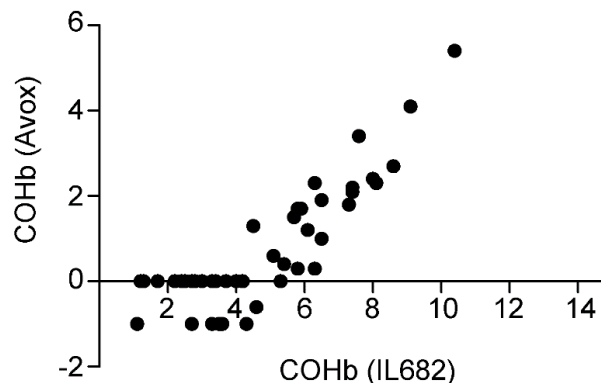


Figure 6. Measurement of COHb levels by AVOX4000 versus IL682 Co-oximeters.

Using the IL 682 Co-oximeter, we found that venous and arterial COHb levels were highly correlated ($r = 0.9904$, $p < 0.0001$)⁴⁸. Modeling these data with type II linear regression, we found that the regression line was a near-perfect diagonal with a slope of 1.003 (95% CI [0.952 – 1.05]) and a y-intercept of -0.064 (95% CI [-0.2941 – 0.1661])⁴⁸ (**Figure 5**). This tight correlation argues that venous measurements are as accurate and reliable as arterial measurements. To determine the accuracy and precision of the point-of-care AVOXimeter 4000 Co-oximeter relative to the gold-standard IL 682 Co-oximeter, we measured COHb levels using both devices on synchronously drawn arterial blood. Compared with the IL 682 Co-Oximeter, the AVOXimeter 4000 Co-oximeter was substantially less sensitive as COHb was not detected until levels had reached 4-5%. Furthermore, the AVOXimeter consistently reported COHb levels that were four percentage points lower than the gold standard (**Figure 6**).

Ambient CO levels in the experiment room remained at 0-1 ppm during assembly, calibration, and use of the CO delivery device. These levels are well below the OSHA permissible exposure limit of 50 ppm as an 8 hour time-weighted average (U.S. Department of Labor, Occupational Safety & Health Administration, <https://www.osha.gov/chemicaldata/chemResult.html?RecNo=462>).

3.2.9 CO DOSING STRATEGY USING CFK EQUATION

In order to develop a safe and effective dosing strategy based on an initial short exposure to inhaled CO, we also evaluated the accuracy of the Coburn-Forster-Kane (CFK) equation (**below**)^{106,111} to predict COHb levels in baboons using measured COHb levels following a 10 and 20 minute CO exposure. Using the 10 minute COHb, we found that there was good correlation between measured COHb levels and the COHb levels predicted by the CFK equation ($r = 0.9038$, $p < 0.0001$)⁴⁸. However, there was superior correlation between

$$\frac{A[\text{HbCO}]_t - B\dot{V}_{\text{CO}} - P_{\text{ICO}}}{A[\text{HbCO}]_0 - B\dot{V}_{\text{CO}} - P_{\text{ICO}}} = \exp(-tA/VbB)$$

measured COHb levels and COHb levels predicted by the CFK equation using the 20 minute COHb ($r=0.9828$, $p<0.0001$)⁴⁸ (Figure 7A).

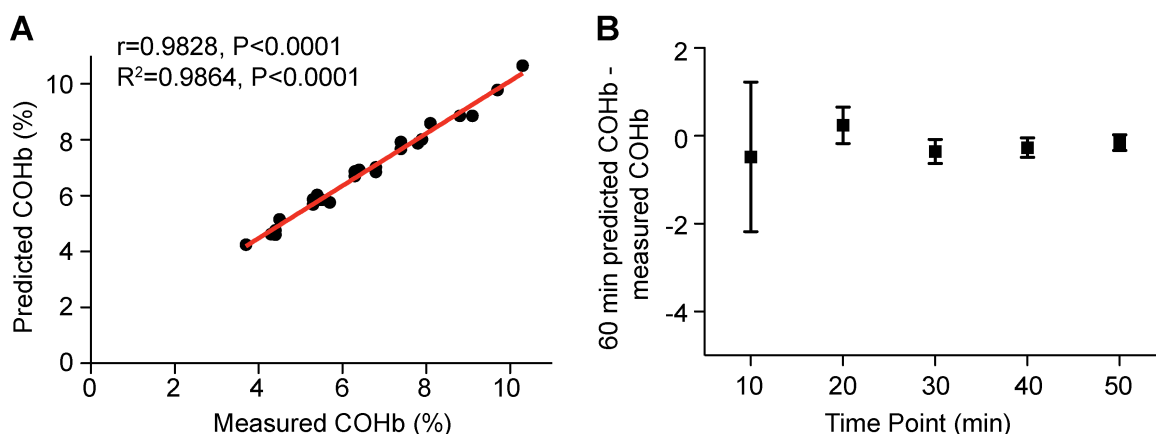


Figure 7. Exposure to CO for 20 min allows for accurate prediction of 60 min COHb using CFK equation.

Modeling these data with linear regression, we found that the regression line was a near-perfect diagonal with a slope of 0.9373 (95% CI [0.8885 – 0.9860]), a y-intercept of 0.7291 (95% CI [0.3976 – 1.061]), and goodness-of-fit $R^2=0.9864$ ($p<0.0001$)⁴⁸. Furthermore, the 20-minute measured COHb was highly accurate in predicting the 60 minute COHb with a difference between predicted and measured COHb of $0.24 \pm 0.33\%$ (95% CI [-0.17 – 0.66])⁴⁸ (Figure 7B). Taken together, by inputting the 20-minute measured COHb level into the CFK equation, we can predict the COHb level after 60 minutes of CO exposure with high accuracy.

In our recently completed Phase I trial, we also found an excellent correlation between measured and predicted COHb levels using the CFK equation. We found an excellent correlation in participants treated with 100 ppm iCO (Spearman $r=0.8614$; $p<0.0001$), but an even stronger correlation in subjects treated with 200 ppm iCO (Spearman $r=0.916$; $p<0.0001$) (Figure 8A). Modeling the 200 ppm Cohort 2 data with linear regression revealed a slope of 1.112 (95% confidence interval [CI] [1.003, 1.22]), a y-intercept of -0.6632 (95% CI [-1.174, -0.1522]), and goodness-of-fit $R^2=0.9204$ ($p<0.0001$). Bland-Altman plots also demonstrated excellent agreement between measured and CFK equation-predicted COHb levels (Figure 8B) with a mean difference between measured and predicted COHb levels of 0.1467 ± 0.2738 in Cohort 2 (200 ppm).

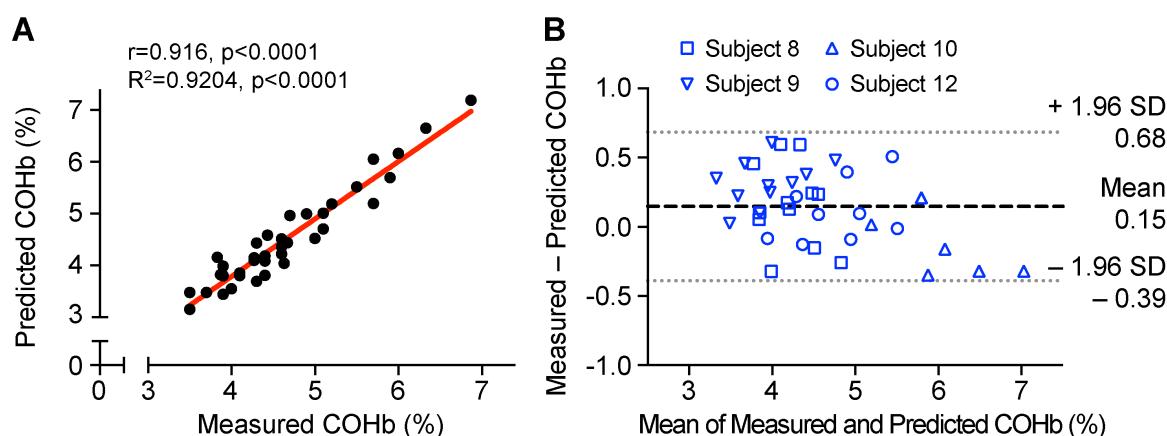


Figure 8. CFK equation accurately predicts COHb levels in ARDS patients.

The above predictions using the CFK equation were made using a MATLAB-generated computer program to estimate DLCO using the baseline and 20 minute COHb. The estimated DLCO was then input into the

programmed CFK equation and used to predict the 60, 75, and 90 minute COHb levels. This program was validated by generating the previous published curves (**Figure 1**) that were derived from predicted values using the CFK equation¹¹¹. In addition, we used the programmed CFK equation and published values for CFK variables in ARDS (DLCO, V_A , Hb, weight, FiO_2) to predict COHb levels in ARDS patients (average and severe) for a given CO concentration and duration of exposure.

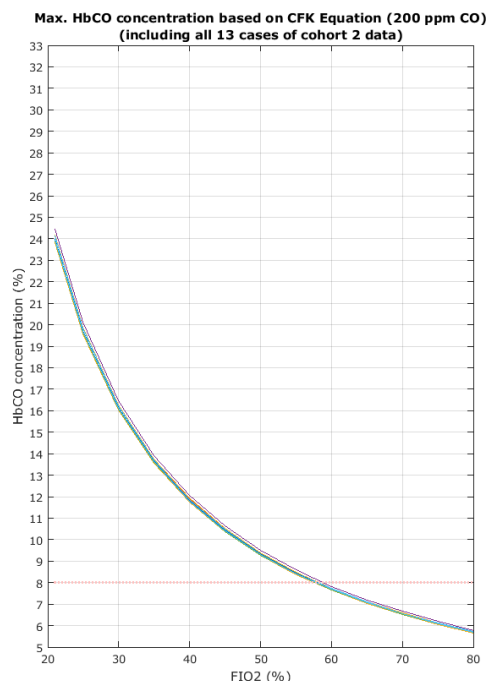


Figure 9. Maximum COHb level vs. FiO_2 at 200 ppm CO inhalation.

Based on these data and to simplify the CFK equation-based dosing in this Phase II study, we generated CFK equation-based tables (**Appendix B or electronic equivalent version in StudyTRAX**), which will be used to look up the time at which the COHb level is predicted to be 8%. We used the CFK equation and mean values (V_A , Hb, weight) from our Phase I Cohort 2 ARDS subjects to generate tables for a range of FiO_2 (21-55%) and a range of baseline (0-3%) and 20 min COHb levels (0-8%) (**Appendix B or electronic equivalent version in StudyTRAX**).

As blood has a maximum concentration of COHb that depends on FiO_2 and would be reached asymptotically during a prolonged exposure to CO, we also calculated the maximum COHb for inhalation of 200 ppm CO at a range of possible FiO_2 using the CFK equation¹¹¹ and our Cohort 2 measurements. Duration of exposure was set to an extreme value (10^6 min = 694 days, almost 2 years) to ensure that the equilibrium would be reached. Results were plotted as maximum COHb vs. FiO_2 for all Cohort 2 subject data ($n=13$) from our prior Phase I study (**Figure 9**). These data reveal that for $FiO_2 > 55\%$, the COHb level will never reach 8% at 200 ppm CO inhalation.

3.3 RISK/BENEFIT ASSESSMENT

3.3.1 KNOWN POTENTIAL RISKS

Risk of Active Study Drug

Potential risks of active study drug (CO) include headache and tachycardia. In cases of overdose, patients can have nausea, vomiting, seizures, problems thinking, coma, cardiopulmonary arrest, and death. These adverse effects are seen at doses much higher than those proposed in this study. Subjects will be vigilantly monitored for side effects during drug administration and COHb and lactate levels will be carefully monitored as outlined in the study protocol. There may be other risks of inhaled CO in patients with ARDS that are currently unknown. Subjects will be monitored closely throughout their participation in the trial. In our Phase I sepsis-induced ARDS study, no subjects exceeded a COHb level of 10%, and there were no administration-associated AEs or study-related SAEs.

Risk of Blood Draws

All enrolled subjects will have blood drawn for both safety monitoring and research purposes. Most blood will be drawn through indwelling catheters. Risks of drawing blood percutaneously are uncommon and include bleeding and bruising.

Risk of Radiation Exposure

Most chest X-rays will be performed as part of usual ICU care. However, if not performed, we will obtain chest X-rays for endpoint assessment on the day after extubation (if the participant is extubated prior to day 7) and on day 7. The risk of ionization radiation from routine chest X-rays is generally considered minimal due to the low dose (~0.1 milliSievert [mSv]).

3.3.2 KNOWN POTENTIAL BENEFITS

Study subjects may or may not receive any direct benefits from their participation in this study. Our preclinical data demonstrate that low dose iCO is protective in experimental models of ALI. Numerous studies have shown CO to be cytoprotective in animal models of lung injury including hyperoxia and endotoxin exposure, bleomycin, ischemia/reperfusion, pneumococcal pneumonia, and VILI^{34,35,40,55-60,64}. Furthermore, CO has been shown to decrease inflammation, enhance phagocytosis, and improve mortality in models of sepsis including endotoxemia^{36,61,127}, hemorrhagic shock^{63,128-130}, and CLP^{42,43,53}. In addition, CO has been shown to have beneficial therapeutic effects in pre-clinical models of other diseases including traumatic brain injury (TBI)¹³¹, kidney fibrosis¹⁰⁵, colitis^{100,102}, arthritis¹⁰⁴, organ transplantation^{58,59,76-84}, hepatitis^{98,99}, and vasculopathies of the heart and lung^{67,72-74,94}.

Potential benefits from the administration of iCO include decreased requirement for ventilatory support, decreased days spent in the ICU, increased organ failure free days, and enhanced survival. Finally, there are potential benefits to society as the discovery of effective therapies that can reduce the substantial morbidity and mortality of ARDS or the identification of biomarkers that might improve prediction and monitoring of ARDS disease severity would enhance the health of society.

In addition to the potential benefit of iCO administration, all subjects, including those randomized to placebo, will benefit from protocolized low tidal volume ventilation and weaning protocols, as these have been shown to be beneficial in prior studies and are recommended in evidence-based consensus guidelines as best practice. As this protocol incorporates best practice guidelines, participation in the study will reduce the unexplained or potentially harmful variability in the application of such guidelines, and thus likely improve care, for all participants in our trial, including those participants randomized to placebo. Furthermore, there may be salutary effects on all participants given the additional clinical personnel and monitoring that will occur during administration of the study products.

3.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

Federal regulations at 45 CFR 46.111 (a)(2) require that “the risks to subjects are reasonable in relation to anticipated benefits, if any, to subjects, and the importance of the knowledge that may reasonably be expected to result.” There are several elements of study design in the present protocol that meets this human subject protection requirement. Several of the exclusion criteria prohibit participation of patients who might be at increased risk from the effects of iCO. These include individuals with severe hypoxemia, acute myocardial infarction (MI), stroke within 1 month, cardiac arrest requiring CPR within 72 hours, or inability to assess mental status following cardiac arrest, angina pectoris with activities of daily living, cardiopulmonary disease (NYHA class IV), as well as women who are pregnant or breastfeeding. In addition, to ensure the safety of subjects and minimize potential risks related to the study drug administration, our study protocol includes stringent monitoring of subjects, provisions for daily hold parameters, criteria for interruption of dosing, and permanent discontinuation of the study drug.

Based on the preceding assessment of risks and potential benefits, the risks to subjects are reasonable in relation to anticipated benefits, and commensurate with those inherent in the subjects' expected critical illness

requiring ICU care. There are currently no alternative available approaches to treat ARDS itself, besides supportive care, treatment of the underlying disease (eg. infection), and lung protective ventilator strategies. In addition, there are currently no biomarkers to assess disease severity and outcomes in ARDS. The potential risks of participation include those related to blood draws, chest X-rays, and those related to iCO treatment.

Blood draws: All enrolled subjects will have blood drawn for both safety monitoring and research purposes. Most blood will be drawn through indwelling catheters. Risks of drawing blood percutaneously are uncommon and include bleeding and bruising. The risks associated with this common clinical practice are small, whereas the knowledge gained in furthering our understanding of ARDS and development of a potential new therapy for patients with ARDS may be substantial.

Radiation Exposure: Most chest X-rays will be performed as part of usual ICU care. The risk of ionization radiation from routine chest X-rays is generally considered minimal due to the low dose (~0.1 milliSievert [mSv]).

CO Treatment: Data from animal studies demonstrate that low dose iCO has beneficial effects on outcomes in models of ALI. There is potential benefit to society and individual patients should iCO treatment prove to be beneficial for future patients with ARDS. Potential risks of high doses of the active study drug include headache and tachycardia. In cases of overdose, patients can have nausea, vomiting, seizures, problems thinking, coma, cardiopulmonary arrest, and death. These adverse effects are seen at doses much higher than those proposed in this study. Although there is a risk of toxicity associated with inhalation of high concentrations of CO, low dose iCO has been shown to be protective in animal models and safe in humans. No protocol-specified iCO administration-related AEs occurred in 8 subjects treated with low dose iCO in our prior Phase I trial.

Subjects will be vigilantly monitored for side effects during drug administration and COHb and lactate levels will be carefully monitored as outlined. There may be other risks of iCO in patients with ARDS that are currently unknown. Subjects will be monitored closely throughout their participation in the trial.

4 OBJECTIVES AND ENDPOINTS

4.1 PRIMARY OBJECTIVE

To evaluate the safety, tolerability, and efficacy of inhaled carbon monoxide (iCO), as well as to examine the biologic readouts of low dose iCO therapy in patients with ARDS.

4.2 HYPOTHESES

- Low dose iCO will be safe and well-tolerated and will reduce mtDNA levels in ARDS patients.
- Low dose iCO will reduce the severity of lung injury and non-pulmonary organ failure in ARDS patients.
- Low dose iCO will reduce mitochondrial dysfunction, attenuate inflammasome activation, and promote resolution of inflammation in ARDS patients. Furthermore, modulation of these pathways will predict improvements in the clinical outcomes.

4.3 PRIMARY SAFETY ENDPOINT

The primary safety endpoint is to evaluate the safety of inhaled CO, by determining COHb levels and the incidence of pre-specified administration-related adverse events.

- Safety of inhaled CO, defined by the incidence of pre-specified administration-related AEs (as defined below) and spontaneously reported AEs through study day 7.
 - Acute MI within 48 hours of study drug administration
 - Acute cerebrovascular accident (CVA) within 48 hours of study drug administration
 - New onset atrial or ventricular arrhythmia requiring DC cardioversion within 48 hours of study drug administration
 - Increased oxygenation requirements defined as: an increase in FiO_2 of ≥ 0.2 **AND** increase in $\text{PEEP} \geq 5$ cm H_2O within 6 hours of study drug administration
 - Increase in COHb $\geq 10\%$
 - Increase in lactate by ≥ 2 mmol/L within 6 hours of study drug administration

4.4 PRIMARY EFFICACY ENDPOINT

The primary efficacy endpoint is to compare the effects of iCO versus placebo on mtDNA levels from baseline to day 5 (or death, whichever comes first) in patients with ARDS.

4.5 SECONDARY ENDPOINTS

The secondary endpoints will examine the effects of iCO versus placebo on the following outcomes:

- Lung Injury Score (LIS) on days 1-5, and 7
- $\text{PaO}_2/\text{FiO}_2$ ratio on days 1-5, and 7
- Oxygenation Index (OI) on days 1-5, and 7
- Dead Space Fraction (Vd/Vt) on days 1-3, and 7
- Sequential Organ Failure Assessment (SOFA) score on days 1-5, 7, 14, 28

Additional secondary endpoints will examine the effects of iCO versus placebo on biomarkers of mitochondrial dysfunction, inflammasome activation, and lipid mediators in patients with ARDS.

- Autophagy markers (eg. LC3B)
- Inflammasome-dependent cytokines (eg. IL-18)
- Lipid mediators (LM) and specialized pro-resolving mediators (SPMs)
- Mitochondrial quality control biomarkers (eg. Pink1, Wipi1)
- Biomarkers of inflammation (eg. IL-6, IL-8, IL-10, IL-1Ra)
- To enhance the biologic analytic power, samples from 12 subjects enrolled in a similar, already completed study will be similarly analyzed for these biomarkers, as well as in more extensive biologic 'omics profiling that will permit more detailed analyses of these pathways in correlation with clinical data.

4.6 EXPLORATORY ENDPOINTS

- Ventilator-free days at day 28
- ICU-free days at day 28
- Hospital-free days at day 60
- Hospital mortality to day 28 and 60

4.7 FOCUSED SAFETY ANALYSIS

The incidence of elevation in plasma COHb $\geq 10\%$ measured on study days 1-3 and pre-specified administration-associated adverse events (**Section 7.1.8**) and serious adverse events (**Section 9.7**).

5 STUDY DESIGN

5.1 OVERALL DESIGN

We will perform a multi-center, prospective, randomized, partially double-blind, placebo-controlled Phase II clinical trial of inhaled CO for the treatment of ARDS. Thirty-two intubated subjects with ARDS will be randomized in a 1:1 ratio to receive inhaled CO versus inhaled air placebo for up to 90 minutes daily for a total of 3 days. Subjects randomized to receive iCO will be administered 200 ppm CO to achieve a COHb level of 5-8%.

5.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

We have designed our study to optimally balance risks and potential benefits of iCO treatment, recognizing that we are still early in the developmental pathway. We chose a partially double-blind design so that there would always be an unblinded assessment of COHb levels in order not to exceed potentially toxic thresholds. The 1:1 randomization between iCO and placebo will provide us the maximum statistical power to identify both any adverse effects and any potential beneficial outcomes in our critically ill study population.

5.3 JUSTIFICATION FOR DOSE

Subjects randomized to CO will be administered iCO daily at 200 ppm for up to 90 minutes for three consecutive days. The rationale for fixed 200 ppm dosing is based on our Phase I trial demonstrating safety and the ability to achieve COHb levels which do not exceed 8%, a level which has been shown to be safe in prior human studies^{45,111,114,115,117,118,132}, and protective in animal models of sepsis and ALI^{34-36,38,40,42,43,48,55-65}. Although direct exposure of lung epithelial cells to CO gas at low levels may evoke local cytoprotective effects, it is unlikely that systemic effects will be achieved without elevation of tissue CO levels, for which COHb is a surrogate. Prior studies have demonstrated safety of iCO at 500-1000 ppm in humans¹¹⁵, a dose associated with reduced inflammation in NHPs⁶⁴, therefore we do not anticipate epithelial toxicity at a dose of 200 ppm.

The rationale for three consecutive days of treatment is based on our prior work in healthy individuals demonstrating safety and activation of mitochondrial biogenesis after CO inhalation at 200 ppm for 1 hour/day for 5 consecutive days^{6,45}. The rationale for once-daily dosing as opposed to continuous inhalation, as with inhaled nitric oxide (NO) or other inhaled pulmonary vasodilators, is based on pre-clinical studies showing that iCO treatment for short periods of time induces transcriptional programs of mitochondrial biogenesis^{45,46,50,51,133} and induces autophagy^{43,49,50,134-136} leading to downstream protection without the need for continuous CO inhalation. In addition, once-daily iCO dosing will optimize subject safety by ensuring that COHb levels do not exceed our target range of 5-8%, and that CO accumulation will not occur as treatments are separated by 4-6 half-lives for CO elimination¹³⁷⁻¹³⁹.

5.4 END OF STUDY DEFINITION

A participant is considered to have completed the study if he or she has completed all phases of the study including the six month telephone neurocognitive assessment as shown in the **Time-Events Schedule, Section 1.3**.

6 STUDY POPULATION

6.1 INCLUSION CRITERIA

All intubated patients ≥ 18 years old with ARDS according to the Berlin criteria¹⁵ will be eligible for inclusion. Subjects will be recruited from the medical, surgical, cardiac, and burn ICUs at each center with a specific focus on patients with ARDS secondary to infection, trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.

1. ARDS is defined when all four of the following criteria are met:
 - a. A $\text{PaO}_2/\text{FiO}_2$ ratio ≤ 300 with at least 5 cm H_2O positive end-expiratory airway pressure (PEEP)
 - b. Bilateral opacities on frontal chest radiograph (not fully explained by effusions, lobar/lung collapse, or nodules) within 1 week of a known clinical insult or new or worsening respiratory symptoms
 - c. A need for positive pressure ventilation by an endotracheal or tracheal tube
 - d. Respiratory failure not fully explained by cardiac failure or fluid overload; need objective assessment (e.g., echocardiography) to exclude hydrostatic edema if no risk factor present
(Appendix C)
2. ARDS onset is defined as the time the last of criteria 1-4 are met. ARDS must persist through the enrollment time window of 168 hours.

6.2 EXCLUSION CRITERIA

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Age less than 18 years
2. Greater than 168 hours since ARDS onset
3. Pregnant or breastfeeding
4. Prisoner
5. Patient, surrogate, or physician not committed to full support (exception: a patient will not be excluded if he/she would receive all supportive care except for attempts at resuscitation from cardiac arrest)
6. No consent/inability to obtain consent or appropriate legal representative not available
7. Physician refusal to allow enrollment in the trial
8. Moribund patient not expected to survive 24 hours
9. No arterial or central line/no intent to place an arterial or central line
10. No intent/unwillingness to follow lung protective ventilation strategy
11. Severe hypoxemia defined as $\text{SpO}_2 < 95$ or $\text{PaO}_2 < 90$ on $\text{FiO}_2 \geq 0.9$
12. Hemoglobin < 7.0 g/dL
13. Subjects who are Jehovah's Witnesses or are otherwise unable or unwilling to receive blood transfusions during hospitalization
14. Acute myocardial infarction (MI) or acute coronary syndrome (ACS) within the last 90 days
15. Coronary artery bypass graft (CABG) surgery within 30 days

16. Angina pectoris or use of nitrates with activities of daily living
17. Cardiopulmonary disease classified as NYHA class IV
18. Stroke (ischemic or hemorrhagic) within the prior 1 month, cardiac arrest requiring CPR within the prior 72 hours, or inability to assess mental status following cardiac arrest
19. Burns > 40% total body surface area (TBSA)
20. Severe airway inhalational injury
21. Use of high frequency oscillatory ventilation
22. Use of extracorporeal membrane oxygenation (ECMO)
23. Concomitant use of inhaled pulmonary vasodilator therapy (eg. nitric oxide [NO] or prostaglandins)
24. Diffuse alveolar hemorrhage from vasculitis
25. Concurrent participation in other investigational drug study

Reasons for Exclusions:

Patients less than 18 years of age are excluded because the participating ICUs do not typically admit pediatric patients and we believe the benefit/risk ratio for children to receive iCO is not appropriate at this early stage. Patients with ARDS for more than 168 hours are excluded in order to evaluate more clearly the effects of iCO early in the course of lung injury. Patients with severe hypoxemia or need for ECMO are excluded because they may not have adequate reserve to tolerate the reduction in oxygen carrying capacity. The parameters of $\text{SpO}_2 < 95$ or $\text{PaO}_2 < 90$ on $\text{FiO}_2 \geq 0.9$ are used rather than a simpler $\text{PaO}_2/\text{FiO}_2$ ratio of 100 to define severe hypoxemia for both enrollment and daily treatment to better account for factors affecting arterial oxygen content and to ensure an additional safety margin during treatment of enrolled subjects. These criteria exclude patients with $\text{PaO}_2/\text{FiO}_2 \leq 100$ (PaO_2 90/ FiO_2 0.9), but also explicitly specify a safety margin of 0.1 for FiO_2 . These criteria are currently approved by the Institutional Review Boards (IRBs) at our participating clinical centers, our DSMB, and the FDA for our Phase I CO ARDS trial (NCT02425579).

Patients with hemoglobin < 7.0 g/dL, Jehovah's witnesses, or patients otherwise unable or unwilling to receive blood transfusions during hospitalization are excluded because of the volume of blood drawn for monitoring during iCO therapy may place these patients at greater risks from complications of anemia. Moribund patients and patients with extensive body surface area burns have a high incidence of AEs and lactic acidosis that will confound the safety assessment. Pregnancy, recent stroke, or cardiac arrest are exclusions because iCO may reduce oxygen delivery to the fetus and recently injured brain respectively. Patients with alveolar hemorrhage from vasculitis are excluded because the mechanism of lung injury is different from ARDS and diffuse alveolar damage. Patients with acute MI within 90 days, recent CABG, and angina pectoris are excluded because of a potential excess risk of reducing oxygen delivery to the myocardium. Patients with congestive heart failure and NYHA class IV cardiopulmonary disease are excluded because of concerns about ventricular arrhythmias and high mortality. Patients ventilated with high frequency oscillatory ventilation are excluded because administration of iCO with this mode of ventilation may be potentially unreliable. Patients on ECMO are also excluded because the effects of CO uptake and elimination from the blood during ECMO are unknown. Patients on concurrent inhaled pulmonary vasodilator therapy are excluded as these inhaled medications may interfere with the dosing of iCO.

Patients who are cigarette smokers, but otherwise eligible for inclusion, will not be excluded from potential enrollment in the study. Rather, enrolled subjects will be screened with daily COHb levels and study drug treatment held if $\text{COHb} \geq 3\%$. This criterion was implemented for our Phase I trial and this Phase II trial as smoking history obtained from surrogate decision makers may be unreliable and the average COHb half life is

240 minutes when breathing room air and less on supplemental oxygen¹³⁷⁻¹³⁹. Since current smoking rates amongst military (24%)¹⁴⁰ and veteran populations (20%)¹⁴¹ are higher than for civilians (15%)¹⁴², exclusion on the basis of smoking history would preclude many patients from receiving this potentially beneficial treatment. Instead, this protocol incorporates treatment holds on the basis of daily COHb levels, which will allow otherwise eligible patients to be treated safely and have the opportunity to benefit from this potentially new promising therapy. We anticipate enrolling smokers at a frequency similar to their proportion in our target populations.

6.3 STUDY INITIATION TIME WINDOW

All ARDS criteria must occur concurrently within the same 24 hour period. The onset of ARDS is when the last criterion is met. Patients must be enrolled within 168 hours of ARDS onset. Information for determining when these time window criteria were met may come from either the site hospital or a referring hospital reports. Following randomization, the low tidal volume protocol for mechanical ventilation must be initiated within two hours.

The first treatment of study drug should be given within 24 hours of randomization. The day of randomization will be considered study day zero.

6.4 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention or entered in the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any SAE.

Individuals who do not meet the criteria for participation in this trial (screen failure) because of severe hypoxemia (Exclusion Criterion 11), low hemoglobin (Exclusion Criterion 12), use of high frequency oscillatory ventilation (Exclusion Criterion 21), or use of inhaled pulmonary vasodilators (Exclusion Criterion 22) may be rescreened. Rescreened participants will be assigned the same participant number as for the initial screening.

6.5 STRATEGIES FOR RECRUITMENT AND RETENTION

One hundred inpatient subjects will be recruited for this study at seven enrolling medical centers. Subjects will be identified by prospective medical record review. We anticipate that the distribution of gender, race, and ethnicity of the subjects enrolled will be similar to the demographic characteristics of ICU patients admitted to each of our participating centers and be proportional to the enrollment distribution. No gender, race, and/or ethnicity group will be excluded from participation in our proposed study.

Study coordinators at each site will screen inpatient ICUs daily to identify all potential subjects based on the pre-defined inclusion and exclusion criteria. The recruitment process will consist of electronic medical record (EMR) review of all intubated patients in the ICUs at each site to determine if a potential subject meets study eligibility criteria. EMR alerts will also be used to assist research coordinators in the early identification of potential participants that meet study eligibility criteria. The research coordinators at each site will identify potential participants and review the study eligibility criteria in detail with the site PI or designee. No recruitment or advertisement materials will be utilized to recruit patients, however flyers will be posted in ICU staff areas to educate colleagues and other health care providers about study eligibility criteria and the importance of participation in clinical research. We will also engage the ICU clinical communities through

conferences and other educational resources at each site as our ICU colleagues will play a critical role in the recruitment, retention, and overall conduct of the clinical trial.

Participants will be hospitalized in the ICU at the time of informed consent and throughout the treatment period of the study protocol, thus we do not foresee difficulties in adherence with the study drug treatment. For participants who are extubated prior to completion of the 3 day treatment portion of the protocol, all data will be analyzed on a modified intention-to-treat (MITT) basis. For long-term follow-up outcomes, we will use web-based investigator tools for messaging and scheduling reminders to contact participants.

Several engagement strategies will be adopted to ensure retention of study participants for assessment of long-term outcomes following hospitalization. First, we have designed the study protocol to minimize participant burden in order to optimize subject retention in the trial. We have designed the protocol such that the long-term follow up requires no travel or costs and entails three brief (2-5 minute) telephone calls at day 28, day 60, and 6 months. Second, we have established an efficient tracking system for long-term follow-up. At the time of consent, participants and/or their legally authorized representative (LAR) will be asked to provide email addresses and several phone numbers (home, work, cell) and to indicate the best times to contact them and whether voice and/or text messages can be left.

As many ICU patients are discharged to long-term care facilities following hospitalization, we will consent study participants for permission to contact them at a long-term care facility if applicable. The names and contact information of up to three people who can be contacted for assistance with locating participants will also be requested. Written informed consent to contact these individuals will be obtained, including a description of the scope of the information to be obtained (eg. forwarding addresses versus questions about the clinical status of the participant). In the event of being unable to contact study subjects, we will send letters and/or emails to participants who are unresponsive to personal contact. We will also obtain consent to use social security numbers to determine vital status of participants lost to follow-up.

To mitigate retention risks at the time of consent, we will educate subjects (and their LARs) about their important role as research participants, the requirements of the study, and clearly orient participants to the study demands, tasks, and responsibilities.

Most of the patients approached for participation in this research protocol will have limitations of decision-making abilities due to their critical illness or need for sedating medications. Hence, most patients will not be able to provide informed consent and accordingly, informed consent will be sought from the potential subject's LAR. For subjects for whom consent was initially obtained from a surrogate, but who subsequently regain decision-making capacity while in hospital, we will obtain formal written consent for continuing participation, inclusive of continuance of data acquisition. The initial consent form signed by the surrogate will reflect that such continuing consent will be obtained when possible. All patients who recover decision-making capacity will also be approached for written re-consent for genetic testing. Subjects will not be compensated for participation in the study.

6.5.1 SCREENING

Federal regulations at 45 CFR 46(a)(3) require the equitable selection of subjects. The ICUs will be screened to determine if any patient meets the inclusion and exclusion criteria. Data that have been collected as part of the routine management of the patient will be reviewed to determine eligibility. No protocol-specific tests or procedures will be performed as part of the screening process. If any patient meets criteria for study enrollment, then the attending physician will be asked for permission to approach the patient or his/her surrogate for

informed consent. Justifications of exclusion criteria are given in **Section 6.2**. These exclusion criteria neither unjustly exclude classes of individuals from participation in the research nor unjustly include classes of individuals from participation in the research. Hence, the recruitment of subjects conforms to the principle of distributive justice.

A screening log will be kept to track subjects that could potentially meet inclusion criteria. All patients meeting the inclusion criteria will be entered into the StudyTRAX database. If the patient is not enrolled, information explaining why enrollment did not occur will be entered into StudyTRAX (exclusion criteria, attending physician denial, patient refusal, etc. see **Appendix D** for a listing of the de-identified data to be collected on screened, non-enrolled subjects).

6.5.2 JUSTIFICATION OF INCLUDING VULNERABLE SUBJECTS

The present research aims to evaluate the safety and efficacy of iCO as a treatment for patients with ARDS who are intubated and mechanically ventilated. Due to the nature of this illness, the vast majority of these patients will have impaired decision-making capabilities. This study cannot be conducted if enrollment is limited to only those subjects with decision-making capacity. Potential benefits to participation in this study are increased survival and ventilator free days (VFDs).

7 STUDY INTERVENTION

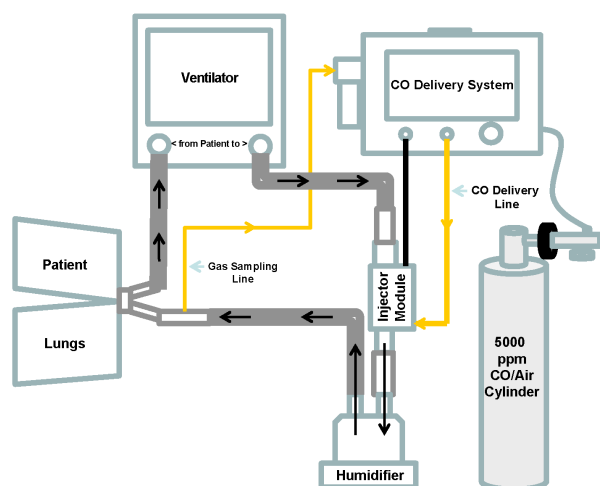


Figure 10. Schema of CO Delivery System and ventilator.

of the ventilator and inlet port of the humidifier. The gas sampling line will be placed between the outlet port of the humidifier and the patient wye as shown. The study drug will be administered by an RT and a physician study staff member OR a physician study staff member alone. A physician study staff member will be present at the bedside during and immediately available for an additional 90 minutes after administration of the study drug for any clinical concerns that arise. The administering RT and physician study staff member OR the physician study staff member alone will be unblinded to the treatment assignments and will conceal the gas cylinders and COventDS to assure that the study coordinator and treating ICU team remain blinded to the study drug assignment. A physician investigator will be at the bedside of the study subject during and immediately available after the administration of the study drug for an additional 90 minutes to provide any necessary emergency care in conjunction with the patient's clinical ICU team.

7.1 STUDY INTERVENTION(S) ADMINISTRATION

7.1.1 CO DELIVERY SYSTEM

Inhaled CO or placebo air will be administered to mechanically ventilated subjects using a mechanical ventilator approved for NO delivery and the CO Delivery System developed by 12th Man Technologies (**Figure 3, Appendix A**). See **Appendix A** for details of the CO Delivery Device testing, assembly, calibration, and standard operating procedures (SOP). The CO Delivery System will be calibrated and connected to the ventilator (**Figure 10**) as described in **Appendix A**. As per the SOP and illustrated in the schema in **Figure 10**, the injector module will be connected between the inspiration port

7.1.2 CO OR PLACEBO CYLINDERS

The gas cylinders proposed for this clinical trial are AG aluminum cylinders with a CGA 500 valve and will be supplied by Praxair. The cylinder contains approximately 360 liters of 5000 ppm (0.5%) CO in room air (21% oxygen) and poses no increased flammability risk. Placebo tanks will contain medical grade air. The gas cylinder's nominal size is 5 inches in diameter by 17 inches tall. Given the flow limitation from the regulator of ~7 liters per minute and a minimum of 6 air exchanges per hour in an average 15x15x10 ICU room, we do not expect ambient CO levels to exceed the OSHA permissible exposure limit (PEL) of 50 ppm as an 8 hour time-weighted average. We measured ambient CO levels during our animal studies using the COventDS and were unable to detect increases in ambient CO levels throughout assembly, calibration, and delivery of CO to the animals. In addition, we simulated CO administration with an ICU ventilator (Puritan-Bennett 840), CO Delivery System, and lung model in an ICU room at Massachusetts General Hospital at 500 ppm for over 2 hours and ambient CO levels were near zero and well below the OSHA PEL.

7.1.3 DOSING AND ADMINISTRATION

Subjects will be treated with inhaled CO at a dose of 200 ppm or placebo. Placebo will consist of medical-grade air in identical appearing aluminum AG gas cylinders.

The study drug will be administered for up to 90 minutes daily for three days following randomization or until discontinuation of mechanical ventilation, whichever occurs first. For patients who have a tracheostomy, the equivalent of extubation for the purposes of this protocol will be breathing via tracheostomy with unassisted breathing.

7.1.4 CO MONITORING

The study drug concentration will be monitored continuously with the COventDS built-in analyzer to assure that the accurate dose is delivered. A physician investigator will assess subjects prior to daily iCO treatment. Subjects will have blood drawn daily for measurement of COHb and lactate prior to study drug administration. If COHb \geq 3% (assessed by unblinded physician) or lactate \geq 4 mmol/L, the study drug will be held and the subject will be reassessed for treatment the following day. An electrocardiogram (EKG) will also be performed daily prior to study drug administration to evaluate for cardiac exclusion criteria. Blood COHb levels will be measured with an IL GEM Premier Co-oximeter and SpCO measured with a noninvasive pulse oximeter (Masimo Radical-7).

Blood (arterial or venous) will also be drawn at 20, 60, 75, 90, and 180 minutes for safety monitoring of COHb levels. If an arterial line is not available, venous blood will be used for COHb monitoring as we⁴⁸ and others¹⁴³⁻¹⁴⁵ have shown that venous levels are as accurate and reliable as arterial measurements. Blood (arterial or venous) will also be drawn for blood gas analysis (using the local clinical lab) at 90 minutes or at the completion of treatment, if discontinued sooner than 90 minutes. If arterial blood is not available and venous blood gas is used, PaO₂/FiO₂ will be estimated using the formula $SpO_2/FiO_2 = 64 + 0.84 \times PaO_2/FiO_2$, as long as SpO₂ \leq 97% and subject remains intubated as described in the Statistical Analysis Plan.

The unblinded physician will review and adjudicate COHb measurements and communicate any study drug treatment holds to the administering study staff. COHb levels will be entered into the electronic case report forms (eCRFs) in a separate limited-access project in StudyTRAX by the unblinded RT or physician. The study drug will be administered for up to 90 minutes daily for up to 3 days following randomization or until discontinuation of mechanical ventilation, whichever occurs first.

The concentration of the study drug will be measured by the built-in gas monitor in the COventDS. The CO Delivery System contains an inhaled gas monitor, which is an electrochemical device that monitors the inhaled gas for concentrations of CO (0-800 ppm) and O₂ (15-100%) to assure that safe levels are inhaled. The sample pump maintains a constant flow of gas to the sensors. Samples of inspired gases are taken with a continuous ~400 mL/min sample pump just proximal to the patient's airway to reflect actual inspired gases. Alarms will sound on high or low CO or O₂ concentrations.

Ambient air CO concentrations will be measured in real time with a Dräger Pac 7000 CO detector to assure that ambient levels are maintained within the recommended limits for occupational exposure of a maximum of 50 ppm. Ambient air CO detectors will be calibrated every 6 months per the manufacturer's instructions to ensure proper functioning.

All CO monitoring will be carried out by the administering RT and physician study staff member OR physician study staff member alone and concealed from the study coordinator and clinical team. The physician study staff member will be responsible for maintaining a separate password-protected limited-access project within the database with the subject identification number and CO-related measurements (SpCO, COHb, ambient CO) which the study coordinator will not have access to.

7.1.5 CO MONITORING USING THE CFK EQUATION

Subjects will be administered iCO at 200 ppm or placebo (medical grade air) for up to 90 minutes on each study day for up to 3 consecutive days.

COHb levels will be measured at baseline prior to treatment and after 20 minutes of iCO administration. These two COHb levels will be used to look up the time at which the COHb level is predicted to be 8% using a CFK equation-based table (**Appendix B or electronic equivalent version in StudyTRAX**). If the COHb level is predicted to be 8% prior to 90 minutes, the study drug will be stopped at that time to ensure that subjects do not exceed a COHb level of 10% and safely achieve a target COHb level of 5-8%.

Blood will be drawn for baseline COHb level prior to iCO or placebo administration using the bedside IL GEM Premier Co-oximeter. Arterial or venous blood will be drawn for pre-treatment blood gas analysis performed at the local clinical laboratory.

1. Subjects will be treated with placebo air or iCO at 200 ppm.
2. After 20 minutes, blood will be drawn for COHb measurement using the bedside IL GEM Premier Co-oximeter.
3. The CFK equation-based table will be used to look up the time at which the COHb level is predicted to be 8%.
4. Blood will be drawn for COHb measurements at 60 min, 75 min, 90 min, and 180 min. Blood will also be drawn for blood gas analysis at the local clinical laboratory at 90 min at the completion of the study drug.

On days 1-3, a total of approximately 2.5 mL of blood will be drawn during the study drug administration for safety monitoring of COHb levels and blood gas analysis.

7.1.6 DAILY HOLD PARAMETERS PRIOR TO DRUG ADMINISTRATION

Subjects will be assessed daily prior to study drug administration as described in the **Time-Events Schedule (Section 1.3)**.

Administration of the study drug will be held if the following criteria are met.

- COHb level $\geq 3\%$
- Lactate ≥ 4 mmol/L
- ST elevation MI or unstable angina/non ST elevation MI concerning for ACS
- Unstable atrial or ventricular arrhythmia

Subjects will have blood drawn daily for measurement of COHb and lactate prior to administration of study drug. If COHb $\geq 3\%$ or lactate ≥ 4 mmol/L, the study drug will be held until the next scheduled dose the following day. Lactate and COHb will be measured the following day to determine whether the study drug will be administered. If the study drug is being held for another reason, COHb levels will not be measured on days the study drug is being held.

A 12 lead EKG will also be performed and reviewed daily by the study staff physician prior to study drug administration to evaluate for cardiac exclusion criteria (eg. acute MI, acute coronary syndrome (ACS), or unstable atrial or ventricular arrhythmia). If a subject meets the following criteria for ST elevation MI or unstable angina/non ST elevation MI concerning for ACS according to the American College of Cardiology Foundation/American Heart Association guidelines¹⁴⁷⁻¹⁴⁹, they will be excluded from enrollment or further study drug administration according to exclusion criteria.

ST elevation MI (STEMI) Criteria: New ST elevation at the J point in at least 2 contiguous leads of ≥ 2 mm (0.2 mV) in men or ≥ 1.5 mm (0.15 mV) in women in leads V2–V3 and/or of ≥ 1 mm (0.1 mV) in other contiguous chest leads or the limb leads; New or presumably new left bundle branch block (LBBB)¹⁴⁷⁻¹⁴⁹.

Unstable angina/non ST elevation MI (NSTEMI): Ischemic ST-segment depression ≥ 0.5 mm (0.05 mV) or dynamic T-wave inversion with pain or discomfort; Nonpersistent or transient ST-segment elevation ≥ 0.5 mm for < 20 minutes. Threshold values for ST-segment depression consistent with ischemia are J-point depression 0.05 mV (-0.5 mm) in leads V2 and V3 and -0.1 mV (-1 mm) in all other leads (men and women)¹⁴⁷⁻¹⁴⁹.

Note, troponin (I or T) may be increased in patients with sepsis and ARDS¹⁵⁰ in the absence of an acute MI or ACS from coronary artery disease. If, in the judgment of the clinical team, an ARDS patient with elevated troponin levels has no other indication of an MI or ACS, the patient may still be eligible for enrollment.

In addition, if a subject develops the following criteria during the study, the study drug will be held until resolved:

- Severe hypoxemia defined as $\text{SpO}_2 < 95$ or $\text{PaO}_2 < 90$ on $\text{FiO}_2 \geq 0.9$
- Hemoglobin < 7.0 g/dL
- Diffuse alveolar hemorrhage from vasculitis
- Use of high frequency ventilation
- Use of inhaled pulmonary vasodilator therapy (eg. NO or prostaglandins)
- Use of ECMO

If study drug hold criteria are met, the study drug will be held and subjects will be evaluated on a daily basis and assessed for whether a given criteria has resolved and whether study drug treatment may be resumed.

7.1.7 INTERRUPTION OF DOSING DURING STUDY DRUG ADMINISTRATION

Subjects will have blood drawn for measurement of COHb levels prior to study drug treatment and 20 min, 60 min, 75 min, 90 min, and 180 min after the start of study drug treatment. It is anticipated that the predicted and measured COHb levels will not exceed the COHb target range of 5-8% given the impaired diffusion capacity

in ARDS patients. However, the following parameters will be used to shorten the 90 minute treatment duration should CO uptake be higher than anticipated:

The study drug will be stopped prior to 90 minutes:

1. If the measured COHb level is > 8% at any time during study drug treatment.
2. If the COHb level is predicted to be 8% prior to 90 minutes according to the CFK equation-based table. If this occurs, study drug treatment will be stopped at the time the COHb level is predicted to be 8% by the CFK equation-based table and blood COHb level will be measured at that time.
3. If the investigator, attending physician, the patient, or their surrogate decides that the study drug should be discontinued.

If COHb levels unexpectedly exceed our target level, in addition to discontinuation of the study drug, subjects will be placed on 100% O₂ to accelerate CO elimination until COHb levels normalize.

7.1.8 PERMANENT DISCONTINUATION OF STUDY DRUG ADMINISTRATION

Permanent discontinuation of the study drug is defined as cessation of the study drug without the intent of restarting the study drug during the three-day treatment period.

Permanent discontinuation of the study drug inhalation will occur in the following situations:

- **Occurrence of pre-specified administration related adverse events:**
 - Acute MI within 48 hours of study drug administration
 - Acute CVA within 48 hours of study drug administration
 - New onset atrial or ventricular arrhythmia requiring DC cardioversion within 48 hours of study drug administration
 - Increased oxygenation requirements defined as: an increase in FiO₂ of ≥ 0.2 **AND** increase in PEEP ≥ 5 cm H₂O within 6 hours of study drug administration
 - Increase in any protocol-specified measurement of COHb to ≥ 10%
 - Increase in lactate by ≥ 2 mmol/L within 6 hours of study drug administration
- If the patient experiences serious adverse events ***related to the study drug (Section 9.7)***.
- If the investigator, attending physician, the patient or their surrogate decides that the study drug should be discontinued. If this decision is made because of an AE, then appropriate AE reporting procedures will be followed (**Section 9.7**).
- Daily baseline COHb levels greater than 3% leading to three missed drug doses.
- Three or more missed drug administrations due to AEs.

Subjects who have their study drug permanently discontinued will continue their participation in the study, and will be followed to determine their vital status and perform neurocognitive testing at 6 months, as outlined in the **Time-Events Schedule (Section 1.3)**.

7.1.9 COMPLETION OF THE STUDY DRUG ADMINISTRATION

Subjects will be considered to have completed the study drug administration portion of the study when one of the following conditions is met, whichever comes first:

1. Three days after study drug administration
2. Discontinuation of mechanical ventilation
3. Death

7.1.10 TREATMENT IN COVID-19 SUBJECTS

For enrolled ARDS patients with COVID-19 infection, all research and clinical staff will conform to each site's hospital infection control guidelines for COVID-19 positive patients with appropriate personal protective equipment (PPE) for enhanced respiratory isolation precautions. All research and clinical staff will have appropriate training on PPE, be fit-tested, and wear an N95 respirator or powered air-purifying respirator (PAPR) if staff cannot be fit-tested, face shield or other eye protection, gloves, and gown when entering the subject's room for treatment or sample collection. Gown, gloves, and eye protection will be doffed upon exiting the subject's room either just inside or just outside the door, and hand hygiene will be performed. Respiratory protection should be removed after exiting the subject's room unless extended use is being practiced.

During study treatment, all staff will conform to each site's hospital infection control guidelines regarding treatment of COVID-19 positive mechanically ventilated patients with inhaled therapies. Staff will be limited to essential personnel in the room. All equipment will be single-use or dedicated to use of the subject to avoid sharing with other subjects. Reusable equipment will be disinfected with a hospital-approved disinfectant before use in another subject.

Blood sample collection and handling will be performed according to each site's hospital infection control guidelines for COVID-19. Samples obtained from a subject will be put in a tube by research or clinical staff, and the tube will be wiped down with a hospital-approved disinfectant. While holding the tube with the wipe, the specimen tube will be carefully placed into a clean specimen bag held by a monitor or nurse outside the room. The specimen bag will in turn be wiped down with a hospital-approved disinfectant. A member of the research team will pick up the research specimens and transport them to a BSL2+ laboratory in a secondary container. Research samples will only be processed in hospital biosafety committee approved BSL2+ laboratory facilities.

7.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

7.2.1 ACQUISITION AND ACCOUNTABILITY

Each participating site is responsible for the ordering, storing and dispensing of the investigational agent(s). Each study team will keep a record of CO/placebo tanks received, administered, and returned at the study site in a Site Drug Accountability Log. This log will include Lot #, date and amount/concentration received, subject ID, date and time administered, date and time administration ended, date and amount/concentration returned.

7.2.2 FORMULATION, APPEARANCE, PACKAGING, AND LABELING

The study drug will be provided for this study by Praxair Healthcare Services (Morrisville, PA). Gas mixtures will be manufactured with United States Pharmacopeia (USP)/Ultra High Purity (UHP)/National Formulary (NF) components. The mixtures will be prepared as a Certified Standard with a blend tolerance of $\pm 10\%$ and analytical uncertainty of 2%.

The gas mixture provided to subjects randomized to CO will be 5000 ppm CO (balance air) and will be provided to study sites by Praxair Healthcare Services (Morrisville, PA) in AG aluminum cylinders with a CGA 500 valve. The gas cylinder's nominal size is 5 inches in diameter by 17 inches tall, contains 360 liters of 5000 ppm (0.5%) CO in room air (21% oxygen), and poses no increased flammability risk. The 5000 ppm CO source gas will be administered via the CO Delivery System (12th Man Technologies, Garden Grove, CA) and delivered to subjects at a dose of 200 ppm.

The mixture components include: **Carbon Monoxide UHP Grade 3.0** (Assay 99.9%; O₂ <10 ppm, H₂O <5 ppm, N₂ <900 ppm and CO₂ <20 ppm); and **Medical Air USP**. The source materials for **Medical Air USP** include: **Oxygen USP** (Assay ≥ 99.2%; Odor- none; Oxygen produced by air liquefaction process, does not require CO and CO₂ analysis); and **Nitrogen NF USP** (Assay ≥ 99.2%; Identification- OK, Paramagnetic Assay Method; CO ≤ 10 ppm; Odor- none).

The gas mixture provided to subjects randomized to placebo will be medical grade air and will be provided to study sites by Praxair Healthcare Services (Corona, CA) in AG aluminum cylinders with a CGA 346 valve. With the exception of the 346 valve, the placebo gas cylinders will be identical in size and color as the CO cylinders. The placebo gas will be administered via the CO Delivery System (12th Man Technologies, Garden Grove, CA) and delivered to subjects in an identical fashion as the CO gas.

7.2.3 PRODUCT STORAGE AND STABILITY

The CO and placebo air cylinders will be stored at room temperature in a secure area at each center. The cylinders will be stored and handled according to each site's Compressed Gas Cylinder Storage Guidelines. The CO and placebo air cylinders will be returned to Praxair upon expiration. The expiration date is generally three years from the date of fill and is clearly indicated on the certificate of analysis for each cylinder.

7.2.4 PREPARATION

As described in the SOP, the CO Delivery System will be calibrated and performance testing conducted daily prior to use. The calibration will be performed as described in the Operator's Manual in **Appendix A**. The calibration consists of a low (21%) and high (100%) range O₂ calibration, followed by a low (100 ppm) and high (400 ppm) range CO calibration. After completion of the calibration, performance testing will be performed as described in the SOP. The CO Delivery System will be assembled, the appropriate gas cylinder (CO or air) connected, and the delivery system will be connected to the ventilator as described in **Appendix A**. As shown in **Figure 7**, the injector module will be connected between the inspiration port of the ventilator and inlet port of the humidifier. The gas sampling line will be placed between the outlet port of the humidifier and the patient wye as shown.

7.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

Eligible participants will be randomized to one of the two treatment arms using a permuted block method¹⁵¹ with random block sizes. The randomization ratio will be 1:1 iCO:placebo. The randomization will not be stratified by any factors. The study biostatistician will verify all the necessary randomization information (blinding method, randomization method, block size, and number of treatment groups) in the StudyTRAX randomization configuration module.

Site investigators will review all potential study participants with one of the Data Coordinating Center (DCC) physician members. Following informed consent, the unblinded administering RT or physician or blinded study coordinator will randomize subjects in the database using the StudyTRAX randomization tool. The unblinded administering RT or physician will obtain the subject's treatment assignment through the "View Treatment" button in the blinded database, a privilege granted to unblinded RTs and physicians only. CO-related measurements (SpCO, COHb, ambient CO) and drug-related information will be in a separate project within the database, which the blinded study coordinators will not have access to. Permission to this limited-access project will only be granted to unblinded study personnel by the PI, as it contains treatment-specific information such as CO monitoring and study drug administration data.

The study drug assignment will be blinded to the subject, clinical team, study coordinators, and other study staff with the exception of the administering study staff (RT and physician OR physician alone), who will be unblinded to the treatment assignment to ensure subject safety. We chose this partially double-blind design to ensure safe iCO administration and an unblinded assessment of COHb levels in order not to exceed potentially toxic thresholds.

To maintain blinding, the placebo gas cylinders are identical in appearance as the CO cylinders (with the exception of different CGA valves required by the manufacturer Praxair). Cylinders will also be shrouded in dark sleeves to ensure blinding. The placebo gas will be administered via inhalation via the CoventDS and delivered to subjects in an identical fashion as those randomized to CO. To ensure that the subject, study coordinator, and clinical staff remain blinded to the study drug assignment, the administering RT and physician study staff member OR physician study staff member alone will conceal the gas cylinders, CO delivery device, and measurements of COHb, SpCO, and ambient CO levels. Unblinded test results will be kept in a separate location from the blinded test results and concealed from the study coordinators.

The unblinded administering physician will be responsible for maintaining a separate password-protected limited-access project within the database with the subject identification number and CO-related measurements (SpCO, COHb, ambient CO) which the study coordinator will not have access to. While the administering investigator will be unblinded throughout the study due to safety monitoring, they will only be unblinded to the treatment assignment for subjects enrolled at their own site. Investigators will otherwise be blinded to the study treatment assignment for subjects enrolled at the other sites. Two lead RTs (Mr. Hess and Mr. Davies) may become unblinded to the treatment assignments at other sites to assist with study drug administration procedures if necessary.

If the clinical team believes that unblinding is medically necessary in order to properly treat the patient, the unblinded administering physician can disclose study product assignment. If there is time to do so, the unblinded administering physician should discuss this unblinding with the unblinded DCC physician prior to the disclosure. All episodes of unblinding, whether deliberate or inadvertent, will be reported to the DSMB at the next routine report.

A blinded study physician at each site will review all AEs and will assess their relationship to the study intervention. Additional AE adjudication will be provided the Medical Monitor (Dr. Thompson). In addition to blinded adjudication of AEs, investigators blinded to treatment allocation will assess chest X-rays required for the calculation of the LIS score, the primary efficacy endpoint of this study. De-identified chest X rays at each site will be reviewed centrally by blinded investigators at BWH.

7.4 STUDY INTERVENTION COMPLIANCE

To assess protocol adherence during monitoring visits, the DCC will review the Study Drug Administration and CO Administration Monitoring forms, in addition to the Study Drug Accountability Log. The administering RT and physician study staff member OR physician study staff member alone will keep track of study drug assignment and dosages, time and duration of treatment, tank lot number, and COHb results on Subject Drug Administration and CO Administration Monitoring forms. These forms should be kept in a secure place and confidential from blinded study staff.

Each participating site is responsible for the ordering, storing and dispensing of the investigational agent(s). Each study team will keep a record of CO/placebo tanks received, administered, and returned at the study site in a Study Drug Accountability Log. This log will include lot number, date and amount/concentration received,

subject ID, date and time administered, date and time administration ended, date and amount/concentration returned.

7.5 CONCOMITANT THERAPY

Subjects will be excluded from participation in the study if they are receiving concomitant inhaled pulmonary vasodilators continuously (such as inhaled nitric oxide [NO] or prostacyclin), as these inhaled medications may interfere with the dosing of inhaled CO. If a decision is made by the clinical team to initiate these medications in enrolled subjects following randomization and within the 3-day study treatment period, the study drug will be held until subjects are no longer receiving these medications concomitantly. The study drug may be reinitiated following cessation of these inhaled medications if there are no other protocol-specified reasons that preclude ongoing treatment in the study. Potential subjects will also be excluded if they are participating in another interventional clinical trial. There are otherwise no contraindications to concomitant medications in the study.

8 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

8.1 DISCONTINUATION OF STUDY INTERVENTION

Discontinuation from study drug does not mean discontinuation from the study, and remaining study procedures should be completed as indicated in the study protocol. If a clinically significant finding is identified (including, but not limited to changes from baseline) after enrollment, the site investigator or designee will determine if any change in participant management is needed. Any new clinically relevant finding will be reported as an AE.

Permanent discontinuation of the study drug is defined as cessation of the study drug without the intent of restarting the study drug during the three-day treatment period. Subjects who have their study drug permanently discontinued will continue their participation in the study, and will be followed to determine their vital status and perform neurocognitive testing at 6 months as outlined in the **Time-Events Schedule (Section 1.3)**.

Criteria for permanent discontinuation of the study drug are described in **Section 7.1.8**.

In some instances, the study drug may be held and the subject may be re-evaluated for treatment on subsequent days. The criteria for a study drug hold are described in **Section 7.1.6**.

If study drug hold criteria are met, the study drug will be held and subjects will be evaluated on a daily basis and assessed for whether a given criteria has resolved and whether study drug treatment may be resumed.

8.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request.

An investigator may discontinue or withdraw a participant from the study for the following reasons:

- If any clinical AE, laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant
- Disease progression which requires discontinuation of the study intervention

- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation
- Participant unable to receive at least one dose of the study drug

If a patient or surrogate requests withdrawal from the study, the investigators will seek explicit permission to continue data collection.

The reason for participant discontinuation or withdrawal from the study will be recorded on the Case Report Form (CRF). Subjects who sign the informed consent form and are randomized but do not receive the study intervention may be replaced. Subjects who sign the informed consent form, and are randomized and receive at least one dose of the study product, and subsequently withdraw, or are withdrawn or discontinued from the study will not be replaced.

8.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if the study staff is unable to verify vital status after the subject has been discharged from the hospital.

Before a participant is deemed lost to follow-up, the site investigator or designee will make every effort to regain contact with the participant or surrogate (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts should be documented in the participant's medical record or study file.

We will verify duration of survival for participants lost to follow-up or noted to have died using the Centers for Disease Control and Prevention's National Death Index (National Death Index, 2000). The subject's social security number (SSN) will be used for an exact NDI match. Contact information will be collected for the subject and alternative contact information will be collected on up to 3 individuals. Contact information and the subject's SSN will be collected on paper at the time of consent. Contact information and SSN will be maintained on paper locally and will not appear in the DCC database.

9 STUDY ASSESSMENTS AND PROCEDURES

9.1 DESCRIPTION OF STUDY PROCEDURES

Study assessments described below will be done according to the **Time-Events Schedule** in **Section 1.3**.

Vital Signs: Heart rate (beats/min), systemic systolic, diastolic, mean arterial blood pressure (mm Hg), and body temperature (°C), will be recorded in the eCRF.

Central Venous Pressure (CVP): CVP will be recorded if available.

Ventilator Parameters: The following settings will be recorded:

- a) Mode of ventilation
- b) FiO₂ and PEEP
- c) Tidal volume, Respiratory rate (set rate and total rate), Minute ventilation, Inspiratory flow rate
- d) Peak pressure, Plateau pressure, Compliance, and Mean airway pressure. If on a pressure-cycling mode, peak pressure during inspiration will be assumed to be the plateau pressure.
- e) Pressure during inspiration if on a pressure targeted mode (PSV, PCV, etc)

EKG: A 12 lead EKG will be performed and reviewed by a study investigator. The EKG will also be officially read

locally at each site.

Chest X-ray (CXR): The baseline frontal CXR will be reviewed by two study physician investigators to determine study eligibility. Study physician investigators blinded to treatment allocation will review and perform the assessment for the Lung Injury Score (LIS). All 4 lung quadrants will be assessed for infiltrates and pneumothoraces. If the subject is extubated on days 1-5, the study team will order a chest X-ray the day following extubation, if not ordered by the clinical team. Chest X-rays will also be ordered by the study team for endpoint analysis on Day 7, if not ordered by the clinical team.

Lung Injury Score (LIS): The lung injury score will be calculated based on the chest x-ray assessment, PaO₂, FiO₂, PEEP, and ventilator compliance measurement as described in **Appendix E1**.

Oxygenation Index (OI): The OI will be calculated based on the PaO₂, FiO₂, and mean airway pressure obtained from the ventilator parameters.

APACHE II Score: The APACHE II consists of 12 physiological variables, age, and underlying health. The score will be calculated based on the worst values in the 24 hours prior to randomization. Calculation variables are described in **Appendix E2**.

Sequential Organ Failure Assessment (SOFA): The SOFA score will be calculated based on the worst values recorded in the past 24 hours. Calculation variables are described in **Appendix E3**.

- a) Worst PaO₂/FiO₂ for that date
- b) Worst creatinine (or urine output), bilirubin, and platelet count for that date
- c) Worst Glasgow Coma Scale for that date
- d) Vasopressor use and maximal dose for that date

Glasgow Coma Scale (GCS): The GCS is a determination of the best eye opening, motor, and verbal responses at any given time. The GCS is documented by the clinical team as part of routine ICU nursing assessment. If not available, the GCS will be assessed by the study staff as described in **Appendix E4**.

Richmond Agitation Scale (RASS): RASS is a scale used to measure the agitation or sedation level of a patient. RASS scoring is documented by the clinical team as part of routine ICU nursing assessment. If not available, the RASS will be assessed by the study staff as described in **Appendix E5**.

Blood gas analysis: The following values will be recorded from the arterial blood gas (ABG) results: pH, PCO₂, PO₂, and base excess or deficit. ABG is required for baseline assessment. If no ABG is available on subsequent days, record blood gas analysis results from the venous blood gas (VBG).

ScvO₂: ScvO₂ will be recorded if available.

Vasopressors and Inotropes Review: The following vasopressors/inotropes will be recorded in the study eCRFs. (epinephrine, norepinephrine, phenylephrine, vasopressin, dopamine, dobutamine, phosphodiesterase inhibitors, including dose).

Fluid Intake/Output: Fluid intake and output (most recent 24 hour value) or mean hourly value for most recently available period. Diuretic administration will also be recorded if applicable.

Renal replacement therapy status: Renal replacement therapy status within the last 24 hours will be recorded.

Concomitant Medications Review: The following medications will be recorded in the study eCRFs: aspirin,

angiotensin converting enzyme inhibitors (ACEIs), steroids, neuromuscular blockade, inhaled NO or prostaglandins.

Microbiological Results: Microbiological results will be recorded in the eCRF when ordered for clinical purposes.

- a) Blood cultures
- b) Urine cultures
- c) Sputum cultures
- d) BAL cultures
- e) CSF cultures if available
- f) Stool
- g) Other

Vital Status: Vital status will be assessed at Days 28, 60, and 6 months. Subjects who have been discharged from the hospital will be contacted by phone. Additional information will be collected on discharge status, ventilator status, and vasopressor status at Days 28 and 60.

Neurocognitive Testing: Neurocognitive testing will be performed via telephone interviews using the Montreal Cognitive Assessment (MoCA)-BLIND and Hayling sentence completion test as (**Appendix F**).

Clinical Laboratory Assessments: Clinical labs will be processed at each site's local laboratory and reviewed by a study investigator. If labs are required according to the **Time-Event Schedule (Section 1.3)** and are not available as part of standard of care testing, they will be drawn for research. A urine or serum pregnancy test will be done for women of childbearing potential. A subject with a positive pregnancy result will not be eligible for randomization.

9.1.1 STUDY SAMPLE COLLECTION

Sample collection will be done according to the **Time-Events Schedule** in **Section 1.3**.

COHb Levels: COHb levels will be measured using the IL GEM Premier Co-oximeter at specified time points on Days 1 through 3. See **Section 9.5.2**.

Plasma Samples: Blood (4 mL on days 1-3) will be collected pre-treatment in EDTA anti-coagulated tubes for plasma isolation and measurement of biomarkers of mitochondrial dysfunction, inflammasome activation, lipid mediators, and cytokines. Blood will be collected (4 mL on days 1-3) post-treatment (at 90 min and 3 hrs). Blood (4 mL) will also be collected for plasma isolation on Day 5. Plasma will be obtained and divided immediately after centrifugation into equal aliquots in specified tubes and frozen at -80°C .

RNA Samples: On days 1-3, blood (2.5 mL) will be collected in Paxgene tubes for RNA isolation. Blood will be collected in Paxgene tubes before and after (at 90 min and 3 hrs) study drug administration, and frozen at -80°C . Blood (2.5 mL) will also be collected in Paxgene tubes for RNA isolation on Day 5, and frozen at -80°C .

DNA Samples: Cell pellets will be obtained for future DNA isolation from the blood collected in EDTA tubes on days 1-3 and on day 5. Cell pellets will be isolated pre-treatment and post-treatment (at 90 min and 3 hrs) on days 1 through 3. On days 1 and 5, the 8 mL tube may be used to isolate cell pellets if unable to obtain an adequate pellet from the 4 mL tube on that day.

Primary Cell Isolation: An additional 8 mL of blood will be drawn for isolation of peripheral blood mononuclear cells (PBMCs) pre-treatment on day 1 and post-treatment on day 3.

Urine Sample Collection: Between 50-100 mL of urine will be collected in a sterile specimen container on days 1-3 and on Day 5. Cell pellets will be isolated pre-treatment and post-treatment (at 90 min and 3 hours) to obtain RNA and protein, as well as mtDNA.

Discarded Specimens: Discarded bronchoalveolar lavage (BAL) fluid and plasma will be obtained (when available) for levels of cytokines, mediators/biomarkers, and protein. Plasma and BAL fluid will be divided into equal aliquots in specified tubes and frozen at -80°C .

A total of approximately 88.5 mL of blood will be collected for research samples during the study from each study subject as follows:

Sample collection	Day 1	Day 2	Day 3	Day 5	Total Volume
ABG, lactate, and COHb analysis	2.5 mL	2.5 mL	2.5 mL		7.5 mL
4 mL EDTA tube (plasma and DNA)- pre and post-treatment	12 mL	12 mL	12 mL	4 mL	40 mL
2.5 mL PAXgene tube (RNA)- pre and post-treatment	7.5 mL	7.5 mL	7.5 mL	2.5 mL	25 mL
Primary Cell Isolation	8 mL			8 mL	16 mL
Total Volume	30 mL	22 mL	22 mL	14.5 mL	88.5 mL

Additional blood (29 mL) may be drawn during the study for research purposes as below if not drawn as part of usual clinical care.

Sample collection	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 14	Day 28	Total Volume
ABG, lactate	2 mL									2 mL
CBC	2 mL	2 mL	2 mL	2 mL						8 mL
Metabolic panel	3 mL									3 mL
Bilirubin		2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	16 mL
Total Volume	7 mL	4 mL	4 mL	4 mL	2 mL	2 mL	2 mL	2 mL	2 mL	29 mL

Samples will be sent to the central repository at Brigham and Women's Hospital (BWH) to be stored. Study subject ID numbers will identify samples during shipment and storage in the central repository. In the future, the DCC will instruct the repository to prepare the appropriate samples for shipment. The DCC will not record or store unique patient identifiers (such as initials, date of birth, hospital record numbers, addresses, phone numbers, etc.) in the database. All data released by the DCC for studies will be linked to the specimen but will be de-identified. The link (key) between the de-identified database and the patient will be removed two years after the primary publication. Samples collected for this trial will be frozen and stored in the biorepository for

future research related to carbon monoxide, ARDS, sepsis, trauma and burn injuries, critical illness or other lung diseases.

To enhance the biologic analytic power, samples from 12 subjects enrolled in a similar, already completed study will be similarly analyzed for these biomarkers, as well as in more extensive biologic 'omics profiling that will permit more detailed analyses of these pathways in correlation with clinical data.

9.2 SCREENING PROCEDURES

No evaluations will be required to determine eligibility for study participation and the diagnostic criteria for entry. Patients will be screened based on laboratory values (eg. arterial blood gas analysis) and radiologic tests (eg. CXR) that are otherwise conducted as part of routine clinical care in the ICU. No procedures will be required to determine subject eligibility. No study procedures will be initiated prior to obtaining written informed consent.

All subjects who meet the inclusion criteria of the CO ARDS protocol will be included in a screening log. Potential subjects who appear to meet eligibility criteria by chart review, will be approached for informed consent.

Subjects who are screened, but not enrolled into the study, either because they meet one or more exclusion criteria or decline participation, will be assigned a screening ID. De-identified data outlined in **Appendix D** of the CO ARDS protocol will be collected for subjects that have been screened, but not enrolled.

9.2.1 ASSIGNING A SCREENING ID

All subjects who are screened will be given a Reference ID and a Project ID. The Reference ID is automatically generated as a consecutive number by StudyTRAX (eg. Ref-0001, Ref-0002). The Reference ID carries with the subject throughout projects; it is universal to the database and does not contain any project-specific information. Additionally, a specific Project ID will be assigned to each subject that contains the site and a sequence unique to each site as follows (eg. BWH-0001, MGH-0001, BWH-0002 ... WCM-0001). Sites will be identified with a three-letter code as follows: BWH (Brigham and Women's Hospital), MGH (Massachusetts General Hospital), Duke University Hospital (DUH), Weill Cornell Medicine (WCM), Brooklyn Methodist Hospital (BMH), Durham Regional Hospital (DRH), Washington University (WU). The sites operate as separate projects, therefore coordinators and physicians only have access to the subjects at their site, unless additional privileges/permissions are granted in the database. All subjects who have been screened will be included in the Subject Screening Log.

9.2.2 BACKGROUND ASSESSMENTS

Background assessment information data may be collected any time after informed consent has been obtained.

1. Demographic and Admission Data
2. Pertinent Medical History and Physical Examination
3. Height; gender, measured body weight (MBW); calculated predicted body weight (PBW).
4. Time on ventilator prior to enrollment
5. Type of Admission
 - a. Medical
 - b. Surgical scheduled

- c. Surgical unscheduled
- d. Trauma
- 6. Risk factors for ARDS (infection, aspiration, trauma, pneumonia, drug overdose, other)
- 7. Presumed site of infection, if infection is the risk factor for ARDS
- 8. Acute or Chronic renal failure and use of dialysis
- 9. Presence of the following chronic diseases:
 - a. Cancer
 - b. Hematological malignancy
 - c. AIDS
 - d. Diabetes mellitus
 - e. COPD
 - f. Asthma
 - g. Liver cirrhosis
 - h. Hypertension
 - i. Coronary artery disease
 - j. Congestive heart failure
 - k. Peripheral vascular disease
 - l. Dementia
 - m. Prior stroke with sequelae
- 10. Survey of smoking history including:
 - a. Ever smoker (> 100 cigarettes in lifetime)?
 - b. If yes, current smoker?
 - Estimate of pack years (# packs per day) x (# years smoked)
 - c. If former smoker, when did the subject quit smoking?
- 11. Pregnancy test (serum or urine) for women of childbearing potential

9.2.3 BASELINE ASSESSMENTS

The following information will be recorded within the 24-hour interval preceding initiation of study drug treatment.

If more than one value is available for this 24-hour period, the appropriate values for the APACHE II calculator will be recorded. If no values are available from the 24 hours prior to study drug administration, then values must be measured prior to initiation of study drug. A full description of the below data collection is in **Section 9.1**.

Baseline Assessments

- 1. Vital Signs
- 2. CVP if available
- 3. Electrocardiogram (EKG)
- 4. Ventilator Parameters
- 5. Frontal Chest Radiograph (qualifying radiograph)
- 6. Lung Injury Score (LIS)

7. APACHE II Score
8. SOFA Score
9. Glasgow Coma Score (GCS)
10. Richmond Agitation Sedation Scale (RASS)
11. ABG (qualifying arterial blood gas)
12. SpO₂
13. ScvO₂ if available
14. Complete blood count (CBC)
15. Basic metabolic panel
16. PT/PTT/INR if available
17. Serum CK, AST, ALT, Albumin, Total Protein if available
18. Bilirubin
19. Lactate
20. Vasopressors and Inotropes Review
21. Fluid intake, fluid output (most recent 24 hour value) or mean hourly value for most recently available period
22. Renal replacement therapy status
23. Concomitant Medications Review
24. Discarded bronchoalveolar lavage (BAL) fluid and plasma if available

9.3 RANDOMIZATION PROCEDURES

All ARDS criteria must occur within the same 24 hour period. The onset of ARDS is when the last criterion is met. Patients must be enrolled within 168 hours of ARDS onset. Information for determining when these time window criteria were met may come from either the site hospital or reports from a referring hospital.

Following randomization, the low tidal volume protocol for mechanical ventilation must be initiated within two hours.

The first treatment of study drug should be given within 24 hours of randomization. The day of randomization will be considered study day zero.

Eligible participants will be randomized to one of the two treatment arms using a permuted block method¹⁵¹ with random block sizes. The randomization ratio will be 1:1 iCO:placebo. The randomization will not be stratified by any factors. The study biostatistician will verify all the necessary randomization information (blinding method, randomization method, block size, and number of treatment groups) in the StudyTRAX randomization configuration module.

Site investigators will review all potential study participants with one of the DCC physician members. Following informed consent, the unblinded administering RT or physician or blinded study coordinator will randomize subjects in the database using the StudyTRAX randomization tool. The unblinded administering RT or physician will obtain the subject's treatment assignment through the "View Treatment" button in the blinded database, a privilege granted to unblinded RTs and physicians only. CO-related measurements (SpCO, COHb, ambient CO) and drug-related information will be in a separate project within the database, which the blinded study

coordinators will not have access to. This central review of randomization by the DCC unblinded physician will ensure that no subjects are enrolled in this multi-center trial during planned or unplanned study holds.

9.3.1 ASSIGNING A RANDOMIZATION

All subjects will be randomized through the StudyTRAX database using a permuted block method with random block sizes. The unblinded physicians or RTs or blinded coordinators will randomize the subject after enrollment. Subjects will receive a Randomization ID (RI-SiteCode-Seq) which includes a Site Code and Sequence starting with 0001 per site (eg. RI-BWH-0001, RI-MGH-0001, RI-DUH-0001, RI-WCM-0001, RI-DVA-0001, RI-BMH-0001, RI-DRH-0001).

9.3.2 VENTILATOR PROCEDURES

FiO₂ will be increased prior to study drug administration in order to achieve a PaO₂ ≥ 80 or SpO₂ ≥ 95%. Ventilator management, including weaning, will follow the modified ARDS Network lower tidal volume (6 mL/kg PBW) protocol (**Appendix G**)⁹. If not already being utilized, this low tidal volume protocol for mechanical ventilation must be initiated within two hours of randomization. Since the time a patient achieves unassisted ventilation affects a secondary endpoint, VFDs, and because recent evidence-based consensus recommendations have identified a best practice for weaning, weaning strategy will also be controlled by protocol rules in accordance with these evidence-based recommendations. This newer weaning strategy is a simplified version of the protocolized weaning strategy used in prior ARDS Network studies (**Appendix G**). Study drug administration will be continued in patients undergoing weaning from mechanical ventilation as in **Appendix G** unless the subject is deemed ready for extubation by the clinical team.

9.4 REFERENCE MEASUREMENTS (DAYS 1 – 3)

Reference Measurements (Days 1 – 3)

The following parameters will be measured and recorded daily on study Days 1 through 3 from 3:00-10:00 am using the values closest to 8:00 am on the days specified in the **Time-Events Schedule (Section 1.3)**. On days when the study drug is administered, the ventilator parameters will be recorded using the values closest to the study drug administration. Details regarding the below assessments are found in **Section 9.1**.

The following conditions will be ensured prior to reference measurements:

- No endobronchial suctioning for 10 minutes
- No invasive procedures or ventilator changes for 30 minutes
- All vascular pressures will be zero-referenced to the mid-axillary line with the patient supine.

Reference Measurements

1. Vital Signs
2. CVP if available
3. EKG
4. Ventilator Parameters Assessment (closest to the time of study drug administration)
5. Frontal Chest Radiograph
6. Lung Injury Score (LIS)
7. SOFA Score
8. GCS
9. RASS

10. ScvO₂ if available
11. CBC
12. Basic metabolic panel if available and requested by treating physician
13. PT/PTT/INR if available and requested by treating physician
14. Serum CK, AST, ALT, Albumin, Total Protein if available and requested by treating physician
15. Bilirubin
16. Lactate
17. Vasopressors and Inotropes Review
18. Fluid intake, fluid output (most recent 24 hour value) or mean hourly value for most recently available period
19. Renal replacement therapy status
20. Concomitant Medications Review
21. Adverse Event Monitoring
22. Blood for Biomarker Collection
23. Urine Sample Collection
24. Microbiological results when available
25. Discarded BAL fluid and plasma if available

9.5 STUDY DRUG ADMINISTRATION ASSESSMENTS (DAY 1-3)

The following assessments will be done on Days 1 – 3 prior to study drug administration. **No treatment will be started before these assessments are performed and the pre-treatment checklist is completed by the study investigator**

Calibration of IL GEM Premier Co-oximeter and COventDS: Calibration will be performed according to device manuals and SOP. Documentation of calibration will be kept in study files.

COHb Level: The baseline COHb level will be drawn and assessed by an unblinded physician investigator, just prior (less than 15 minutes) to starting the study drug administration. COHb will be measured by study investigators using an IL GEM Premier Co-oximeter at the patient's bedside. These results are obtained within less than 5 minutes. The study drug will not be started until this result is obtained and reviewed for a potential study hold. All COHb level result printouts should be filed in the unblinded subject file.

Lactate Level: Lactate level will be measured and assessed daily by the local laboratory at each center prior to study drug administration. The study drug will not be started until this result is obtained and reviewed by the study physician investigator for a potential study hold.

Hemoglobin Level: Hemoglobin will be measured and assessed daily prior to study drug administration. The hemoglobin level will be measured by the local laboratory at each center. The study drug will not be started until this result is obtained and reviewed by the study physician investigator for a potential study hold.

EKG: A 12 lead EKG will be obtained and reviewed daily by the study staff physician prior to study drug administration to evaluate for cardiac exclusion criteria ie. acute MI, ACS, or unstable atrial or ventricular arrhythmia. The study drug will not be started until the EKG is obtained and reviewed by the study physician investigator for a potential study hold. The EKG will also be officially read locally at each site.

Review Ventilator Parameters: The study staff will record ventilator parameters prior to starting study drug administration.

Calculate Deadspace (Vd/Vt): Dead space fraction will be measured daily by the administering RT or physician prior to study drug administration using a NICO or NM3 monitor (Respironics) and the PCO₂ obtained from an arterial or venous blood gas.

Blood Gas Analysis (arterial or venous): Arterial or venous blood will be drawn for blood gas analysis by the local laboratory at each center. The blood gas will be assessed daily prior to starting study drug administration. The study drug will not be started until this result is obtained and reviewed by the study physician investigator for a potential study hold. The following values will be recorded from the ABG or VBG results: pH, PCO₂, PO₂, and base excess or deficit.

Monitor SpCO and SpO₂ with pulse oximeter: SpCO and SpO₂ will be measured prior to treatment, recorded at intervals as described below, and monitored throughout study drug treatment using a noninvasive pulse oximeter (Masimo Radical-7).

Ambient air CO levels: Ambient air CO levels will be measured before and throughout study drug treatment using a calibrated Dräger Pac 7000 CO detector. This will ensure that ambient levels are maintained within acceptable limits for occupational exposure.

Ensure blinding: All CO monitoring will be carried out by the unblinded administering RT and/or investigator and concealed from the blinded study team members.

Review for Adverse Events: The blinded study coordinator will review events over the past 24 hours that may meet criteria for adverse events.

9.5.1 REVIEW PARAMETERS PRIOR TO ADMINISTRATION OF STUDY DRUG

Review the Daily Hold Parameters (detailed information in **Section 7.1.6**). Administration of the study drug will be held if the following criteria are met.

Daily Hold Parameters:

- COHb level $\geq 3\%$
- Lactate ≥ 4 mmol/L
- ST elevation MI or unstable angina/non ST elevation MI concerning for ACS
- Unstable atrial or ventricular arrhythmia
- Severe hypoxemia defined as SpO₂ < 95 or PaO₂ < 90 on FiO₂ ≥ 0.9
- Hemoglobin < 7.0 g/dL
- Diffuse alveolar hemorrhage from vasculitis
- Use of high frequency ventilation
- Use of inhaled pulmonary vasodilator therapy (eg. NO or prostaglandins)
- Use of ECMO
- Occurrence of an AE meeting criteria for study drug hold or discontinuation (**Sections 7.1.6, 7.1.8.**).

9.5.2 STUDY DRUG ADMINISTRATION AND MONITORING

The study drug will be administered for up to 90 minutes daily for up to 3 days following randomization or until discontinuation of mechanical ventilation, whichever occurs first according to **Section 7.1.9**. See **Sections 7.1.6, 7.1.7, and 7.1.8** for information about study drug hold, interruption, and permanent discontinuation criteria.

Monitoring during Study Drug Administration:

Subjects will be monitored with continuous pulse oximetry, cardiac monitoring, and either intermittent (at least every 15 minutes) or continuous blood pressure monitoring (if an arterial line is in place) throughout the course of the 90 minute study drug treatment.

SpCO and SpO₂ will be monitored continuously using a Masimo Rad-7 non-invasive pulse oximeter, and recorded at the time points specified below. COHb levels will be measured using an IL GEM Premier Co-oximeter and recorded at the time points specified below. Arterial or venous blood will be drawn for blood gas analysis prior to treatment and at the completion of study drug administration (90 min or earlier if discontinued prior to 90 min). If study drug treatment is discontinued prior to 90 min, all of the scheduled 90-minute measurements will be made and recorded at the time of study drug discontinuation.

Ambient CO levels will be monitored continuously using a Dräger Pac 7000 CO detector, and recorded at the time points specified below.

	ABG or VBG (pH, PCO ₂ , PO ₂)	FiO ₂	COHb level (IL GEM Premier co-oximeter)	CFK look-up table (Appendix B or StudyTRAX)	SpCO (Masimo Rad-7)	SpO ₂ (Masimo Rad-7)	Ambient CO Level (Dräger Pac CO detector)
Pre-treatment	X	X	X		X	X	X
20 min			X	X	X	X	X
60 min			X		X	X	X
75 min			X		X	X	X
90 min*	X	X	X		X	X	X
180 min			X		X	X	X

*90 min or upon completion of study product if discontinued sooner than 90 min.

CFK Equation-Based Table: The unblinded administering physician and/or RT will use the CFK equation-based table (**Appendix B or electronic equivalent version in StudyTRAX**) to look up the time at which the COHb level is predicted to be 8%. If the CFK-equation predicted time is 90 min or greater, the study drug will be administered for 90 minutes. If the CFK-equation predicted time is less than 90 min, the study drug will be discontinued at the time the COHb level is predicted to be 8%. The required 90 minute measurements will be replaced by measurements done at the completion of the study drug administration if study product administration is discontinued prior to 90 min. Note that for FiO₂ > 55%, the COHb level will never reach 8% with administration of 200 ppm CO and therefore the study drug treatment should be administered for the complete 90 minutes.

9.6 POST-TREATMENT ASSESSMENTS

Day 4, 5 and 7 Assessments

The following assessments will be done on Days 4, 5 and 7. Details about the below assessments are found in **Section 9.1**.

1. Vital Signs
2. EKG on day 7 and when available
3. Ventilator Parameters Assessment
4. Frontal Chest Radiograph on day 7 and when available
5. ABG; VBG if ABG is not available
6. SpO₂
7. Lung Injury Score (LIS)
8. Dead space fraction (Vd/Vt) on day 7
9. SOFA Score
10. GCS
11. RASS
12. CBC if available and requested by treating physician
13. Basic metabolic panel if available and requested by treating physician
14. PT/PTT/INR if available and requested by treating physician
15. Serum CK, AST, ALT, Albumin, Total Protein if available and requested by treating physician
16. Bilirubin
17. Lactate if available
18. Vasopressors and Inotropes Review
19. Fluid intake, fluid output (most recent 24 hour value) or mean hourly value for most recently available period
20. Renal replacement therapy status
21. Concomitant Medications Review
22. Adverse Event Monitoring
23. Blood for Biomarker Collection
24. Urine Sample Collection
25. Microbiological results when available
26. Discarded BAL fluid and plasma if available

Day 14 Assessments

The following assessments will be done on Day 14. Details about the below assessments are found in **Section 9.1**.

1. Ventilator Parameters Assessment
2. SOFA Score
3. GCS
4. Total Bilirubin
5. Vasopressors and Inotropes Review
6. Renal Replacement Therapy Status
7. Microbiological results when available

Day 28 Assessments

The following assessments will be done on Day 28. Details about the below assessments are found in **Section 9.1**.

1. SOFA Score
2. GCS
3. Bilirubin
4. Microbiological results when available
5. Vital Status
6. Discharge, Ventilator, and Vasopressor Status

Day 60 Assessments

The following assessments will be done on Day 60. Details about the below assessments are found in **Section 9.1**.

1. Vital Status
2. Discharge, Ventilator, and Vasopressor Status

6 Month Follow-up Assessments

The following assessments will be done at 6 months. Details about the below assessments are found in **Section 9.1**.

1. Vital Status
2. Neurocognitive Testing – Montreal Cognitive Assessment (MoCA)-BLIND, Hayling sentence completion test

9.6.1 ASSESSMENTS AFTER HOSPITALIZATION

Vital status will be collected at 28 days, 60 days, and 6 months through telephone interviews with participants. Surrogates will be contacted in the case that subjects cannot be reached. The site investigator or designee will make every effort to contact the participant or surrogate (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts should be documented in the participant's medical record or study file.

In addition, we will verify duration of survival for participants lost to follow-up or noted to have died using the Centers for Disease Control and Prevention's National Death Index (National Death Index, 2000). We will use each subject's SSN for an exact NDI match. We will collect contact information for the subject and alternative contact information on up to 3 individuals. This information and the SSN will be collected on paper at the time of consent. Contact information and SSN will be maintained on paper and will not appear in the DCC database. In addition to vital status, long term cognitive function will also be assessed at 6 months. Cognitive impairment is highly prevalent among survivors of critical illness¹⁵²⁻¹⁵⁷. In addition, neurocognitive deficits observed at 3 months after hospitalization persist at 12 months following critical illness in the majority of patients¹⁵⁷. In survivors of ARDS, cognitive impairment has been observed in approximately 30-55% of patients following one year of hospitalization^{152,156}. Similarly, moderate to severe cognitive impairment has been observed in survivors of severe sepsis up to one year following hospitalization¹⁵³.

Several studies have demonstrated the safety of CO, including lack of adverse neurocognitive effects, at levels of COHb < 10%, which exceed the levels anticipated in our study. Neurocognitive effects of CO have been extensively evaluated in previous human studies^{116,158}, our endotoxin study in healthy volunteers (NCT00094406), and our recent trial of CO treatment in IPF patients (NCT01214187)¹²⁰. Stewart et al.

demonstrated no impairment in performance testing in healthy humans exposed to CO at 100 ppm for 8 hours with COHb levels of 11-13%¹¹⁶. In addition, we demonstrated lack of adverse neurocognitive effects in healthy volunteers exposed to CO at 100 ppm for 6 hours with COHb 6.5% ± 1.7%. In addition, a recent study by Linde Gas Therapeutics assessed the safety of inhaled CO in 32 healthy subjects. CO was well tolerated with no significant neurocognitive effects observed in subjects with COHb levels of 2-8.8%. In our CO IPF trial, neurocognitive function was evaluated using the Montreal Cognitive Assessment (MoCA) tool. Subjects with IPF receiving biweekly treatment with inhaled CO at 100-200 ppm for 2 hours demonstrated no impairment in neurocognitive function after 2.5 weeks of follow-up. In addition to these studies, a recent review of the literature suggests that neurocognitive effects are only seen once COHb rises above 15-20%¹⁵⁸.

In this study, we will use a telephone version of the MoCA^{159,160}, which consists of 4 items examining attention, verbal learning and memory, executive functions/language, and orientation and can be administered in 5 minutes. The MoCA has been shown to be a valid and feasible screening tool to assess cognitive function in patients following CVA and acute coronary syndrome¹⁵⁹⁻¹⁶³. We will also use a telephone version of the Hayling sentence completion test to further evaluate executive function^{164,165} which is commonly impaired in ARDS survivors¹⁵²⁻¹⁵⁷.

9.7 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

9.7.1 DEFINITION OF ADVERSE EVENTS (AE)

Adverse event means any untoward medical occurrence associated with the use of an intervention in humans, whether or not considered intervention-related (21 CFR 312.32 (a)).

9.7.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

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

- results in death;
- is life threatening (places the subject at immediate risk of death from the event as it occurred);
- requires inpatient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant disability/incapacity;
- results in a congenital anomaly/birth defect; or
- any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the outcomes listed above.

Organ failures related to ARDS or the patient's underlying condition that are systematically captured by the protocol should not be reported as adverse events ***unless they are considered to be study related.***

If the suspected adverse reaction is unexpected, it will be classified as a serious unexpected suspected adverse reaction (SUSAR).

9.7.3 CLASSIFICATION OF AN ADVERSE EVENT

9.7.3.1 SEVERITY OF EVENT

For adverse events (AEs) not included in the protocol defined grading system, the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0 guidelines will be used to describe severity.

- **Grade 1 Mild** - asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
- **Grade 2 Moderate** - minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL).
- **Grade 3 Severe** - medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.
- **Grade 4 Life-threatening** - urgent intervention indicated.
- **Grade 5 Death**

9.7.3.2 RELATIONSHIP TO STUDY INTERVENTION

All adverse events (AEs) will have their relationship to study intervention assessed by a designated blinded study physician at each site. Additional AE adjudication will be provided by the Medical Monitor (Dr. Thompson) who will review all AEs in an unblinded fashion as is currently recommended by the FDA for SAEs (Guidance for Industry and Investigators Safety: Reporting Requirements for INDs and BA/BE Studies (Section VI.C); December 2012). The Medical Monitors at the DCC will work collaboratively with the reporting investigator to determine if a serious adverse event has a reasonable possibility of having been caused by the study drug or procedure.

The degree of certainty about causality will be graded using the categories below. In a clinical trial, the study product must always be suspect.

- **Definitely Related** – There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.
- **Probably Related** – There is evidence to suggest a causal relationship, and the influence of other factors is unlikely.
- **Possibly Related** – There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the trial medication). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events).
- **Probably not related** – A clinical event, including an abnormal laboratory test result, whose temporal relationship to study intervention administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study intervention) and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).
- **Not Related** – The AE is completely independent of study intervention administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician.

Administration related adverse events, as outlined in **Section 9.7.4** of the protocol will always be considered suspected adverse reactions if they occur within 48 hours of study drug exposure.

9.7.3.3 EXPECTEDNESS

The designated blinded study physician at each site and the Medical Monitors will be responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study product as described in the Investigator's Brochure.

9.7.4 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

Designated blinded study physicians will determine daily if any clinical adverse events occur during the period from enrollment to study day 7. Adverse event collection begins after the patient or surrogate has signed informed consent and has received study drug or undergone study procedures. If a patient experiences an adverse event after consent, but prior to receiving study product, the event will NOT be collected unless the investigator feels the event may have been caused by a protocol procedure. The designated blinded study physician will evaluate any changes in laboratory values and physical signs and will determine if these changes are clinically important. All clinically important adverse events will be recorded in the case report form regardless of attribution to study product. After study day 7, adverse events are not required to be reported unless the investigator feels the events were related to either study product or a protocol procedure.

All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate case report form (CRF). Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution. Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the pre-existing medical condition deteriorates at any time between the first study product administration and study day 7, it will be recorded as an AE. Organ failures related to ARDS or the patient's underlying condition that are systematically captured by the protocol should not be reported as adverse events ***unless they are considered to be study related.***

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed.

AEs will be followed for outcome information until resolution or stabilization or until 28 days (or hospital discharge whichever occurs first) after enrollment.

For this trial, a subset of adverse events will be considered to be "administration related adverse events". These "administration related adverse events" will by definition be considered suspected adverse reactions, as outlined in **Section 7.1.8**. These events are:

- New onset atrial or ventricular arrhythmias requiring DC cardioversion within 48 hours of study drug administration
- Myocardial infarction within 48 hours of study drug administration
- CVA within 48 hours of study drug administration
- Increase in O₂ requirements defined as: an increase in FiO₂ of ≥ 0.2 **AND** increase in PEEP ≥ 5 cm H₂O within 6 hours of study drug administration
- Increase in lactate by 2 mmol/L within 6 hours of study drug administration
- Increase in any protocol specified measurement of COHb $\geq 10\%$

9.7.5 ADVERSE EVENT REPORTING

All clinically important AEs as described in **Section 9.7.4**, will be recorded in the StudyTRAX database. For each AE, an email will be sent to the DCC, site PI, and blinded physician adjudicating AEs at each site. AEs will be summarized using System Organ Class and preferred terms. Tables will show by treatment group the number and percentage of participants experiencing an AE within a System Organ Class or within preferred term category by severity or by relationship to study product. For the calculations in these tables, a participant with

multiple AEs within a category will be counted once under the maximum severity or the strongest recorded causal relationship to study product. Formal statistical testing comparing groups is not planned since interpretation of differences must rely heavily upon clinical judgment.

The DSMB will review all AEs during scheduled interim analyses. The DCC will distribute the written summary of the DSMB's periodic review of adverse events to investigators for submission to their respective IRBs.

9.7.6 SERIOUS ADVERSE EVENT REPORTING

Site investigators will report all **serious AND unexpected adverse events or reactions**, regardless of relationship to the study intervention to the DCC by phone, fax, or email within 24 hours of becoming aware of the event. The site investigator should include an assessment of whether there is a reasonable possibility that the study intervention caused the event.

Investigators will also report all **serious AND administration related adverse events** (as described in **Section 7.1.8**) to the DCC by phone, fax or email within 24 hours of becoming aware of the event.

The DCC will review the event and may inform the site to permanently discontinue study drug administration to the subject (**Section 7.1.8**), and may hold enrollment pending Scientific Review Committee (SRC) and DSMB review (**Section 11.1.5**). The local IRBs will also be notified according to local requirements. The investigator will then submit a detailed written report to the DCC and the IRB no later than 7 calendar days after the investigator discovers the event.

The DCC will report all **unexpected and study-related deaths or life-threatening suspected serious adverse events (SUSAR)** to the FDA within 7 calendar days. The DCC will report **all deaths occurring during the study hospitalization and all serious, unexpected, and study-related adverse events and all administration related adverse events** to the DSMB, by email, or telephone, within 7 calendar days of the DCC being notified of the event. A written report will be sent to the DSMB and the FDA within 15 calendar days, and these reports will be sent to investigators for submission to their respective IRBs. The DSMB will also review all adverse events during scheduled interim analyses. The DCC will distribute the written summary of the DSMB's periodic review of adverse events to investigators for submission to their respective IRBs.

9.8 UNANTICIPATED PROBLEMS

9.8.1 DEFINITION OF UNANTICIPATED PROBLEMS (UP)

The USAMRMC Human Research Protection Office (HRPO) considers unanticipated problems involving risks to participants or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected, in terms of nature, severity, or frequency, given the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol, the Investigator's Brochure, or the informed consent document; and the characteristics of the subject population being studied;
- Related or possibly related to participation in the research, in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research;
- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

9.8.2 UNANTICIPATED PROBLEM REPORTING

Site investigators will report unanticipated problems (UPs) to their reviewing IRB as well as to the DCC and the lead PI. The UP report will include the following information:

- Protocol identifying information: protocol title and number, site investigator's name, and the IRB project number;
- A detailed description of the event, incident, experience, or outcome;
- An explanation of the basis for determining that the event, incident, experience, or outcome represents an UP;
- A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the UP.

To satisfy the requirement for prompt reporting, UPs will be reported using the following timeline:

- All UPs will be reported to the IRB and to the DCC/PI within 24 hours of the investigator becoming aware of the event.

All UPs should be promptly reported to HRPO by phone (301-619-2165), or by facsimile (301-619-7803). A complete written report will follow the initial notification. In addition to the methods above, the complete report can be sent to the US Army Medical Research and Materiel Command, ATTN: MCMR-RP, 810 Schreider Street, Fort Detrick, Maryland 21702-5000.

10 STATISTICAL CONSIDERATIONS

10.1 STATISTICAL HYPOTHESES

- **Primary Safety Endpoint:**

The primary safety endpoint is to evaluate the safety of inhaled CO, by determining COHb levels and the incidence of pre-specified administration-related AEs.

- Safety of inhaled CO, defined by the incidence of pre-specified administration-related AEs (as defined below) and spontaneously reported AEs through study day 7.
 - Acute MI within 48 hours of study drug administration
 - Acute CVA within 48 hours of study drug administration
 - New onset atrial or ventricular arrhythmia requiring DC cardioversion within 48 hours of study drug administration
 - Increased oxygenation requirements defined as: an increase in FiO_2 of ≥ 0.2 **AND** increase in $\text{PEEP} \geq 5$ cm H_2O within 6 hours of study drug administration
 - Increase in COHb $\geq 10\%$
 - Increase in lactate by ≥ 2 mmol/L within 6 hours of study drug administration

Hypothesis: We hypothesize that low dose iCO will be safe and well-tolerated in patients with ARDS.

- **Primary Efficacy Endpoint:**

The primary efficacy endpoint is to compare the effects of iCO versus placebo on mtDNA levels from baseline to day 5 (or death, whichever comes first) in patients with ARDS.

Hypothesis: We hypothesize that, compared to placebo, low dose iCO will lead to a reduction in mtDNA levels on day 5 compared with baseline.

- **Secondary Efficacy Endpoints:**

The secondary endpoints will examine the effects of iCO versus placebo on the following outcomes:

- Lung Injury Score (LIS) on days 1-5, and 7
- PaO₂/FiO₂ ratio on days 1-5, and 7
- Oxygenation Index (OI) on days 1-5, and 7
- Dead Space Fraction (Vd/Vt) on days 1-3, and 7
- Sequential Organ Failure Assessment (SOFA) score on days 1-5, 7, 14, 28

Hypothesis: We hypothesize that low dose iCO will reduce the severity of lung injury and non-pulmonary organ failure in patients with ARDS. We hypothesize that treatment with iCO will reduce the severity of ARDS as measured by LIS over the first 7 days of the trial, as well as other physiologic measures of lung injury, including PaO₂/FiO₂, OI, and Vd/Vt. We hypothesize that treatment with iCO will reduce non-pulmonary organ failure in patients with ARDS as measured by the SOFA score.

Additional secondary endpoints will examine the effects of iCO versus placebo on biomarkers of mitochondrial dysfunction, inflammasome activation, and lipid mediators in patients with ARDS.

- Autophagy markers (eg. LC3B)
- Inflammasome-dependent cytokines (eg. IL-18)
- Lipid mediators (LM) and specialized pro-resolving mediators (SPMs)
- Mitochondrial quality control biomarkers (eg. Pink1, Wipi1)
- Biomarkers of inflammation (eg. IL-6, IL-8, IL-10, IL-1Ra)
- To enhance the biologic analytic power, samples from 12 subjects enrolled in a similar, already completed study will be similarly analyzed for these biomarkers, as well as in more extensive biologic 'omics profiling that will permit more detailed analyses of these pathways in correlation with clinical data.

Hypothesis: We hypothesize that, compared to placebo, low dose iCO will reduce mitochondrial dysfunction, attenuate inflammasome activation, and promote resolution of inflammation in patients with ARDS. Furthermore, we hypothesize that modulation of these pathways will predict improvements in the clinical outcomes of our Phase II trial.

- **Exploratory Endpoints:**

- Ventilator-free days at day 28
- ICU-free days at day 28
- Hospital-free days at day 60
- Hospital mortality to day 28 and 60

Focused Safety Analysis:

The incidence of elevation in plasma COHb \geq 10% measured on study days 1-3 and pre-specified administration-associated adverse events (**Section 7.1.8**) and serious adverse events (**Section 9.7**).

10.2 SAMPLE SIZE DETERMINATION

A total of 32 subjects will be enrolled accounting for a 10% of loss of follow up. Sample size is based on 80% power to detect a difference in change from baseline to day 5 (or death whichever comes first) in mtDNA. With 16 subjects in the placebo arm and 16 subjects in the iCO arm, we will have 80% power to detect a 17225 absolute difference in change in mtDNA (copies/ μ l) from baseline to day 5 (or death whichever comes first) between treatment arms, assuming a conservative 14011 standard deviation (SD)¹⁶⁶ (Table 4). We will also have 80% power to detect a 0.98 difference in LIS, 81 mm Hg difference in PaO₂/FiO₂, and a 0.147 difference in Vd/Vt between treatment arms, assuming an SD of 0.8, 66, and 0.12 respectively^{167,168} (Table 5). With 16 patients randomized to iCO (Table 6), there will be a 77% probability of observing at last one AE that has a 10% background rate and a 96% probability of observing at least one AE that has a 20% background rate.

With 16 patients to each arm, we demonstrated differences in proportions with 2-sided 5% alpha (Table 7).

Table 4. Minimal Detectable Difference at 80% Power for Primary Outcome

Endpoint	Expected Mean Change in Placebo Arm	Expected Mean Change in iCO Arm	Standard Deviation	Detectable Absolute Difference (alpha=5%)
Change in mtDNA (copies/ μ l)	13348	-3877	14011	17225

Table 5. Minimal Detectable Difference on Day 7 at 80% Power for Secondary Endpoints

Endpoint	Detectable Absolute Difference on Day 7 (alpha=5%)	Standard Deviation
LIS	0.98	0.8
PaO ₂ /FiO ₂	81	66
OI	12.4	10.097
Vd/Vt	0.147	0.120
Vasopressor free days	5.2	4.2
Ventilator free days	13.7	11.2
ICU free days	12.6	10.3

Table 6. Probability of observing 1 or more events on the iCO arm for different background AE rates.

n	Background AE rate	Probability to detect ≥ 1 AE
16	0.010	0.13
16	0.020	0.25
16	0.035	0.39
16	0.050	0.51
16	0.100	0.77
16	0.150	0.90
16	0.200	0.96

Table 7. Adverse Events: Differences in proportions that can be demonstrated with 16 on each arm, 2-sided 5% alpha.

Placebo Proportion	iCO Proportion	Difference	Power
0.40	0.0191	0.3809	0.80
0.40	0.0042	0.3958	0.85
0.40	0.0000	0.4000	0.90
0.45	0.0459	0.4041	0.80
0.45	0.0293	0.4207	0.85
0.45	0.0103	0.4397	0.90
0.50	0.0762	0.4238	0.80
0.50	0.0580	0.4420	0.85
0.50	0.0370	0.4630	0.90

PASS version16, test for two proportions, unpooled
variance

10.3 POPULATIONS FOR ANALYSES

The primary analysis population will be the modified intention-to-treat (MITT) population. All randomized subjects who complete at least one dose of the study drug will be included in the analysis. Because some patients may withdraw or have a change in clinical status precluding dosing of study drug (eg. post-randomization lactic acidosis), we may randomize additional patients to achieve the enrollment goal of 32 patients who are randomized and treated with at least one dose of the study drug. We expect this to be an uncommon occurrence. Patients who are randomized but do not undergo any study procedures or receive at least one dose of the study product do not contribute any data and will not be included in the study analysis.

10.4 STATISTICAL ANALYSES

10.4.1 GENERAL APPROACH

We will use the Mann-Whitney U or t-test, chi-square (χ^2) or Fisher's exact tests to analyze baseline demographics and clinical characteristics between the treatment groups. Distribution of numerical outcomes will be evaluated prior to any analysis. All statistical analyses will be performed using SAS v 9.4 or newer versions (Cary, NC) or equivalent statistical packages. The detailed statistical analysis of this study can be found in the Statistical Analysis Plan.

10.4.1 ANALYSIS OF THE PRIMARY SAFETY ENDPOINT

The primary analysis population for the primary safety endpoint will be the MITT population. All randomized subjects treated with at least one dose of the study drug will be included in the analysis.

We will examine AEs by the type of event, as well as by body system class, and we will compare the occurrence of specific AEs in each group, as well as the number of AEs per patient in each group.

10.4.2 ANALYSIS OF THE PRIMARY EFFICACY ENDPOINT

We will use the Mann-Whitney U test to compare changes in mtDNA (copies/ μ l) from baseline to day 5 (or death whichever comes first) between treatment arms. Log transformed mtDNA will be analyzed using repeated measures analysis of covariance (ANCOVA) to assess the effects of treatment, time, and the interaction between time and treatment, while incorporating between-patient variation in baseline measurements and important covariates including age and severity of ARDS (SAS PROC MIXED). Interaction contrasts will include polynomial trends across time, focusing on a linear trend suggesting improvement and a quadratic trend suggesting that improvement is leveling off. As part of our analysis, we will also include the interaction between treatment and ARDS severity in the model. Because the study is not powered for the interaction between treatment and severity of ARDS, this test will be exploratory. We will also perform post-hoc analyses within each severity group to generate hypotheses for future study.

10.4.3 ANALYSIS OF THE SECONDARY AND EXPLORATORY ENDPOINTS

LIS, PaO₂/FiO₂, oxygenation index (OI), dead space fraction (Vd/Vt), and SOFA will be analyzed using repeated measures analysis of covariance (ANCOVA) to assess the effects of treatment, time, and the interaction between time and treatment, while incorporating between-patient variation in baseline measurements and important covariates including severity of ARDS (SAS PROC MIXED). Interaction contrasts will include polynomial trends

across time, focusing on a linear trend suggesting improvement and a quadratic trend suggesting that improvement is leveling off. As part of our analysis, we will also include the interaction between treatment and ARDS severity in the model. Because the study is not powered for the interaction between treatment and severity of ARDS, this test will be exploratory. We will also perform post-hoc analyses within each severity group to generate hypotheses for future study. For analysis of secondary endpoints, we will compare biological outcomes between treatment arms using the Mann-Whitney U or t-test for numerical endpoints and either χ^2 or Fisher's exact test for categorical outcomes.

Based on our prior work^{3,169}, the SD of the log-IL-18 is approximately 0.7. With 16 subjects in the placebo arm and 16 in the iCO arm, we will have > 80% power ($\alpha = 0.05$) to detect a difference in IL-18 levels of 0.5 log which corresponds to a 1.65-fold difference in plasma IL-18.

Based on an interim analysis of Cohort 1 in our Phase I trial, we will also have >80% power to detect a difference in a number of LM and SPMs including PGD₂, PGE₂, PGF_{2 α} , TXB₂, RvD2, and Mar1. Briefly, for multivariate statistical analysis, partial least square discriminant analysis (PLS-DA) will be performed as we have done previously¹⁷⁰. As we are testing multiple secondary and exploratory endpoints, a conservative 1% α level (ie. $p < 0.01$) will be used to accept statistical significance.

Ventilator-free days (VFDs), hospital- and ICU-free days will each be analyzed separately using ANCOVA with treatment arm as the main effect, however we will only be powered to see large changes in these outcomes (**Table 5**). Mortality will be analyzed using Cox regression models for time-to-death with treatment arm as the main covariate, and with important covariates including age in the model. HR and 95% CI for CO relative to placebo will be reported, along with estimated proportion surviving at 28, 60, and 180 days.

We will assess correlations between biomarkers and clinical and lung physiology outcomes including LIS, SOFA, VFDs, as well as 28- and 60-day hospital mortality. We will use multivariable linear regression as the primary model with LIS as the primary outcome variable. Biomarker levels tend to exhibit right skewedness and large outliers, so data will likely require log transformation. Additional predictor variables (such as demographic variables and comorbidities) will be specified *a priori* and interactions between those variables and biomarkers will be included in the model with adjustment for age as an important biological variable.

To evaluate the predictive ability of biomarkers for binary outcomes, we will use two methods. We will use c-statistics obtained from a logistic regression model to evaluate the effect of biomarker measurements on increasing the area under the receiver operating characteristic (ROC) curves. In addition, we will test if including measured biomarkers to models including important covariates results in an improvement in the net reclassification index as we have done previously for mtDNA³.

For secondary and exploratory endpoints, subjects may also be analyzed according to number of study drug doses completed.

11 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

11.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

11.1.1 INFORMED CONSENT PROCESS

11.1.1.1 CONSENT/ASSENT AND OTHER INFORMATIONAL DOCUMENTS PROVIDED TO PARTICIPANTS

Federal regulations 45 CFR 46.111(a)(5) require that informed consent will be sought from each prospective subject or the subject's legally authorized representative (LAR). The investigator is responsible for ensuring that the patient understands the risks and benefits of participating in the study and answering any questions the patient may have throughout the study and sharing any new information in a timely manner that may be relevant to the patient's willingness to continue his or her participation in the trial. All study participants or their surrogates will be informed of the objectives of the study and the potential risks. The informed consent document will be used to explain the risks and benefits of study participation to the patient in simple terms before the patient is entered into the study, and to document that the patient is satisfied with his or her understanding of the risks and benefits of participating in the study and desires to participate in the study. The investigator is responsible for ensuring that informed consent is given by each patient or legal representative. This includes obtaining the appropriate signatures and dates on the informed consent document prior to the performance of any protocol procedures and prior to the administration of study agent.

11.1.1.2 CONSENT PROCEDURES AND DOCUMENTATION

A licensed physician investigator not involved in the medical care of the patient will obtain written informed consent after a detailed review of the consent form with the patient, or more likely, the LAR of the potential study participant. The majority of the patients approached for participation in this study will have limitations of decision-making abilities due to their critical illness or need for sedating medications. Hence, most patients will not be able to provide informed consent. Accordingly, informed consent will be sought from the potential subject's LAR.

The physician investigator will be responsible for ensuring that the subject or LAR understands the risks and benefits of participating in the study and answering any questions the subject or LAR may have throughout the study and sharing in a timely manner any new information that may be relevant to the subject's willingness to continue his or her participation in the trial. All study participants or their surrogates will be informed of the objectives of the study and the potential risks. The informed consent document will be used to explain the risks and benefits of study participation to the patient or LAR in simple terms before the patient is entered into the study, and to document that the patient or LAR is satisfied with his or her understanding of the risks and benefits of participating in the study and desires to participate in the study. The investigator is responsible for ensuring that informed consent is given by each patient or legal representative. This includes obtaining the appropriate signatures and dates on the informed consent document prior to the performance of any protocol procedures and prior to the administration of the study agent.

The subject or LAR will be given ample opportunity to read the consent form and ask questions during the initial consent visit and afterwards prior to the signing of the consent form. The physician investigator obtaining informed consent will explain to the subject or LAR that questions can be addressed at any point throughout the trial and the PI's contact information will be made available during the consent visit for any questions that arise prior to or throughout the study. The subject or LAR will be given a signed copy of the informed consent form, which will include the site PI contact information and the subject or LAR will be encouraged to call with any questions.

The consent process will take place with the subject or LAR in person in a private room within the ICU or in close proximity to the ICU. Privacy and ample time for questions and decision-making will be provided prior to written consent by the subject or LAR. The subject or LAR will be allowed to discuss the study with anyone they choose before making a decision.

For subjects for whom consent was initially obtained from a surrogate, but who subsequently regain decision-making capacity while in hospital, we will obtain formal consent for continuing participation, inclusive of

continuance of data acquisition. The initial consent form signed by the surrogate will reflect that such continuing consent will be obtained when possible.

The following categories of surrogates (listed in general order of preference) may provide written informed consent on behalf of potential subjects incapable of providing informed consent themselves, however the exact order will be dependent on the local IRB at each site.

- court appointed guardian with specific authority to consent to participation in research or authority to make health care decisions for a class of diagnostic and therapeutic decisions inclusive of the proposed research;
- health care proxy/person with durable power of attorney with specific authority for making health care decisions inclusive of the proposed research; or
- spouse, adult child, or other close family member who knows the subject well and has been involved in their care.

Subjects and/or surrogates will be given a written copy of the consent form, as well as ample time to read and understand the written material. The surrogate will be asked to consider the potential subject's own views prior to providing surrogate consent. An investigator will be available to answer any additional questions. Subjects and/or their surrogates can withdraw consent at any time. If the attending ICU physician is one of the study investigators, this individual would not be permitted to obtain consent from a potential subject; one of the other study team physician members would obtain consent in this instance.

If consent from a LAR or surrogate cannot be obtained in person on behalf of a subject with impaired decision-making capacity, a licensed physician investigator may call the subject's LAR to perform consent by phone using an IRB-approved telephone script. Consent obtained by telephone must comply with all regulatory requirements about the process, the consent elements, and documentation of consent.

Due to infection control measures and hospital visitor policy restrictions during the COVID-19 pandemic, physician investigators will obtain and document wet consent from potential subjects or their LARs following each site's IRB policy and FDA guidance on the conduct of clinical trials during the COVID-19 pandemic. If unable to obtain wet consent from the subject's LAR in person, the physician investigator will obtain consent remotely via telephone call in accordance with each site's IRB policy and FDA guidance on the conduct of clinical trials during the COVID-19 pandemic. The consent form will be provided to the LAR via secure email or other electronic means including REDCap, Adobe E-Sign, or using the COVID MyStudies application and asked to electronically sign the document, scan or take a picture of the consent signature page, and then return electronically to the research team, in accordance with each site's institutional policy.

The physician investigator will electronically sign or print and sign their own copy of the paper consent form and document the consent process in a note to file or informed consent process checklist. Documentation will include that the consent form was provided to the LAR, consent was obtained, the method used to obtain consent, that the LAR signed a separate copy of the consent form, and the date/time of consent. A compiled copy of the consent including the document with the physician investigator's signature and the LAR's signature consent document will be retained in the study record and a copy of the compiled consent form will be uploaded to subject's medical record. Consent obtained electronically must comply with all regulatory requirements about the process, the consent elements, and documentation of consent.

11.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping

Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, investigators, USAMRMC ORP HRPO, the Investigational New Drug (IND) sponsor and regulatory authorities. If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants, the IRB, and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

If the trial is suspended, all enrollments and study product administration will cease, but other study procedures will continue. The SRC and DSMB will convene for a conference call within seven days of the suspension to determine whether the suspension can be lifted or permanent discontinuation of study product administration is appropriate. Lifting the suspension requires the unanimous approval of all members of the SRC and DSMB.

11.1.3 CONFIDENTIALITY AND PRIVACY

All subjects or surrogates must provide written informed consent and signed HIPAA authorization prior to the performance of any screening or main study procedures. Federal regulations at 45 CFR 46 111 (a) (7) requires that when appropriate, there are adequate provisions to protect the privacy of subjects and to maintain the confidentiality of data. Subject confidentiality will be protected throughout the study and no subject-identifying information will be released to anyone outside the study. Confidentiality will be secured through several mechanisms. To maintain confidentiality, all laboratory specimens, evaluation forms, and reports will be identified only by a coded number. The coded number will be generated at random by a computer, and only the study investigators will have access to the codes. All records will be kept in a locked, password protected computer. All computer entry and networking programs will be done with coded numbers only. Any study forms and paper records containing personal identifier information (e.g., address, phone number) will be kept secured and locked at each clinical center. No personal identifiers will be placed on biological samples and other documents forwarded to central labs. All paper case report forms will be maintained in a locked cabinet inside a locked office. No personal identifiers, such as name, address, or social security number will be entered into the study database. Any subject-specific data reported to any study committees will only be identified by subject ID number. Access to all subject data and information at the clinical centers, including biological samples, will be restricted to authorized personnel. Finally, subjects will not be identified by name in any reports or publications, nor will the data be presented in such a way that the identity of individual subjects can be inferred. Analysis files created for further study by the scientific community will have no subject identifiers. Clinical information will not be released without the written permission of the patient, except as necessary for monitoring by the Department of Defense, the Federal Drug Administration or other authorized Federal Agencies, local IRBs, and the DCC.

11.1.4 FUTURE USE OF STORED SPECIMENS AND DATA

With the participant's approval and as approved by local IRBs, coded biological samples will be stored at a central repository located at BWH, along with coded study data, for use by other researchers including those outside of the study. These samples could be used for research related to carbon monoxide, ARDS, trauma, critical illness or other lung diseases. The central repository at BWH will also be provided with a code that will allow linking the biological specimens with the data from each participant, maintaining the blinding of the

identity of the participant. Only the originating clinical trial site will be able to link coded samples or data to specific individuals.

Samples may also be stored in the central repository at BWH for future genetic research related to carbon monoxide, ARDS, critical illness or other lung diseases. Specific consent for future genetic research will be required.

During the conduct of the study, an individual participant can choose to withdraw consent to have biological specimens stored for future research. However, withdrawal of consent with regards to biosample storage may not be possible after the study is completed.

11.1.5 KEY ROLES AND STUDY GOVERNANCE

Principal Investigator	Independent Research Monitor
Rebecca M. Baron, MD	Souheil El-Chemaly, MD
Brigham and Women's Hospital	Brigham and Women's Hospital
75 Francis Street, Boston, MA	75 Francis Street, Boston, MA
Tel: 617-525-6642	Tel: 617-732-7420
Email: rbaron@bwh.harvard.edu	Email: sel-chemaly@partners.org

Medical Monitor
B. Taylor Thompson, MD
Massachusetts General Hospital
55 Fruit Street, Boston, MA
Tel: 617-643-2411
E-mail: tthompson1@mgh.harvard.edu

Scientific Review Committee (SRC)

The Scientific Review Committee (SRC) will consist of the site PIs from all the enrolling clinical trial sites, as well as the IND Sponsor (Dr. Perrella) and the overall Project PI (Dr. Choi).

Overall Study Principal Investigator

The Study Principal Investigator has ultimate responsibility for the conduct of the study, and for monitoring its safety and progress, at all participating sites.

Data Coordinating Center (DCC) Personnel

The DCC will serve as the overall coordinating center for all study sites and will be located at Brigham and Women's Hospital. In collaboration with the Study PI, the DCC is also responsible for the coordination, development, submission, and approval of the protocol, and its subsequent amendments. The DCC will also ensure that all participating sites are using the correct version of the protocol. As part of the DCC, Dr. Thompson will serve as the Medical Monitor and will be responsible for safety reporting to the IND Sponsor, the Study PI, DSMB, DoD, and FDA. The DCC will assist the IND Sponsor in preparing reports for the FDA, including annual reports, protocol amendments, and safety reports, as well as reports to the DSMB and SRC.

11.1.6 SAFETY OVERSIGHT

Data and Safety Monitoring Board (DSMB)

The principal role of the DSMB is to regularly monitor data from this trial, review and assess the performance of its operations, and make recommendations, as appropriate, to the Study PI, IND Sponsor, and Project PI with respect to:

- Benefit/risk ratio of procedures and participant burden
- Selection, recruitment, and retention of participants
- Adherence to protocol requirements
- Completeness, quality, and analysis of measurements
- Amendments to the study protocol and consent forms
- Performance of individual centers and core labs
- Participant safety
- Notification of and referral for adverse events

The DSMB will consist of four members with expertise in acute lung injury, biostatistics, ethics, and clinical trials. Ad hoc members will be appointed with particular expertise where necessary. Appointment of all members is contingent upon the absence of any conflicts of interest. All the members of the DSMB are voting members. The DSMB will review data prepared by the DCC on a quarterly basis. The Study PI and the DCC physician members will be responsible for the preparation of DSMB and adverse event reports. The DSMB meetings will be scheduled every six months during the study and the DSMB will review the protocol during its first meeting. Conference calls may be held in place of face-to-face meetings.

Recommendations to end, modify, or continue the trial will be prepared by the DSMB executive secretary for review by the Study PI and IND Sponsor no more than two working days after a DSMB meeting. Recommendations for major changes, such as stopping, will be communicated immediately, and followed by a written summary. The Study PI and IND Sponsor will act on recommendations expeditiously in consultations with the SRC. The executive secretary of the DSMB will be responsible for preparing the minutes for each meeting or conference call.

Scientific Review Committee

The SRC will meet and independently review the safety data every six months or after every tenth enrollment (whichever interval is shorter) and make recommendations to the DSMB. The SRC review will be blinded to treatment allocation with respect to participants not at an Investigator's specific site.

Independent Research Monitor

Our Independent Research Monitor, Dr. El-Chemaly, will independently evaluate all safety and monitoring data from the trial on a quarterly basis and report his findings to the IND Sponsor, the Study PI, USAMRMC HRPO, as well as the site PIs. Research monitor functions will include:

- may discuss the research protocol with the investigators, interview human subjects, and consult with others outside of the study about the research;
- shall have authority to stop the research in progress, remove individual human subjects from the research protocol, and take whatever steps are necessary to protect the safety and well-being of human subjects until the IRB can assess the Monitor's report;
- shall have the responsibility to promptly report their observations and findings to the IRB or other designated official and the HRPO.

Summary Guidelines for SRC and DSMB Assessment

The DSMB will review reports every three months prepared by the DCC and meet approximately every six months. The DSMB will be provided with summary statistics of baseline and on-study vital signs and laboratory values as well as tabulations of all the study endpoints. **The SRC and DSMB may halt enrollment in the study at any time during the trial.**

11.1.7 CLINICAL MONITORING

Clinical site monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with International Conference on Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s).

External site monitoring visits will be performed remotely or in-person every 6 months by the DCC, to ensure that all regulatory requirements are met and to monitor the quality of the data collected. Records of IRB approvals and subjects' charts will be examined on a spot check basis by study monitors under the supervision of our Independent Research Monitor, Dr. El-Chemaly, to evaluate the accuracy of the data entered into the database. Dr. El-Chemaly will supervise the site monitoring plan and will review the reports generated from each monitoring visit. He will share the reports with each site PI, the IND Sponsor, the Study PI, the site's IRB (if necessary), as well as with HRPO, along with suggestive corrective actions and a timeline for implementation of the recommendations. Dr. El-Chemaly can recommend findings be reported to site IRBs and the DSMB and will suggest revisions to the protocol or standard operating procedures (SOPs) if needed to address safety or procedural findings. Compliance oversight will also be provided by HRPO site visits.

Each clinical trial site will provide direct access to all source data/documents, and reports for the purpose of monitoring and auditing by the DCC on behalf of the IND Sponsor, and inspection by local and regulatory authorities.

11.1.8 QUALITY ASSURANCE AND QUALITY CONTROL

Each clinical site will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. Each site will operate under a comprehensive Quality Management Plan, which incorporates internal Quality Control (QC) activities prior to data entry and regular internal Quality Assurance (QA) review of subjects' charts.

QC procedures will be implemented beginning with the data entry system and data QC checks that will be run on the database will be generated. Any missing data or data anomalies will be communicated to the site(s) for clarification/resolution.

11.1.9 DATA HANDLING AND RECORD KEEPING

11.1.9.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site investigator. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

Source documents will contain no personal identifiers and will be identified with the study subject Reference ID and a Project ID numbers. All source documents will be maintained at the enrollment site in a locked cabinet

inside a locked office. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Study visit worksheets will be provided for use as source document worksheets for recording data for each participant enrolled in the study. Data should be entered in StudyTRAX as directed on the source document worksheets.

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into StudyTRAX, a 21 CFR Part 11-compliant data capture system provided by the DCC. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

The StudyTRAX database has the capacity for data entry and transfer from a wide variety of existing databases, tracking data manipulation and export into standard statistical packages, as well as advanced dataset analysis. Data will be entered and managed using quality control (QC) checks, including random data checks, use of at least two separate identifiers for each DCF or biospecimen, and manual and computer program checks for missing, incorrect, out-of-range data or logical inconsistencies. The DCC will provide each site with a monthly QC report to verify or correct any potential data discrepancies. The DCC will coordinate regular data locks in order to prepare reports for the DSMB, local IRBs, DoD, and the FDA. In addition, regular on-site monitoring will ensure that local source documentation matches the data in the StudyTRAX database.

11.1.9.2 STUDY RECORDS RETENTION

The data will be stored for at least three years following FDA approval of inhaled carbon monoxide as a treatment for adults with ARDS or for five years after study completion, whichever is longer. No records will be destroyed without the written consent of the IND Sponsor. It is the responsibility of the IND Sponsor to inform the site investigators when these documents no longer need to be retained.

11.1.10 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the clinical trial protocol, International Conference on Harmonisation Good Clinical Practice (ICH GCP) requirements. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

It is the responsibility of the site investigator to use continuous vigilance to identify and report deviations in a timely manner. All deviations must be addressed in study source documents and will be reported to the DCC. Major protocol deviations, such as enrollment violations or any deviation that involves risks to participants or others, will be reported to the DCC within seven days of awareness. Minor deviations will be reported to the DCC and the Independent Research Monitor as part of the site monitoring visits.

Protocol deviations must be sent to the reviewing IRB per their policies. The site investigator is responsible for knowing and adhering to the reviewing IRB requirements.

11.1.11 PUBLICATION AND DATA SHARING POLICY

Results from this study will be reported according to guidelines established by the National Institutes of Health (NIH) Public Access Policy, which ensures that the public has access to the published results of NIH funded

research. The final peer-reviewed journal manuscripts will be submitted to the digital archive [PubMed Central](#) upon acceptance for publication.

This study will comply with DoD policy for making data and research resources publicly available. The investigators will make all the information, data, and research resources generated from this DoD-funded Clinical Trial Award available to the research community, including both scientific and consumer advocacy communities, as well as to the public at large. All metabololipidomics and biomarker data generated in this DoD Award will be shared with the scientific community following the appropriate guidelines for data deposition. The investigators and their respective institutions acknowledge the importance of making model organisms and research resources available to the broader scientific community. The investigators will comply with the CDMRP Policy on Sharing Data and Research Resources. The investigators will comply with the policies and guidelines established by the NIH with respect to sharing research results and model organisms, including the NIH Policy on Sharing of Model Organisms for Biomedical Research (2004), the NIH Grants Policy Statement (2003), and the Principles and Guidelines for Recipients of NIH Research Grants and Contracts on Obtaining and Disseminating Biomedical Research Resources (1999). In accordance with these policies, the investigators intend to release and share final research data and materials from DoD-supported studies for use by other researchers in a timely manner. The investigators will register the proposed Phase II clinical trial on the registry and results database [ClinicalTrials.gov](#).

11.1.12 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by any outside party, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial. All persons involved in study are expected to take the appropriate actions to ensure that they are in compliance with the financial disclosure requirements of their respective institutions.

Protocol Amendment History

Version	Date	Description of Change	Brief Rationale
2.0	10/21/18	Clarification of eligibility and study drug hold criteria. Potential benefits to study participants elaborated. Clarification of study sample collection, pre-treatment assessment, and vital signs monitoring during treatment.	Patients with anoxic/hypoxic brain injury and on ECMO may be at increased risk and are thus excluded from participation/treatment. Protocolized low tidal volume ventilation will benefit all study participants. Additional details included on pre-treatment assessment, monitoring, and sample collection.
3.0	02/21/19	Clarification of how PaO ₂ /FiO ₂ will be estimated when a venous blood gas is used, placebo tank CGA valves, randomization procedures and subjects' identification within StudyTRAX, AE adjudication and time period for AE monitoring and that DSMB has 4 members.	When an arterial blood gas is not available, the Rice method will be used to calculate PaO ₂ /FiO ₂ . Praxair was required to change CGA valves for placebo tanks. Randomization and subject's identification has been set up through the StudyTRAX database. AEs will be adjudicated by blinded study physicians at each site. A fourth member was added to DSMB
4.0	06/28/19	Edits to Time-Events schedule based on protocol content. Addition of urine sample collection. Clarification of language regarding study staff and administration of the study drug. Clarification of screening log for subjects that may potentially meet inclusion criteria and are not included in the database. Blood collection amount for primary cell isolation was changed from 10 mL to 8 mL. Clarification of concomitant medications list. Clarification of scoring of the cardiovascular component of the SOFA score when on multiple vasopressors and when on vasopressin.	Time-Events schedule was not consistent with protocol. Urine will be collected for measurement of mtDNA and other biomarkers. A screening log will be kept for subjects who may potentially meet inclusion criteria. CPT tubes allow a maximum volume of 8 mL. It is not necessary to collect information on statins, nitroprusside, methylene blue, nitrates. Clarification that if a subject is on two concomitant vasopressors (or more) will be scored as 4 points and any patient on vasopressin (at any dose) will be scored as 3 points.
5.0	02/15/20	External site monitoring visits will be performed remotely or in-person every 6 months. Enrollment time	Due to enrollment goals, semi-annual monitoring provides adequate oversight of trial

		window modified from 120 hours to 168 hours. Time period for reference measurements modified from 04:00-10:00 to 03:00-10:00.	activities. A longer enrollment time window may enhance enrollment without raising any additional safety concerns. Time period for reference measurements modified to align with time closest to study drug administration and standard of care testing.
6.0	06/21/2020	Expansion of electronic options to obtain remote consent from LARs including REDCap, Adobe E-Sign, or using the COVID MyStudies application. Addition of New York-Presbyterian Brooklyn Methodist Hospital and Durham Regional Hospital as study sites.	Due to infection control measures and hospital visitor policy restrictions during the COVID-19 pandemic, it may not be possible to obtain wet consent from a subject's LAR and therefore, it may be necessary to obtain electronic consent from a subject's LAR. To enhance enrollment, two additional sites have been added.
7.0	2/12/2021	Revision of exclusion criteria #11, #12, and #18. Revision of hypoxia and anemia study drug daily hold parameters.	Revision of hypoxemia, anemia and stroke exclusion criteria may enhance participant enrollment without raising any additional safety concerns.
7.0	2/12/2021	Decrease duration of study drug treatment from 5 days to 3 days.	This will enhance feasibility of the protocol. In phase I, no subjects received 5 days of treatment (median 3.5 days) and circulating mtDNA levels were reduced after 2 doses. In baboons with pneumococcal pneumonia, one iCO treatment attenuated ALI on Day 8.
7.0	2/12/2021	Samples will not be collected on Study Days 4 and 7.	Due to the decreased days of treatment from 5 days to 3 days, blood will no longer be drawn from study participants on days 4 and 7. This will enhance feasibility of the study and decrease blood volume from 136.5 mL to 88.5 mL.
7.0	2/12/2021	Infection control guidelines included for treatment of enrolled subjects with ARDS secondary to COVID-19.	COVID-19 is a common etiology of hypoxic respiratory failure and ARDS. Each site will follow local hospital infection

			guidelines regarding treatment of COVID-19 mechanically ventilated patient with inhaled therapies as well as hospital infection control guidelines regarding blood sample collection/handling.
8.0	10/28/2021	The primary efficacy endpoint was revised to compare the effect of low dose iCO therapy on mitochondrial DNA (mtDNA) levels in patients with ARDS. Effects on Lung injury score (LIS), PaO ₂ /FiO ₂ ratio, Oxygenation Index (OI), and SOFA score were made secondary endpoints. The sample size was changed from 100 to 32 participants. The statistics sections were revised to describe the revised study endpoints.	The Phase I trial of CO in ARDS found significant differences in mtDNA levels. Given the recruitment challenges, the study was repowered based on mtDNA as the primary endpoint. The study endpoints were revised and thus the statistics sections were revised accordingly.
9.0	4/19/2022	The PI was changed from Dr. Laura Fredenburgh to Dr. Rebecca Baron. Dr. Baron was removed as Medical Monitor. The language regarding stratification by site was revised.	Dr. Rebecca Baron is the new PI of the study and will no longer serve as Medical Monitor. Dr. Taylor Thompson will serve as the Medical Monitor. An error was noted in the protocol. The randomization is not stratified by site or any other factor.

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