CLINICAL STUDY PROTOCOL

Methodological trial to investigate the dose-response relationship, test-retest reliability, and tolerance to repeated exposure of the 35% carbon dioxide experimental panic challenge in CO2-sensitive healthy volunteers.

Short Title:	Methodological optimization of the CO2 challenge
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Date:	13-Sep-2023
CHDR number:	CHDR2344
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Study Title

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I acknowledge accountability for this protocol in accordance with CHDR's current procedures.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee; in Dutch, ABR = Algemene Beoordeling en Registratie
AE	Adverse Event
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AUC	Area under the concentration – time curve
AUC_{inf}	Area under the concentration – time curve from time zero to infinity
AUC_{last}	Area under the concentration – time curve from time zero to time of last measurable concentration
AUC_{tau}	Area under the concentration – time curve between consecutive dosing
CA	Competent authority (also CCMO)
CHDR	Centre for Human Drug Research
CI	Confidence Interval
CV	Coefficient of variation
EC	Ethics Committee (also Medical Research Ethics Committee (MREC); in Dutch: Medisch Ethische Toetsing Commissie (METC).
ECG	Electrocardiogram
GCP	Good Clinical Practice
ICH	International Conference on Harmonization
Max	Maximum
MedDRA	Medical Dictionary for Regulatory Activities
min	Minutes
Min	Minimum
SAE	Serious Adverse Event
SD	Standard Deviation
SEM	Standard Error of the Mean
SOC	System Organ Class
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
$t_{½}$	Terminal Elimination Half-life
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen.

PROTOCOL SYNOPSIS

Title

Methodological trial to investigate the dose-response relationship, test-retest reliability, and tolerance to repeated exposure of the 35% carbon dioxide experimental panic challenge in CO2-sensitive healthy volunteers.

Short Title

Methodological optimization of the CO2 challenge

Principal investigator & Trial Site

G.E. (Gabriël) Jacobs, MD, PhD, Centre for Human Drug Research, Zernikedreef 8, 2333 CL Leiden, The Netherlands

Background & Rationale

Panic attacks (PAs) are human fear-related phenomena characterized by episodic feelings of dread and discomfort, cognitive misinterpretation of potential threat and physical symptoms related to autonomic nervous system (ANS) activation. Since PAs usually occur without warning, they are not amenable to real-time assessment, and as a result, are assessed retrospectively by structurally administering patient and clinician-rated psychometric questionnaires in a research setting. The validity of such methodologies is obviously questionable and creates the urgent need for alternative methodologies to investigate PAs in health and disease and to establish efficacy of anxiolytic drugs.

In the past, different methodologies have been utilized to characterize PAs in humans. Fear challenges with for instance compounds such as lactate, yohimbine and cholecystokinin tetrapeptide (CCK-4) or self-induced hyperventilation yielded inconsistent results and has led to their abandonment in recent years. Currently, the acute intrapulmonary administration of carbon dioxide (CO2) is widely considered a reliable challenge model to experimentally provoke a panic reaction that phenomenologically and physiologically resembles PAs. Acute CO2 inhalation therefore experimentally induces a panic reaction in humans that displays adequate face-validity as a panic challenge in experimental research.

The CO2 inhalation model for panic has undergone both technical innovation and relatively extensive validation since the 1980's. Panic disorder (PD) patients consistently display the highest sensitivity to CO2, followed by first degree relatives of PD patients and healthy volunteers, which show a concentration dependent sensitivity to inhaled CO2. Additionally, registered anxiolytic drugs administered in clinically effective therapeutic doses reduce CO2 sensitivity in healthy volunteers and patients over time.

In the past, various CO2 regimens have been applied to induce PAs in human populations. Although both single and double vital capacity inhalations of 35% CO2/65% O2 consistently demonstrate panicogenic effects in healthy volunteers and PD patients or their first-degree relatives, the CO2 sensitivity of both similar patient and healthy groups tend to vary between research groups. Since varying CO2 administration protocols which include both single and double vital capacity administrations are being applied across research groups, differences in CO2 sensitivity could very well be the result of unintended methodological variability. The lack of a standardized procedure to test sensitivity to CO2 therefore hampers accurate comparisons between tests performed under different protocols. A better understanding of whether a single or double breath 35% CO2/65% O2 is sufficient to induce PAs is expected to contribute to the validity of acute CO2 inhalation as tool in pathophysiological research and in early CNS drug development.

The differential response to single versus double 35% CO2 vital capacity inhalation of CO2 remains to be examined systematically to further validate it as experimental paradigm for future use. To the best of our knowledge no study has been previously published that compares single and double vital capacity 35% CO2 inhalation in a single study. Therefore, we aim to investigate the panicogenic effects of a single vs. a double vital capacity method 35% CO2 in healthy volunteers. We hypothesize that 35% CO2 double vital capacity inhalation is associated with a higher percentage of subjects experiencing a panic attack compared to single vital capacity inhalation.

Additionally, this study also seeks to systematically investigate the test-retest reliability of the CO2 inhalation challenge in healthy volunteers who are sensitive to the anxiogenic effects of CO2 inhalation. Previous research has only examined intervals of up to a few weeks, leaving it unclear whether individuals who are sensitive to the 35% CO2 inhalation challenge remain sensitive for longer periods, possibly years later. Leibold et al. (2015[1]) found that a single vital capacity breath of 35% CO2 inhalation demonstrated good reliability of subjective fear in PD patients, but only used an interval of one week between inhalations. These previous studies were conducted in patients with PD, who exhibit greater sensitivity to the anxiogenic effects of the CO2 challenge compared to healthy volunteers. By analysing this effect in a different population, this study also aims to provide a more comprehensive understanding of the test-retest reliability of the CO2 inhalation challenge.

Finally, this study seeks to investigate whether tolerance or desensitization occurs in healthy volunteers who were previously sensitive to the CO2 challenge. While past research has explored this topic in patients with panic disorders, studies in healthy volunteers who were screened for sensitivity to the anxiogenic effects of CO2 challenges are non-existent. Previous tolerance studies have been conducted in healthy volunteers, but these participants were not selected based on their sensitivity to the anxiogenic effects of CO2 inhalation. If tolerance does not occur after four challenges administered one week apart over the course of a month, this would justify conducting future three-way crossover studies.

Objective(s) and endpoints

Primary objectives	**Endpoints**
To investigate the difference in panic response (i.e., “dose-response”) between single and double vital capacity 35% CO_2/65% O_2 inhalation in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack between the single and double vital capacity 35% CO_2/65% O_2 inhalation as measured by the difference in pre- and post CO2 challenge questionnaires Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
To investigate the test-retest reliability of repeated double vital capacity 35% CO2/65% O2 inhalation in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack between the test-retest double vital capacity 35% CO_2/65% O_2 inhalation as measured by the difference in pre- and post CO2 challenge questionnaires Panic Symptoms List-IV (PSL-IV) and VAS subjective fear, and VAS discomfort.
To investigate whether repeated double vital capacity 35% CO2/65% O2 inhalation leads to tolerance/desensitization in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack in the repeated 35% CO_2/65% O_2 inhalation as measured by the difference in pre- and post CO2 challenge questionnaires Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
To establish whether CO2 sensitivity represents a stable trait phenomenon in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack in the repeated 35% CO_2/65% O_2 inhalation as measured by the difference in pre- and post CO2 challenge questionnaires Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
Secondary objectives	**Endpoints**
Explore the effects of single and (repeated) double breath 35% CO_2/65% O_2 on heart rate and blood pressure in CO2-sensitive healthy volunteers.	Difference in mean change from pre-CO2 to post-CO2 challenge in vital sign measurements (systolic blood pressure, diastolic blood pressure, heart rate).

Explore the effects of single and (repeated) double breath 35% CO2/65% O2 on neuro-endocrine mediated autonomic nervous system activation and orexin/hypocretin release in CO2-sensitive healthy volunteers.	Differences in plasma ACTH, prolactin, orexin-1 and cortisol, and saliva amylase and cortisol from pre-CO2 to post-CO2 challenge.
To explore the relationship between baseline personality and temperament characteristics and individual response to CO2 in CO2-sensitive healthy volunteers.	Dutch Personality Questionnaire (DPQ), Cloninger Temperament Character Inventory (TCI), and Spielberger State-Trait Anxiety Inventory (STAI)

Design

Five visit trial to investigate the dose-response relationship, test-retest reliability, and tolerance to repeated exposure of the 35% carbon dioxide experimental panic challenge in 20 CO2-sensitive healthy volunteers.

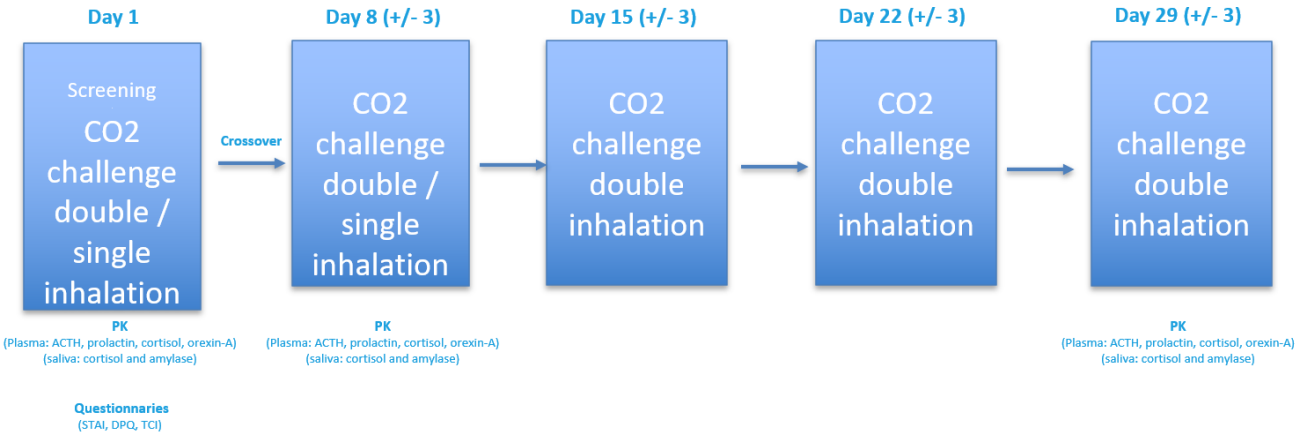
Out of the five scheduled visits (Figure 1):

- single or a double vital capacity inhalation of 35% CO2/65% O2 will be randomized over a 2-way cross-over part consisting of visit 1 and visit 2. During the first visit, half of the participants (n=10) will be randomized to receive a single vital capacity inhalation of CO2 while the other half (n=10) will be randomized to double vital capacity inhalation of CO2. Approximately a week later, the groups will switch, with the group originally assigned the single vital capacity inhalation now receiving the double vital capacity inhalation, and vice versa.
- visits 3, 4 and 5 will follow a fixed pattern, with all participants receiving only double vital capacity 35% CO2/65% O2 inhalation roughly a week apart.

On the first day, prior to the CO2 challenge, the Spielberger State-Trait Anxiety Inventory (STAI), Dutch Personality Questionnaire (DPQ), and Cloninger Temperament Character Inventory (TCI) will be administered according to the schedule of assessments (Table 1).

To evaluate the severity of panic symptoms, each subject will complete the PSL-IV, VAS Fear and VAS Discomfort within five minutes before and as soon as possible (but no later than 15 minutes) after the CO2 challenge according to the schedule of assessments (Table 1). In addition, the STAI Y1 will be administered within 20 minutes before and as soon as possible after the CO2 challenge. Throughout the procedure, vital signs such as blood pressure and heart rate will be continuously monitored. Lastly, biomarkers for neuroendocrine autonomic nervous system activation (plasma ACTH, cortisol, and prolactin; saliva alpha-amylase and cortisol) and plasma orexin-1 will be assessed according to the schedule of assessments (Table 1).

Figure 1: Schematic overview of design.



Investigational drug

Not applicable.

Subjects / Groups

A total of 20 healthy subjects, who previously have shown to be sensitive to the panicogenic effects of the CO2 challenge, of either gender will be enrolled into the study following satisfactory completion of a screening (ECG and medical history) where eligibility for the study will be checked.

Inclusion criteria

1. Healthy male or female aged between 18 and 65 years (inclusive) at screening who have been demonstrated to be sensitive to the panicogenic effects of the CO2 challenge in previous studies.
2. Sensitivity to the fear-inducing effects of 35% CO2 double-breath inhalation is defined as an increase from pre-CO2 to post-CO2 challenge in the following: PSL-IV total scores ≥4 with at least 1-point increase for at least 4 of the symptoms specified in the PSL-IV and an increase on the Visual Analog Scale (VAS) Fear of at least 25 mm.
3. BMI of 18-32 kg/m_2 (inclusive).
4. Non-smoker for at least 3 months.

Exclusion criteria

1. Subjects with a clinically significant current or past personal or family history of any psychiatric disorder as classified by DSM-4 or DSM-5 criteria.
2. Current or past history of alcohol or any substance abuse or dependence disorder within the past 12 months.
3. Clinically significant ECG abnormalities.
4. Clinically significant abnormality of the lungs (e.g., COPD, asthma, lung fibrosis) and hematologic diseases concerning hemoglobin (e.g., thalassemia and sickle cell disease).
5. Important cardiovascular history, or suspicion of infarct, cardiomyopathy, cardiac failure, TIA, angina pectoris, cardiac arrhythmias, CVA.
6. Personal or familial history of cerebral aneurysm.

Concomitant medications

Certain concomitant medications are allowed, only after the rationale is clearly documented by the investigator.

Tolerability / safety endpoints

(Serious) adverse events ((S)AEs) will be collected throughout the study at every study visit. ECG will be obtained during the first visit of the study according to the Visit and Assessment Schedule

Sample Size Justification

This is a methodological study; therefore, the sample size is not based on statistical considerations. We've chosen a sample size of 20 participants because our current database includes 180 individuals sensitive to the panic-inducing effects of the CO2 challenge. Based on our previous experiences, selecting 20 participants from this group for the study is realistic

Statistical methodology

The study would involve employing descriptive statistics to summarize and understand the data, followed by inferential statistics to compare means between challenges. Repeated measures mixed model ANCOVA with challenge (single/double or number of double) as fixed factor, subject as

random factor and baseline as covariate will be used to analyze test-retest reliability, and tolerance effects of induced panic attacks. For the panic attacks repeated measures logistic regression will be used with challenge as fixed factor and subject as random factor. Correlation/regression will also be used to examine relationships among variables and the outcomes.

Table 1 Visit and Assessment Schedule

Assessment \ Time point	Day 1	Day 8 (+/-3 days)	Day 15 (+/-3 days)	Day 22 (+/-3 days)	Day 29 (+/-3 days)
Informed consent	X				
MRSAD/BRMO screening	X				
Demography	X				
Inclusion and exclusion criteria	X				
Medical and psychiatry history	X				
Concomitant medication	X	X	X	X	X
ECG	X				
Randomization[a]	X				
STAI-Questionnaire (STAI) Y1[b] and Y2	X				
STAI-Questionnaire (STAI) Y1[b]		X	X	X	X
Dutch Personality Questionnaire (DPQ)	X				
Cloninger Temperament, Character Inventory (TCI)	X				
CO2 challenge (single or double vital capacity inhalation)	X	X			
CO2 challenge (double vital capacity inhalation)			X	X	X
Finapres assessment[c]	X	X	X	X	X
PSL-IV[d]	X	X	X	X	X
VAS Fear and Discomfort[d]	X	X	X	X	X
ACTH, cortisol, prolactin, and Orexin-1 in plasma[e]	X	X			X
Cortisol and alpha-amylase in saliva[f]	X	X			X
Adverse event and concomitant medication monitoring	<----------→				

CO2: Carbon dioxide; ECG: Electrocardiogram; MRSA/BRMO: Methicillin-Resistant Staphylococcus Aureus / Bacteroides fragilis group multi-drug resistant organisms; STAI: State-Trait Anxiety Inventory; PSL-IV: Panic Symptom List -IV; VAS: Visual Analogues Scale.

a. Subject will be randomized on the same day once all previously performed assessments allow for the subject to be included in the study.

b. The STAI Y1 will be completed by the subject within 20 mins before and as soon as possible, but within 15 minutes after the CO2 Challenge.

c. The Finapres system will be used to measure vital signs (systolic blood pressure, diastolic blood pressure and heart rate). The system will obtain vital sign measurements starting 15 minutes prior to and for 15 minutes following the CO2 Challenge.

d. The PSL-IV, VAS Fear, and VAS Discomfort will be completed by the subject within 5 minutes before and as soon as possible, but within 15 minutes, after the CO2 Challenge.

e. During each visit, ACTH, prolactin, cortisol, and orexin-1 in blood plasma will be collected at specific timepoints surrounding the CO2 challenge: one will be taken 15 minutes before the challenge, another within the 5-minute window immediately preceding the challenge, a third one directly within the 5-minutes following the challenge, and the remaining at 15-, 30-, 60-, and 120-minutes intervals post-challenge.

f. During each visit, cortisol and amylase in saliva will be collected at specific timepoints surrounding the CO2 challenge: one will be taken 10 minutes before the challenge, another within 1-minute window immediately preceding the challenge, a third one directly within the 1-minutes following the challenge, and the remaining at 10-, 20-, 40-, and 60-minutes intervals post-challenge

1 BACKGROUND AND RATIONALE

1.1 Context

Panic attacks (PAs) are human fear-related phenomena characterized by episodic feelings of dread and discomfort, cognitive misinterpretation of potential threat and physical symptoms related to autonomic nervous system (ANS) activation. Since PAs usually occur without warning, they are not amenable to real-time assessment, and as a result, are assessed retrospectively by structurally administering patient and clinician-rated psychometric questionnaires in a research setting. The validity of such methodologies is obviously questionable and creates the urgent need for alternative methodologies to investigate PAs in health and disease and to establish efficacy of anxiolytic drugs.

In the past, different methodologies have been utilized to characterize PAs in humans. Fear challenges with for instance compounds such as lactate, yohimbine and cholecystokinin tetrapeptide (CCK-4) or self-induced hyperventilation yielded inconsistent results and has led to their abandonment in recent years. Currently, the acute intrapulmonary administration of carbon dioxide (CO2) is widely considered a reliable challenge model to experimentally provoke a panic reaction that phenomenologically and physiologically resembles PAs. Acute CO2 inhalation therefore experimentally induces a panic reaction in humans that displays adequate face-validity as a panic challenge in experimental research.

The CO2 inhalation model for panic has undergone both technical innovation and relatively extensive validation since the 1980's. Panic disorder (PD) patients consistently display the highest sensitivity to CO2, followed by first degree relatives of PD patients and healthy volunteers, which show a concentration dependent sensitivity to inhaled CO2. Additionally, registered anxiolytic drugs administered in clinically effective therapeutic doses reduce CO2 sensitivity in healthy volunteers and patients over time.

In the past, various CO2 regimens have been applied to induce PAs in human populations. Although both single and double vital capacity inhalations of 35% CO2/65% O2 consistently demonstrate panicogenic effects in healthy volunteers and PD patients or their first-degree relatives, the CO2 sensitivity of both similar patient and healthy groups tend to vary between research groups. Since varying CO2 administration protocols which include both single and double vital capacity administrations are being applied across research groups, differences in CO2 sensitivity could very well be the result of unintended methodological variability. The lack of a standardized procedure to test sensitivity to CO2 therefore hampers accurate comparisons between tests performed under different protocols. A better understanding of whether a single or double breath 35% CO2/65% O2 is sufficient to induce PAs is expected to contribute to the validity of acute CO2 inhalation as tool in pathophysiological research and in early CNS drug development.

The differential response to single versus double 35% CO2 vital capacity inhalation of CO2 remains to be examined systematically to further validate it as experimental paradigm for future use. To the best of our knowledge no study has been previously published that compares single and double vital capacity 35% CO2 inhalation in a single study. Therefore, we aim to investigate the panicogenic effects of a single vs. a double vital capacity method 35% CO2 in healthy volunteers. We hypothesize that 35% CO2 double vital capacity inhalation is associated with a higher percentage of subjects experiencing a panic attack compared to single vital capacity inhalation.

Additionally, this study also seeks to systematically investigate the test-retest reliability of the CO2 inhalation challenge in healthy volunteers who are sensitive to the anxiogenic effects of CO2 inhalation. Previous research has only examined intervals of up to a few weeks, leaving it unclear whether individuals who are sensitive to the 35% CO2 inhalation challenge remain sensitive for longer periods, possibly years later. Leibold et al. (2015[1]) found that a single vital capacity breath of 35% CO2 inhalation demonstrated good reliability of subjective fear in PD patients, but only used an interval of one week between inhalations. These previous studies were conducted in patients with PD, who exhibit greater sensitivity to the anxiogenic effects of the CO2 challenge compared to

healthy volunteers. By analysing this effect in a different population, this study also aims to provide a more comprehensive understanding of the test-retest reliability of the CO2 inhalation challenge.

Finally, this study seeks to investigate whether tolerance or desensitization occurs in healthy volunteers who were previously sensitive to the CO2 challenge. While past research has explored this topic in patients with panic disorders, studies in healthy volunteers who were screened for sensitivity to the anxiogenic effects of CO2 challenges are non-existent. Previous tolerance studies have been conducted in healthy volunteers, but these participants were not selected based on their sensitivity to the anxiogenic effects of CO2 inhalation. If tolerance does not occur after four challenges administered one week apart over the course of a month, this would justify conducting future three-way crossover studies.

1.2 Study rationale

1.2.1 Benefit and risk assessment

The CO2 challenge has previously been established as a safe and effective method to investigate panicogenic effects in healthy volunteers by multiple research groups. No serious adverse events have been reported, nor has there been evidence of increased risk of developing panic disorder (PD) as a result of these tests. Therefore, the potential CO2-related carry-over effects are non-existent.

The robust fear-like behaviour CO2 induces in preclinical models mirrors the respiratory and cardiovascular effects observed in humans, making acute CO2 inhalation a valid translational fear challenge. The physiological nature of CO2 allows for real-time assessment of panic attacks (PAs) in experimental settings. This model also offers the possibility of demonstrating panicolytic effects of novel central nervous system (CNS) active compounds in human subjects. Several studies conducted at the Centre for Human Drug Research (CHDR) have verified the panicolytic effects of compounds with different mechanisms of action in CO2-sensitive healthy volunteers, confirming the reliability of the CO2 experimental model [2,3].

In over 300 challenges with healthy volunteers at CHDR, the CO2 challenge was deemed safe with no serious adverse events. However, some limitations were identified that needed further exploration to make this translational model more applicable and less invasive in phase 1 clinical trials. It remains uncertain whether a single and double vital capacity inhalation yield equivalent panicogenic effects. Tolerance to the CO2 challenge might seems to develop after repeated exposure in healthy subjects known to be sensitive to its panicogenic effects. Furthermore, it is unknown whether this test-retest reliability persists over extended periods (years).

Addressing these methodological issues is of importance for two primary reasons. Firstly, it expected to enhance the reliability of the CO2 challenge model for future proof of mechanism studies, thereby potentially avoiding unnecessary exposure of individuals to the CO2 challenge in such trials. Secondly, resolving these uncertainties might potentially further minimize the burden on the subjects undergoing the CO2 challenge, further ensuring their safety and well-being throughout the research process.

1.2.2 Study population

A total of 20 healthy subjects, who previously have shown to be sensitive to the panicogenic effects of the CO2 challenge, of either gender will be enrolled into the study following satisfactory completion of a screening (ECG and medical history) where eligibility for the study will be checked.

1.2.3 Comparative drug(s) and/or placebo

Not applicable.

1.2.4 Treatment duration

The total study duration will be 29 (+/-3) days, and subjects will undergo the CO2 inhalation challenge in total five times on Day 1, Day 8 (+/-3), Day 15 (+/-3), Day 22 (+/-3) and Day 29 (+/-3) of the study. No treatment will be administered.

1.2.5 Primary endpoint

The primary endpoint will be based on the PSL-IV total Score, VAS Fear, and VAS Discomfort as these are the endpoint to objectify the panicogenic effect of the CO2 Challenge. This study aims to illuminate the primary endpoints of the following three objectives as follows:

1. The objective is to discern and contrast the frequency and intensity of panic attacks induced by a single vs. a double vital capacity inhalation of 35% CO2/65% O2. We will accomplish this by quantifying the difference in pre- and post-CO2 challenge metrics – namely PSL-IV, VAS Fear, and VAS Discomfort. The goal is to reveal any statistically significant distinctions in the frequency and intensity of panic reactions between these two inhalation scenarios.

2. The objective is to determine whether the test-retest outcomes of double vital capacity 35% CO2/65% O2 inhalation remain consistent in CO2-sensitive healthy volunteers. We will accomplish this by quantifying the difference in pre- and post-CO2 challenge metrics – namely PSL-IV, VAS Fear, and VAS Discomfort. The goal is to identify whether the panic response stays steady across repeated exposures, thus measuring the consistency of the test-retest.

3. The objective is to scrutinize the consistency and reliability of panic reactions induced by the repeated inhalation of double vital capacity 35% CO2/65% O2 in CO2-sensitive, healthy volunteers. We will accomplish this by quantifying the difference in pre- and post-CO2 challenge metrics - specifically PSL-IV, VAS Fear, and VAS Discomfort, between the initial and subsequent inhalation tests. The goal is to determine whether repeated exposures yield statistically significant consistency in the frequency and intensity of panic responses among the study participants.

4. The objective is to discern and contrast the persistence of sensitivity to the panicogenic effects of CO2 in healthy participants, previously identified as CO2-sensitive, over an extended period. We plan to accomplish this by quantifying the variation in pre- and post-CO2 challenge metrics, specifically PSL-IV, VAS Fear, and VAS Discomfort, between these two timepoints - initial assessment and years later. The aim is to establish whether there exist statistically significant distinctions in the frequency and intensity of panic reactions over time, indicating a maintained sensitivity or alteration to the CO2 challenge.

1.2.6 Statistical hypotheses and sample size

This is an observational, methodological study; therefore, the sample size is not based on statistical considerations. We've chosen a sample size of 20 participants because our current database includes 180 individuals sensitive to the panic-inducing effects of the CO2 challenge. Based on our previous experiences, selecting 20 participants from this group for the study is realistic.

2 STUDY OBJECTIVES AND ENDPOINTS

Primary objectives	Endpoints
To investigate the difference in panic response (i.e.. “dose-response”) between single and double vital capacity 35% CO_2/65% O_2 inhalation in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack between the single and double vital capacity 35% CO_2/65% O_2 inhalation as measured by the Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
To investigate the test-retest reliability of repeated double vital capacity 35% CO2/65% O2 inhalation in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack between the test-retest double vital capacity 35% CO_2/65% O_2 inhalation as measured by the Panic Symptoms List-IV (PSL-IV) and VAS subjective fear, and VAS discomfort.
To investigate whether repeated double vital capacity 35% CO2/65% O2 inhalation leads to tolerance/desensitization in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack in the repeated 35% CO_2/65% O_2 inhalation as measured by the Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
To establish whether CO2 sensitivity represents a stable trait phenomenon in CO2-sensitive healthy volunteers.	The difference in the occurrence and severity of a panic attack in the repeated 35% CO_2/65% O_2 inhalation as measured by the Panic Symptoms List-IV (PSL-IV), VAS subjective fear, and VAS discomfort.
Secondary objectives	**Endpoints**
Explore the effects of single and (repeated) double breath 35% CO_2/65% O_2 on heart rate and blood pressure in CO2-sensitive healthy volunteers.	Difference in mean change from pre-CO2 to post-CO2 challenge in vital sign measurements (systolic blood pressure, diastolic blood pressure, heart rate).
Explore the effects of single and (repeated) double breath 35% CO2/65% O2 on neuro-endocrine mediated autonomic nervous system activation and orexin/hypocretin release in CO2-sensitive healthy volunteers.	Differences in plasma ACTH, prolactin, orexin-1 and cortisol, and saliva amylase and cortisol from pre-CO2 to post-CO2 challenge.
To explore the relationship between baseline personality and temperament characteristics and individual response to CO2 in CO2-sensitive healthy volunteers.	Dutch Personality Questionnaire (DPQ), Cloninger Temperament Character Inventory (TCI), and Spielberger State-Trait Anxiety Inventory (STAI)

3 STUDY DESIGN

3.1 Overall study design and plan

Five visit trial to investigate the dose-response relationship, test-retest reliability, and tolerance to repeated exposure of the 35% carbon dioxide experimental panic challenge in 20 CO2-sensitive healthy volunteers.

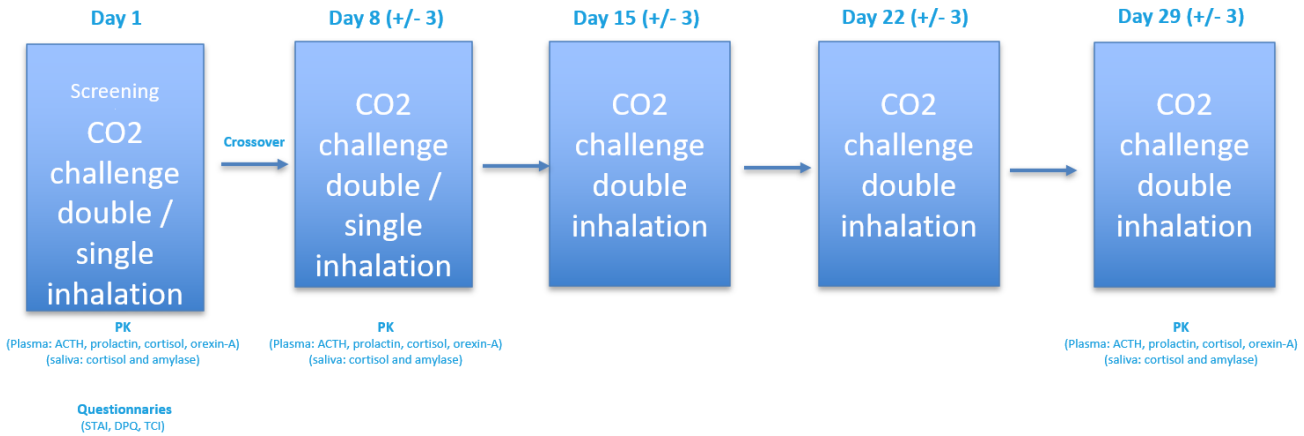
Out of the five scheduled visits (Figure 1):

- single or a double vital capacity inhalation of 35% CO2/65% O2 will be randomized over a 2-way cross-over part consisting of visit 1 and visit 2. During the first visit, half of the participants (n=10) will be randomized to receive a single vital capacity inhalation of CO2 while the other half (n=10) will be randomized to double vital capacity inhalation of CO2. Approximately a week later, the groups will switch, with the group originally assigned the single vital capacity inhalation now receiving the double vital capacity inhalation, and vice versa.
- visits 3, 4 and 5 will follow a fixed pattern, with all participants receiving only double vital capacity 35% CO2/65% O2 inhalation roughly a week apart.

On the first day, prior to the CO2 challenge, the Spielberger State-Trait Anxiety Inventory (STAI), Dutch Personality Questionnaire (DPQ), and Cloninger Temperament Character Inventory (TCI) will be administered according to the schedule of assessments (Table 1).

To evaluate the severity of panic symptoms, each subject will complete the PSL-IV, VAS Fear and VAS Discomfort within five minutes before and as soon as possible (but no later than 15 minutes) after the CO2 challenge according to the schedule of assessments (Table 1). In addition, the STAI Y1 will be administered within 20 minutes before and as soon as possible after the CO2 challenge. Throughout the procedure, vital signs such as blood pressure and heart rate will be continuously monitored. Lastly, biomarkers for neuroendocrine autonomic nervous system activation (plasma ACTH, cortisol, and prolactin; saliva alpha-amylase and cortisol) and plasma orexin-1 will be assessed according to the schedule of assessments (Table 1).

Figure 2: Schematic overview of design.



4 STUDY POPULATION

4.1 Subject population

A study population of healthy volunteers who have been demonstrated to be sensitive to the panicogenic effects of the CO2 challenge between the age of 18 and 65. The CO2 challenge has been the subject of two studies conducted by CHDR (1614 and 1935), resulting in a database of approximately 180 healthy volunteers. For the purposes of this study, we aim to recruit 20 out of the known 180 CO2 sensitive participants.

4.2 Inclusion criteria

1. Healthy male or female aged between 18 and 65 years (inclusive) at screening who have been demonstrated to be sensitive to the panicogenic effects of the CO2 challenge in previous studies.
2. Sensitivity to the fear-inducing effects of 35% CO2 double-breath inhalation is defined as an increase from pre-CO2 to post-CO2 challenge in the following: PSL-IV total scores ≥4 with at least 1-point increase for at least 4 of the symptoms specified in the PSL-IV and an increase on the Visual Analog Scale (VAS) Fear of at least 25 mm.
3. BMI of 18-32 kg/m_2 (inclusive).
4. Non-smoker for at least 3 months.

4.3 Exclusion criteria

1. Subjects with a clinically significant current or past personal or family history of any psychiatric disorder as classified by DSM-4 or DSM-5 criteria.
2. Current or past history of alcohol or any substance abuse or dependence disorder within the past 12 months.
3. Clinically significant ECG abnormalities.
4. Clinically significant abnormality of the lungs (e.g., COPD, asthma, lung fibrosis) and hematologic diseases concerning hemoglobin (e.g., thalassemia and sickle cell disease).
5. Important cardiovascular history, or suspicion of infarct, cardiomyopathy, cardiac failure, TIA, angina pectoris, cardiac arrhythmias, CVA.
6. Personal or familial history of cerebral aneurysm.

4.4 Concomitant medications

4.4.1 Allowed concomitant medications

Certain concomitant medications are allowed, only after the rationale is clearly documented by the investigator.

4.4.2 Prohibited concomitant medications

This study prohibits the use of benzodiazepines, non-benzodiazepine sleep medications, as well as any serotonergic medications. Any subjects who have used these medications within the last 90 days are ineligible to participate.

4.5 Lifestyle restrictions

- Subjects are not required to fast before the performance of the CO2 inhalation challenge.
- During the first, second, and fifth visits, the subjects is asked not to brush their teeth because of the saliva collection. Also, no drinking or eating 30 minutes before the saliva collection.

- The use of (illicit) drugs can influence the measurements. Therefore, using 'drugs' is not permitted from 30 days before conducting the first CO2 challenge and until the last CO2 inhalation challenge has been completed.
- Alcohol will not be allowed from at least 48 hours before each scheduled visit, and whilst in the study unit until the last CO2 challenge has been completed. Subjects may undergo an alcohol breath test at the discretion of the investigator.
- Subjects will abstain from the use of tobacco-or nicotine-containing products (including e-cigarettes and patches) for three months prior to conducting the first CO2 challenge until the last CO2 inhalation challenge has been completed.

5 INVESTIGATIONAL MEDICINAL PRODUCT

5.1 Treatment assignment and blinding

5.1.1 Randomization and treatment assignment

Study type	Cross-over for visits 1 and 2
Number of treatments *	NA
Total number of subjects	20
Number of cohorts	2
Ratio of active:placebo per cohort	NA
Sentinel	NA
Stratification	No
Randomise in blocks	No
Block size	NA
Subject numbering	1 to 20, replacements 91 to 921

The randomization code will be generated by a study independent, CHDR statistician. The randomization code will be unblinded/broken and made available for data analysis only after study closure, i.e., when the study has been completed, the protocol deviations determined, and the clinical database declared complete, accurate and locked. The randomization code will be kept strictly confidential. Sealed individual randomization codes, per subject and per treatment, will be placed in a sealed envelope with the label 'emergency decoding envelopes' in a safe cabinet at CHDR.

5.1.2 Blinding

Not applicable.

6 STUDY ASSESSMENTS

See Table 1 for the time points of the assessments.

6.1 Safety and tolerability assessments

The definitions, reporting and follow-up of AEs, SAEs and potential pregnancies are described in section 7.

6.1.1 Electrocardiography

ECGs will be obtained during the study using Marquette 2000/5500 and stored using the MUSE Cardiology Information System. ECGs will be taken after at least 5 minutes in the supine position. The investigator will assess the ECG recording as 'normal', 'abnormal - not clinically significant', or 'abnormal - clinically significant' and include a description of the abnormality as required. The ECG parameters assessed will include heart rate, PR, QRS, QT, and QTcF (calculated using Fredericia's method).

6.1.2 Safety laboratory assessments

No laboratory safety assessments will be done for this study.

6.2 Pharmacokinetic assessments

Not applicable.

6.3 Pharmacodynamic assessments and questionnaires

6.3.1 CO2 Challenge

Intrapulmonary administration of carbon dioxide (CO2) reliably induces panic-related symptoms in human subjects. Past reports after inhaling CO2 have described symptoms like palpitations, sweating, shivering or trembling, feeling of shortness of breath, increased breathing rate, increased blood pressure and heart rate, chest pain, nausea, dizziness, tingling in the fingers, headache, deterioration of vision and hearing, feeling of losing control, or having disturbing thoughts. Some people also feel as though they are suffocating or dying. The acute inhalation of either single or double breath vital capacity CO2 has been validated as a challenge test developed to experimentally provoke symptoms of panic in healthy volunteers, high risk individuals and patients with anxiety disorders. As a result, acute CO2 inhalation can be applied in pathophysiological research and in assessing potential panicolytic effects of novel compounds in drug development. The CO2 inhalation challenge is a translational model used as an instrument that safely and reliably induces panic attacks (PAs) by the protocolized administration of inhaled 35% CO2. In addition, the CO2 inhalation challenge simultaneously measures cardiovascular changes associated with CO2-induced autonomic nervous system activation such has heart rate and blood pressure. In contrast to previous experimental CO2 set ups, the CO2 inhalation challenge accompanying instrument (Finapres) yields integrated real time information on autonomic nervous system panic-related parameters following acute CO2 inhalation which can be readily combined with subjective fear-related phenomena. Please refer to the Standard Operational procedure of CHDR for more information. User experience and subjective burden questionnaire.

6.3.2 Panic Symptom List-IV

The PSL-IV is used to evaluate panic symptomatology. It consists of a questionnaire containing 13 items, each item derived from those listed for panic disorder in the Diagnostic and Statistical Manual of Mental Disorders, version 4 (DSM-4). The PSL-IV uses an ordinal scale, ranging from 0 (not at all) to 4 (very severe).

6.3.3 Visual Analog Scale – Fear

Feelings of fear will be rated by subjects using a VAS, consisting of a horizontal line 100 mm in length. Subjects will indicate their fear level along the line, with 0 corresponding to “no fear” and 100 corresponding to “the most fear possible”. The fear intensity will also be measured continuously after

the CO2 challenge with subjects rating their fear intensity using a 100% electronic VAS (eVAS)-slider, with 0 and 100 defined as ‘no fear’ and ‘most fear possible’ respectively. The VAS provides a good estimate of rapid changes of aspects of mood states.

6.3.4 Visual Analog Scale – Discomfort

Feelings of discomfort will be rated by subjects using a VAS, consisting of a horizontal line 100 mm in length. Subjects will indicate their fear level along the line, with 0 corresponding to “no discomfort” and 100 corresponding to “the most discomfort possible.” The VAS provides a good estimate of rapid changes of aspects of mood states.

6.3.5 Finapres NanoCore Physiological Measurements

The Finapres NanoCore system is a non-invasive, continuous system that measures blood pressure and heart rate with a sampling frequency of up to 200 Hz. This system will be utilized to obtain blood pressure and heart rate measurements 15 minutes prior to and for 15 minutes following the CO2 challenge to be used in physiological change PD analyses.

6.3.6 State-Trait Anxiety Inventory (STAI)

The ‘Zelfbeoordelingsvragenlijst’, the Dutch version of the Spielberger State-Trait Anxiety Inventory (Ploeg et al., 1979 and Spielberger et al., 1970) includes two sets of alternate self- rating scales, each consisting of 20 statements, which are used by the subject to describe his/her feelings. The scales use a four-point score system, with steps: 4 ‘not at all’- 3 ‘somewhat’- 2 ‘quite a bit’- 1 ‘very much’. The steps are translated into numerical scores, according to the log provided in the Appendix Scoring Keys. The same key is used to calculate scores for the STAI-DY-1 and –2. The scores allow an assessment of two distinct concepts of anxiety:

State anxiety: a transient emotional condition, characterised by subjective tension or stress (varying in intensity and fluctuating in time) and by a state of arousal of the autonomous nervous system.

Trait anxiety: a general disposition to anxiety, characterised by a tendency to react with increasing intensities of state anxiety, in response to perceived threatening experiences.

STAI-DY1 is used as a biomarker for anxiolytic or potentially anxiogenic agents (Gijsman et al., 1998). State anxiety trait scores are used to measure baseline propensity to anxious reactions. This can affect reactivity to sedative agents and may interfere with general CNS-performance. STAI-DY2 is measured as background information for STAI-DY1, e.g., to study the influence of anxiety levels on the PK/PD-relationships of benzodiazepines in chronic benzodiazepine-users (Van Steveninck et al., 1997).

6.3.7 The Dutch Temperament and Character Inventory (TCI)

The Dutch Temperament and Character Inventory (TCI – Duijsens et al., 2004) consists of 240 bi-directional true-false alternatives, allowing a detailed assessment of personality traits, with a good internal consistency (Duijsens and Spinhoven, 2004; Duijsens et al 200029, Otani et al. 200830). Temperament is characterized on four dimensions: novelty seeking, harm avoidance, reward dependence, and persistence; character on three dimensions: self-directedness, cooperativeness, and self-transcendence. The four temperament dimensions are assumed to be underlined by specific neurotransmission systems (Evren et al., 2007).

6.3.8 The Dutch Personality Questionnaire (DPQ)

The Dutch Personality Questionnaire-2-Revised (DPQ-2-R) is the most frequently used (clinical) personality questionnaire in The Netherlands. The DPQ-2-R has satisfactory reliability, validity, and internal consistencies, and consists of 140 items (Barelds et al., 2014). All items are answered on a bi-directional three-point scale (true -? - false), equally divided into seven scales of personality aspects: neuroticism, social anxiety, rigidity, hostility, egoism, dominance, and self-esteem. The DPQ-2-R also has subscales; neuroticism has the subscales: depression and anxiety, social anxiety has

the subscales shyness and social avoidance, rigidity has subscales for neatness and inflexibility and dominance has subscales for leadership and independence.

6.3.9 ACTH, Cortisol, Prolactin, and Orexin-A in plasma

Blood samples for determination of plasma concentrations of ACTH, cortisol, prolactin and orexin-A will be collected at the timepoints identified in the Schedule of Assessments.

6.3.10 Cortisol and alpha-amylase in saliva

Saliva samples for determination of saliva concentrations of alpha-amylase and cortisol will be collected at the timepoints identified in the Schedule of Assessments.

6.4 Sequence of assessments and time windows

When the following assessments are scheduled to be performed at the same time-point, the order of priority will be as follows: ECG, efficacy endpoints, laboratory assessments.

The deviations of actual time points from the expected time points will be within ten percent, calculated from the zero point (time of drug administration). The expected timepoints are defined as the timepoints in Promasys. Deviations of more than 10% will be explained in a note.

7 SAFETY REPORTING

7.1 Definitions of adverse events

An Adverse Event (AE) is any untoward medical occurrence in a subject who is participating in a clinical study performed. An AE can therefore be any unfavourable and unintended sign (including a vital sign finding), symptom, or disease temporally associated with the study participation, whether or not it is related to the study procedure.

7.1.1 Intensity of adverse events

The intensity of clinical AEs is graded three-point scale as defined below:

- Mild: discomfort noticed but no disruption of normal daily activity;
- Moderate: discomfort sufficient to reduce or affect normal daily activity;
- Severe: inability to work or perform daily activity.

7.1.2 Relationship to study procedure

For each AE the relationship to study procedure as judged by the investigator:

- Probable;
- Possible;
- Unrelated.

7.1.3 Chronicity of adverse events

The chronicity of the AE will be classified by the investigator on a three-item scale as defined below:

- Single occasion: single event with limited duration;
- Intermittent: several episodes of an event, each of limited duration;
- Persistent: event which remained indefinitely.

7.1.4 Action

Eventual actions taken will be recorded.

7.1.5 Serious adverse events

A Serious Adverse Event (SAE) is defined by the International Conference on Harmonization (ICH) guidelines as any AE fulfilling at least one of the following criteria:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a SAE.

7.1.6 Suspected unexpected serious adverse reactions

A SUSAR (Suspected Unexpected Serious Adverse Reaction) is a SAE that is unexpected, (nature or severity of which is not consistent with the applicable product information (e.g., investigator's brochure for an unauthorised investigational product or summary of product characteristics for an

authorised product)) and suspected (a reasonable possibility of causal relationship with the CO2 challenge).

7.1.7 Reporting of serious adverse events

SAEs and SUSAR's will be reported according to the following procedure.

The investigator will report expedited the following SUSARs through the web portal ToetsingOnline to the EC:

- SUSARs that have arisen in the clinical trial that was assessed by the EC;

The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the EC, CA and the Dutch Medicines Evaluation Board, a separate notification is not necessary. To prevent a double notification, it must be indicated in ToetsingOnline if the SUSAR is reported in the EMA EudraVigilance database, this will prevent the notification of the CA and the Dutch Medicines Evaluation Board through the web portal ToetsingOnline.

The expedited reporting will occur not later than 15 days after the first knowledge of the adverse reactions. For fatal or life-threatening cases, the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

7.1.8 Follow-up of adverse events

All AEs will be followed until they have abated, returned to baseline status or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

7.2 Concomitant medications

Concomitant medications initiated, stopped, up-titrated or down-titrated for an AE will be recorded.

7.3 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the investigator will inform the subjects and the EC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the EC, except insofar as suspension would jeopardise the subjects' health. The investigator will ensure that all subjects are kept informed.

7.4 Annual safety report or development safety update report

In addition to the expedited reporting of SUSARs, the investigator will submit, once a year throughout the clinical trial, a safety report to the EC, CA and CAs of the concerned Member States. This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

Submission of an annual safety update report is not deemed applicable to this study. Besides the inhalation of CO_2 as challenge model, no pharmacological compounds will be administered.

7.5 Pregnancy

If a woman becomes pregnant during the study, the investigator must counsel the subject and discuss the risks of continuing with the pregnancy and the possible effects on the foetus. Monitoring of the subject should continue until the outcome of the pregnancy is known. The subject must discontinue with the study when the subject is pregnant.

8 STATISTICAL METHODOLOGY AND ANALYSES

8.1 Statistical analysis plan

All safety and statistical programming are conducted with SAS 9.4 for Windows or newer (SAS Institute Inc., Cary, NC, USA).

8.2 Protocol violations/deviations

Protocol deviations will be identified based on conditions related to the categories below:

- Protocol entry criteria
- Forbidden concomitant medications
- Missing evaluations for relevant endpoints
- Other protocol deviations occurring during study conduct.

Major protocol deviations will be identified before the study closure and listed where appropriate.

8.3 Power calculation

Not applicable.

8.4 Missing, unused and spurious data

All missing or incomplete safety and PD data, including dates and times, are treated as such. Missing test results or assessments will not be imputed. Missing PD data, indicated as 'M' in the data listing, will be estimated within the statistical mixed model using SAS PROC MIXED.

The handling of missing, unused, and spurious data will be documented in the study report.

8.5 Analysis sets

Data of all subjects participating in the study will be included in the analyses if the data can meaningfully contribute to the objectives of the study.

8.5.1 Safety set

The safety population will be defined as all subjects who were validated (randomised) and received at least 1 dose of study treatment.

8.5.2 Pharmacodynamic analysis set

The analysis population for pharmacodynamics is defined as all subjects who were validated (randomised), performed at least one CO2 challenge, and have at least one post-baseline assessment of the parameter been analysed.

8.6 Subject disposition

Subject disposition will be listed by subject.

The following subject data will be summarized:

- number and percentage of subjects screened,
- number and percentage of subjects enrolled,
- number and percentage of subjects completed,
- number and percentage of subjects included in safety population and
- number and percentage of subjects included in the PD analysis population.

A subject who completed the study is defined as a subject which performed 5 CO_2 challenges adequately.

8.7 Baseline parameters and concomitant medications

8.7.1 Demographics and baseline variables

Continuous demographic variables (e.g., age, height, weight, BMI) will be summarized by descriptive statistics (n, mean, SD, median, Min, Max).

Qualitative demographic characteristics (sex, race/ethnicity) will be summarized by counts and percentages.

8.7.2 Medical history

Medical history will only be listed.

8.7.3 Concomitant Medications

Previous and concomitant medications will be listed by International Nonproprietary Names, dose, regimen, route and for which indication it was prescribed.

8.8 Safety and tolerability endpoints

The safety set is used to perform all safety analyses.
Baseline is defined as the average value prior to conducting the CO2 inhalation challenge. Change from baseline will be calculated for all continuous safety parameters.

8.8.1 Adverse events

The AE coding dictionary for this study will be Medical Dictionary for Regulatory Activities (MedDRA). It will be used to summarize AEs by primary system organ class (SOC) and preferred term (PT).
All adverse events will be displayed in listings.

A treatment-emergent adverse event (TEAE) is defined as an adverse event observed after starting the CO2 challenge. If a subject experiences an event both prior to and after starting administration of the CO2 challenge, the event will be considered a TEAE only if it has worsened in severity (i.e., it is reported with a new start date) after starting administration of the specific treatment, and prior to the start of another treatment, if any. All TEAEs collected during the investigational period will be summarized.

The number of and the number of subjects with treatment emergent AEs will be summarized by:

1. treatment, MedDRA SOC and PT;
2. treatment, MedDRA SOC, PT and severity;
3. treatment, MedDRA SOC, PT and drug relatedness.

8.8.2 ECG

At each time point, absolute values and change from baseline of ECG numeric variables will be summarized with the number of samples (n), mean, SD, SEM, median, Min, and Max values. The number of available observations and out-of-range values (absolute and in percentage) will be presented. Values outside the investigator's normal range will be flagged in the listing. 'H' and 'L', denoting values above or below the investigator reference range (when present), will flag out-of-range results.

8.9 Pharmacodynamic endpoints

The final analysis will be preceded by a data review which consists of individual graphs per visit by time of all pharmacodynamic measurements by time. The graphs will be used to detect outliers and measurements unsuitable for analysis.
The PD parameters will be listed by treatment, subject, visit and time. Individual graphs by time will be generated.

All repeatedly measured PD endpoints will be summarized (n, mean, SD, SEM, median, Min and Max values) by treatment and time, and will also be presented graphically as mean over time, with standard deviation as error bars. The occurrence of PAs will be summarized in a frequency table.

Parameters will initially be analysed without transformation, but if the data suggest otherwise, log-transformation may be applied. Log-transformed parameters will be back-transformed after analysis where the results may be interpreted as percentage change.

8.9.1 Occurrence of PAs

The difference in response between single and double vital capacity 35% CO2/65% O2 in terms of the occurrence and intensity of PAs in CO2 sensitive healthy subjects, which is assessed with the Panic Symptoms List-IV (PSL-IV), VAS Discomfort, and VAS subjective fear.

For the occurrence of PAs repeated measures logistic regression will be used with challenge (single/double) as fixed factor and subject as random factor.

For the intensity of Pas repeated measures mixed model ANCOVA with challenge (single/double) as fixed factor, subject as random factor and baseline as covariate will be used.

8.9.2 Test-Retest Reliability

The test-retest reliability of the double vital capacity 35% CO2/65% O2 in healthy subjects who have previously been tested sensitive to the panicogenic effects of the CO2 challenge, which is assessed with the Panic Symptoms List-IV (PSL-IV), VAS Discomfort, and VAS subjective anxiety and fear.

Test-retest reliability will be analyzed with repeated measures mixed model ANCOVA with challenge number as fixed factor, subject as random factor and baseline as covariate.

8.9.3 Occurrence of tolerance after repeated exposure

The occurrence and intensity of PAs in CO2 sensitive healthy subjects caused by a double (and single) vital capacity inhalation of 35% CO2/65% O2.

For the occurrence of PAs repeated measures logistic regression will be used with challenge number as fixed factor and subject as random factor.

The intensity of PAs will be analyzed with repeated measures mixed model ANCOVA with challenge number as fixed factor, subject as random factor and baseline as covariate.

8.9.4 Stable trait phenomenon

The occurrence and intensity of PAs in CO2 sensitive healthy subjects caused by a double (and single) vital capacity inhalation of 35% CO2/65% O2 now and some time ago.

For the occurrence of PAs repeated measures logistic regression will be used with challenge number as fixed factor and subject as random factor.

The intensity of PAs will be analyzed with repeated measures mixed model ANCOVA with challenge number as fixed factor, subject as random factor and baseline as covariate.

8.10 Exploratory analyses and deviations

Exploratory data-driven analyses can be performed with the caveat that any statistical inference will not have any confirmatory value.
Deviations from the original statistical plan will be documented in the clinical study report.

8.10.1 Changes in Heart Rate, Blood Pressure, and Respiratory Rate

Explore the effects of single and double breath 35% CO2/65% O2 on heart rate and blood pressure. This is assessed with the difference in mean change from pre-CO2 to post-CO2 challenge in vital sign measurements (systolic blood pressure, diastolic blood pressure, heart rate) related to the cardiovascular response to CO2 inhalation challenge using Finapres Assessments. These endpoints will be analyzed with repeated measures mixed model ANCOVA with challenge (single/double) as fixed factor, subject as random factor and baseline as covariate.

8.10.2 Relationship between Baseline Personality and Temperament Characteristics and Individual Response to CO2

Explore the relationship between baseline personality and temperament characteristics and individual response to CO2 in healthy CO2-sensitive subjects. This is assessed with the Dutch Personality

Questionnaire (DPQ), Cloninger Temperament Character Inventory (TCI), and Spielberger State-Trait Anxiety Inventory (STAI). Correlation/regression will be used to examine relationships among variables and the outcomes.

8.11 Interim analyses

No interim analysis is planned.

9 GOOD CLINICAL PRACTICE, ETHICS AND ADMINISTRATIVE PROCEDURES

9.1 Good clinical practice

9.1.1 Ethics and good clinical practice

The investigator will ensure that this study is conducted in full compliance with the protocol, the principles of the Declaration of Helsinki (www.wma.net), ICH GCP guidelines (http://www.ich.org/products/guidelines.html), and with the laws and regulations of the country in which the clinical research is conducted.

9.1.2 Ethics committee

The investigator will submit this protocol and any related documents to an Ethics Committee (EC) and the Competent Authority (CA). Approval from the EC and the statement of no objection from the CA must be obtained before starting the study and should be documented in a dated letter/email to the investigator, clearly identifying the trial, the documents reviewed and the date of approval. A list of EC members must be provided, including the functions of these members. If study staff were present, it must be clear that none of these persons voted.

Modifications made to the protocol after receipt of the EC approval must also be submitted as amendments by the investigator to the EC in accordance with local procedures and regulations.

9.1.3 Informed consent

It is the responsibility of the investigator to obtain written informed consent from each individual participating in this study after adequate information of the study.

Potential subjects will reach out to CHDR when they are interested in participation. After a brief explanation as stated in E3. Document, we plan a meeting where we thoroughly explain the ICF to the subjects – the information visit. This information includes the aims, methods, objectives and potential hazards of the study and an explanation that subjects are completely free to refuse to enter the study or to withdraw from it at any time for any reason. After this information visit, subjects can sign the Informed Consent if they are still interested in the study participation and their study participation can begin. Subjects always have the opportunity to discuss their study participation and questions with a CHDR physician before the information visit by phone or during the information visit, screening or clinical conduct with the CHDR physician. In accordance with regulations, sufficient time is given for subjects to understand what their participation means, but no minimum amount of time is specified or required.

The Informed Consent and Subject Information will be provided in Dutch.

9.1.4 Insurance

The investigator has a liability insurance which is in accordance with article 7, subsection 6 of the WMO.
The investigator also has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23rd June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

- € 650,000.- (i.e., six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
- € 5,000,000.- (i.e., five million Euro) for death or injury for all subjects who participate in the Research;
- € 7,500,000.- (i.e., seven million and five hundred thousand Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as ‘verrichter’ in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

9.2 Study funding

CHDR is the sponsor of the study and is funding the study.

9.3 Data handling and record keeping

9.3.1 Data collection

Data will be recorded on electronic data collection forms in Promasys for subsequent tabulation and statistical analysis. The data will be handled confidentially.

A Subject Screening and Enrolment Log will be completed for all eligible or non-eligible subjects with the reasons for exclusion.

Data will be recorded on paper data collection forms and will be entered after quality control in a Promasys database for tabulation and statistical analysis. The data will be handled confidentially.

A Subject Screening and Enrolment Log will be completed for all eligible or non-eligible subjects with the reasons for exclusion.

9.3.2 Database management and quality control

All data from paper source will be entered into the Promasys database twice, by two different individuals. A quality control check will be done by CHDR staff on all data entered in the Promasys database, using data entry progress checks and database listings (blind data review). Errors with obvious corrections will be corrected before database lock.
After the database has been declared complete and accurate, the database will be locked. Any changes to the database after that time can only be made by joint written agreement between the investigator and the statistician.

9.3.3 Access to source data and documents

All study data will be handled confidential. The investigator will retain the originals of all source documents generated at CHDR until at least database lock, after which all study-related documents will be archived in an outside storage location. Administrative files are archived when a study is finished, defined as when the protocol status is FIN in the study life cycle of the Promasys. Clinical files are archived after the database lock, this is when the phase is transferred from DAT to ANA in Promasys. Financial files are archived after final completion. This outside storage facility has appropriate environmental controls and adequate protection from fire, flood and unauthorized access to secure long-term archiving. Study data will be archived for 25 years, after which it will be destroyed.

The investigator will permit trial-related monitoring, audits, EC review and regulatory inspections, providing direct access to source data and documents.

9.4 Quality control and quality assurance

This study will be conducted according to applicable Standard Operating Procedures (SOPs). Quality assurance will be performed under the responsibility of CHDR's Quality Assurance manager.

9.4.1 Monitoring

An initiation visit will be performed before the first subject is included. Monitoring visits and contacts will occur at regular intervals thereafter, according to a frequency defined in the study-specific monitoring plan. A close-out visit will be performed after study closure.

9.5 Protocol amendments

Any change to a protocol has to be considered as an amendment.

9.5.0 Substantial amendment

Significant changes that affect subject safety and/or the scientific value of a trial require a substantial amendment. Examples of significant changes are given in EU guidelines on the request to the competent authorities for authorisation of a clinical trial on a medicinal product for human use, the notification of substantial amendments and the declaration of the end of the trial (CT-1, 2010/C 82/01). Substantial amendments are to be approved by the appropriate EC and the CA will need to provide a 'no grounds for non-acceptance' notification prior to the implementation of the substantial amendment.

9.5.1 Non-substantial amendment

Non substantial amendments do not affect subject safety or the scientific integrity of the trial. Non-substantial amendments will be approved (signed) by the investigator(s) and will be recorded and filed by the investigator. Non-substantial amendments will be submitted to the EC for information only. The CA will only be notified by changes in Eudract form and ABR form (if applicable) at toetsingonline. The implementation of a non-substantial amendment can be done immediately.

The EU guideline CT-1 2010/C 82/01 stipulates the importance of preventing over-reporting. Therefore the following changes are by definition non-substantial in this study:

- change in amount and timing of the samples (maximum of 2 samples without a > 50 ml increase in the amount of blood taken and not exceed 500 ml of blood in total)
- changes in assay-type and / or institution where an assay will be performed, provided that validated assays will be used;
- editorial changes to documents in the submission dossier including the volunteer information sheets and the protocol. An editorial change is defined as a modification in the documents of typographical errors and other modifications that in no way alter the meaning or content of the document
- determination of additional parameters in already collected materials, which are in agreement with the study objectives and do not provide prognostic or genetic information;
- other statistical analyses than described in the protocol.
- A change in clinical staff, including the principal investigator, when this concerns regular staff members of CHDR who comply with internal regulations for training and authorisation.
- A change in dosing schedule in an ascending dose trial, provided the expected exposure of the subjects does not exceed the preset values indicated in this protocol.

9.5.2 Urgent amendment

An urgent amendment might become necessary to preserve the safety of the subjects included in the study. The requirements for approval should in no way prevent any immediate action being taken by the investigators in the best interests of the subjects. Therefore, if deemed necessary, an investigator can implement an immediate change to the protocol for safety reasons. This means that, exceptionally, the implementation of urgent amendments will occur before submission to and approval by the EC(s) and CA.

9.6 End of study report

The investigator will notify the EC of the end of the study within a period of 8 weeks. The end of the study is defined as the last subject's last visit. In case the study is ended prematurely, the investigator will notify the EC within 15 days, including the reasons for the premature termination.

The investigator will notify the EC immediately of a temporary halt of the study, including the reason of such an action.

Within one year after the end of the study, the investigator will submit a final study report with the results of the study, including any publications/abstracts of the study, to the EC. The principal investigator will be the signatory for the study report.

9.7 Public disclosure and publication policy

In accordance with standard editorial and ethical practice, the results of the study will be published. The authorship guidelines of the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals will be followed regarding co-authorship.

The list of authors of any formal publication or presentation of study results may include, as appropriate, and will be determined by mutual agreement.

In accordance with standard editorial and ethical practice, the results of the study will be published. The authorship guidelines of the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals will be followed regarding co-authorship.

10 REFERENCES

[1] Leibold, N. K., van den Hove, D. L. A., Viechtbauer, W., Buchanan, G. F., Goossens, L., Lange, I., Knuts, I., Lesch, K. P., Steinbusch, H. W. M., & Schruers, K. R. J. (2016). CO2 exposure as translational cross-species experimental model for panic. Translational Psychiatry, 6(9), e885–e885. https://doi.org/10.1038/tp.2016.162

[2] Salvadore, G., Bonaventure, P., Shekhar, A. *et al.* Translational evaluation of novel selective orexin-1 receptor antagonist JNJ-61393215 in an experimental model for panic in rodents and humans. *Transl Psychiatry,* 10, 308 (2020). https://doi.org/10.1038/s41398-020-00937-9

[3] Rachel Gurrell, Ih Chang, Ann Dandurand, Sridhard Duvvuri, Amy Guigliano, Gina Pastino, Theresa Pham, Stacey Versavel, Gabriel Jacobs, Koshar Safai Pour, Rob Zuiker, Raymond Sanchez, John Renger. Phase 1 Trial of Darigabat for the Reduction of Acute Psychological and Physiological Panic and Fear Symptoms Following CO2 Inhalation in Healthy Participants (S8.005). Neurology Apr 2023, 100 (17 Supplement 2) 1752; DOI: 10.1212/WNL.0000000000202097

C1. CHDR2344_Research_Protocol_NL84999.0566_v1_12Sep2023

Final Audit Report 2023-09-13

Created:	2023-09-13 (Central European Summer Time)
By:	Ivo Tielbeek (itielbeek@chdr.nl)
Status:	Signed
Transaction ID:	CBJCHBCAABAAaRPDJaXSef_7MFD-s7DiB0uBdRdGjV5j

"C1. CHDR2344_Research_Protocol_NL84999.0566_v1_12Sep 2023" History

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Email viewed by K. Safai Pour (KSafaiPour@chdr.nl)
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K. Safai Pour (KSafaiPour@chdr.nl) authenticated with Adobe Acrobat Sign.
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K. Safai Pour (KSafaiPour@chdr.nl) authenticated with Adobe Acrobat Sign.
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K. Safai Pour (KSafaiPour@chdr.nl) authenticated with Adobe Acrobat Sign.

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Ivo Tielbeek (itielbeek@chdr.nl) replaced signer G. Jacobs (Gjacobs@chdr.nl) with G.J. Groeneveld (ggroeneveld@chdr.nl)

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Agreement completed.

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