CLINICAL STUDY PROTOCOL

Assessing Variability of the Ventilatory Response to Duffin's Rebreathing Procedure

PROTOCOL NO. SCR-013 Lead-in

Sponsor:	U.S. Food and Drug Administration White Oak Building #64, Room 2072 10903 New Hampshire Avenue Silver Spring, MD 20993
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Version of Protocol:	1.0
Date of Protocol:	27th October, 2021

CONFIDENTIAL

SPONSOR SIGNATURE PAGE

This study will be conducted with the highest respect for the individual participants in accordance with the requirements of this clinical study protocol and also in accordance with the following:

- The ethical principles that have their origin in the Declaration of Helsinki;
- International Council for Harmonisation (ICH) harmonised tripartite guideline E6 (R2): Good Clinical Practice; and
- All applicable laws and regulations, including without limitation, data privacy laws and compliance with appropriate regulations, including human subject research requirements set forth by the Institutional Review Board (IRB).

DocuSigned by:
David Strauss
Signer Name: David Strauss
Signing Reason: I approve this document
Signing Time: 10/28/2021 | 8:02:03 AM PDT
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10/28/2021

David Strauss, MD, PhD
Director, Division of Applied
Regulatory Science
U.S. Food and Drug Administration

Date

INVESTIGATOR SIGNATURE PAGE

I confirm that I have read and that I understand this protocol, the investigator brochure, and other product information provided by the sponsor. I agree to conduct this study in accordance with the requirements of this protocol and also protect the rights, safety, privacy, and well-being of study subjects in accordance with the following:

- The ethical principles that have their origin in the Declaration of Helsinki;
- ICH harmonised tripartite guideline E6 (R2): Good Clinical Practice;
- All applicable laws and regulations, including without limitation data privacy laws and regulations;
- Human subject research requirements set forth by the IRB;
- Regulatory requirements for reporting of serious adverse events (SAEs) defined in Section 4.7.3.1 of this protocol; and
- Terms outlined in the Clinical Study Site Agreement.

I further authorize that my personal information may be processed and transferred in accordance with the uses contemplated in Section 6 of this protocol.

DocuSigned by:
Jan Matousek
Signer Name: Jan Matousek
Signing Reason: I have reviewed this document
Signing Time: 10/28/2021 | 8:09:51 AM PDT
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10/28/2021

Jan Matousek, D.O.
Principal Investigator

Date

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AE	adverse event
CFR	Code of Federal Regulations
COVID-19	coronavirus disease of 2019
ECG	electrocardiogram
eCRF	electronic case report form
FDA	Food and Drug Administration
GCP	Good Clinical Practice
HBsAg	hepatitis B surface antigen
HepC	hepatitis C
HIPAA	Health Insurance Portability and Accountability Act
HIV	human immunodeficiency virus
ICH	International Council for Harmonisation
IRB	Institutional Review Board
IV	intravenous
MedDRA	Medical Dictionary for Regulatory Activities
NSAID	nonsteroidal anti-inflammatory drug
OTC	over-the-counter
QA	quality assurance
Rebreathing procedure	Refers to the method consisting of three stages: relaxation, hyperventilation, and rebreathing assessment
Rebreathing assessment	Refers to a single assessment at an isoxic end tidal PO_2 level after hyperventilation
SAE	serious adverse event
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	standard deviation

PROTOCOL SYNOPSIS

Protocol Number:	SCR-013 (Lead-in)
Title:	Assessing Variability of the Ventilatory Response to Duffin's Rebreathing Procedure
Investigators:	Principal Investigator: Jan Matousek Study Physician: Jan Matousek
Study Phase:	Phase 1
Study Period:	The duration of study participation will be approximately 3 days (excluding the screening period).
Study Site:	Spaulding Clinical Research Unit, West Bend, Wisconsin
Background and Motivation:	In order to study the effects of opioids alone or in combination with other sedative psychotropic drugs on ventilation, we previously implemented the Read rebreathing procedure.[1] With Read rebreathing, a study participant rebreathes through a circuit with a bag that initially contains 93% oxygen (O_2) and 7% carbon dioxide (CO_2). The 7% CO_2 results in relatively rapid equilibration between arterial partial pressure of carbon dioxide (PCO_2), end tidal PCO_2, and mixed venous PCO_2. As the study participant continues to breathe, CO_2 accumulates and triggers an increase in ventilation referred to as the "hypercapnic ventilatory response." Drugs can decrease the hypercapnic ventilatory response at drug concentrations that may have minimal-to-no detectable effect on ventilation when breathing room air.[2-3] However, this method is limited in that all measurements are performed under hyperoxic conditions, which can have independent effects on ventilation, and the data cannot necessarily be used to extrapolate to the dynamic changes in PO_2 and PCO_2 that occur during real-world drug-induced respiratory depression when a patient is breathing room air. Duffin modified the Read rebreathing procedure to include 1) hyperventilation (primarily through deep breathing) prior to rebreathing to achieve an end tidal PCO_2 or approximately 20-25 mm Hg prior to rebreathing,[4] 2) maintaining an isoxic end tidal PO_2 during rebreathing,[5] and 3) repeating rebreathing at 2 different isoxic end tidal PO_2 levels (i.e., at 50 mm Hg and 150 mm Hg).[6] These modifications allow for critical physiological measurements and thresholds to be captured that can then be used to model the effects of drugs when there are dynamic changes in PO_2 and PCO_2, such as during a real-world opioid overdose. The Duffin rebreathing procedure has been used for multiple decades by different investigators studying different populations with and without drugs that can affect ventilation.[1-14]

	Baseline minute ventilation is captured when end tidal PCO_2 is less than the ventilatory recruitment threshold. The *ventilatory recruitment threshold* is the end tidal PCO_2 above which minute ventilation starts to increase linearly with further increases in end tidal PCO_2. The *slope of the PCO_2-ventilatory response curve* reflects the increase in minute ventilation relative to the increase in end tidal PCO_2. Changing the PO_2 can affect each of these variables, with hypoxia increasing baseline minute ventilation, decreasing the ventilatory recruitment threshold, and increasing the slope of the PCO_2-ventilatory response curve, while hyperoxia has the opposite effects. The reproducibility of Duffin rebreathing has been assessed previously by Mahamed and Duffin (2001)[7] performing hyperoxic and hypoxic rebreathing procedures measured once daily for 14 consecutive days and then by Jensen et al. (2010)[8] performing 4 pairs of hyperoxic and hypoxic rebreathing procedures in 1 day followed by 1 pair on 4 additional days separated by weeks to more than a month. The present study will combine Duffin's rebreathing procedure with some additional study procedures, such as quantitative pupillometry, that are planned for subsequent clinical pharmacology studies in order to confirm feasibility and gather reproducibility data.
Objectives and Endpoints:	**Objective:** The objective of this study is to evaluate variability of the ventilatory response to Duffin's rebreathing procedure. The endpoints of this study are below and will be determined for each rebreathing procedure: **Primary Endpoints** 1. *Baseline minute ventilation* when end tidal PCO_2 is less than the ventilatory recruitment threshold (represents non-chemoreflex drives to breathe) 2. *Ventilatory recruitment threshold* (end tidal PCO_2 above which minute ventilation starts to increase linearly with further increases in end tidal PCO_2) 3. *Slope of the PCO_2-ventilatory response curve* that reflects the increase in minute ventilation relative to the increase in end tidal PCO_2 (represents chemoreflex sensitivity) 4. *Extrapolated ventilatory recruitment threshold* (intersection with X axis) **Exploratory Endpoints** 1. Additional physiological assessments as specified in the protocol 2. Between occasion (within a day) and between day variability for

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Healthy subjects will be enrolled in this study. Subjects will report to the study site for screening from Days -28 to -2 and then will return to the site on Day -1 for baseline assessments and check-in. After check-in (Day -1), subjects will remain in study site for PD assessments on Day 1 and check out on Day 2.

Paired rebreathing procedures (i.e., at two different isoxic end tidal PO_2 levels) will be performed on Day 1 at 0, 2, 4, and 6 hours. An additional pair of rebreathing procedures will be performed on Day 2 before checkout. Subjects will not be administered any drugs in this lead-in study.

A summary of all procedures is described in the Schedule of Events (Appendix A). Subjects will be discharged from the study after completion of all study procedures. If a subject discontinues from the study prematurely, all procedures scheduled for the end of the study will be performed.</td></tr>
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<tr><td>Inclusion Criteria:</td><td>Subjects who meet all the following inclusion criteria will be eligible to participate in the study:
1. Subject signs an IRB-approved written informed consent and privacy language as per national regulations (e.g., Health Insurance Portability and Accountability Act authorization) before any study related procedures are performed.
2. Subject is a healthy, non-smoking man or woman, 18 to 50 years of age, inclusive, who has a body mass index of 18.5 to 32 kg/m^2, inclusive, at Screening.</td></tr>
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	3. Subject has normal medical history findings, clinical laboratory results, vital sign measurements, 12 lead ECG results, and physical examination findings at screening or, if abnormal, the abnormality is not considered clinically significant (as determined and documented by the investigator or designee). 4. Subject must have a negative test result for alcohol and drugs of abuse at screening and Check-in (Day -1). 5. Subject must test negative for severe acute respiratory syndrome coronavirus (SARS-CoV-2) by a molecular diagnostic test at check-in (Day -1). If a subject's test comes back inconclusive, it can be repeated at Check-in. 6. Female subjects must be of non-childbearing potential or, if they are of childbearing potential, they must: 1) have been strictly abstinent for 1 month before Check in (Day -1) and agree to remain strictly abstinent for the duration of the study and for at least 1-month after the last rebreathing procedure; OR 2) be practicing 2 highly effective methods of birth control (as determined by the investigator or designee; one of the methods must be a barrier technique) from at least 1 month before Check in (Day -1) until at least 1 month after the end of the study. 7. Male subjects must agree to practice 2 highly effective methods of birth control (as determined by the investigator or designee) from at least 1 month before Check In (Day -1) until at least 1 month after the last rebreathing procedure. 8. Subject is highly likely (as determined by the investigator) to comply with the protocol defined procedures and to complete the study.
Exclusion Criteria:	Subjects who meet any of the following exclusion criteria will not be eligible to participate in the study: 1. Subject has non-reactive or misshapen pupil(s) or damaged orbit structure or surrounding soft tissue is edematous or has an open lesion. 2. Subject Duffin rebreathing data is of poor quality or subject does not agree to remain clean-shaven for all days when the Duffin rebreathing procedure is being performed. 3. Subject has used any prescription or nonprescription drugs (including aspirin or NSAIDs and excluding oral contraceptives and acetaminophen) within 14 days or 5 half-lives (whichever is

	longer) or complementary and alternative medicines within 28 days before check-in. 4. Subject has used nicotine-containing products (e.g., cigarettes, cigars, chewing tobacco, snuff) within 6 weeks of Screening 5. Subject has a history or evidence of a clinically significant disorder, condition, or disease (e.g., cancer, human immunodeficiency virus [HIV], hepatic or renal impairment) that, in the opinion of the investigator would pose a risk to subject safety or interfere with the study evaluation, procedures, or completion. This includes subjects with any underlying medical conditions that the Investigator believes would put subjects at increased risk of severe illness from COVID-19 based on the Centers for Disease Control and Prevention (CDC) guidelines. 6. Subject has any signs or symptoms that are consistent with COVID-19. These include subjects with fever or chills, cough, shortness of breath or difficulty breathing, fatigue, muscle or body aches, headache, new loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, or diarrhea may have COVID-19. In addition, the subject has any other findings suggestive of COVID-19 risk in the opinion of the investigator. 7. Subject tests positive for SARS-CoV-2 by a molecular diagnostic test performed prior to admission. 8. Subject has clinical laboratory test results (hematology, serum chemistry and urinalysis) at Screening or Check-In that are outside the reference ranges provided by the clinical laboratory and considered clinically significant by the investigator. 9. Subject has a positive test result at Screening for HIV 1 or 2 antibody, hepatitis C virus antibodies, or hepatitis B surface antigen. 10. Female subject is pregnant or lactating before enrollment in the study. 11. Subject has a history of or currently has hypoventilation syndrome, sleep apnea, or chronic obstructive pulmonary disease (COPD) and is on non-invasive ventilation. 12. Subject has a history of asthma that has required medication within the last five years. 13. Subject has a history of sleep disorders, Panic Disorder, Panic Attacks, Generalized Anxiety Disorder, or any associated DSM diagnosis or condition.

Assessments:	The following physiologic measurements will be assessed multiple times throughout the study: • Minute ventilation • Tidal volume • Respiratory rate • End-tidal PCO_2 • End-tidal O_2 • Oxygen saturation (SpO_2) • Heart rate • Maximum pupil diameter before constriction
Safety Assessments:	Safety will be evaluated in terms of adverse events (AEs), clinical laboratory results (hematology, serum chemistry, and urinalysis), vital sign measurements (blood pressure, heart rate, respiratory rate, pulse oximetry and oral body temperature), 12-lead ECG, and physical examination findings.
Sample Size and Threshold Determination:	Up to 10 healthy volunteer participants are planned for enrollment in this study. The sample size was selected empirically to acquire experience for the research staff with use of the rebreathing equipment and to assess performance over repeated assessments in a participant over multiple days.
Statistical Methods:	All data will be presented in data listings. Data from subjects excluded from an analysis population will be presented in the data listings but not included in the calculation of summary statistics. The number of subjects who enroll in the study and the number and percentage of subjects who complete each assessment will be presented. The frequency and percentage of subjects who withdraw or discontinue from the study and the reason for withdrawal or discontinuation will be summarized. Demographic and baseline characteristics will be summarized overall for all subjects. Descriptive statistics will be used to summarize demographic and baseline subject characteristics. For continuous variables, the mean, median, standard deviation (SD), minimum, and maximum values will be reported. For categorical (nominal) variables, the number and percentage of subjects (or observations) will be reported. **Primary Analysis** Baseline minute ventilation, ventilatory recruitment threshold, slope of the PCO_2-ventilatory response curve, and the extrapolated ventilatory recruitment threshold will be calculated for each subject at each

	rebreathing assessment. Mean, within- and between-day variability, between-subject variability of the ventilatory response to modified Duffin's rebreathing will be examined using mixed-effects approaches. Coefficients of variation (CV) will be calculated using mean within- and between-day values of each end point. Variability will be assessed separately for each isoxic gas condition (i.e. end tidal PO_2 of 50 mmHg and 150 mmHg). Full details will be described in a separate Statistical Analysis Plan. **Exploratory Analysis** Additional physiological assessments (e.g. ventilation, respiratory rate, heart rate, maximum pupil diameter before constriction) as specified in the protocol will be reported with standard descriptive statistics. **Safety** The safety population will include all subjects who undergo rebreathing procedures starting on Day 1. All AEs will be coded using the Medical Dictionary for Regulatory Activities (MedDRA). The incidence of AEs, organized by system organ class and preferred term, will be summarized with a focus on treatment-emergent AEs. Vital sign measurements will be summarized using descriptive statistics by time point. All values will be evaluated for clinically notable results. Data for additional safety parameters (e.g., physical examination findings) will be listed.
Date of Protocol:	27th October 2021

1. INTRODUCTION

In order to study the effects of opioids alone or in combination with other sedative psychotropic drugs on ventilation, we previously implemented the Read rebreathing procedure.[1] With Read rebreathing, a study participant rebreathes through a circuit with a bag that initially contains 93% oxygen (O_2) and 7% carbon dioxide (CO_2). The 7% CO_2 results in relatively rapid equilibration between arterial partial pressure of carbon dioxide (PCO_2), end tidal PCO_2, and mixed venous PCO_2. As the study participant continues to breathe, CO_2 accumulates and triggers an increase in ventilation referred to as the "hypercapnic ventilatory response." Drugs can decrease the hypercapnic ventilatory response at drug concentrations that may have minimal-to-no detectable effect on ventilation when breathing room air.[2-3] However, this method is limited in that all measurements are performed under hyperoxic conditions, which can have independent effects on ventilation, and the data cannot necessarily be used to extrapolate to the dynamic changes in PO_2 and PCO_2 that occur during real-world drug-induced respiratory depression when a patient is breathing room air.

Duffin modified the Read rebreathing method to include 1) hyperventilation (primarily through deep breathing) prior to rebreathing to achieve an end tidal PCO_2 or approximately 20-25 mm Hg prior to rebreathing,[4] 2) maintaining an isoxic end tidal PO_2 during rebreathing,[5] and 3) repeating rebreathing at 2 different isoxic end tidal PO_2 levels (i.e., at 50 mm Hg and 150 mm Hg).[6] These modifications allow for critical physiological measurements and thresholds to be captured that can then be used to model the effects of drugs when there are dynamic changes in PO_2 and PCO_2, such as during a real-world opioid overdose. The Duffin rebreathing procedure has been used for multiple decades by different investigators studying different populations with and without drugs that can affect ventilation.[1-14]

Baseline minute ventilation is captured when end tidal PCO_2 is less than the ventilatory recruitment threshold. The *ventilatory recruitment threshold* is the end tidal PCO_2 above which minute ventilation starts to increase linearly with further increases in end tidal PCO_2. The *slope of the PCO_2-ventilatory response curve* reflects the increase in minute ventilation relative to the increase in end tidal PCO_2. Changing the PO_2 can affect each of these variables, with hypoxia increasing baseline minute ventilation, decreasing the ventilatory recruitment threshold, and increasing the slope of the PCO_2-ventilatory response curve, while hyperoxia has the opposite effects.

The reproducibility of Duffin rebreathing has been assessed previously by Mahamed and Duffin (2001)[7] performing hyperoxic and hypoxic rebreathing procedures measured once daily for 14 consecutive days and then by Jensen et al. (2010)[8] performing 4 pairs of hyperoxic and hypoxic rebreathing procedures in 1 day followed by 1 pair on 4 additional

days separated by weeks to more than a month. The present study will combine Duffin's rebreathing procedure with some additional study procedures, such as quantitative pupillometry, that are planned for subsequent clinical pharmacology studies in order to confirm feasibility and gather reproducibility data.

2. STUDY OBJECTIVES

2.1 Primary Objective

The objective of this study is to evaluate variability of the ventilatory response to Duffin's rebreathing procedure.

3. STUDY ENDPOINTS

The endpoints of this study are below and will be determined for each rebreathing procedure:

3.1 Primary Endpoints

1. *Baseline minute ventilation* when end tidal PCO_2 is less than the ventilatory recruitment threshold (represents non-chemoreflex drives to breathe)
2. *Ventilatory recruitment threshold* (end tidal PCO_2 above which minute ventilation starts to increase linearly with further increases in end tidal PCO_2)
3. *Slope of the PCO_2-ventilatory response curve* that reflects the increase in minute ventilation relative to the increase in end tidal PCO_2 (represents chemoreflex sensitivity)
4. *Extrapolated ventilatory recruitment threshold* (intersection with X axis)

3.2 Exploratory Endpoints

1. Additional physiological assessments as specified in the protocol
2. Between occasion (within a day) and between day variability for physiological assessments as specified in the protocol

4. INVESTIGATIONAL PLAN

4.1 Study Design

This study is an unblinded lead-in reproducibility assessment to assess variability of the ventilatory response to Duffin's rebreathing procedure.

Table 4-1: Study Schedule

Day -1	Day 1	Day 2

Check-In	Lead-in PD Assessments	Check-Out

Healthy subjects will be enrolled in this study. Subjects will report to the study site for screening from Days -28 to -2 and then will return to the site on Day -1 for baseline assessments and check-in. After check-in (Day -1), subjects will remain in study site for PD assessments on Day 1 and check out on Day 2.

Paired rebreathing procedures (i.e., at two different isoxic end tidal PO_2 levels) will be performed on Day 1 at 0, 2, 4, and 6 hours. An additional pair of rebreathing procedures will be performed on Day 2 before checkout. Subjects will not be administered any drugs in this lead-in study.

A summary of all procedures is described in the Schedule of Events (Appendix A). Subjects will be discharged from the study after completion of all study procedures. If a subject discontinues from the study prematurely, all procedures scheduled for the end of the study will be performed.

4.1.1 Risk/Benefit

The Duffin rebreathing procedure has been used for multiple decades by different investigators studying different populations with and without drugs that can affect ventilation.[1-14] This study will utilize a lower isoxic level of 50 mmHg, which has been used in the majority of Duffin rebreathing studies, however some have safely used a lower level of 40 mmHg.[9,10] When conducting studies with PO2 at 50 mmHg, SpO2 will be approximately 84% when end tidal pCO2 is 40 mmHg and will decrease as end-tidal PCO2 increases (SpO2 approximately 80% when PCO2 is 55 mmHg). Some subjects are likely to have SpO2 below 80%, however this is only for brief periods as the total rebreathing time is approximately 5 minutes. Prior studies with other methods have targeted an SpO2 of 80% for one hour[11] (or other time limits longer than 25 minutes[11, 15-19]) and thresholds for ending hypoxic challenge studies have been SpO2 of 70% in multiple studies.[12-14] With rebreathing at isoxic level of 50 mmHg, rebreathing will end when PCO2 reaches 55 mmHg or if SpO2 is less than 75% for 3 minutes or if SpO2 is less than 70% (after confirming is not due to noise). This is well within what has been performed safely in prior studies.

Subjects will be informed that participation in a human physiological measurement study like the present one cannot be of benefit to healthy volunteers. Nevertheless, the information from the physical examination, vital sign measurements, and ECG results may be shared with the subject's personal physician if this is the subject's choice. Subjects will be informed that it is also their choice to inform their personal physician that they are participating in this research study.

Subjects will be informed that their contribution to the study is of major importance to agencies like the FDA to understand the ventilatory response to Duffin's rebreathing procedure. However, since this is a study involving healthy volunteers, subjects will be informed that they have the option not to participate.

All women must take a pregnancy test in this study. All women of childbearing potential enrolled on this study will be informed that they must use effective birth control methods (abstinence, intrauterine device, or contraceptive foam and a condom [i.e., double-barrier method]) during the study participation. Subjects will be informed that they must notify the investigator if they or their female partners become pregnant during the course of the study. If a subject becomes pregnant, she will be informed that neither Spaulding Clinical Research nor the sponsor will be responsible for the cost of any obstetric or related care, or for the child's care.

Subjects will be informed that they will be participating in the Duffin's rebreathing procedure where they will alternatively breathe a hyperoxic-hypercapnic (24% O_2, 6% CO_2, N_2 balanced) or hypoxic-hypercapnic (6% O_2, 6% CO_2, N_2 balanced) gas mixture through a mask. They will be informed that this mixture of oxygen, carbon dioxide and nitrogen provided will be different than is normally found in room air (21% O_2, 0.04% CO_2, N_2 balanced with minor amounts of other gasses). Subjects will be informed that the different gas concentrations, in particular the higher level of carbon dioxide breathed in, may result in a feeling of needing to breathe faster or more deeply. Subjects will be informed that this procedure has been used in many previous physiologic measurement studiesand is non-invasive. During screening, subjects will be shown the Duffin's rebreathing equipment and will be trained how to use it to understand if the procedure is tolerable.

Subjects will be informed that the confidentiality of their data will be respected at all times according to state law, and the study personnel handling their study data are bound by confidentiality agreements.

Subjects will be informed that extra precautions will be put in place, including required screening tests, that will limit the risk of COVID-19. Precautions will be documented in a COVID-19 risk management plan. Subjects will be informed that despite the extra precautions, there is still a risk of them contracting COVID-19. Any changes to the COVID-19 precautions (e.g. due to updated CDC recommendations or new testing becoming available) will be documented in the COVID-19 risk management plan.

Subjects will be informed that all tests, procedures, and visits required by the study are provided at no cost to them. If subjects become ill or physically injured because of participation in this study, they will be informed that costs of treatment will not be covered by the sponsor.

4.2 Selection of Study Population

Subjects will be screened, and the data collected will be reviewed by the principal investigator. Only those subjects who meet all the eligibility criteria will be enrolled. The lead-in reproducibility assessment will have up to 10 healthy volunteer participants. Interim data analyses may be performed after each cohort of participants have completed. If reproducibility is acceptable and no procedural changes are needed, additional lead-in cohorts may not be enrolled. If any study procedure element changes are needed, they may be made and additional participants may be enrolled. Every effort will be made to maintain an approximate 50:50 male-to-female sex distribution.

4.2.1 Inclusion Criteria

Subjects who meet all of the following inclusion criteria will be eligible to participate in the study:

1. Subject signs an IRB approved written informed consent and privacy language as per national regulations (e.g., Health Insurance Portability and Accountability Act authorization) before any study related procedures are performed.
2. Subject is a healthy, non-smoking man or woman, 18 to 50 years of age, inclusive, who has a body mass index of 18.5 to 32 kg/m^2, inclusive, at Screening.
3. Subject has normal medical history findings, clinical laboratory results, vital sign measurements, 12 lead ECG results, and physical examination findings at screening or, if abnormal, the abnormality is not considered clinically significant (as determined and documented by the investigator or designee).
4. Subject must have a negative test result for alcohol and drugs of abuse at screening and Check-in (Day -1).
5. Subject must test negative for severe acute respiratory syndrome coronavirus (SARS-CoV-2) by a molecular diagnostic test at check-in (Day -1). If a subject's test comes back inconclusive, it can be repeated at Check-in.
6. Female subjects must be of non-childbearing potential or, if they are of childbearing potential, they must: 1) have been strictly abstinent for 1 month before Check in (Day -1) and agree to remain strictly abstinent for the duration of the study and for at least 1-month after the last rebreathing procedure; OR 2) be practicing 2 highly effective methods of birth control (as determined by the investigator or designee; one of the methods must be a barrier technique) from at least 1 month before Check in (Day -1) until at least 1 month after the end of the study.

7. Male subjects must agree to practice 2 highly effective methods of birth control (as determined by the investigator or designee) from at least 1 month before Check In (Day -1) until at least 1 month after the end of the study.
8. Subject is highly likely (as determined by the investigator) to comply with the protocol defined procedures and to complete the study.

4.2.2 Exclusion Criteria

1. Subject has non-reactive or misshapen pupil(s) or damaged orbit structure or surrounding soft tissue is edematous or has an open lesion.
2. Subject Duffin rebreathing data is of poor quality or subject does not agree to remain clean-shaven for all days when the Duffin rebreathing procedure is being performed.
3. Subject has used any prescription or nonprescription drugs (including aspirin or NSAIDs and excluding oral contraceptives and acetaminophen) within 14 days or 5 half-lives (whichever is longer) or complementary and alternative medicines within 28 days before Check in (Day -1).
4. Subject has used nicotine-containing products (e.g., cigarettes, cigars, chewing tobacco, snuff) within 6 weeks of Screening
5. Subject has a history or evidence of a clinically significant disorder, condition, or disease (e.g., cancer, human immunodeficiency virus [HIV], hepatic or renal impairment) that, in the opinion of the investigator would pose a risk to subject safety or interfere with the study evaluation, procedures, or completion. This includes subjects with any underlying medical conditions that the Investigator believes would put subjects at increased risk of severe illness from COVID-19 based on the Centers for Disease Control and Prevention (CDC) guidelines.
6. Subject has any signs or symptoms that are consistent with COVID-19. These include subjects with fever or chills, cough, shortness of breath or difficulty breathing, fatigue, muscle or body aches, headache, new loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, or diarrhea may have COVID-19. In addition, the subject has any other findings suggestive of COVID-19 risk in the opinion of the investigator.
7. Subject tests positive for SARS-CoV-2 by a molecular diagnostic test performed prior to admission.

8. Subject has clinical laboratory test results (hematology, serum chemistry and urinalysis) at Screening or Check-In that are outside the reference ranges provided by the clinical laboratory and considered clinically significant by the investigator.
9. Subject has a positive test result at Screening for HIV 1 or 2 antibody, hepatitis C virus antibodies, or hepatitis B surface antigen.
10. Female subject is pregnant or lactating before enrollment in the study.
11. Subject has a history of or currently has hypoventilation syndrome or sleep apnea and is on non-invasive ventilation.
12. Subject has a history of sleep disorders, Panic Disorder, Panic Attacks, Generalized Anxiety Disorder, or any associated DSM diagnosis or condition.

4.3 Screening Failures

Subjects who sign and date the informed consent form but who fail to meet the inclusion and exclusion criteria are defined as screening failures. A screening log, which documents the subject initials and reason(s) for screening failure, will be maintained by the investigator for all screening failures. A copy of the log should be retained in the investigator's study files.

If a subject fails the screening process because of an abnormal laboratory result, they can receive a copy of the results upon request. The investigator will determine if follow-up for the abnormal laboratory result is needed and will encourage the subject to follow-up with his or her personal physician as appropriate. All subjects will be informed as to the reason(s) they are excluded from study participation, even if follow-up is not required. If a subject fails the screening process because of a positive test result for human immunodeficiency virus or hepatitis, the positive result will be reported to local health authorities as required by law.

4.4 Termination of Study or Investigational Site

4.4.1 Criteria for Termination of the Study

The study will be completed as planned unless one of the following criteria is satisfied that requires early termination of the study.

- Significant violation of Good Clinical Practice (GCP) that compromises the ability to achieve the primary study objective or compromises subject safety.

4.4.2 Criteria for Termination of the Investigational Site

The study site may be terminated if the site (including the investigator) is found in significant violation of GCP, the protocol, the contractual agreement, or is unable to ensure adequate performance of the study.

In the event that the sponsor elects to terminate the study or the investigational site, a study-specific procedure for early termination will be provided by the sponsor; the procedure will be followed by the applicable investigational site during the course of termination.

4.5 Criteria for Subject Withdrawal

Subjects may withdraw from the study at any time at their own request, or they may be withdrawn by the investigator without the approval of the subject based on the investigator's clinical judgment. A subject is not required to provide a written request to withdraw from the study; however, a written request is required if a subject withdraws consent for his or her personal data to be used for study-related purposes.

A subject may be discontinued for any of the following reasons:

- AE: The subject has experienced an AE that, in the opinion of the investigator, requires early termination. The appropriate electronic case report form (eCRF) must be completed for each AE. If a subject is discontinued from the study due to an AE, the investigator is required to follow-up with the subject until the event resolves or becomes stable. If a subject dies during the study, the cause of death must be reported as a serious AE (SAE), with an outcome of death noted in the eCRF.
- Protocol Violation: The subject failed to meet protocol entry criteria or did not adhere to protocol requirements, and continued participation poses an unnecessary risk to the subject's health.
- Withdrawal by Subject: The subject (or other responsible individual [e.g., caregiver]) wishes to withdraw from the study in the absence of a medical need.

  NOTE: Withdrawal due to an AE should not be recorded in the "voluntary withdrawal" category.
- Study Terminated by Sponsor: The sponsor, IRB, FDA, or other regulatory agency terminates the study.
- Pregnancy: The subject is found to be pregnant.

  NOTE: If the subject is found to be pregnant, the subject must be withdrawn immediately. The pregnancy will be followed-up to term, and the outcome, including any premature termination will be recorded. All live births must be followed for a minimum of 30 days or until the first well-baby visit.

- Other.

  NOTE: This category records withdrawals caused by an accidental or a medical emergency, and other rare cases. The specific reason should be recorded in the comment space of the eCRF.

4.5.1 Handling of Withdrawals

The investigator may terminate a subject's study participation at any time during the study when the subject meets the criteria described in Section 4.5. In addition, a subject may discontinue his or her participation without giving a reason at any time during the study. Subjects will be informed that their participation in the study is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Should a subject's participation be discontinued, the primary reason for termination must be recorded. In addition, efforts should be made to perform all procedures scheduled for the early termination visit. Any data and samples collected before subject withdrawal will become the property of the sponsor.

4.5.2 Replacement of Subjects

The lead-in reproducibility assessment will have up to 10 healthy volunteer participants. Interim data analyses may be performed after each cohort of participants have completed. If reproducibility is acceptable and no procedural changes are needed, additional lead-in cohorts may not be enrolled and the lead-in would end with fewer than 10 participants. If any study procedure element changes are needed, they may be made and additional participants may be enrolled. While they are not expected to be needed, up to 5 replacement participants may be enrolled.

4.6 Study Visits

4.6.1 Recruitment

Recruitment materials (e.g., internet, radio, and print advertisements, social media posts) will be approved by the local IRB before telephone screening. The sponsor is responsible for registration of the study on clinicaltrials.gov. Recruitment may not occur until the study is fully registered on clinicaltrials.gov.

4.6.2 Compensation

Subjects will be offered payment for Screening; however, if the results of their alcohol and drug screening tests are positive, they will not be compensated. Subjects who

complete the entire study will receive payment according to the schedule provided in the informed consent form. No special incentives are offered. Final payment will not be released until all follow-up procedures have been completed and accepted by the investigator.

If a subject chooses to withdraw from the study prematurely, he or she will only be compensated for completed days. If subjects are withdrawn for medical reasons or if the study is halted temporarily or permanently, the subjects will receive compensation proportional to the time spent in the study. No compensation will be provided if a subject is dismissed from the study for noncompliance (e.g., improper conduct, ingesting alcohol and/or drugs [including recreational drugs], consuming any prohibited foods or beverages).

If subjects are required to stay in the clinic for a longer period for safety reasons, they will be compensated at a rate proportional to the entire compensation for the study. If a subject becomes ill or physically injured because of participation in this study, the subject will be referred for treatment.

4.6.3 Screening

The following procedures and assessments will be performed at Screening (Day -28 to -2):

- Obtain informed consent/HIPAA authorization. The informed consent process will be performed by a clinical research nurse in a private room. The subject will be given unlimited time to ask questions regarding study participation, and each subject will be questioned to ensure their understanding.

After informed consent is obtained:

- Review inclusion/exclusion criteria to confirm subject eligibility
- Record demographic information
- Perform serology screening (HIV antigen/antibody [Ag/Ab] Combo 1/2, HepC antibody, HBsAg)
- Record medical history
- Perform alcohol and drug screening (amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, alcohol, opiates, phencyclidine, propoxyphene, and methadone)
- Perform a serum pregnancy test (female subjects only)

- Assess follicle-stimulating hormone (FSH) for postmenopausal (i.e., without menses for two years) female subjects
- Record prior medications
- Monitor for AEs
- Perform clinical laboratory tests (hematology, serum chemistry, and urinalysis)
- Measure vital signs (blood pressure, heart rate, respiratory rate, oral body temperature, and pulse oximetry)
- Perform a safety 12-lead ECG
- Perform a complete physical examination
- Duffin's rebreathing procedure training

4.6.4 Study Periods

This study is an unblinded lead-in reproducibility assessment where subjects will check-in on Day -1 and remain in the study site until checkout on Day 2.

4.6.4.1 Check-In

The following procedures and assessments will be performed at Check-in [Day -1]:

- Perform/review results from SARS-CoV-2 molecular test (may be performed ~2 days before check-in to allow time for results)
- Review inclusion/exclusion criteria to confirm subject eligibility
- Review medical history
- Perform alcohol and drug screening (amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, alcohol, opiates, phencyclidine, propoxyphene, and methadone)
- Perform a serum pregnancy test (female subjects only)
- Assess follicle-stimulating hormone (FSH) for postmenopausal (i.e., without menses for two years) female subjects
- Record concomitant medications
- Monitor for AEs
- Perform clinical laboratory tests (hematology, serum chemistry, and urinalysis)

- Measure vital signs (blood pressure, heart rate, respiratory rate, oral body temperature, pulse oximetry)
- Perform a safety 12-lead ECG
- Perform a comprehensive physical examination
- Admit subject to the study clinic

4.6.4.2 Study Procedure (Day 1)

The following procedures and assessments will be performed during the study procedure period (day 1) according to the Schedule of Events (Table 9-1):

- Monitor for AEs
- Record concomitant medications
- Measure vital signs (blood pressure, heart rate, respiratory rate, oral body temperature, and pulse oximetry).
- Collect samples for estrogen and progesterone (female subjects only)
- Perform Duffin's rebreathing procedure at the approximate time points 0, 2, 4, 6 and 24 hours (last time point on morning of Day 2, Discharge day). Preparatory steps for the Duffin's rebreathing procedure (i.e., relaxation, hyperventilation) should begin prior to the scheduled time. At each time point, the pair of rebreathing assessments (1 at 150 mmHg end tidal PO_2 and 1 at 50 mmHg end tidal PO_2) will be separated by approximately 30 min.
- Quantitative pupillometry will be performed before and after each rebreathing procedure.

4.6.5 Discharge (or Early Termination)

The following procedures and assessments will be performed before the subject is discharged from the study or at early termination:

- Perform a serum pregnancy test (female subjects only)
- Record concomitant medications
- Monitor for AEs
- Perform clinical laboratory tests (hematology, serum chemistry, and urinalysis)
- Measure vital signs (blood pressure, heart rate, respiratory rate, pulse oximetry and oral body temperature)

- Perform Duffin's rebreathing procedure at the 24 hour time point (last time point of the procedure). Preparatory steps for the Duffin's rebreathing procedure (i.e., relaxation, hyperventilation) should begin prior to the scheduled time. At each time point, the pair of rebreathing assessments (1 at 150 mmHg end tidal PO2 and 1 at 50 mmHg end tidal PO2) will be separated by approximately 30 min.
- Quantitative pupillometry will be performed before and after each rebreathing procedure.
- Perform a safety 12-lead ECG
- Perform a complete physical examination
- Discharge subject from the study clinic after completion of all study procedures

4.7 Study Procedures

4.7.1 Duffin's Rebreathing Procedure

Training on the Duffin's rebreathing procedure will be performed to exclude any subjects who cannot tolerate the procedure.

During Duffin's rebreathing procedure, subjects will be placed in a semi-supine position on a bed and connected to the respiration measurement set up. Subjects will wear a facemask and breathe from a pre-filled rebreathing bag containing approximately 4 L of either a hyperoxic-hypercapnic (24% O_2, 6% CO_2, N_2 balanced) or hypoxic-hypercapnic (6% O_2, 6% CO_2, N_2 balanced) gas mixture. During this study, subjects will be asked to breath room air for 5 minutes (relaxation stage) and then to hyperventilate (primarily through deep breathing) room air voluntarily for 5 minutes (hyperventilation stage). Thereafter, subjects will be switched to the rebreathing bag and perform rebreathing assessments at one of the 2 different isoxic end tidal PO_2, i.e., 150 mmHg and 50 mmHg. The isoxia at 150 mmHg or 50 mmHg end-tidal PO_2 will be maintained by providing a computer controlled flow of 100% O_2 to the rebreathing bag.

Subjects will perform 4 pairs of rebreathing procedures (i.e., 8 procedures in total) on Day 1 followed by 1 pair of rebreathing procedures on Day 2 (24-hours after the first rebreathing procedure on Day 1). Each pair of rebreathing procedures will be separated by approximately 30 min. Participants can communicate to staff to discontinue an assessment due to discomfort at any time. Additional considerations for discontinuing a rebreathing procedure are described in a separate Standard Operating Procedure for the Rebreathing Procedure.

Each rebreathing procedure (relaxation, hyperventilation, and rebreathing) should take approximately 13-15 minutes. Data collected throughout the procedure includes

ventilatory flow and volumes as well as percent gas compositions of O_2 and CO_2, which are used to calculate multiple physiological variables.

Data from the rebreathing procedure will be acquired using the Hans Rudolph SmartLab™ Data Acquisition System. Collected data will be analyzed using separate statistical software. Interim data analyses may be performed after each cohort of participants have completed. If reproducibility is acceptable and no procedural changes are needed, additional lead-in cohorts may not be enrolled. If any study procedure element changes are needed, they may be made and additional participants may be enrolled.

The clock on the rebreathing recorder will be confirmed to be consistent with the study clock at the clinical site; this will be confirmed on each study day. Preparatory steps for the Duffin's rebreathing procedure (i.e., relaxation, hyperventilation) should begin prior to the scheduled sample time. Timing of sample collection is planned so that the rebreathing assessment begins at the specified time. Additional details on the Duffin's rebreathing procedure is described in a separate SOP.

The following physiologic measurements will be assessed multiple times throughout the study:

- Minute ventilation
- Tidal volume
- Respiratory rate
- End-tidal PCO_2
- End-tidal O_2
- Oxygen saturation (SpO_2)
- Heart rate
- Maximum pupil diameter before constriction

4.7.2 Pupillary Assessments

At Screening, subjects will be evaluated to ensure they meet requirements necessary for pupillary assessments (e.g., pupils are reactive and not misshapen). During the study, a pupillometer (a hand-held optical scanner) will be used to measure pupil size and dynamics in response to a light stimulus. Pupillary assessments will be performed before and after each rebreathing procedure while the ambient light is kept at a consistent level. The rubber cup of the pupillometer will be placed sequentially on the right eye followed by the left eye (i.e., one pupillary assessment includes measurement of both eyes). The actual data recording only takes seconds on each eye. If the pupillometer device reports

the assessment was incomplete or inaccurate (based on device error codes), the assessment for that eye will be repeated up to three times.

4.7.3 Safety Assessments

Safety will be evaluated in terms of AEs, clinical laboratory results (hematology, serum chemistry, and urinalysis), vital sign measurements (blood pressure, heart rate, respiratory rate, pulse oximetry, and oral body temperature), safety 12-lead ECG results, and physical examination findings.

4.7.3.1 Adverse Events

4.7.3.1.1 Adverse Event Definitions

An AE is defined as any untoward and/or unintended sign, including an abnormal clinical laboratory finding, symptom, or disease. No study drugs are being administered in this "lead-in" protocol to assess variability of noninvasive physiological measurements.

A serious adverse event (SAE) is defined as any AE occurring at any assessment that meets the following criteria:

- Results in death,
- Is life threatening,
- Requires hospitalization or prolongation of existing hospitalization,
- Results in persistent or significant disability or incapacity,
- Results in a congenital anomaly/birth defect due to exposure prior to conception or during pregnancy, or
- Is an important medical event that may not meet the previous criteria but, based upon appropriate medical judgment, jeopardizes the subject or requires medical or surgical intervention to prevent one of the outcomes listed previously.

4.7.3.1.2 Adverse Event Reporting

The recording of AEs will begin after the subject signs the informed consent form and will continue until discharge (or early termination). All AEs, whether serious or nonserious, must be recorded in the eCRF.

Any SAE must be entered into the eCRF system and reported by email to the medical monitor or designee using the SAE Reporting Form within 24 h of the investigator or study clinic staff becoming aware of the event. It is the responsibility of the investigator to report all SAEs to the medical monitor and to provide the most complete report possible. The investigator is responsible for obtaining follow-up information on all SAEs

and submitting follow-up SAE data. Any unexpected SAEs must be reported promptly to the investigator's IRB as per the IRB's requirements.

Adverse events that are assessed by the investigator as possibly or probably related to the study procedures will be followed until they resolve or stabilize. All SAEs will be followed until resolution.

4.7.3.1.3 Assessment of Severity

The investigator will assess the severity of each AE using the following scale:

- Mild: The subject is aware of the AE but is still able to perform all activities; minimal or no medical intervention or therapy is required.
- Moderate: The subject has to discontinue some activities due to the AE; minimal or no medical intervention or therapy is required.
- Severe: The subject is incapacitated by the AE and is unable to perform normal activities; significant medical intervention or therapy is required, and hospitalization is possible.

4.7.3.1.4 Pregnancy

A serum pregnancy test will be performed for female subjects as presented in the Schedule of Events (Table 9-1). If a subject becomes pregnant while on the study, this should be reported immediately to the investigator, the subject will be withdrawn from the study and the medical monitor and the subject will be instructed to follow-up with his or her personal physician. All pregnancies are to be reported as an AE and followed for outcome.

4.7.3.2 Clinical Laboratory Tests

Clinical laboratory and diagnostic screening tests will be performed as presented in the Schedule of Events (Table 9-1) and will be collected in accordance with acceptable laboratory procedures. Clinical laboratory testing will be performed by the clinical study contractor(s). Tests can be repeated once in-case of isolated elevation or contamination of sample. The clinical laboratory tests that will be performed are presented in Table 4-2. Unused clinical laboratory test samples will not be stored for future use.

Table 4-2: Clinical Laboratory Tests & Diagnostic Screening Tests

Hematology	Serum Chemistry	Urinalysis
Hematocrit Hemoglobin Platelet count Red blood cell count White blood cell count (with automated differential)	Alanine aminotransferase Albumin Alkaline phosphatase Aspartate aminotransferase Bicarbonate Bilirubin (total, direct, and indirect) Blood urea nitrogen Calcium Chloride Creatinine (including calculated creatinine clearance by Cockcroft-Gault) Glucose Lactate dehydrogenase Magnesium Phosphorus Potassium Sodium Total protein Uric acid	Appearance Bilirubin Blood Color Glucose Ketones Leukocyte esterase Microscopic examination: red blood cells, white blood cells, epithelial cells, bacteria, crystals, and casts (if present) Nitrite pH Protein Specific gravity Urobilinogen
Diagnostic Screening Tests:		
Serum	**Urine**	**Other**
Serology (human immunodeficiency virus Ag/Ab Combo 1/2, hepatitis C virus antibody, and hepatitis B surface antigen) **Female Subjects Only** Human chorionic gonadotropin (for pregnancy) Estrogen Progesterone	Drug screen including: amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, alcohol, opiates, phencyclidine, propoxyphene, and methadone	SARS-CoV2 molecular test

4.7.3.3 Vital Sign Measurements

Vital signs (blood pressure, heart rate, respiratory rate, pulse oximetry, and oral body temperature) will be measured as in the Schedule of Events (Table 9-1). The subject should be in a supine position, if possible, for a minimum of 5 minutes before vital signs are measured.

4.7.3.4 Safety 12-lead Electrocardiograms

12-lead ECGs will be obtained with the subjects in the supine position for a minimum of 5 minutes before recording. ECGs will be overread by the Principal Investigator or designee (e.g., a medically qualified subinvestigator). If an abnormality is observed, the subject will be instructed to follow-up with his or her personal physician.

4.7.3.5 Physical Examinations

A complete physical examination will be performed as presented in the Schedule of Events (Table 9-1).

The complete physical examination will include, but not be limited to, assessments of the head, eyes, ears, nose, throat, skin, thyroid, nervous system, respiratory system, cardiovascular system, abdomen (liver and spleen), lymph nodes, and extremities. Height, weight (without shoes and wearing the lightest possible clothing), and calculation of body mass index will be performed at Screening and check-out.

If a clinically significant abnormality is observed upon physical examination, the subject will be instructed to follow-up with his or her personal physician.

4.7.4 Demographics and Medical History

Demographic data (date of birth, gender, race, and ethnicity) will be collected at Screening.

Each subject will provide a complete medical history at Screening that will be reviewed at Check-in. Specific information relating to any prior or existing medical conditions/surgical procedures will be recorded in the subject's eCRF.

4.7.5 Prior and Concomitant Medications

Subjects are prohibited from using any prescription or nonprescription drugs (including aspirin or non-steroidal anti-inflammatory drugs [NSAIDs] and excluding oral contraceptives and acetaminophen) within 14 days or 5 half-lives (whichever is longer), or complementary and alternative medicines within 28 days before Day 1 of the study. Subjects will be asked if they have used any of these substances and their responses will be recorded on the eCRF.

Subjects are also prohibited from currently participating in another clinical study of an investigational drug and may not have been treated with any investigational drug within 30 days or 5 half-lives (whichever is longer) of the compound.

Subjects must be instructed not to take any medications, including over-the-counter products, without first consulting with the investigator. If needed for an on-study adverse event, acetaminophen could be used if deemed necessary by the Principal Investigator or designee (e.g., a medically qualified subinvestigator).

4.7.6 Subject Restrictions

Subjects are not allowed to use nicotine containing products (e.g., cigarettes, cigars, chewing tobacco, snuff) within 6 weeks before Screening Subjects are not allowed to use aspirin or NSAIDs within 14 days before Day 1 of the study.

Subjects must be able to tolerate a controlled, quiet study conduct environment, including avoidance of music, television, movies, games, and activities that may cause excitement, emotional tension, or arousal during prespecified times (e.g., before and during rebreathing procedures) throughout the duration of the study.

Subjects must be willing to comply with study rules; attempting to void at specified times (e.g., before rebreathing procedures); remaining quiet, awake, undistracted, motionless, and supine during specified times; and avoiding vigorous exercise as directed throughout the duration of the study. Subjects will not be allowed to sleep during any rebreathing procedure periods.

Standardized meals will be served at consistent times relative to the first rebreathing procedure, and no food or fluids will be served containing caffeine. Subjects will be required to fast during the intensive rebreathing procedure portions of the study. Subjects will be provided their first meal after the 6 h rebreathing procedure on Day 1 and an optional meal after the 24 h procedure on Day 2. Subjects must only eat meals and snacks that are provided during their stay in the study clinic, and must consume all of each meal that is served at a reasonable pace. Outside of meal times, the subjects will only be allowed to intake water, which will be available ad libitum.

Subject must agree to remain clean-shaven for all days when the Duffin rebreathing procedure is being performed.

Due to current precautions being taken for COVID-19, subjects must be willing to:

- Always wear masks except when in a private room without anyone else present or for a limited time for a study procedure (e.g. rebreathing procedure or eating) when instructed by staff.
- Practice social distancing. Subjects will spend most of their time in their rooms except for specified times for walking in the halls (with masks).
- Practice regular handwashing with soap and water, scrubbing hands for at least 20 seconds or with approved hand sanitizer as supplied by study staff.

Designated isolation rooms will be set up to segregate any participant(s) that develop any symptoms of concern while housed in the unit and COVID-19 testing will be done when deemed necessary by the Investigator. If new information becomes available, there could be other precautions that lead to additional restrictions that will be documented in the COVID-19 Risk Management Plan and/or the study specific COVID-19 Procedure Plan.

4.8 Statistical Methods

4.8.1 Sample Size

Up to 10 healthy volunteer participants are planned for enrollment in this study. The sample size was selected empirically to acquire experience for the research staff with use of the rebreathing equipment and to assess performance over repeated assessments in a participant over multiple days.

4.8.2 Analysis Populations

For this study the analysis population will include all subjects who complete at least one rebreathing procedure on Day 1. The analysis population may be defined further in the Statistical Analysis Plan.

4.8.3 General Statistical Considerations

All data will be presented in data listings. Data from subjects excluded from an analysis population will be presented in the data listings, but not included in the calculation of summary statistics. Demographic and baseline characteristics will be summarized overall for all subjects.

4.8.4 Subject Disposition

The number of subjects who enroll in the study and the number and percentage of subjects who complete each assessment will be presented. The frequency and percentage of subjects who withdraw or discontinue from the study and the reason for withdrawal or discontinuation will be summarized.

4.8.5 Demographics and Baseline Characteristics

Descriptive statistics will be used to summarize demographic and baseline subject characteristics. For continuous variables, the mean, median, standard deviation (SD), minimum, and maximum values will be reported. For categorical (nominal) variables, the number and percentage of subjects (or observations) will be reported.

4.8.6 Primary Analysis

Baseline minute ventilation, ventilatory recruitment threshold, slope of the PCO_2-ventilatory response curve, and the extrapolated ventilatory recruitment threshold will be calculated for each subject at each rebreathing assessment. Mean, within- and between-day variability, between-subject variability of the ventilatory response to modified Duffin's rebreathing will be examined using mixed-effects approaches. Coefficients of variation (CV), which will be calculated using mean within- and between-day values of

each end point. Variability will be assessed separately for each isoxic gas condition (i.e. 50 mmHg and 150 mmHg). Full details will be described in a separate Statistical Analysis Plan.

4.8.7 Additional Analyses

Additional physiological assessments (e.g. ventilation, respiratory rate, heart rate, maximum pupil diameter before constriction) as specified in the protocol will be reported with standard descriptive statistics.

4.8.8 Safety Analyses

4.8.8.1 Adverse Events

All AEs will be coded using the latest version of the Medical Dictionary for Regulatory Activities. The incidence of AEs, organized by system organ class and frequency, will be summarized by seriousness. A detailed listing of serious AEs leading to withdrawal will also be provided.

4.8.8.2 Clinical Laboratory Tests

Clinical laboratory results will be reviewed by the investigator or designee together with data in the eCRF. Any values outside the reference range will be evaluated for clinical significance. If a value is determined to be clinically significant, the subject will be instructed to follow-up with his or her personal physician. The investigator or designee may repeat the clinical laboratory tests if deemed appropriate. The investigator will maintain a copy of the laboratory accreditation and the reference ranges for the laboratory used.

Clinical laboratory results (hematology, serum chemistry, and urinalysis) will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum). Clinical laboratory results will be classified as normal or abnormal, according to the reference ranges of the individual parameter. The number and percentage of subjects with abnormal laboratory results will be provided. No statistical testing will be performed on clinical laboratory data.

4.8.8.3 Vital Sign Measurements

Vital sign measurements and changes from baseline will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum) by time point.

4.8.8.4 Safety 12-lead Electrocardiograms

Safety 12-lead ECGs will be obtained with the subjects in the supine position for a minimum of 5 minutes before recording. ECGs will be overread by the Principal Investigator or designee (e.g., a medically qualified subinvestigator). If an abnormality is observed, the subject will be instructed to follow-up with his or her personal physician.

4.8.8.5 Physical Examinations

Physical examination findings will be presented in a data listing, and abnormal physical examination findings will be recorded as AEs.

4.8.8.6 Other Safety Data

All concomitant medication usage and medications that changed in daily dose, frequency, or both since the subject provided informed consent will be summarized for each subject.

4.8.9 Interim Analyses

No formal interim analyses are planned for this study. Rebreathing results for individual subjects will be reviewed and analyzed as each subject completes the study. Feedback will be provided so that research staff can incorporate accrued experience into how the rebreathing equipment is used and how rebreathing procedures are conducted.

4.8.10 Missing Data

Missing data will not be imputed. Data that are excluded from the descriptive or inferential analyses will be included in the subject data listings. This will include data from subjects not in the particular analysis population, measurements from unscheduled visits, or extra measurements that may arise from 2 or more analyses of the biofluid sample at the same time point. Details on the handling of missing data will be further described in the Statistical Analysis Plan.

4.9 Data Quality Assurance

Completed eCRFs are required for each subject participating in study procedures. Electronic data entry will be accomplished through the ClinSpark® remote electronic data capture system, which allows for on-site data entry and data management. This system provides immediate, direct data transfer to the database, as well as immediate detection of discrepancies, enabling site coordinators to resolve and manage discrepancies in a timely manner. Each person involved with the study will have an individual identification code and password that allows for record traceability. Thus, the system, and subsequently any investigative reviews, can identify coordinators, investigators, and individuals who have entered or modified records.

Furthermore, the investigator retains full responsibility for the accuracy and authenticity of all data entered into the electronic data capture system. The completed dataset and their associated files are the sole property of the sponsor and should not be made available in any form to third parties, except for appropriate governmental health or regulatory authorities, without written permission of the sponsor.

4.10 Data Sharing

De-identified subject-level data may be released to other researchers (including through a data warehouse or as a part of a publication) to enable secondary research. Additional secondary research may also be performed by the sponsor.

5. ETHICAL CONSIDERATIONS

5.1 Ethical Conduct of the Study

This study will be performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects adopted by the 18th World Medical Association General Assembly, Helsinki, Finland, 1964 and later revisions, as well as, United States Title 45 Code of Federal Regulations (CFR) Part 46 GCP, and International Council for Harmonisation (ICH) guidelines describing technical requirements for registration of pharmaceuticals for human use.

5.2 Institutional Review Board (IRB)

The FDA Project Lead or investigator will provide the designated IRB with all required documents, including the study protocol and informed consent form. The study will not be initiated until appropriate IRB approval is obtained from the designated IRB. The investigator will provide the FDA Project Lead with copies of the approval documents for the protocol, informed consent form, and all recruiting materials. The designated IRB will also receive copies of any original or amended information sheets or pamphlets given to the study subject in support of the informed consent process and any advertisements or other recruitment material. Such materials will not be employed in the study before approval by the designated IRB.

Subjects will be informed that they have the right to contact the IRB or Office for Human Research Protections if they have any questions, concerns, complaints, or believe they have been harmed by the participation in this research study as a result of investigator negligence. Subjects will be given the address and phone number of the IRB.

6. ADMINISTRATIVE PROCEDURES

6.1 Responsibilities of the Investigator

The following administrative items are meant to guide the investigator in the conduct of the study but may be subject to change based on industry and government standard operating procedures, working practice documents, or guidelines. Changes may be reported to the IRB but will not result in protocol amendments.

6.1.1 Form FDA 1572

The investigator will complete and sign the Form FDA 1572.

6.1.2 Adherence to Protocol

The investigator agrees to conduct the study as outlined in this protocol in accordance with the ICH E6(R2) and all applicable guidelines and regulations.

6.1.3 Reporting Requirements

By participating in this study, the investigator agrees to submit reports of SAEs according to the time line and method outlined in the protocol (Section 4.7.3.1.2). In addition, the investigator agrees to submit reports to the IRB as appropriate. The investigator also agrees to provide the sponsor with an adequate report shortly after completion of the investigator's participation in the study.

6.1.4 Source Documentation

By participating in this study, the investigator agrees to maintain adequate case histories for the subjects treated as part of the research under this protocol. The investigator agrees to maintain accurate eCRFs and source documentation as part of the case histories.

6.1.5 Retention of Records

The investigator agrees to keep the records stipulated in this protocol and those documents that include (but are not limited to) the study-specific documents, identification log of all participating subjects, medical records, source worksheets, all original signed and dated informed consent forms, subject authorization forms regarding the use of personal health information (if separate from the informed consent form), copies of all eCRFs, query responses, and detailed records of drug disposition, to enable evaluations or audits from regulatory authorities, the sponsor, or its designees.

Furthermore, ICH 4.9.5 requires the investigator to retain essential documents specified in Section 8 of ICH E6(R2) until at least 2 years after the last approval of a marketing

application for a specified drug indication being investigated or, if an application is not approved, until at least 2 years after the investigation is discontinued and regulatory authorities are notified. In addition, ICH 4.9.5 states that the study records should be retained until an amount of time specified by applicable regulatory requirements or for a time specified in the clinical study site agreement between the investigator and sponsor.

Refer to the clinical study site agreement for the sponsor's requirements on record retention. The investigator should contact and receive written approval from the sponsor before disposing of any such documents.

6.1.6 Financial Disclosure and Obligations

The investigator is required to provide financial disclosure information to allow the sponsor to submit the complete and accurate certification or disclosure statements required under 45 CFR 46. In addition, the investigator must provide to the sponsor a commitment to update this information promptly if any relevant changes occur during the course of the investigation and for 1 year after the completion of the study.

Neither the sponsor nor the study clinic is financially responsible for further testing or treatment of any medical condition that may be detected during the screening process.

6.2 Confidentiality and Disclosure of Data

All subjects will sign a HIPAA-compliant authorization form containing the mandated core elements and requirements before participation in this clinical study. The sponsor and designees affirm and uphold the principle of the subject's right to protection against invasion of privacy. Throughout this study, a subject's source data will only be linked to the clinical site's electronic data capture system database or documentation via a unique identification number. As permitted by all applicable laws and regulations, limited subject attributes such as gender, age or date of birth, and subject initials are maintained by the clinical site and may be used to verify the subject and accuracy of the subject's unique identification number. The key which allows the clinical site to link the unique identification number to the individual subject will be maintained by the clinical site and will not be released to the sponsor. The sponsor will not request or attempt to re-identify subjects and all personally identifiable information will be removed from the data when shared with the sponsor.

To comply with ICH Guidelines for GCP and to verify compliance with this protocol, the sponsor requires that the investigator allow review of the subject's original medical records (source data or documents) by the study monitor, representatives from any regulatory authority (e.g., FDA), the sponsor's designated auditors, and the appropriate IRB. These medical records will include, but will not be limited to, clinical laboratory test result reports, ECG reports, admission and discharge summaries for hospital

admissions occurring during a subject's study participation, and autopsy reports. Access to a subject's original medical records requires the specific authorization of the subject as part of the informed consent process.

Copies of any subject source documents that are provided to the sponsor must have certain personally identifiable information removed (i.e., subject name, address, and other identifier fields not collected in the subject's eCRF).

Data will be maintained and backed up in the electronic data capture system. All access to the data is protected by username and password, and each staff member and all sponsor staff will have separate access that requires a separate username and password. Access is only given to site staff and requested sponsor staff who have completed the appropriate training.

6.3 Subject Consent

Written informed consent in compliance with 45 CFR 46 will be obtained from each subject before entering the study or performing any unusual or nonroutine procedure that involves risk to the subject. An informed consent template may be provided by the sponsor to the study clinic. If any institution-specific modifications to study-related procedures are proposed or made by the study clinic, the consent should be reviewed by the sponsor or its designee or both before IRB submission. Once reviewed, the consent will be submitted by the investigator to the IRB for review and approval before the start of the study. If the informed consent form is revised during the course of the study, all active participating subjects must sign the revised form.

Before enrollment, each prospective subject will be given a full explanation of the study and be allowed to read the approved informed consent form. The informed consent process will be performed by a clinical research nurse in a private room. The subject will be given unlimited time to ask questions regarding study participation, and each subject will be questioned to ensure their understanding. Once the investigator is assured that the subject understands the implications of participating in the study, the subject will be asked to give consent to participate in the study by signing the informed consent form.

The investigator will provide a copy of the signed informed consent form to the subject. The original form will be maintained in the subject's medical records at the site.

6.4 Data Collection

Full details of procedures for data collection and handling will be documented in the data management plan, which is initiated with the final protocol receipt. The data management plan is a changing document that evolves over the course of the study.

6.5 Publications

No information related to or generated by this study will be released to the public until it has been reviewed by the sponsor. The sponsor shall own intellectual rights for the data and analysis resulting from this study. Authorship on publications will be determined by standard journal requirements.

7. STUDY MANAGEMENT

7.1 Monitoring

The sponsor or its designee will monitor the study to ensure that it is being conducted according to the protocol, GCP standards, and applicable region-specific requirements, and to ensure that study initiation, conduct, and closure are adequate. The investigators and the study clinic staff will be expected to cooperate fully with the study monitors and personnel or agents of the sponsor and be available during monitoring visits to answer questions sufficiently and to provide any missing information. The investigators and their institutions will permit direct access to source data/documents for study-related monitoring activities, audits, IRB reviews, and regulatory inspections.

During any on-site visits, the study monitor will:

- Check and assess the progress of the study
- Review all informed consent forms
- Review study data collected
- Conduct source document verification
- Identify any issues and address their resolution
- Verify that the facility remains acceptable

These monitoring activities will be done in order to verify that the:

- Data are authentic, accurate, and complete.
- The safety and rights of the subjects are being protected.
- The study is being conducted in accordance with the currently approved protocol (including any amendments), GCP, and all applicable regulatory requirements.

In addition, the sponsor, designated auditors, and government inspectors must be allowed access to eCRFs, source documents, and other study files that may be required to evaluate the conduct of the study.

7.2 Management of Protocol Amendments and Deviations

7.2.1 Modification of the Protocol

Any changes in this research activity, except those necessary to remove an apparent immediate hazard to the subject, must be submitted to the sponsor or designee and reviewed and approved by the local IRB before implementation. Amendments to the protocol must be submitted in writing to the investigator's IRB for approval before subjects are enrolled into an amended protocol.

7.2.2 Protocol Violations and Deviations

Any significant protocol deviations that the investigator or study clinic staff believes are of major importance (e.g., subject enrolled but not eligible) should be reported to the sponsor and the investigator's IRB as soon as possible. Significant protocol deviations may include the following:

- Deviations from the inclusion/exclusion criteria that may affect subject safety
- Deviations (omission or delay) of safety monitoring procedures
- Deviations in obtaining informed consent

8. REFERENCE LIST

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9. APPENDICES

9.1 Appendix A – Schedule of Events

Table 9-1: Schedule of Events

SCR-013 Opioid Agonist Antagonist Study - Lead In				
	Screening	Check-In	Treatment Period	Check-out
Study Day	-28 to -2	-1	1	2
In house residency		X	X	
Informed consent process	X			
Medical history – healthy	X	X		
Comprehensive physical exam	X	X		X
Vital signs (including pulse oximetry)	X	X	X	X
ECG - single[1]	X	X		X
Chemistry/hematology[1]	X	X		X
Urinalysis	X	X		X
Urine drug screen + alcohol screen	X	X		
Pregnancy test[2]	X	X		X
FSH Test (females only)	X	X		
SARS-CoV-2 Molecular Test		X		
HIV test	X			
Hepatitis test	X			
Estrogen and progesterone			X	
Duffin's Rebreathing Procedure (See protocol for time points)	2		8	2
Pupillometry Assessments (See protocol for time points)			16	4
Con Med/AE assessment	X	X	X	X

1 Clinically notable results are repeated
2 Serum test

9.2 Appendix B – Protocol Revision History

PROTOCOL REVISION HISTORY			
Protocol Number	**Version**	**Effective Date**	**Summary of Changes**
SCR-013	1.0	27th October 2021	Developed initial protocol

Statistical Analysis Plan

SCR-013 Lead-in: Assessing Variability of the Ventilatory Response to Duffin's Rebreathing Procedure

Sponsor: U.S. Food and Drug Administration
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Version of SAP: 1.0

Date of SAP: 9 Jan 2022

CONFIDENTIAL

Sponsor Signatures Page

Prepared by

Celine Wang, PharmD ORISE Fellow, Division of Applied Regulatory Science U.S. Food and Drug Administration	Date

Prepared by

Jeffry Florian, PhD Associate Director, Division of Applied Regulatory Science U.S. Food and Drug Administration	Date

Approved by

David Strauss, MD, PhD Director, Division of Applied Regulatory Science U.S. Food and Drug Administration	Date

Contents

Abbreviations and definitions

AE	Adverse event
CV	Coefficient of variation
ECG	Electrocardiogram
eCFRs	Electronic case report forms
FDA	Food and Drug Administration
mmHg	Millimeter's mercury
PD	Pharmacodynamic
PCO_2	Partial pressure carbon dioxide
PO_2	Partial pressure oxygen
SAP	Statistical analysis plan
SD	Standard deviation
TEAE	Treatment-emergent adverse event

Change Log

Version / Date	Section	Summary of Changes
1.0 / 9 Jan 2022	General	Developed initial statistical analysis plan

1. Introduction

This document outlines the proposed statistical methods for data analysis of data collected from Protocol 'SCR-013 Lead-in: Assessing variability of the ventilatory response to Duffin's rebreathing procedure'.

The following statistical analysis plan (SAP) provides the framework for the summarization of the data from this study. The SAP may change due to unforeseen circumstances. Any changes made to the SAP will be documented in a change log within the SAP along with a rationale for the changes.

2. Study Objectives

The objective of this study is to evaluate variability of the ventilatory response to Duffin's rebreathing procedure.

3. Study Endpoints

3.1 Primary Endpoints

1. *Baseline minute ventilation* when end tidal PCO_2 is less than the ventilatory recruitment threshold (represents non-chemoreflex drives to breathe)
2. *Ventilatory recruitment threshold* (end tidal PCO_2 above which minute ventilation starts to increase linearly with further increases in end tidal PCO_2)
3. *Slope of the PCO_2-ventilatory response curve* that reflects the increase in minute ventilation relative to the increase in end tidal PCO_2 (represents chemoreflex sensitivity)
4. *Extrapolated ventilatory recruitment threshold* (intersection with X axis)

3.2 Exploratory Endpoints

1. Between occasion (within a day) and between day variability for primary endpoints
2. Additional physiological assessments
    a. Pharmacodynamics (respiratory and cardiovascular) (separate hyperoxic and hypoxic)
        i. Minute ventilation, tidal volume, respiratory rate, end tidal PCO_2, oxygen saturation and heart rate during each stage of the rebreathing assessment
        ii. Number of apneic events lasting > 10 s
    b. Pharmacodynamics (pupillary) (before and after rebreathing)
        i. Maximum pupil diameter before constriction
        ii. Dynamic pupillary measurements after a light stimulus
            1. Minimum diameter at peak constriction
            2. Percent change between min/max diameter
            3. Latency of constriction
            4. Average constriction velocity
            5. Maximum constriction velocity

6. Dilation velocity after peak constriction
7. Time to reach 75% recovery of maximum diameter

4. Study Overview

4.1 Study Design

This study is an unblinded lead-in reproducibility assessment to assess variability of the ventilatory response to Duffin's rebreathing procedure.

Table 3--1: Study Schedule

Day -1	Day 1	Day 2
Check-In	Lead-in PD Assessments	Check-Out

Healthy subjects will be enrolled in this study. Subjects will report to the study site for screening from Days -28 to -2 and then will return to the site on Day -1 for baseline assessments and check-in. After check-in (Day -1), subjects will remain in study site for PD assessments on Day 1 and check out on Day 2.

Paired rebreathing procedures (i.e., at two different isoxic end tidal PO_2 levels) will be performed on Day 1 at 0, 2, 4, and 6 hours. An additional pair of rebreathing procedures will be performed on Day 2 before checkout. Subjects will not be administered any drugs in this lead-in study.

A summary of all procedures is described in the Protocol, Schedule of Events. Subjects will be discharged from the study after completion of all study procedures. If a subject discontinues from the study prematurely, all procedures scheduled for the end of the study will be performed.

4.2 Sample Size

The lead-in reproducibility assessment will have up to 10 healthy volunteer participants. This sample size was determined empirically and was considered sufficient to gain sufficient experience with conducting the rebreathing procedure under different isoxic conditions and to characterize variability in measurements obtained using the procedure. No power calculation was performed with regards to variability for the study endpoints.

A published study (Jensen et al., 2010) that also evaluated variability in the Duffin rebreathing procedure included 20 subjects under the same isoxic conditions. In that study, the between occasion (four assessments on day 1 over a period of 6 hours) and between day (five assessments at approximately the same time of day on day 1, 8, 15, 22, and 61) variability was evaluated for various rebreathing measures, including the baseline minute ventilation, ventilatory recruitment threshold, slope of the PCO_2-ventilatory response curve, and extrapolated ventilatory recruitment threshold (i.e., same primary endpoints as proposed in the current study). Between occasion and

between day variability across all measures proposed as primary endpoints was similar under hyperoxic and hypoxic conditions (Table 4-1).

Table 4-1: Between Occasion and Between Day Variability Reported in Jensen et al for Study Primary Endpoints

Variable	Hyperoxia		Hypoxia	
	Between Occasion CV (%)	Between Day CV (%)	Between Occasion CV (%)	Between Day CV (%)
Baseline minute Ventilation (L/min)	41.1	40.0	39.8	33.2
Ventilatory recruitment threshold (mmHg)	4.0	4.4	3.8	4.2
Slope of PCO_2-ventilatory response curve (L/min/mmHg)	37.8	44.9	40.4	46.4
Extrapolated ventilatory recruitment threshold (mmHg)	5.9	5.7	4.3	5.1

As the primary aim of the study is to evaluate reproducibility in Duffin rebreathing procedure, interim data analyses may be performed after each cohort of participants has completed. If reproducibility is acceptable and no procedural changes are needed, additional lead-in cohorts may not be enrolled. If any study procedure changes are needed, these study procedure changes may be made, and additional participants may be enrolled.

5. Analysis Populations

The safety population will include all subjects who enrolled in the study.

The rebreathing analysis population will include all subjects who enrolled in the study and who completed at least one rebreathing assessment on any day or at any timepoint. A rebreathing assessment will be considered complete if the subject makes it through the entire procedure (e.g., subject makes it to the rebreathing stage with sufficient data to estimate the slope of the PCO_2-ventilatory response curve) and if there are no identifiable issues with how the procedure was conducted.

Potential issues with how the procedure was conducted can include, but are not limited to, a leak from the system (e.g., substantially decreasing O_2 during rebreathing or no evidence of an increase in CO_2 during rebreathing), inaccurate readings from the pneumotach (e.g., non-physiologic baseline minute ventilation readings), failure in the oxygen control loop (oxygen is not maintained at proper setpoint during rebreathing procedure), or a subject suddenly increasing minute ventilation without subsequent increases in minute ventilation as end tidal pCO_2 increases (suggesting stress-associated or other non-CO_2 mediated hyperventilation confounding the ventilatory response to hypercapnia through $[H^+]$ chemoreceptors). Determination of the

completeness of each rebreathing assessment and its individual components/measurements will be performed manually by a member of the data analysis team.

6. Data Screening and Acceptance

6.1 Handling of Missing or Incomplete Data

The following approaches will be used for the handling of missing or incomplete data:

- Missing PD data (e.g., subject discontinued from study; subject could not successfully complete rebreathing at a time point) will not be imputed.
- Pupillometry data collected from the PLR®-3000 pupillometer device flagged as being anomalous based on device error codes will be excluded from analyses

7. General Statistical Considerations

All data will be presented in data listings. Data from subjects excluded from an analysis population will be presented in the data listings, but not included in the calculation of summary statistics. Demographic and baseline characteristics will be summarized overall and by treatment for all subjects.

7.1 Subject Disposition

The number of subjects who enroll in the study and the number and percentage of subjects who complete each assessment will be presented. The frequency and percentage of subjects who withdraw or discontinue from the study and the reason for withdrawal or discontinuation will be summarized.

7.2 Demographic and Baseline Characteristics

Continuous demographic and baseline characteristic variables (age, height, weight, and body mass index) will be summarized overall and by treatment using descriptive statistics (number of subjects, mean, standard deviation [SD], median, minimum, and maximum). The number and percentage of subjects in each class of categorical demographic and baseline characteristic variables will also be summarized.

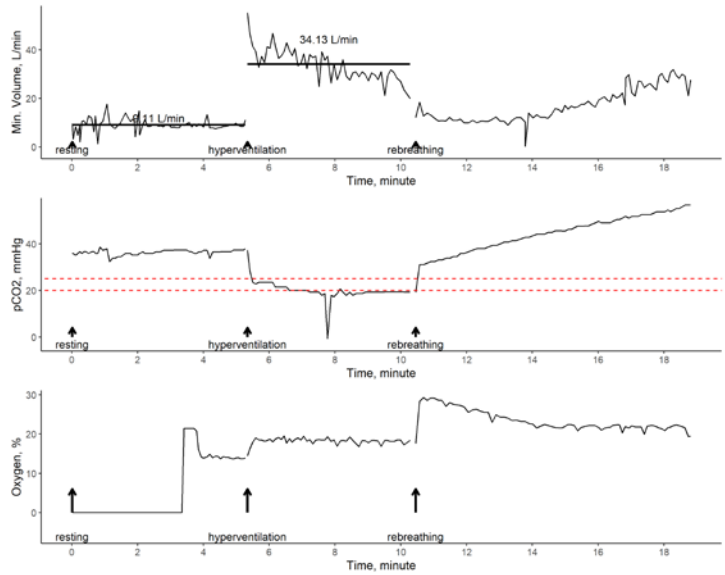
7.3 Pharmacodynamic Analyses

7.3.1 Rebreathing Analyses

Figure 7-1 below shows representative time-course results from the rebreathing procedure, with minute ventilation, oxygen percentage, and end-tidal PCO_2 shown from top to bottom. Throughout the procedure subjects will undergo different steps designed to reduce noise and variability. Initially, subjects breathe room air for 5 minutes and relax as much as possible. Around the 5-minute mark, the subject is instructed to begin hyperventilating (primarily through deep breathing) for 5 min to achieve an end tidal PCO_2 of approximately 20-25 mmHg prior to

rebreathing. During this rebreathing test, subjects will be maintained at one of 2 different isoxic end tidal PO_2. The isoxia at a hyperoxic (150 mmHg) or hypoxic (50 mmHg) end tidal PO_2 will be maintained by providing a computer-controlled flow of 100% O_2 to the rebreathing bag.

Each of these steps can be noted on the example figure. In this example, relaxation while breathing room air was performed for 5-minutes. From 5 to 10 minutes, the test subject began hyperventilation and slight decreases in O_2% and PCO_2 with an increase in minute ventilation are observed. Finally, the test subject begins rebreathing at 10 minutes. After taking deep breaths, PCO_2 in the rebreathing circuit and the lungs equilibrate and then PCO_2 increases linearly to approximately 55 mm Hg at the end of rebreathing in this subject. An increase in minute ventilation is triggered at a certain point during rebreathing (ventilatory recruitment threshold), and both measures increase until completion of the procedure based on subject tolerability or until other procedure stopping criteria are reached (see protocol and rebreathing SOP for full list). If the subject's data does not follow these trends due to a leak in the apparatus (e.g., incomplete seal between the mask and subject's face), inaccurate readings from the pneumotach, issues in operator recording of the data, or the subject not having a linear increase in minute ventilation as end tidal PCO_2 increases prior to reaching PCO2 of 55 mm Hg, the run will not be considered completed and will be excluded from subsequent data analysis that is dependent on the minute ventilation / end tidal PCO_2 regression line.



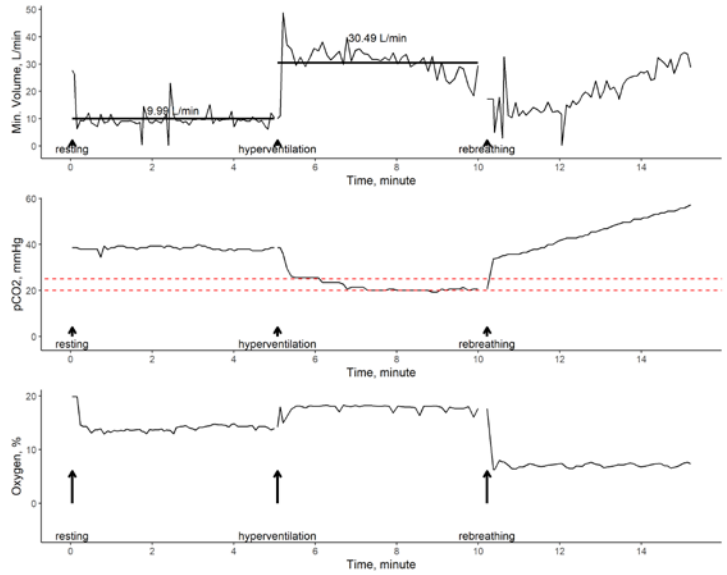


Figure 7-1: Sample data from a test subject during relaxation, hyperventilation, and rebreathing, with minute ventilation, O_2 percentage, and PCO_2 mm-Hg over time during these experimental steps. A representative hyperoxic test is shown at the top (oxygen percentage maintained at ~21% during rebreathing) and a representative hypoxic test is shown at the bottom (oxygen percentage maintained at ~7.1% during rebreathing).

From these results, minute ventilation and end tidal PCO_2 levels over time will be matched. End tidal PCO_2 will be calculated as % CO_2 • (barometric pressure – 47 mmHg [water vapor]). Separate linear and nonlinear (hockey stick model) regressions will be fit to the experiment data. The nonlinear and linear regressions will be fit to data from the rebreathing stage of the rebreathing procedure (i.e., during isoxic hypercapnia). This functional relationship for the nonlinear model assumes a constant relationship between minute ventilation and end tidal PCO_2 up until a threshold value is reached, after which minute ventilation and end tidal PCO_2 both increase linearly. Example code for the nonlinear fit can be found in the Appendix, and a sample result from the test case can be seen in Figure 7-2. Because of potential inconsistencies in when the stage transition may have been flagged by the device operator (pressed while hyperventilation was still ongoing) and as the isoxic setpoint is being achieved, the first 15 seconds of data from the rebreathing stage will be removed when calculating the linear regression. In addition, all other exploratory pharmacodynamic endpoints will be automatically calculated.

Because of the potential for outlying data points in the minute ventilation and/or end-tidal PCO_2 signal at each individual breath, it is sometimes necessary to exclude outlying data points from the regression. Such outlying data points can be introduced by subject postural changes, sighing, hiccups, talking, or faulty sensors. In addition, there may be a need to remove additional data at

the beginning of rebreathing or to remove data at the end of rebreathing (e.g., nonlinearity as subject approaches or exceeds end tidal PCO_2 55 mmHg).

Outlying values for the regressions will be identified automatically through evaluation of standardized residuals after performing the initial regression (i.e., standardized residuals > 2). Then, the data analysis team will be provided with time course plots from the full assessment (all rebreathing procedure stages with stage transitions marked) and regression results using data from the rebreathing stage. The data analysis team will evaluate the completeness of the rebreathing assessment and visually assess the automatically calculated values in comparison to the underlying data. Any automatically calculated values that do not accurately capture the underlying data will be flagged, additional outliers will be removed, and the automated calculations will be re-run. A subject may have incomplete rebreathing data not supporting calculation of one or more primary endpoints, but other endpoint measures would still be used for analyses.

If the nonlinear regression converges, the regression results will be used to derive all four primary endpoints for that subject at that assessment. Baseline minute ventilation when end tidal PCO2 is less than the ventilatory recruitment threshold and the slope of the PCO2-ventilatory response curve are V_0 and s_{slope} from the regression, respectively. The ventilatory recruitment threshold is calculated as the intersection between the two line segments. The extrapolated ventilatory recruitment threshold is calculated as the intersection of the PCO2-ventilatory response curve and the x-axis. If the nonlinear regression does not converge, results from the linear regression will be used to determine the slope of the PCO2-ventilatory response curve and data from the relaxation stage will be used to determine baseline minute ventilation. The ventilatory recruitment threshold and the intersection of the PCO2-ventilatory response curve and the x-axis will then be calculated as described above. All modeling analyses for the regressions will be performed in statistical software.

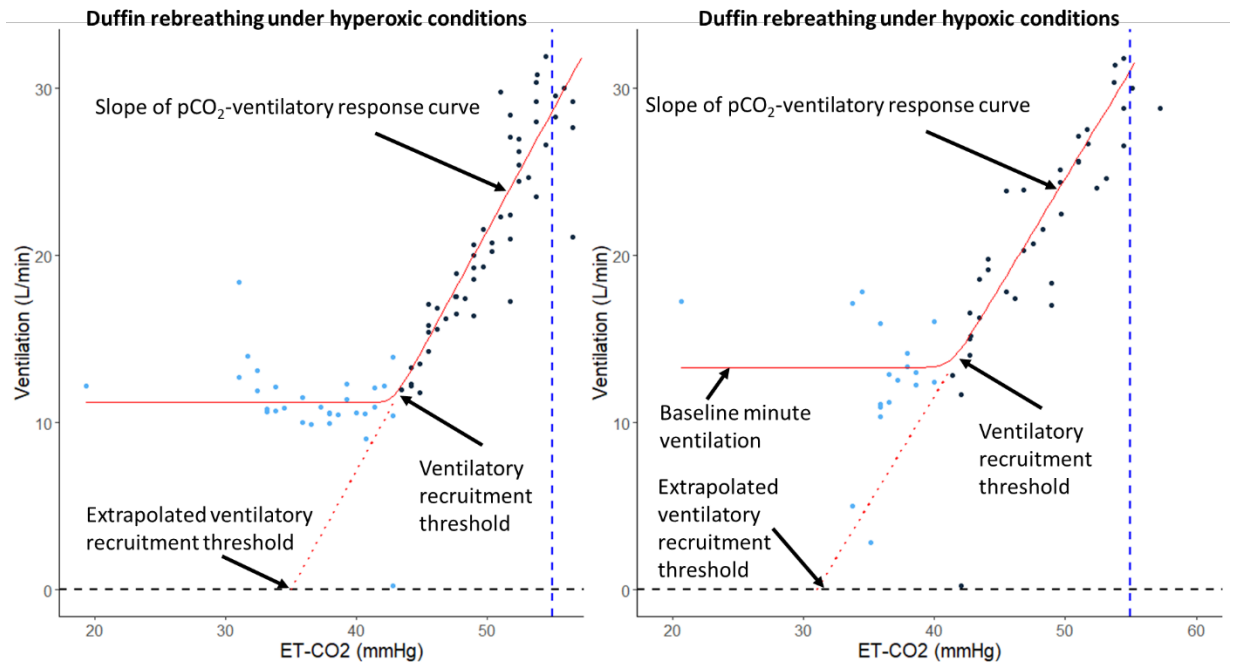


Figure 7-2: Sample nonlinear least squares fit between minute ventilation and end tidal PCO_2 for test subject under hyperoxic (left) or hypoxic (right) conditions. The four primary endpoints are annotated on each figure.

7.3.1.1 Assessment of Between Occasion and Between Day Variability

Each of the primary endpoints (denoted as $end_{primary}$) will be summarized by day and time point using descriptive statistics (number of subjects, mean, SD, median, minimum, and maximum). Time course profiles for all primary endpoints will be calculated and plotted for the overall group and individuals. Linear-mixed effect modeling will be used to determine between occasion, between day, and between subject variability, where USUBJID, OCC, DAY are the subject identifier and measurements obtained at different times across the study. The model will not include any fixed effects. In the event that all variabilities cannot be simultaneously estimated, separate analyses will be run for data from the first 6 hours (between occasion variability) and for data from time 0 at the start of day 1 and at the start of day 2 (i.e., 24 hours) (between day variability):

$$end_{primary} \sim (1|USUBJID/OCC/DAY)$$

7.3.2 Exploratory Ventilation Measures

Other exploratory respiratory measures will be calculated and summarized from information collected during planned rebreathing procedures. Data from the initial relaxation/resting and hyperventilation stages of the procedure will be used to calculate ventilation, tidal volume and end tidal PCO_2. All measures will be summary by day, treatment, and time using descriptive

statistics (number of subjects, mean, SD, median, minimum, and maximum). Time course profiles for all measures will be calculated and plotted.

Apneic events lasting > 10 seconds will be determined using data collected during the rebreathing procedure. An event is defined as the absence of inspiratory flow (as measured by the pneumotachograph) for at least 10 s during this period. These parameters will be summarized using descriptive statistics (number of subjects, number of events) by study part, treatment, day, and time, as appropriate.

7.3.3 Pupillometry Analyses

Maximum pupil diameter before constriction and dynamic pupillary measurements after a light stimulus will be measured during relaxation and after each rebreathing assessment. This will include measurements before and after the rebreathing assessment and from the left and right eye. These exploratory parameters will be summarized using descriptive statistics (number of subjects, mean, SD, median, minimum, and maximum), and time courses summaries of each pupillary measurement will be generated. Pupillary changes will be compared to baseline measurements. In addition, time course pupillary changes will be compared to time course ventilatory changes across treatments to evaluate concordance between these measures.

7.4 Additional Exploratory Analyses

7.4.1 Sex Hormone Analyses

Estrogen and progesterone levels will be collected at the start of the study (Day 1 prior to the first rebreathing assessment). These parameters will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum) and may be used to explore variation in ventilation across the menstrual cycle.

7.5 Safety Analyses

7.5.1 Adverse Events

All adverse events (AEs) will be coded using the latest version of the Medical Dictionary for Regulatory Activities. The incidence of treatment-emergent adverse event (TEAEs), organized by system organ class and frequency, will be summarized by seriousness, severity, relationship to treatment, and by treatment at onset of the TEAE. A detailed listing of serious AEs and TEAEs leading to withdrawal will also be provided.

7.5.2 Clinical Laboratory Tests

Clinical laboratory results (hematology, serum chemistry, and urinalysis) will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum). Clinical laboratory results will be classified as normal or abnormal, according to the reference ranges of the individual parameter. The number and percentage of subjects with abnormal

laboratory results will be provided. No statistical testing will be performed on clinical laboratory data.

7.5.3 Vital Sign Measurements

Vital sign measurements and changes from baseline will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum) by treatment and time point.

7.5.4 Safety 12-lead Electrocardiograms

Safety 12-lead ECG data will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum) by treatment and time point.

7.5.5 Physical Examinations

Physical examination findings will be presented in a data listing, and abnormal physical examination findings will be recorded as AEs.

7.5.6 Other Safety Data

All concomitant medication usage and medications that changed in daily dose, frequency, or both since the subject provided informed consent will be summarized for each subject.

8. Data Quality Assurance

Completed electronic case report forms (eCRFs) are required for each subject randomly assigned to the study drug. Electronic data entry will be accomplished through the ClinSpark® remote electronic data capture system, which allows for on-site data entry and data management. This system provides immediate, direct data transfer to the database, as well as immediate detection of discrepancies, enabling site coordinators to resolve and manage discrepancies in a timely manner. Each person involved with the study will have an individual identification code and password that allows for record traceability. Thus, the system, and subsequently any investigative reviews, can identify coordinators, investigators, and individuals who have entered or modified records.

Furthermore, the investigator retains full responsibility for the accuracy and authenticity of all data entered into the electronic data capture system. The completed dataset and their associated files are the sole property of the sponsor and should not be made available in any form to third parties, except for appropriate governmental health or regulatory authorities, without written permission of the sponsor.

9 Appendices

9.1 Nonlinear Regression Model

The rebreathing procedure consists of three separate periods: i) breathing room air, termed 'relaxation' period; ii) hyperventilation (primarily through deep breathing); and iii) rebreathing at one of 2 different isoxic end tidal PO_2 ($PetO_2$) (i.e., at 50 mm Hg and 150 mm Hg). For the purposes of data analysis and determination of the primary endpoints, data from iii) will be used to estimate the ventilatory response to hypercapnia curve. This relationship, which is characterized by a flat portion between minute ventilation and end-tidal PCO_2 followed by a period of linear increase, can be represented using a hockey-stick function. For purposes of these analyses, the following equation is pre-specified to describe data from the procedure:

$V_i = V_0$ (if $PCO_2 \leq$ inflection point)

$V_i = V_0 * (1 + ((PCO_{2,i} - PCO_{2,inflection}) * s_{slope} / V_0)^g)^{1/g}$ (if $PCO_2 >$ inflection point)

Here, V_i is the minute ventilation that corresponds to an end-tidal CO_2 of $PCO_{2,i,}$ V_0 is baseline minute ventilation rate on the flat portion of the curve, $PCO_{2,inflection}$ is the end-tidal CO_2 where increase from baseline minute ventilation begins, s_{slope} is the slope of the curve, and g is a shape parameter in the hockey stick function that is set to 20 to provide the desired curvature. Outlier measures, introduced by subject postural changes, sighing, talking, hiccups and other subject behaviors, will be identified based on evaluation of standardized residuals from the nonlinear regression (i.e., standardized residuals > 2 SD). Coding for the nonlinear function will be performed in R (version 3.6.3 or later). An example of the coded function is provided below.

```
ff <- function(p, v0, b, s) {
  vs <- (p-b)*s
  vs[which(vs<0)] <- 0
  a <- 0.95
  g <- 1/(1-a)
  v <- v0*(1 + (vs/v0)**g)**(1/g)
}

fits<-function (x){
  nls(v ~ ff(p,v0,b,s),
      lower=list(v0=5,  b=0,  s=0),
      start=list(v0=8,  b=30,  s=1),
      upper=list(v0=15, b=100, s=5),
      trace=F, algorithm="port", data=x)
}
```

10 References

Jensen D, Mask G, Tschakovsky ME. Variability of the ventilatory response to Duffin's modified hyperoxic and hypoxic rebreathing procedure in healthy awake humans. Respir Physiol Neurobiol. 2010;170(2):185-197.