# Supplemental Material

Table of Contents

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item # | Description | Page |
|---|---|---|---|
| **Administrative information** | | | |
| Title | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym | M, 1 |
| Trial registration | 2a | Trial identifier and registry name. If not yet registered, name of intended registry | M, 3 |
| | 2b | All items from the World Health Organization Trial Registration Data Set | SP, 4 |
| Protocol version | 3 | Date and version identifier | SP, 4 |
| Funding | 4 | Sources and types of financial, material, and other support | SP, 4 |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors | SP, 5 |
| | 5b | Name and contact information for the trial sponsor | SP, 5 |
| | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities | SP, 5 |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) | SP, 5 |
| **Introduction** | | | |
| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | M, 5 |
| | 6b | Explanation for choice of comparators | SP, 6 |
| Objectives | 7 | Specific objectives or hypotheses | SP, 7 |

| | | | |
|---|---|---|---|
| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | SP, 7 |
| **Methods: Participants, interventions, and outcomes** | | | |
| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | SP, 7 |
| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | SP, 7 |
| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | SP, 8 |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | SP, 10 |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | SP, 10 |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | SP, 10 |
| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | SP, 12 |
| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | SP, 12 |
| Sample size | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations | SP, 13 |

| | | | |
|---|---|---|---|
| Recruitment | 15 | Strategies for achieving adequate participant enrolment to reach target sample size | SP, 13 |
| **Methods: Assignment of interventions (for controlled trials)** | | | |
| Allocation: | | | |
| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions | SP, 14 |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned | SP, 14 |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions | SP, 14 |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how | SP, 15 |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial | SP, 15 |
| **Methods: Data collection, management, and analysis** | | | |
| Data collection methods | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol | SP, 15 |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols | SP, 15 |
| Data management | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol | SP, 16 |

| | | | |
|---|---|---|---|
| Statistical methods | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol | SP, 17 |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) | SP, 17 |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) | SP, 17 |
| **Methods: Monitoring** | | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed | SP, 18 |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial | SP, 18 |
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct | SP, 18 |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor | SP, 18 |
| **Ethics and dissemination** | | | |
| Research ethics approval | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval | SP, 18 |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) | SP, 18 |
| Consent or assent | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) | SP, 19 |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable | SP, 19 |

| | | | |
|---|---|---|---|
| Confidentiality | 27 | How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial | SP, 19 |
| Declaration of interests | 28 | Financial and other competing interests for principal investigators for the overall trial and each study site | SP, 19 |
| Access to data | 29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators | SP, 19 |
| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation | SP, 19 |
| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions | SP, 19 |
| | 31b | Authorship eligibility guidelines and any intended use of professional writers | SP, 19 |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code | SP, 19 |
| **Appendices** | | | |
| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates | SP, 20 |
| Biological specimens | 33 | Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable | SP, 20 |

Abbreviations: M, manuscript; SP, supplemental study protocol documentation.
Chan A et al. SPIRIT 2013 Statement: Defining standard protocol items for clinical trials. *Ann Intern Med* 2013; 158:200-207.

# DETAILED STUDY PROTOCOL

## TABLE OF CONTENTS

## DETAILED STUDY PROTOCOL

### I. List of Abbreviations

**AIH:** acute intermittent hypoxia

**AIS:** American Spinal Injury Association Impairment Scale

**α:** alpha level, established Type I error rate for power analyses

**ANOVA:** analysis of variance

**ANCOVA:** analysis of covariance

**BP:** blood pressure

**BL:** baseline

**BDNF:** brain-derived neurotrophic factor

**$C_2$:** spinal cord cervical level 2

**C:** caffeine

**CI:** confidence interval

**CRP:** c-reactive protein

**$D_i$:** $i^{th}$ day of intervention

**ERK MAP:** extracellular signal-regulated kinases; mitogen-activated protein kinases

**ECG:** electrocardiography

**5-$HT_2$:** receptor that binds endogenous serotonin neurotransmitter

**$FiO_2$:** fraction of inspired oxygen

**F:** follow-up

**Gq:** guanosine nucleotide-binding protein

**HIPAA:** Health Insurance Portability and Accountability Act

**HR:** heart rate

**iSCI:** incomplete spinal cord injury

**LEMS:** lower extremity motor score

**$L_5$:** spinal cord lumbar level 5

**MAP:** mitogen-activated protein kinases

**m:** meter

**min:** minute

**mmHg:** millimeter of mercury

**P:** placebo

**RR:** respiratory rate

**SCI:** spinal cord injury

**s:** second

**6MWT:** 6-minute walk test

**SBP:** systolic blood pressure

**SCATS:** spinal cord assessment tool for spastic reflexes

**SCI-FAI:** spinal cord injury functional ambulatory index

**SDB:** sleep-disordered breathing

**$SpO_2$:** blood oxygen saturation level

**10MWT:** 10-meter walk test

**TrkB:** tyrosine kinase B receptor

**TUG:** timed up-and-go test

**WISCI:** walking index for spinal cord injury

## II. Administration Information

### 1. Title

Mechanisms of intermittent hypoxia-induced motor recovery in persons with SCI

### 2. Trial registration

ClinicalTrials.gov Identifier: NCT02323698

Registry name: Effects of caffeine and intermittent hypoxia on leg function in human spinal cord injury

### 3. Protocol version

Detailed Protocol Summary, Last updated: September 27, 2021

### 4. Funding

National Institutes of Health, HD081274

Wings for Life Foundation WFL US-026/14

## 5. Roles and responsibilities (delegation of authority)

**Study Title:** Mechanisms of intermittent hypoxia-induced motor recovery in persons with SCI

**IRB #:** 2017P001940
Principal Investigator: Randy Trumbower, PT, PhD
Sponsors**:** Spaulding, NIH-NICHD, Wings for Life International
Study Site**:** Spaulding Rehabilitation Hospital

List delegated study related tasks and dates of involvement for each staff member in accordance with institutional guidelines and Good Clinical Practice (GCP). All IRB approved study staff should sign and initial this log. The PI should acknowledge delegation by signing his/her initials after each entry and at study 'close out' to attest to the fact that the list is complete, accurate and that all staff are accounted for. We will update this log in a timely manner as new personnel are added and/or study roles change.

| First Name | Last Name | Title | Start Date | End Date | Activities* |
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Activity codes:

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|---|---|---|---|---|---|---|---|
| 1 | Obtain Informed Consent | 2 | Assess Eligibility Criteria | 3 | Maintain IRB & Regulatory Documents | 4 | Essential Documentation |
| 5 | Obtain Medical History | 6 | Concomitant Medication Review | 7 | Dispense Study Drug/Device | 8 | Perform Protocol Tasks with Study Subjects |
| 9 | Review Lab and Procedure Results | 10 | Safety Monitoring | 11 | Data Monitoring | 12 | Collection, Handling, and Shipment of Samples |
| 13 | AE Report to Sponsor and/or IRB | 14 | CRF Queries | 15 | Perform Physical Exam | 16 | AE Grading and Attribution |

# III. Introduction

## 6. Background and Rationale

Spinal cord injury (SCI) disrupts connections between the brain and spinal cord, causing life-long paralysis and reduced mobility. As such, restoring walking ability remains a highly valued goal for persons with SCI.[1] Even small improvements in walking skills that enable a person with SCI to stand, walk within the home, or negotiate spaces not accessible to wheelchairs can translate into significant empowerment, health benefits, and improvement in quality of life. Because most spinal

injuries are incomplete, there are at least some spared neural pathways to the motor neurons that can initiate and coordinate movements necessary for walking recovery. Although injury-induced plasticity in spared spinal pathways enables partial spontaneous recovery of respiratory and somatic motor function,[2, 3] the extent of this recovery is slow, variable and frustratingly limited.[4] Thus, there is a critical need for new strategies that augment spinal plasticity and subsequently improve walking ability in persons with SCI.

Acute intermittent hypoxia (AIH) induces spinal plasticity, strengthening connections to motor neurons.[5-9] Considerable progress has been made towards an understanding of cellular mechanisms giving rise to AIH-induced respiratory plasticity.[7] Repetitive exposure to AIH enhances the expression of plasticity-promoting proteins in respiratory motor nuclei[9-11] and elicits profound recovery of breathing capacity in spinally injured rats.[12] Key steps in AIH-induced plasticity include episodic release of serotonin near respiratory motor neurons, initiating new synthesis of brain derived neurotrophic factor (BDNF) and subsequently activating its high affinity receptor, TrkB [5]. "Downstream" events include the activation of extracellular signal-regulated kinases (ERK); mitogen-activated protein (MAP) kinases and postulated insertion of glutamate receptors at the synapse between respiratory pre-motor and motor neurons. Excitingly, these changes extend beyond the respiratory system. Colleagues also observed that AIH enhances expression of these same proteins in *non-respiratory* motor neurons.[8]

Although dAIH may be a viable method to enhance walking after iSCI, the mechanisms underlying dAIH-induced motor recovery may be constrained by competing neural mechanisms of motor facilitation. While dAIH induces facilitation predominantly via serotonin-dependent mechanisms [13], even mild hypoxia causes ATP release from glia and other sources in the nervous system, leading to an accumulation of extracellular adenosine. Subsequent activation of $A_{2A}$ receptors elicits a distinct form of motor facilitation [14] that is known to compete with the serotonin-dependent pathway via cross-talk inhibition [15, 16]. Consequently, systemic administration of a selective $A_{2A}$ receptor antagonist, MSX-3, reduced cross-talk inhibition and increased AIH-induced long-term motor facilitation in the phrenic and hypoglossal nerves by nearly 50% and 20%, respectively, in rats [16]. The impact of adenosine $A_{2A}$ receptor antagonists on AIH-induced motor recovery have not yet been studied in persons with incomplete SCI (iSCI).

Our working hypothesis is that reducing crosstalk inhibition during dAIH with a well-tolerated $A_{2A}$ antagonist (caffeine) will enhance AIH-induced improvement of walking function in persons with chronic iSCI.

## 7. Objectives

The main objective of this clinical trial is to evaluate the effects of a common, safe adenosine $A_{2A}$ antagonist, caffeine, on recovery of walking following dAIH in persons with chronic iSCI. While highly specific $A_{2A}$ receptor antagonists, such as MXS-3 and ZM2412385, block cross-talk in rats,[16] their safety in SCI is not known. On the other hand, caffeine offers the ideal blend of known safety, metabolism, and efficacious $A_{2A}$ receptor antagonism.[17] Even at low plasma concentrations (1-10μM) after one cup of coffee, caffeine antagonizes $A_{2A}$ receptors,[17] and the efficacy of moderate caffeine in blocking neural $A_{2A}$ receptors is illustrated by the neuroprotective effects of regular caffeine consumption in dopaminergic transmission in Parkinson's Disease.[18] Although caffeine has many neurobiological actions, caffeine's actions at physiologically relevant doses (1-100 μM), including the proposed dose of up to 6 mg/kg body weight, are mediated by adenosine receptor antagonism versus phosphodiesterase inhibition, release of intracellular calcium stores,

or other site.[19] Our study will quantify the daily (5 consecutive days) effects of caffeine (e.g., 4 mg/kg) as an adjunctive therapy to dAIH for restoring walking function in persons with chronic iSCI. This dose has known safety in iSCI and substantial antagonism to $A_{2A}$ receptors.[17, 20, 21]

### 8. Trial Design

We will conduct a placebo-controlled, cross-over, block-randomized intervention study to assess the efficacy of daily caffeine prior to AIH on improving walking function in persons with chronic, motor-incomplete SCI.

## IV. Methods: Participants, interventions, and outcomes

### 9. Study setting

The following laboratory will be used as the primary site of this study:

INSPIRE Lab at Spaulding Hospital
1575 Cambridge Street
Cambridge MA 02138 USA

### 10. Eligibility criteria

Potential subjects will be informed about the objectives of the study and must meet all inclusion and exclusion criteria (see Appendix 1 for sample Informed Consent Form). They will be required to read and sign approved consent and HIPAA forms prior to participation to allow access to medical information from their physicians.

Inclusion criteria: Participation requirements for SCI participants include: 1) 18 to 75 years old; 2) medically stable with medical clearance from physician to participate; 3) SCI at or below $C_2$ and at or above $L_5$ with at least some lower extremity motor function preserved below the neurologic level; 4) non-progressive etiology of spinal injury; 4) AIS A-D at initial screen; 5) at least 6 months post-injury (chronic). We plan to choose subjects greater that 6 months post-injury to ensure minimal confounding effects of spontaneous neurological recovery during the experiments. This will mean that changes in sensorimotor performance are more likely due to the interventions associated with the research study.

Exclusion criteria: Individuals will be excluded due to: 1) severe concurrent illness or pain, including unhealed decubiti, severe neuropathic or chronic pain syndrome, infection (e.g. bladder), hypertension, cardiovascular disease, pulmonary disease, severe osteoporosis (history of fractures), active heterotopic ossification in the lower extremities, or history of peripheral nerve injury in the legs; 2) < 24 on Mini-Mental Exam [22]; 3) severe autonomic dysreflexia; 4) history of cardiovascular/pulmonary complications; 5) pregnancy because of unknown effects of AIH on a fetus, although women of childbearing potential will not otherwise be excluded; 6) undergoing concurrent physical therapy; 7) history of diabetes. Caffeine can potentially alter sugar metabolism and, thus, persons diagnosed with diabetes will be excluded from participating in the caffeine portion of this study; 8) history of cirrhosis. Cirrhosis is known to increase the elimination time of caffeine and, thus, persons diagnosed with cirrhosis will be excluded from participating in the caffeine portion of this study; and 9) history of caffeine allergies or intolerances. Participants with a history of caffeine allergies or intolerances will be excluded from participating in the caffeine study.

Inclusion of women and minorities: We will recruit persons with SCI to include women and minorities. To protect against possible unknown side effects of dAIH, women who are pregnant

may not take part in this study as the effects of dAIH on the developing fetus have not been studied. If a woman is of childbearing ability, birth control will be recommended for use throughout the study.

Our study coordinator, study physicians, physical therapists, PI (Trumbower), and research collaborators recruited subjects using a variety of strategies shown effective in the past. First, the team identified potential subjects using Institutional Review Board (IRB) approved resources to inform persons with SCI that are currently admitted to inpatient rehabilitation/outpatient units of Spaulding Rehabilitation Hospital, Boston University Medical Center, Massachusetts General Hospital, Gaylord hospital, Albany Medical Center, and Maine Medical Center. Subjects also will be recruited by word of mouth and flyers at these facilities. Eligible subjects screened at INSPIRE Laboratory, Spaulding Rehabilitation Hospital Cambridge, MA. At the screening visit, subjects informed of this project, their potential involvement, the possible benefits, and risks, and their right to terminate participation at any time without penalty. Qualifying subjects in agreement with the study details asked to sign the informed consent and HIPAA form approved by Partners IRB. All potential subjects entered the study according to the inclusion/exclusion criteria above and following medical clearance. Experiments will be conducted at the Spaulding Rehabilitation Hospitals.

## 11. Interventions

a. Intervention Procedures: This placebo-controlled study will examine the combined effects of caffeine (vs. placebo) and AIH (vs. SHAM) on walking performance in persons with chronic, iSCI. Participants will receive three combinatorial interventions in random order: placebo+AIH, caffeine+AIH, caffeine+SHAM. The order of interventions will be block randomized. The initial screening and evaluation procedures are similar for all participants. For each intervention, participants will complete up to 8 visits/intervention (1 baseline/intervention + 5 treatment days/intervention + 2 follow-up days/intervention). Participants will complete a total of 24 visits (8 visits/intervention x 3 interventions) after the initial screen. Participant enrollment and allocation will be completed during the screening visit. Baseline will be recorded up to 3 days prior to the first intervention.

   Caffeine intervention (caffeine or placebo): Before each intervention round, subjects will be asked to avoid caffeine-containing substances for 48 hrs. (half-life ~7 hrs.)[21, 23] prior to arrival to control for baseline plasma levels of caffeine.[20] Subjects will then ingest capsules containing either placebo (sucrose) or caffeine (up to 400 mg). USP and FCC grade caffeine and placebo capsules will be sourced to a private independent compounding pharmacy (Johnson Compounding Pharmacy, Waltham MA, USA). The study PI (Trumbower) will hold a Class VI Drug License and the study physician (Slocum) will write the prescription for caffeine or placebo and have the prescription sent to the compounding pharmacy. The compounding pharmacy will then dispense the caffeine or placebo to the subject for use in the study. Administration will also be overseen by a physician member of the study team.

   Persons experiencing caffeine-induced adverse side effects (e.g., dizziness) will be excluded. Since caffeine is a diuretic, we will encourage subjects to empty bladders prior to AIH (or SHAM). To monitor caffeine levels and identify subject-to-subject differences in absorption, blood samples will be collected before capsule consumption and post-AIH or SHAM intervention on days 1 and 5. [17, 24, 25]

*Safety considerations for caffeine:* Caffeine is commonly used on a daily basis, with an average adult consumption of up to ~6 mg of caffeine per kg of body weight and heavy consumers consuming ≥ 9 mg/kg,[19, 26] including in persons with SCI [27]. High doses of > 6 mg/kg caffeine have been tested in humans for athletic performance enhancement without reported adverse effects.[20, 28, 29] In persons with SCI, 6 mg/kg has been shown enhance athletic performance without reported adverse effects [20, 27] and no significant or unsafe changes in blood pressure, heart rate, or respiration were seen.[27] Thus, up to 6 mg/kg is within a reasonable and safe range, and that caffeine was safe and well-tolerated by persons with SCI.

Breathing intervention (AIH or SHAM): Breathing interventions will begin 30 min after caffeine or placebo to approximately coincide with peak plasma caffeine concentrations.[30] Participants will don a latex-free, full non-rebreather mask with a custom neoprene head strap while they sit comfortably in a semi-reclined chair. Intermittent gas mixtures will be delivered via automatic adjustment of one-way valves attached to a hypoxia generator [HYP123, Hypoxico Inc., USA].[31] We will deliver preset air mixtures of $FIO_2$=0.09±0.02 (hypoxia) or $FIO_2$=0.21±0.02 (normoxia) for 1.5min at 1 min intervals of room air breathing. The generator will fill reservoir bags attached to a non-rebreathing facemask worn by subjects. Oxygen concentration will be continuously monitored [OM-25RME; Maxtec Inc, USA]. We will take precautions to ensure safety during interventions by monitoring 5-lead electrocardiography (ECG) and recording oxyhemoglobin saturation via pulse oximetry ($SpO_2$) and heart rate (HR) at 2-second intervals. Respiratory rate (RR) and blood pressure (BP) will be recorded every 5$^{th}$ breathing interval [Dash 4000 Patient Monitor, GE]. We also will record $SpO_2$, HR, RR, and BP at the start and end of walking practice and again at the end of each treatment session.

*Safety considerations for AIH:* The physiological effects of AIH in humans are well studied and the methods proposed here deemed safe. Daily AIH-induced enhancements in breathing capacity, limb strength, and walking function occurred without significant adverse events in humans with chronic SCI.[32-34] Although prolonged chronic exposure to more severe and longer duration hypoxia causes serious morbidity,[35] considerable data show that repetitive exposure to brief mild AIH elicits meaningful functional benefits without pathology.[8, 36, 37] With mild AIH, the severity of hypoxemia, number of episodes, and exposure duration are low (15 episodes per day, 5 days) versus the chronic hypoxia experienced in obstructive sleep apnea (80-400 episodes per day for years [35, 38]). Daily AIH (10 episodes/day, 7 days) showed no evidence for hypertension or weight loss [9] or hippocampal gliosis or cell death in rats with SCI.[8] Prior study in persons with SCI found no adverse events associated with mild exposures of single-day and dAIH (5 days), and all subjects tolerated exposures well.[32, 33] No changes in mean arterial blood pressure or heart rate were found before and after each treatment or between baseline and the end of the exposure week. No maladaptive changes in cognitive or spasticity were found either, as measured by the Mini-Mental State Examination and Spinal Cord Assessment Tool for Spasticity.

We have taken great care and consideration in establishing a safe AIH protocol for persons with SCI. Rodent studies were developed using modest protocols of dAIH (10, 5 min episodes; 7 days [8, 9]). In humans, we selected even more modest dosing (15, 1.5 min episodes; up to 5 days).[32, 33] Our mild AIH dose of 1.5-min duration is based on human studies that delivered short doses of hypoxia (SaO2=80-85%) without report of adverse events.[32, 33, 39, 40] We also based our AIH protocol on the pattern-sensitive effects of dAIH on respiratory motor control that were more dependent on the intermittency and repetition than the duration of the

episodes.[5, 7, 41-43] Such pattern-sensitivity is common to many models of neuroplasticity [44] including serotonin-dependent plasticity.[45] Daily exposures in rats (7 days) allowed for further amplification of the gains without eliciting maladaptive changes, such as cell death, hypertension, or weight loss.[8, 9]

b. Criteria for discontinuing or modifying allocated interventions: The interventions will be stopped, and immediate care provided if any significant adverse effects, such as rapid heart rate, dizziness, nausea, or a significant change in blood pressure, are detected or self-reported by the study participant. Participants with diabetes, cirrhosis, a history of autonomic dysreflexia, caffeine allergies or sensitivities, or females who are pregnant will be excluded from this portion of the study. Due to the diuretic effects of caffeine, subjects will be asked to empty their bladders prior to consumption. Throughout the 30 min wait time and experimentation, blood pressure and heart rate will be monitored. Any adverse effects will be reported and assessed. Our study team will be prepared to discontinue treatment and offer immediate care if any significant adverse effects, such as rapid heart rate, systemic hypertensive event, dizziness, or nausea are detected or self-reported by the subject. We will define a systemic hypertensive event as a systolic pressure exceeding 140 mmHg and/or diastolic pressure exceeding 90 mmHg.[46, 47]

c. Strategies to improve adherence to intervention protocols: To improve adherence to intervention protocols, participants will complete these protocols within the INSPIRE Laboratory. We will provide participants with a stipend and reimburse them for travel and lodging costs.

d. Relevant concomitant care and interventions permitted or prohibited during the trial: Patient Monitoring Equipment. Patient Monitor Systems will be used to monitor hemodynamic changes during and following intermittent hypoxia [Smith Medical Systems, USA; Nonin, USA; Welsh-Allyn, USA; Biopac Systems Inc, USA and Masimo; Irvine, CA, USA]. These devices provide estimates of blood pressure, heart rate, oxygen saturation, and pulse rate variability. These measures will be monitored continuously during breathing protocols to ensure subject safety. The blood pressure cuff transducer will be secured around the upper arm during pressure measurements.

We realize concomitant medication use (e.g., bowel/bladder dysfunction, systemic hypertension, pain, spasticity, anti-depressant medications) may confound the effects of our interventions. Due to participant safety and comfort, we will not limit prescribed medication use. However, we will require those receiving medication to maintain dose 1 week prior to and during our interventions as we did in our previous study.[33, 48] We will work closely with physicians to ensure medication dosing is stable before enrollment and during interventions. We also will log daily medication intake for each participant throughout study and assess for possible drug-AIH interactions and changes in prescribed drug dosages at follow-ups. If a patient must change medication for safety, they can readily withdraw from the study at any time.

## 12. Outcomes

The primary outcome measure for this clinical trial is walking speed. We will measure walking speed using the 10-meter walk test (10MWT). The 10MWT is considered the best available measure to assess walking function in persons with iSCI.[49] Speed also is the gold-standard measure of improved walking function in other clinical populations and use of this measure will allow us

to assess outcomes relative to other published studies. At the end of each training session, we will assess the 10MWT speed relative to baseline to determine if the participants achieved a minimum clinically important difference (MCID) of 0.06 m/s change on the 10MWT.[50] We will also use standardized functional assessments to evaluate functional walking capacity via the 6MWT, and functional mobility via the TUG test.[51] The TUG and 10MWT have also been used to distinguish functional ambulation status and progression from non-ambulatory to ambulatory in the acute setting.[52] Our team has experience with all of these measures and testing and analysis will be performed according to our previously published reports.[33, 48]

## 13. Participant timeline

Participants will be randomized to a sequence of three interventions (Table 1). Participants will participate in a single-day screening and 24 visits (1 baseline/intervention + 5 treatment days/intervention + 2 follow-up days/intervention). Participant enrollment and allocation will be completed during the screening visit. Baseline visits will occur up to 3 days prior to the first treatment. A two-week washout will follow the last treatment day. This washout duration is more conservative than washout durations reported in previous animal and human intermittent hypoxia studies.[53, 54]

**Table 1.** Timing Schedule for enrollment, interventions, and assessments

| | Enrollment | Baseline | Intervention | | | | | Follow-ups/Washout | |
|---|---|---|---|---|---|---|---|---|---|
| Timepoints (days) | -5 | 0 | 1 | 2 | 3 | 4 | 5 | 8 | 14 |
| **Screening and Allocation Timing** | | | | | | | | | |
| Eligibility screen | x | | | | | | | | |
| Informed consent | x | | | | | | | | |
| Medical clearance | x | | | | | | | | |
| Allocation | | x | | | | | | | |
| Drug script | | x | | | | | | | |
| **Intervention Timing** | | | | | | | | | |
| Placebo+AIH | | | x | x | x | x | x | | |
| Caffeine+AIH | | | x | x | x | x | x | | |
| Caffeine+SHAM | | | x | x | x | x | x | | |
| **Assessment Timing** | | | | | | | | | |
| **Primary outcome** | | | | | | | | | |
| • Walking speed (10MWT, s) | | x | | | | | x | x | x |
| **Other outcomes** | | | | | | | | | |
| • Voluntary leg strength | | x | | | | | x | x | x |
| • Spasticity | | x | | | | | x | x | x |
| • Spasm frequency | | x | | | | | x | x | x |
| • Walking function | | x | | | | | x | x | x |
| • Walking distance (6MWT, m) | | x | | | | | x | x | x |
| • Walking Balance (TUG, s) | | x | | | | | x | x | x |
| • Blinding Questionnaire | | | x | x | x | x | x | | |
| • Weight (kg) | | x | | | | | | x | x |
| **Safety Outcomes** | | | | | | | | | |
| • Pain (0-10) | | x | x | x | x | x | x | x | x |
| • Blood pressure (mmHg) | | x | x | x | x | x | x | x | x |
| • Heart rate (BPM) | | x | x | x | x | x | x | x | x |
| • Oxygen saturation (%) | | x | x | x | x | x | x | x | x |
| • Memory (0-30) | | x | x | x | x | x | x | x | x |
| **Biomarkers** | | | | | | | | | |
| • CYP1A2 Polymorphism | | x | | | | | | | |
| • Caffeine serum level (mg/L) | | | x | | | | x | | |
| • Inflammation (CRP) | | | x | | | | x | | |

## 14. Sample size

Twenty individuals with chronic (> 6 months post injury), motor-incomplete SCI (i.e., American Spinal Injury Association Impairment Scale (AIS) C or D) will be recruited for the study. Prior to the start of this clinical trial, we estimated the number of participants needed to achieve study objectives. In particular, our sample size computation focused on treatments: caffeine+dAIH versus placebo+dAIH alone. Our preliminary study on the effects of caffeine prior to single AIH sequence (vs placebo+SHAM) on 10MWT showed a decrease of 2.4s±1.2s. Under the hypothesis of an additive effect of caffeine+dAIH (vs. placebo+dAIH), we anticipate the difference in walking speed (10MWT time) change between treatments would be approximately 0.06m/s. With N=20 cross-over participants (includes 17% attrition rate), a repeated measures ANOVA comparing 4 treatments across 4 time points, using pooled SD of 1.3s across days, will be sensitive to detect a difference at power of $1 - \beta = 0.80$ ($f=0.7$, $F_{3,12}=3.5$; $\rho=0.4$, $\alpha=.05$).

## 15. Recruitment

Our study coordinator (Barth), study physicians (Slocum, Zafonte), physical therapists, PI (Trumbower), and research collaborators will recruit subjects using a variety of strategies shown effective in the past. First, the team will identify potential subjects using Institutional Review Board approved resources to inform persons with SCI that are currently admitted to inpatient and outpatient settings at Spaulding Rehabilitation Hospital, Boston University Medical Center, Massachusetts General Hospital, Gaylord Hospital, Albany Medical Center, and Maine Medical Center. Subjects will be recruited by word of mouth and flyers at these facilities. This project also will have access to clinical resources at Spaulding Rehabilitation Network.

Prospective participants will be contacted initially by phone or in-person and informed of the methods, inclusion/exclusion criteria, and purpose of the study. We also will provide a description of this study on ClinicalTrials.gov (NCT02323698), as required by U.S. law. In all cases, recruitment will not be coercive, will not involve undue inducements, and will accurately reflect the study.

Potential subjects will be identified by the following sources:

- Attending physicians or therapists may refer their subjects to the study. We will provide physicians, therapists, and clinics with study information sheets, letters, and flyers. Prospective subjects will be encouraged to contact the study co-investigators.
- Flyers posted in public areas across the Boston-land region, in the outpatient specialist clinics, or other private locations with given permission.
- Internet, e-mail, digital signage, and newspaper advertisements.
- Advertisings posted in Boston public transportation (The T).
- Patients who have received care within the Mass General Brigham Healthcare system will be recruited Patient Gateway. We will use the Research Patient Data Registry (RPDR) to identify potential participants using strict search criteria which will include spinal cord injury diagnosis, age (18 to 75), and date of injury; we will exclude individuals who have "opted out" of receiving research announcements.
- Possible participants may also be identified through their medical records and their physicians might be asked to inform the subjects about the study.

- Presentations at local, state hospitals, and rehabilitation facilities that include public and VA medical centers in the U.S. Northeast (such as to rehabilitation staff and support groups at Spaulding and Partners Network, Boston University Medical Center, Gaylord Hospital, Albany Medical Center, and Maine Medical Center).
- Attending public forums, conferences, or events at which the co-investigator will distribute IRB approved recruitment materials.
- Social Media: Google Ads, Facebook, and Twitter – all advertisements will be submitted to (the way they appear on any social media site) and approved by the IRB prior to posting.
- Partners clinicaltrials.partners.org site.
- We will contact patients who have consented to be added to the Spaulding Rehabilitation Hospital's Spinal Cord Injury Model Systems (SCIMS) Database who have identified that they wished to be contacted for additional research studies. Research staff recruiting from the SCIMS database are also added as study staff on the SCIMS protocol.

Eligible participants will be screened at the designated site facilities (i.e., INSPIRE Laboratory, Spaulding Rehabilitation Hospital at Boston and at Cambridge sites). At the screening visit, subjects will be informed of this project, their potential involvement, the possible benefits and risks, and their right to terminate participation at any time without penalty. Qualifying subjects in agreement with the study details will be asked to sign the informed consent and HIPAA form approved by Spaulding Rehabilitation Hospital's IRB. All potential subjects will enter the study according to the inclusion/exclusion criteria above and following medical clearance. Experiments will be conducted at the Spaulding Hospital in Cambridge.

Prospective subjects will be contacted by telephone, mail or email and briefly informed of the methods, inclusion criteria, and purpose of the study. We also will provide a description of this study on http://www.ClinicalTrials.gov, as required by U.S. law. In all cases, recruitment will not be coercive, will not involve undue inducements, and will accurately reflect the study.

We will recruit individuals who reflect the population of persons with SCI in the Northeast region of the United States, which is 19% women, 81% men, 67% Caucasian, 24% African American, 8% Hispanic, 2% Asian, and 1% Native American. We do not yet know of any differences in responsiveness to AIH or caffeine according to the gender or race of persons with SCI.

Children will not be included in this study. The proposed research is aimed at understanding the plasticity-inducing mechanisms of caffeine and repetitive acute intermittent hypoxia exposure in adults with chronic, incomplete spinal cord injury. A separate study would be required to address the confounding effects of childhood growth and development after spinal injury on the proposed intervention study – a topic best suited for future research once the underlying mechanisms of the intervention are better characterized.

**16. Assignment of interventions (for controlled trials)**

Assignment of interventions for study participants will be done using allocation sequence generation, concealment, and double-blinded implementation.

- Allocation sequence generation – This study will consist of three blocks: caffeine+AIH, caffeine+SHAM, and placebo+AIH. We will generate the blocked randomization for the study participants using a random number list randomizer (Random.org).[55] Participants will

randomly receive each intervention block with a minimum of 2-week wash-out between blocks. We will analyze *post hoc* differences in age, impairment score, assistive device used, baseline lower extremity motor scores, and male-female ratio using independent t-tests.

- Concealment mechanism – We will conceal the intervention allocation from the evaluators performing the assessments, treatment administrators, and the study participant.
- Implementation – The PI and biostatistician will generate the allocation sequences for the cross-over study. The trial coordinator will enroll participants and study staff who are not training or assessing the participants will assign the block randomized interventions.

**17. Blinding (masking)**

The PI, participants, care provider, and clinical evaluators will be made aware of possible air mixtures and caffeine interventions, but not made aware of the combinatorial intervention delivered. Based upon reports from participants (N=6) blinded during our prior study,[33] 4 of 6 guessed incorrectly or were uncertain of the intervention received, suggesting our prior blinding were effective.

**18. Data collection, management, and analysis**

Data collection methods

- Assessment and collection plans: Physical evaluations will be completed by the study's licensed physical therapists or occupational therapists and/or trained research study staff. Functional ability of subjects will be evaluated using standard clinical assessments with high inter-rater reliability: American Spinal Injury Association Impairment Scale (AIS) lower extremity motor scores (LEMS) to quantify strength,[56] and Spinal Cord Assessment Tool for Spastic Reflexes (SCATS), and the Penn Spasm Frequency Scale (PSFS) which is a self-report assessment of the frequency and severity of muscle spasms[57] and modified Ashworth Scale to quantify spasticity.[58] We also will record pain severity using the well-established Wong-Baker FACES scale, which shows high test-retest reliability and interpretability as well as good content and construct validity.[59, 60] Clinical ratings of walking ability, including Walking Index for Spinal Cord Injury (WISCI) II [61] and/or SCI Functional Ambulation Index (SCI-FAI),[62] will be assessed based on video recordings of the 10MWT.

  We will quantify overground walking ability using the 10MWT.[49] Subjects will perform 2 trials each of the 10MWT at their fastest but safe speed with a minimum of 1-minute rest between trials. Average speed across the two 10MWT trials will be used for analyses. The validated assessment tool will provide an indication of return of our subject population to pre-morbid activities following the initial neurological insult. The 10MWT has high inter-rater reliability, and the rating procedure can be accomplished in approximately 30 minutes.

  *The screening and physical assessment procedures will last approximately 1.5 hours.*

  To ensure safety, we also will assess pain, spasticity, systemic hypertension, and autonomic dysreflexia prior to and following caffeine and AIH interventions. We routinely measure pain, spasticity, and incidence of hypertension and autonomic dysreflexia as part of our study protocols in persons with chronic SCI.[32, 33] We will make measurements at baseline, after session 5, and one-week follow-up, and again at two-weeks follow-ups.

  Quantify pain: We will assess pain severity using the Wong-Baker FACES scale of 0 (no pain) to 5 (extreme pain).[59, 60]

Quantify spasticity: We will assess spasticity using the Spinal Cord Assessment Tool for Spastic Reflexes (SCATS).[58] We will quantify total lower extremity spasticity score using the cumulative sum of 3 SCATS subscales: clonus (0=no spasticity; 3=severe), flexor (0=no spasticity; 3=severe), and extensor (0=no spasticity; 3=severe).

Quantify systemic hypertension incidence rate: In addition to monitoring during the intervention, we will measure systolic and diastolic BP at baseline, after sessions 5, and again at follow-ups. We will define a systemic hypertensive event as a systolic pressure exceeding 140 mmHg and/or diastolic pressure exceeding 90 mmHg.[46, 47]

We will compute hypertension incident rate for each intervention group as the number of hypertensive events divided by the total person-time. We define person-time in units of person-measures (the sum of the total number of BP measurements) taken for each person. Person-measures accounts for the total number of chances for detecting a hypertensive event and accounts for measurements not made due to drop-out or a disqualifying adverse event [63]. Finally, we will compute Relative Risk for each Aim as the incidence rate in the dAIH groups over the incidence rate in the dSHAM groups.[64]

Quantify autonomic dysreflexia incidence rate: We will assess the occurrence of autonomic dysreflexia in all intervention groups. Systolic blood pressure (SBP) will be measured as detailed in common methods. We will assess SBP at baseline, and before, during and immediately after breathing interventions, and again at follow-ups. Additional SBP measurements will be made if participants first present with symptoms characteristic of autonomic dysreflexia (e.g., headache, diaphoresis, blurred vision). An autonomic dysreflexia event will constitute a participant having a SBP increase from baseline of 20 mmHg or SBP greater than 150 mmHg with complaints of headache, diaphoresis, and/or blurred vision and will be diagnosed by our study team clinicians (physicians and physical therapists). An autonomic dysreflexia event will result in termination of study participation and require prompt evaluation by the team physicians and emergency medical team.

We will compute autonomic dysreflexia incident rate for each group as the number of autonomic dysreflexia events divided by the total person-time. We define person-time in units of person-days (the number of days a person remains in the study). Person-days accounts for the total number of chances for detecting autonomic dysreflexia and accounts for days on which measurements were not made due to drop-out or a disqualifying adverse event.[63] Finally, we will compute Relative Risk as the ratio of incidence rates between groups.[64]

We previously demonstrated that daily exposures of AIH (up to 5 consecutive days) do not elicit adverse clinical events in persons with chronic SCI.[32, 33] Similarly, rodents exposed to daily exposure for 7 consecutive days did not induce hippocampal cell death, changes in body mass, or hypertension,[8, 9] including in animals early after injury.[8] Determining whether dAIH or in combination protocols lead to the onset of significant pathology (pain, spasms, hypertension, autonomic dysreflexia) in persons with SCI is essential for establishing safe delivery of caffeine combined with AIH in this earlier stage of injury.

Quantify inflammation: We will quantify inflammation using measures of C-reactive protein concentrations in the blood.

- Participant retention plans: Whether or not you complete the whole study, we will pay you $25 for each visit you do complete. We will also reimburse for up to $100 per visit in travel

expenses. Receipts for travel expenses are required. If eligible, we will reimburse for up to $150 per night for up to 12 nights in lodging expenses. Receipts for lodging expenses are required. Eligibility depends on travel distance of more than 60 miles to Spaulding Hospital.

**19. Data management**

Research materials will consist of data from human subjects including: (1) recordings of subject muscle activity, kinematics, and kinetics during overground walking (2) subject anthropometric measurements; (2) video images of the experiments; (3) biological (i.e., blood, saliva) samples; and (4) personal health information collected during screening. Assessment scores will be obtained during an initial screening conducted by the team physician (Slocum) and physical therapist.

The clinical and physiological data will be obtained only for research purposes. Upon enrollment, study subjects will be assigned an ID number. The ID number will identify data in reports, public or private, and personal information kept confidential. Data will not be labeled with subject names; no code sheets or linkages between subject name and code number will be made on data collection sheets. Biological samples will be collected, de-identified, and immediately stored according to biohazard safety protocols. Testing results will not be provided to the subjects.

Consent forms and HIPAA waivers that contain subject names will be stored separate from data sheets in a locked file cabinet in the PI's laboratory. Coded data, which include computer files and computer video records, will be kept on a password protected local network in a secured lab area. Any forms/computer files containing the subject's personal information and their ID number will be housed in a locked office/laboratory space and accessed only by the investigators or other study personnel unless required by law. Study records can be opened by court order or produced in response to a subpoena or a request for production of documents unless a Certificate of Confidentiality is in place for this study.

**20. Statistical methods**

All data will be managed using REDCap[65], MS 365 Excel 16.54 (Microsoft, USA), and MATLAB R2020a (Mathworks Inc, USA) and will be analyzed using SPSS® Statistics 26 (64-bit edition, IBM, USA). Descriptive statistics, including means, standard deviations and 95% confidence intervals of the within-participant pre-post change for each intervention will calculated for all measures. The baseline characteristics of the participants will be compared using parametric and non-parametric inferencing.

Hypothesis testing: To evaluate our main hypothesis that caffeine prior to AIH enhances walking recovery, we will perform a linear mixed model with fixed effects at a significance at the $p=0.05$ level.[66] Intervention (caffeine+dAIH, caffeine+SHAM, placebo+dAIH) and time (day) will be the fixed main effects, with subject as random effect and walking speed (10MWT) as repeat measures. Differences from BL will be compared between and within interventions at $D_5$, $F_1$, and $F_2$. Bonferroni corrections will be made for multiple comparisons. If distributions are not normal, we will perform comparable nonparametric tests. If baseline measures are significantly different between sessions (interventions), an analysis of co-variance (ANCOVA) will be used to analyze the data. We predict walking speed (10MWT) will be greater after daily caffeine+AIH as compared to after daily placebo+AIH and daily caffeine+SHAM.

Blinding integrity analysis: we plan to rigorously quantify blinding integrity here. We will ask participants and raters to guess the intervention received at the end of each intervention and indicate guess confidence using a Likert scale.[67, 68] We will use a contingency table and Fischer's

Exact Test to determine if the probability of correct guessing is different from chance. Using multivariate logistic regressions, we also will assess factors that may influence guessing (i.e., adverse events, perceived effects, and sensorimotor changes). In the event of a medical emergency, we will unblind the PI, study participants, and care provider. We also will notify IRB of treatment allocation during adverse event reporting as necessary.

## V. Monitoring

### 21. Data monitoring

While this study is not under FDA review, all investigators have completed/will complete training requirements at Spaulding Rehabilitation Hospital. Staff training will include Protection of Human Subjects that will familiarize our team with updates to Good Clinical Practices (i.e., CITI certification), and other principles related to the ethical and appropriate conduct of clinical research. The Principal Investigator will regularly review study procedures with the Medical Monitor (monthly) and study staff (bi-weekly), and annual reviews will be conducted by the sites' Office of Clinical Research to ensure compliance with applicable regulations. The monitoring plan will include review of elements required by U.S. 21 CFR 50 and ICH E6, Section 4.8. Onsite monitoring visits will be conducted annually. Adverse event reporting and corrective actions will be reported to the Institutional Review Boards and DOD Sponsor as soon as possible to ensure participant safety.

The PI (Trumbower) will be responsible for monitoring the completeness of all data and source documents. The Principal Investigator will monitor the informed consent procedures in accordance with the Informed Consent Compliance Checklist of Partners HealthCare Systems. Subject data/protocol adherence will be monitored by the research coordinator at each step in the study including.

### 22. Harms

Checklists and note pages are used to note any deviations or omissions from the protocols. Any clinical/health related issues would be immediately presented to the subject to determine appropriate notification (i.e., current physician or appropriate specialist). Based on the seriousness of the situation Drs. Slocum or Zafonte may be contacted to provide clinical guidance on the appropriate course of action as per our Laboratory Medical Emergency Safety Plan.

### 23. Auditing

The MGB performs routine internal audits of laboratories on an annual basis. The audits will be performed independent from the investigators and sponsors.

## VI. Ethics and Dissemination

### 24. Research ethics approval

The study will be conducted in accordance with the principles of GCP. A favorable ethical opinion will be obtained from the appropriate REC and local R&D approval will be obtained prior to starting the study. The study will not start until a Clinical Trial Authorization is obtained from the appropriate Regulatory Authority.

### 25. Protocol amendments

Procedures defined in the study protocol will be carefully reviewed by the PI and research staff prior to the time of study initiation. Any changes to the protocol in the form of an amendment must be submitted to the IRB. We also plan to communicate important protocol modifications such as

changes to eligibility criteria, outcomes, and analyses to trial participants, trial registry (clinicaltrials.gov), and journals.

**26. Consent or assent**

The research physician or physical therapist may be responsible for identifying suitable patients and obtaining consent the patient. However, the PI must confirm that the patient fulfills the eligibility criteria. The PI is responsible for ensuring informed consent is obtained before any protocol specific procedures are carried out.

**27. Confidentiality**

Assessment forms, reports, and other medical records must be identified in a manner that ensures participant confidentiality. All records will be kept in a secure storage area with limited access. The PI and study site staff involved with this study may not use for any purpose other than performance of the study, any data, confidential information disclosed to those individuals for the purpose of the study. Only authorized personnel will have access to these confidential files. Authorized FDA personnel have the right to inspect and copy all records pertinent to this trial. Use of study data when reporting results will be without identifiable reference to the participants.

**28. Declaration of interests**

There are no financial or competing interests for the PI at the start of this clinical trial.

**29. Access to data**

The study staff and PI will comply with the requirements of the Data Protection Act 1998 with regard to the collection, storage, processing, and disclosure of personal information and will uphold the Act's core principles. Published results will not contain any personal data that could allow identification of individual participants.

**30. Ancillary and post-trial care**

We will offer the care needed to treat any injury that directly results from taking part in this research study. We reserve the right to bill the participant's insurance company or other third parties, if appropriate, for the care they receive for the injury. We will try to have these costs paid for, but the participant may be responsible for some of them. For example, if the care is billed to your insurer, the participant will be responsible for payment of any deductibles and co-payments required by their insurer.

**31. Dissemination policy**

The clinical trial report will be used for dissemination within clinicaltrials.gov, scientific journals, and meetings. The PI has the right to publish orally or in writing the results of the study. On completion of the trial, a clinical study report will be prepared in accordance with GCP guidelines. The success of this trial depends entirely on the collaboration of a large number of doctors, therapists, neuroscientists, engineers, and other healthcare professionals. Those included in the Delegation Log will be included in any listing of collaborators. The primary trial publication will be drafted by a writing committee who will approve submission for publication.

## VII. Appendices

### 32. Informed consent materials

Participants will receive a copy of their signed informed consent forms.

### 33. Biological specimen protocols

We will collect blood samples from eligible participants by venipuncture for the purpose of accurately assessing caffeine dose, as well as C-Reactive Protein (CRP) levels. A licensed phlebotomist will draw blood using venipuncture per WHO guidelines (WHO, 2010) before and after combinatorial treatment on day one, and once after combinatorial treatment day five, totaling no more than 3 total venipuncture blood draws or 2 tablespoons of blood per time point. Collection of blood samples by venipuncture poses minimal risk to subjects. To minimize the number of venipunctures and total volume of blood drawn, we will only perform venipuncture at critical time points during the study. All samples to assess caffeine and CRP levels will be de-identified then allowed to coagulate and spun via centrifuge if needed. Serum samples will be transported to Quest Diagnostics, Inc. for analysis. Biohazard safety protocols will be strictly followed. Samples drawn to assess for additional markers will be de-identified, spun via centrifuge to extract and aliquot serum, frozen and securely stored at -80 °C until future analyses.

Blood collection protocols: To monitor caffeine plasma concentration and levels of inflammation, we collected blood samples at the beginning and end of the study visit intervention, with a total of fewer than 50mL of blood taken during a single visit day.

Genetic biomarker protocol: Subjects will participate in identifying genetic markers that may contribute to intervention responsiveness and motor recovery after SCI. Saliva samples will be collected after subjects are screened and deemed eligible to participate as defined by our inclusion and exclusion criteria. Samples will be collected using a standard passive drool protocol. The subject will be asked to collect saliva in their mouth and drool into a vial (DNA genotek OGR-500 Vials, distributed by DNA Genotek Inc, USA) via a saliva collection aide (DNA Genotek Inc, USA). The vial will be sealed tightly and stored at ambient temperature, until transported, plated, and stored at the Partners Biobank until further analysis. BDNF, ApoE, and other genotypes will be determined. The results of genotyping will be evaluated in relation to performance levels achieved following treatments. All samples will be de-identified and stored and transported according to biohazard safety protocols. Genetic testing results will not be provided to the subjects.

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# TABLE OF CONTENTS

# I. Administration Information

## 1. Title and trial registration

Title: Caffeine combined with low oxygen therapy boosts walking recovery in people with spinal cord injury

Trial registration: ClinicalTrials.gov Identifier: NCT02323698, registry name: Effects of caffeine and intermittent hypoxia on leg function in human spinal cord injury

## 2. Statistical analysis plan (SAP) Version 4.0

Recruitment to the study ended on 19 March 2021

Follow-up for the main results ended on 22 September 2021

## 3. Study Protocol Version 3.0

Last updated: September 27, 2021

## 4. Statistical analysis plan revisions

Revision history and justification

| Revision | Date | Modification to SAP | Reason |
|---|---|---|---|
| 1.0 | 11/29/2017 | Change in primary study site | PI changed institutions |
| 2.0 | 11/29/2017 | Change in administration | Change in roles and responsibilities of initial study staff due to relocation of study site. |
| 3.0 | 11/21/2019 | Change in trial population | Extended age and level of injury inclusion criterion due to new evidence of intervention benefits.[1] |
| 4.0 | 05/11/2020 | Change in study methods | Modify SAP to accommodate COVID-19 pandemic safety mandates |

## 5. Roles and responsibility

Names, affiliations, and roles of SAP contributors

| Name | Affiliation | Role of SAP Contributor |
|---|---|---|
| Randy Trumbower | Harvard Medical School & Spaulding Rehabilitation Hospital | Principal Investigator, wrote all version of SAP |
| Stella Barth | Spaulding Rehabilitation Hospital | Lab Manager, contributed to final version of SAP |
| Heather Hayes | Emory University | Co-Investigator, contributed to the first version of SAP |
| Michael Epstein | Emory University | Statistician, contributed to the first version of study methods, statistical principles, and analyses |
| Chris Tuthill | Harvard Medical School & Spaulding Rehabilitation Hospital | Postdoctoral Fellow, contributed to the final version of SAP |
| Gordon Mitchell | University of Florida | Co-Investigator, contributed to the initial version of introduction and study methods |

## 6. Statistical analysis plan approval

Final SAP Approval

Version 4.0

Date Finalized: 3 November 2021

PI Name: Randy D Trumbower

PI Email Address: randy.trumbower@mgh.harvard.edu

| | |
|---|---|
| Randy D Trumbower, Principal Investigator | 3 November 2021 |
| Print first and last name | Date |
|  | 3 November 2021 |
| Signature | Date |

## II. Introduction

### 7. Background and Rationale

Spinal cord injury (SCI) disrupts connections between the brain and spinal cord, causing life-long paralysis and reduced mobility. As such, restoring walking ability remains a highly valued goal for persons with SCI.[2] Even small improvements in walking skills that enable a person with SCI to stand, walk within the home, or negotiate spaces not accessible to wheelchairs can translate into significant empowerment, health benefits, and improvement in quality of life. Because most spinal injuries are incomplete, there are at least some spared neural pathways to the motor neurons that can initiate and coordinate movements necessary for walking recovery. Although injury-induced plasticity in spared spinal pathways enables partial spontaneous recovery of respiratory and somatic motor function,[3, 4] the extent of this recovery is slow, variable and frustratingly limited.[5] Thus, there is a critical need for new strategies that augment spinal plasticity and subsequently improve walking ability in persons with SCI.

Acute intermittent hypoxia (AIH) induces spinal plasticity, strengthening connections to motor neurons.[6-10] Considerable progress has been made towards an understanding of cellular mechanisms giving rise to AIH-induced respiratory plasticity.[8] Repetitive exposure to AIH enhances the expression of plasticity-promoting proteins in respiratory motor nuclei[10-12] and elicits profound recovery of breathing capacity in spinally injured rats.[13] Key steps in AIH-induced plasticity include episodic release of serotonin near respiratory motor neurons, initiating new synthesis of brain derived neurotrophic factor (BDNF) and subsequently activating its high affinity receptor, TrkB [6]. "Downstream" events include the activation of extracellular signal-regulated kinases (ERK); mitogen-activated protein (MAP) kinases and postulated insertion of glutamate receptors at the synapse between respiratory pre-motor and motor neurons. Excitingly, these changes extend beyond the respiratory system. Colleagues also observed that AIH enhances expression of these same proteins in *non-respiratory* motor neurons.[9]

Although dAIH may be a viable method to enhance walking after iSCI, the mechanisms underlying dAIH-induced motor recovery may be constrained by competing neural mechanisms of motor facilitation. While dAIH induces facilitation predominantly via serotonin-dependent mechanisms [14], even mild hypoxia causes ATP release from glia and other sources in the nervous system, leading to an accumulation of extracellular adenosine. Subsequent activation of $A_{2A}$ receptors elicits a distinct form of motor facilitation [15] that is known to compete with the serotonin-dependent pathway via cross-talk inhibition [16, 17]. Consequently, systemic administration of a selective $A_{2A}$ receptor antagonist, MSX-3, reduced cross-talk inhibition and increased AIH-induced long-term motor facilitation in the phrenic and hypoglossal nerves by nearly 50% and 20%, respectively, in rats [17]. The impact of adenosine $A_{2A}$ receptor antagonists on AIH-induced motor recovery have not yet been studied in persons with incomplete SCI (iSCI).

Our working hypothesis is that reducing crosstalk inhibition during dAIH with a well-tolerated $A_{2A}$ antagonist (caffeine) will enhance AIH-induced improvement of walking function in persons with chronic iSCI.

### 8. Objectives

The main objective of this clinical trial is to evaluate the effects of a common, safe adenosine $A_{2A}$ antagonist, caffeine, on recovery of walking following dAIH in persons with chronic iSCI. While highly specific $A_{2A}$ receptor antagonists, such as MXS-3 and ZM2412385, block cross-talk in rats,[17] their safety in SCI is not known. On the other hand, caffeine offers the ideal blend of known

safety, metabolism, and efficacious $A_{2A}$ receptor antagonism.[18] Even at low plasma concentrations (1-10μM) after one cup of coffee, caffeine antagonizes $A_{2A}$ receptors,[18] and the efficacy of moderate caffeine in blocking neural $A_{2A}$ receptors is illustrated by the neuroprotective effects of regular caffeine consumption in dopaminergic transmission in Parkinson's Disease.[19] Although caffeine has many neurobiological actions, caffeine's actions at physiologically relevant doses (1-100 μM), including the proposed dose of up to 6 mg/kg body weight, are mediated by adenosine receptor antagonism versus phosphodiesterase inhibition, release of intracellular calcium stores, or other site.[20] Our study will quantify the daily (5 consecutive days) effects of caffeine (e.g., 4 mg/kg) as an adjunctive therapy to dAIH for restoring walking function in persons with chronic iSCI. This dose has known safety in iSCI and substantial antagonism to $A_{2A}$ receptors.[18, 21, 22]

## III. Methods: Participants, interventions, and outcomes

### 9. Trial Design

We will conduct a placebo-controlled, cross-over, block-randomized intervention study to assess the efficacy of daily caffeine prior to AIH on improving walking function in persons with chronic, motor-incomplete SCI.

### 10. Randomization

Assignment of interventions for study participants will be done using allocation sequence generation, concealment, and double-blinded implementation.

- Allocation sequence generation – This study will consist of three blocks: caffeine+AIH, caffeine+SHAM, and placebo+AIH. We will generate the blocked randomization for the study participants using a random number list randomizer (Random.org).[23] Participants will randomly receive each intervention block with a minimum of 2-week wash-out between blocks. We will analyze *post hoc* differences in age, impairment score, assistive device used, baseline lower extremity motor scores, and male-female ratio using independent t-tests.
- Concealment mechanism – We will conceal the intervention allocation from the evaluators performing the assessments, treatment administrators, and the study participant.
- Implementation – The PI and biostatistician will generate the allocation sequences for the cross-over study. The trial coordinator will enroll participants and study staff who are not training or assessing the participants will assign the block randomized interventions.

### 11. Sample size

Twenty individuals with chronic (> 6 months post injury), motor-incomplete SCI (i.e., American Spinal Injury Association Impairment Scale (AIS) C or D) will be recruited for the study. Prior to the start of this clinical trial, we estimated the number of participants needed to achieve study objectives. In particular, our sample size computation focused on treatments: caffeine+dAIH versus placebo+dAIH alone. Our preliminary study on the effects of caffeine prior to single AIH sequence (vs placebo+SHAM) on 10MWT showed a decrease of 2.4s±1.2s. Under the hypothesis of an additive effect of caffeine+dAIH (vs. placebo+dAIH), we anticipate the difference in walking speed (10MWT time) change between treatments would be approximately 0.06m/s. With N=20 cross-over participants (includes 17% attrition rate), a repeated measures ANOVA comparing 4 treatments across 4 time points, using pooled SD of 1.3s across days, will be sensitive to detect a difference at power of $1 - \beta = 0.80$ ($f=0.7$, $F_{3,12}=3.5$; $\rho=0.4$, $\alpha=.05$).

## 12. Framework

This placebo-controlled study will examine the combined effects of caffeine and AIH results in greater walking performance than either intervention alone. However, this trial will not evaluate the potential superiority or equivalence of caffeine+AIH to standard of care treatments for persons with chronic iSCI.

## 13. Statistical interim analyses and stopping guidance

We do not plan to conduct an interim analysis. However, we will impose criteria for discontinuing or modifying allocated interventions. The interventions will be stopped, and immediate care provided if any significant adverse effects, such as rapid heart rate, dizziness, nausea, or a significant change in blood pressure, are detected or self-reported by the study participant. Participants with diabetes, cirrhosis, a history of autonomic dysreflexia, caffeine allergies or sensitivities, or females who are pregnant will be excluded from this portion of the study. Due to the diuretic effects of caffeine, subjects will be asked to empty their bladders prior to consumption. During and between interventions, blood pressure and heart rate will be monitored. Any adverse effects will be reported and assessed. Our study team will be prepared to discontinue treatment and offer immediate care if any significant adverse effects, such as rapid heart rate, systemic hypertensive event, dizziness, or nausea are detected or self-reported by the subject. We will define a systemic hypertensive event as a systolic pressure exceeding 140 mmHg and/or diastolic pressure exceeding 90 mmHg.[24, 25] We will stop this trial if serious adverse events are directly attributed to the interventions under study.

## 14. Timing of analysis

All outcomes will be analyzed collectively. We will maintain concealment of the intervention allocation until the completion of data collection.

## 15. Timing of outcome assessments

Timing of outcome assessments will occur during specified visits. The timepoints of these assessments along with the timepoints for enrollment and interventions are detailed in the timing schedule (Table 1).

**Table 1.** Timing Schedule for enrollment, interventions, and assessments

| | Enrollment | Baseline | Intervention | | | | | Follow-ups/Washout | |
|---|---|---|---|---|---|---|---|---|---|
| Timepoints (days) | -5 | 0 | 1 | 2 | 3 | 4 | 5 | 8 | 14 |
| **Screening and Allocation Timing** | | | | | | | | | |
| Eligibility screen | x | | | | | | | | |
| Informed consent | x | | | | | | | | |
| Medical clearance | x | | | | | | | | |
| Allocation | | x | | | | | | | |
| Drug script | | x | | | | | | | |
| **Intervention Timing** | | | | | | | | | |
| Placebo+AIH | | | x | x | x | x | x | | |
| Caffeine+AIH | | | x | x | x | x | x | | |
| Caffeine+SHAM | | | x | x | x | x | x | | |
| **Assessment Timing** | | | | | | | | | |
| **Primary outcome** | | | | | | | | | |
| • Walking speed (10MWT, s) | | x | | | | | x | x | x |
| **Other outcomes** | | | | | | | | | |
| • Voluntary leg strength | | x | | | | | x | x | x |
| • Spasticity | | x | | | | | x | x | x |
| • Spasm frequency | | x | | | | | x | x | x |
| • Walking function | | x | | | | | x | x | x |
| • Walking distance (6MWT, m) | | x | | | | | x | x | x |
| • Walking Balance (TUG, s) | | x | | | | | x | x | x |
| • Blinding Questionnaire | | | x | x | x | x | x | | |
| • Weight (kg) | | x | | | | | | x | x |
| **Safety Outcomes** | | | | | | | | | |
| • Pain (0-10) | | x | x | x | x | x | x | x | x |
| • Blood pressure (mmHg) | | x | x | x | x | x | x | x | x |
| • Heart rate (BPM) | | x | x | x | x | x | x | x | x |
| • Oxygen saturation (%) | | x | x | x | x | x | x | x | x |
| • Memory (0-30) | | x | x | x | x | x | x | x | x |
| **Biomarkers** | | | | | | | | | |
| • CYP1A2 Polymorphism | | x | | | | | | | |
| • Caffeine serum level (mg/L) | | | x | | | | x | | |
| • Inflammation (CRP) | | | x | | | | x | | |

## IV. Statistical Principles

We plan to use a parametric linear mixed model since this method is sensitive to within subject changes and robust against variability, small sample sizes, and missing/unbalanced data sets as compared to other statistical models.[26] If the results do not meet these model assumptions, we will apply alternative non-parametric tests.

### 16. Level of significance

All applicable statistical tests will be 2-sided and will be performed using a 5% significance level.

### 17. Adjustment for multiplicity

we do not plan to adjust for multiplicity.

### 18. Confidence intervals

Statistical comparisons will include 95% (2-sided) confidence intervals (CIs).

### 19. Adherence and protocol deviations

We will record a deviation (if the even does not significantly affect a participant's rights, safety, or well-being, or trial outcomes), or a violation (if the deviation may potentially significantly impact the completeness, accuracy, and/or reliability of the trial data or that may significantly affect a patient's rights, safety, or well-being). All protocol deviations/violations will be logged, but no formal statistical testing will be performed.

Adverse events will be reported on an IRB-approved electronic Case Report Form (eCRF). Details will include the type of event, date of onset, duration, intensity, causality relationship to the study drug(s), and outcome. If an adverse event should occur, every attempt will be made to obtain as much information as possible about event evaluation and outcome. If a serious adverse event occurs, the treatment will be interrupted or discontinued at the physician investigator's discretion. All protocol deviations will be reported to the PI (Trumbower) and the IRB.

Stella Barth will fulfill the responsibilities identified in the study's delegation log. These responsibilities include collecting and tracking data forms and instituting quality control measures for data entry verification and study compliance. She will request further documentation such as physician and/or procedure notes in the event of observed or reported adverse events or deviations. They also will be responsible for auditing the database and confirming the overall integrity of the data. The PI (Trumbower) will ensure that all information pertaining to significant new developments and unanticipated adverse events are provided to the appropriate regulatory authorities, the Investigators, and to the IRB. Quality control measures for this study will be conducted at regular intervals ensure the highest standard of clinical research conduct. These inspections are conducted in order to verify adherence to the protocol and the completeness and accuracy of the data.

### 20. Analysis populations

In this cross-over trial, participants will receive the primary intervention (caffeine+AIH) as well as the control conditions (placebo+AIH, caffeine+SHAM). We will perform safety-related assessments on each study participant. We anticipate all participants will participate in the 3 interventions as prescribed, will attend all test visits, and will not have any serious protocol violations.

## V. Trial Population

### 21. Screening data

There will be no statistical testing completed for clinical characteristics. We will summarize the sex, duration of SCI, level and severity of SCI, age, weight, as well as biomarker measures (see Table below). The categorical data will be presented using counts and percentages. The continuous variables (e.g., outcome measures) will be presented using the mean, median, standard deviation (SD), minimum, maximum, and number of participants with an observation (n).

### 22. Eligibility

Potential subjects will be informed about the objectives of the study and must meet all inclusion and exclusion criteria (see Appendix 1 for sample Informed Consent Form). They will be required to read and sign approved consent and HIPAA forms prior to participation to allow access to medical information from their physicians.

Inclusion criteria: Participation requirements for SCI participants include: 1) 18 to 75 years old; 2) medically stable with medical clearance from physician to participate; 3) SCI at or below $C_2$ and at or above $L_5$ with at least some lower extremity motor function preserved below the neurologic level; 4) non-progressive etiology of spinal injury; 4) AIS A-D at initial screen; 5) at least 6 months post-injury (chronic). We plan to choose subjects greater that 6 months post-injury to ensure minimal confounding effects of spontaneous neurological recovery during the experiments. This will mean that changes in sensorimotor performance are more likely due to the interventions associated with the research study.

Exclusion criteria: Individuals will be excluded due to: 1) severe concurrent illness or pain, including unhealed decubiti, severe neuropathic or chronic pain syndrome, infection (e.g. bladder), hypertension, cardiovascular disease, pulmonary disease, severe osteoporosis (history of fractures), active heterotopic ossification in the lower extremities, or history of peripheral nerve injury in the legs; 2) < 24 on Mini-Mental Exam [27]; 3) severe autonomic dysreflexia; 4) history of cardiovascular/pulmonary complications; 5) pregnancy because of unknown effects of AIH on a fetus, although women of childbearing potential will not otherwise be excluded; 6) undergoing concurrent physical therapy; 7) history of diabetes. Caffeine can potentially alter sugar metabolism and, thus, persons diagnosed with diabetes will be excluded from participating in the caffeine portion of this study; 8) history of cirrhosis. Cirrhosis is known to increase the elimination time of caffeine and, thus, persons diagnosed with cirrhosis will be excluded from participating in the caffeine portion of this study; and 9) history of caffeine allergies or intolerances. Participants with a history of caffeine allergies or intolerances will be excluded from participating in the caffeine study.

Inclusion of women and minorities: We will recruit persons with SCI to include women and minorities. To protect against possible unknown side effects of dAIH, women who are pregnant may not take part in this study as the effects of dAIH on the developing fetus have not been studied. If a woman is of childbearing ability, birth control will be recommended for use throughout the study.

Our study coordinator, study physicians, physical therapists, PI (Trumbower), and research collaborators recruited subjects using a variety of strategies shown effective in the past. First, the team identified potential subjects using Institutional Review Board (IRB) approved resources to inform persons with SCI that are currently admitted to inpatient rehabilitation/outpatient units of Spaulding Rehabilitation Hospital, Boston University Medical Center, Massachusetts General

Hospital, Gaylord hospital, Albany Medical Center, and Maine Medical Center. Subjects also will be recruited by word of mouth and flyers at these facilities. Eligible subjects screened at INSPIRE Laboratory, Spaulding Rehabilitation Hospital Cambridge, MA. At the screening visit, subjects informed of this project, their potential involvement, the possible benefits, and risks, and their right to terminate participation at any time without penalty. Qualifying subjects in agreement with the study details asked to sign the informed consent and HIPAA form approved by Partners IRB. All potential subjects entered the study according to the inclusion/exclusion criteria above and following medical clearance. Experiments will be conducted at the Spaulding Rehabilitation Hospitals.

**23. Recruitment**

Our study coordinator (Barth), study physicians (Slocum, Zafonte), physical therapists, PI (Trumbower), and research collaborators will recruit subjects using a variety of strategies shown effective in the past. First, the team will identify potential subjects using Institutional Review Board approved resources to inform persons with SCI that are currently admitted to inpatient and outpatient settings at Spaulding Rehabilitation Hospital, Boston University Medical Center, Massachusetts General Hospital, Gaylord Hospital, Albany Medical Center, and Maine Medical Center. Subjects will be recruited by word of mouth and flyers at these facilities. This project also will have access to clinical resources at Spaulding Rehabilitation Network.

Prospective participants will be contacted initially by phone or in-person and informed of the methods, inclusion/exclusion criteria, and purpose of the study. We also will provide a description of this study on ClinicalTrials.gov (NCT02323698), as required by U.S. law. In all cases, recruitment will not be coercive, will not involve undue inducements, and will accurately reflect the study.

Potential subjects will be identified by the following sources:

- Attending physicians or therapists may refer their subjects to the study. We will provide physicians, therapists, and clinics with study information sheets, letters, and flyers. Prospective subjects will be encouraged to contact the study co-investigators.
- Flyers posted in public areas across the Boston-land region, in the outpatient specialist clinics, or other private locations with given permission.
- Internet, e-mail, digital signage, and newspaper advertisements.
- Advertisings posted in Boston public transportation (The T).
- Patients who have received care within the Mass General Brigham Healthcare system will be recruited Patient Gateway. We will use the Research Patient Data Registry (RPDR) to identify potential participants using strict search criteria which will include spinal cord injury diagnosis, age (18 to 75), and date of injury; we will exclude individuals who have "opted out" of receiving research announcements.
- Possible participants may also be identified through their medical records and their physicians might be asked to inform the subjects about the study.
- Presentations at local, state hospitals, and rehabilitation facilities that include public and VA medical centers in the U.S. Northeast (such as to rehabilitation staff and support groups at Spaulding and Partners Network, Boston University Medical Center, Gaylord Hospital, Albany Medical Center, and Maine Medical Center).

- Attending public forums, conferences, or events at which the co-investigator will distribute IRB approved recruitment materials.
- Social Media: Google Ads, Facebook, and Twitter – all advertisements will be submitted to (the way they appear on any social media site) and approved by the IRB prior to posting.
- Partners clinicaltrials.partners.org site.
- We will contact patients who have consented to be added to the Spaulding Rehabilitation Hospital's Spinal Cord Injury Model Systems (SCIMS) Database who have identified that they wished to be contacted for additional research studies. Research staff recruiting from the SCIMS database are also added as study staff on the SCIMS protocol.

Eligible participants will be screened at the designated site facilities (i.e., INSPIRE Laboratory, Spaulding Rehabilitation Hospital at Boston and at Cambridge sites). At the screening visit, subjects will be informed of this project, their potential involvement, the possible benefits and risks, and their right to terminate participation at any time without penalty. Qualifying subjects in agreement with the study details will be asked to sign the informed consent and HIPAA form approved by Spaulding Rehabilitation Hospital's IRB. All potential subjects will enter the study according to the inclusion/exclusion criteria above and following medical clearance. Experiments will be conducted at the Spaulding Hospital in Cambridge.

Prospective subjects will be contacted by telephone, mail or email and briefly informed of the methods, inclusion criteria, and purpose of the study. We also will provide a description of this study on http://www.ClinicalTrials.gov, as required by U.S. law. In all cases, recruitment will not be coercive, will not involve undue inducements, and will accurately reflect the study.

We will recruit individuals who reflect the population of persons with SCI in the Northeast region of the United States, which is 19% women, 81% men, 67% Caucasian, 24% African American, 8% Hispanic, 2% Asian, and 1% Native American. We do not yet know of any differences in responsiveness to AIH or caffeine according to the gender or race of persons with SCI.

Children will not be included in this study. The proposed research is aimed at understanding the plasticity-inducing mechanisms of caffeine and repetitive acute intermittent hypoxia exposure in adults with chronic, incomplete spinal cord injury. A separate study would be required to address the confounding effects of childhood growth and development after spinal injury on the proposed intervention study – a topic best suited for future research once the underlying mechanisms of the intervention are better characterized.

### 24. Withdrawal/follow-up

We will compute the completeness of follow-up as a ratio depicting the proportion of participants with a complete follow-up 10MWT time after randomization relative to the number of participants enrolled. There will be no statistical testing completed for assessing the adherence to allocated treatments. Reasons for lost data will be documented and detailed in the study's CONSORT flow diagram.

### 25. Baseline patient characteristics

## VI. Analysis

### 26. Outcome definitions

The primary outcome measure is change in walking speed (10MWT time, s) relative to baseline (BL). For all analyses, we will use the average of two 10MWT times at BL, $D_5$, $F_1$, and $F_2$. Change

in walking speed will correspond to the difference between post-intervention 10MWT and BL times. Please review Table 1 for timing of outcome assessments.

All the summaries in this section will be equivalent to the main and the final results reports. There will be statistical testing completed for baseline testing of the primary outcome measure (10MWT). Since we plan to use a cross-over study design, we will compare the 10MWT baseline measurements between the interventions to evaluate the effects of the washout period.

Assessment and collection plans: Physical evaluations will be completed by the study's licensed physical therapists or occupational therapists and/or trained research study staff. Functional ability of subjects will be evaluated using standard clinical assessments with high inter-rater reliability: American Spinal Injury Association Impairment Scale (AIS) lower extremity motor scores (LEMS) to quantify strength,[28] and Spinal Cord Assessment Tool for Spastic Reflexes (SCATS), and the Penn Spasm Frequency Scale (PSFS) which is a self-report assessment of the frequency and severity of muscle spasms[29] and modified Ashworth Scale to quantify spasticity.[30] We also will record pain severity using the well-established Wong-Baker FACES scale, which shows high test-retest reliability and interpretability as well as good content and construct validity.[31, 32] Clinical ratings of walking ability, including Walking Index for Spinal Cord Injury (WISCI) II [33] and/or SCI Functional Ambulation Index (SCI-FAI),[34] will be assessed based on video recordings of the 10MWT.

We will quantify secondary measures of walking function using the timed up-and-go test (TUG, walking initiation and balance), six-minute walk test (6MWT, walking endurance). These tests capture functional ambulation as well as the ability to initiate walking, exhibit high reliability and construct validity.[35] Participants will perform 2 trials each of the TUG at their fastest but comfortable and safe speed with a minimum of 1-minute rest between trials. Average speed across the 2 TUG trials will be used for analyses. Subjects will perform the 6MWT once at their fastest, comfortable walking speed sustainable for 6 minutes. Distances will be recorded at 2 and 6 minutes. Participants will use their least restrictive assistive device during these walking assessments.

**27. Analysis methods**

We plan to analyze within-participant pre-post changes in walking speed (10MWT) for each intervention. We plan to use a parametric linear mixed model for comparing our outcome measure since this method is sensitive to within subject changes and robust against variability, small sample sizes, and missing/unbalanced data sets as compared to other statistical models.[26] If the results do not meet these model assumptions, we will apply alternative non-parametric tests.

All data will be managed using REDCap[36], MS 365 Excel 16.54 (Microsoft, USA), and MATLAB R2020a (Mathworks Inc, USA) and will be analyzed using SPSS® Statistics 26 (64-bit edition, IBM, USA). Descriptive statistics, including means, standard deviations and 95% confidence intervals of the within-participant pre-post change for each intervention will calculated for all measures. The baseline characteristics of the participants will be compared using parametric and non-parametric inferencing.

Hypothesis testing: To evaluate our main hypothesis that caffeine prior to AIH enhances walking recovery, we will perform a linear mixed model with fixed effects at a significance at the $p=0.05$ level.[37] Intervention (caffeine+dAIH, caffeine+SHAM, placebo+dAIH) and time (day) will be the fixed main effects, with subject as random effect and walking speed (10MWT) as repeat measures. Differences from BL will be compared between and within interventions at $D_5$, $F_1$, and $F_2$.

Bonferroni corrections will be made for multiple comparisons.

Alternative statistical methods: If distributions are not normal, we will perform comparable nonparametric tests. If baseline measures are significantly different between sessions (interventions), an analysis of co-variance (ANCOVA) will be used to analyze the data.

Blinding integrity analysis: we plan to rigorously quantify blinding integrity here. We will ask participants and raters to guess the intervention received at the end of each intervention and indicate guess confidence using a Likert scale.[38, 39] We will use a contingency table and Fischer's Exact Test to determine if the probability of correct guessing is different from chance. Using multivariate logistic regressions, we also will assess factors that may influence guessing (i.e., adverse events, perceived effects, and sensorimotor changes). In the event of a medical emergency, we will unblind the PI, study participants, and care provider. We also will notify IRB of treatment allocation during adverse event reporting as necessary.

CYP1A2 analysis: we will perform subgroup analysis on our study cohort to determine the extent to which differences in caffeine metabolism due to CYP1A2 polymorphisms may affect responsiveness to the combinatorial treatment of caffeine+AIH.

**28. Missing data**

Rigorous efforts will be made to ensure complete outcome data. However, we anticipate that participants in this study cohort may be unable to complete the study requirements. Missing data will be reported and documented as we did in our previous clinical trial.[40] We will document reasons for missing data and our approach to handle missing data during statistical analysis. We will account for potential outliers in our results using a conservative outlier labeling technique set forth by Hoaglin and Tukey.[41]

**29. Additional analyses**

We do not anticipate any additional statistical analyses methods.

**30. Harms**

Checklists and note pages are used to log any deviations or omissions from the protocols. Any clinical/health related issues would be immediately presented to the subject to determine appropriate notification (i.e., current physician or appropriate specialist). Based on the seriousness of the situation Drs. Slocum or Zafonte may be contacted to provide clinical guidance on the appropriate course of action as per our Laboratory Medical Emergency Safety Plan.

**31. Statistical software**

All data will be managed using REDCap[36], MS 365 Excel 16.54 (Microsoft, USA), and MATLAB R2020a (Mathworks Inc, USA) and will be analyzed using SPSS® Statistics 26 (64-bit edition, IBM, USA).

**32. Links to additional supporting documentation**

Link to Data Management Plan

Link to the Trial Master File and Statistical Master File

Link to other standard operating procedures

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**Supplemental Figure.** Flowchart of prospective participant enrollment

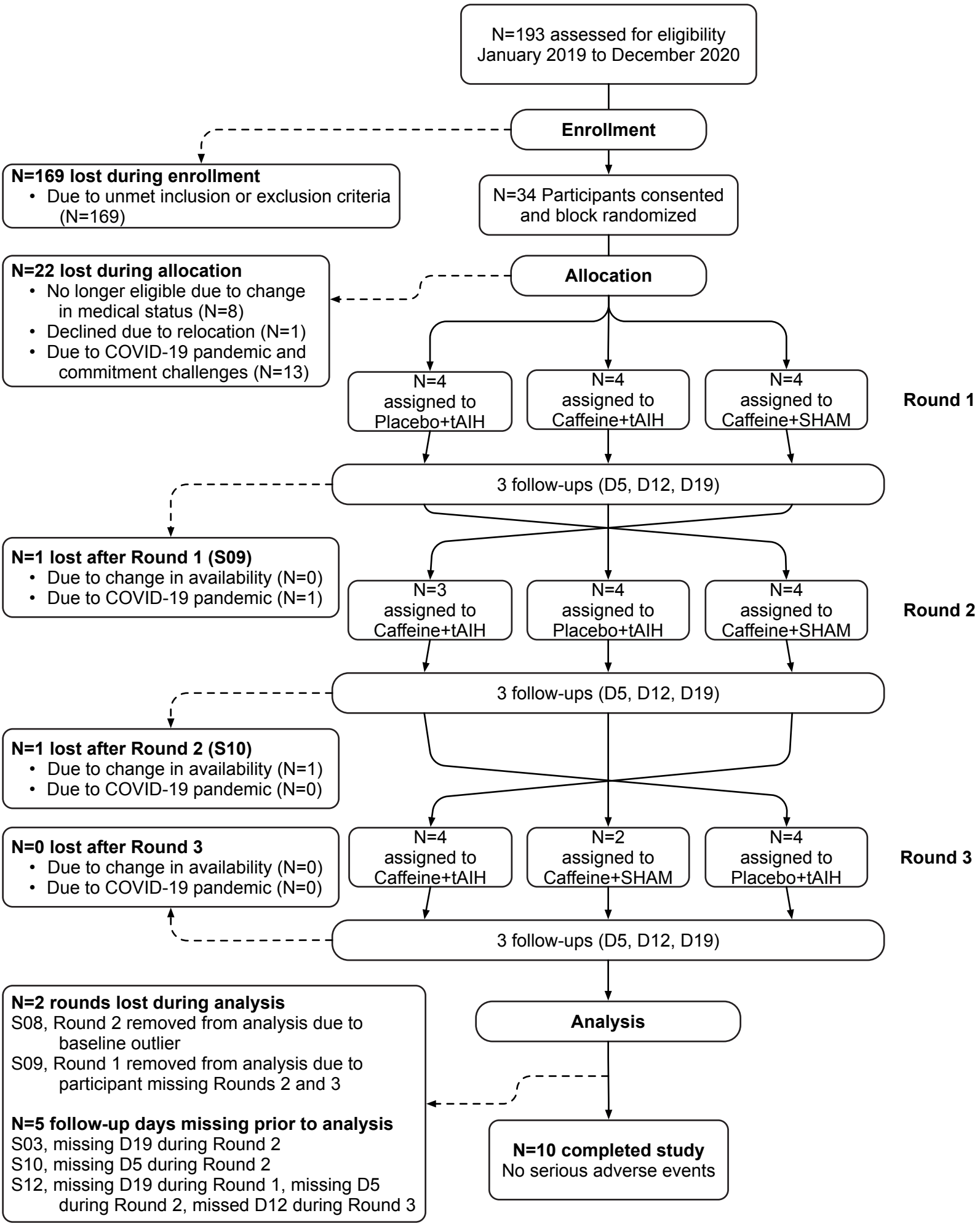

**Table 1.** Timing Schedule for enrollment, interventions, and assessments

| | Enrollment | Baseline | Intervention | | | | | Follow-ups/Washout | |
|---|---|---|---|---|---|---|---|---|---|
| Timepoints (days) | -5 | 0 | 1 | 2 | 3 | 4 | 5 | 8 | 14 |
| **Screening and Allocation Timing** | | | | | | | | | |
| Eligibility screen | x | | | | | | | | |
| Informed consent | x | | | | | | | | |
| Medical clearance | x | | | | | | | | |
| Allocation | | x | | | | | | | |
| Drug script | | x | | | | | | | |
| **Intervention Timing** | | | | | | | | | |
| Placebo+AIH | | | x | x | x | x | x | | |
| Caffeine+AIH | | | x | x | x | x | x | | |
| Caffeine+SHAM | | | x | x | x | x | x | | |
| **Assessment Timing** | | | | | | | | | |
| **Primary outcome** | | | | | | | | | |
| • Walking speed (10MWT, s) | | x | | | | | x | x | x |
| **Other outcomes** | | | | | | | | | |
| • Voluntary leg strength | | x | | | | | x | x | x |
| • Spasticity | | x | | | | | x | x | x |
| • Spasm frequency | | x | | | | | x | x | x |
| • Walking function | | x | | | | | x | x | x |
| • Walking distance (6MWT, m) | | x | | | | | x | x | x |
| • Walking Balance (TUG, s) | | x | | | | | x | x | x |
| • Blinding Questionnaire | | | x | x | x | x | x | | |
| • Weight (kg) | | x | | | | | | x | x |
| **Safety Outcomes** | | | | | | | | | |
| • Pain (0-10) | | x | x | x | x | x | x | x | x |
| • Blood pressure (mmHg) | | x | x | x | x | x | x | x | x |
| • Heart rate (BPM) | | x | x | x | x | x | x | x | x |
| • Oxygen saturation (%) | | x | x | x | x | x | x | x | x |
| • Memory (0-30) | | x | x | x | x | x | x | x | x |
| **Biomarkers** | | | | | | | | | |
| • CYP1A2 Polymorphism | | x | | | | | | | |
| • Caffeine serum level (mg/L) | | | x | | | | x | | |
| • Inflammation (CRP) | | | x | | | | x | | |

**Supplemental Table.** Physiological markers of caffeine dosage, absorption, and metabolism during first treatment day

| ID | Body Weight (kg) | Caffeine Dosage (mg) | Placebo+AIH [Caffeine, mg/L] Pre | Placebo+AIH [Caffeine, mg/L] Post | Caffeine+AIH [Caffeine, mg/L] Pre | Caffeine+AIH [Caffeine, mg/L] Post | Caffeine+SHAM [Caffeine, mg/L] Pre | Caffeine+SHAM [Caffeine, mg/L] Post | CYP1A2 Genotype |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 81 | 300 | 1.4 | 1.1 | 2.8 | 8.3 | 2.5 | 6.8 | CA |
| 2 | 77 | 300 | 0.0 | 0.0 | 2.5 | 9.8 | 2.8 | 8.5 | CA |
| 3 | 45 | 200 | 3.6 | 2.9 | 1.0 | 9.6 | 5.3 | 12.0 | AA |
| 4 | 83 | 300 | - | - | 0.0 | 6.4 | 0.0 | 7.6 | CA |
| 5 | 58 | 200 | - | - | 0.0 | 5.2 | 0.0 | 7.9 | CA |
| 6 | 102 | 400 | 1.0 | 3.4 | 2.8 | 12.6 | 2.8 | 7.0 | CA |
| 7 | 90 | 300 | 0.0 | 0.0 | 0.0 | 6.0 | 0.0 | 5.7 | AA |
| 8 | 93 | 400 | 0.0 | 0.0 | 0.0 | 7.0 | 1.2 | 6.0 | CA |
| 9 | 86 | - | - | - | - | - | - | - | - |
| 10 | 68 | - | - | - | - | - | - | - | - |
| 11 | 73 | 300 | 0.0 | 0.0 | 0.0 | 5.7 | 0.0 | 4.7 | CA |
| 12 | 74 | 300 | 0.0 | 0.0 | 0.0 | 5.6 | 0.0 | 5.8 | AA |
| Mean±SD | | | 0.7±12 | 0.8±1.4 | 0.9±1.3 | 7.6±2.4* | 1.4±1.7* | 7.1±2.0 | |

CYP1A2: cytochrome P450 family 1, subfamily A, member 2; SNP: Single nucleotide polymorphism; CA: CYP1A2 heterozygous, CA alleles carrier; AA: homozygous, CYP1A2 AA alleles carrier. *Significant difference in serum caffeine concentration between post-treatment of Caffeine and Placebo at the Bonferroni-corrected, p=0.05 level.