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Hyperbaric Oxygen for Treatment of Long COVID syndrome (HOT-LoCO); Protocol for a Randomised, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

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Hyperbaric Oxygen for Treatment of Long COVID Syndrome (HOT-LoCO); Protocol for a Randomised, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

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Abstract

Introduction Long COVID, where symptoms persist 12 weeks after the initial SARS-CoV-2-infection, is a substantial problem for individuals and society in the surge of the pandemic. Common symptoms are fatigue, post-exertional malaise, and cognitive dysfunction. There is currently no effective treatment, and the underlying mechanisms are unknown although several hypotheses exist, with chronic inflammation as a common denominator. In prospective studies, hyperbaric oxygen therapy (HBOT) has been suggested to be effective for the treatment of similar syndromes such as chronic fatigue syndrome and fibromyalgia. A case series has suggested positive effects of HBOT in Long COVID. This randomised placebo-controlled clinical trial will explore HBOT as a potential treatment for Long COVID. The primary objective is to evaluate if HBOT improves health related quality of life (HRQoL) for patients with Long COVID compared to placebo/sham. The main secondary objectives are to evaluate whether HBOT improves endothelial function, objective physical performance, and short term HRQoL.

Methods and Analysis A randomised, placebo-controlled, double-blind, phase II clinical trial in 80 previously healthy subjects debilitated due to Long COVID, with low HRQoL. Clinical data, HRQoL-questionnaires, blood samples, objective tests and activity meter data will be collected at baseline. Subjects will be randomised to a maximum of 10 treatments with hyperbaric oxygen or sham treatment over six weeks. Assessments for safety and efficacy will be performed at six, 13, 26 and 52 weeks, with the primary endpoint (physical domains in RAND-36) and main secondary endpoints defined at 13 weeks after baseline. Data will be reviewed by an independent Data Safety Monitoring Board.

Ethics and Dissemination The trial is approved by The Swedish National Institutional Review Board (2021-02634) and the Swedish Medical Product Agency (5.1-2020-36673). Positive, negative, and inconclusive results will be published in peer-reviewed scientific journals with open access.

Trial Registration NCT04842448. EudraCT: 2021-000764-30

Strengths and limitations of this trial

Strengths

- Randomised placebo-controlled, double-blind, parallel groups, clinical trial in compliance with ICH-GCP

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- Evaluation of safety and efficacy, including objective and explanatory endpoints
- Independent Data Safety Monitoring Board (DSMB)

Limitations

- New syndrome with unknown mechanisms
- Power calculation is based on similar syndromes
- Selection bias as patients are enrolled from the same post-COVID clinic

Introduction/Background

In the wake of the first wave of the SARS-CoV-2 pandemic, a new set of often debilitating post-infectious symptoms have arisen. Such symptoms that persist for more than three months, even after mild SARS-CoV infection, have become a major burden for the individuals affected, health care providers, and society in general[1]. The prevalence of long COVID is difficult to determine due to a plethora of symptoms and different definitions[2]. A recent estimation from a UK cohort of 508,707 patients suggests that more than 30% had experienced at least one symptom with "significant impact on my daily life" giving an overall prevalence of 1.72%[3]. Most patients experiencing lingering symptoms are women, of which many have experienced only mild if any respiratory symptoms, and seldom required hospital care during the acute phase of their SARS-CoV-2 infection [4]. Reported long-term symptoms include shortness of breath, fatigue, post-exertional malaise, and cognitive dysfunction, frequently leading to reduced working capability [2]. Some patients are also diagnosed with autonomic dysfunction, including Postural Orthostatic Tachycardia Syndrome (POTS) and inappropriate sinus tachycardia[5 6].

As the pandemic continues to spread, with new mutations and resulting variants of SARS-CoV-2 appearing, effective treatments are needed to quell infection rates as well as mitigate lingering long-term symptoms. There is still not a uniform definition or name of the syndrome, but post-acute COVID-19 syndrome (PACS), post COVID syndrome (PCS), or Long COVID are commonly used[7]. An attempt to achieve a global definition of Post COVID condition, the name suggested by World Health organisation (WHO), was recently made by a Delphi consensus process[8]. Post COVID condition is previously listed in International Classification of Diseases (ICD-10) with code U09.9, which includes all commonly used names. Experts in the field have recently suggested management guidelines for monitoring and follow-up, but to date there is no effective treatment[9]. The underlying mechanisms are not understood but several hypotheses including endothelial dysfunction, oxidative stress, and

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chronic inflammation have been proposed[10 11]. In fact, a recent study demonstrated persistent microvascular endothelial dysfunction for four months following COVID-19 infection[12].

Hyperbaric oxygen therapy (HBOT) is administered by delivering 100% oxygen at raised pressure to patients in a hyperbaric chamber. HBOT has previously been used as an adjunctive treatment for COVID-19, resulting in faster recovery in prospective trials, case series[13], and a randomised controlled trial (RCT)[14], with additional RCTs ongoing[15]. The rationale for treatment of COVID-19 with HBOT is the treatment's well-established anti-inflammatory effects[16 17]. Furthermore, a small retrospective cohort study has shown promising results in alleviating symptoms of Long COVID in patients treated with HBOT[18]. The safety profile of HBOT is well established and is considered both safe and effective for the treatment of several chronic inflammatory diseases such as soft tissue radiation injury[19]. HBOT has been shown to improve symptoms and quality of life in other syndromes associated with chronic fatigue[20 21]. We explore HBOT administered within a randomised placebo-controlled clinical trial as a potential treatment for patients suffering from Long COVID. The purpose of this manuscript is to provide a summary of our protocol that complies with International Council for Harmonisation-Good Clinical Practice (ICH-GCP), with a detailed description and rationale for the primary and main secondary endpoints, including patient reported outcomes (PRO) in line with Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) SPIRIT-PRO Extension Guidelines[22].

Hypothesis and objectives

The overall hypothesis to be evaluated is that HBOT reduces oxidative stress and chronic inflammation, improves endothelial dysfunction, and thereby alleviates symptoms associated with Long COVID.

The primary objective is to evaluate whether HBOT improves Health related quality of life (HRQoL) for patients compared to placebo. The main secondary objectives are to evaluate whether HBOT improves endothelial dysfunction, objective physical performance, and improvement of short term HRQoL. Other secondary objectives are to evaluate if HBOT improves autonomic dysfunction, restorative sleep, the health-economic benefits of the treatment and evaluate biomarkers for the HBO effect on inflammation and chronic hypoxia. Furthermore, we aim to evaluate the safety profile of HBOT for Long COVID patients.

Methods and analysis

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Trial design

The trial is designed as a prospective, randomised, placebo-controlled, double-blind, phase II clinical trial. The trial consists of 5 visits for 52 weeks. At Visit 1 the participant eligibility will be established, and baseline data collected. Block randomisation will be performed, stratified by gender and disease severity as determined by the RAND-36-questionnaire. Eligible subjects are randomised a maximum of two weeks before the first treatment and will receive a maximum of ten treatments over six weeks from randomisation. Treatment is conducted by designated staff not involved in assessment or data collection, subjects and investigators are blinded to the treatment allocation. The randomisation and blinding process is described in a standard operating procedure (SOP). Visit 2 is conducted on the day of the last treatment. The primary and main secondary endpoints will be assessed at 13 weeks from baseline at Visit 3. Visits 4 and 5 are long term follow-up. Subjects will also be asked to participate in a post-trial follow up over 4 years. A flowchart of the trial design is depicted in Figure 1. and the Consolidated Standards of Trials (CONSORT) flow diagram is depicted in Figure 2.

Patient and Public Involvement

The trial design and consent form were discussed with and approved by a patient representative.

We thank Svenska Covidföreningen through chairman Åsa Kristofferson-Hedlund for their support.

Setting

The trial is investigator initiated and will take place in a single center. The sponsor is Region Stockholm via the Karolinska University Hospital in collaboration with Karolinska Institutet, both in Stockholm, Sweden. Patients will be recruited through the post-COVID outpatient clinic and/or advertisement. Measurements and treatments will take place at the hyperbaric unit. If included in the trial, all patients regardless of intervention or control will be treated at the hyperbaric treatment facility, staffed by anesthesiologists and intensivists as well as nurses specifically trained in HBOT. All personnel involved in the trial are designated to specific duties and trained in ICH-GCP. As per protocol at Karolinska University Hospital, each treatment in the hyperbaric chambers must be overseen by a minimum of two staff members. Local, national, and international guidelines for clinical trials and HBOT during the COVID-19 pandemic will be followed.

Trial population

80 patients aged 18–60, previously generally healthy (defined as American Society of Anesthesiologists (ASA) class I-II), will be recruited. They must have had symptoms consistent with

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Long COVID for a minimum of 12 weeks, as well as a Long COVID diagnosis with ICD- 10 code U09.9. Subjects must have been working or studying before the diagnosis. A HBOT specific questionnaire with focus on HBOT contraindications will be filled in by all subjects, contraindications include pregnancy, claustrophobia, obstructive lung disease and history of spontaneous pneumothorax. All inclusion and exclusion criteria are listed in **Table 1**. Subjects who are diagnosed with Long COVID through the Karolinska University Hospital Post-COVID outpatient clinic will be evaluated by a multidisciplinary team consisting of an infectious disease specialist, pulmonologist, cardiologist as well as a physiotherapist. All patients will be assessed with a battery of questionnaires, physical tests, laboratory tests and radiology including MRI's.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
• Aged 18–60 years • Healthy or mild systemic disease (ASA I-II) prior to COVID-19 • Symptoms consistent with Long COVID for at least 12 weeks • Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9) • Working or studying prior to COVID-19 • Documented informed consent according to ICH-GCP and national regulations	• Known pregnancy or positive pregnancy test in women of childbearing age • ASA III or more from other cause than Long COVID • Score above 70 in RAND-36 Role Limitation Physical Health (RP) or Physical Functioning (PF) • Diabetes mellitus • Diagnosed with hypertension prior to COVID-19 • Contraindication for HBOT treatment according to local guidelines • Participation or recent participation in a clinical trial with an investigational product • Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trial participation

Treatment/interventions

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The HBOT group will undergo HBOT at 2.4 Atmospheres absolute (ATA), approximately 240kPa for 90 minutes with two airbrakes, with a maximum of 10 treatments within 6 weeks of randomisation. The placebo group will undergo 'Sham treatment' with air-breathing at 1.34 ATA, approximately 134kPa to equate the sensation of HBOT and airbrakes will be simulated. They will undergo a maximum of 10 treatments within 6 weeks of randomisation. Both treatment protocols and Blinding SOP are available as supplementary material.

The hyperbaric chambers to be used are designed for a single patient (monoplace chamber) or for multiple patients (multi-place chamber). In the case of the monoplace chamber, it is pressurized with 100% oxygen and staff and equipment are located outside the chamber. However, multi-place chambers are pressurized with air, allowing staff and equipment to be inside the same chamber where the patient breathes oxygen through a mask. The latter is suitable for patients requiring a high level of medical care or groups of patients that can sit in a chair for 90 minutes, whereas the monoplace chamber is more comfortable but requires the patient to be fully alert and stable.

Procedures

The patients will be informed about the trial orally and in writing and given the chance to ask questions. If they agree to participate, an informed consent form (ICF) will be signed by the patient and an investigator before any study-specific procedures occur. Subjects will then be scheduled for a screening visit (Visit 1) where baseline data will be collected, and inclusion/exclusion criteria are verified. Subjects eligible for inclusion in the trial will subsequently enter the trial, be randomised, and allocated to treatment. After the treatment period of six weeks, the subjects will be scheduled for follow-up visits at 13 +/- 2 weeks and 26 and 52 weeks +/- 4 weeks after randomisation.

All procedures in the trial are described in detail in the full protocol that is availible as supplementary material. For treatments, blinding procedures, and assessments, standard operating procedures (SOPs) will be followed. A list of procedures is depicted in **Table 2**.

Table 2 List of procedures (Trial specific procedures are marked with **bold X**, data collected from medical records are marked with narrow X)

*Exclusion criteria includes a pregnancy test (if applicable), RAND 36 questionnaire, a HBOT specific questionnaire, review of medical records and a medical examination if needed.

** Medical history includes COVID-19 specific history, routine blood tests, questionnaires, physical tests, and radiology, medical records will be reviewed and recorded.

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*** Socio-demography that may change over time such as sick-leave, weight, activity, smoking habits.

Activity	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Week	0	6	13	26	52
Signed Informed consent Form	**X**				
Inclusion/exclusion criteria	**X***				
Randomisation	**X**				
Medical history	**X**	X**	X**	X**	X**
Socio-demography	**X**	X***	X***	X***	X***
Concomitant medications	**X**	**X**	**X**	X	X
RAND 36	**X**	X	**X**	**X**	**X**
EQ-5D	**X**	**X**	**X**	**X**	**X**
RHI	**X**		**X**		
6 min walk test	**X**	X	**X**	X	X
30/60 s chair-stand	**X**	X	**X**	X	
Nexfin	**X**		**X**		
Treatment (HBOT/Placebo)		**X (1-10)**			
Treatment planned		**X (1-10)**			
AE/ADR	**X**	**X**	X	X	X
Trial-specific biochemistry	**X**	**X**	**X**	X	X
Biobanking (PBMC, Plasma, EPR)	**X**	**X, X**	**X**	**X**	
Activity meter	**X**	**X**	**X**	**X**	**X**

Assessments/measurements

Prior to inclusion subjects will have undergone extensive tests, including radiology with different modalities such as computer tomography (CT), magnetic resonance imaging (MRI), dual-energy computer tomography (DECT), cardiac ultrasound and chest X-rays, and objective physical measurements such as handgrip strength, spirometry and head-up-tilt test and questionnaires used in clinical practice to confirm the diagnosis and rule out any differential diagnosis. This data will be obtained from medical records.

Blood-based biochemical values for kidney function, liver function, cardiac enzymes, haematology, and blood glucose will be obtained from patients' medical records. Trial-specific biochemistry will

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include ferritin, D-dimer, LDH, troponin T, and a pregnancy test for any woman of childbearing age; blood for biobanking will be collected from fasting subjects.

During the screening visit (Visit 1) subjects will fill out the RAND 36-item Health Survey (RAND-36), EuroQol-5 Dimensions Questionnaire (EQ-5D) and undergo physical tests including the 6-minute walk test (6MWT) and 30/60 sec chair stand test (CST), and other objective evaluations including endothelial function with pulse amplitude tonometry (PAT), measurements of cardiac function, and activity, heart rate variability and sleep patterns with an activity meter.

Patient Reported Outcome (PRO) Measures

RAND-36-item health survey (RAND-36)

RAND 36 is a self-reporting questionnaire that contains 36 items that measure eight concepts of health in general terms, at present and past four weeks: physical functioning (ten items), role limitations due to physical health (four items), role limitations due to emotional problems (three items), energy/fatigue (four items), emotional well-being (five items), social functioning (two items), pain (two items) and general health (five items). It also includes a single item that provides an indication of perceived change in health over the last year. Scoring RAND 36 is a two-step process. First, numeric values from the survey are coded so that all items are scored from 0 (lowest score) to 100 (highest possible score). Scores then represent the percentage of total possible score achieved. In step two, items in the same scale are averaged together to create the eight-scale scores. Items that are left blank (missing data) are not considered when calculating the scale scores. Hence, scale scores represent the average for all items in the scale that the respondent answered. RAND 36 is well documented in terms of reliability and variability also for Swedish translation [23]. National gender and age normative data are availible for comparison[23] The questionnaire will be sent out digitally to the subjects on the day of the visit, and when filled out uploaded to the medical records. The dimensions in RAND-36 are presented separately and we have chosen the physical domains RP and PF as primary endpoint for two reasons:

1. The physical domains seem to be severely affected in conditions associated with chronic fatigue and POTS[24 25].
2. We expect the physical domains to be least affected by placebo.

EuroQol-5 Dimensions Questionnaire (EQ-5D)

EQ-5D is a widely used patient-reported questionnaire aimed at measuring five different dimensions of present health with three or five levels of severity: no problems, some/moderate problems, and

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severe/extreme problems. The five different dimensions are mobility, self-care, usual activities, pain/discomfort, anxiety/depression. It also uses a visual analogue scale (VAS) 0-100 for quantifying measures of overall health. EQ-5D is a well-validated tool and the index that is calculated from the dimensions gives an estimate of Quality Adjusted Life Years (QALY), with a low index indicating a low HRQoL[26]. We will use five levels of severity (EQ-5D-5L) in our trial. One of the strengths of EQ-5D is that gender and age normative data for the Swedish population is available for use in health economic evaluation[27], and the index can be used to predict ability to work or study. The questionnaire will be sent out digitally to the subjects on the day of the visit and when filled out, uploaded to the medical records.

The rationale for choosing RAND-36 is that it is well validated and used in previous studies with similar methodology to enable power calculations. EQ-5D was chosen to provide an evaluation of HRQoL in a shorter perspective, as it is easier to fill in and may therefore be a better option for long term follow-up, to enable a simple health economic evaluation.

Physical tests

6-minute walk test (6MWT)

The 6MWT will be performed in a corridor with a measured distance of 30 m, with markings for every meter. The subject will carry a pulse oximeter with a probe attached to their forehead. The test will be monitored by an experienced instructor recording parameters every minute, the total number of meters walked in six minutes, the subject's graded and subjective feeling of leg-fatigue and dyspnea according to the Borg CR-10-scale, as well as the feeling of general exertion according to the Borg-RPE-scale, both at baseline and at the end of the tests[28].

30/60 seconds chair stand test (CST)

Here the subject will stand up straight and sit down completely as many times as possible for 30/60 seconds (s). An instructor will record the number of times the subject manages to perform the movement, as well as the subject's graded and subjective feeling of general exertion according to the Borg-RPE-Scale, and dyspnea and leg fatigue according to the Borg CR-10-scale at baseline and the end of the test. The rationale for recording 30/60 s is that some subjects may not be able to perform the full 60 s test.

Objective measurements

Nexfin

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The *Nexfin* monitor will be connected to a fasting subject. This is a non-invasive measurement of cardiovascular indices, with a beat-to-beat pulse wave analyzer. The Nexfin device (ClearSight, Edwards Lifesciences) is placed on the middle phalanx of the middle finger on the right hand. The Nexfin device comprises a pneumatic plethysmograph that provides advanced hemodynamic parameters and continuous noninvasive blood pressure (BP) from a finger cuff, with a redesigned self-coiling mechanism that reconstructs the clinical standard brachial arterial waveform from the finger arterial pressure waveform; it has been validated towards invasive measurements in several clinical trials[29].

Reactive Hyperemia Index (RHI)

Endothelial function will be determined in fasting state using an *EndoPAT 2000* device (Itamar Medical, Caesarea, Israel). The subjects will be connected to the pulse amplitude tonometry (PAT) device for non-invasive determination of digital endothelial function. The PAT device comprises a pneumatic plethysmograph that allows measurements of pulse amplitude at baseline and during hyperemia following a five minutes arterial occlusion of the forearm [30]. The change in the PAT signal is used for calculating the reactive hyperemia index (RHI), which has been shown to reflect microvascular endothelial dysfunction, reduced NO bioavailability and to predict cardiovascular events [31].

Activity meter

The *OURA™ ring* (Oura Health Oy) will be used as an activity tracker that registers heart rate variability, body temperature, physical activity, and sleep patterns. Subjects will wear the ring for at least 1 week before and after each visit with data being synced in OURA's smartphone application which subsequently will be uploaded to an encrypted database [32]. The weekly mean of each variable will be collected.

Randomisation

Subjects who meet the inclusion criteria will be randomised using a digital tool, Randomizer.at, version 2.0.0 (Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz). The system has a complete electronic audit trail for all activities involved with the randomisation. Randomisation is stratified for gender and 'illness severity'. Illness severity is determined as the mean of RAND-36 score for RP and PF into three strata: 1. <30, 2. 30-50 and 3. >50. Investigators access the randomisation system through a web portal with access control. Staff designated to treatment allocation have user-specific access to the unblinded treatment schedule.

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Study treatment is allocated according to protocol, 10 treatments over six weeks, a maximum of two weeks after randomisation.

Subjects as well as all personnel participating in assessments of symptoms and any objective findings will be blinded to the treatment. The placebo 'Sham treatment' protocol is well established and even experienced divers cannot differ between Sham treatment and HBOT [33]. Designated personnel, experienced in HBOT and trained in GCP and the specific protocols will administer the assigned treatments. All subjects will furthermore be asked during the first week of treatment whether they believe they received the placebo treatment or HBOT, to validate the blinding process.

Trial endpoints

The primary endpoints are the mean change from baseline to 13 weeks in RAND 36 domains RP and PF respectively. The main secondary endpoints are mean change from baseline to 13 weeks in RHI, 6MWT, 30/60 s CST, EQ-5D and proportion of subjects with a normalisation of levels in RAND-36 domains RP and PF respectively, at 13 weeks. Primary-, Main secondary-, Selected other- and Safety endpoints are listed in **Table 3**.

Table 3. HOT-LoCO: Trial endpoints

Primary endpoints	Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health and physical functioning respectively.
Main secondary efficacy endpoints	I. Mean change from baseline to 13 weeks in RHI. II. Mean change from baseline to 13 weeks in the 6MWT. III. Mean change from baseline to 13 weeks in the 30/60 s CST. IV. Mean change from baseline to 13 weeks in EQ-5D scores. V. Proportion of subjects with a normalisation* of levels in RAND-36 domains RP and PF respectively, at 13 weeks.

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Other efficacy endpoints	I. Mean change in other RAND-36 domains at 13, 26 and 52 weeks compared to baseline. II. Mean change in EQ-5D at 6, 26 and 52 weeks compared to baseline. III. Mean change in physical activity using an activity meter at 6, 13 and 26 weeks compared to baseline IV. Mean change in HRV using an activity meter at 6, 13 and 26 weeks compared to baseline V. Mean change in sleeping pattern using an activity meter at 6, 13 and 26 weeks compared to baseline.
Explorative/Descriptive endpoints	I. Mean change from baseline in hypoxia pathways in PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks. II. Mean change from baseline in inflammatory PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks III. Mean change from baseline of reactive oxygen species in red blood cells measured by EPR, at, 6 and 13 weeks. IV. Mean change from baseline of microRNA in plasma, at 6 and 13 weeks. V. Mean change from baseline in trial-specific clinical biochemistry at 6 and 13 weeks. a. D-Dimer b. Ferritin c. LDH d. Troponin T VI. Long term post-trial follow-up of HRQoL using EQ-5D as variable up to 4 years post trial.
Safety and compliance endpoints	I. Number of subjects, proportion of subjects and number of adverse events (AEs) at 13 weeks. II. Number of subjects, proportion of subjects that have completed planned treatments after 6 weeks.

* According to Swedish normative data[23]

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Safety and adverse events

Collection of Adverse events (AE) and Serious Adverse Events (SAE) data will start directly after inclusion and will be recorded until Visit 3. Only SAE will be collected outside the treatment period (Visit 2). Ongoing AE and SAE at the end of Visit 3 will be followed up during long-term follow-up until the subject's last visit. The definition, handling, follow-up and reporting of AEs are defined in the original protocol (p.34–38). The safety endpoints will be evaluated by an independent Data Safety Monitoring Board (DSMB) in the context of the trial design and currently existing information about Long COVID and HBOT. The DSMB is composed of three experts in their respective disciplines of medicine, clinical trial methodology and conduct. The DSMB will review the data at the predetermined interim analyses and at the end of trial, a charter delineating their guidelines for operating and stopping rules for terminating individual subjects, a portion or all the trial prematurely, was drawn up and agreed upon before the trial started. The members of the DSMB, meeting plan and responsibilities are specified in the original protocol (p.6 and 44).

Statistical analyses

This section is a short summary of the planned statistical analyses of the most important endpoints including primary and main secondary endpoints. A longer summary is availible in the full protocol (p.38-42). A more technical and detailed elaboration of the principal features will be written in a separate Statistical Analysis Plan (SAP). The SAP will be finalised prior to Data Base Lock (DBL).

Analysis population

Full analysis set (FAS), per-protocol (PP) and safety population (SP) will be employed. The FAS population will be defined as: all randomized subjects who were exposed at least once to the study intervention.

Sample size calculation

The primary endpoint is mean change from baseline to week 13 in the RAND-36 score. A ten-point higher mean change in the HBOT group compared to the placebo group is considered as a clinically relevant difference. Sample size calculation was performed using t-test for independent groups, with an 80% power), and a type-I error rate of 0.05 (5%), assuming a common SD of 15 from prior studies, to detect a 10-unit difference between groups. Power calculations indicates that at least 37 subjects per group are needed. Subsequently, we aim to recruit 80 subjects. nQuery, version 7 was used for sample size calculation.

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Hypothesis testing and adjustment for multiplicity

Hypothesis testing will be controlled at the type-I error rate of 0.05 and adequately adjusted for multiplicity in the two primary endpoints. However, there will be no adjustment for multiplicity in main secondary endpoints as this is an exploratory study, but nominal p-values will be presented, and results will be interpreted as exploratory findings. All hypothesis tests will be two-sided. Details of the multiplicity adjustment in terms of the selection of endpoints to include in the testing sequence and the criteria for rejecting (or not rejecting) individual hypotheses will be provided in the SAP.

Subgroups

Subgroup analysis will be done and presented for gender and disease severity defined as the mean of RAND-36 RP and PF and divided into 'RP and PF below 30', 'RP and PF 30-50' and 'RP and PF above 50'.

Statistical methodology

Primary and secondary endpoints will be evaluated using the FAS population and sensitivity analyses performed using the PP population. The primary objective of the study is to confirm a superior efficacy for the active treatment compared to placebo in the primary endpoints. The null hypothesis to be tested is that there is no difference between HBO treatment and placebo, i.e., the mean change in (HBOT) = mean change (placebo). The same statistical hypothesis will be used for key secondary endpoints.

All continuous variables will be described using standard statistical measures, i.e., number of observations, mean and median value, standard deviation, minimum and maximum value. All categorical variables will be summarised in frequency tables.

In general, for continuous outcome variables including the primary endpoint, they will be analysed using ANCOVA, unless otherwise specified, including stratification factors and treatment as fixed factors in the model. Estimates will be presented using least-square means for differences between treatment arms. In addition, continuous endpoints measured repeatedly over time, such as EQ5D and RAND-36 domains, the change from baseline will be analyzed using a linear mixed-effect model including baseline, treatment group, sex, symptom severity, visit, and treatment group by visit interaction, and subjects as random effects, in the models. An unstructured covariance matrix will be assumed.

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Analysis for categorical data in terms of binary data (Yes/No) will be presented as the proportion of subjects with the frequency of presence or absence, by treatment group of the characteristics of interest and analysed using the CMH Chi-square test including stratification factors, where the parameter used for the statistical hypothesis testing will be the odds ratio (OR), as a measure of the relative difference in odds between treatment arms. An OR>1 indicates efficacy in favor of HBOT compared to placebo.

Missing data will be adequately imputed for all subjects in the FAS population. In addition, the observed cases population will be evaluated as a sensitivity analysis.

An interim safety analysis will be performed when 20 subjects have available data for the safety endpoints, and a second interim analysis when 40 subjects have data available for primary endpoint to adjust the sample size if needed. The trial will also be evaluated for futility regarding the primary endpoints, i.e., the predictive probability of success at the end of the trial.

Safety analysis

The number and percentage of patients reporting AEs, and the number of AEs reported will be presented. The events will be tabulated by system organ class and preferred term by treatment group. In addition, summaries by relationship to trial drug and severity will be presented. AEs will also be presented in separate tabulations.

The number of patients experiencing an AE will be compared descriptively between groups. All patients with AEs will be listed individually with the patient number in addition to the type of event, start and stop time, duration, seriousness, severity, any action taken, relationship to trial drug and outcome of AE.

Discussion

This manuscript presents the trial design and rationale for the HOT-LOCO trial. The trial is conducted in compliance with ICH-GCP to protect the safety and well-being of the subjects as well as the integrity and validity of the data. HBOT has been used for almost a century for other chronic inflammatory conditions with well documented safety profiles for accepted indications [34]. However, the intervention is not without risk. The nature of the disease, which provokes multiple symptoms and a low quality of life make the risk group a 'vulnerable group' and it is important to make sure

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that the subjects are not unduly influenced by the expectation or benefits associated with participation.

The randomised, double-blinded design is gold standard, and thus is a strength considering primary endpoints being PRO. The trial design involves multiple exploratory and descriptive endpoints, which may provide valuable data regarding the disease regardless of clinical outcomes. Should HBOT prove clinically effective for the efficacy endpoints the trial design also allows further investigation into possible causal mechanisms.

Limitations

The current trial has some important limitations. Long COVID is a novel disease with unknown mechanisms. The prevalence is continuously being revised and it is not known how symptoms and best practice treatment will evolve over time. The treatment protocol in this trial is novel and thus considered a limitation. Normally, HBOT is administered five days a week, with 30–40 sessions over six to eight weeks. The protocol in this trial is based on experience from severe COVID-19 where five treatments seem to be sufficient. However, more research on the dose is needed. Further limitations lie in the possible selection bias of patients being referred through the same outpatient clinic; most patients are severely debilitated (a prerequisite for referral was at least 50% sick-leave) and due to long waiting times, most patients have been ill for more than one year. The power calculation for the primary endpoint is extrapolated from studies of similar design and diseases with similar symptoms but have not been based on a pilot trial and thus is considered as an increased risk of type II error. However, interim analyses will be performed when 20 patients have data available for safety endpoints, and when 40 patients have available for primary endpoint to minimize the risk of an underpowered trial. Furthermore, 'sham treatment' may have up to 58% efficacy[35]. We did not take this into account when we performed our power calculation, which could result in the trial being underpowered. Both EQ-5D and RAND-36 are the most widely used PRO measures for HRQoL and have been used in the setting of long COVID and similar conditions such as ME/CFS and fibromyalgia but due to the novelty of the condition we do not know what to expect from our population and our 'clinically relevant' estimation may be set too high. Three to five points have been proposed as a minimally clinically important difference (MCID) for RAND-36 when used in health economic evaluation[36]. This assumption in our power calculation may also cause a type II error.

Ethics and dissemination

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The trial is conducted in accordance with The Declaration of Helsinki, ICH-GCP, local and national regulations. The trial was approved by The Swedish ethical review board (EPM no 2021-02634, amendment 2021-04572), approval 2021-05-25 and 2021-09-22 and The Swedish medical products agency (LV no 5.1-2020-36673), approval 2021-07-06. The trial was registered online (NCT04842448) and EudraCT number: 2021-000764-30 before start of the trial.

The trial is monitored by the Karolinska Trial Alliance (KTA) before the trial started, during the trial, and after trial completion. A designated monitor will monitor the randomisation and blinding process. The monitoring is performed to ensure that the trial is conducted in compliance with the protocol, detailed in a separate monitoring plan and that data is handled according to ICH-GCP.

The first publication will report the results of the interim safety analysis to help other researchers in trial designs and health care providers in decision making. The main publication will report the primary and main secondary endpoints together with the full safety and compliance report at 13 weeks. Separate publications will report exploratory endpoints: 1. Descriptive results from the Oura-ring, 2. Health economic analysis, 3. Exploratory biomarkers and biochemical analyses. 4. Descriptive results from medical history that is collected during the trial. 5. Depending on the outcome of the primary endpoint at 13 weeks, follow-up on HRQoL at 26 and 52 weeks. 6. Long time, post-trial follow-up on HRQoL, 4 years.

Current trial status

The first subject was included in September 2021. Nineteen subjects have been randomized, 14 have completed the intervention by February 1, 2022. The first safety analysis will be performed when 20 subjects have completed the interventions, according to the plan Q1 2022.

Authors contribution

AK is the principal investigator who wrote the hypothesis and developed most of the protocol together with PL. AK and PL wrote the applications to Swedish IRB and MPA. LAH drafted the manuscript together with AK. AH, SEG, SAE and EB are sub-investigators, enrolling and evaluating subjects and collecting data. MNB, JB, MS, and MR are trial chairs involved in writing the protocol and applications. JK wrote the statistical analysis plan together with AK and designed the randomisation. All authors including CJS, KRW, SBC, XZ and JP contributed to the current submission and critically reviewed the manuscript. AK is corresponding author for this work and attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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Competing interest

AK and PL disclose funding from HLF and ALF for the present trial. AK disclose funding from Oura Health Oy with complimentary hardware and software for the Oura rings. MS discloses funding from Swedish Research Council and Dysautonomia International during the trial and previously from HLF. MS also disclose consulting fee from Swedish agency for health technology assessment of social services, speaker honoraria from Orion Pharma, Werfen and has filed a patent for pharmacological treatment in post-COVID POTS. JK declares consulting fee for statistical work in this trial.
LAH, AH, SEG, SAE, EB, CJS, JP, KM, KRW, XZ, SBC, MR, JB, MNB declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Patient consent for publication

Not required.

Data sharing

The full trial protocol, statistical plan and consent form will be publicly available. Data will be available on patient level; data will be pseudonymised, the full dataset and statistical code will be available upon request. All publications will be made available on Open Access. Source data will be described in a Meta-data repository. A full description of the intended use of the data must be sent to the corresponding author for review and approval. Participant consent for data sharing is conditioned and new ethics approval may be required.

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Figure 1. Trial Flowchart

Figure 2. Consolidated Standards of Trials (CONSORT) flow diagram

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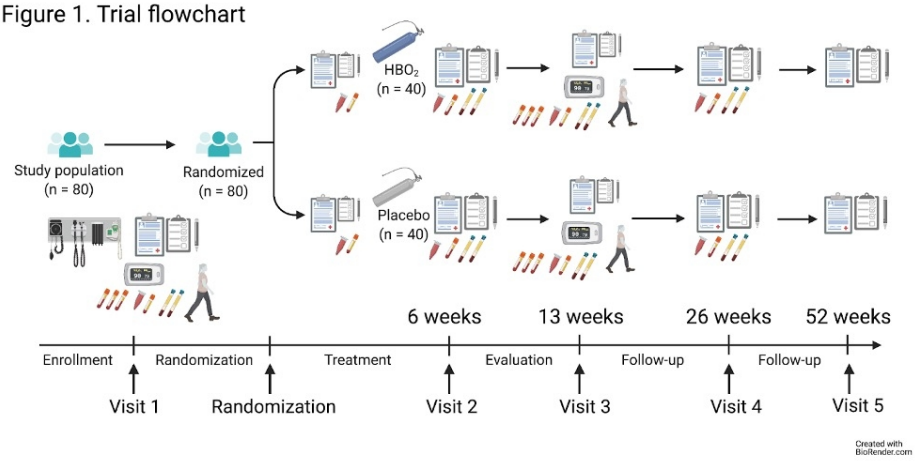
Figure 1. Trial flowchart



Trial flowchart

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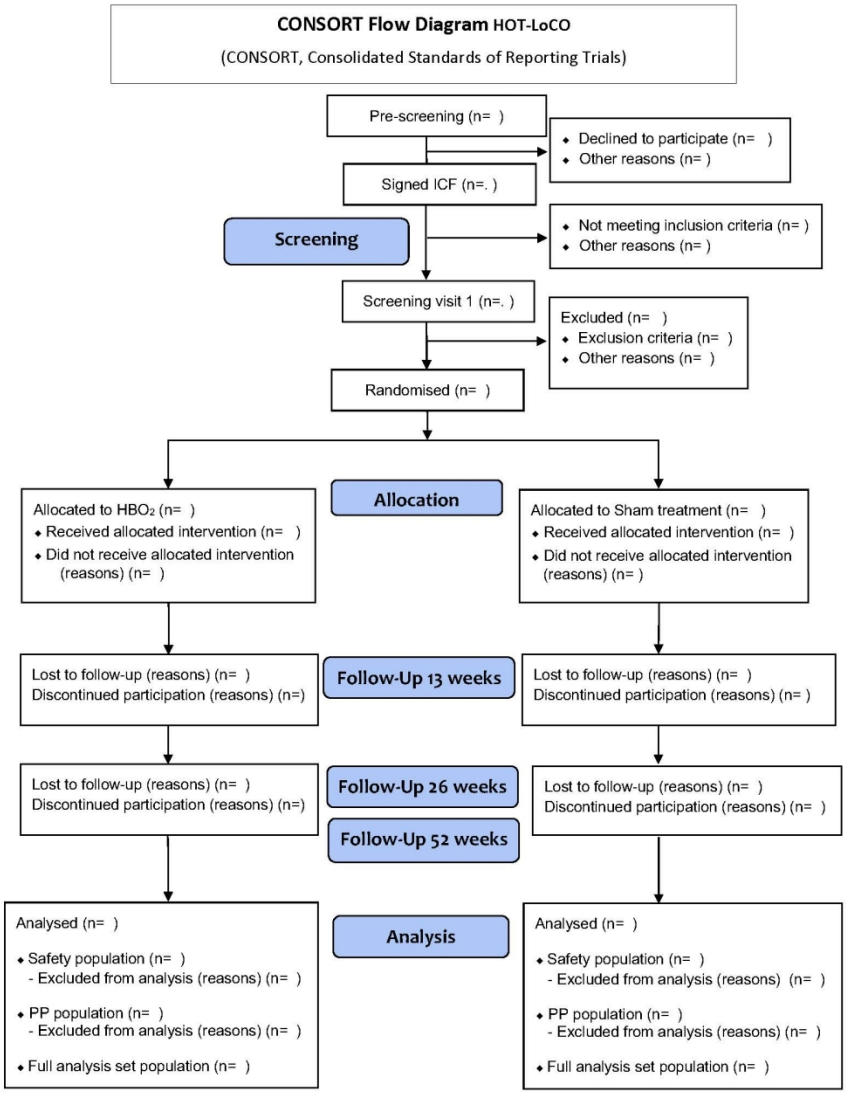

Consolidated Standards of Trials (CONSORT) flow diagram

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Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

CLINICAL TRIAL PROTOCOL

Hyperbaric Oxygen for Treatment of Long COVID syndrome; A Randomized, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

Safety and Efficacy of Hyperbaric Oxygen Therapy for Long COVID Syndrome

Trial code:	HOT-LOCO
EudraCT number:	2021-000764-30
ClinicalTrials.gov Identifier:	NCT04842448
Version number:	4
Date:	2022-01-03
Sponsor:	Karolinska University Hospital, Solna
Principal Investigator	Anders Kjellberg, MD

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Trial Code:	HOT-LOCO
Version No:	v.4
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EudraCT No:	2021-000764-30

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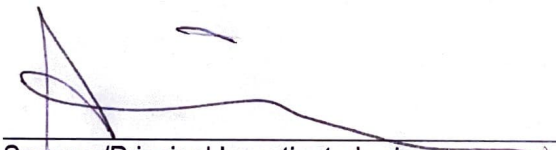
Signature page

Sponsor/Principal Investigator

I am responsible for ensuring that this protocol includes all essential information for the conduct of this trial. By signing my name below, I agree to conduct the trial in compliance with this protocol, the Declaration of Helsinki, ICH GCP (Good Clinical Practice) guidelines and the current hospital, national and international regulations governing the conduct of this clinical trial.

I will submit this protocol and all other important trial-related information to the staff members and investigators who participate in this trial, so that they can conduct the trial correctly. I am aware of my responsibility to continuously keep the staff members and investigators who work with this trial informed and trained.

I am aware that quality control of this trial will be performed in the form of monitoring, audit, and possibly inspection.



2022-01-03

Sponsor/Principal Investigator's signature Date

Anders Kjellberg MD

Printed name

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Trial Code: HOT-LOCO
Version No: v.4
Date: 2022-01-03
EudraCT No: 2021-000764-30

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List of used acronyms and abbreviations

Abbreviation	Term/Explanation
6 min walk test	6 minutes walk test (assessment of physical endurance)
30/60 s chair stand	30/60 seconds chair stand (assessment of functional mucscle strength)
AE	Adverse Event = any untoward medical occurrence
ANCOVA	Analysis of Covariance
AR	Adverse Reaction = adverse event, that is each unfavorable and unexpected reaction to a trial treatment, regardless of dose
ASA Class	ASA Physical Status Classification System
BP	Blood Pressure
CAT	COPD Assessement Test
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Corona Virus Disease 2019
CRA	Clinical Research Associate
CRO	Contract Research Organization
CT	Computerized Tomography
CXR	Chest X-Ray
DECT	Dual Energy Computed Tomography
DSMB	Data Safety Monitoring Board
DSUR	Development Safety Update Report = annual safety report
ECG	Electrocardiogram
eCRF	Electronic Case Report Form
EndoPAT	Endothelial assessement of Pulse Amplitude Tonometry
EPM	Etikprövningsmyndigheten (Swedish Ethical Review Authority)
EPR	Electron Paramagnetic Resonance Spectroscopy
EQ-5D	EuroQol 5 Dimensions questionnaire
FAS	Full Analys Set = including all data from all subjects who have participated in the trial
Frändin-Grimby	Frändin-Grimby activity scale
FSS	Fatigue Severity Scale
GAD-7	Generalised Anxiety Disorder 7-item scale
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Council for Harmonization
IEC	Independent Ethics Comittee
IRB	Institutional Review Board

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HBO_2	Hyperbaric Oxygen
HBOT	Hyperbaric Oxygen Therapy/Treatment
HIF	Hypoxia Inducible Factor
HRV	Heart Rate Variability (assessement for autonomic dysfunction)
HRQoL	Health-Related Quality of Life
HUT	Head Up Tilt test (assessment for POTS)
Jamar	Jamar (assessment of hand muscle strength)
kPa	kilo Pascal (SI unit for pressure, 100 kPa= 1 bar)
KSB	Kognitiva Screening Batteriet (Cognitive Screening Battery)
Long COVID	Long COVID Syndrome = PCS = PACS
LVFS	Läkemedelsverkets författningssamling (Swedish Medical Products Agency's statutes)
MIP/MEP	Maximal inspiratory and expiratory muscle strength
microRNA	Micro-Ribonucleic acid
MFS	Mental Fatigue Scale
mMRC	The Modified Medical Research Council Dyspnea Scale
MOCA	The Montreal Cognitive Assessement
MPA	Medical Products Agency
MRI	Magnetic Resonance Imaging
Nexfin	Nexfin noninvasive cardiovascular monitoring
PACS or PCS	Post (Acute) COVID-19 Syndrome = PCS = Long COVID
PBMC	Peripheral Blood Mononuclear Cells
PCL-5	Posttraumatic Stress Disorder Checklist (version 5)
PE	Pulmonary Embolism
PHQ-9	Patient Health Questionnaire-9
POTS	Postural Orthostatic Tachycardia Syndrome
PP	Per Protocol analysis = including only data from subjects who have completed the trial completely in accordance with the protocol, with no deviations from the protocol
RAND 36	RAND 36-Item Short Form Health Survey 1.0
RHI	Reactive Hyperemia Index
RNA	Ribonucleic acid
SAE	Serious Adverse Event = serious untoward medical occurrence
SAP	Statistical Analysis Plan
SPC or SmPC	Summary of (medical) Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Corona Virus 2
SOP	Standard Operation Procedure
SpO_2	Peripheral Oxygen Saturation
TMF	Trial Master File
WAI	Work Ability Index (assessement of self reported work ability)

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1. Synopsis

EudraCT number:	2021-000764-30
Title:	Hyperbaric Oxygen for Treatment of Long COVID Syndrome; A Randomized, Placebo-Controlled, Double-Blind, Phase II Clinical Trial
Trial code:	HOT-LOCO
ClinicalTrials.gov identifier:	NCT04842448
Short background/ Rationale/Aim:	Long COVID Syndrome (Long COVID), Post Acute COVID-19 Syndrome (PACS) or Post COVID-19 Syndrome (PCS) is defined as 'signs and symptoms that develop during or following an infection consistent with COVID-19, continue for more than 12 weeks and are not explained by an alternative diagnosis'. Common symptoms are fatigue, post-exertional malaise, brain fog, neurologic sensation, headaches, memory issues, insomnia, muscle aches, palpitations, shortness of breath, dizziness and speech issues. Many patients report very low Health Related Quality of Life (HRQoL) One in ten infected individuals may suffer persistent symptoms, and we are facing an emerging problem that will severely affect individuals, health care systems and society for years to come. Subjects will be recruited once they have been diagnosed with Long COVID through assessment by a multidisciplinary team with a thorough diagnostic work up including medical history, routine blood tests, questionnaires, physical tests and radiology. We explore hyperbaric oxygen administered in a randomized placebo-controlled clinical trial as a potential treatment for patients suffering from Long COVID. The overall hypothesis to be evaluated is that hyperbaric oxygen **(**HBO_2) treatment (HBOT) reduces oxidative stress and chronic inflammation, improves endothelial dysfunction and thereby alleviates symptoms associated with Long COVID.
Trial objectives:	**Primary objective:** To evaluate if HBOT improves HRQoL (role limitations due to physical health and physical functioning for patients with Long COVID compared to placebo (sham treatment). **Main secondary objectives:** To evaluate if HBOT improves endothelial dysfunction in Long COVID. To evaluate if HBOT improves objective physical performance in Long COVID. To evaluate if HBOT improves HRQoL short term.

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	To evaluate if HBOT can normalise physical functioning in Long COVID. **Other secondary objectives (in selection):** To evaluate if HBOT improves autonomic dysfunction. To evaluate if HBOT improves restorative sleep. To evaluate if HBOT has a long-term effect on subjective symptoms, HRQoL and objective physical performance in Long COVID To evaluate the potential health-economic benefits of the treatment.
Trial design:	Randomized, placebo-controlled, double-blind, phase II
Trial population:	Previously healthy adult patients with Long COVID syndrome
Number of subjects:	80
Inclusion criteria:	1) Aged 18–60 years 2) Healthy or mild systemic disease (ASA 1-2) prior to COVID-19 3) Symptoms consistent with Long COVID for at least 12 weeks 4) Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9) 5) Working or studying prior to COVID-19 6) Documented informed consent according to ICH-GCP and national regulations
Exclusion criteria:	1) Known pregnancy or positive pregnancy test in women of childbearing age 2) ASA 3 or more from other cause than Long COVID 3) Score above 70 in RAND-36 Role Limitation Physical Health (RP) or Physical Functioning (PF) 4) Diabetes 5) Diagnosed with Hypertension prior to COVID-19 6) Contraindication for HBO_2 treatment according to local guidelines 7) Participation or recent participation in a clinical trial with an investigational product 8) Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trialstudy participation
Investigational product(s), dosage, administration:	Hyperbaric oxygen (HBO_2) compared with placebo HBO_2: HBO_2 240 kPa for 90 min, maximum 10 treatments

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	Placebo: Air 134 kPa for 90 min, maximum 10 treatments
Trial endpoints:	**Primary endpoint:** Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health and physical functioning. **Secondary endpoints (in selection)** Main Secondary Efficacy Endpoints: I. Mean change from baseline to 13 weeks in Reactive Hyperemia Index (RHI). II. Mean change from baseline to 13 weeks in the 6-min walk test. III. Mean change from baseline to 13 weeks in the 30/60 sec chair stand. IV. Mean change from baseline to 13 weeks in EQ-5D. V. Proportion of subjects with a normalisation of levels in RAND-36 domains role limitations due to physical health and physical functioning respectively, at 13 weeks. Safety and Compliance Endpoints I. Number of subjects, proportion of subjects and number of AEs at 13 weeks. II. Number of subjects, proportion of subjects that have completed planned treatments and number of treatments after 6 weeks.
Trial period:	Q3 2021 – Q4 2023
Statistical analyses	The analysis of the primary endpoint will be conducted on the Full Analys Set (FAS) and the Per Protocol Set (PPS). The primary analysis of the primary endpoints will be performed using the ANCOVA, including randomisation strata of gender and disease severity together with treatment as fixed factors in the model. The two primary endpoitns will be adequately adjusted for multiplicity.The p-value for testing the null hypotheses, no difference between treatment groups, must be less than 0.05 to be considered to have met the primary objective.

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2. Introduction

2.1 Background

Post COVID-19 Syndrome (PCS), Post Acute COVID-19 Syndrome (PACS) or Long COVID Syndrome (Long COVID) has been defined as 'signs and symptoms that develop during or following an infection consistent with COVID-19, continue for more than 12 weeks and are not explained by an alternative diagnosis' (Venkatesan, 2021).

Common symptoms are fatigue, post-exertional malaise, brain fog, neurologic sensation, headaches, memory issues, insomnia, muscle aches palpitations, shortness of breath, dizziness and speech issues. Nearly 50% have reduced working capability and 22% cannot work at all. A majority are women and have never been hospitalized for acute COVID-19 (Davis et al., 2020).

The most common organ affected by the SARS-CoV-2 virus is the lung due to its main site of entry, binding to the angiotensin-converting enzyme 2 (ACE-2) receptor; resulting in damage to the cells of the alveolar-capillary membrane (Bourgonje et al., 2020). COVID-19 is associated with endothelial dysfunction, tissue edema and a pro-coagulant state in various organs including the lungs, liver, heart, kidney and small bowel (Varga et al., 2020).

Many of these changes may become chronic, which have been observed at post-COVID follow up (Halpin et al., 2021). COVID-19 should not only be viewd upon as an acute infection but as an inductor of a chronic inflammatory disease. Chronic oxidative stress, inflammation and endothelial dysfunction may explain many of the symtoms and objective findings associated with post-acute and long COVID even after recovery from the viral infection (Chang et al., 2020). These features are also hallmarks of other post-viral syndromes such as Myalgic Encepalomyelitis/Chronic Fatigue Syndrome (ME/CFS)(Scherbakov et al., 2020)

Endothelial dysfunction is a potentially reversible condition that serves as an independent predictor of cardiovascular events(Bonetti et al., 2003). Reactive Hyperemia Pulse Amplitude Tonometry (RH-PAT) is a non-invasive, user-independent tool for assessement of endothelial dysfunction. RH-PAT can be used in a clinical setting for monitoring of treatment effect in subjects with this condition (Bonetti et al., 2004). Autonomic dysfunction is an early marker of endothelial damage and is associated with cardivovascular morbidity and mortality (Khemani and Mehdirad, 2020). Endothelial dysfuncton is common in patients with ME/CFS and is associated with severity of symptoms and immune response (Scherbakov et al., 2020). Heart rate variability (HRV) is widely used as a standard method of measuring autonomic dysfunction in cardiovascular and neurological disorders (Rajendra Acharya et al., 2006).

Hyperbaric oxygen (HBO_2) (PO_2 240–280kPa) delivered by inhalation in a hyperbaric chamber in daily treatments over several weeks, has several anti-inflammatory effects. Hyperbaric

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Oxygen Therapy/Treatment (HBOT) has been used safely for a century to treat other chronic inflammatory conditions such as radiation cystitis (Oscarsson et al., 2019), fibromyalgia (Efrati et al., 2015) and acute inflammatory conditions such as ulcerative colitis (Dulai et al., 2020). HBOT has shown beneficial effects on endothelial function in patients with slow coronary flow (Li et al., 2018). Usefully, it is possible to perform a placebo-controlled double-blind trial with HBO_2 (Lansdorp and van Hulst, 2018).

HBO_2 has been used off-label as one of few potentially curative treatments for acute COVID-19. Case series and a case control-study using HBO_2 have shown faster recovery and reduced need for ICU treatment (Guo et al., 2020, Thibodeaux et al., 2020, Gorenstein et al., 2020). RCTs are ongoing, including one at the Karolinska University Hospital (Clincaltrials.gov identifier: NCT04327505). Multiple hypotheses have been proposed for the effect of the therapy, with the common denominator being normalization of hypoxic- and inflammatory response (De Maio and Hightower, 2020, Kjellberg et al., 2020, Paganini et al., 2021).

One of the most studied effects of HBO_2 is attributed to Hypoxia Inducible Factor-1 (HIF-1) and target genes (Thom, 2011). One target for HIF-1 regulation, which is known to be associated with COVID-19, is Angiotensin Converting Enzyme 2 (ACE2). Hospitalized patients with COVID-19 have a three-fold higher expression of ACE2 in the lungs compared to healthy controls (Chua et al., 2020), suggesting a susceptibility for severe infection or an adaptive response. HIF-1 has been show to suppress ACE2, making HIF-1 modulation an interesting therapeutical target in COVID-19 (Serebrovska et al., 2020). Agents that stabilize HIF-1 have been proposed for COVID-19 (Afsar et al., 2020). A major challange in translating HIF-regulation into clinical practice is the complex adaptation to hypoxia and the intricate interplay between three different HIFs. The crosstalk between hypoxia and and inflammatory pathways adds further complexity to the system (D'Ignazio et al., 2016).

2.2 Research hypothesis

- HBO_2 can induce HIF signalling independent of heart, lung and brain function, thus has the potential to reduce inflammation, restore normal hypoxic response and thereby reduce morbidity in Long COVID.
- HBO_2 is safe and tolerable for Long COVID patients and the effect is accociated with relief in symptoms and thereby improve HRQoL .
- The effect can be monitored by markers of oxidative stress in blood and by non invasive assessement of endothelial dysfunction and autonomic dysfunction.
- Treatment results are not transient and thereby also cost efficient.
- The effect is related to regulation of hypoxia and inflammatory pathways.

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2.3 Rationale for conducting the trial

Long COVID seems to affect approximately 10% of people infected with SARS-CoV-2, most of them are young women (Sivan and Taylor, 2020). To date, few treatment options are available. With over 100 million confirmed COVID-19 cases globally (600 000 in Sweden), the healthcare systems and their infrastructure are at risk of collapse if we cannot find an effective way of treating these patients. Karlolinska Universtity Hospitals was one of the first centers in the world to set up a multidisciplinary clinic for post covid sequaele and is now beening overwhelmed with referrals of suspected Long COVID.

The most common symtoms in Long COVID is chronic fatigue and autonomic dysregulation that are also hallmarks of Fibromyalgia (Sarzi-Puttini et al., 2020) and Myalgic Encepalomyelitis/Chronic Fatigue Syndrome (ME/CFS)(Lim et al., 2020) and some patients are also diagnosed with Postural Orthostatic Tachycardia Syndrome (POTS) and they may all be different semblance of the same chronic inflammatory disease. HBOT has been shown to have positive effect on ME/CFS and fibromyalgia in small clinical trials (Efrati et al., 2015, Yildiz et al., 2004, Akarsu et al., 2013).

If HBO_2 is effective for relieving symtoms in Long COVID there would be an obvious benefit for the individual patient. There is also a potential significant health-economic benefit if there is a lasting effect. The multiple explanatory endpoints may add valuble information about the disease for future interventional trials even with a negative result on the primary endpoint.

3. Benefit-risk evaluation

3.1 The risk group

There is currently no effective treatment available for Long COVID and since this is a new disease, there remain uncertainties regarding diagnosis, prognosis and mechanisms of action. There is emerging evidence that this may be an enormous problem for individuals, health-care and society. Diagnosis of Long COVID is mainly a clinical definition based on symptoms and it is difficult to find objective measurements. Patients that have been suffering from Long COVID since the first wave have often been misunderstood by the health care society and are desperate to find a cure for the disease. HBO_2 has the theoretical potential to reverse or reduce symptoms in Long COVID. The nature of the disease, which provokes multiple symptoms and a low quality of life make the risk group a ‘vulnerable group’ and it is important to make sure that the subjects are not unduly influenced by the expectation or benefits associated with participation. Therefore, we will conduct a placebo-controlled, double-blind, clinical trial in compliance with Good Clinical Practice (GCP), the Declaration of Helsinki and national regulatory requirements. The written information has a neutral language explaining both risks and potential benefits and the investigators are instructed to keep a neutral tone when delivering oral information. The cause of Long COVID is still not known and optimal

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management is far from defined. We present a plausible hypothesis of the mechanism and a possible cure. Since there are no better options than 'expectation', and HBOT has been safely and effectively used in other chronic inflammatory conditions, the potential benefit for the subjects outweigh the risk.

3.2 General risks with HBO_2

HBOT is a well-established method that has been used for almost a century for several different indications. The mechanisms for its efficacy are not fully understood but it is generally regarded as safe, with few adverse events; serious adverse events are extremely rare. The Undersea and Hyperbaric Medical Society (UHMS) have reported a total of 40 complications per 10,000 treatments during 463,293 treatments over two years (Moon, 2019). Adverse events per 10,000 treatments include: ear pain 20, confinement anxiety 8, hypoglycaemic event 5, shortness of breath 2, oxygen-induced seizure 2, sinus pain, 1, chest pain. HBOT has very few contraindications that are all relative to the treatment environment; they include claustrophobia, medical history of spontaneous pneumothorax, severe COPD, and pregnancy.

3.3 Blood sampling

Blood sampling may have negative impact on the subject as a large number of samples will be necessary for the clinical investigation and may be needed for other trials. We aim to use blood tests already collected as much as possible. The blood sampling serves three purposes:

1. Safety, which is of benefit for the subject.

2. Explanatory, which may be beneficial for the placebo subjects in particular, if the trial results are positive and HBOT for Long COVID is adopted into clinical practice. Samples will serve as a quality control measure to ensure the validity of the data upon presentation of results.

3. Exploratory, which may benefit the subjects even if the HBOT is not successful, as the trial may generate hypotheses for alternative treatments.

Explanatory and Exploratory objectives are important for public health.

3.4 Handling of sensitive personal data

We will handle personal data, including gene expression analyses on the subjects, and there is a risk of personal integrity involved. The trial will be performed according to ICH-GCP; all staff involved will be educated in GCP. All information about the protocol and data will be entered into an eCRF. The data will not identify any person taking part in the trial in accordance with the EU Data Protection Directive (95/46/EU). An external monitor will help us assess the risks by assessing quality of trial design, data collection and informed consent.

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3.5 Safety and logistics

The HBO_2 treatments will be performed in a hyperbaric chamber at the Karolinska University Hospital. Depending on availability, either monoplace or multiplace chambers will be used. The principal investigator is head of the unit with more than 20 years experience in HBOT. All staff are trained and certified for operating the chambers. Standard Operating Procedures for treatment will be used. Local, national and international guidelines for clinical trials and HBOT during the COVID-19 pandemic will be followed.

Monitoring will be conducted before, during and after the trial according to the monitoring plan. Interim analyses for safety and efficacy will be conducted by an independent Data Safety Monitoring Board (DSMB).

In summary, we believe the benefits for subjects, the risk-group and public health will outweigh the risks.

4. Trial objectives and endpoints

The overall hypothesis to be evaluated is that HBOT reduces oxidative stress and chronic inflammation, improves endothelial dysfunction and thereby alleviates symtoms associated with Long COVID.

4.1 Primary objective

To evaluate if HBOT improves HRQoL (role limitations due to physical health and physical functioning) for patients with Long COVID compared to placebo (sham treatment).

4.2 Secondary objective(s)

4.2.1 Main secondary objective

To evaluate if HBOT improves endothelial dysfunction in Long COVID.

To evaluate if HBOT improves objective physical performance in Long COVID.

To evaluate if HBOT improves HRQoL short term.

To evaluate if HBOT can normalise physical function in Long COVID

4.2.2 Other secondary objectives

To evaluate if HBOT improves autonomic dysfunction.

To evaluate if HBOT improves restorative sleep.

To evalute if HBOT has a long term effect on subjective symptoms, HRQoL and objective physical performance in Long COVID

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To evaluate the potential health-economic benefits of the treatment.

To explore changes in general and organ-specific questionaires, physical tests and radiology used in clinical follow-up before and after treatment

To explore biomarkers in plasma, erythrocytes and PBMCs for HBO_2 effect on inflammation, endothelial function and chronic hypoxia.

4.3 Primary endpoint:

Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health (RP) and physical functioning (PF).

4.4 Secondary endpoints:

4.4.1 Main Secondary Efficacy Endpoints

I. Mean change from baseline to 13 weeks in Reactive Hyperemia Index (RHI).
II. Mean change from baseline to 13 weeks in the 6-min walk test.
III. Mean change from baseline to 13 weeks in the 30/60 sec chair stand.
IV. Mean change from baseline to 13 weeks in EQ-5D.
V. Proportion of subjects with a normalisation of levels in RAND-36 domains RP and PF respectively, at 13 weeks.

4.4.2 Other Efficacy Endpoints

I. Mean change in other RAND-36 domains at 13, 26 and 52 weeks compared to baseline.
II. Mean change in EQ-5D at 6, 26 and 52 weeks compared to baseline.
III. Mean change in physical activity using an activity meter at 6, 13 and 26 weeks compared to baseline
IV. Mean change in HRV using an activity meter at 6, 13 and 26 weeks compared to baseline
V. Mean change in sleeping pattern using an activity meter at 6, 13 and 26 weeks compared to baseline.

4.4.3 Explorative/Descriptive Endpoints

I. Mean change from baseline in hypoxia pathways in PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks.
II. Mean change from baseline in inflammatory PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks
III. Mean change from baseline of reactive oxygen species in red blood cells measured by EPR, at, 6 and 13 weeks.

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IV. Mean change from baseline of microRNA in plasma, at 6 and 13 weeks.
V. Mean change from baseline in study specific clinical biochemistry at 6 and 13 weeks.
   a. D-Dimer
   b. Ferritin
   c. LDH
   d. Troponin T
VI. Mean change from baseline in objective organ specific findings on imaging at 13 and 26 weeks (from medical records).
VII. Mean change from baseline in objective organ specific and general physical tests (6-min walk test, 30/60-sec chair stand, HUT, Jamar, MIP/MEP and Spirometry at 13 and 26 weeks (from medical records).
VIII. Mean change from baseline in subjective rating of physical and cognitive symptoms evaluated by self-reported questionnaires (CAT, Frändin-Grimby, FSS, GAD-7, MFS, mMRC, MOCA, PCL-5, PHQ-9, WAI) at 13 and 26 weeks (from medical records).
IX. Long term post-trial follow-up of HRQoL using EQ-5D as variable up to 4 years post trial.

4.4.4 Safety and Compliance Endpoints

I. Number of subjects, proportion of subjects and number of AEs at 13 weeks.
II. Number of subjects, proportion of subjects that have completed planned treatments after 6 weeks.

5. Trial design and procedures

5.1 Overall Trial design and flowchart

Phase II Clinical Trial

Prospective randomized, placebo-controlled, double-blind, clinical trial, estimated enrolment: 80 subjects

Parallel groups

Intervention: HBO_2: 240 kPa for 90 min, maximum 10 treatments within 6 weeks from randomization.

Control: Placebo treatment with 'sham' air breathing at a moderately higher pressure (134 kPa) for 90 min to simulate hyperbaric chamber treatment, maximum 10 treatments within 6 weeks from randomization.

The population will comprise of previously generally healthy patients diagnosed with Long COVID (U09.9). All patients are clinically assessed by a multidisciplinary team with a battery of questionnaires, physical tests, laboratory tests and radiology. After their first assessment,

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individuals may have further organ specific work up for diagnosis, such as diagnosis of Postural Orthostatic Tachycardia Syndrome (POTS).

Once the patient has been diagnosed with Long COVID, they will be informed and asked to participate in the trial. No study specific procedures will take place before an informed consent form (ICF) has been signed. Some study specific procedures will be performed before inclusion (screening), such as HRQoL questionnaires and pregnancy test (if applicable). The patients will be included once they fulfil the inclusion criteria and exhibit none of the exclusion criteria. Baseline medial history, medical examination and study specific tests, blood samples and questionnaires will be collected during visit 1. If patients have already entered or gone through follow-up in clinical routine, some data from the last visit, no more than three months prior can be used for visit 1. If less than two weeks since last follow up, study specific procedures do not need to be repeated. Eligible subjects will be randomized within two weeks of the planned first treatment. Subjects will be randomized in a 1:1 alloction to HBO_2 or placebo (sham treatment). Scheduling of the HBOT will depend on available resources but the first treatment should be given within two weeks after randomization, and a maximum ten treatments should be given within 6 weeks from randomization. Physical tests, blood tests and questionnaires are repeated after the last treatment. Saftety and secondary endpoints are evaluated at visit 2. Efficacy evaluation of the primary endpoints will be made on assessments at visit 3 (three months), questionnaires and bloodtests. Subjects will be asked to use an activity meter in conjunction with each visit. Visit 4 and 5 are long term follow up, includes questionnaires, bloodtests and activity meter.

Clinical equipoise: The rationale for 1:1 randomization is that this is a new disease and that it will maximise the statistical power to detect a statistically significant efficacy between treatment groups.

Main efficacy and safety endpoints will be evaluated at one and three months after randomization, but all subjects will be asked to participate in a one-year follow-up after inclusion.

Subjects will also be asked to participate in a post-trial long-term follow-up with EQ-5D Questionnaire that will be sent out once a year for up to four years after visit 5.

Figure 1 and Table 1 show the trial overview and procedures

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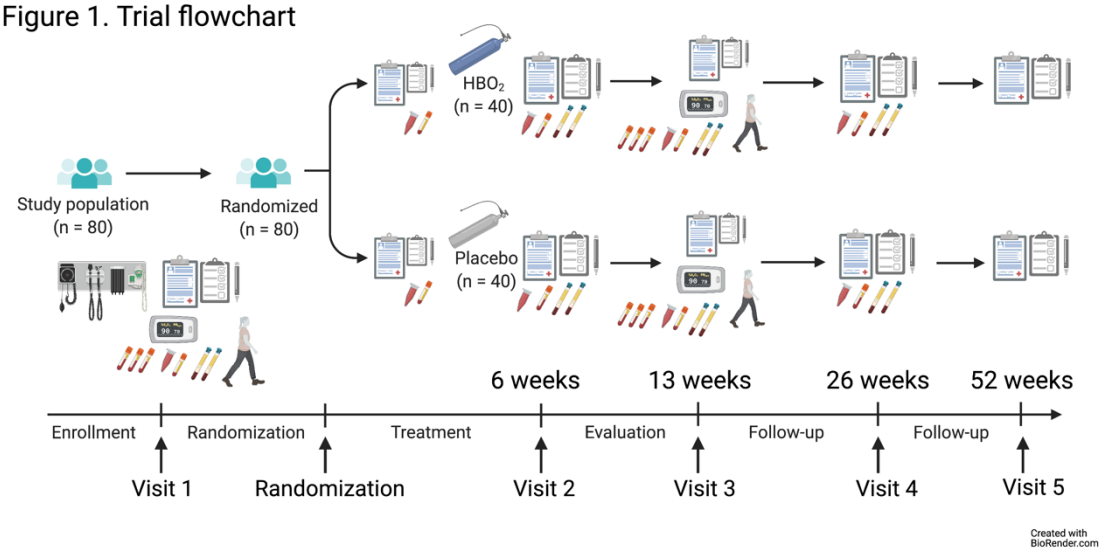
Figure 1. Trial flowchart



5.2 Procedures

Table 1. **List of procedures (Bold letters indicate study specific procedures**; other procedures may vary depending on symptoms and availability from medical records.)

Activity	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Week	0	6	13	26	52
Signed Informed consent Form	**X**				
Inclusion/exclusion criteria	**X***				
Randomization	**X**				
Medical history	**X**	X**	X**	X**	X**
Socio-demography	**X**	X***	X***	X***	X***
Concomitant medications	**X**	**X**	**X**	X	X
RAND 36	**X**	X	**X**	**X**	**X**
EQ-5D	**X**	**X**	**X**	**X**	**X**
RHI	**X**		**X**		
6 min walk test	**X**	X	**X**	X	X
30/60 s chair-stand	**X**	X	**X**	X	
Nexfin	**X**		**X**		
Treatment (HBOT/Placebo)		**X (1-10)**			
Treatment planned		**X (1-10)**			
AE/ADR	**X**	**X**	X	X	X
Study-specific biochemistry	**X**	**X**	**X**	X	X
Biobanking (PBMC, Plasma, EPR)	**X**	**X, X**	**X**	**X**	
Activity meter	**X**	**X**	**X**	**X**	**X**

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*Exclusion criteria includes a pregnancy test (if applicable), RAND 36 questionnaire, a HBOT specific questionnaire, review of medical records and a medical examination if needed.
**Medical history includes COVID-19 specific history, routine blood tests, questionnaires, physical tests, and radiology, medical records will be reviewed and recorded.
***Socio-demography that may change over time such as sick-leave, weight, activity, smoking habits.

Trial schedule

Visit 1: (Minimum 12 weeks post COVID-19)

a) After the patient has been informed about the trial and if agreed to participate, an **informed consent** form (ICF) will be **signed** off before any study-specific procedures occur.
During the **Screening**, procedures to assure the patient´s eligibility for trial participation will be performed, this includes a serum **pregnancy test** for females of childbearing potential, **RAND-36** and **EQ-5D questionnaires**, a **HBOT specific questionnaire**, review of medical records and a medical examination if needed for all. **Socio-demography**, **medical history** including COVID-19 specific history, adverse events, routine blood tests, questionnaires, physical tests, and radiology will be reviewed and recorded. **Questionnaires will be sent digitally and if eligible, subjects are booked for the physical tests.**

b) **Blood** samples for future biochemical research will be collected, and **study-specific chemistry** supplemented if necessary. **Study-specific procedures** will be conducted (not repeated if less than two weeks since last clinical visit and other relevant procedures will be recorded if less than 12 weeks since last clinical visit.

c) Subjects will be **randomized** to either HBO_2 or placebo when the first treatment is planned. Time, date and randomization group are recorded (blinded to subjects and all assessors of outcome variables).

Visit 2: (Starts within 4 weeks after visit 1, within 2 weeks of randomization, ends after last HBO_2 treatment)

Subjects are booked for the treatment.
a) **Review of medical records** and medical history. Advererse events, changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded.

b) **Blood** samples for future biochemical research may be collected before and after the first and the last treatment, **study-specific biochemistry** supplemented if necessary. Data from **activity meter** is registred. RAND 36 and EQ-5D questionnaires are sent digitally.

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c) Subject will be introduced to the **Hyperbaric chamber** and given a **maximum 10 treatments within six weeks from randomization**. If planned but not given, this will be recorded with the reason for not giving the treatment.

Visit 3: (13 weeks after randomization +/- 2 weeks)

Questionnaires will be sent digitally and subjects are booked for physical tests.
a) **Review of medical records** and medical history. Adverse events, changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded.

b) **Blood** samples for future biochemical research will be collected, and **study-specific chemistry** supplemented if necessary.

c) **Study-specific procedures** will be conducted.

Visit 4: (26 weeks after randomization +/- 4 weeks)

Questionnaires wil be sent digitally to subjects.
a) **Review of medical records** and medical history. Changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded. Adverse events will be followed up.

b) **Study-specific blood** samples for future biochemical research will be collected, and **routine chemistry** supplemented if necessary. Data from **activity meter** is registred.

c) **Long term follow-up.**

Visit 5: (52 weeks after randomization +/- 4 weeks)

Questionnaires wil be sent digitally to subjects.
a) **Review of medical records** and medical history. Changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded. Adverse events will be followed up.

b) Data from **activity meter** is registred.

c) **Long-term follow-up.**

Unscheduled visits:

Any variables outside the timeframe of scheduled visits may be recorded as unscheduled visits during the trial.

End of Trial

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A final visit in the electronic case report form (eCRF) should be completed for every randomised patient whether the patient completed the trial or not. The reason for any early discontinuation should be indicated on this form.

5.2.1 Assessments and procedures

Medical history

Relevant medical history will be recorded at Visit 1. The medical history will include a review of past and current relevant diseases/diagnoses/symptoms, for female subjects this includes information regarding menstrual cycle and pregnancies. Symptoms, signs and the start date of COVID-19, Long COVID and vaccination status will be collected. For concomitant diagnoses start year will be collected. Findings and/or abnormalities detected will be recorded in the eCRF. Other medical history, not relevant for the trial will be documented in medical records. Records and medical history will be reviewed for update/change in significantly changed parameters such as symptoms/signs or new diagnoses.

HBO_2 specific questionnaire

A HBO_2 specific questionnaire with focus on HBO_2 contraindications will be filled in by all subjects, contraindications include pregnancy, claustrophobia, obstructive lung disease and history of spontaneous pneumothorax. If anything in the questionnaire renders further examination, a review of medical records, an interview and a medical exam will be conducted. Findings and/or abnormalities detected will be documented in medical records with a statement “No contraindications for HBOT” or else the reason for contraindication.

Questionnaires

Change in RAND 36-item Health Survey (RAND-36), EQ-5D(euroquol.org) are used as primary and secondary enpoints , other questionaires may vary depending on clinical evaluation and main symptoms. Multiple questionnaries are used in clinical assessment including: RAND 36, EQ-5D, Frändin-Grimby activity sale, The Montreal Cognitive Assessment (MOCA), Work Ability Index (WAI), Mental Fatigue Scale (MFS), Fatigue Secerity Scale(FSS), Patient Health Questionnaire (PHQ-9), and the Generalized Anxiety Disorder (GAD-7), COPD Assessemnet Test(CAT), Medical Research Council(mMRC).

Medical records will be reviewed, time of questionnaire, reason for questionnaire and finding will be recorded. Baseline and change is categorized and described according to the reference in the specific questionnaire. SOPs for the study-specific questionnaires are available in the TMF, short description below:

RAND 36-item Health Survey 1.0 (RAND 36)

RAND 36 is a self-reporting questionnaire that contains 36 items that measure eight concepts of health in general terms, at present and past four weeks: physical functioning (10 items), role limitations due to physical health (4 items), role limitations due to emotional problems (3 items), energy/fatigue (4 items), emotional well-being (5 items), social functioning (2 items),

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pain (2 items) and general health (5 items). It also includes a single item that provides an indication of perceived change in health over the last year. Scoring RAND 36 is a two-step process. First, numeric values from the survey are coded so that all items are scored from 0 (lowest score) to 100 (highest possible score). Scores then represent the percentage of total possible score achieved. In step 2, items in the same scale are averaged together to create the 8 scale scores. Items that are left blank (missing data) are not taken into account when calculating the scale scores. Hence, scale scores represent the average for all items in the scale that the respondent answered. RAND 36 is well documented in terms of reliability and variability also for Swedish translation (Orwelius et al., 2017).

EuroQol-5 Dimensions questionnaire (EQ-5D)

EuroQol-5 Dimensions questionnaire is a widely used self-reporting questionnaire that measure 5 dimensions of health TODAY at three or five levels (EQ-5D-3L or EQ-5D-5L) of severity; no problems, some/moderate problems and extreme problems/unable.The health dimensions are mobility, self-care, usual activities, pain/discomfort, anxiety/depression and a visual analogue scale (VAS) 0-100 which it used as a quantitative measure of overall health status. EQ-5D is the most widely used questionnaire for health-economy evaluation. Swedish population norm data for age and gender are available and can also be used for determining ability to work/study.

Physical tests

The 6 min walk test (American Thoracic Society), 30/60 sec chair stan (Jones et al 1997) , EndoPAT for measurement of RH-PAT and Nexfin (Edward Lifesiences) for measurement of cardiac indicies and activity meter for activity, heart rate variability (HRV) and sleep pattern are study-specific, other physical tests used in clinical practice may vary depending on main symptoms.
Multiple different physical tests are used in the clinical assessement including: 30/60-sec chair stand, Handgrip (Jamar), Spirometry, Maximal Inspiratory and Expiratory muscle strength (MIP/MEP), 6-min walk test, Head-Up-Tilt test (HUT).

Medical records will be reviewed, time of test, reason for test and finding will be recorded. Baseline and change is categorized and described according to the reference in the specific test. SOPs for the study-specific tests are available in the TMF, short description below:

6 minute walk test
The test is conducted in a corridor without obstacles with a measured distance of 30 meters (a cone is placed for start and turn) with markings every meters and double marknings every 5 meters. The subject carries a portable pulse/saturation meter.

- If the subject uses a walking aid the same should be used during the test, type of aid, if used is documented in the protocol.
- Periferal oxygen saturation (SpO_2) and pulse are recorded each minute.
- Any pauses during the test is noted, how long and posture during paus is recorded.

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- A timer is started when the subject starts walking. The instructor only walks with the subject if deemed necessary from a safety perspective.
- Fatigue (general and leg) and dyspnea is self-rated with a Borg CR-10 scale immediately when the subject stops.
- The test is stopped it the subject experiences chest pain, SpO_2 below 80%, severe dyspnéa, cramping legs, staggering or wobbleing gait, perspiration or pale face. Time of disontinuation, cause and primary limiting factor is noted in the protocol.

30/60 sec chair stand

A red chair (44 cm high) is used, placed against a wall to minimise risk of falling.
The subject sits on the seat with a staight back, feet sholder wide with close to 90 degree angle in the knees, one foot slightly in front of the other. Arms crossed over chest.
The instructor demonstrates once and the subject practice once.

- The subject in instructed to stand up straight and sit down completely as many times possible during 60 seconds.
- A timer is started when the subjects back side lifts for the seat. The number of straight stands at 30 and 60 seconds is noted in the protocol. The subject is cheered on.The last stand is counted if the subject has risen more than half way at 60 seconds.
- Pulse, SpO_2, fatigue (general and leg) and dyspnea is self-rated with a Borg CR-10 scale immediately when the subject stops.

Nexfin

Subject is fasting, no beverage with caffein or sugar within 2 hours. The monitor is connected before 5 min rest in supine position without distraction. Non-invasive measurement of cardiovascular indicies with a beat-to-beat pulse wave analyzer placed on the middle phalang of one finger by Nexfin teqnology (ClearSight, Edwards Lifeciences). The ClearSight device comprices a pneumoatic pletysmograph that provides advanced hemodynamic parameters and continuous noninvasive blood pressure (BP) from a finger cuff with a redesigned self-coiling mechanism that reconstructs the clinical standard brachial arterial waveform from the finger arterial pressure waveform; it has been validated towards invasive measurements in a number of clinical trials.

- Measurement of beat-to-beat blood pressure and pulse including pulse-contour analysisis at rest and during physical tests.
- Registration of Heart rate, estimated Stroke volume, Cardiac index and Systemic vascular resistance index is recorded in the protocol.

EndoPAT

Subject is fasting, no beverage with caffein or sugar within 2 hours. The monitor is connected before 10 min rest in supine position without distraction. Non-invasive determination of digital endothelial function is measured with a pulse amplitude tonometry (PAT) device placed on the tip of each index finger (Endo-PAT2000; Itamar Medical, Caesarea, Israel). The PAT device comprises a pneumatic plethysmograph that allows measurement of pulse volume changes.

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The PAT signal is recorded at baseline and following 5 min arterial occlusion using an inflatable blood pressure cuff placed on the forearm of one arm, while the contralateral arm serves as a control. The blood pressure cuff is inflated to 30 mmHg higher than the systolic pressure or a maximum of 200 mmHg for 5 min. The post-occlusive hyperemia stimulates endothelium-dependent vasodilatation causing an increase in digital pulse amplitude. The change from the baseline measurement is expressed as the reactive hyperemia index (RHI) which reflects vasodilator function of the digital microcirculation (Hamburg and Benjamin, 2009). Previous evaluation has demonstrated that reduced RHI reflects microvascular endothelial dysfunction, predicts cardiovascular events and reflects reduced NO bioavailability (Alexander et al., 2020).

Activity meter

The commercially available OURA™ ring will be used. The OURA™ ring is worn like a finger ring and has a number of sensors that register heart rate, temperature and physical activity. With the OURA™ ring it is possible to monitor HRV, level of physical activity, changes in body temperature and sleeping pattern. Subjects will be asked to wear the OURA™ ring at a minimum 1 week before and after each visit. Data will be automatically registred in a smartphone application and then uploaded to a secure encrypted database.

Radiology

Multiple different modalities of imaging are usesd in the clinical assessement including: Dual Energy Computed Tomography (DECT), Magnetic Resonance Imaging (MRI), Computed Tomography (CT) and normal chest X-ray (CXR). Review of records, document the time of radiology, reason for radiology and finding if it coincides with an interval of 12 weeks of visit 1, 3 4 and 5.

Socio-demography

Demographic data such as gender, age, level of education, rate of employment/studies, level/rate of exercise, country of origin, body weight, height, and smoking habits/ nicotine use will be collected at Visit 1. Records and medical history will be reviewed for update/change in parameters at each visit.

Concomitant and post-trial treatment(s)

Since Long COVID is a new syndrome, that may be chronic, without any definiete cure, “best practice” for symptomatic medications and other treatments are likely to change over the course of trial. Subjects are also likely to have tried or may try other remedies.

Medications and treatments that are considered “best practice” may be given to the subjects at the discretion of their attending physician/physiotherapist/phycologist. Subjects will be discouraged to try new medications, treatments or remedies that are not evidence based during the course of the trial.

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Information regarding relevant regular concomitant medications, including vitamins, anti-oxidants, treatments and other remedies will be collected at Visit 1. Only relevant medications taken regularly, suspected to have caused an AE or used for treatment of an AE will be recorded. Changes in concomitant medications will be assessed (e.g. stop date or entry of a new treatment), throughout the trial by reviewing the patient's medical records and taking their medical history. Any changes will be recorded in the eCRF.

Blood samples

Routine biochemistry for kidney function, liver function, cardiac insult, haematology and blood glucose will be registered from the hospitals electronic system if they are outside normal range.

Study-specific blood tests that will be collected are: Ferritin, D-Dimer, LDH, Troponin T and a pregnancy test for women of childbearing age.

Date and time of collection and results from routine and study specific blood tests are recorded in the eCRF.

Details regarding the handling of blood sampling for laboratory analysis are found in section 5.3.

HBO_2 SOP and assessment

A standard operation procedure (SOP) will be attatched in the Trial Master File (TMF) but in general terms:

Subjects will be introduced to the hyperbaric unit; if required the subject may visit the unit before the first treatment. Treatment will be conducted in the multiplace (HAUX-STARMED-QUADRO 3500-2400) or monoplace chamber (SECHRIST 3300) depending on availability and number of subjets, at the discretion of the responsible physiscian. Subjects will be treated for 90 minutes; the treatment protocol is as follows - HBO_2 240 kPa with 10 min compression time and 10 minutes decompression time, and two air breakes, while placebo entails - 134kPa air, with 5 min compression time, and 5 min decompression to 120 kPa, and two air breakes will be reported to the subjects. Pressure gauges that can be seen by subjects will be covered. The frequency of the treatments and timing will depend on available resources at the discretion of the responsible physician but should be 2–5 treatments per week for 2–4 weeks. No treatment must be given more than 6 weeks after randomization.

Date and time for treatment will be recorded. Any planned treatment that could not be delivered and reason for the cancellation will be recorded. The treatment will be recorded on a separate CRF accessible only to staff designated to the treatment but blinded for the investigators performing assessements. Treatment type will be recorded in the eCRF and medical records once the code is broken or at the end of trial.

AE and ADR

Adverse events (AEs) and collection of AEs and Serious Adverse Events (SAEs) data.

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Collection of AEs will start directly after inclusion and will be recorded until visit 3. Only SAEs will be collected outside the treatment period (visit 2). Ongoing AEs and SAEs at the end of visit 3 will be followed up during long-term follow-up until the subjects last visit. Definitions, documentation and reporting of AEs are described in detail in the AE section below.

5.3 Biological sampling procedures

5.3.1 Handling, storage, and destruction of biological samples

Study-specific routine biochemistry will be analysed at the Karolinska University Hospital laboratory (KUL).

Study-specific biobanking includes collection ot 4 extra tubes:

1x4ml EDTA plasma will be bio-banked for later analysis

2x8ml Citrate plasma (CPT-tubes) will be bio-banked for PBMC isolation and later analysis.

1x4ml heparin blood will be centrifuged and erythrocyte fraction will be incubated with CPH spin probe, bio-banked for later analysis of ROS in erythrocytes by EPR, plasma will be biobanked for later analysis.

CPT and EDTA tubes will be collected by a research nurse and transported immediately to the research laboratory Studiecenter Karolinska where PBMCs are isolated, half are prepared with RNA-later® for later RNA extraction and gene expression analysis and half is cryopreserved for later functional analysis of the monocytes. The monocytes, citrate-, EDTA- and heparin plasma will be stored in a sub-biobank at Bioclinicum Karolinska University Hospital. The biological samples will be saved until all analyses are performed.

5.3.2 Total volume of blood per subject

The study-specific blood will be maximum 40 ml (24 ml for all and additionally 16ml for some subjects). A maximum total amount of 200 ml blood is collected from each subject at five visits over nine months. This volume should be related to a blood donator that donates 450ml at one occation that can be repeated every four months for women.

5.3.3 Biobank

Plasma, erythrocytes and PBMCs collected in this trial are registered in a regional biobank with an agreement with *Stockholms Medicinska Biobank (IVO reg nr 914)* and handled according to the current biobank laws and regulations. The samples are coded/psedonymized to protect the subject´s identification. All samples and the identification/code list are stored securely and separately to prevent unauthorized access.

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5.3 End of Trial

The end of trial is defined as the last subject's final follow-up at visit 5 (week 52).

Premature termination of this clinical trial may occur because of a regulatory authority decision or at the discretion of the sponsor/the steering comittee.

The sponsor/steering comittee reserves the right to discontinue the trial at any time point in the following cases:

- Unexpected high proportion of AEs that are possibly or probably related to the trial drug.
- Trial protocol is difficult to cope with.
- Recruitment of eligible subjects is too low.

The end of the trial will be reported to the regulatory authority within 90 days, or 15 days if the trial is terminated prematurely. The Investigators will inform participants and ensure that the appropriate follow up is arranged for all involved.

6. Subject selection

6.1 Inclusion criteria:

To be included in the trial, subjects must meet the following criteria:

1) Aged 18–60 years
2) Healthy or mild systemic disease prior to COVID-19
3) Symptoms consistent with Long COVID for at least 12 weeks
4) Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9)
5) Working or studying prior to COVID-19
6) Documented informed consent according to ICH-GCP and national regulations

6.2 Exclusion criteria:

Subjects must not be included in the trial if any of the following criteria are met:

1) Known pregnancy or positive pregnancy test in women of childbearing age
2) ASA 3 or more from other cause than Long COVID
3) Score above 70 in RAND-36 domain Role Limitation Physical Health (RP) or Physical Functioning (PF)
4) Diabetes
5) Diagnosed with Hypertension prior to COVID-19
6) Contraindication for HBO_2 treatment according to local guidelines
7) Participation or recent participation in a clinical trial with an investigational product
8) Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trial participation

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6.3 Screening

Patients that have been assessed for Long COVID and that are likely to fullfil the inclusion criteria will be screened. Subjects will be informed about the trial by a study nurse during pre-screening and in detail about the trial by an investigator and after written informed consent, additional medical record review, HRQoL questionnaires, a HBOT specific questionnaire, physical examination (and pregnancy test if applicable) will be conducted. Subject eligibility (that subjects fulfill all inclusion criteria and do not meet any exclusion criteria) will be established before randomization to treatment.

6.4 Withdrawal Criteria

Subject participation: A subject will be considered to have completed the trial when he or she completes the assessment at 52 weeks (visit 5). Subjects should be encouraged to continue the trial but have the right to withdraw their consent or part of their consent regarding the trial participation e.g. to discontinue a study-specific blood test, but still participate in follow-up visits with questionnaires or not participate in further trial visits. The subject has no obligation to explain why he/she does not want to continue. The investigator also has the right to stop the subjects treatment in the event of AEs, protocol deviations, administrative reasons or any other reasons. It is understood by all concerned that an excessive rate of discontinues can render the trial uninterpretable. Therefore, unnecessary discontinuation should be avoided.

Irrespective of the reason for not continuing with the treatmentsl and whenever possible, the patient should be examined. Relevant laboratory test samples should be obtained and all relevant assessments should be completed if applicable. All AEs should be followed up until they have returned to baseline status or stabilised.
A termination visit (End of trial) in the electronic case report form (eCRF) should be completed for every randomised subject whether the subject completed the trial or not. The reason for any early discontinuation should be indicated on this form.

Subjects may be discontinued from the trial at the discretion of the Investigator. Specific reasons for discontinuing a subject from further assessments are:

AEs: Clinical or laboratory events that in the judgment of the investigator, DSMB or the Sponsor and in the best interest of the subject constitute grounds for discontinuation. This includes serious and non-serious AEs regardless of relation to trial drug.

Withdrawal of Consent: If a subject withdraws consent for disclosure of future information at the discontinuation of the trial or after completion of the trial, no further evaluations should be performed and no additional data should be collected. The Sponsor may retain and continue to use data collected before subject withdrew his/her consent. The Withdrawal of Consent reason is only applicable if the subject denies any further contact with site and no further data collection.

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Lack of Efficacy/Treatment Failure: Subjects experiencing deterioration or no improvement of disease as judged by the investigator, may be discontinued from the trial at any time during the trial, offered alternative treatment and scored as treatment failures. Treatment failures includes significant disease worsening, requirement for surgical intervention and HBOT related SAE. Patients may be discontinued for sustained non-response at the discretion of investigator.

Protocol Violation: If the subject's findings, or conduct, fails to meet protocol entry criteria or fails to adhere to the protocol requirements that make it impossible to derive sound scientific or medical conclusions from the primary endpoint data generated on a subject, (e.g. diagnose is changed after randomization or wrong treatment is given according to randomization).

Lost to Follow-Up: The subject does not show up for further visits and study personnel cannot reach the patient.

Other: Termination of other reason

If the subject discontinues the trial, follow-up of this subject will be performed according to the clinic's routine but will be included in the Safety population if he/she have received at least one treatment.

7. Trial treatments

7.1 Description of investigational product(s)

Oxygen 100%, medical grade (Conoxia cryogen)

Placebo Air, compressed air medical grade

7.2 Dose and administration

Hyperbaric oxygen 240 kPa for 90 minutes (with 10 min compression time, two air breakes and 10 minutes decompression time). The number and frequency of treatments and timing will depend on the subject´s tolerance and available resources at the discretion of the attending physician, but the recommended interval is 2–5 treatments per week with a maximum of 10 treatments within 6 weeks from randomization. To satisfy the treatment compliance, a subject need to complete at least 5 treatments.

Placebo (134 kPa Air, with 5 min compression time, and 5 min decompression to 120 kPa, two air breakes will be reported to the subjects). The number and frequency of treatments and timing will depend on the subject´s tolerance and available resources at the discretion of the attending physician, but the recommended interval is 2–5 treatments per week with a maximum of 10 treatments within 6 weeks from randomization. To satisfy the treatment compliance, a subject need to complete at least 5 treatments.

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7.3 Packaging, labeling, and handling of investigational products(s)

Treatment: 100% oxygen for medical use, cryogenic gas from hospital supply system. There will be no study-specific packaging or labeling.

Placebo: Compressed air from hospital supply system. There will be no study-specific packaging or labeling.

Treatments will be recorded in the eCRF, the code will be unblinded for staff administering the treatments but assessor-blinded. After the subjects end of study, the code will be broken and recorded in the medical records.

7.4 Drug accountability and treatment compliance

HBO_2 is delivered inside a hyperbaric chamber by inhaling 100% oxygen through a tight facemask (in selected cases a hood) attended by medical staff, or inside a monoplace chamber filled with oxygen. If the mask/hood is tight the inspired oxygen pressure is 233.7–240kPa (range depending on 100% saturated - dry gas) at 240 kPa pressure, hence there is no uncertainty about compliance. During compression/decompression patients may need to remove the mask in order to equalize the middle ears and the time might differ slightly between monoplace and multiplace chambers. The difference in dose during this period is neligleble. The date and time of treatment will be recorded in the eCRF. Compliance will be measured as the number and fraction of treatments planned vs given. Subjects that have been given at least 5 treatments will be analysed in the PP population. Any discrepancies from the protocol should be recorded in the eCRF.

7.5 Randomization

Subjects will be enrolled consecutively, as they are found to be eligible for inclusion in the trial, and randomized but after the treatment has been scheduled. Treatment should start within two weeks of randomization.

If a subject discontinues their trial participation, their subject code will not be reused, and the subject will not be allowed to re-enter the trial again. There will be no replacement for these subjects.

Eligible subjects will be randomized in a 1:1 allocation, stratified by disease severity in relation to RAND 36 and gender in blocks (blinded to all study personnel) to either HBO_2 or Placebo. There will be a computer generated randomization.

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7.6 Blinding

This is a double-blind placebo-controlled trial where subjects and all study personell that participate in the asessement of sympoms and objective findings will be blinded to the treatment. The placebo protocol is well established and even experienced divers cannot differ between “sham treatment” and HBO_2 (Lansdorp and van Hulst, 2018). It is not unlikely that some sujects may have problems equalizing the ears even during placebo. Pressure gauges that can be seen by subjects will be covered and all staff will be informed not to discuss the treatment settings when subjects can hear and they will report two air brakes in the same manner as a normal treatment. To validate the blinding process all subjects will be asked at the end of the treatment if they believe they received placebo or HBO_2 and AE directly attributed to equalization problems will be compared.

7.7 Code breaking

The code is kept in the TMF in sealed envelopes, only accessed by staff designated to the hyperbaric unit if needed for safety reasons. If an AE or an SAE is reported, the PI should immediately assess the casual relationship and if an AR or SUSAR is suspected the code may be broken. Treatment type will be recorded in the medical records once the code is broken or at the end of trial.

7.8 Concomitant Medication

Medications that are considered necessary for the safety and well-being of the subject can be given at the discretion of the investigator, unless otherwise specified as an exclusion criterion.

All medications that the subject has taken regularly during the trial must be recorded in the eCRF. Non prescribed food supplements such as vitamins and anti-oxidants should also be recorded in the eCRF if taken regularly. Any changes need to be reported. Concomitant prescribed medications since start of symptoms shall be recorded at Visit 1.

7.9 Treatment after trial end

After an interval of six weeks no more HBO_2 must be given. The total dose during the trial will be recorded until six weeks after first treatment. At trial end, the participants will be treated according to routine clinical praxis.

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8. Handling of Adverse Events

8.1 Definitions

8.1.1 Adverse Event (AE)

Adverse Events constitute any untoward medical occurrence in a clinical investigation subject administered a medicinal product and, which does not necessarily have a causal relationship with the treatment, can be an unfavorable and unintended sign (including an abnormal laboratory discovery), symptom or disease temporally associated with the use of the medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

8.1.2 Adverse Reaction (AR)

In the new use of a medicinal product all noxious and unintended reactions to the medicinal product related to any dose should be considered an adverse reaction (AR). The phrase 'reaction to a medicinal product' means that the causal relationship between the medical product and an adverse event is at least a reasonable possibility, that is the relationship cannot be ruled out.

8.1.3 Serious Adverse Event (SAE)

Serious adverse events constitute any untoward medical occurrence that at any dose:

- results in death
- is life-threatening
- requires inpatient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- results in a congenital anomaly/malformation
- regarded as medically important without meeting the above mentioned criteria

Medical and scientific assessment will be made to determine if an event is 'serious' and whether it would prompt reporting in other situations, for example important medical events that may not be directly life-threatening or result in death or hospitalization but may compromise the subject or may require intervention to prevent one of the other results set forth in the definitions above. These should also normally be considered as SAEs.

8.1.4 Suspected Unexpected Serious Adverse Reaction (SUSAR)

SUSAR comprise a reaction/event that is unexpected, serious, and suspected to be caused by the treatment, i.e. adverse events that are not included in the SPC.

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8.2 Assessment of Adverse Events

8.2.1 Assessment of causal relationship

The investigator is responsible for determining whether there is a causal relationship between the AE/SAE and use of the investigational product.

Those AEs which are suspected of having a relationship to the investigational product will be followed up until the subject has recovered or is well taken care of and on their way to good recovery (see also section 8.4, Follow-up of Adverse Events).

All AEs will be categorized either as related, probably related, possibly related, unlikely related or not related, in accordance with the definitions below:

Related: Clinical event, including abnormal results from laboratory analyses, occurring in a plausible temporal sequence in relation to drug administration. The observed event matches with the known adverse reactions scheme for the drug involved. The event cannot be attributed to underlying disease or other medications.

Probably related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the investigational product. The observed event match with the known adverse reactions scheme for the drug involved. It is unlikely attributable to underlying disease or other drugs.

Possibly related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the intervention/investigational product. The event could be explained by the investigational product and its emergence is reasonable in relationship with use of the investigational product, but there is insufficient information to determine the relationship. The event could be explained by an underlying disease or other medications.

Unlikely related: Clinical event, including abnormal results from laboratory analyses, with a with a temporal relationship with respect to drug exposure that makes a relationship improbable (but not impossible). The event could be plausibly explained by an underlying disease or other medications.

Not related: Clinical event, including abnormal results from laboratory analyses that do not meet any of the above criteria for relatedness.

8.2.2 Assessment of intensity

Each adverse event shall be classified by an investigator as mild, moderate or severe.

Mild: Transient symptoms that are relatively tolerable and does not affect the subject's normal life.

Moderate: Marked symptoms, sufficiently unpleasant that interfere with the subject's normal life. Deterioration of function but is transient.

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Severe: Unacceptable or incapacitating symptoms that causes deterioration of function to the extent that the subject is unable to perform normal activities.

8.2.3 Assessment of seriousness

The investigator is responsible for assessing the seriousness (serious or non-serious). If the incident is considered serious, this should be reported as a serious adverse event (SAE) by the investigator to the sponsor. See also section 8.3.2, Reporting of Serious Adverse Events (SAE).

8.3 Reporting and registration of Adverse Events

At each trial visit, AE are registered. Collection of AE data will start directly after inclusion and continue until 13 weeks (Visit 3) which is 7 weeks after the subject has ended their treatment with the investigational product. All AEs that occur during the trial and that are observed by the investigator/study-nurse or reported by the subject will be registered in the eCRF regardless of whether they are related to the investigational product or not. Assessment of causal relationship, severity, and whether the AE is considered to be an SAE or not will be done by the investigator directly in the eCRF. At minimum, for each AE/SAE, a description of the event is recorded (diagnosis/symptom if diagnosis is missing), start and stop times, causal relationship, severity, if the AE is considered to be an SAE or not, measures and outcome.

The following situations will not be reported as AE/SAE:

- Symptoms judged by the investigator as associated with Long COVID will not be recorded as an AE.
- A change in routine biochemistry will not be reported as AE unless detected during the treatment period.
- Non-serious adverse events outside the treatment period (visit 2) will not be recorded.

8.3.1 Reporting of Adverse Events (AE)

All AEs to be reported shall be registered in the eCRF continously.

8.3.2 Reporting of Serious Adverse Events (SAE)

Serious adverse events are reported to the sponsor on a special SAE form (included in the eCRF) within 24 hours of the investigator being informed of the SAE.

Follow-up information describing the outcome and handling of the SAE is reported as soon as this information is available.

The sponsor will in a timely manner assess whether the adverse event was expected for the investigational product or not, using the reference safety information. Serious AEs must be

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collected, registered in the CRFs and an assessment of causality of the SAE should be performed. Also, discontinuations due to AEs will be collected.

8.3.3 Reporting of Suspected Unexpected Serious Adverse Reactions (SUSAR)

Those SAEs in Sweden which are assessed by the sponsor to be SUSARs are reported via a CIOMS form to the MPA that are submitting the CIOMS report to the to the European Medicines Agency (EudraVigilance database) according to the specified time frames.

SUSARs that are fatal or life-threatening are reported as soon as possible and no later than 7 days after the incident has become known to the sponsor. Relevant follow-up information is sent thereafter within an additional 8 days. Other SUSARs are reported as soon as possible and no later than 15 days after they have come to the sponsor's knowledge.

Any SUSAR will also be notified to the EPM by the sponsor.

Information about SUSARs occurring during the study is compiled by the sponsor and sent out to the principal investigator at all participating centers in connection to the event.

SUSARs in other participating countries will be reported to respective CA and EC according to applicable procedures

8.4 Follow-up of Adverse Events

All AEs should be followed up until they have returned to baseline status or stabilized until End of trial. AEs suspected to have a causal relationship with the trial intervention are followed until recovered or until the subject is on good way to recovery, follow-up will be done at the planned visits regadless of withdrawal from the trial.

8.5 Safety Report (Development Safety Update Report, DSUR)

During the trial period an annual Development and Safety Update Report (DSUR) will be submitted to the Swedish MPA and EPM .

The report includes a summary of all reported SAEs and SUSARs, a summarized safety assessment for trial subjects and information regarding potential updates of the risk-benefit assessment since trial approval.

8.6 Procedures in case of emergencies, overdose or pregnancy

The sponsor and investigator are obliged to immediately take the urgent safety measures necessary to protect the subjects from immediate danger. Examples of such measures are to temporarily suspend the clinical trial or to introduce supplementary monitoring measures.

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The sponsor shall inform the MPA and EPM as soon as possible about the urgent safety measures taken by the investigator or sponsor.

If a subject who participates in a clinical trial for investigational products becomes pregnant, this person must be followed up until the birth has taken place. If the fetus/child has any congenital malformation, this must be reported as a serious adverse event or side effect (SAE).

8.7 Reference Safety Information

For reference safety information, reference is given in the SmPC.

9. Statistics

9.1 Statistical Analysis Plan

The principal features of the statistical analysis of the data are described in this section. A more technical and detailed elaboration of the principal features will be written in a separate Statistical Analysis Plan (SAP). The SAP will be finalised prior to Data Base Lock (DBL) and will include a more technical and detailed description of the statistical analyses described in this section. This section is a summary of the planned statistical analyses of the most important endpoints including primary and key secondary endpoints.

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9.1.1 Analysis population

9.1.1.1 Definition of Trial Populations

9.1.1.1.1 The Full Analysis Set (FAS) Population; All randomized subjects who were exposed at least once to the study intervention will be included in the FAS population.

9.1.1.1.2 Per-Protocol (PP) Population; All randomized subjects with no major protocol violations will be included in the PP population. The final decisions regarding the PP population will be taken at the Clean File meeting before the database lock.

9.1.1.1.3 Safety Population; All randomized subjects that have received at least one treatment will be included in the safety population.

9.2 Statistical analyses

9.2.1 Sample size calculations

The assessment of the primary endpoints in this trial are based on the RAND 36-item health survey at baseline and 3 months where the domains of physical functioning and role functioning/physical defines the primary endpoints.

The primary endpoints have been used for long COVID (Garratt et al., 2021). There are norm data available for Sweden which enable us to determine a threshold for normalisation of individual RAND 36 domain levels. Using data from a few studies with similar methodology where RAND 36 has been previously used, we have assumed the standard deviation (SD) of Role Physical (RP), Physical Functioning (PF) 15.0. We expect the quality of life to be generally low in our cohort, especially in the RAND 36 RP and PF domains. We consider a ten points higher RAND 36 score in the HBO group compared to the placebo group to constitute a clinically relevant difference to be detected. Sample size calculation using t-test for independent groups, with 80% power, assuming a common SD of 15, and with a 5% significance level, reveals that at least 37 subjects per group are needed. We aim to recruit 80 subjects. An interim analysis will be made after 20 have undergone visit 2 to evaluate safety and when 40 subjects have undergone visit 3 in order to stop for futility and adjustment of sample-size if needed.

Sample size calculation was done in nQuery version 7.

9.2.2 General statistical methodology

Primary and secondary endpoints will be evaluated using the FAS population and sensitivity analyses performed using the PP population.

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9.2.2.1 Statistical Hypotesis

The primary objective of the study is to confirm a superior efficacy for the active treatment compared to placebo in the primary endpoints. The null hypothesis to be tested is that there is no difference between HBO treatment and placebo, i.e., the mean change in (HBOT) = mean change (placebo). The same statistical hypothesis will be used for key secondary endpoints.

9.2.2.2 Adjustment for Multiplicity

The overall type I error rate for testing the primary efficacy endpoints will be controlled at the type I error rate of 0.05 using appropriate methods for adjustment of multiplicity in the primary. There will be no adjustment for multiplicity in main secondary endpoints but nominal p-values will be presented and results will be interpreted as exploratory findings.

All hypothesis tests will be two-sided. Details of the methods for adjustment in terms of the selection of endpoints to include in the testing sequence and the criteria for rejecting (or not rejecting) individual hypotheses are provided in the SAP.

9.2.2.3 Subgroups

The following subgroups will be evaluated for this study:

- Gender
- Disease severity
    - RAND-36 RP and PF below 30
    - RAND-36 RP and PF 30-50
    - RAND-36 RP and PF above 50

9.2.3 Patient Demographic and Baseline Characteristics

Baseline values and patient characteristics will be presented in tables by group and in total. All continuous variables will be described using standard statistical measures, i.e., number of observations, mean and median value, standard deviation, minimum and maximum value. All categorical variables will be summarised in frequency tables.

In general, continuous outcome variables will be analysed using ANCOVA, unless otherwise specified. Estimates will be presented using least square means for differences between treatment arms. For continuous endpoints that are measured repeatedly over time, such as EQ5D, RAND-36 domains, the change from baseline will be analyzed using a linear mixed effect model including baseline, treatment group, sex, symptom severity, visit, and treatment

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group by visit interaction, and subjects as random effects, in the models. An unstructured covariance matrix will be assumed.

Analysis for categorical data in terms of binary data (Yes/No) will be presented as the proportion of participants with the frequency of presence or absence by treatment group of the characteristics of interest and analysed using the CMH Chi-square test, where the parameter used for the statistical hypothesis testing will be the OR, as a measure of the relative difference in odds between treatment arms. An OR>1 indicates an efficacy in favour of HBOT compared to placebo.

9.2.4 Primary Endpoint Analysis

The analysis of the primary endpoint will be conducted on the Full Analys Set (FAS) and the Per Protocol Set (PPS).

The primary analysis of the primary endpoints will be performed using the ANCOVA, including randomisation strata of main symptom and gender together with treatment as fixed factors in the model.

The two primary endpoitns will be adequately adjusted for multiplicity.The p-value for testing the null hypotheses, no difference between treatment groups, must be less than 0.05 to be considered to have met the primary objective.

9.2.5 Secondary Endpoints Analysis

The same analysis approach used for the primary efficacy endpoint will be applied to the secondary efficacy and exploratory endpoints as for the primary endpoints referred to as a 'Proportion endpoints'.

For categorical secondary endpoints, the CMH Chi-square test adjusting for disease severity and gender will be used to test for differences between treatments. Results will be presented using the frequency and the proportion by treatment group and the OR together with its corresponding 95% confidence interval.

All tests for the secondary endpoints will be two-sided on the 0.05 significance level. There will be no adjustment for multiplicity in main secondary endpoints.

All analysis will be done for the FAS population using observed data.

9.2.6 Safety analyses

Safety analyses will be performed on the Safety population.

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9.2.6.1 Analysis of Adverse Events

The number and percentage of patients reporting AEs, and the number of AEs reported will be presented. The events will be tabulated by system organ class and preferred term. In addition, summaries by relationship to trial drug and severity will be presented. SAEs will also be presented in separate tabulations.

The number of patients experiencing an AE will be compared descriptively between groups. All patients with AEs will be listed individually with patient number in addition to type of event, start and stop time, duration, seriousness, severity, action taken, relationship to trial drug and outcome of AE.

9.2.7 Interim Analysis

Safety will be monitored continuously by the DSMB throughout the trial, an interim safety analysis will be performed when 20 subjects have available data for the safety endpoints.

There will be an interim analysis performed after 40 subjects have available data for the primary endpoint. The purpose of the interim analysis is to evaluate the assumption used for the sample size calculation and if necessary, to adjust the sample-size if needed. Also, the study will be evaluated for futility regarding the primary endpoints, to stop the study for futility (i.e., the predictive probability of success at the end of the study, given the data at the interim analysis) is less than 20%.

The DSMB will perform both interim analyses. A separate DSMB protocol will be created.

9.2.8 Handling of Dropouts and Missing Data

For the primary endpoint efficacy analyses, missing data will be adequately imputed for all subjects in the FAS population. In addition, the observed cases population will be evaluated as a sensitivity analysis. For secondary endpoints, only observed data will be analysed.

10. Quality Control and Quality Assurance

10.1 Quality Assurance and Sponsor oversight

The sponsor is responsible for having oversight of the trial´s quality. Steps to be taken to ensure the accuracy and reliability of data include the selection of qualified investigators and review of protocol procedures with the site personnel before the trial. eCRF completion guidelines will be provided and reviewed with study-personnel before the start of the trial.

10.2 Monitoring

The trial will be monitored by an independent monitor before the trial begins, during the trial conduct, and after the trial has been completed, so as to ensure that the trial is carried out

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according to the protocol and that data is collected, documented, and reported according to ICH-GCP and applicable ethical and regulatory requirements. Monitoring is performed as per the trial's monitoring plan for which the sponsor is responsible and is intended to ensure that the subject's rights, safety, and well-being are met as well as data in the CRF are complete, correct, and consistent with the source data. The monitoring will be performed by an independent experienced monitor qualified in ICH GCP, applicable national and international regulations and the Declaration of Helsinki.

10.3 Source data

The investigator must keep source documents for each subject in the trial. Data in the eCRF can be source data, such as for certain demography parameters, AEs and assessment of SAEs. Source data is defined before trial start and a document describing what has been classified as source data in the trial should be included in the TMF. The investigator must ensure that all source documents are accessible for monitoring and other quality control activities.

10.4 Deviations or serious breaches

Serious breaches and deviations from the trial protocol, GCP and other regulations that significantly and directly affects, or with high likelihood could affect, the subjects or the scientific value of the trial, shall be reported within seven days (from knowledge) to the Swedish MPA. It is the sponsor's responsibility to judge the consequences of deviations that have occurred, and thus also to decide whether the Swedish MPA should be informed.

For major protocol deviations i.e violations see also section 6.4.

Minor deviations that do not affect subjects' integrity or safety, nor significantly affect the trial's scientific value, are documented in the trial documentation of the principal investigator and the sponsor.

10.5 Audits and inspections

Authorized representatives for the sponsor and Competent Authorities (CA) may carry out audits or inspections at the trial site, including source data verification. The investigator must ensure that all source documents are available for audits and inspections. The purpose of an audit or inspection is to systematically and independently review all study-related activities and documents, so as to determine whether these activities were performed, registered, analyzed and reported correctly according to protocol, Good Clinical Practice (GCP) and applicable regulations.

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10.6 Data Safety Monitoring Board

An independent DSMB will evaluate the safety data in the context of the overall trial and the currently existing information about the trial drug. The DSMB will be composed of representatives from and experts in their respective disciplines of medicine, statistics and clinical trial methodology and conduct.

The DSMB will review the data during the course of the trial, as specified in table 2 below, and will draw up a charter delineating their guidelines for operating and stopping rules for terminating individual subjects, a portion or all of the trial prematurely. However, the DSMB may for any safety concerns recommend stopping of the trial even if these criteria are not fulfilled. It is the responsibility of the Sponsor to decide whether premature end of trial will be made, based on the advice provided by the DSMB

The DSMB will have access to all trial data. It may request and will be provided with whatever data is deemed necessary or useful for it to carry out its duties. The data provided will be blinded to treatment group unless specific unblinding is requested by the DSMB.

Table 2. DSMB meeting schedule	**Time of meeting**
Before trial start	Before first subject is included
Safety Interim analysis	When 20 subjects have completed visit 2
Interim analysis	When 40 subjects have completed visit 3
Efficacy analysis	When all 80 subjects have completed visit 3
End of the trial	Final visit has been done by the last subject.

10.7 Data protection

If any part of the data is handled by any other organization, inside or outside the EU, appropriate agreements and/or other documentation will be established, to ensure that the data processing is performed in accordance with the provisions of the General Data Protection Regulation (GDPR) and other relevant legislation, before any data transfer takes place.

The content of the informed consent form complies with relevant integrity and data protection legislation. In the subject information and the informed consent form, the subject will be given complete information about how collection, use and publication of their trial data will take place. The subject information and the informed consent form will explain how trial data are stored to maintain confidentiality in accordance with national data legislation. All information processed by the sponsor will be pseudonymized and identified with a Study-ID.

The informed consent form will also explain that for verification of the data, authorized representatives of the sponsor, as well as relevant authority, may require access to parts of

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medical records or study records that are relevant to the trial, including the subject's medical history.

11. Ethics

11.1 Compliance to the protocol, GCP and regulations

The trial will be performed in compliance with the trial protocol, the Declaration of Helsinki, ICH-GCP (Good Clinical Practice) guidelines and current hospital, national and international regulations governing this clinical trial. This is to ensure the safety and integrity of the subjects as well as the quality of the data collected.

11.2 Ethical review of the study

The final trial protocol must be approved, as a part of the application for a permit for clinical trials, by both the Swedish Ethical Review Authority (Etikprövningsmyndigheten, EPM) and the Swedish Medical Products Agency (MPA) before the trial can be conducted. The final version of the informed consent form and other information provided to subjects, must be approved or given a written positive opinion by EPM. EPM and MPA must be informed of any changes in the trial protocol in accordance with current requirements.

11.3 Procedure for obtaining informed consent

The principal investigator shall ensure that the subject is given full and adequate oral and written information about the trial, its purpose, any risks and benefits as well as inclusion and exclusion criteria. Subjects must also be informed that they are free to discontinue their participation in the trial at any time without having to provide a reason. Subjects should be given the opportunity to ask questions and be allowed time to consider the provided information. If the person chooses to participate, both the subject and the investigator (qualified physician) shall sign the informed consent form. A copy of the subject information as well as the informed consent form shall be provided to the subject. The subject's signed and dated informed consent must be obtained before performing any study-specific activity in the trial. Each subject who participated in the trial will be identified by a subject number and if randomized, indentified by a randomization number on a subject identification list. The subject agrees that monitors, auditors, and inspectors may have access to their medical records and other source data. If new information is added to the trial, the subject has the right to reconsider whether he/she will continue their participation.

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12. Insurances

Trial subjects are covered by the Swedish patient insurance and the Swedish pharmaceutical insurance.

13. Substantial changes to the trial

Substantial changes to the signed trial protocol are only possible through approved protocol amendments and by agreement from all responsible persons. Information on non-substantial changes should be clearly noted in the amended protocol.

In the event that substantial changes to the protocol (e.g., changing of the main objective, primary or secondary variables, method to measure the primary variable, changing of the investigational product or dosage) will be made during the course of the trial, approval from the MPA and EPM shall be obtained before any changes are implemented. A change that concerns a new site, new investigator or a new subject information sheet shall only be approved by the EPM, as applicable.

Non-substantial changes will be recorded and later entered in documentation that is submitted, for example in any subsequent notifications of a substantial change or in connection with End of Trial reporting.

14. Collection, handling and archiving data

Subjects who participate in the trial are coded with a specific trial identification number (Study-ID). All subjects are registered in a subject identification list (subject enrolment and identification list) that connects the subject's name and personal identity number with the Study-ID. When randomized a separate randomization number will be added.

All data will be registered, managed, and stored in a manner that enables correct reporting, interpretation, and verification. The complete Trial Master File, as well as source documents, will be archived for at least 10 years after the trial is completed. Source data in the medical records system is stored and archived in accordance with hospital regulations.

14.1 Case Report Form

An electronic Case Report Form (eCRF) is used for data collection. The investigator must ensure that data is registered and any corrections in the eCRF are made as stated in the trial protocol and in accordance with the instructions. The investigator must ensure that the registered data is correct, complete, and that reporting takes place according to the timelines that have been predefined and agreed. The investigator signs the completed eCRF. A copy of the completed eCRF will be archived at the trial site.

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If an examination/test is not performed and data does not exist, ND (Not done) or NK (Not known) is marked. If the question is irrelevant NA (Not applicable) is written. Corrections in paper work sheets are done by striking out the incorrect information and adding the correct information next to the incorrect information, signing, and dating the correction.

15. Notification of trial completion, reporting, and publication

The MPA and EPM shall be informed of the trial's completion at latest 90 days after trial end, through submission of a "Declaration of End of Trial Notification" form.

Within one year after the trial is completed, the results shall be analyzed, a clinical trial report with individual data shall be prepared, and the trial results shall also be reported in the EudraCT database. The sponsor is responsible for the preparation of the clinical trial report. The statistical analyses will be performed and the results will be presented to the Investigator(s). Based on these data, the Principal investigator, in cooperation with the Co-Investigator(s), will prepare a clinical trial report. The report will be submitted to the competent authorities and will form the basis for a manuscript intended for publication in a medical/scientific journal. All personnel who have contributed significantly with the planning and performance of the trial may be included in the list of authors.

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Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

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Trial Code: HOT-LOCO
Version No: v.4
Date: 2022-01-03
EudraCT No: 2021-000764-30

17. Amendments and Administrative changes

The following amendments and Administrative changes have been made to this protocol since day of preparation:

Amendment	Section/Page	Date	Type/comment
Version 1		2021-05-05	EPM submission
Version 2 addition of sponsor representative signature addition of DSMB members addition of COVID-19 pandemic statement minor layout and typos	Signature page/5 Contact information/6 3.5/16 Full protocol	2021-06-30	MPA submission/non substantial change
Version 3 specification of activity meter used minor layout and typos	 5.2/21-26 Full protocol	2021-08-16	EPM amendment/non substantial change
Version 4 minor layout and typos Change of treatment interval	 Full protocol 7.9	2022-01-03	Non substantial change Incoherent with section 5

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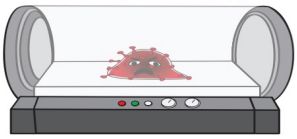
SOP Randomization-Blinding



PURPOSE: The purpose of this Standard Operations Procedure (SOP) is to describe procedures that protect the health and welfare of participants and data integrity in a double-blinded, randomized clinical trial

SCOPE: This SOP applies to all study personnel involved in the conduct of the trial, development and implementation of the randomization and/or blinding process.

RESPONSIBILITY: The PI and designated trial staff are responsible for following the randomization and blinding procedures described in the clinical trial protocol. All personnel, including staff designated to treatment, needs to participate in GCP training and be delegated to each task.

DEFINITIONS:

Blinding: The procedure in which both the subject and the assessors are kept unaware of the treatment assignment. Double blinding refers to that also the subjects and assessors being unaware of the treatment assignments. Assessors includes anyone determining subject eligibility, evaluating endpoints, or assessing compliance with the protocol, i.e. investigators, monitor and data manager.

Randomization: The process of assigning trial subjects to treatment or sham-treatment group using an element of chance to determine the assignments in order to reduce bias.

Randomization tool: Web-based tool used to allocate patients to a particular treatment arm. In this trial Randomizer.at is used.

Unblinding: Identification of the treatment code for a subject; by the subject or investigators involved in assessment. Also referred to as “code breaking”. Code breaking may be intentional in case of emergency or evaluation of an AE or can be accidental. Examples of accidental code breaking is that the subject or assessors see the treatment protocol or that the treatment group is discussed with an assessor.

PROCEDURE:

1. The Randomization tool is programmed by the senior statistician Jan Kowalski at EDC Scandinavia AB. The study staff member(s) responsible for the randomization will have training in and access to the randomization tool prior to participant recruitment.

2. **Blinding:**

2.1 The PI and trial staff will review the protocol and determine who should be designated to treatment/randomization and assessment respectively.

2.2 A printout of the randomization code is kept together with the treatment protocol in the subject’s treatment portfolio. The portfolio is stored in a locked cabinet with limited access.

2.3 During treatment it is important that the treating staff use normal routines for treatment in both groups, such as: ask normal questions regarding equalization problems, notify when it is time for “air break” even in “placebo”, remind about oxygen safety etc.

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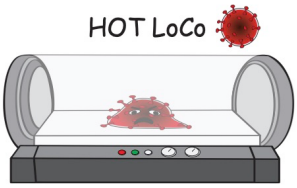
SOP Randomization-Blinding



2.4 Assessors should avoid discussing the treatment with the subjects and should not enter the treatment rooms unless a case of emergency that cannot be solved by designated staff.

2.5 When the treatment is finalized the treatment protocol is stored together with the randomization code in a sealed envelope in the TMF.

3. Unblinding:

3.1 Randomization and treatment procedures must be followed to ensure the code is broken only in accordance with the protocol. Circumstances for unblinding and procedures thereof must be known by all study staff before the first patient is randomized.

3.2 In general, the code should only be broken in the case of an adverse event where it is necessary for the Principal Investigator to know which treatment the patient is receiving before the participant can be treated. This is a very unlikely in this trial.

3.3 The sponsor/PI should be notified immediately, preferably by telephone and then by email, regarding the necessity of code breaking.

3.4 When it is necessary to break the blind, the PI must notify the IRB.

3.5 If the code is broken for a participant, this must be documented in the eCRF, together with the reasons for breaking the code. The reason for breaking the code should also be written on the randomization printout.

3.6 Justification for premature unblinding of the investigational product should be documented (e.g. accidental unblinding, unblinding due to serious adverse event) in the source document as well as the Regulatory File.

REFERENCES:	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH): ICH-E8 General considerations for clinical trials ICH-E9 Statistical Principles for Clinical Trials (CPMP/ICH/363/96)
RELATED SOPs:	Treatment protocol (Behandlingsordination HOT-LOCO) Treatment tables (HOT-LOCO Behandlingstabell HBO, HOT-LOCO Behandlingstabell Placebo)

REVISION HISTORY:

Amendment	Date	Type/comment
Version 1 En	2021-06-26	MPA submission
Version 2 En	2021-09-25	Change of randomization tool

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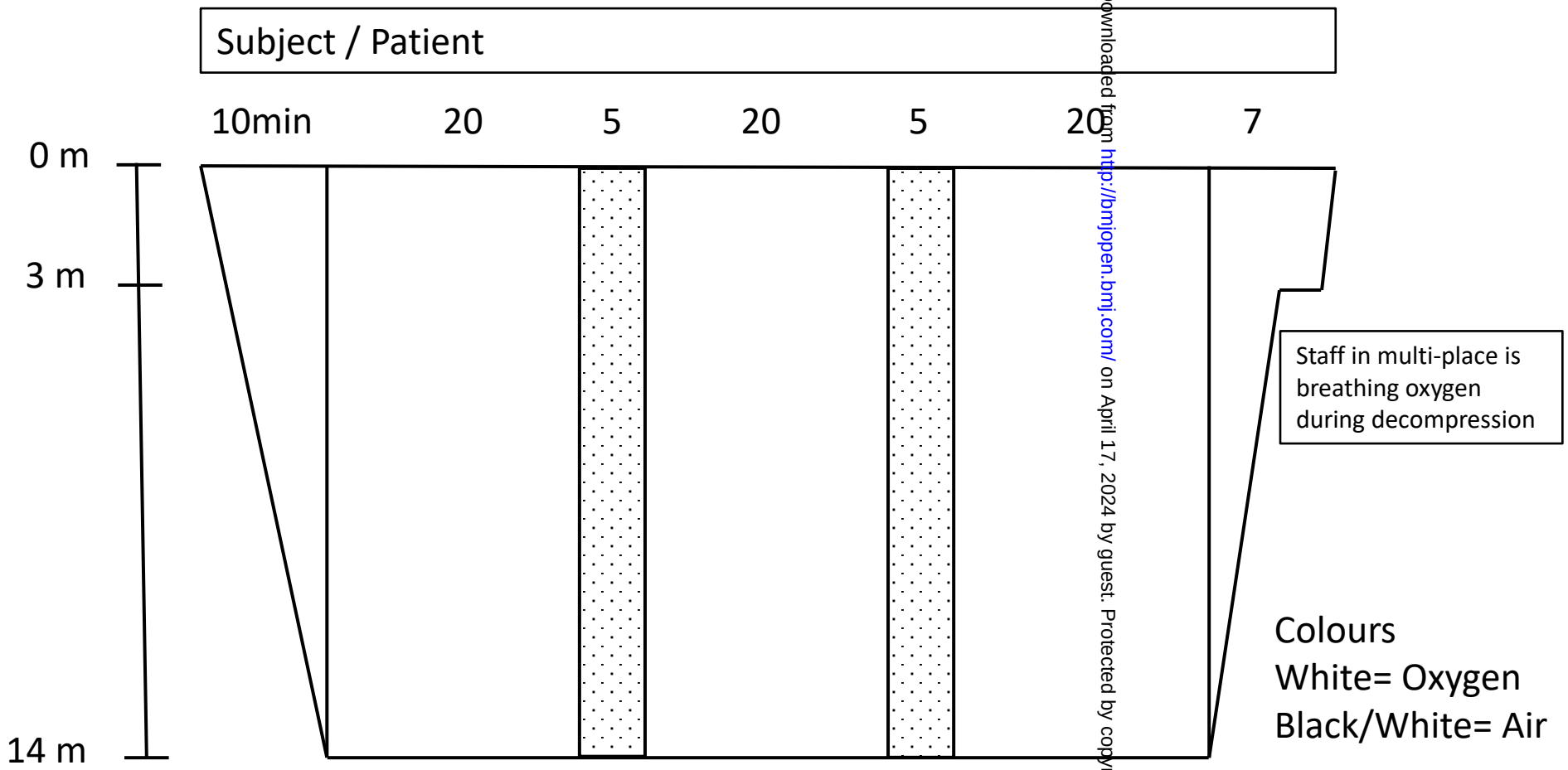
HBO treatment (IMP)

14:80:7 (meter):(bottom time, minutes):(decompression time, minutes) Total time: 87 minutes



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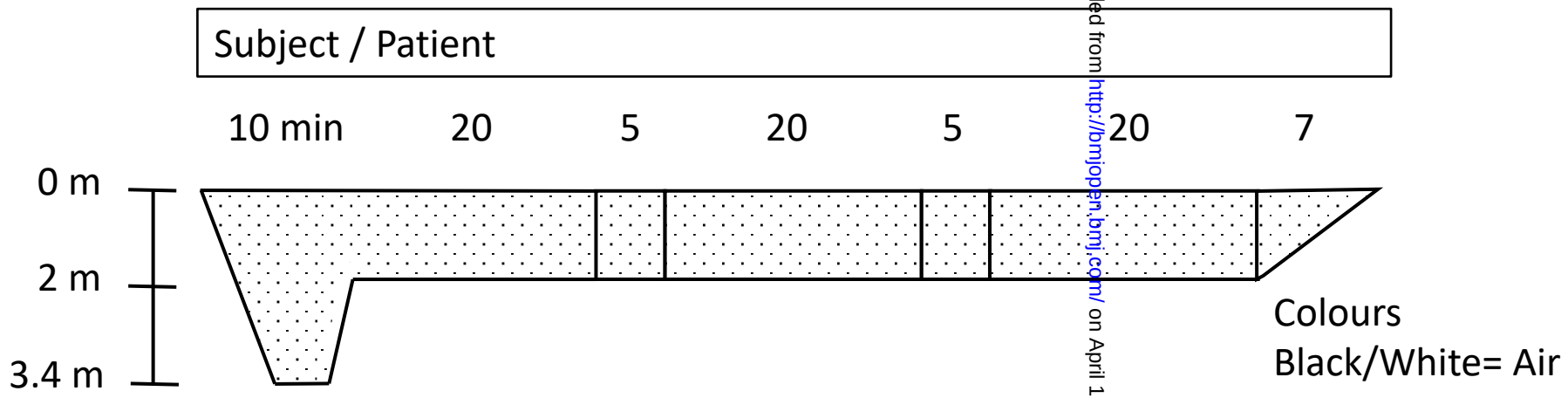
Sham treatment (placebo)

3.4-2:80:7 (meter):(decompression time, minutes) Total time: 87 minutes



Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines with the PRO-extension

Calvert M, Kyte D, Mercieca-Bebber R, et al. Guidelines for Inclusion of Patient-Reported Outcomes in Clinical Trial Protocols: The SPIRIT-PRO Extension. *JAMA : the journal of the American Medical Association* 2018;319(5):483-94. doi: 10.1001/jama.2017.21903 [published Online First: 2018/02/08]

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	N/A, full protocol
Protocol version	#3	Date and version identifier	N/A, full protocol
Funding	#4	Sources and types of financial, material, and other support	19
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1, 19
Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	NA, full protocol
Roles and responsibilities: sponsor and funder	#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	19

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		decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	
Roles and responsibilities: committees	#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)	N/A, full protocol
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	3-4
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators (PRO extension)	9-11, 17-18
Objectives	#7	Specific objectives or hypotheses	4
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, non-inferiority, exploratory)	5
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	5
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	6

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		individuals who will perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6-7
Interventions: modifications	#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)	N/A full protocol
Interventions: adherence	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	N/A full protocol
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A full protocol
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	7-14
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)	Figure1
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	15
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5-6

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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	11-12
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	11-12
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	12
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7, 12
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A, full protocol
Methods: Data collection, management, and analysis			
Data collection plan	#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	7

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		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 (PRO extension)	
Data collection plan: retention	#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 (PRO extension)	9-10
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	N/A, full protocol
Statistics: outcomes	#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	14-17
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15
Statistics: analysis population and missing data	#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)	15
Methods: Monitoring			
Data monitoring: formal committee	#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	N/A, full protocol

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		found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
Data monitoring: interim analysis	#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	N/A, full protocol
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 (PRO extension)	14
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A, full protocol
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	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)	N/A, full protocol
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	N/A, full protocol
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A, full protocol
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	N/A, full protocol

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Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	20
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	N/A, full protocol
Dissemination policy: trial results	#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	18
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	N/A, full protocol
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	20
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	N/A can be sent on request
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	N/A Separate Laboratory manual can be

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sent on request

Notes:

- 2b: N/A, In full protocol p. 9-11
- 3: N/A, In full protocol p. 1
- 5b, 5d: N/A, In full protocol p. 6
- 11b: N/A, In full protocol p. 34-38
- 11c: N/A, In full protocol p. 42-44
- 11d: N/A, In full protocol p. 33
- 17b: N/A, In full protocol p. 33
- 19: N/A, In full protocol p. 42-45
- 21a, 21b: N/A, In full protocol p. 44
- 23: N/A, In full protocol p. 42-44
- 25: N/A, In full protocol p. 46
- 26a, 26b: N/A, In full protocol p. 45
- 27: N/A, In full protocol p. 46-47
- 30: N/A, In full protocol p. 46
- 31b: N/A, In full protocol p. 47
- The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 1. February 2022 using https://www.goodreports.org/, a tool made by the EQUATOR Network in collaboration with Penelope.ai

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BMJ Open

Hyperbaric Oxygen for Treatment of Long COVID syndrome (HOT-LoCO); Protocol for a Randomised, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-061870.R1
Article Type:	Protocol
Date Submitted by the Author:	25-Aug-2022

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	Karolinska University Hospital, ME Cardiology, Heart, vascular and Neuro Theme Bruchfeld, Judith; Karolinska University Hospital, Department of Infectious Diseases; Karolinska Institutet, Department of Medicine Solna, Division of Infection Diseases Nygren-Bonnier, Malin; Karolinska Institutet, Department of Neurobiology, Care Sciences and Society, Division of Physiotherapy.; Karolinska University Hospital, Women's Health and Allied Health Professionals Theme, Medical unit Occupational Therapy and Physiotherapy Lindholm, Peter; Karolinska Institutet, Department of Physiology and Pharmacology ; UCSD, Department of Emergency Medicine, Division of Hyperbaric medicine
Primary Subject Heading:	Pharmacology and therapeutics
Secondary Subject Heading:	Cardiovascular medicine, Immunology (including allergy), Respiratory medicine, Rehabilitation medicine
Keywords:	COVID-19, RESPIRATORY MEDICINE (see Thoracic Medicine), VASCULAR MEDICINE, CARDIOLOGY, REHABILITATION MEDICINE, IMMUNOLOGY

SCHOLARONE™
Manuscripts

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Hyperbaric Oxygen for Treatment of Long COVID Syndrome (HOT-LoCO); Protocol for a Randomised, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

Anders Kjellberg[1,2], Lina Abdel-Halim[1], Adrian Hassler[1,3], Sara El Gharbi[1,3], Sarah Al-Ezerjawi[1,3], Emil Boström[1,3], Carl Johan Sundberg[1,4], John Pernow[5,6], Koshiar Medson[1,7], Jan Kowalski[8], Kenny A Rodriguez-Wallberg[9,10], Xiaowei Zheng[11], Sergiu-Bogdan Catrina[11,12], Michael Runold[13,14], Marcus Ståhlberg[5,6], Judith Bruchfeld[15,16], Malin Nygren-Bonnier[17,18] and Peter Lindholm[1,19]

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Abstract

Introduction Long COVID, where symptoms persist 12 weeks after the initial SARS-CoV-2-infection, is a substantial problem for individuals and society in the surge of the pandemic. Common symptoms are fatigue, post-exertional malaise, and cognitive dysfunction. There is currently no effective treatment, and the underlying mechanisms are unknown although several hypotheses exist, with chronic inflammation as a common denominator. In prospective studies, hyperbaric oxygen therapy (HBOT) has been suggested to be effective for the treatment of similar syndromes such as chronic fatigue syndrome and fibromyalgia. A case series has suggested positive effects of HBOT in Long COVID. This randomised placebo-controlled clinical trial will explore HBOT as a potential treatment for Long COVID. The primary objective is to evaluate if HBOT improves health related quality of life (HRQoL) for patients with Long COVID compared to placebo/sham. The main secondary objectives are to evaluate whether HBOT improves endothelial function, objective physical performance, and short term HRQoL.

Methods and Analysis A randomised, placebo-controlled, double-blind, phase II clinical trial in 80 previously healthy subjects debilitated due to Long COVID, with low HRQoL. Clinical data, HRQoL-questionnaires, blood samples, objective tests and activity meter data will be collected at baseline. Subjects will be randomised to a maximum of 10 treatments with hyperbaric oxygen or sham treatment over six weeks. Assessments for safety and efficacy will be performed at six, 13, 26 and 52 weeks, with the primary endpoint (physical domains in RAND-36) and main secondary endpoints defined at 13 weeks after baseline. Data will be reviewed by an independent Data Safety Monitoring Board.

Ethics and Dissemination The trial is approved by The Swedish National Institutional Review Board (2021-02634) and the Swedish Medical Product Agency (5.1-2020-36673). Positive, negative, and inconclusive results will be published in peer-reviewed scientific journals with open access.

Trial Registration NCT04842448. EudraCT: 2021-000764-30

Strengths and limitations of this trial

Strengths

- Randomised placebo-controlled, double-blind, parallel groups, clinical trial in compliance with ICH-GCP

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- Evaluation of safety and efficacy, including objective and explanatory endpoints

Limitations

- New syndrome with unknown mechanisms
- Power calculation is based on similar syndromes
- Selection bias as patients are enrolled from the same post-COVID clinic

Introduction/Background

In the wake of the first wave of the SARS-CoV-2 pandemic, a new set of often debilitating post-infectious symptoms have arisen. Such symptoms that persist for more than three months, even after mild SARS-CoV infection, have become a major burden for the individuals affected, health care providers, and society in general[1]. The prevalence of long COVID is difficult to determine due to a plethora of symptoms and different definitions[2]. A recent estimation from a UK cohort of 508,707 patients suggests that more than 30% had experienced at least one symptom with "significant impact on my daily life" giving an overall prevalence of 1.72%[3]. Most patients experiencing lingering symptoms are women, of which many have experienced only mild if any respiratory symptoms, and seldom required hospital care during the acute phase of their SARS-CoV-2 infection [4]. Reported long-term symptoms include shortness of breath, fatigue, post-exertional malaise, and cognitive dysfunction, frequently leading to reduced working capability [2]. Some patients are also diagnosed with autonomic dysfunction, including Postural Orthostatic Tachycardia Syndrome (POTS) and inappropriate sinus tachycardia[5, 6].

As the pandemic continues to spread, with new mutations and resulting variants of SARS-CoV-2 appearing, effective treatments are needed to quell infection rates as well as mitigate lingering long-term symptoms. There is still not a uniform definition or name of the syndrome, but post-acute COVID-19 syndrome (PACS), post COVID syndrome (PCS), or Long COVID are commonly used[7]. An attempt to achieve a global definition of Post COVID condition, the name suggested by World Health organisation (WHO), was recently made by a Delphi consensus process[8]. Post COVID condition is previously listed in International Classification of Diseases (ICD-10) with code U09.9, which includes all commonly used names. Experts in the field have recently suggested management guidelines for monitoring and follow-up, but to date there is no effective treatment[9]. The underlying mechanisms are not understood but several hypotheses including endothelial dysfunction, oxidative stress, and chronic inflammation have been proposed[10, 11]. In fact, a recent study demonstrated persistent microvascular endothelial dysfunction for four months following COVID-19 infection[12].

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Hyperbaric oxygen therapy (HBOT) is administered by delivering 100% oxygen at raised pressure to patients in a hyperbaric chamber. HBOT has previously been used as an adjunctive treatment for COVID-19, resulting in faster recovery in prospective trials, case series[13], and a randomised controlled trial (RCT)[14], with additional RCTs ongoing[15]. The rationale for treatment of COVID-19 with HBOT is the treatment's well-established anti-inflammatory effects[16, 17]. Furthermore, a small retrospective cohort study has shown promising results in alleviating symptoms of Long COVID in patients treated with HBOT[18]. The safety profile of HBOT is well established and is considered both safe and effective for the treatment of several chronic inflammatory diseases such as soft tissue radiation injury[19]. HBOT has been shown to improve symptoms and quality of life in other syndromes associated with chronic fatigue[20, 21]. We explore HBOT administered within a randomised placebo-controlled clinical trial as a potential treatment for patients suffering from Long COVID. The purpose of this manuscript is to provide a summary of our protocol that complies with International Council for Harmonisation-Good Clinical Practice (ICH-GCP), with a detailed description and rationale for the primary and main secondary endpoints, including patient reported outcomes (PRO) in line with Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) SPIRIT-PRO Extension Guidelines[22].

Hypothesis and objectives

The overall hypothesis to be evaluated is that HBOT reduces oxidative stress and chronic inflammation, improves endothelial dysfunction, and thereby alleviates symptoms associated with Long COVID.

The primary objective is to evaluate whether HBOT improves Health related quality of life (HRQoL) for patients compared to placebo. The main secondary objectives are to evaluate whether HBOT improves endothelial dysfunction, objective physical performance, and improvement of short term HRQoL. Other secondary objectives are to evaluate if HBOT improves autonomic dysfunction, restorative sleep, the health-economic benefits of the treatment and evaluate biomarkers for the HBO effect on inflammation and chronic hypoxia. Furthermore, we aim to evaluate the safety profile of HBOT for Long COVID patients.

Methods and analysis

Trial design

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The trial is designed as a prospective, randomised, placebo-controlled, double-blind, phase II clinical trial. The trial consists of 5 visits for 52 weeks. At Visit 1 the participant eligibility will be established, and baseline data collected. Block randomisation will be performed, stratified by gender and disease severity as determined by the RAND-36-questionnaire. Eligible subjects are randomised a maximum of two weeks before the first treatment and will receive a maximum of ten treatments over six weeks from randomisation. Treatment is conducted by designated staff not involved in assessment or data collection, subjects and investigators are blinded to the treatment allocation. The randomisation and blinding process is described in a standard operating procedure (SOP), (See supplementary file 1). Visit 2 is conducted on the day of the last treatment. The primary and main secondary endpoints will be assessed at 13 weeks from baseline at Visit 3. Visits 4 and 5 are long term follow-up. Subjects will also be asked to participate in a post-trial follow up over 4 years. A flowchart of the trial design is depicted in Figure 1. And the Consolidated Standards of Trials (CONSORT) flow diagram is depicted in Figure 2.

Patient and Public Involvement

The trial design and consent form were discussed with and approved by a patient representative.

We thank Svenska Covidföreningen through chairman Åsa Kristofferson-Hedlund for their support.

Setting

The trial is investigator initiated and will take place in a single center. The sponsor is Region Stockholm via the Karolinska University Hospital in collaboration with Karolinska Institutet, both in Stockholm, Sweden. Patients will be recruited through the post-COVID outpatient clinic and/or advertisement. Measurements and treatments will take place at the hyperbaric unit. If included in the trial, all patients regardless of intervention or control will be treated at the hyperbaric treatment facility, staffed by anesthesiologists and intensivists as well as nurses specifically trained in HBOT. All personnel involved in the trial are designated to specific duties and trained in ICH-GCP. As per protocol at Karolinska University Hospital, each treatment in the hyperbaric chambers must be overseen by a minimum of two staff members. Local, national, and international guidelines for clinical trials and HBOT during the COVID-19 pandemic will be followed.

Trial population

80 patients aged 18–60, previously generally healthy (defined as American Society of Anesthesiologists (ASA) class I-II), will be recruited. They must have had symptoms consistent with Long COVID for a minimum of 12 weeks, as well as a Long COVID diagnosis with ICD- 10 code U09.9.

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Subjects must have been working or studying before the diagnosis. A HBOT specific questionnaire with focus on HBOT contraindications will be filled in by all subjects, contraindications include pregnancy, claustrophobia, obstructive lung disease and history of spontaneous pneumothorax. All inclusion and exclusion criteria are listed in **Table 1**. Subjects who are diagnosed with Long COVID through the Karolinska University Hospital Post-COVID outpatient clinic will be evaluated by a multidisciplinary team consisting of an infectious disease specialist, pulmonologist, cardiologist as well as a physiotherapist. All patients will be assessed with a battery of questionnaires, physical tests, laboratory tests and radiology including MRI's.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
• Aged 18–60 years • Healthy or mild systemic disease (ASA I-II) prior to COVID-19 • Symptoms consistent with Long COVID for at least 12 weeks • Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9) • Working or studying prior to COVID-19 • Documented informed consent according to ICH-GCP and national regulations	• Known pregnancy or positive pregnancy test in women of childbearing age • ASA III or more from other cause than Long COVID • Score above 70 in RAND-36 Role Limitation Physical Health (RP) or Physical Functioning (PF) • Diabetes mellitus • Diagnosed with hypertension prior to COVID-19 • Contraindication for HBOT treatment according to local guidelines • Participation or recent participation in a clinical trial with an investigational product • Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trial participation

Treatment/interventions

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The HBOT group will undergo HBOT at 2.4 Atmospheres absolute (ATA), approximately 240kPa for 90 minutes with two airbrakes (See supplementary file 2), with a maximum of 10 treatments within 6 weeks of randomisation. The placebo group will undergo 'Sham treatment' with air-breathing at 1.34 ATA, approximately 134kPa (See supplementary file 3) to equate the sensation of HBOT and airbrakes will be simulated. They will undergo a maximum of 10 treatments within 6 weeks of randomisation.

The hyperbaric chambers to be used are designed for a single patient (monoplace chamber) or for multiple patients (multi-place chamber). In the case of the monoplace chamber, it is pressurized with 100% oxygen and staff and equipment are located outside the chamber. However, multi-place chambers are pressurized with air, allowing staff and equipment to be inside the same chamber where the patient breathes oxygen through a mask. The latter is suitable for patients requiring a high level of medical care or groups of patients that can sit in a chair for 90 minutes, whereas the monoplace chamber is more comfortable but requires the patient to be fully alert and stable.

Procedures

The patients will be informed about the trial orally and in writing and given the chance to ask questions. If they agree to participate, an informed consent form (ICF) will be signed by the patient and an investigator before any study-specific procedures occur. Subjects will then be scheduled for a screening visit (Visit 1) where baseline data will be collected, and inclusion/exclusion criteria are verified. Subjects eligible for inclusion in the trial will subsequently enter the trial, be randomised, and allocated to treatment. After the treatment period of six weeks, the subjects will be scheduled for follow-up visits at 13 +/- 2 weeks and 26 and 52 weeks +/- 4 weeks after randomisation.

All procedures in the trial are described in detail in the full protocol (See supplementary File 4). For treatments, blinding procedures, and assessments, standard operating procedures (SOPs) will be followed. A list of procedures is depicted in **Table 2**.

Table 2 List of procedures (Trial specific procedures are marked with **bold X**, data collected from medical records are marked with narrow X)

*Exclusion criteria includes a pregnancy test (if applicable), RAND 36 questionnaire, a HBOT specific questionnaire, review of medical records and a medical examination if needed.

** Medical history includes COVID-19 specific history, routine blood tests, questionnaires, physical tests, and radiology, medical records will be reviewed and recorded.

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*** Socio-demography that may change over time such as sick-leave, weight, activity, smoking habits.

Activity	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Week	0	6	13	26	52
Signed Informed consent Form	**X**				
Inclusion/exclusion criteria	**X***				
Randomisation	**X**				
Medical history	**X**	X**	X**	X**	X**
Socio-demography	**X**	X***	X***	X***	X***
Concomitant medications	**X**	**X**	**X**	X	X
RAND 36	**X**	X	**X**	**X**	**X**
EQ-5D	**X**	**X**	**X**	**X**	**X**
RHI	**X**		**X**		
6 min walk test	**X**	X	**X**	X	X
30/60 s chair-stand	**X**	X	**X**	X	
Nexfin	**X**		**X**		
Treatment (HBOT/Placebo)		**X (1-10)**			
Treatment planned		**X (1-10)**			
AE/ADR	**X**	**X**	X	X	X
Trial-specific biochemistry	**X**	**X**	**X**	X	X
Biobanking (PBMC, Plasma, EPR)	**X**	**X, X**	**X**	**X**	
Activity meter	**X**	**X**	**X**	**X**	**X**

Assessments/measurements

Prior to inclusion subjects will have undergone extensive tests, including radiology with different modalities such as computer tomography (CT), magnetic resonance imaging (MRI), dual-energy computer tomography (DECT), cardiac ultrasound and chest X-rays, and objective physical measurements such as handgrip strength, spirometry and head-up-tilt test and questionnaires used in clinical practice to confirm the diagnosis and rule out any differential diagnosis. This data will be obtained from medical records.

Blood-based biochemical values for kidney function, liver function, cardiac enzymes, haematology, and blood glucose will be obtained from patients' medical records. Trial-specific biochemistry will

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include ferritin, D-dimer, LDH, troponin T, and a pregnancy test for any woman of childbearing age; blood for biobanking will be collected from fasting subjects.

During the screening visit (Visit 1) subjects will fill out the RAND 36-item Health Survey (RAND-36), EuroQol-5 Dimensions Questionnaire (EQ-5D) and undergo physical tests including the 6-minute walk test (6MWT) and 30/60 sec chair stand test (CST), and other objective evaluations including endothelial function with pulse amplitude tonometry (PAT), measurements of cardiac function, and activity, heart rate variability and sleep patterns with an activity meter.

Patient Reported Outcome (PRO) Measures

RAND-36-item health survey (RAND-36)

RAND 36 is a self-reporting questionnaire that contains 36 items that measure eight concepts of health in general terms, at present and past four weeks: physical functioning (ten items), role limitations due to physical health (four items), role limitations due to emotional problems (three items), energy/fatigue (four items), emotional well-being (five items), social functioning (two items), pain (two items) and general health (five items). It also includes a single item that provides an indication of perceived change in health over the last year. Scoring RAND 36 is a two-step process. First, numeric values from the survey are coded so that all items are scored from 0 (lowest score) to 100 (highest possible score). Scores then represent the percentage of total possible score achieved. In step two, items in the same scale are averaged together to create the eight-scale scores. Items that are left blank (missing data) are not considered when calculating the scale scores. Hence, scale scores represent the average for all items in the scale that the respondent answered. RAND 36 is well documented in terms of reliability and variability also for Swedish translation [23]. National gender and age normative data are availible for comparison[23] The questionnaire will be sent out digitally to the subjects on the day of the visit, and when filled out uploaded to the medical records. The dimensions in RAND-36 are presented separately and we have chosen the physical domains RP and PF as primary endpoint for two reasons:

1. The physical domains seem to be severely affected in conditions associated with chronic fatigue and POTS[24, 25].
2. We expect the physical domains to be least affected by placebo.

EuroQol-5 Dimensions Questionnaire (EQ-5D)

EQ-5D is a widely used patient-reported questionnaire aimed at measuring five different dimensions of present health with three or five levels of severity: no problems, some/moderate problems, and

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severe/extreme problems. The five different dimensions are mobility, self-care, usual activities, pain/discomfort, anxiety/depression. It also uses a visual analogue scale (VAS) 0-100 for quantifying measures of overall health. EQ-5D is a well-validated tool and the index that is calculated from the dimensions gives an estimate of Quality Adjusted Life Years (QALY), with a low index indicating a low HRQoL[26]. We will use five levels of severity (EQ-5D-5L) in our trial. One of the strengths of EQ-5D is that gender and age normative data for the Swedish population is available for use in health economic evaluation[27], and the index can be used to predict ability to work or study. The questionnaire will be sent out digitally to the subjects on the day of the visit and when filled out, uploaded to the medical records.

The rationale for choosing RAND-36 is that it is well validated and used in previous studies with similar methodology to enable power calculations. EQ-5D was chosen to provide an evaluation of HRQoL in a shorter perspective, as it is easier to fill in and may therefore be a better option for long term follow-up, to enable a simple health economic evaluation.

Physical tests

6-minute walk test (6MWT)

The 6MWT will be performed in a corridor with a measured distance of 30 m, with markings for every meter. The subject will carry a pulse oximeter with a probe attached to their forehead. The test will be monitored by an experienced instructor recording parameters every minute, the total number of meters walked in six minutes, the subject's graded and subjective feeling of leg-fatigue and dyspnea according to the Borg CR-10-scale, as well as the feeling of general exertion according to the Borg-RPE-scale, both at baseline and at the end of the tests[28].

30/60 seconds chair stand test (CST)

Here the subject will stand up straight and sit down completely as many times as possible for 30/60 seconds (s). An instructor will record the number of times the subject manages to perform the movement, as well as the subject's graded and subjective feeling of general exertion according to the Borg-RPE-Scale, and dyspnea and leg fatigue according to the Borg CR-10-scale at baseline and the end of the test. The rationale for recording 30/60 s is that some subjects may not be able to perform the full 60 s test.

Objective measurements

Nexfin

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The *Nexfin* monitor will be connected to a fasting subject. This is a non-invasive measurement of cardiovascular indices, with a beat-to-beat pulse wave analyzer. The Nexfin device (ClearSight, Edwards Lifesciences) is placed on the middle phalanx of the middle finger on the right hand. The Nexfin device comprises a pneumatic plethysmograph that provides advanced hemodynamic parameters and continuous noninvasive blood pressure (BP) from a finger cuff, with a redesigned self-coiling mechanism that reconstructs the clinical standard brachial arterial waveform from the finger arterial pressure waveform; it has been validated towards invasive measurements in several clinical trials[29].

Reactive Hyperemia Index (RHI)

Endothelial function will be determined in fasting state using an *EndoPAT 2000* device (Itamar Medical, Caesarea, Israel). The subjects will be connected to the pulse amplitude tonometry (PAT) device for non-invasive determination of digital endothelial function. The PAT device comprises a pneumatic plethysmograph that allows measurements of pulse amplitude at baseline and during hyperemia following a five minutes arterial occlusion of the forearm [30]. The change in the PAT signal is used for calculating the reactive hyperemia index (RHI), which has been shown to reflect microvascular endothelial dysfunction, reduced NO bioavailability and to predict cardiovascular events [31].

Activity meter

The *OURA™ ring* (Oura Health Oy) will be used as an activity tracker that registers heart rate variability, body temperature, physical activity, and sleep patterns. Subjects will wear the ring for at least 1 week before and after each visit with data being synced in OURA's smartphone application which subsequently will be uploaded to an encrypted database [32]. The weekly mean of each variable will be collected.

Randomisation

Subjects who meet the inclusion criteria will be randomised using a digital tool, Randomizer.at, version 2.0.0 (Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz). The system has a complete electronic audit trail for all activities involved with the randomisation. Randomisation is stratified for gender and 'illness severity'. Illness severity is determined as the mean of RAND-36 score for RP and PF into three strata: 1. <30, 2. 30-50 and 3. >50. Investigators access the randomisation system through a web portal with access control. Staff designated to treatment allocation have user-specific access to the unblinded treatment schedule.

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Study treatment is allocated according to protocol, 10 treatments over six weeks, a maximum of two weeks after randomisation.

Subjects as well as all personnel participating in assessments of symptoms and any objective findings will be blinded to the treatment. The placebo 'Sham treatment' protocol is well established and even experienced divers cannot differ between Sham treatment and HBOT [33]. Designated personnel, experienced in HBOT and trained in GCP and the specific protocols will administer the assigned treatments. All subjects will furthermore be asked during the first week of treatment whether they believe they received the placebo treatment or HBOT, to validate the blinding process.

Trial endpoints

The primary endpoints are the mean change from baseline to 13 weeks in RAND 36 domains RP and PF respectively. The main secondary endpoints are mean change from baseline to 13 weeks in RHI, 6MWT, 30/60 s CST, EQ-5D and proportion of subjects with a normalisation of levels in RAND-36 domains RP and PF respectively, at 13 weeks. Primary-, Main secondary-, Selected other- and Safety endpoints are listed in **Table 3**.

Table 3. HOT-LoCO: Trial endpoints

Primary endpoints	Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health and physical functioning respectively.
Main secondary efficacy endpoints	I. Mean change from baseline to 13 weeks in RHI. II. Mean change from baseline to 13 weeks in the 6MWT. III. Mean change from baseline to 13 weeks in the 30/60 s CST. IV. Mean change from baseline to 13 weeks in EQ-5D scores. V. Proportion of subjects with a normalisation* of levels in RAND-36 domains RP and PF respectively, at 13 weeks.

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Other efficacy endpoints	I. Mean change in other RAND-36 domains at 13, 26 and 52 weeks compared to baseline. II. Mean change in EQ-5D at 6, 26 and 52 weeks compared to baseline. III. Mean change in physical activity using an activity meter at 6, 13 and 26 weeks compared to baseline IV. Mean change in HRV using an activity meter at 6, 13 and 26 weeks compared to baseline V. Mean change in sleeping pattern using an activity meter at 6, 13 and 26 weeks compared to baseline.
Explorative/Descriptive endpoints	I. Mean change from baseline in hypoxia pathways in PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks. II. Mean change from baseline in inflammatory PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks III. Mean change from baseline of reactive oxygen species in red blood cells measured by EPR, at, 6 and 13 weeks. IV. Mean change from baseline of microRNA in plasma, at 6 and 13 weeks. V. Mean change from baseline in trial-specific clinical biochemistry at 6 and 13 weeks. a. D-Dimer b. Ferritin c. LDH d. Troponin T VI. Long term post-trial follow-up of HRQoL using EQ-5D as variable up to 4 years post trial.
Safety and compliance endpoints	I. Number of subjects, proportion of subjects and number of adverse events (AEs) at 13 weeks. II. Number of subjects, proportion of subjects that have completed planned treatments after 6 weeks.

* According to Swedish normative data[23]

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Safety and adverse events

Collection of Adverse events (AE) and Serious Adverse Events (SAE) data will start directly after inclusion and will be recorded until Visit 3. Only SAE will be collected outside the treatment period (Visit 2). Ongoing AE and SAE at the end of Visit 3 will be followed up during long-term follow-up until the subject's last visit. The definition, handling, follow-up and reporting of AEs are defined in the original protocol (p.34–38). The safety endpoints will be evaluated by an independent Data Safety Monitoring Board (DSMB) in the context of the trial design and currently existing information about Long COVID and HBOT. The DSMB is composed of three experts in their respective disciplines of medicine, clinical trial methodology and conduct. The DSMB will review the data at the predetermined interim analyses and at the end of trial, a charter delineating their guidelines for operating and stopping rules for terminating individual subjects, a portion or all the trial prematurely, was drawn up and agreed upon before the trial started. The members of the DSMB, meeting plan and responsibilities are specified in the original protocol (p.6 and 44).

Statistical analyses

This section is a short summary of the planned statistical analyses of the most important endpoints including primary and main secondary endpoints. A longer summary is availible in the full protocol (p.38-42). A more technical and detailed elaboration of the principal features will be written in a separate Statistical Analysis Plan (SAP). The SAP will be finalised prior to Data Base Lock (DBL).

Analysis population

Full analysis set (FAS), per-protocol (PP) and safety population (SP) will be employed. The FAS population will be defined as: all randomized subjects who were exposed at least once to the study intervention.

Sample size calculation

The primary endpoint is mean change from baseline to week 13 in the RAND-36 score. A ten-point higher mean change in the HBOT group compared to the placebo group is considered as a clinically relevant difference. Sample size calculation was performed using t-test for independent groups, with an 80% power), and a type-I error rate of 0.05 (5%), assuming a common SD of 15 from prior studies, to detect a 10-unit difference between groups. Power calculations indicates that at least 37 subjects per group are needed. Subsequently, we aim to recruit 80 subjects. nQuery, version 7 was used for sample size calculation.

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Hypothesis testing and adjustment for multiplicity

Hypothesis testing will be controlled at the type-I error rate of 0.05 and adequately adjusted for multiplicity in the two primary endpoints. However, there will be no adjustment for multiplicity in main secondary endpoints as this is an exploratory study, but nominal p-values will be presented, and results will be interpreted as exploratory findings. All hypothesis tests will be two-sided. Details of the multiplicity adjustment in terms of the selection of endpoints to include in the testing sequence and the criteria for rejecting (or not rejecting) individual hypotheses will be provided in the SAP.

Subgroups

Subgroup analysis will be done and presented for gender and disease severity defined as the mean of RAND-36 RP and PF and divided into 'RP and PF below 30', 'RP and PF 30-50' and 'RP and PF above 50'.

Statistical methodology

Primary and secondary endpoints will be evaluated using the FAS population and sensitivity analyses performed using the PP population. The primary objective of the study is to confirm a superior efficacy for the active treatment compared to placebo in the primary endpoints. The null hypothesis to be tested is that there is no difference between HBO treatment and placebo, i.e., the mean change in (HBOT) = mean change (placebo). The same statistical hypothesis will be used for key secondary endpoints.

All continuous variables will be described using standard statistical measures, i.e., number of observations, mean and median value, standard deviation, minimum and maximum value. All categorical variables will be summarised in frequency tables.

In general, for continuous outcome variables including the primary endpoint, they will be analysed using ANCOVA, unless otherwise specified, including stratification factors and treatment as fixed factors in the model. Estimates will be presented using least-square means for differences between treatment arms. In addition, continuous endpoints measured repeatedly over time, such as EQ5D and RAND-36 domains, the change from baseline will be analyzed using a linear mixed-effect model including baseline, treatment group, sex, symptom severity, visit, and treatment group by visit interaction, and subjects as random effects, in the models. An unstructured covariance matrix will be assumed.

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Analysis for categorical data in terms of binary data (Yes/No) will be presented as the proportion of subjects with the frequency of presence or absence, by treatment group of the characteristics of interest and analysed using the CMH Chi-square test including stratification factors, where the parameter used for the statistical hypothesis testing will be the odds ratio (OR), as a measure of the relative difference in odds between treatment arms. An OR>1 indicates efficacy in favor of HBOT compared to placebo.

Missing data will be adequately imputed for all subjects in the FAS population. In addition, the observed cases population will be evaluated as a sensitivity analysis.

An interim safety analysis will be performed when 20 subjects have available data for the safety endpoints, and a second interim analysis when 40 subjects have data available for primary endpoint to adjust the sample size if needed. The trial will also be evaluated for futility regarding the primary endpoints, i.e., the predictive probability of success at the end of the trial.

Safety analysis

The number and percentage of patients reporting AEs, and the number of AEs reported will be presented. The events will be tabulated by system organ class and preferred term by treatment group. In addition, summaries by relationship to trial drug and severity will be presented. AEs will also be presented in separate tabulations.

The number of patients experiencing an AE will be compared descriptively between groups. All patients with AEs will be listed individually with the patient number in addition to the type of event, start and stop time, duration, seriousness, severity, any action taken, relationship to trial drug and outcome of AE.

Discussion

This manuscript presents the trial design and rationale for the HOT-LOCO trial. The trial is conducted in compliance with ICH-GCP to protect the safety and well-being of the subjects as well as the integrity and validity of the data. HBOT has been used for almost a century for other chronic inflammatory conditions with well documented safety profiles for accepted indications [34]. However, the intervention is not without risk. The nature of the disease, which provokes multiple symptoms and a low quality of life make the risk group a ‘vulnerable group’ and it is important to

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make sure that the subjects are not unduly influenced by the expectation or benefits associated with participation.

The randomised, double-blinded design is gold standard, and thus is a strength considering primary endpoints being PRO. The trial design involves multiple exploratory and descriptive endpoints, which may provide valuable data regarding the disease regardless of clinical outcomes. Should HBOT prove clinically effective for the efficacy endpoints the trial design also allows further investigation into possible causal mechanisms.

Limitations

The current trial has some important limitations. Long COVID is a novel disease with unknown mechanisms. The prevalence is continuously being revised and it is not known how symptoms and best practice treatment will evolve over time. The treatment protocol in this trial is novel and thus considered a limitation. Normally, HBOT is administered five days a week, with 30–40 sessions over six to eight weeks. The protocol in this trial is based on experience from severe COVID-19 where five treatments seem to be sufficient. However, more research on the dose is needed. Further limitations lie in the possible selection bias of patients being referred through the same outpatient clinic; most patients are severely debilitated (a prerequisite for referral was at least 50% sick-leave) and due to long waiting times, most patients have been ill for more than one year. The power calculation for the primary endpoint is extrapolated from studies of similar design and diseases with similar symptoms but have not been based on a pilot trial and thus is considered as an increased risk of type II error. However, interim analyses will be performed when 20 patients have data available for safety endpoints, and when 40 patients have available for primary endpoint to minimize the risk of an underpowered trial. Furthermore, 'sham treatment' may have up to 58% efficacy[35]. We did not take this into account when we performed our power calculation, which could result in the trial being underpowered. Both EQ-5D and RAND-36 are the most widely used PRO measures for HRQoL and have been used in the setting of long COVID and similar conditions such as ME/CFS and fibromyalgia but due to the novelty of the condition we do not know what to expect from our population and our 'clinically relevant' estimation may be set too high. Three to five points have been proposed as a minimally clinically important difference (MCID) for RAND-36 when used in health economic evaluation[36]. This assumption in our power calculation may also cause a type II error.

Ethics and dissemination

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The trial is conducted in accordance with The Declaration of Helsinki, ICH-GCP, local and national regulations. The trial was approved by The Swedish ethical review board (EPM no 2021-02634, amendment 2021-04572), approval 2021-05-25 and 2021-09-22 and The Swedish medical products agency (LV no 5.1-2020-36673), approval 2021-07-06. The trial was registered online (NCT04842448) and EudraCT number: 2021-000764-30 before start of the trial.

The trial is monitored by the Karolinska Trial Alliance (KTA) before the trial started, during the trial, and after trial completion. A designated monitor will monitor the randomisation and blinding process. The monitoring is performed to ensure that the trial is conducted in compliance with the protocol, detailed in a separate monitoring plan and that data is handled according to ICH-GCP.

The first publication will report the results of the interim safety analysis to help other researchers in trial designs and health care providers in decision making. The main publication will report the primary and main secondary endpoints together with the full safety and compliance report at 13 weeks. Separate publications will report exploratory endpoints: 1. Descriptive results from the Oura-ring, 2. Health economic analysis, 3. Exploratory biomarkers and biochemical analyses. 4. Descriptive results from medical history that is collected during the trial. 5. Depending on the outcome of the primary endpoint at 13 weeks, follow-up on HRQoL at 26 and 52 weeks. 6. Long time, post-trial follow-up on HRQoL, 4 years.

Current trial status

The first subject was included in September 2021. Nineteen subjects have been randomized, 14 have completed the intervention by February 1, 2022. The first safety analysis will be performed when 20 subjects have completed the interventions, according to the plan Q1 2022.

Authors contribution

AK is the principal investigator who wrote the hypothesis and developed most of the protocol together with PL. AK and PL wrote the applications to Swedish IRB and MPA. LAH drafted the manuscript together with AK. AH, SEG, SAE and EB are sub-investigators, enrolling and evaluating subjects and collecting data. MNB, JB, MS, and MR are trial chairs involved in writing the protocol and applications. JK wrote the statistical analysis plan together with AK and designed the randomisation. All authors including CJS, KRW, SBC, XZ, KM and JP contributed to the current submission and critically reviewed the manuscript. AK is corresponding author for this work and

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attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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Competing interest

AK and PL disclose funding from HLF and ALF for the present trial. AK disclose funding from Oura Health Oy with complimentary hardware and software for the Oura rings. MS discloses funding from Swedish Research Council and Dysautonomia International during the trial and previously from HLF. MS also disclose consulting fee from Swedish agency for health technology assessment of social services, speaker honoraria from Orion Pharma, Werfen and has filed a patent for pharmacological treatment in post-COVID POTS. JK declares consulting fee for statistical work in this trial.
LAH, AH, SEG, SAE, EB, CJS, JP, KM, KRW, XZ, SBC, MR, JB, MNB declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Patient consent for publication

Not required.

Data sharing

The full trial protocol, statistical plan and consent form will be publicly available. Data will be available on patient level; data will be pseudonymised, the full dataset and statistical code will be available upon request. All publications will be made available on Open Access. Source data will be described in a Meta-data repository. A full description of the intended use of the data must be sent to the corresponding author for review and approval. Participant consent for data sharing is conditioned and new ethics approval may be required.

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Study coordinator Felicia Doeser for invaluable help with managing subjects and collecting data. The doctors and nurses at the hyperbaric unit at Karolinska University Hospital involved in the

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treatments and allocation to the treatment groups; Doctors: Karl-Fredrik Sjölund, Johan Thelaus and Georgios Sidiras Nurses: Carola Lernbäck, Birgitta Johansson and Johan Ohlberger and Annelie Kruthammar. Medical student: Lovisa Liwenborg. The director of Intensive care, Björn Persson, director of Health professions, Emma Sjölund and director of Cardiology Frieder Braunschweig for supporting this project. The research nurses at KFE for planning and help with blood sampling; Anna Schening, Anna Granström, Ola Friman and Pia Zetterqvist. Physiotherapists Anna Svensson-Raskh and Ulrika Holdar for planning and performing the physical tests. Staff at Studiecenter Karolinska for setting up the laboratory manual and handling blood samples.

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Figure 1. Trial Flowchart

Figure 2. Consolidated Standards of Trials (CONSORT) flow diagram

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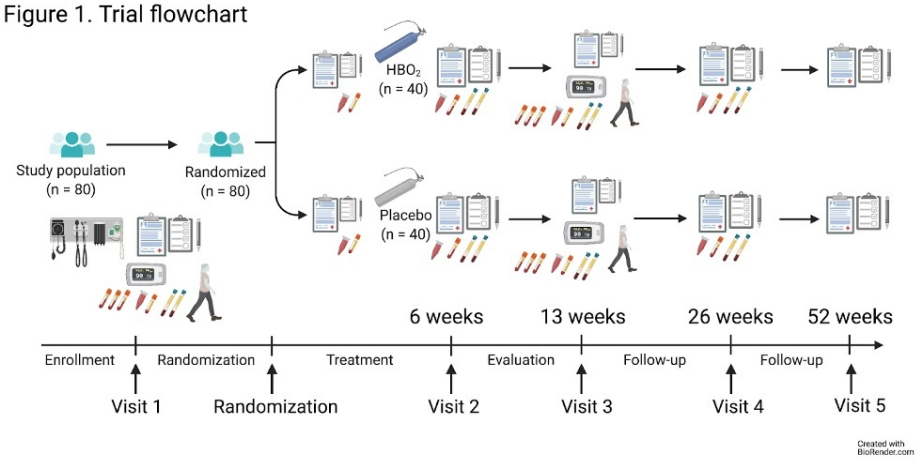
Figure 1. Trial flowchart



Trial flowchart

187x94mm (144 x 144 DPI)

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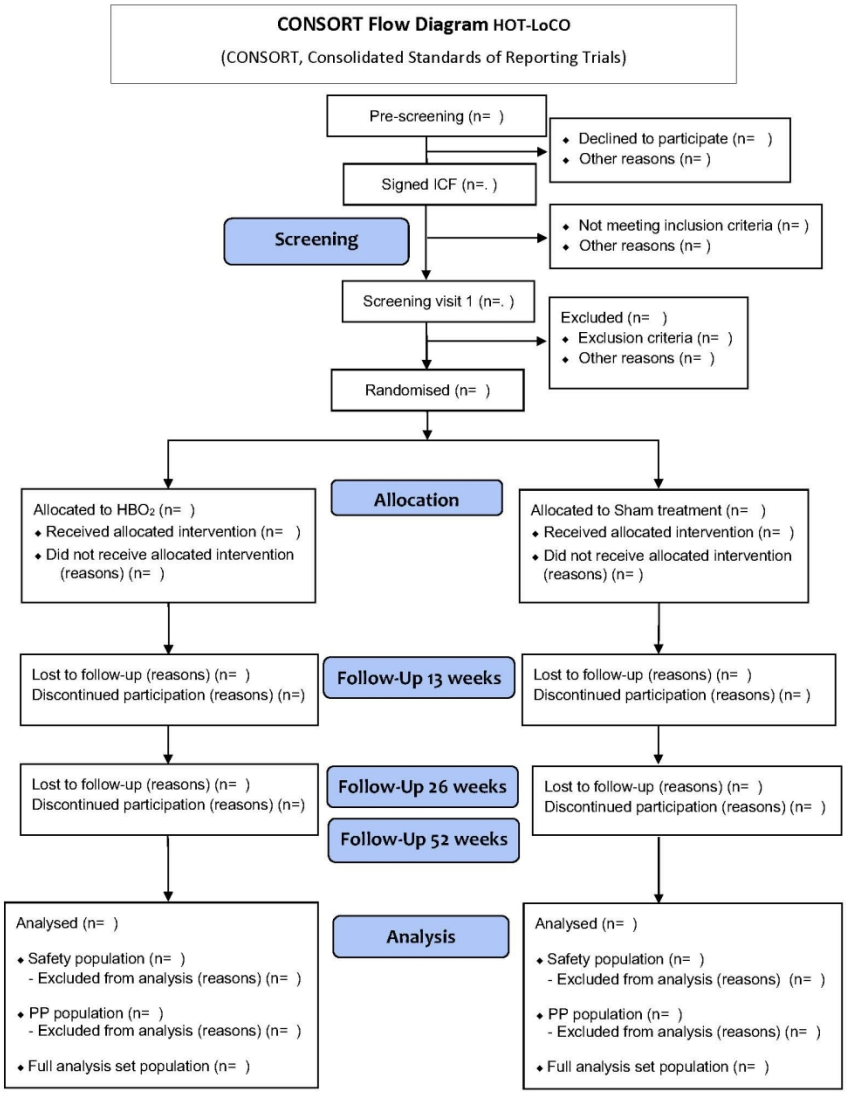

Consolidated Standards of Trials (CONSORT) flow diagram

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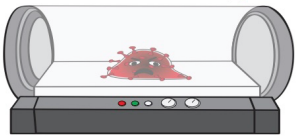
SOP Randomization-Blinding



PURPOSE: The purpose of this Standard Operations Procedure (SOP) is to describe procedures that protect the health and welfare of participants and data integrity in a double-blinded, randomized clinical trial

SCOPE: This SOP applies to all study personnel involved in the conduct of the trial, development and implementation of the randomization and/or blinding process.

RESPONSIBILITY: The PI and designated trial staff are responsible for following the randomization and blinding procedures described in the clinical trial protocol. All personnel, including staff designated to treatment, needs to participate in GCP training and be delegated to each task.

DEFINITIONS:

Blinding: The procedure in which both the subject and the assessors are kept unaware of the treatment assignment. Double blinding refers to that also the subjects and assessors being unaware of the treatment assignments. Assessors includes anyone determining subject eligibility, evaluating endpoints, or assessing compliance with the protocol, i.e. investigators, monitor and data manager.

Randomization: The process of assigning trial subjects to treatment or sham-treatment group using an element of chance to determine the assignments in order to reduce bias.

Randomization tool: Web-based tool used to allocate patients to a particular treatment arm. In this trial Randomizer.at is used.

Unblinding: Identification of the treatment code for a subject; by the subject or investigators involved in assessment. Also referred to as “code breaking”. Code breaking may be intentional in case of emergency or evaluation of an AE or can be accidental. Examples of accidental code breaking is that the subject or assessors see the treatment protocol or that the treatment group is discussed with an assessor.

PROCEDURE:

1. The Randomization tool is programmed by the senior statistician Jan Kowalski at EDC Scandinavia AB. The study staff member(s) responsible for the randomization will have training in and access to the randomization tool prior to participant recruitment.

2. **Blinding:**

2.1 The PI and trial staff will review the protocol and determine who should be designated to treatment/randomization and assessment respectively.

2.2 A printout of the randomization code is kept together with the treatment protocol in the subject’s treatment portfolio. The portfolio is stored in a locked cabinet with limited access.

2.3 During treatment it is important that the treating staff use normal routines for treatment in both groups, such as: ask normal questions regarding equalization problems, notify when it is time for “air break” even in “placebo”, remind about oxygen safety etc.

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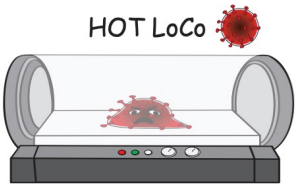
SOP Randomization-Blinding



2.4 Assessors should avoid discussing the treatment with the subjects and should not enter the treatment rooms unless a case of emergency that cannot be solved by designated staff.

2.5 When the treatment is finalized the treatment protocol is stored together with the randomization code in a sealed envelope in the TMF.

3. Unblinding:

3.1 Randomization and treatment procedures must be followed to ensure the code is broken only in accordance with the protocol. Circumstances for unblinding and procedures thereof must be known by all study staff before the first patient is randomized.

3.2 In general, the code should only be broken in the case of an adverse event where it is necessary for the Principal Investigator to know which treatment the patient is receiving before the participant can be treated. This is a very unlikely in this trial.

3.3 The sponsor/PI should be notified immediately, preferably by telephone and then by email, regarding the necessity of code breaking.

3.4 When it is necessary to break the blind, the PI must notify the IRB.

3.5 If the code is broken for a participant, this must be documented in the eCRF, together with the reasons for breaking the code. The reason for breaking the code should also be written on the randomization printout.

3.6 Justification for premature unblinding of the investigational product should be documented (e.g. accidental unblinding, unblinding due to serious adverse event) in the source document as well as the Regulatory File.

REFERENCES:	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH): ICH-E8 General considerations for clinical trials ICH-E9 Statistical Principles for Clinical Trials (CPMP/ICH/363/96)
RELATED SOPs:	Treatment protocol (Behandlingsordination HOT-LOCO) Treatment tables (HOT-LOCO Behandlingstabell HBO, HOT-LOCO Behandlingstabell Placebo)

REVISION HISTORY:

Amendment	Date	Type/comment
Version 1 En	2021-06-26	MPA submission
Version 2 En	2021-09-25	Change of randomization tool

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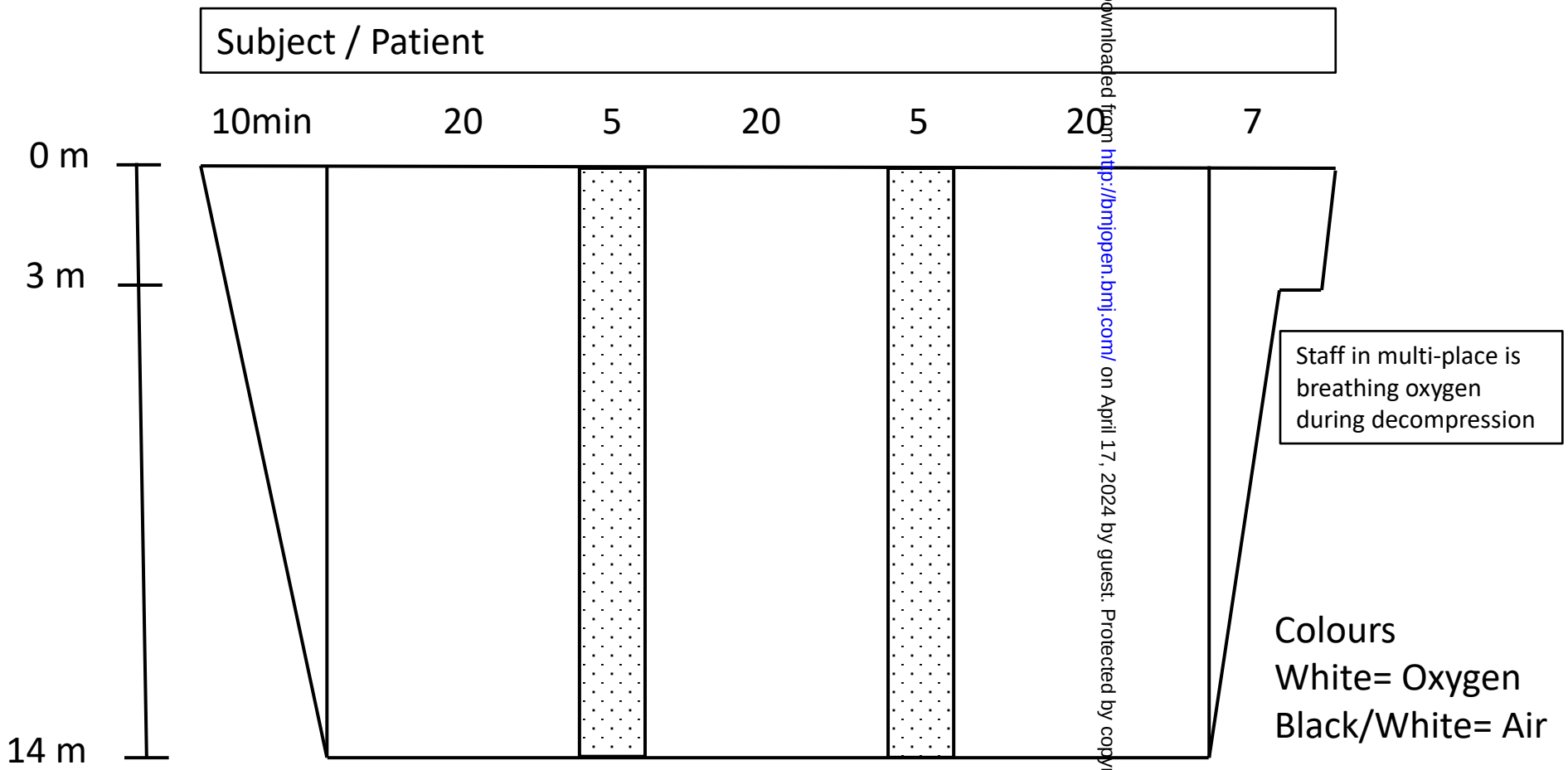
HBO treatment (IMP)

14:80:7 (meter):(bottom time, minutes):(decompression time, minutes) Total time: 87 minutes



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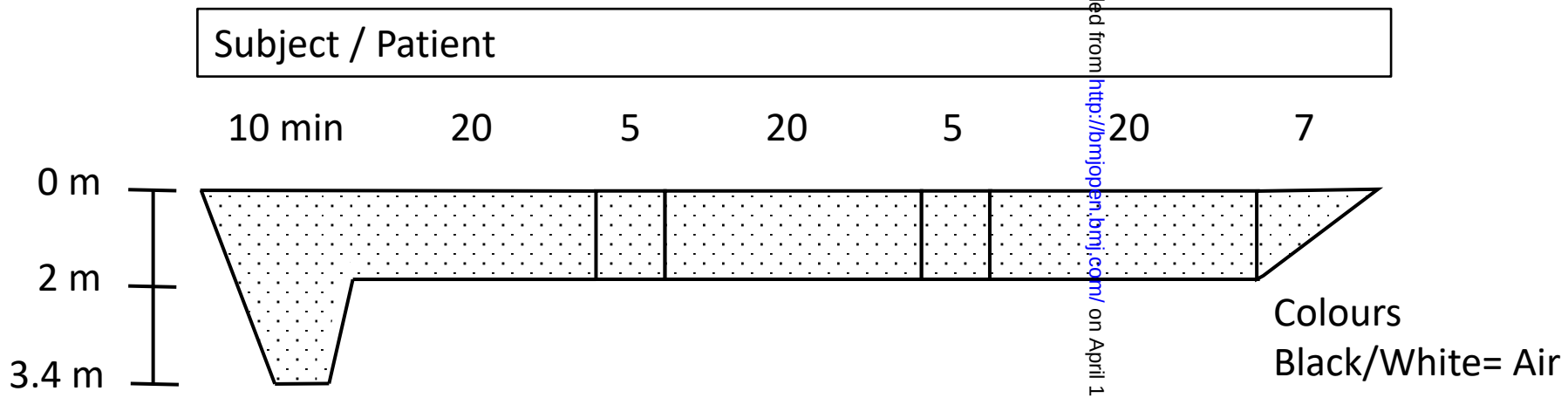
Sham treatment (placebo)

3.4-2:80:7 (meter):(decompression time, minutes) Total time: 87 minutes



Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

CLINICAL TRIAL PROTOCOL

Hyperbaric Oxygen for Treatment of Long COVID syndrome; A Randomized, Placebo-Controlled, Double-Blind, Phase II Clinical Trial

Safety and Efficacy of Hyperbaric Oxygen Therapy for Long COVID Syndrome

Trial code:	HOT-LOCO
EudraCT number:	2021-000764-30
ClinicalTrials.gov Identifier:	NCT04842448
Version number:	4
Date:	2022-01-03
Sponsor:	Karolinska University Hospital, Solna
Principal Investigator	Anders Kjellberg, MD

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Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

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Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

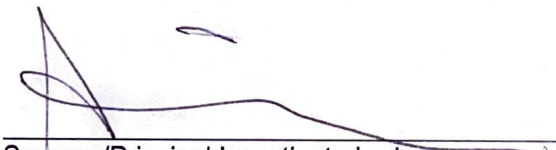
Signature page

Sponsor/Principal Investigator

I am responsible for ensuring that this protocol includes all essential information for the conduct of this trial. By signing my name below, I agree to conduct the trial in compliance with this protocol, the Declaration of Helsinki, ICH GCP (Good Clinical Practice) guidelines and the current hospital, national and international regulations governing the conduct of this clinical trial.

I will submit this protocol and all other important trial-related information to the staff members and investigators who participate in this trial, so that they can conduct the trial correctly. I am aware of my responsibility to continuously keep the staff members and investigators who work with this trial informed and trained.

I am aware that quality control of this trial will be performed in the form of monitoring, audit, and possibly inspection.



2022-01-03

Sponsor/Principal Investigator's signature Date

Anders Kjellberg MD

Printed name

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Trial Code: HOT-LOCO
Version No: v.4
Date: 2022-01-03
EudraCT No: 2021-000764-30

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Data Safety Monitoring Board	Kjell Ahlén, MD Niklas Nielsen, MD, PhD Stefan Grass, MD, PhD

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Trial Code: HOT-LOCO
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List of used acronyms and abbreviations

Abbreviation	Term/Explanation
6 min walk test	6 minutes walk test (assessment of physical endurance)
30/60 s chair stand	30/60 seconds chair stand (assessment of functional mucscle strength)
AE	Adverse Event = any untoward medical occurrence
ANCOVA	Analysis of Covariance
AR	Adverse Reaction = adverse event, that is each unfavorable and unexpected reaction to a trial treatment, regardless of dose
ASA Class	ASA Physical Status Classification System
BP	Blood Pressure
CAT	COPD Assessement Test
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Corona Virus Disease 2019
CRA	Clinical Research Associate
CRO	Contract Research Organization
CT	Computerized Tomography
CXR	Chest X-Ray
DECT	Dual Energy Computed Tomography
DSMB	Data Safety Monitoring Board
DSUR	Development Safety Update Report = annual safety report
ECG	Electrocardiogram
eCRF	Electronic Case Report Form
EndoPAT	Endothelial assessement of Pulse Amplitude Tonometry
EPM	Etikprövningsmyndigheten (Swedish Ethical Review Authority)
EPR	Electron Paramagnetic Resonance Spectroscopy
EQ-5D	EuroQol 5 Dimensions questionnaire
FAS	Full Analys Set = including all data from all subjects who have participated in the trial
Frändin-Grimby	Frändin-Grimby activity scale
FSS	Fatigue Severity Scale
GAD-7	Generalised Anxiety Disorder 7-item scale
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Council for Harmonization
IEC	Independent Ethics Comittee
IRB	Institutional Review Board

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HBO_2	Hyperbaric Oxygen
HBOT	Hyperbaric Oxygen Therapy/Treatment
HIF	Hypoxia Inducible Factor
HRV	Heart Rate Variability (assessement for autonomic dysfunction)
HRQoL	Health-Related Quality of Life
HUT	Head Up Tilt test (assessment for POTS)
Jamar	Jamar (assessment of hand muscle strength)
kPa	kilo Pascal (SI unit for pressure, 100 kPa= 1 bar)
KSB	Kognitiva Screening Batteriet (Cognitive Screening Battery)
Long COVID	Long COVID Syndrome = PCS = PACS
LVFS	Läkemedelsverkets författningssamling (Swedish Medical Products Agency's statutes)
MIP/MEP	Maximal inspiratory and expiratory muscle strength
microRNA	Micro-Ribonucleic acid
MFS	Mental Fatigue Scale
mMRC	The Modified Medical Research Council Dyspnea Scale
MOCA	The Montreal Cognitive Assessement
MPA	Medical Products Agency
MRI	Magnetic Resonance Imaging
Nexfin	Nexfin noninvasive cardiovascular monitoring
PACS or PCS	Post (Acute) COVID-19 Syndrome = PCS = Long COVID
PBMC	Peripheral Blood Mononuclear Cells
PCL-5	Posttraumatic Stress Disorder Checklist (version 5)
PE	Pulmonary Embolism
PHQ-9	Patient Health Questionnaire-9
POTS	Postural Orthostatic Tachycardia Syndrome
PP	Per Protocol analysis = including only data from subjects who have completed the trial completely in accordance with the protocol, with no deviations from the protocol
RAND 36	RAND 36-Item Short Form Health Survey 1.0
RHI	Reactive Hyperemia Index
RNA	Ribonucleic acid
SAE	Serious Adverse Event = serious untoward medical occurrence
SAP	Statistical Analysis Plan
SPC or SmPC	Summary of (medical) Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Corona Virus 2
SOP	Standard Operation Procedure
SpO_2	Peripheral Oxygen Saturation
TMF	Trial Master File
WAI	Work Ability Index (assessement of self reported work ability)

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Trial Code: HOT-LOCO
Version No: v.4
Date: 2022-01-03
EudraCT No: 2021-000764-30

1. Synopsis

EudraCT number:	2021-000764-30
Title:	Hyperbaric Oxygen for Treatment of Long COVID Syndrome; A Randomized, Placebo-Controlled, Double-Blind, Phase II Clinical Trial
Trial code:	HOT-LOCO
ClinicalTrials.gov identifier:	NCT04842448
Short background/ Rationale/Aim:	Long COVID Syndrome (Long COVID), Post Acute COVID-19 Syndrome (PACS) or Post COVID-19 Syndrome (PCS) is defined as 'signs and symptoms that develop during or following an infection consistent with COVID-19, continue for more than 12 weeks and are not explained by an alternative diagnosis'. Common symptoms are fatigue, post-exertional malaise, brain fog, neurologic sensation, headaches, memory issues, insomnia, muscle aches, palpitations, shortness of breath, dizziness and speech issues. Many patients report very low Health Related Quality of Life (HRQoL) One in ten infected individuals may suffer persistent symptoms, and we are facing an emerging problem that will severely affect individuals, health care systems and society for years to come. Subjects will be recruited once they have been diagnosed with Long COVID through assessment by a multidisciplinary team with a thorough diagnostic work up including medical history, routine blood tests, questionnaires, physical tests and radiology. We explore hyperbaric oxygen administered in a randomized placebo-controlled clinical trial as a potential treatment for patients suffering from Long COVID. The overall hypothesis to be evaluated is that hyperbaric oxygen **(**HBO_2) treatment (HBOT) reduces oxidative stress and chronic inflammation, improves endothelial dysfunction and thereby alleviates symptoms associated with Long COVID.
Trial objectives:	**Primary objective:** To evaluate if HBOT improves HRQoL (role limitations due to physical health and physical functioning for patients with Long COVID compared to placebo (sham treatment). **Main secondary objectives:** To evaluate if HBOT improves endothelial dysfunction in Long COVID. To evaluate if HBOT improves objective physical performance in Long COVID. To evaluate if HBOT improves HRQoL short term.

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	To evaluate if HBOT can normalise physical functioning in Long COVID. **Other secondary objectives (in selection):** To evaluate if HBOT improves autonomic dysfunction. To evaluate if HBOT improves restorative sleep. To evaluate if HBOT has a long-term effect on subjective symptoms, HRQoL and objective physical performance in Long COVID To evaluate the potential health-economic benefits of the treatment.
Trial design:	Randomized, placebo-controlled, double-blind, phase II
Trial population:	Previously healthy adult patients with Long COVID syndrome
Number of subjects:	80
Inclusion criteria:	1) Aged 18–60 years 2) Healthy or mild systemic disease (ASA 1-2) prior to COVID-19 3) Symptoms consistent with Long COVID for at least 12 weeks 4) Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9) 5) Working or studying prior to COVID-19 6) Documented informed consent according to ICH-GCP and national regulations
Exclusion criteria:	1) Known pregnancy or positive pregnancy test in women of childbearing age 2) ASA 3 or more from other cause than Long COVID 3) Score above 70 in RAND-36 Role Limitation Physical Health (RP) or Physical Functioning (PF) 4) Diabetes 5) Diagnosed with Hypertension prior to COVID-19 6) Contraindication for HBO_2 treatment according to local guidelines 7) Participation or recent participation in a clinical trial with an investigational product 8) Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trialstudy participation
Investigational product(s), dosage, administration:	Hyperbaric oxygen (HBO_2) compared with placebo HBO_2: HBO_2 240 kPa for 90 min, maximum 10 treatments

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	Placebo: Air 134 kPa for 90 min, maximum 10 treatments
Trial endpoints:	**Primary endpoint:** Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health and physical functioning. **Secondary endpoints (in selection)** Main Secondary Efficacy Endpoints: I. Mean change from baseline to 13 weeks in Reactive Hyperemia Index (RHI). II. Mean change from baseline to 13 weeks in the 6-min walk test. III. Mean change from baseline to 13 weeks in the 30/60 sec chair stand. IV. Mean change from baseline to 13 weeks in EQ-5D. V. Proportion of subjects with a normalisation of levels in RAND-36 domains role limitations due to physical health and physical functioning respectively, at 13 weeks. Safety and Compliance Endpoints I. Number of subjects, proportion of subjects and number of AEs at 13 weeks. II. Number of subjects, proportion of subjects that have completed planned treatments and number of treatments after 6 weeks.
Trial period:	Q3 2021 – Q4 2023
Statistical analyses	The analysis of the primary endpoint will be conducted on the Full Analys Set (FAS) and the Per Protocol Set (PPS). The primary analysis of the primary endpoints will be performed using the ANCOVA, including randomisation strata of gender and disease severity together with treatment as fixed factors in the model. The two primary endpoitns will be adequately adjusted for multiplicity.The p-value for testing the null hypotheses, no difference between treatment groups, must be less than 0.05 to be considered to have met the primary objective.

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2. Introduction

2.1 Background

Post COVID-19 Syndrome (PCS), Post Acute COVID-19 Syndrome (PACS) or Long COVID Syndrome (Long COVID) has been defined as 'signs and symptoms that develop during or following an infection consistent with COVID-19, continue for more than 12 weeks and are not explained by an alternative diagnosis' (Venkatesan, 2021).

Common symptoms are fatigue, post-exertional malaise, brain fog, neurologic sensation, headaches, memory issues, insomnia, muscle aches palpitations, shortness of breath, dizziness and speech issues. Nearly 50% have reduced working capability and 22% cannot work at all. A majority are women and have never been hospitalized for acute COVID-19 (Davis et al., 2020).

The most common organ affected by the SARS-CoV-2 virus is the lung due to its main site of entry, binding to the angiotensin-converting enzyme 2 (ACE-2) receptor; resulting in damage to the cells of the alveolar-capillary membrane (Bourgonje et al., 2020). COVID-19 is associated with endothelial dysfunction, tissue edema and a pro-coagulant state in various organs including the lungs, liver, heart, kidney and small bowel (Varga et al., 2020).

Many of these changes may become chronic, which have been observed at post-COVID follow up (Halpin et al., 2021). COVID-19 should not only be viewd upon as an acute infection but as an inductor of a chronic inflammatory disease. Chronic oxidative stress, inflammation and endothelial dysfunction may explain many of the symtoms and objective findings associated with post-acute and long COVID even after recovery from the viral infection (Chang et al., 2020). These features are also hallmarks of other post-viral syndromes such as Myalgic Encepalomyelitis/Chronic Fatigue Syndrome (ME/CFS)(Scherbakov et al., 2020)

Endothelial dysfunction is a potentially reversible condition that serves as an independent predictor of cardiovascular events(Bonetti et al., 2003). Reactive Hyperemia Pulse Amplitude Tonometry (RH-PAT) is a non-invasive, user-independent tool for assessement of endothelial dysfunction. RH-PAT can be used in a clinical setting for monitoring of treatment effect in subjects with this condition (Bonetti et al., 2004). Autonomic dysfunction is an early marker of endothelial damage and is associated with cardivovascular morbidity and mortality (Khemani and Mehdirad, 2020). Endothelial dysfuncton is common in patients with ME/CFS and is associated with severity of symptoms and immune response (Scherbakov et al., 2020). Heart rate variability (HRV) is widely used as a standard method of measuring autonomic dysfunction in cardiovascular and neurological disorders (Rajendra Acharya et al., 2006).

Hyperbaric oxygen (HBO_2) (PO_2 240–280kPa) delivered by inhalation in a hyperbaric chamber in daily treatments over several weeks, has several anti-inflammatory effects. Hyperbaric

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Oxygen Therapy/Treatment (HBOT) has been used safely for a century to treat other chronic inflammatory conditions such as radiation cystitis (Oscarsson et al., 2019), fibromyalgia (Efrati et al., 2015) and acute inflammatory conditions such as ulcerative colitis (Dulai et al., 2020). HBOT has shown beneficial effects on endothelial function in patients with slow coronary flow (Li et al., 2018). Usefully, it is possible to perform a placebo-controlled double-blind trial with HBO_2 (Lansdorp and van Hulst, 2018).

HBO_2 has been used off-label as one of few potentially curative treatments for acute COVID-19. Case series and a case control-study using HBO_2 have shown faster recovery and reduced need for ICU treatment (Guo et al., 2020, Thibodeaux et al., 2020, Gorenstein et al., 2020). RCTs are ongoing, including one at the Karolinska University Hospital (Clincaltrials.gov identifier: NCT04327505). Multiple hypotheses have been proposed for the effect of the therapy, with the common denominator being normalization of hypoxic- and inflammatory response (De Maio and Hightower, 2020, Kjellberg et al., 2020, Paganini et al., 2021).

One of the most studied effects of HBO_2 is attributed to Hypoxia Inducible Factor-1 (HIF-1) and target genes (Thom, 2011). One target for HIF-1 regulation, which is known to be associated with COVID-19, is Angiotensin Converting Enzyme 2 (ACE2). Hospitalized patients with COVID-19 have a three-fold higher expression of ACE2 in the lungs compared to healthy controls (Chua et al., 2020), suggesting a susceptibility for severe infection or an adaptive response. HIF-1 has been show to suppress ACE2, making HIF-1 modulation an interesting therapeutical target in COVID-19 (Serebrovska et al., 2020). Agents that stabilize HIF-1 have been proposed for COVID-19 (Afsar et al., 2020). A major challange in translating HIF-regulation into clinical practice is the complex adaptation to hypoxia and the intricate interplay between three different HIFs. The crosstalk between hypoxia and and inflammatory pathways adds further complexity to the system (D'Ignazio et al., 2016).

2.2 Research hypothesis

- HBO_2 can induce HIF signalling independent of heart, lung and brain function, thus has the potential to reduce inflammation, restore normal hypoxic response and thereby reduce morbidity in Long COVID.
- HBO_2 is safe and tolerable for Long COVID patients and the effect is accociated with relief in symptoms and thereby improve HRQoL .
- The effect can be monitored by markers of oxidative stress in blood and by non invasive assessement of endothelial dysfunction and autonomic dysfunction.
- Treatment results are not transient and thereby also cost efficient.
- The effect is related to regulation of hypoxia and inflammatory pathways.

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2.3 Rationale for conducting the trial

Long COVID seems to affect approximately 10% of people infected with SARS-CoV-2, most of them are young women (Sivan and Taylor, 2020). To date, few treatment options are available. With over 100 million confirmed COVID-19 cases globally (600 000 in Sweden), the healthcare systems and their infrastructure are at risk of collapse if we cannot find an effective way of treating these patients. Karlolinska Universtity Hospitals was one of the first centers in the world to set up a multidisciplinary clinic for post covid sequaele and is now beening overwhelmed with referrals of suspected Long COVID.

The most common symtoms in Long COVID is chronic fatigue and autonomic dysregulation that are also hallmarks of Fibromyalgia (Sarzi-Puttini et al., 2020) and Myalgic Encepalomyelitis/Chronic Fatigue Syndrome (ME/CFS)(Lim et al., 2020) and some patients are also diagnosed with Postural Orthostatic Tachycardia Syndrome (POTS) and they may all be different semblance of the same chronic inflammatory disease. HBOT has been shown to have positive effect on ME/CFS and fibromyalgia in small clinical trials (Efrati et al., 2015, Yildiz et al., 2004, Akarsu et al., 2013).

If HBO_2 is effective for relieving symtoms in Long COVID there would be an obvious benefit for the individual patient. There is also a potential significant health-economic benefit if there is a lasting effect. The multiple explanatory endpoints may add valuble information about the disease for future interventional trials even with a negative result on the primary endpoint.

3. Benefit-risk evaluation

3.1 The risk group

There is currently no effective treatment available for Long COVID and since this is a new disease, there remain uncertainties regarding diagnosis, prognosis and mechanisms of action. There is emerging evidence that this may be an enormous problem for individuals, health-care and society. Diagnosis of Long COVID is mainly a clinical definition based on symptoms and it is difficult to find objective measurements. Patients that have been suffering from Long COVID since the first wave have often been misunderstood by the health care society and are desperate to find a cure for the disease. HBO_2 has the theoretical potential to reverse or reduce symptoms in Long COVID. The nature of the disease, which provokes multiple symptoms and a low quality of life make the risk group a 'vulnerable group' and it is important to make sure that the subjects are not unduly influenced by the expectation or benefits associated with participation. Therefore, we will conduct a placebo-controlled, double-blind, clinical trial in compliance with Good Clinical Practice (GCP), the Declaration of Helsinki and national regulatory requirements. The written information has a neutral language explaining both risks and potential benefits and the investigators are instructed to keep a neutral tone when delivering oral information. The cause of Long COVID is still not known and optimal

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management is far from defined. We present a plausible hypothesis of the mechanism and a possible cure. Since there are no better options than 'expectation', and HBOT has been safely and effectively used in other chronic inflammatory conditions, the potential benefit for the subjects outweigh the risk.

3.2 General risks with HBO_2

HBOT is a well-established method that has been used for almost a century for several different indications. The mechanisms for its efficacy are not fully understood but it is generally regarded as safe, with few adverse events; serious adverse events are extremely rare. The Undersea and Hyperbaric Medical Society (UHMS) have reported a total of 40 complications per 10,000 treatments during 463,293 treatments over two years (Moon, 2019). Adverse events per 10,000 treatments include: ear pain 20, confinement anxiety 8, hypoglycaemic event 5, shortness of breath 2, oxygen-induced seizure 2, sinus pain, 1, chest pain. HBOT has very few contraindications that are all relative to the treatment environment; they include claustrophobia, medical history of spontaneous pneumothorax, severe COPD, and pregnancy.

3.3 Blood sampling

Blood sampling may have negative impact on the subject as a large number of samples will be necessary for the clinical investigation and may be needed for other trials. We aim to use blood tests already collected as much as possible. The blood sampling serves three purposes:

1. Safety, which is of benefit for the subject.

2. Explanatory, which may be beneficial for the placebo subjects in particular, if the trial results are positive and HBOT for Long COVID is adopted into clinical practice. Samples will serve as a quality control measure to ensure the validity of the data upon presentation of results.

3. Exploratory, which may benefit the subjects even if the HBOT is not successful, as the trial may generate hypotheses for alternative treatments.

Explanatory and Exploratory objectives are important for public health.

3.4 Handling of sensitive personal data

We will handle personal data, including gene expression analyses on the subjects, and there is a risk of personal integrity involved. The trial will be performed according to ICH-GCP; all staff involved will be educated in GCP. All information about the protocol and data will be entered into an eCRF. The data will not identify any person taking part in the trial in accordance with the EU Data Protection Directive (95/46/EU). An external monitor will help us assess the risks by assessing quality of trial design, data collection and informed consent.

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3.5 Safety and logistics

The HBO_2 treatments will be performed in a hyperbaric chamber at the Karolinska University Hospital. Depending on availability, either monoplace or multiplace chambers will be used. The principal investigator is head of the unit with more than 20 years experience in HBOT. All staff are trained and certified for operating the chambers. Standard Operating Procedures for treatment will be used. Local, national and international guidelines for clinical trials and HBOT during the COVID-19 pandemic will be followed.

Monitoring will be conducted before, during and after the trial according to the monitoring plan. Interim analyses for safety and efficacy will be conducted by an independent Data Safety Monitoring Board (DSMB).

In summary, we believe the benefits for subjects, the risk-group and public health will outweigh the risks.

4. Trial objectives and endpoints

The overall hypothesis to be evaluated is that HBOT reduces oxidative stress and chronic inflammation, improves endothelial dysfunction and thereby alleviates symtoms associated with Long COVID.

4.1 Primary objective

To evaluate if HBOT improves HRQoL (role limitations due to physical health and physical functioning) for patients with Long COVID compared to placebo (sham treatment).

4.2 Secondary objective(s)

4.2.1 Main secondary objective

To evaluate if HBOT improves endothelial dysfunction in Long COVID.

To evaluate if HBOT improves objective physical performance in Long COVID.

To evaluate if HBOT improves HRQoL short term.

To evaluate if HBOT can normalise physical function in Long COVID

4.2.2 Other secondary objectives

To evaluate if HBOT improves autonomic dysfunction.

To evaluate if HBOT improves restorative sleep.

To evalute if HBOT has a long term effect on subjective symptoms, HRQoL and objective physical performance in Long COVID

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To evaluate the potential health-economic benefits of the treatment.

To explore changes in general and organ-specific questionaires, physical tests and radiology used in clinical follow-up before and after treatment

To explore biomarkers in plasma, erythrocytes and PBMCs for HBO_2 effect on inflammation, endothelial function and chronic hypoxia.

4.3 Primary endpoint:

Mean change from baseline to 13 weeks in RAND 36 domains role limitations due to physical health (RP) and physical functioning (PF).

4.4 Secondary endpoints:

4.4.1 Main Secondary Efficacy Endpoints

I. Mean change from baseline to 13 weeks in Reactive Hyperemia Index (RHI).
II. Mean change from baseline to 13 weeks in the 6-min walk test.
III. Mean change from baseline to 13 weeks in the 30/60 sec chair stand.
IV. Mean change from baseline to 13 weeks in EQ-5D.
V. Proportion of subjects with a normalisation of levels in RAND-36 domains RP and PF respectively, at 13 weeks.

4.4.2 Other Efficacy Endpoints

I. Mean change in other RAND-36 domains at 13, 26 and 52 weeks compared to baseline.
II. Mean change in EQ-5D at 6, 26 and 52 weeks compared to baseline.
III. Mean change in physical activity using an activity meter at 6, 13 and 26 weeks compared to baseline
IV. Mean change in HRV using an activity meter at 6, 13 and 26 weeks compared to baseline
V. Mean change in sleeping pattern using an activity meter at 6, 13 and 26 weeks compared to baseline.

4.4.3 Explorative/Descriptive Endpoints

I. Mean change from baseline in hypoxia pathways in PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks.
II. Mean change from baseline in inflammatory PBMCs evaluated by RNA sequencing, at 6, 13 and 26 weeks
III. Mean change from baseline of reactive oxygen species in red blood cells measured by EPR, at, 6 and 13 weeks.

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IV. Mean change from baseline of microRNA in plasma, at 6 and 13 weeks.
V. Mean change from baseline in study specific clinical biochemistry at 6 and 13 weeks.
   a. D-Dimer
   b. Ferritin
   c. LDH
   d. Troponin T
VI. Mean change from baseline in objective organ specific findings on imaging at 13 and 26 weeks (from medical records).
VII. Mean change from baseline in objective organ specific and general physical tests (6-min walk test, 30/60-sec chair stand, HUT, Jamar, MIP/MEP and Spirometry at 13 and 26 weeks (from medical records).
VIII. Mean change from baseline in subjective rating of physical and cognitive symptoms evaluated by self-reported questionnaires (CAT, Frändin-Grimby, FSS, GAD-7, MFS, mMRC, MOCA, PCL-5, PHQ-9, WAI) at 13 and 26 weeks (from medical records).
IX. Long term post-trial follow-up of HRQoL using EQ-5D as variable up to 4 years post trial.

4.4.4 Safety and Compliance Endpoints

I. Number of subjects, proportion of subjects and number of AEs at 13 weeks.
II. Number of subjects, proportion of subjects that have completed planned treatments after 6 weeks.

5. Trial design and procedures

5.1 Overall Trial design and flowchart

Phase II Clinical Trial

Prospective randomized, placebo-controlled, double-blind, clinical trial, estimated enrolment: 80 subjects

Parallel groups

Intervention: HBO_2: 240 kPa for 90 min, maximum 10 treatments within 6 weeks from randomization.

Control: Placebo treatment with 'sham' air breathing at a moderately higher pressure (134 kPa) for 90 min to simulate hyperbaric chamber treatment, maximum 10 treatments within 6 weeks from randomization.

The population will comprise of previously generally healthy patients diagnosed with Long COVID (U09.9). All patients are clinically assessed by a multidisciplinary team with a battery of questionnaires, physical tests, laboratory tests and radiology. After their first assessment,

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individuals may have further organ specific work up for diagnosis, such as diagnosis of Postural Orthostatic Tachycardia Syndrome (POTS).

Once the patient has been diagnosed with Long COVID, they will be informed and asked to participate in the trial. No study specific procedures will take place before an informed consent form (ICF) has been signed. Some study specific procedures will be performed before inclusion (screening), such as HRQoL questionnaires and pregnancy test (if applicable). The patients will be included once they fulfil the inclusion criteria and exhibit none of the exclusion criteria. Baseline medial history, medical examination and study specific tests, blood samples and questionnaires will be collected during visit 1. If patients have already entered or gone through follow-up in clinical routine, some data from the last visit, no more than three months prior can be used for visit 1. If less than two weeks since last follow up, study specific procedures do not need to be repeated. Eligible subjects will be randomized within two weeks of the planned first treatment. Subjects will be randomized in a 1:1 alloction to HBO_2 or placebo (sham treatment). Scheduling of the HBOT will depend on available resources but the first treatment should be given within two weeks after randomization, and a maximum ten treatments should be given within 6 weeks from randomization. Physical tests, blood tests and questionnaires are repeated after the last treatment. Saftety and secondary endpoints are evaluated at visit 2. Efficacy evaluation of the primary endpoints will be made on assessments at visit 3 (three months), questionnaires and bloodtests. Subjects will be asked to use an activity meter in conjunction with each visit. Visit 4 and 5 are long term follow up, includes questionnaires, bloodtests and activity meter.

Clinical equipoise: The rationale for 1:1 randomization is that this is a new disease and that it will maximise the statistical power to detect a statistically significant efficacy between treatment groups.

Main efficacy and safety endpoints will be evaluated at one and three months after randomization, but all subjects will be asked to participate in a one-year follow-up after inclusion.

Subjects will also be asked to participate in a post-trial long-term follow-up with EQ-5D Questionnaire that will be sent out once a year for up to four years after visit 5.

Figure 1 and Table 1 show the trial overview and procedures

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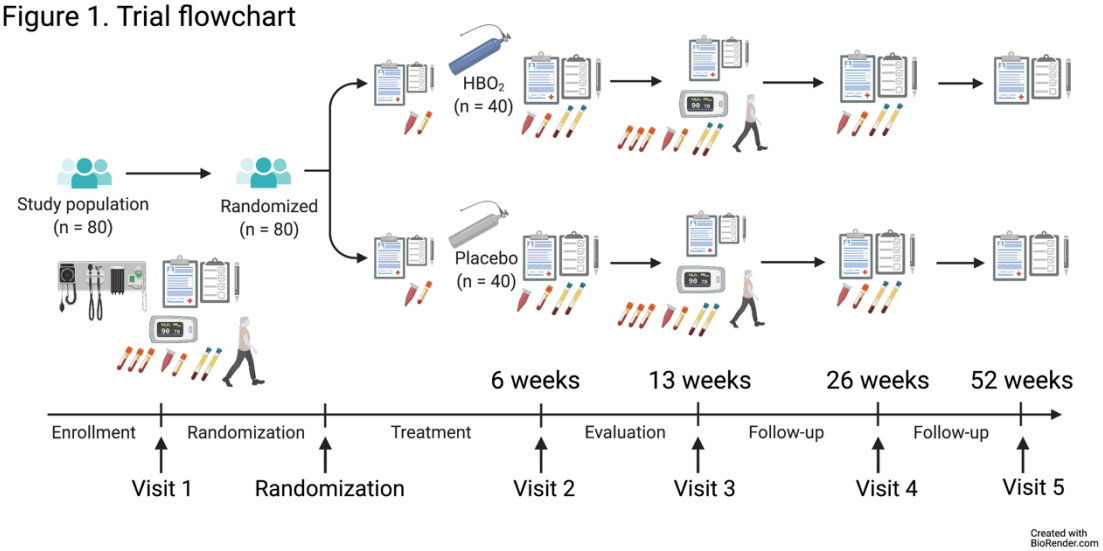
Figure 1. Trial flowchart



5.2 Procedures

Table 1. **List of procedures (Bold letters indicate study specific procedures**; other procedures may vary depending on symptoms and availability from medical records.)

Activity	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Week	0	6	13	26	52
Signed Informed consent Form	**X**				
Inclusion/exclusion criteria	**X***				
Randomization	**X**				
Medical history	**X**	X**	X**	X**	X**
Socio-demography	**X**	X***	X***	X***	X***
Concomitant medications	**X**	**X**	**X**	X	X
RAND 36	**X**	X	**X**	**X**	**X**
EQ-5D	**X**	**X**	**X**	**X**	**X**
RHI	**X**		**X**		
6 min walk test	**X**	X	**X**	X	X
30/60 s chair-stand	**X**	X	**X**	X	
Nexfin	**X**		**X**		
Treatment (HBOT/Placebo)		**X (1-10)**			
Treatment planned		**X (1-10)**			
AE/ADR	**X**	**X**	X	X	X
Study-specific biochemistry	**X**	**X**	**X**	X	X
Biobanking (PBMC, Plasma, EPR)	**X**	**X, X**	**X**	**X**	
Activity meter	**X**	**X**	**X**	**X**	**X**

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*Exclusion criteria includes a pregnancy test (if applicable), RAND 36 questionnaire, a HBOT specific questionnaire, review of medical records and a medical examination if needed.
**Medical history includes COVID-19 specific history, routine blood tests, questionnaires, physical tests, and radiology, medical records will be reviewed and recorded.
***Socio-demography that may change over time such as sick-leave, weight, activity, smoking habits.

Trial schedule

Visit 1: (Minimum 12 weeks post COVID-19)

a) After the patient has been informed about the trial and if agreed to participate, an **informed consent** form (ICF) will be **signed** off before any study-specific procedures occur.
During the **Screening**, procedures to assure the patient´s eligibility for trial participation will be performed, this includes a serum **pregnancy test** for females of childbearing potential, **RAND-36** and **EQ-5D questionnaires**, a **HBOT specific questionnaire**, review of medical records and a medical examination if needed for all. **Socio-demography**, **medical history** including COVID-19 specific history, adverse events, routine blood tests, questionnaires, physical tests, and radiology will be reviewed and recorded. **Questionnaires will be sent digitally and if eligible, subjects are booked for the physical tests.**

b) **Blood** samples for future biochemical research will be collected, and **study-specific chemistry** supplemented if necessary. **Study-specific procedures** will be conducted (not repeated if less than two weeks since last clinical visit and other relevant procedures will be recorded if less than 12 weeks since last clinical visit.

c) Subjects will be **randomized** to either HBO_2 or placebo when the first treatment is planned. Time, date and randomization group are recorded (blinded to subjects and all assessors of outcome variables).

Visit 2: (Starts within 4 weeks after visit 1, within 2 weeks of randomization, ends after last HBO_2 treatment)

Subjects are booked for the treatment.
a) **Review of medical records** and medical history. Advererse events, changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded.

b) **Blood** samples for future biochemical research may be collected before and after the first and the last treatment, **study-specific biochemistry** supplemented if necessary. Data from **activity meter** is registred. RAND 36 and EQ-5D questionnaires are sent digitally.

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c) Subject will be introduced to the **Hyperbaric chamber** and given a **maximum 10 treatments within six weeks from randomization**. If planned but not given, this will be recorded with the reason for not giving the treatment.

Visit 3: (13 weeks after randomization +/- 2 weeks)

Questionnaires will be sent digitally and subjects are booked for physical tests.
a) **Review of medical records** and medical history. Adverse events, changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded.

b) **Blood** samples for future biochemical research will be collected, and **study-specific chemistry** supplemented if necessary.

c) **Study-specific procedures** will be conducted.

Visit 4: (26 weeks after randomization +/- 4 weeks)

Questionnaires wil be sent digitally to subjects.
a) **Review of medical records** and medical history. Changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded. Adverse events will be followed up.

b) **Study-specific blood** samples for future biochemical research will be collected, and **routine chemistry** supplemented if necessary. Data from **activity meter** is registred.

c) **Long term follow-up.**

Visit 5: (52 weeks after randomization +/- 4 weeks)

Questionnaires wil be sent digitally to subjects.
a) **Review of medical records** and medical history. Changes in concomitant medication, demographics, routine blood tests, questionnaires, physical tests and radiology will be reviewed and recorded. Adverse events will be followed up.

b) Data from **activity meter** is registred.

c) **Long-term follow-up.**

Unscheduled visits:

Any variables outside the timeframe of scheduled visits may be recorded as unscheduled visits during the trial.

End of Trial

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Trial Code:	HOT-LOCO
Version No:	v.4
Date:	2022-01-03
EudraCT No:	2021-000764-30

A final visit in the electronic case report form (eCRF) should be completed for every randomised patient whether the patient completed the trial or not. The reason for any early discontinuation should be indicated on this form.

5.2.1 Assessments and procedures

Medical history

Relevant medical history will be recorded at Visit 1. The medical history will include a review of past and current relevant diseases/diagnoses/symptoms, for female subjects this includes information regarding menstrual cycle and pregnancies. Symptoms, signs and the start date of COVID-19, Long COVID and vaccination status will be collected. For concomitant diagnoses start year will be collected. Findings and/or abnormalities detected will be recorded in the eCRF. Other medical history, not relevant for the trial will be documented in medical records. Records and medical history will be reviewed for update/change in significantly changed parameters such as symptoms/signs or new diagnoses.

HBO_2 specific questionnaire

A HBO_2 specific questionnaire with focus on HBO_2 contraindications will be filled in by all subjects, contraindications include pregnancy, claustrophobia, obstructive lung disease and history of spontaneous pneumothorax. If anything in the questionnaire renders further examination, a review of medical records, an interview and a medical exam will be conducted. Findings and/or abnormalities detected will be documented in medical records with a statement “No contraindications for HBOT” or else the reason for contraindication.

Questionnaires

Change in RAND 36-item Health Survey (RAND-36), EQ-5D(euroquol.org) are used as primary and secondary enpoints , other questionaires may vary depending on clinical evaluation and main symptoms. Multiple questionnaries are used in clinical assessment including: RAND 36, EQ-5D, Frändin-Grimby activity sale, The Montreal Cognitive Assessment (MOCA), Work Ability Index (WAI), Mental Fatigue Scale (MFS), Fatigue Secerity Scale(FSS), Patient Health Questionnaire (PHQ-9), and the Generalized Anxiety Disorder (GAD-7), COPD Assessemnet Test(CAT), Medical Research Council(mMRC).

Medical records will be reviewed, time of questionnaire, reason for questionnaire and finding will be recorded. Baseline and change is categorized and described according to the reference in the specific questionnaire. SOPs for the study-specific questionnaires are available in the TMF, short description below:

RAND 36-item Health Survey 1.0 (RAND 36)

RAND 36 is a self-reporting questionnaire that contains 36 items that measure eight concepts of health in general terms, at present and past four weeks: physical functioning (10 items), role limitations due to physical health (4 items), role limitations due to emotional problems (3 items), energy/fatigue (4 items), emotional well-being (5 items), social functioning (2 items),

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pain (2 items) and general health (5 items). It also includes a single item that provides an indication of perceived change in health over the last year. Scoring RAND 36 is a two-step process. First, numeric values from the survey are coded so that all items are scored from 0 (lowest score) to 100 (highest possible score). Scores then represent the percentage of total possible score achieved. In step 2, items in the same scale are averaged together to create the 8 scale scores. Items that are left blank (missing data) are not taken into account when calculating the scale scores. Hence, scale scores represent the average for all items in the scale that the respondent answered. RAND 36 is well documented in terms of reliability and variability also for Swedish translation (Orwelius et al., 2017).

EuroQol-5 Dimensions questionnaire (EQ-5D)

EuroQol-5 Dimensions questionnaire is a widely used self-reporting questionnaire that measure 5 dimensions of health TODAY at three or five levels (EQ-5D-3L or EQ-5D-5L) of severity; no problems, some/moderate problems and extreme problems/unable.The health dimensions are mobility, self-care, usual activities, pain/discomfort, anxiety/depression and a visual analogue scale (VAS) 0-100 which it used as a quantitative measure of overall health status. EQ-5D is the most widely used questionnaire for health-economy evaluation. Swedish population norm data for age and gender are available and can also be used for determining ability to work/study.

Physical tests

The 6 min walk test (American Thoracic Society), 30/60 sec chair stan (Jones et al 1997) , EndoPAT for measurement of RH-PAT and Nexfin (Edward Lifesiences) for measurement of cardiac indicies and activity meter for activity, heart rate variability (HRV) and sleep pattern are study-specific, other physical tests used in clinical practice may vary depending on main symptoms.
Multiple different physical tests are used in the clinical assessement including: 30/60-sec chair stand, Handgrip (Jamar), Spirometry, Maximal Inspiratory and Expiratory muscle strength (MIP/MEP), 6-min walk test, Head-Up-Tilt test (HUT).

Medical records will be reviewed, time of test, reason for test and finding will be recorded. Baseline and change is categorized and described according to the reference in the specific test. SOPs for the study-specific tests are available in the TMF, short description below:

6 minute walk test
The test is conducted in a corridor without obstacles with a measured distance of 30 meters (a cone is placed for start and turn) with markings every meters and double marknings every 5 meters. The subject carries a portable pulse/saturation meter.

- If the subject uses a walking aid the same should be used during the test, type of aid, if used is documented in the protocol.
- Periferal oxygen saturation (SpO_2) and pulse are recorded each minute.
- Any pauses during the test is noted, how long and posture during paus is recorded.

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- A timer is started when the subject starts walking. The instructor only walks with the subject if deemed necessary from a safety perspective.
- Fatigue (general and leg) and dyspnea is self-rated with a Borg CR-10 scale immediately when the subject stops.
- The test is stopped it the subject experiences chest pain, SpO_2 below 80%, severe dyspnéa, cramping legs, staggering or wobbleing gait, perspiration or pale face. Time of disontinuation, cause and primary limiting factor is noted in the protocol.

30/60 sec chair stand

A red chair (44 cm high) is used, placed against a wall to minimise risk of falling.
The subject sits on the seat with a staight back, feet sholder wide with close to 90 degree angle in the knees, one foot slightly in front of the other. Arms crossed over chest.
The instructor demonstrates once and the subject practice once.

- The subject in instructed to stand up straight and sit down completely as many times possible during 60 seconds.
- A timer is started when the subjects back side lifts for the seat. The number of straight stands at 30 and 60 seconds is noted in the protocol. The subject is cheered on.The last stand is counted if the subject has risen more than half way at 60 seconds.
- Pulse, SpO_2, fatigue (general and leg) and dyspnea is self-rated with a Borg CR-10 scale immediately when the subject stops.

Nexfin

Subject is fasting, no beverage with caffein or sugar within 2 hours. The monitor is connected before 5 min rest in supine position without distraction. Non-invasive measurement of cardiovascular indicies with a beat-to-beat pulse wave analyzer placed on the middle phalang of one finger by Nexfin teqnology (ClearSight, Edwards Lifeciences). The ClearSight device comprices a pneumoatic pletysmograph that provides advanced hemodynamic parameters and continuous noninvasive blood pressure (BP) from a finger cuff with a redesigned self-coiling mechanism that reconstructs the clinical standard brachial arterial waveform from the finger arterial pressure waveform; it has been validated towards invasive measurements in a number of clinical trials.

- Measurement of beat-to-beat blood pressure and pulse including pulse-contour analysisis at rest and during physical tests.
- Registration of Heart rate, estimated Stroke volume, Cardiac index and Systemic vascular resistance index is recorded in the protocol.

EndoPAT

Subject is fasting, no beverage with caffein or sugar within 2 hours. The monitor is connected before 10 min rest in supine position without distraction. Non-invasive determination of digital endothelial function is measured with a pulse amplitude tonometry (PAT) device placed on the tip of each index finger (Endo-PAT2000; Itamar Medical, Caesarea, Israel). The PAT device comprises a pneumatic plethysmograph that allows measurement of pulse volume changes.

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The PAT signal is recorded at baseline and following 5 min arterial occlusion using an inflatable blood pressure cuff placed on the forearm of one arm, while the contralateral arm serves as a control. The blood pressure cuff is inflated to 30 mmHg higher than the systolic pressure or a maximum of 200 mmHg for 5 min. The post-occlusive hyperemia stimulates endothelium-dependent vasodilatation causing an increase in digital pulse amplitude. The change from the baseline measurement is expressed as the reactive hyperemia index (RHI) which reflects vasodilator function of the digital microcirculation (Hamburg and Benjamin, 2009). Previous evaluation has demonstrated that reduced RHI reflects microvascular endothelial dysfunction, predicts cardiovascular events and reflects reduced NO bioavailability (Alexander et al., 2020).

Activity meter

The commercially available OURA™ ring will be used. The OURA™ ring is worn like a finger ring and has a number of sensors that register heart rate, temperature and physical activity. With the OURA™ ring it is possible to monitor HRV, level of physical activity, changes in body temperature and sleeping pattern. Subjects will be asked to wear the OURA™ ring at a minimum 1 week before and after each visit. Data will be automatically registred in a smartphone application and then uploaded to a secure encrypted database.

Radiology

Multiple different modalities of imaging are usesd in the clinical assessement including: Dual Energy Computed Tomography (DECT), Magnetic Resonance Imaging (MRI), Computed Tomography (CT) and normal chest X-ray (CXR). Review of records, document the time of radiology, reason for radiology and finding if it coincides with an interval of 12 weeks of visit 1, 3 4 and 5.

Socio-demography

Demographic data such as gender, age, level of education, rate of employment/studies, level/rate of exercise, country of origin, body weight, height, and smoking habits/ nicotine use will be collected at Visit 1. Records and medical history will be reviewed for update/change in parameters at each visit.

Concomitant and post-trial treatment(s)

Since Long COVID is a new syndrome, that may be chronic, without any definiete cure, “best practice” for symptomatic medications and other treatments are likely to change over the course of trial. Subjects are also likely to have tried or may try other remedies.

Medications and treatments that are considered “best practice” may be given to the subjects at the discretion of their attending physician/physiotherapist/phycologist. Subjects will be discouraged to try new medications, treatments or remedies that are not evidence based during the course of the trial.

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Information regarding relevant regular concomitant medications, including vitamins, anti-oxidants, treatments and other remedies will be collected at Visit 1. Only relevant medications taken regularly, suspected to have caused an AE or used for treatment of an AE will be recorded. Changes in concomitant medications will be assessed (e.g. stop date or entry of a new treatment), throughout the trial by reviewing the patient's medical records and taking their medical history. Any changes will be recorded in the eCRF.

Blood samples

Routine biochemistry for kidney function, liver function, cardiac insult, haematology and blood glucose will be registered from the hospitals electronic system if they are outside normal range.

Study-specific blood tests that will be collected are: Ferritin, D-Dimer, LDH, Troponin T and a pregnancy test for women of childbearing age.

Date and time of collection and results from routine and study specific blood tests are recorded in the eCRF.

Details regarding the handling of blood sampling for laboratory analysis are found in section 5.3.

HBO_2 SOP and assessment

A standard operation procedure (SOP) will be attatched in the Trial Master File (TMF) but in general terms:

Subjects will be introduced to the hyperbaric unit; if required the subject may visit the unit before the first treatment. Treatment will be conducted in the multiplace (HAUX-STARMED-QUADRO 3500-2400) or monoplace chamber (SECHRIST 3300) depending on availability and number of subjets, at the discretion of the responsible physiscian. Subjects will be treated for 90 minutes; the treatment protocol is as follows - HBO_2 240 kPa with 10 min compression time and 10 minutes decompression time, and two air breakes, while placebo entails - 134kPa air, with 5 min compression time, and 5 min decompression to 120 kPa, and two air breakes will be reported to the subjects. Pressure gauges that can be seen by subjects will be covered. The frequency of the treatments and timing will depend on available resources at the discretion of the responsible physician but should be 2–5 treatments per week for 2–4 weeks. No treatment must be given more than 6 weeks after randomization.

Date and time for treatment will be recorded. Any planned treatment that could not be delivered and reason for the cancellation will be recorded. The treatment will be recorded on a separate CRF accessible only to staff designated to the treatment but blinded for the investigators performing assessements. Treatment type will be recorded in the eCRF and medical records once the code is broken or at the end of trial.

AE and ADR

Adverse events (AEs) and collection of AEs and Serious Adverse Events (SAEs) data.

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Collection of AEs will start directly after inclusion and will be recorded until visit 3. Only SAEs will be collected outside the treatment period (visit 2). Ongoing AEs and SAEs at the end of visit 3 will be followed up during long-term follow-up until the subjects last visit. Definitions, documentation and reporting of AEs are described in detail in the AE section below.

5.3 Biological sampling procedures

5.3.1 Handling, storage, and destruction of biological samples

Study-specific routine biochemistry will be analysed at the Karolinska University Hospital laboratory (KUL).

Study-specific biobanking includes collection ot 4 extra tubes:

1x4ml EDTA plasma will be bio-banked for later analysis

2x8ml Citrate plasma (CPT-tubes) will be bio-banked for PBMC isolation and later analysis.

1x4ml heparin blood will be centrifuged and erythrocyte fraction will be incubated with CPH spin probe, bio-banked for later analysis of ROS in erythrocytes by EPR, plasma will be biobanked for later analysis.

CPT and EDTA tubes will be collected by a research nurse and transported immediately to the research laboratory Studiecenter Karolinska where PBMCs are isolated, half are prepared with RNA-later® for later RNA extraction and gene expression analysis and half is cryopreserved for later functional analysis of the monocytes. The monocytes, citrate-, EDTA- and heparin plasma will be stored in a sub-biobank at Bioclinicum Karolinska University Hospital. The biological samples will be saved until all analyses are performed.

5.3.2 Total volume of blood per subject

The study-specific blood will be maximum 40 ml (24 ml for all and additionally 16ml for some subjects). A maximum total amount of 200 ml blood is collected from each subject at five visits over nine months. This volume should be related to a blood donator that donates 450ml at one occation that can be repeated every four months for women.

5.3.3 Biobank

Plasma, erythrocytes and PBMCs collected in this trial are registered in a regional biobank with an agreement with *Stockholms Medicinska Biobank (IVO reg nr 914)* and handled according to the current biobank laws and regulations. The samples are coded/psedonymized to protect the subject´s identification. All samples and the identification/code list are stored securely and separately to prevent unauthorized access.

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5.3 End of Trial

The end of trial is defined as the last subject's final follow-up at visit 5 (week 52).

Premature termination of this clinical trial may occur because of a regulatory authority decision or at the discretion of the sponsor/the steering comittee.

The sponsor/steering comittee reserves the right to discontinue the trial at any time point in the following cases:
• Unexpected high proportion of AEs that are possibly or probably related to the trial drug.
• Trial protocol is difficult to cope with.
• Recruitment of eligible subjects is too low.

The end of the trial will be reported to the regulatory authority within 90 days, or 15 days if the trial is terminated prematurely. The Investigators will inform participants and ensure that the appropriate follow up is arranged for all involved.

6. Subject selection

6.1 Inclusion criteria:

To be included in the trial, subjects must meet the following criteria:

1) Aged 18–60 years
2) Healthy or mild systemic disease prior to COVID-19
3) Symptoms consistent with Long COVID for at least 12 weeks
4) Diagnosed with Long COVID, PACS, PCS (ICD-10 U09.9)
5) Working or studying prior to COVID-19
6) Documented informed consent according to ICH-GCP and national regulations

6.2 Exclusion criteria:

Subjects must not be included in the trial if any of the following criteria are met:

1) Known pregnancy or positive pregnancy test in women of childbearing age
2) ASA 3 or more from other cause than Long COVID
3) Score above 70 in RAND-36 domain Role Limitation Physical Health (RP) or Physical Functioning (PF)
4) Diabetes
5) Diagnosed with Hypertension prior to COVID-19
6) Contraindication for HBO_2 treatment according to local guidelines
7) Participation or recent participation in a clinical trial with an investigational product
8) Mental inability, reluctance or language difficulties that result in difficulty understanding the meaning of trial participation

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6.3 Screening

Patients that have been assessed for Long COVID and that are likely to fullfil the inclusion criteria will be screened. Subjects will be informed about the trial by a study nurse during pre-screening and in detail about the trial by an investigator and after written informed consent, additional medical record review, HRQoL questionnaires, a HBOT specific questionnaire, physical examination (and pregnancy test if applicable) will be conducted. Subject eligibility (that subjects fulfill all inclusion criteria and do not meet any exclusion criteria) will be established before randomization to treatment.

6.4 Withdrawal Criteria

Subject participation: A subject will be considered to have completed the trial when he or she completes the assessment at 52 weeks (visit 5). Subjects should be encouraged to continue the trial but have the right to withdraw their consent or part of their consent regarding the trial participation e.g. to discontinue a study-specific blood test, but still participate in follow-up visits with questionnaires or not participate in further trial visits. The subject has no obligation to explain why he/she does not want to continue. The investigator also has the right to stop the subjects treatment in the event of AEs, protocol deviations, administrative reasons or any other reasons. It is understood by all concerned that an excessive rate of discontinues can render the trial uninterpretable. Therefore, unnecessary discontinuation should be avoided.

Irrespective of the reason for not continuing with the treatmentsl and whenever possible, the patient should be examined. Relevant laboratory test samples should be obtained and all relevant assessments should be completed if applicable. All AEs should be followed up until they have returned to baseline status or stabilised.
A termination visit (End of trial) in the electronic case report form (eCRF) should be completed for every randomised subject whether the subject completed the trial or not. The reason for any early discontinuation should be indicated on this form.

Subjects may be discontinued from the trial at the discretion of the Investigator. Specific reasons for discontinuing a subject from further assessments are:

AEs: Clinical or laboratory events that in the judgment of the investigator, DSMB or the Sponsor and in the best interest of the subject constitute grounds for discontinuation. This includes serious and non-serious AEs regardless of relation to trial drug.

Withdrawal of Consent: If a subject withdraws consent for disclosure of future information at the discontinuation of the trial or after completion of the trial, no further evaluations should be performed and no additional data should be collected. The Sponsor may retain and continue to use data collected before subject withdrew his/her consent. The Withdrawal of Consent reason is only applicable if the subject denies any further contact with site and no further data collection.

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Lack of Efficacy/Treatment Failure: Subjects experiencing deterioration or no improvement of disease as judged by the investigator, may be discontinued from the trial at any time during the trial, offered alternative treatment and scored as treatment failures. Treatment failures includes significant disease worsening, requirement for surgical intervention and HBOT related SAE. Patients may be discontinued for sustained non-response at the discretion of investigator.

Protocol Violation: If the subject's findings, or conduct, fails to meet protocol entry criteria or fails to adhere to the protocol requirements that make it impossible to derive sound scientific or medical conclusions from the primary endpoint data generated on a subject, (e.g. diagnose is changed after randomization or wrong treatment is given according to randomization).

Lost to Follow-Up: The subject does not show up for further visits and study personnel cannot reach the patient.

Other: Termination of other reason

If the subject discontinues the trial, follow-up of this subject will be performed according to the clinic's routine but will be included in the Safety population if he/she have received at least one treatment.

7. Trial treatments

7.1 Description of investigational product(s)

Oxygen 100%, medical grade (Conoxia cryogen)

Placebo Air, compressed air medical grade

7.2 Dose and administration

Hyperbaric oxygen 240 kPa for 90 minutes (with 10 min compression time, two air breakes and 10 minutes decompression time). The number and frequency of treatments and timing will depend on the subject´s tolerance and available resources at the discretion of the attending physician, but the recommended interval is 2–5 treatments per week with a maximum of 10 treatments within 6 weeks from randomization. To satisfy the treatment compliance, a subject need to complete at least 5 treatments.

Placebo (134 kPa Air, with 5 min compression time, and 5 min decompression to 120 kPa, two air breakes will be reported to the subjects). The number and frequency of treatments and timing will depend on the subject´s tolerance and available resources at the discretion of the attending physician, but the recommended interval is 2–5 treatments per week with a maximum of 10 treatments within 6 weeks from randomization. To satisfy the treatment compliance, a subject need to complete at least 5 treatments.

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7.3 Packaging, labeling, and handling of investigational products(s)

Treatment: 100% oxygen for medical use, cryogenic gas from hospital supply system. There will be no study-specific packaging or labeling.

Placebo: Compressed air from hospital supply system. There will be no study-specific packaging or labeling.

Treatments will be recorded in the eCRF, the code will be unblinded for staff administering the treatments but assessor-blinded. After the subjects end of study, the code will be broken and recorded in the medical records.

7.4 Drug accountability and treatment compliance

HBO_2 is delivered inside a hyperbaric chamber by inhaling 100% oxygen through a tight facemask (in selected cases a hood) attended by medical staff, or inside a monoplace chamber filled with oxygen. If the mask/hood is tight the inspired oxygen pressure is 233.7–240kPa (range depending on 100% saturated - dry gas) at 240 kPa pressure, hence there is no uncertainty about compliance. During compression/decompression patients may need to remove the mask in order to equalize the middle ears and the time might differ slightly between monoplace and multiplace chambers. The difference in dose during this period is neligleble. The date and time of treatment will be recorded in the eCRF. Compliance will be measured as the number and fraction of treatments planned vs given. Subjects that have been given at least 5 treatments will be analysed in the PP population. Any discrepancies from the protocol should be recorded in the eCRF.

7.5 Randomization

Subjects will be enrolled consecutively, as they are found to be eligible for inclusion in the trial, and randomized but after the treatment has been scheduled. Treatment should start within two weeks of randomization.

If a subject discontinues their trial participation, their subject code will not be reused, and the subject will not be allowed to re-enter the trial again. There will be no replacement for these subjects.

Eligible subjects will be randomized in a 1:1 allocation, stratified by disease severity in relation to RAND 36 and gender in blocks (blinded to all study personnel) to either HBO_2 or Placebo. There will be a computer generated randomization.

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7.6 Blinding

This is a double-blind placebo-controlled trial where subjects and all study personell that participate in the asessement of sympoms and objective findings will be blinded to the treatment. The placebo protocol is well established and even experienced divers cannot differ between “sham treatment” and HBO_2 (Lansdorp and van Hulst, 2018). It is not unlikely that some sujects may have problems equalizing the ears even during placebo. Pressure gauges that can be seen by subjects will be covered and all staff will be informed not to discuss the treatment settings when subjects can hear and they will report two air brakes in the same manner as a normal treatment. To validate the blinding process all subjects will be asked at the end of the treatment if they believe they received placebo or HBO_2 and AE directly attributed to equalization problems will be compared.

7.7 Code breaking

The code is kept in the TMF in sealed envelopes, only accessed by staff designated to the hyperbaric unit if needed for safety reasons. If an AE or an SAE is reported, the PI should immediately assess the casual relationship and if an AR or SUSAR is suspected the code may be broken. Treatment type will be recorded in the medical records once the code is broken or at the end of trial.

7.8 Concomitant Medication

Medications that are considered necessary for the safety and well-being of the subject can be given at the discretion of the investigator, unless otherwise specified as an exclusion criterion.

All medications that the subject has taken regularly during the trial must be recorded in the eCRF. Non prescribed food supplements such as vitamins and anti-oxidants should also be recorded in the eCRF if taken regularly. Any changes need to be reported. Concomitant prescribed medications since start of symptoms shall be recorded at Visit 1.

7.9 Treatment after trial end

After an interval of six weeks no more HBO_2 must be given. The total dose during the trial will be recorded until six weeks after first treatment. At trial end, the participants will be treated according to routine clinical praxis.

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8. Handling of Adverse Events

8.1 Definitions

8.1.1 Adverse Event (AE)

Adverse Events constitute any untoward medical occurrence in a clinical investigation subject administered a medicinal product and, which does not necessarily have a causal relationship with the treatment, can be an unfavorable and unintended sign (including an abnormal laboratory discovery), symptom or disease temporally associated with the use of the medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

8.1.2 Adverse Reaction (AR)

In the new use of a medicinal product all noxious and unintended reactions to the medicinal product related to any dose should be considered an adverse reaction (AR). The phrase 'reaction to a medicinal product' means that the causal relationship between the medical product and an adverse event is at least a reasonable possibility, that is the relationship cannot be ruled out.

8.1.3 Serious Adverse Event (SAE)

Serious adverse events constitute any untoward medical occurrence that at any dose:

- results in death
- is life-threatening
- requires inpatient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- results in a congenital anomaly/malformation
- regarded as medically important without meeting the above mentioned criteria

Medical and scientific assessment will be made to determine if an event is 'serious' and whether it would prompt reporting in other situations, for example important medical events that may not be directly life-threatening or result in death or hospitalization but may compromise the subject or may require intervention to prevent one of the other results set forth in the definitions above. These should also normally be considered as SAEs.

8.1.4 Suspected Unexpected Serious Adverse Reaction (SUSAR)

SUSAR comprise a reaction/event that is unexpected, serious, and suspected to be caused by the treatment, i.e. adverse events that are not included in the SPC.

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8.2 Assessment of Adverse Events

8.2.1 Assessment of causal relationship

The investigator is responsible for determining whether there is a causal relationship between the AE/SAE and use of the investigational product.

Those AEs which are suspected of having a relationship to the investigational product will be followed up until the subject has recovered or is well taken care of and on their way to good recovery (see also section 8.4, Follow-up of Adverse Events).

All AEs will be categorized either as related, probably related, possibly related, unlikely related or not related, in accordance with the definitions below:

Related: Clinical event, including abnormal results from laboratory analyses, occurring in a plausible temporal sequence in relation to drug administration. The observed event matches with the known adverse reactions scheme for the drug involved. The event cannot be attributed to underlying disease or other medications.

Probably related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the investigational product. The observed event match with the known adverse reactions scheme for the drug involved. It is unlikely attributable to underlying disease or other drugs.

Possibly related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the intervention/investigational product. The event could be explained by the investigational product and its emergence is reasonable in relationship with use of the investigational product, but there is insufficient information to determine the relationship. The event could be explained by an underlying disease or other medications.

Unlikely related: Clinical event, including abnormal results from laboratory analyses, with a with a temporal relationship with respect to drug exposure that makes a relationship improbable (but not impossible). The event could be plausibly explained by an underlying disease or other medications.

Not related: Clinical event, including abnormal results from laboratory analyses that do not meet any of the above criteria for relatedness.

8.2.2 Assessment of intensity

Each adverse event shall be classified by an investigator as mild, moderate or severe.

Mild: Transient symptoms that are relatively tolerable and does not affect the subject's normal life.

Moderate: Marked symptoms, sufficiently unpleasant that interfere with the subject's normal life. Deterioration of function but is transient.

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Severe: Unacceptable or incapacitating symptoms that causes deterioration of function to the extent that the subject is unable to perform normal activities.

8.2.3 Assessment of seriousness

The investigator is responsible for assessing the seriousness (serious or non-serious). If the incident is considered serious, this should be reported as a serious adverse event (SAE) by the investigator to the sponsor. See also section 8.3.2, Reporting of Serious Adverse Events (SAE).

8.3 Reporting and registration of Adverse Events

At each trial visit, AE are registered. Collection of AE data will start directly after inclusion and continue until 13 weeks (Visit 3) which is 7 weeks after the subject has ended their treatment with the investigational product. All AEs that occur during the trial and that are observed by the investigator/study-nurse or reported by the subject will be registered in the eCRF regardless of whether they are related to the investigational product or not. Assessment of causal relationship, severity, and whether the AE is considered to be an SAE or not will be done by the investigator directly in the eCRF. At minimum, for each AE/SAE, a description of the event is recorded (diagnosis/symptom if diagnosis is missing), start and stop times, causal relationship, severity, if the AE is considered to be an SAE or not, measures and outcome.

The following situations will not be reported as AE/SAE:

- Symptoms judged by the investigator as associated with Long COVID will not be recorded as an AE.
- A change in routine biochemistry will not be reported as AE unless detected during the treatment period.
- Non-serious adverse events outside the treatment period (visit 2) will not be recorded.

8.3.1 Reporting of Adverse Events (AE)

All AEs to be reported shall be registered in the eCRF continously.

8.3.2 Reporting of Serious Adverse Events (SAE)

Serious adverse events are reported to the sponsor on a special SAE form (included in the eCRF) within 24 hours of the investigator being informed of the SAE.

Follow-up information describing the outcome and handling of the SAE is reported as soon as this information is available.

The sponsor will in a timely manner assess whether the adverse event was expected for the investigational product or not, using the reference safety information. Serious AEs must be

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collected, registered in the CRFs and an assessment of causality of the SAE should be performed. Also, discontinuations due to AEs will be collected.

8.3.3 Reporting of Suspected Unexpected Serious Adverse Reactions (SUSAR)

Those SAEs in Sweden which are assessed by the sponsor to be SUSARs are reported via a CIOMS form to the MPA that are submitting the CIOMS report to the to the European Medicines Agency (EudraVigilance database) according to the specified time frames.

SUSARs that are fatal or life-threatening are reported as soon as possible and no later than 7 days after the incident has become known to the sponsor. Relevant follow-up information is sent thereafter within an additional 8 days. Other SUSARs are reported as soon as possible and no later than 15 days after they have come to the sponsor's knowledge.

Any SUSAR will also be notified to the EPM by the sponsor.

Information about SUSARs occurring during the study is compiled by the sponsor and sent out to the principal investigator at all participating centers in connection to the event.

SUSARs in other participating countries will be reported to respective CA and EC according to applicable procedures

8.4 Follow-up of Adverse Events

All AEs should be followed up until they have returned to baseline status or stabilized until End of trial. AEs suspected to have a causal relationship with the trial intervention are followed until recovered or until the subject is on good way to recovery, follow-up will be done at the planned visits regadless of withdrawal from the trial.

8.5 Safety Report (Development Safety Update Report, DSUR)

During the trial period an annual Development and Safety Update Report (DSUR) will be submitted to the Swedish MPA and EPM .

The report includes a summary of all reported SAEs and SUSARs, a summarized safety assessment for trial subjects and information regarding potential updates of the risk-benefit assessment since trial approval.

8.6 Procedures in case of emergencies, overdose or pregnancy

The sponsor and investigator are obliged to immediately take the urgent safety measures necessary to protect the subjects from immediate danger. Examples of such measures are to temporarily suspend the clinical trial or to introduce supplementary monitoring measures.

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The sponsor shall inform the MPA and EPM as soon as possible about the urgent safety measures taken by the investigator or sponsor.

If a subject who participates in a clinical trial for investigational products becomes pregnant, this person must be followed up until the birth has taken place. If the fetus/child has any congenital malformation, this must be reported as a serious adverse event or side effect (SAE).

8.7 Reference Safety Information

For reference safety information, reference is given in the SmPC.

9. Statistics

9.1 Statistical Analysis Plan

The principal features of the statistical analysis of the data are described in this section. A more technical and detailed elaboration of the principal features will be written in a separate Statistical Analysis Plan (SAP). The SAP will be finalised prior to Data Base Lock (DBL) and will include a more technical and detailed description of the statistical analyses described in this section. This section is a summary of the planned statistical analyses of the most important endpoints including primary and key secondary endpoints.

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9.1.1 Analysis population

9.1.1.1 Definition of Trial Populations

9.1.1.1.1 The Full Analysis Set (FAS) Population; All randomized subjects who were exposed at least once to the study intervention will be included in the FAS population.

9.1.1.1.2 Per-Protocol (PP) Population; All randomized subjects with no major protocol violations will be included in the PP population. The final decisions regarding the PP population will be taken at the Clean File meeting before the database lock.

9.1.1.1.3 Safety Population; All randomized subjects that have received at least one treatment will be included in the safety population.

9.2 Statistical analyses

9.2.1 Sample size calculations

The assessment of the primary endpoints in this trial are based on the RAND 36-item health survey at baseline and 3 months where the domains of physical functioning and role functioning/physical defines the primary endpoints.

The primary endpoints have been used for long COVID (Garratt et al., 2021). There are norm data available for Sweden which enable us to determine a threshold for normalisation of individual RAND 36 domain levels. Using data from a few studies with similar methodology where RAND 36 has been previously used, we have assumed the standard deviation (SD) of Role Physical (RP), Physical Functioning (PF) 15.0. We expect the quality of life to be generally low in our cohort, especially in the RAND 36 RP and PF domains. We consider a ten points higher RAND 36 score in the HBO group compared to the placebo group to constitute a clinically relevant difference to be detected. Sample size calculation using t-test for independent groups, with 80% power, assuming a common SD of 15, and with a 5% significance level, reveals that at least 37 subjects per group are needed. We aim to recruit 80 subjects. An interim analysis will be made after 20 have undergone visit 2 to evaluate safety and when 40 subjects have undergone visit 3 in order to stop for futility and adjustment of sample-size if needed.

Sample size calculation was done in nQuery version 7.

9.2.2 General statistical methodology

Primary and secondary endpoints will be evaluated using the FAS population and sensitivity analyses performed using the PP population.

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9.2.2.1 Statistical Hypotesis

The primary objective of the study is to confirm a superior efficacy for the active treatment compared to placebo in the primary endpoints. The null hypothesis to be tested is that there is no difference between HBO treatment and placebo, i.e., the mean change in (HBOT) = mean change (placebo). The same statistical hypothesis will be used for key secondary endpoints.

9.2.2.2 Adjustment for Multiplicity

The overall type I error rate for testing the primary efficacy endpoints will be controlled at the type I error rate of 0.05 using appropriate methods for adjustment of multiplicity in the primary. There will be no adjustment for multiplicity in main secondary endpoints but nominal p-values will be presented and results will be interpreted as exploratory findings.

All hypothesis tests will be two-sided. Details of the methods for adjustment in terms of the selection of endpoints to include in the testing sequence and the criteria for rejecting (or not rejecting) individual hypotheses are provided in the SAP.

9.2.2.3 Subgroups

The following subgroups will be evaluated for this study:

- Gender
- Disease severity
    - RAND-36 RP and PF below 30
    - RAND-36 RP and PF 30-50
    - RAND-36 RP and PF above 50

9.2.3 Patient Demographic and Baseline Characteristics

Baseline values and patient characteristics will be presented in tables by group and in total. All continuous variables will be described using standard statistical measures, i.e., number of observations, mean and median value, standard deviation, minimum and maximum value. All categorical variables will be summarised in frequency tables.

In general, continuous outcome variables will be analysed using ANCOVA, unless otherwise specified. Estimates will be presented using least square means for differences between treatment arms. For continuous endpoints that are measured repeatedly over time, such as EQ5D, RAND-36 domains, the change from baseline will be analyzed using a linear mixed effect model including baseline, treatment group, sex, symptom severity, visit, and treatment

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group by visit interaction, and subjects as random effects, in the models. An unstructured covariance matrix will be assumed.

Analysis for categorical data in terms of binary data (Yes/No) will be presented as the proportion of participants with the frequency of presence or absence by treatment group of the characteristics of interest and analysed using the CMH Chi-square test, where the parameter used for the statistical hypothesis testing will be the OR, as a measure of the relative difference in odds between treatment arms. An OR>1 indicates an efficacy in favour of HBOT compared to placebo.

9.2.4 Primary Endpoint Analysis

The analysis of the primary endpoint will be conducted on the Full Analys Set (FAS) and the Per Protocol Set (PPS).

The primary analysis of the primary endpoints will be performed using the ANCOVA, including randomisation strata of main symptom and gender together with treatment as fixed factors in the model.

The two primary endpoitns will be adequately adjusted for multiplicity.The p-value for testing the null hypotheses, no difference between treatment groups, must be less than 0.05 to be considered to have met the primary objective.

9.2.5 Secondary Endpoints Analysis

The same analysis approach used for the primary efficacy endpoint will be applied to the secondary efficacy and exploratory endpoints as for the primary endpoints referred to as a 'Proportion endpoints'.

For categorical secondary endpoints, the CMH Chi-square test adjusting for disease severity and gender will be used to test for differences between treatments. Results will be presented using the frequency and the proportion by treatment group and the OR together with its corresponding 95% confidence interval.

All tests for the secondary endpoints will be two-sided on the 0.05 significance level. There will be no adjustment for multiplicity in main secondary endpoints.

All analysis will be done for the FAS population using observed data.

9.2.6 Safety analyses

Safety analyses will be performed on the Safety population.

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9.2.6.1 Analysis of Adverse Events

The number and percentage of patients reporting AEs, and the number of AEs reported will be presented. The events will be tabulated by system organ class and preferred term. In addition, summaries by relationship to trial drug and severity will be presented. SAEs will also be presented in separate tabulations.

The number of patients experiencing an AE will be compared descriptively between groups. All patients with AEs will be listed individually with patient number in addition to type of event, start and stop time, duration, seriousness, severity, action taken, relationship to trial drug and outcome of AE.

9.2.7 Interim Analysis

Safety will be monitored continuously by the DSMB throughout the trial, an interim safety analysis will be performed when 20 subjects have available data for the safety endpoints.

There will be an interim analysis performed after 40 subjects have available data for the primary endpoint. The purpose of the interim analysis is to evaluate the assumption used for the sample size calculation and if necessary, to adjust the sample-size if needed. Also, the study will be evaluated for futility regarding the primary endpoints, to stop the study for futility (i.e., the predictive probability of success at the end of the study, given the data at the interim analysis) is less than 20%.

The DSMB will perform both interim analyses. A separate DSMB protocol will be created.

9.2.8 Handling of Dropouts and Missing Data

For the primary endpoint efficacy analyses, missing data will be adequately imputed for all subjects in the FAS population. In addition, the observed cases population will be evaluated as a sensitivity analysis. For secondary endpoints, only observed data will be analysed.

10. Quality Control and Quality Assurance

10.1 Quality Assurance and Sponsor oversight

The sponsor is responsible for having oversight of the trial´s quality. Steps to be taken to ensure the accuracy and reliability of data include the selection of qualified investigators and review of protocol procedures with the site personnel before the trial. eCRF completion guidelines will be provided and reviewed with study-personnel before the start of the trial.

10.2 Monitoring

The trial will be monitored by an independent monitor before the trial begins, during the trial conduct, and after the trial has been completed, so as to ensure that the trial is carried out

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according to the protocol and that data is collected, documented, and reported according to ICH-GCP and applicable ethical and regulatory requirements. Monitoring is performed as per the trial's monitoring plan for which the sponsor is responsible and is intended to ensure that the subject's rights, safety, and well-being are met as well as data in the CRF are complete, correct, and consistent with the source data. The monitoring will be performed by an independent experienced monitor qualified in ICH GCP, applicable national and international regulations and the Declaration of Helsinki.

10.3 Source data

The investigator must keep source documents for each subject in the trial. Data in the eCRF can be source data, such as for certain demography parameters, AEs and assessment of SAEs. Source data is defined before trial start and a document describing what has been classified as source data in the trial should be included in the TMF. The investigator must ensure that all source documents are accessible for monitoring and other quality control activities.

10.4 Deviations or serious breaches

Serious breaches and deviations from the trial protocol, GCP and other regulations that significantly and directly affects, or with high likelihood could affect, the subjects or the scientific value of the trial, shall be reported within seven days (from knowledge) to the Swedish MPA. It is the sponsor's responsibility to judge the consequences of deviations that have occurred, and thus also to decide whether the Swedish MPA should be informed.

For major protocol deviations i.e violations see also section 6.4.

Minor deviations that do not affect subjects' integrity or safety, nor significantly affect the trial's scientific value, are documented in the trial documentation of the principal investigator and the sponsor.

10.5 Audits and inspections

Authorized representatives for the sponsor and Competent Authorities (CA) may carry out audits or inspections at the trial site, including source data verification. The investigator must ensure that all source documents are available for audits and inspections. The purpose of an audit or inspection is to systematically and independently review all study-related activities and documents, so as to determine whether these activities were performed, registered, analyzed and reported correctly according to protocol, Good Clinical Practice (GCP) and applicable regulations.

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10.6 Data Safety Monitoring Board

An independent DSMB will evaluate the safety data in the context of the overall trial and the currently existing information about the trial drug. The DSMB will be composed of representatives from and experts in their respective disciplines of medicine, statistics and clinical trial methodology and conduct.

The DSMB will review the data during the course of the trial, as specified in table 2 below, and will draw up a charter delineating their guidelines for operating and stopping rules for terminating individual subjects, a portion or all of the trial prematurely. However, the DSMB may for any safety concerns recommend stopping of the trial even if these criteria are not fulfilled. It is the responsibility of the Sponsor to decide whether premature end of trial will be made, based on the advice provided by the DSMB

The DSMB will have access to all trial data. It may request and will be provided with whatever data is deemed necessary or useful for it to carry out its duties. The data provided will be blinded to treatment group unless specific unblinding is requested by the DSMB.

Table 2. DSMB meeting schedule	**Time of meeting**
Before trial start	Before first subject is included
Safety Interim analysis	When 20 subjects have completed visit 2
Interim analysis	When 40 subjects have completed visit 3
Efficacy analysis	When all 80 subjects have completed visit 3
End of the trial	Final visit has been done by the last subject.

10.7 Data protection

If any part of the data is handled by any other organization, inside or outside the EU, appropriate agreements and/or other documentation will be established, to ensure that the data processing is performed in accordance with the provisions of the General Data Protection Regulation (GDPR) and other relevant legislation, before any data transfer takes place.

The content of the informed consent form complies with relevant integrity and data protection legislation. In the subject information and the informed consent form, the subject will be given complete information about how collection, use and publication of their trial data will take place. The subject information and the informed consent form will explain how trial data are stored to maintain confidentiality in accordance with national data legislation. All information processed by the sponsor will be pseudonymized and identified with a Study-ID.

The informed consent form will also explain that for verification of the data, authorized representatives of the sponsor, as well as relevant authority, may require access to parts of

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medical records or study records that are relevant to the trial, including the subject's medical history.

11. Ethics

11.1 Compliance to the protocol, GCP and regulations

The trial will be performed in compliance with the trial protocol, the Declaration of Helsinki, ICH-GCP (Good Clinical Practice) guidelines and current hospital, national and international regulations governing this clinical trial. This is to ensure the safety and integrity of the subjects as well as the quality of the data collected.

11.2 Ethical review of the study

The final trial protocol must be approved, as a part of the application for a permit for clinical trials, by both the Swedish Ethical Review Authority (Etikprövningsmyndigheten, EPM) and the Swedish Medical Products Agency (MPA) before the trial can be conducted. The final version of the informed consent form and other information provided to subjects, must be approved or given a written positive opinion by EPM. EPM and MPA must be informed of any changes in the trial protocol in accordance with current requirements.

11.3 Procedure for obtaining informed consent

The principal investigator shall ensure that the subject is given full and adequate oral and written information about the trial, its purpose, any risks and benefits as well as inclusion and exclusion criteria. Subjects must also be informed that they are free to discontinue their participation in the trial at any time without having to provide a reason. Subjects should be given the opportunity to ask questions and be allowed time to consider the provided information. If the person chooses to participate, both the subject and the investigator (qualified physician) shall sign the informed consent form. A copy of the subject information as well as the informed consent form shall be provided to the subject. The subject's signed and dated informed consent must be obtained before performing any study-specific activity in the trial. Each subject who participated in the trial will be identified by a subject number and if randomized, indentified by a randomization number on a subject identification list. The subject agrees that monitors, auditors, and inspectors may have access to their medical records and other source data. If new information is added to the trial, the subject has the right to reconsider whether he/she will continue their participation.

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12. Insurances

Trial subjects are covered by the Swedish patient insurance and the Swedish pharmaceutical insurance.

13. Substantial changes to the trial

Substantial changes to the signed trial protocol are only possible through approved protocol amendments and by agreement from all responsible persons. Information on non-substantial changes should be clearly noted in the amended protocol.

In the event that substantial changes to the protocol (e.g., changing of the main objective, primary or secondary variables, method to measure the primary variable, changing of the investigational product or dosage) will be made during the course of the trial, approval from the MPA and EPM shall be obtained before any changes are implemented. A change that concerns a new site, new investigator or a new subject information sheet shall only be approved by the EPM, as applicable.

Non-substantial changes will be recorded and later entered in documentation that is submitted, for example in any subsequent notifications of a substantial change or in connection with End of Trial reporting.

14. Collection, handling and archiving data

Subjects who participate in the trial are coded with a specific trial identification number (Study-ID). All subjects are registered in a subject identification list (subject enrolment and identification list) that connects the subject's name and personal identity number with the Study-ID. When randomized a separate randomization number will be added.

All data will be registered, managed, and stored in a manner that enables correct reporting, interpretation, and verification. The complete Trial Master File, as well as source documents, will be archived for at least 10 years after the trial is completed. Source data in the medical records system is stored and archived in accordance with hospital regulations.

14.1 Case Report Form

An electronic Case Report Form (eCRF) is used for data collection. The investigator must ensure that data is registered and any corrections in the eCRF are made as stated in the trial protocol and in accordance with the instructions. The investigator must ensure that the registered data is correct, complete, and that reporting takes place according to the timelines that have been predefined and agreed. The investigator signs the completed eCRF. A copy of the completed eCRF will be archived at the trial site.

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If an examination/test is not performed and data does not exist, ND (Not done) or NK (Not known) is marked. If the question is irrelevant NA (Not applicable) is written. Corrections in paper work sheets are done by striking out the incorrect information and adding the correct information next to the incorrect information, signing, and dating the correction.

15. Notification of trial completion, reporting, and publication

The MPA and EPM shall be informed of the trial's completion at latest 90 days after trial end, through submission of a "Declaration of End of Trial Notification" form.

Within one year after the trial is completed, the results shall be analyzed, a clinical trial report with individual data shall be prepared, and the trial results shall also be reported in the EudraCT database. The sponsor is responsible for the preparation of the clinical trial report. The statistical analyses will be performed and the results will be presented to the Investigator(s). Based on these data, the Principal investigator, in cooperation with the Co-Investigator(s), will prepare a clinical trial report. The report will be submitted to the competent authorities and will form the basis for a manuscript intended for publication in a medical/scientific journal. All personnel who have contributed significantly with the planning and performance of the trial may be included in the list of authors.

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Date:	2022-01-03
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Trial Code: HOT-LOCO
Version No: v.4
Date: 2022-01-03
EudraCT No: 2021-000764-30

17. Amendments and Administrative changes

The following amendments and Administrative changes have been made to this protocol since day of preparation:

Amendment	Section/Page	Date	Type/comment
Version 1		2021-05-05	EPM submission
Version 2 addition of sponsor representative signature addition of DSMB members addition of COVID-19 pandemic statement minor layout and typos	Signature page/5 Contact information/6 3.5/16 Full protocol	2021-06-30	MPA submission/non substantial change
Version 3 specification of activity meter used minor layout and typos	 5.2/21-26 Full protocol	2021-08-16	EPM amendment/non substantial change
Version 4 minor layout and typos Change of treatment interval	 Full protocol 7.9	2022-01-03	Non substantial change Incoherent with section 5

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Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines with the PRO-extension

Calvert M, Kyte D, Mercieca-Bebber R, et al. Guidelines for Inclusion of Patient-Reported Outcomes in Clinical Trial Protocols: The SPIRIT-PRO Extension. *JAMA : the journal of the American Medical Association* 2018;319(5):483-94. doi: 10.1001/jama.2017.21903 [published Online First: 2018/02/08]

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	N/A, full protocol
Protocol version	#3	Date and version identifier	N/A, full protocol
Funding	#4	Sources and types of financial, material, and other support	19
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1, 19
Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	NA, full protocol
Roles and responsibilities: sponsor and funder	#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	19

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		decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	
Roles and responsibilities: committees	#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)	N/A, full protocol
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	3-4
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators (PRO extension)	9-11, 17-18
Objectives	#7	Specific objectives or hypotheses	4
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, non-inferiority, exploratory)	5
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	5
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	6

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		individuals who will perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6-7
Interventions: modifications	#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)	N/A full protocol
Interventions: adherence	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	N/A full protocol
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A full protocol
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	7-14
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)	Figure1
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	15
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5-6

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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	11-12
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	11-12
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	12
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7, 12
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A, full protocol
Methods: Data collection, management, and analysis			
Data collection plan	#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	7

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		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 (PRO extension)	
Data collection plan: retention	#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 (PRO extension)	9-10
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	N/A, full protocol
Statistics: outcomes	#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	14-17
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	15
Statistics: analysis population and missing data	#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)	15
Methods: Monitoring			
Data monitoring: formal committee	#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	N/A, full protocol

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		found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
Data monitoring: interim analysis	#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	N/A, full protocol
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 (PRO extension)	14
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A, full protocol
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	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)	N/A, full protocol
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	N/A, full protocol
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A, full protocol
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	N/A, full protocol

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Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	20
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	N/A, full protocol
Dissemination policy: trial results	#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	18
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	N/A, full protocol
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	20
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	N/A can be sent on request
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	N/A Separate Laboratory manual can be

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Notes:

- 2b: N/A, In full protocol p. 9-11
- 3: N/A, In full protocol p. 1
- 5b, 5d: N/A, In full protocol p. 6
- 11b: N/A, In full protocol p. 34-38
- 11c: N/A, In full protocol p. 42-44
- 11d: N/A, In full protocol p. 33
- 17b: N/A, In full protocol p. 33
- 19: N/A, In full protocol p. 42-45
- 21a, 21b: N/A, In full protocol p. 44
- 23: N/A, In full protocol p. 42-44
- 25: N/A, In full protocol p. 46
- 26a, 26b: N/A, In full protocol p. 45
- 27: N/A, In full protocol p. 46-47
- 30: N/A, In full protocol p. 46
- 31b: N/A, In full protocol p. 47
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