Study Protocol

Assessment of chemoreflex control of respiratory and cardiovascular systems in Post-COVID-19 syndrome

IRAS Project ID: 296727

Contents

1. STUDY SUMMARY

Around 4 million individuals have tested positive for COVID-19 in the United Kingdom (UK) up to February 2021.[1] Those who have recovered from the acute effects of COVID-19 often describe ongoing symptoms including decreased exercise tolerance, fatigue, chest pain, and dizziness; so-called 'long covid'.[2,3] Recently the Office for National Statistics showed that ~20% of people diagnosed with COVID-19 still have symptoms after 5 weeks, and that approximately 1 in 10 people have symptoms for 12 weeks or longer[4]. Post COVID-19 syndrome is emerging as a prevalent syndrome, encompassing a plethora of debilitating symptoms (including breathlessness, chest pain, palpitation, and orthostatic intolerance) which can last for weeks or more following mild to moderate illness.[2,3,5] Whilst this is partially due to direct lung injury, increasing evidence points to an ongoing, multi-system disorder involving the brain and the carotid body.[5,6,7] The carotid body, a small organ in the carotid arteries, monitors oxygen levels in the blood and keeps tight control over breathing, heart rate, and blood pressure. This organ has a high distribution of angiotensin converting enzyme 2, the enzyme by which coronavirus enters cells. The carotid body is also sensitive to inflammation, which is triggered by local infection.[8,9] It is possible this inflammation drives symptoms such as breathlessness, inappropriate increases in heart rate, and dizziness.[10] We aim to investigate the longer-term effects of COVID-19 on autonomic and peripheral chemoreflex control of respiratory and cardiovascular systems. This will be achieved by recruiting patients from two populations both of whom have had acute COVID-19: Firstly, a group with Post-COVID-19 Syndrome and secondly a matched control group with COVID-19 symptoms lasting no longer than 4 weeks as per NICE guidelines. We will assess carotid body chemosensitivity at rest and during submaximal exercise. We will also assess autonomic function at rest and during exercise. These data will provide an insight into whether the carotid body is involved in post-COVID-19 syndrome and whether it is linked to autonomic dysfunction. The carotid body could be targeted to help treat post-COVID-19 syndrome.

2. DETAILS OF SPONSOR

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4. BACKGROUND INFORMATION

4.1 The clinical problem of COVID-19

As of February 2021, over 100 million COVID-19 cases and 2 million deaths have been reported to the World Health Organisation (WHO). In the UK, over 4 million cases and 110,000 deaths have been identified since the first two cases of COVID-19 were confirmed in the UK on January the 31st, 2020.[1,11] The WHO, in collaboration with national authorities, institutions, and researchers, continues to monitor public health events associated with SARS-COV-2 variants. The European Centre for Disease Control and Prevention (ECDC) highlights that while several European countries have been reporting an overall decrease in the incidence of COVID-19, likely due to a strong combination of public health and social measures, most countries in Europe continue to experience high or increasing notification rates among older age groups and/or high death rates.[12] Moreover, among samples tested in Europe by PCR-based screening and whole genome sequencing, the proportion of cases infected with COVID-19 variant, VOC 202012/01, has increased, indicating community transmission in several countries.[12]

COVID-19 is characterised by clinical features of pneumonia, and in some individuals, this progresses to a severe multisystem disorder. A meta-analysis by Rodriguez-Morales et al (2020) analysed 656 hospital patients with COVID-19 and reported fever (88.7%, 95%CI 84.5-92.9), cough (57.6%, 95%CI 40.8-74.4), and dyspnoea (45.6%, 95%CI 10.9-80.4) as the most prevalent manifestations. Among these patients, 32.8% presented with Acute Respiratory Distress Syndrome (ARDS) (95%CI 13.7–51.8), 20.3% (95%CI 10.0–30.6%) required mechanical ventilation in an Intensive Care Unit (ICU), and 13.9% (95%CI 6.2–21.5%) ultimately died. However, most individuals who contract COVID-19 develop milder symptoms.[13]

Post COVID-19 syndrome is emerging as a prevalent syndrome, encompassing a plethora of debilitating symptoms (including breathlessness, chest pain, palpitation, and orthostatic intolerance) which can last for weeks or more following mild to moderate illness.[2,3,5]

4.2 Post-COVID-19 Syndrome

According to the National Institute for Clinical Excellence (NICE), the term Post-COVID-19 syndrome includes ongoing symptoms of COVID-19 for more than 12 weeks which are not explained by alternative diagnoses.[6] The scale of the problem is increasing in the UK, where the office for national

statistics recently showed that 22% of respondents to a COVID-19 survey were still experiencing symptoms >5 weeks post infection and 9.1% of respondents had ongoing symptoms >12 weeks post infection.[4] Furthermore, data from the ZOE study published in a pre-print indicates that 4.5% of COVID-19 patients had ongoing symptoms for >8 weeks 2.3% had ongoing symptoms for >12 weeks. Recent literature has suggested underlying impaired autonomic physiology post COVID-19 infection, which could be secondary to deconditioning, hypovolaemia, or immune- or virus-mediated neuropathy.[3,7] Development of chronic fatigue, cognitive slowing, and symptoms of autonomic impairment such as orthostatic intolerance, exaggerated postural tachycardia, and episodic hyperadrenergic surges have also been described in the literature.[14,15]

4.3 COVID-19, chemoreflex impairment, and the carotid body

Autonomic dysregulation and sympathetic hyperreactive responses to COVID-19 have been associated with increased morbidity and mortality.[16] SARS-CoV-2 infects host cells through angiotensin converting enzyme 2 (ACE2) receptors in respiratory endothelium and myocardium, leading to COVID pneumonitis, as well as causing acute myocardial injury, myocarditis, and chronic damage to the cardiovascular system.[6] COVID-19 both directly and indirectly has effects on the brain and the autonomic nervous system. Viral invasion of chemo-sensing neural cells in the brainstem impairs respiratory and cardiovascular regulation and disrupts the blood brain barrier.[9] Areas of the brain responsible for cardiovascular and respiratory homeostasis are infected, including the nucleus tractus solitarius (NTS; abundant in ACE2), which mediates the autonomic nervous system and taste; and the rostral ventrolateral medulla, which mediates the ventilatory response of the chemoreflex.[17,18,19]

ACE2 is also widely expressed in other organs such as the brain, kidneys, and carotid body (CB).[8,9,10] Peripheral chemoreceptor cells are located in the CB. These detect low blood oxygen levels and have important regulatory functions controlling the autonomic nervous system, ventilation, heart rate, and blood pressure at rest and in response to stressors like exercise.[20,21,22] The CB sends afferent chemo-reflex neural signals to the brainstem via the petrosal ganglion where they travel alongside nerves carrying signals responsible for taste and smell. The CB has the highest blood flow per gram of tissue mass versus any other organ and strongly expresses ACE2. Therefore local viral invasion of the carotid bodies is feasible; in fact Porzionato et al propose that local CB invasion with SARS-CoV-2 could also be a site for entry of the virus into the central nervous system (CNS).[10] They propose that axonal transport of SARS-CoV-2 in afferent chemoreceptor fibres may occur and cause translocation into the NTS.[10,23] The CB also express its own renin-angiotenisn system; thus disruption of this system could cause chemoreceptive dysfunction, provoking sympathetic hyperreactivity by increasing local ACE1/ACE2 imbalance and angiotensin 2 (AngII) stimulation.[9] Systemic ACE1/ACE2 imbalance has been shown to be proportional to COVID-19 viral load.[8,10] and circulating AngII may increase the sympathetic output both centrally, at the level of the circumventricular organs (area postrema and subfornical organ), and peripherally, by acting on the CB.[9,10]

In addition to modulating ventilation, heart rate, and blood pressure in response to oxygen levels the carotid body also mediates ventilatory responses to exercise and feelings of breathlessness[24]. In healthy people, when blood oxygen saturation (SpO2) drops, the chemo-reflex increases ventilation. In hypertensive individuals[25,26] and in heart failure[27,28] the chemoreflex becomes sensitised as a compensatory and protective mechanism. An over-active chemoreflex is associated with a worse prognosis.[29,30] Recently, a study showed that patients hospitalised with COVID-19 experienced

hyperventilation during exercise on discharge. The authors postulate that the carotid bodies may contribute to this hyperventilation because pulmonary blood flow was not a limiting factor[31].

In severe COVID-19, leading to hospitalisation or requiring non-invasive or invasive ventilation, the occurrence of a 'cytokine storm', a sudden acute increase in circulating level of pro-inflammatory cytokines, including interleukin (IL)-6, IL-10, and tumour necrosis factor-alpha (TNF-α)) has been reported.[32] AngII may also activate macrophages and other immune cells to produce inflammatory cytokines, such as IL-6 and TNF-α.[32,33] Circulating and local cytokines activate the carotid body, which could also cause chemoreceptor dysfunction and thus autonomic imbalance.

We propose that carotid body dysfunction occurs in Post-COVID-19 Syndrome, which contributes to autonomic dysfunction and dysregulation of ventilation (feelings of breathlessness) and cardiovascular control, especially during exercise.

5. AIMS AND HYPOTHESES

Aim 1: To determine whether sympathetic nerve activity is elevated at rest and in response to exercise in people diagnosed with Post-COVID-19 Syndrome.

Hypothesis 1: There will be a difference in the level of sympathetic nerve activity at rest and during exercise between people diagnosed with Post-COVID-19 Syndrome and a control group (people who have had acute COVID-19 but without symptoms lasting more than 4 weeks).

Aim 2: To determine whether the carotid body is hyperactive to hypoxia at rest and during exercise in people diagnosed with Post-COVID-19 Syndrome.

Hypothesis 2: There will be a difference in carotid body chemoreflex sensitivity to hypoxia between a group of people diagnosed with Post-COVID-19 Syndrome versus a control group (people who have had acute COVID-19 but without symptoms lasting longer than 4 weeks).

Aim 3: To examine the inflammatory response to exercise in people diagnosed with Post-COVID-19 Syndrome versus controls.

Hypothesis 3: There will be a difference in the levels of inflammatory markers during exercise in people with Post-COVID-19 Syndrome versus a control group (people who have had acute COVID-19 but without symptoms lasting more than 4 weeks).

6. EXPECTED VALUE OF RESULTS

The results of this study will be of immediate benefit to the NHS by identifying a novel mechanism that contributes to ongoing symptoms when recovering from COVID-19. Understanding whether the CB might be driving some of the symptoms in Post-COVID-19 Syndrome will help clarify its pathophysiology and guide future therapies for COVID-19. CB receptors are targetable for various drug treatments. For example, P2X3 receptors have been shown to be involved in hyper-reactivity in hypertensive animals[34,35]. There is already a Medicines and Healthcare products Regulatory Agency (MHRA) approved drug that can target them[36]. Further work could include testing the efficacy of exercise and breathing control techniques in these populations.

7. STUDY SITE

There will be 2 study visits for all participants, which will be carried out at the Clinical Research Facility (CRF), part of University Hospitals Bristol and Weston NHS Foundation Trust. The CRF is situated in St. Michael's Hospital. Dr's Hart and Nightingale have a dedicated experimental physiology lab in this facility.

8. STUDY DESIGN

This study will be a case-control study.

- Group 1 will include participants who have had acute COVID-19 and have now been formally diagnosed with Post-COVID-19 Syndrome i.e. Symptoms lasting >12 weeks, not explained by an alternative diagnosis as per NICE guidelines. These participants will have had prior positive PCR swab or a positive anti-body test BEFORE vaccination.

- Group 2 are age and sex-matched controls with history of acute COVID-19 (confirmed via PCR or antibody test before vaccination), who were asymptomatic or had symptoms ongoing for <4 weeks. I.e. No evidence of long COVID-19 or Post-COVID-19 Syndrome.

9. PARTICIPANTS AND RECRUITMENT

We will recruit 54 people to 2 groups: firstly, a group with Post-COVID-19 Syndrome and secondly a matched control group with COVID-19 symptoms lasting no longer than 4 weeks as per NICE guidelines. See sample size calculation on page 21.

9.1 Inclusion criteria

All participants

- Aged 18-80 years

Post-COVID-19 Syndrome Participants

- As per NICE guidelines https://www.nice.org.uk/guidance/ng188

Age & sex-matched controls

- Positive SARS-CoV-2 antibody test before vaccination, or a positive COVID-19 PCR antigen swab test
- Asymptomatic or symptoms <4 weeks after COVID-19 infection

9.2 Exclusion criteria

All participants

- Body mass index ≥35 kg/m^2
- Pregnancy/breastfeeding women
- Ongoing requirement of oxygen therapy
- Taking antihypertensive, nitrate, steroid or immunosuppressant medication or medication
- Major illness e.g., cancer, inflammatory disease (including vasculitis) or receiving palliative care
- History of organ transplantation or are candidates for organ transplantation at the time of screening
- History of Chronic Fatigue Syndrome prior to COVID-19 infection
- Diagnosed cardiovascular disease (including current non-benign arrhythmia, chronic heart failure)
- History of major psychiatric disorder including bipolar disorders, schizophrenia, schizoaffective disorder, major depression.
- Diagnosis of structural lung disease (such as COPD or pulmonary fibrosis)
- Diagnosed renal disease
- Congenital or acquired neurological conditions (including dementia), language disorders, repeated or chronic pain conditions (excluding menstrual pain and minor sporadic headaches)
- Diabetes Mellitus
- Symptoms of febrile illness 2 weeks before experiment
- Excessive alcohol consumption (>28 units/week) or use of illicit drugs
- History of smoking within 2 months
- Inability to understand instructions given in English
- Surgery under general anaesthesia within 3 months
- History of stroke
- Heart transplant

- Coronary revascularisation
- Haemodialysis or peritoneal dialysis
- Participating in another study for an investigational medicinal product

Controls

- Symptoms lasting >4 weeks following acute, confirmed, COVID-19 infection

9.3 Recruitment:

***Patients diagnosed with* Post-COVID-19 Syndrome** will be recruited via long COVID-19 clinics at the University Hospitals Bristol and Weston NHS Trust.

Participants for the control group will be recruited via posters and leaflets displayed around the University of Bristol and public spaces in the wider area (e.g. local shops, cafes, community centres and GP reception areas); study adverts placed in community/University newsletters; study adverts emailed through University Faculty email; information on our research groups website, http://www.uhbristol.nhs.uk/hypertension; and finally social media posts.

Permission will be gained before posters/leaflets are left in public spaces and before adverts are emailed through University email.

Additionally, individuals that have given permission for the research group to store their contact details and to be contacted about taking part in studies (for example after review in the long COVID clinic) may be invited to take part in the study.

NB: We will encourage involvement from members of the Black, Asian, and Minority Ethnic (BAME) community.

9.4 Selection and screening

1. **Invitation:** Group 1 will be given a letter of invitation with a participant information sheet via the Long COVID clinics. Group 2 will be asked to directly contact a member of the research team if interested in participating in this study. An invitation letter and participant information sheet will then be sent to the potential volunteer. A minimum of a week will be left before contacting volunteers, to allow time for the information to be read and understood.

2. **Screening telephone call:** A telephone call from the research staff to ask screening questions to ensure that the volunteer meets the inclusion/exclusion criteria, answer questions from the potential participant and to schedule study visits will be completed.

Depending on the availability of the participant, the study visits will take place over a 3-month period. Overall duration of the study for participants will be no more than 3 months.

10. EXPERIMENTAL DESIGN AND PROCEDURES

Data for all aims will be collected in two study visits. Depending on the availability of the participant, the study visits will take place over a 3-month period. Overall duration of the study for participants will be no more than 3 months. Table 1 summarises the participants and procedures involved throughout the study.

We will have 3 separate workstreams to address our aims above:

Workstream 1/aim 1: Is sympathetic nerve activity at rest and during exercise elevated in people with Post-COVID-19 Syndrome versus a control group?

To address aim 1, we will measure sympathetic nerve activity via microneurography, which is a direct measure of the sympathetic nerve activity at 1) rest and 2) during isometric handgrip exercise.

Workstream 2/aim 2: Is the peripheral chemoreflex hyperreactive at rest and during exercise in people with Post-COVID-19 Syndrome versus a control group?

To address aim 2, we will measure the hypoxic ventilatory response at rest and during moderate dynamic exercise.

Workstream 3/aim 3: Is the inflammatory response to exercise higher in people with Post-COVID-19 Syndrome versus a control group?

We will measure inflammatory responses at rest and following peak exercise.

Table 1. Participant study pathway showing the order of activities

Order of activities	Time taken (mins)
Pre-visit:	
1. Invitation to study- letter given / sent to potential participants	n/a
2. Screening Telephone call - Relevant medical history taken	~30
Visit 1:	
3. Consent obtained	~15
4. Screening: 12 lead ECG	~15
5. Screening: Urinary pregnancy test in pre-menopausal women	~5
6. Screening: Height, weight, office BP	~10
7. Handgrip familiarisation & calculation of MVC	~10
8. MSNA & handgrip	~90
9. Break	~15-30
10. Cardiopulmonary exercise test to calculate VO_2Peak	~20
Visit 2:	
11. Hypoxic Ventilatory Response at rest	~30
12. Break	~60
13. Hypoxic Ventilatory Response during exercise, including venous blood sampling	~30

MSNA, muscle sympathetic nerve activity; MVC, maximal voluntary contraction; ABPM, ambulatory blood pressure monitor; ECG, electrocardiography; VO_2Peak, maximal oxygen uptake

10.1 Screening

Participants will be screened to ensure they are able to participate in the study and to correctly group participants as with and without Post-COVID-19 Syndrome.

Screening procedures:

- Full, detailed medical history will be assessed by a screening questionnaire.
- Height and weight measurements.
- Office blood pressure will be measured twice on each arm with an automated blood pressure cuff, followed by two further readings from the arm with higher blood pressure, two minutes apart. An average of the final two readings will be used.
- Ambulatory blood pressure monitoring (ABPM) will be used to measure blood pressure over 24 hours. Participants will be issued with an ambulatory blood pressure monitor after the screening visit. Participants will be asked to keep a blood pressure diary to record waking/sleeping times, which will be used to calculate daytime ambulatory blood pressure. Activities completed during blood pressure readings will also be noted.
- 12 lead electrocardiography (ECG) will be performed to exclude obvious cardiovascular disease (assessed by a Doctor).
- A urine pregnancy test will be performed for premenopausal women.

10.2 Handgrip exercise and recovery

During screening, participants will squeeze a handgrip dynamometer three times with maximal effort with the dominant hand. The highest value will be taken as maximal voluntary contraction (MVC). During microneurography recordings, participants will perform handgrip exercise at 40% of their MVC for two minutes.

10.3 Microneurography

Sympathetic nerve activity (nerve signals that control the diameter of the peripheral vessels) can be measured in humans using a technique called microneurography. Microneurography measures muscle sympathetic nerve activity (MSNA) in the peroneal nerve in the lower leg. This is the one of most superficial nerves in the human body making it easy to locate.

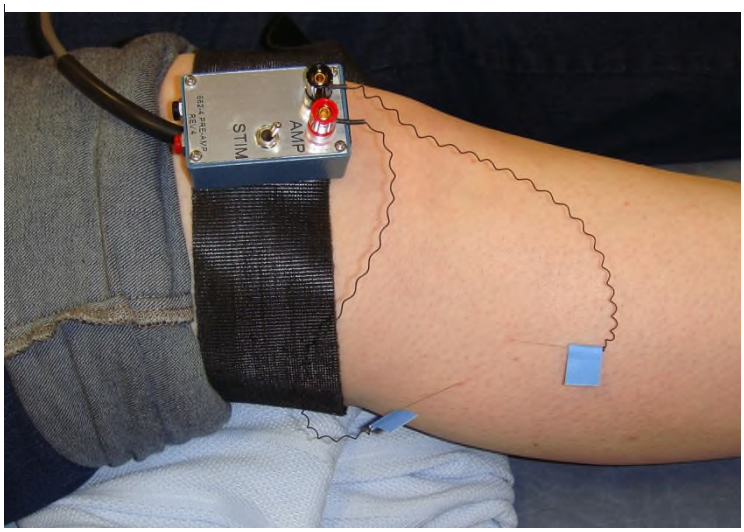
This technique has been used worldwide since its development in the 1960's by Hagbarth, Vallbo, Sundlof and Wallin, and is now well validated.[37] This procedure has been completed in over 10,000 studies worldwide, in normal healthy participants to patients with neuronal disorders and cardio-respiratory diseases. We have completed various studies and have several ongoing studies that have used this technique and it has been well tolerated by all the participants who have been happy to return for a second visit.[38,39,40,41]

10.3.1 Details of the procedure

The microneurography procedure is illustrated in Figure 1. To perform this technique, two micro-electrodes are used. These are very much like acupuncture needles but have a fine tip which is less than the width of a human hair (< 1 micron). One electrode is the reference electrode and is inserted into the surface of the skin. This is not moved until the end of the nerve recording. The other electrode is inserted into the peroneal nerve to record nerve activity.

Sympathetic nerve activity will be recorded for a maximum of 90 minutes, similar to other studies measuring sympathetic nerve activity.

Figure 1. Microneurography procedure. Two tungsten microelectrodes inserted into the skin and the peroneal nerve.



10.3.2 Measurements during microneurography

Equipment used: tungsten microelectrode using standard validated criteria for locating a site in the peroneal nerve yielding discrete pulse-synchronised MSNA bursts[42]. Data presented as bursts per minute from mean voltage neurograms. Results will be analysed by second independent practitioner blinded as to status of the participant.

Setting: quiet room at 19-25^{o}C. Lying supine (light breakfast allowed, but caffeine/tobacco free for at least 4 hours)

• Step 1: Identification of peroneal nerve using anatomical landmarks over the head of the fibula and non-invasive stimulating electrode. This is not painful and is well tolerated by patients.

• Step 2: Siting of the reference and recording electrodes with identification of spontaneous sympathetic nerve firing as described and shown in the diagram above.

Comparing the mechanistic role of carotid bodies in human heart failure with and without preserved ejection fraction.

• Step 3: 10-minute baseline recording with the patient lying still and quiet at rest. During this period continuous measurements will be made of:

- Beat to beat BP using a Finapres device
- 3 lead ECG recording
- Non-invasive oxygen saturations
- Respiratory rate using a respiratory belt
- Sympathetic nerve activity/MSNA
- HR variability can be calculated using the data above

10.4 Incremental exercise test: Ramped bike exercise tolerance testing

A graded exercise test performed on an upright cycle ergometer using a participant-tailored protocol. ECG, blood pressure, heart rate, tidal volume, respiratory rate, and partial pressures of inspired and expired gases will be monitored throughout (ranges from 6-12 minutes of exercise). Rating of perceived breathlessness and exertion using a Borg scale[43] will be assessed intermittently. This test will be used to assess the chemo-reflex sensitivity at submaximal intensity (30%-40% of the VO_2 peak).

10.5 Venous blood sampling

At rest and at the end of peak exercise venous blood will be taken from an antecubital vein to measure markers of inflammation and venous blood catecholamines ($\leq$20 mL of venous blood).

Venous blood will be collected by a Research Nurse or Doctor. Samples will be labelled with Study name and ID number and participant age and sex. Labelling with age and sex facilitates quicker identification of results outside the normal range for the age/sex of the participant. Venous blood samples will immediately be transported by a member of the study team to the Unit for the Support of Trials and Research in the Department of Clinical Biochemistry at the Bristol Royal Infirmary. The sample for catecholamines will be transported on ice and plasma will be extracted from this sample by staff in Clinical Biochemistry. Plasma samples will be labelled with Study ID and stored at -70 degrees Celsius. When ready to conduct analysis of catecholamine hormone levels, a member of the study team will transport the plasma samples to the University of Bristol, Biomedical Sciences Building, to complete the analysis. All samples will be discarded after testing.

10.6 Chemoreflex assessment at rest and during moderate exercise.

NB: we will leave at least one week between visit one and visit two to allow for recovery.

10.6.1 Carotid body chemoreflex assessment: the hypoxic ventilatory response

Chemoreflex assessment will be completed at rest (semi-supine position) and during submaximal exercise.

The techniques and equipment for assessing carotid body sensitivity[44,45] are established in our research group.[46,36] Participants will be monitored throughout with a 3-lead ECG, ear O_2 saturation monitor for SpO2%, finger BP cuff, and a spirometer using a two-way non-rebreathe valve connected to a facemask. Participants will initially breathe room air. To measure carotid peripheral chemoreflex sensitivity the transient hypoxia method will be used.[44] Briefly, participants are silently switched to breathing pure pharmaceutical grade nitrogen gas for 10–45 s. This procedure is repeated 5–8 times per study participant with a 2 min gap in between each burst, achieving transient falls in SpO2% to 99–65% and brief compensatory increases in ventilation. The primary outcome variable, the chemo-reflex sensitivity, is calculated by plotting the minute ventilation against the SpO_2% for each burst of nitrogen and also including a baseline value taken as the mean of the minute before hypoxia; this linear regression is termed the hypoxic ventilatory response (HVR) and expressed as L/min/SpO2%. This measurement of HVR is a prognostic indicator in patients with chronic heart failure.[47] The protocol is shown below in Figure 2 with example recordings form a participant with heart failure in Figure 3 and example chemoreflex plots from participants with heart failure and a healthy control in Figure 4.

This is a safe measurement of chemoreflex sensitivity as bursts of hypoxia last for no longer than 45 seconds. The technique was established originally for patients with chronic heart failure.[45,47]

10.6.2 Moderate exercise

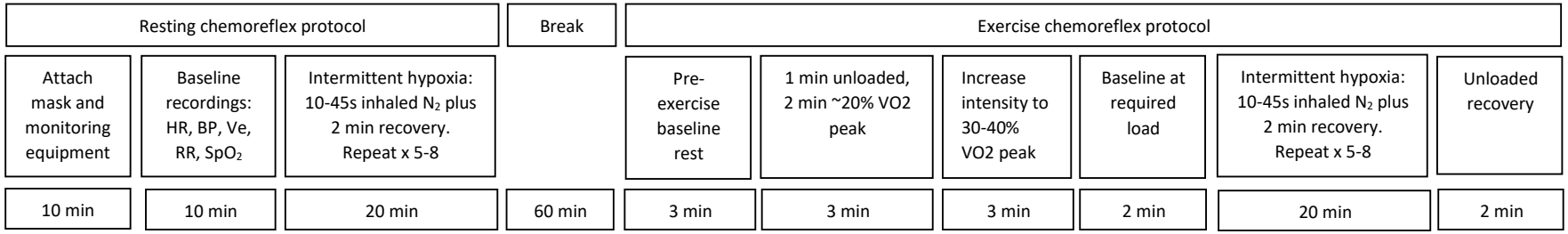
Participants will continuously cycle at an exercise intensity of 30-40% of their VO_2 peak. Figure 2 shows the exercise plus chemoreflex testing protocol. During cycle ergometry, heart rate (12 lead ECG), ventilation (spirometer), respiratory gases, and blood pressure (intermittent, automated brachial arm cuff) will be monitored.

Participants will rest on the bike for 3 mins without cycling and resting variables collected. Participants will begin unloaded cycling for 1 min and then increase exercise intensity to ~20% of their VO2 peak. Participants will then start cycling at 30-40% of VO2 peak for 2 mins. This will be the exercise baseline for the chemoreflex testing. Hypoxic ventilatory testing will begin using the intermittent hypoxia protocol described in section 10.6.1, followed by unloaded recovery and resting recovery.

10.6.3 Capillary blood lactate

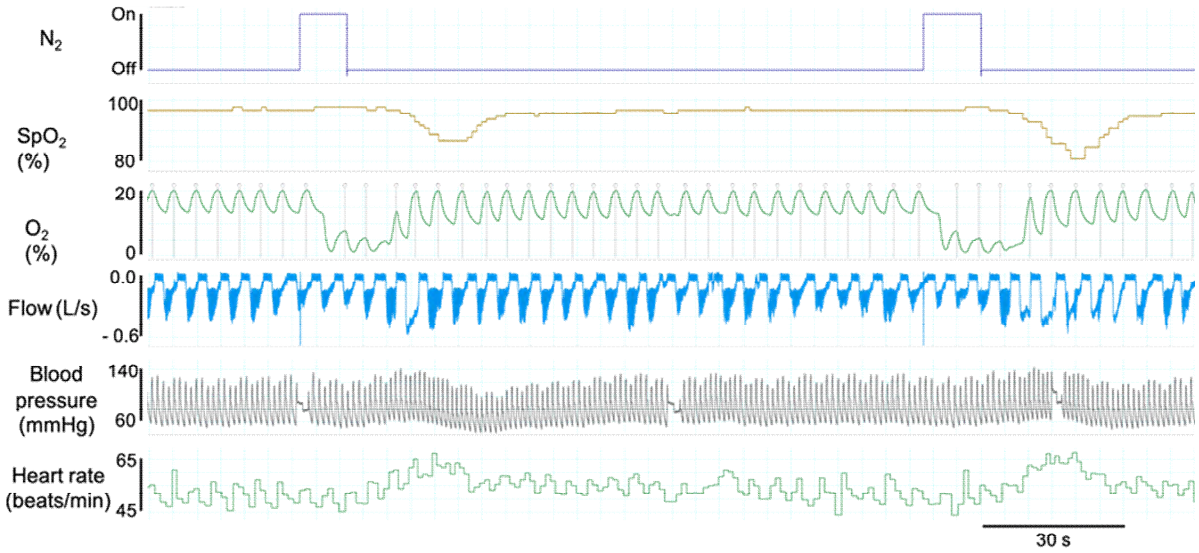
Will be sampled from the ear or finger at rest, start of exercise at 30-40% of VO2 peak, and at the end of intermittent hypoxia using the Lactate Pro 2 blood lactate analyser.

Figure 2. Protocol flow chart showing the chemoreflex intermittent hypoxia protocol that will be used to test the hypoxic ventilatory response



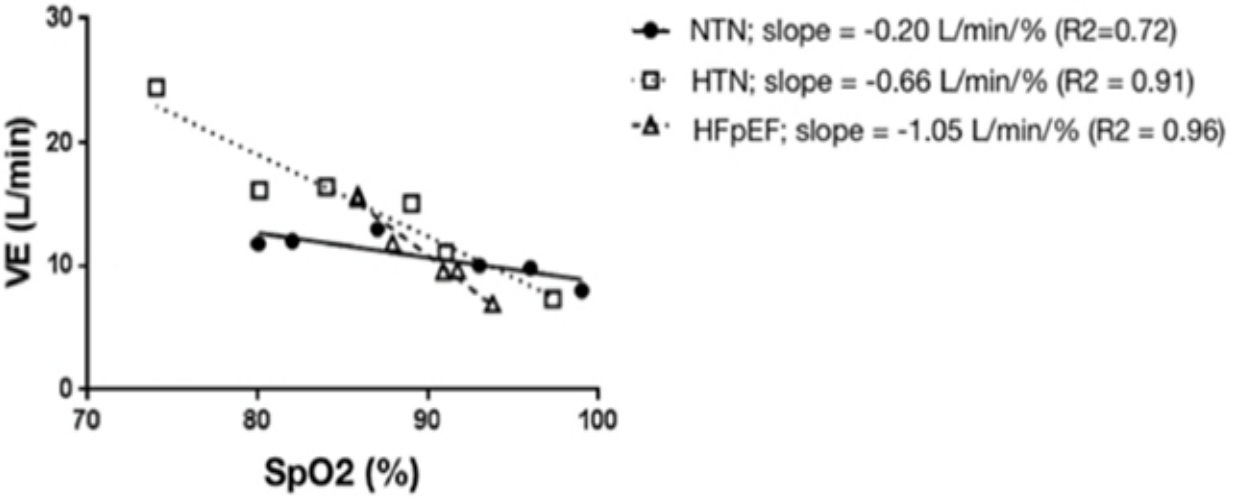
BP, blood pressure; HR, heart rate; RR, respiratory rate; SpO_2, oxygen saturations; N_2, Nitrogen gas; Ve, spirometry; VO_2Peak, maximal oxygen uptake

Figure 3. Chemo-reflex intermittent hypoxia protocol measurements in a patient with heart failure



mmHg, millimetres mercury; L/s, litres per second; SpO_2, oxygen saturations; N_2, Nitrogen gas; O_2, Oxygen gas

Figure 4. Chemo-reflex plots from participants with heart failure and a healthy control.



HFpEF, heart failure with preserved ejection fraction; HTN, hypertension; NTN normotension.

11. OUTCOMES

11.1 Aim 1

11.1.1 Primary outcome measures

- Level of MSNA at rest
- Level of MSNA during exercise

11.1.2 Secondary outcome measures

- Spontaneous sympathetic baroreflex sensitivity (data taken from resting MSNA recording)
- Spontaneous parasympathetic baroreflex sensitivity (data taken from resting ECG recording)
- 24-hour ambulatory blood pressure and office blood pressure
- Beat-to-beat blood pressure variability at rest (data taken from resting beat-to-beat finger blood pressure recording)
- Change in BP in response to handgrip exercise
- Sympathetic-respiratory coupling at rest (data taken from MSNA and respiratory belt recordings at rest)
- VO_2 peak
- VE/VCO_2 slope
- Rating of perceived exertion (Borg scale)
- Change in heart rate from peak exercise to recovery (within first 2 mins of stopping exercise).

11.2 Aim 2

11.2.1 Primary outcome measures

- Hypoxic ventilatory response at rest
- Hypoxic ventilatory response during exercise

11.2.2 Secondary outcome measures

- Blood pressure response to hypoxia at rest
- Blood pressure response to hypoxia during exercise
- Heart rate response to hypoxia at rest
- Heart rate response to hypoxia during exercise
- Capillary blood lactate at rest
- Capillary blood lactate at end of moderate exercise

11.3 Aim 3

11.3.1 Primary outcome measures

- Change in the inflammatory biomarker IL-6 from rest to peak exercise

11.3.2 Secondary outcome measures

- Change in other inflammatory biomarkers from rest to peak exercise
- Change in catecholamines from rest to peak exercise

12. STATISTICS AND POWER CALCULATIONS

Data will be checked for normal distribution. To test for group differences in endpoints at rest an analysis of covariance (ANCOVA) will be completed (unless non-parametric tests are required), with adjustments for body mass and other potential confounders (e.g. age). Changes in endpoints over time and in response to stressors will be compared using a two-mixed model ANCOVA (time-group analysis). Alpha will be set at 0.05. We will also complete a false positive analysis which provides information on the likelihood a result with a 'significant' p-value is a false positive. Feasibility outcomes including the recruitment rate, retention rate, data completeness will also be reported.

12.1 Sample size and power calculation

Based on power calculation for aim 1; specifically, is there a greater increase in MSNA from rest to exercise in people with long COVID, a total of 54 people will provide a moderate effect size (f=0.39) to detect a difference in MSNA between the 2 groups. We have then calculated the maximum achieved power for 54 participants (Table 1) to assess outcomes (response variables) for aims 2 and 3. From previous experience we expect a 20% attrition rate, so we will aim to recruit 65 people with an aim to get 54 complete data sets.

Table 2: Sample size and power calculations for aims 1 to 3

Aim	Response variable	Expected statistical test	Minimum estimated effect sizes	Min. N for >0.80 power	* power achieved
1	MSNA (rest to exercise)	MANCOVA	Cohen's f for patient groups = 0.39	54 (α=0.05)	0.80 (α=0.05)
2	HVR (rest to exercise)	MANCOVA	Cohen's f for patient groups=0.39	54 (α=0.05)	0.80 (α=0.05)
3	IL-6 (rest to exercise)	MANCOVA	Cohen's f for patient groups=0.39	54(α=0.05)	0.80 (α=0.05)

HVR, hypoxic ventilatory response; IL-6, interleukin-6; MANCOVA, multivariate analysis of covariance; MSNA, muscle sympathetic nerve activity

13. Ethical Considerations

13.1 Safety

13.1.1 Screening procedures

The risk associated with conducting screening procedures is identification of incidental findings. Should abnormalities in ECG and blood pressure monitoring and / or pregnancy test identified, the participant will be informed, as they may be unable to participate in the rest of the study. With the participant's permission, their GP will be informed of the findings in writing and the participant will be encouraged to see their GP about the results.

13.1.2 Potential exposure to COVID-19

There is a risk to participants, of becoming infected with COVID-19 from the study visits. We will mitigate this by following national and local guidelines on the use of personal protective equipment (PPE). Participants will always wear a face mask unless they are connected to the face mask system for the chemo-reflex test. All equipment will be cleaned after each participant according to the manufacturer's instructions and local policies. Our laboratory has had an airflow analysis, which is required for aerosol generating procedures, such as exercise tests. We have specific guidelines for our lab, including time between separate visits. We can do a maximum of 2 exercise tests per day, separated by 3 hours, where the lab cannot be entered between visits.

13.1.3 Chemo-reflex assessment

The risk to participants from participating in the chemoreflex test is low. Side effects of breathing nitrogen include transient dizziness or light-headedness. The nitrogen can be immediately switched off and clears from the breathing circuit in seconds. SpO2 returns to normal quickly (as shown in Figure 3). We will mitigate the risks to the participants by carrying out the procedures in line with our established protocols. We have performed over 100 chemoreflex studies over the last 10 years with no adverse events from the test. We will adjust the number and duration of nitrogen given to induce hypoxia to keep SpO2 above 65%, starting with short exposures to assess the patient's response. We are experienced in varying the degree of nitrogen exposure in participants who are breathless with reduced cardio-respiratory reserve from our studies in people with chronic heart failure.

13.1.4 Microneurography

In 1989, Eckberg et al. published a prospective study of symptoms occurring after microneurography[38]. The study followed 1000 patient recordings and found that minor aftereffects such as deep transient aches in specific muscles (onset is usually 2-3 days after the experiment and resolve within 3-7 days after the study), were reported in less than 10% of the studies[38]. Microneurography has been performed in healthy individuals to individuals with neuropathies without major complications. Eckberg et al. reported that only one major adverse event occurred in the 10's of 1000 's of individuals participating in microneurography experiments, which was a case of small fibre neuropathy[38]. Based on their data, Eckberg et al. recommended that the time manipulating the microelectrode to look for nerve activity should be limited to 1 hour[38]. Dr. Emma Hart is an expert in microneurography and has set-up the technique in her Physiology Lab at the (former) CRIC-Bristol. Dr. Hart has trained several researchers involved in the study to complete microneurography. Dr. Hart and/or one of the trained research staff will perform the microneurography recordings. Dr. Hart and her group have now performed this procedure in over 350 participants ranging from participants who are healthy to participants with obesity, diabetes mellitus, heart failure and/or hypertension[44,45,46,47]. At the University of Bristol we have not to date experienced any adverse events associated with microneurography.

13.1.5 Handgrip exercise

There are no risks associated with handgrip exercise.

13.1.6 Venepuncture

Three venous blood samples will be obtained from all participants to measure levels of inflammatory and fibrotic markers, metabolites, and some hormones.

Risks to the participant of venepuncture include phlebitis, failure to access the vein, thrombophlebitis, bruising and localised pain at the insertion site, clot formation and introduction of flora to site precipitating infection. To minimise these risks, venepuncture will be performed by a Research Nurse or Doctor, according to Trust guidelines. The risk of venepuncture to the qualified

healthcare professional performing venepuncture is needle-stick injury. This risk will be minimised by following trust guidelines on venepuncture, using the appropriate sharps disposal units in accordance with guidance and by following Trust guidance in the incidence of needle-stick injury.

13.1.7 Physiological and blood pressure monitoring

There are no risks associated with heart rate monitoring with 3-lead ECG. Blood pressure monitoring can cause mild discomfort on the finger and arm. To minimise this, unnecessary blood pressure readings will be avoided, and cuffs will be adjusted should they cause more than mild discomfort. For the 24-hour ambulatory blood pressure monitoring, participants are advised to remove the cuff if they experience excessive discomfort or bruising from the cuff inflation; however, this is rare.

13.1.8 Ramped exercise tolerance testing

Exercise-induced changes in blood pressure may be experienced by some participants. Either an increase or decrease, during and / or after exercise may occur. A research nurse or doctor will be always present, with continuous ECG and blood pressure monitoring. Any adverse changes will be acted upon. The protocol will be stopped immediately should any participant experience discomfort or distress.

13.1.9 Pregnancy testing

Premenopausal females will be asked to confirm that they are not pregnant and will be offered a pregnancy test prior to participating in the study to confirm they are not pregnant. This test is mandatory. The urine pregnancy test will be completed on both days (before commencing any procedures) by a researcher or research nurse.

13.2 Research Materials

The data collected in this study will be for research purposes only. Data will be stored in linked-anonymised format and collected and kept in accordance with ICH-GCP guidelines in a secure, locked location. Participants will be able to view their results and receive an explanation from the investigator upon request.

13.3 Specimen Handling and Laboratory Procedures

Venous blood will be collected by a Research Nurse or Doctor. Samples will be labelled with Study name and ID number and participant age and sex. Labelling with age and sex facilitates quicker identification of results outside the normal range for the age/sex of the participant. Venous blood samples for metabolites and inflammatory and fibrotic markers will be stored on ice in the study room until the study visit is completed, at which point the samples will be processed at Bristol Royal Infirmary Research Level 7 or serum will be extracted and stored for later analysis at -70°C. Blood to be tested for cholesterol, full blood count and CRP will be immediately placed in the appropriate

sample tubes, labelled, and stored on ice within a box. These will be stored within the study room until the study visit is completed at which point, the samples will be delivered by a researcher or research nurse to the UH Bristol and Weston Clinical Biochemistry department where they will be processed immediately. All blood samples will be discarded after testing and urine samples discarded after pregnancy testing is completed.

13.4 Informed Consent

Individuals expressing interest in participating in the study will be given a copy of the participant information sheet to read, which details the procedures involved and the associated risks. Those interested in taking part will receive a pre-screening telephone call from Hazel Blythe or a Research Nurse, to rule out obvious exclusion criteria. This is also an opportunity for potential participants to ask questions about the study. Those still interested will then be booked into a visit at the CRF. At this visit, the procedures and risks will be explained in full to participants, who will then have the opportunity to ask questions. Those wanting to take part will be asked to sign a consent form, a copy of which will be given to the participant.

13.5 Right to Withdraw

Participants will be able to withdraw from the study at any point without providing an explanation. This will be explained to participants in the participant information sheet, on the consent form and verbally prior to the start of the experiment. Withdrawal from the study will not affect the care or legal rights of the participant, or their relationship with the University of Bristol.

13.6 Confidentiality

Participant confidentiality will be maintained using Study ID codes on samples and records.

13.7 Sex/Minority Mix

Males and females of all ethnic groups will be eligible to participate in the study. As recent literature has suggested worse clinical outcomes in BAME individuals with COVID-19[48], we will aim to recruit significant numbers from the BAME community. Bristol has a large BAME community, and this is represented in our healthcare workforce. We will engage with local representatives of the BAME community through the staff BAME committee in the hospital to identify any concerns and find ways to maximise participation.

14. REPORTING INCIDENTAL FINDINGS

It is possible that incidental findings concerning participants' health could be identified during the study, for example abnormality in the ECG, blood pressure or pregnancy test. Should incidental findings be identified, both the participant and their GP will be informed. Permission will be gained

before a participant's GP is informed of incidental findings. If the participant does not want their GP informed, only the participant will be informed of any findings; however, a letter will be written to the participant to inform them of the findings.

15. DATA HANDLING, CONFIDENTIALITY AND DISPOSAL

Personalised study data will be maintained at the University of Bristol in paper and/or electronic format. Both paper and electronic records will be kept in a locked cupboard in a locked room in a department with security-limited access. Access to the records is restricted to researchers working on the study. Password protection will be used for electronic data and, for the purposes of data analysis, anonymised data will be held on an encrypted flash drive, to be locked as above when not in use. No identifiable data will be stored on laptop computers or portable electronic devices. Analysis will take place by the study team led by Dr. Emma Hart and collaborators (using anonymised data). Data will be collected and retained in accordance with the Data Protection Act 2018. Study documents (paper and electronic) will be retained in a secure location during and after the trial has finished. All source documents will be retained for a period of fifteen years following the end of the study. Where trial related information is documented in the medical records – those records will be identified by a 'Do not destroy before dd/mm/yyyy' label where date is fifteen years after the last patient last visit.

The Chief Investigator, Dr. Angus Nightingale, will have control of and act as custodian of the data on behalf of the University of Bristol and University Hospitals Bristol and Weston NHS Foundation Trust.

Personal data will be stored for 15 years at the University of Bristol in electronic and hard copy. Access will be controlled by Dr. Angus Nightingale who will continue to act as custodian.

16. ADVERSE EVENTS AND REPORTING PROCEDURES

Any adverse incidents or events that occur during the study will be recorded in the site file. Any serious adverse events will be recorded and reported to the University Hospitals Bristol and Weston NHS Foundation Trust R&I team who undertake safety reporting on behalf of the Sponsor.

16.1 Definitions

16.1.1 Adverse incident

An adverse incident is an event or circumstance that could or did lead to unintended or unexpected harm, loss, or damage (this can apply to patients, staff and visitors). Adverse incidents will be reported to the NHS site (UH Bristol) according to the UH Bristol Organisation-wide Policy for the Management of Incidents (including Serious Incidents).

16.1.2 Adverse Events (AE)

AEs are defined as any untoward medical occurrence in a study participant. An AE does not necessarily have to have a causal relationship with the study treatment/intervention.

An AE can therefore be any unfavourable and unintended sign (e.g. abnormal lab result), symptom or disease temporally associated with the use of the medicinal product/medical device/intervention, whether or not this is related to the medicinal product/ medical device/intervention. This includes any occurrence that is new in onset or aggravated in severity or frequency from the participant's baseline condition, or abnormal results of diagnostic procedures (e.g. abnormalities in the ECG found during exercise), including laboratory test abnormalities. All AEs will be recorded in the case report form (CRF) for the duration of the participant's involvement in the study. Before the study begins participants will have given consent for us to contact their GP if any abnormal findings are discovered.

The expected adverse events for this study have been listed and explained in section 13.1.

16.1.3 Adverse Reaction (AR)

ARs are defined as any untoward and unintended response in a subject to an investigational medicinal product/medical device/intervention, which is related to any dose administered to that subject.

Any event that is judged by the reporting investigator as having a causal relationship to the medicinal product/medical device or intervention is classified as an AR.

An unexpected AR is where the nature and severity of the adverse reaction is not coherent with the information available regarding the medicinal product/medical device/intervention in question. (See section 13.1 for list of expected adverse events/reactions).

16.1.4 Serious Adverse Event (SAE) and Adverse Reaction (SAR)

A SAE/SAR is defined as serious if it:

1. *Results in the death of the subject,*
2. *Is life-threatening,*

The term "life-threatening" refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

3. *Requires inpatient hospitalisation or prolongation of existing hospitalisation,*
4. *Results in persistent or significant disability / incapacity, or*

Any event that seriously disrupts the ability of the participant to lead a normal life, in other words leads to a persistent or permanent significant change, deterioration, injury or perturbation of the participant's body functions or structure, physical activity and/or quality of life.

5. *Is a congenital anomaly / birth defect.*

It should be noted that during the screening process for this study, all women of childbearing age will take a pregnancy test. All pregnant women are excluded from this study.

Medical judgement should be exercised in deciding whether an SAE/SAR is serious in other situations. Important SAE/SARs that are not be immediately life-threatening or result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of these outcomes (there is minimal risk that the procedures outlined in this study will result in a SAE), should also be considered serious.

16.1.5 Suspected serious reactions (SSAR) and suspected unexpected serious adverse reactions (SUSAR)

A SSAR is any serious adverse reaction that is suspected (possibly or probably) to be related to the investigation medicinal product/medical device/intervention.

A SUSAR is unexpected, meaning that the exact extent of the nature and severity of the AR is not coherent with the information available regarding the medicinal product/medical device/intervention.

16.2 Procedures

16.2.1 Adverse Events

Adverse events will be assessed and acted upon by the research team. All AE's will be recorded in the study or project file with a note that will identify when the event occurred, the details of the AE, any potential study relation, action taken and resolution/closure of the AE. An assessment of seriousness will be made and if it is regarded as an SAE and will reported as stated below.

Prior to taking part in this research participants will have given consent for us to report any abnormal findings during the study to their GP. For example, if an abnormal ECG reading was obtained from a participant, the information would be passed on to the participants GP, with their consent.

The following are expected AEs that may be encountered:

- Chemo-reflex testing: Transient dizziness or light-headedness following the inhalation of Nitrogen
- COVID-19 infection

16.2.2 Serious Adverse Events (SAEs)

All SAEs will be reported to University Hospitals Bristol and Weston NHS Foundation Trust R&I team (email: research@uhbristol.nhs.uk) by investigational staff within 24 hours of their knowledge of the event in accordance with University Hospitals Bristol and Weston NHS Foundation Trust research related adverse event reporting policy.

All SAEs that have not been resolved by the end of the study, or that have not been resolved upon discontinuation of the participant's involvement in the study, will be monitored until one of the following occurs:

- the event resolves
- the event stabilizes
- the event returns to baseline, if a baseline value is available
- the event can be attributed to agents other than the study drug or to factors unrelated to study conduct
- when it becomes unlikely that any additional information can be obtained (participant or health care practitioner refusal to provide additional information, lost to follow-up after demonstration of due diligence with follow-up efforts)

The death of a participant is considered an SAE, as is any event requiring hospitalization (or prolongation of hospitalization) that occurs during the course of a participant's participation. Exceptions to this are hospitalizations for:

- social reasons in absence of an adverse event
- the in-clinic protocol procedures
- surgery or procedure planned before entry into the study (must be documented in the CRF)

16.2.3 Suspected Unexpected Serious Adverse Reaction (SUSAR)

All relevant information regarding a SUSAR that occurs during the time course of the study that is fatal or life-threatening will be reported immediately to UH Bristol. The expectedness of an adverse event will be determined by whether it is coherent with the information regarding the characteristics of the medicinal product/medical device/intervention.

17. MONITORING PROCEDURE

17.1 Monitoring and Quality Assurance

Good Clinical Practice (GCP) is an international ethical and scientific quality standard for designing, conducting, recording, and reporting studies that involve the participation of human participants. Compliance with this standard provides public assurance that the rights, safety, and well-being of

study participants are protected, consistent with the principles that originated in the Declaration of Helsinki and that the clinical study data are credible. This research study will be run in accordance with GCP.

The University of Bristol monitors 10% of its studies. Monitoring is carried out by University Hospitals Bristol and Weston NHS Foundation Trust under a service level contract.

17.2 Direct Access to Source Data / Documents

The chief investigator will allow monitors persons responsible for the audit, representatives of the Ethics Committee and of the Regulatory Authorities to have direct access to source data / documents. This is reflected in the Participant Information Sheet (PIS). The study will be monitored and audited in accordance with Sponsor procedures and undertaken by University Hospitals Bristol and Weston NHS Foundation Trust R&I team on behalf of the Sponsor.

18. PUBLICATION PROCEDURE

18.1 Definition of authorship

An author is considered to be someone who has made substantive intellectual contribution to a study. Many journals consider it best practice that everyone who is listed as an author should have made a substantial, direct, intellectual contribution to the work. Honorary or guest authorship is not acceptable.

18.2 Procedure

The baseline criteria for this research for both authorship and acknowledgments for peer reviewed publications and conference contributions is that:

1. Authors must meet all of the following criteria:

- substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data
- drafting the article or revising it critically for important intellectual content
- final approval of the version to be published

2. No-one should be omitted from the authorship list if he/she meets the three criteria in 1 above.

3. Some journals allow authorship of multi-centre projects to be attributed to a group. However, all members of the group who are named as authors must still fully meet the above criteria for authorship in 1 above.

4. Other collaborators or members of the research group who may have contributed to some but not all of the criteria in 1 above will be listed in the Acknowledgments (see 6 below).

5. The individual authors will jointly make decisions about authorship before submitting the manuscript for publication. The lead author, corresponding author or the guarantor must be prepared to explain the presence and order of these individuals to the editor of a journal. Authorship and order of authorship (see 7 below) will be agreed in advance, in the early stages of the research.

6. All contributors who do not meet the criteria for authorship will be listed in an Acknowledgments section. Examples of those who might be acknowledged include:

- persons who have contributed materially to the paper but whose contributions do not justify authorship. These may be listed under such headings as “participating investigators” and their function or contribution should be described - for example, “served as scientific advisors,” “critically reviewed the study proposal,” or “collected data/material”. Because readers may infer their endorsement of the data and conclusions, these persons must give written permission to be acknowledged
- a person who provided purely technical help, provided general comments on the manuscript or writing assistance, or a departmental chair who provided general support
- editors can ask corresponding authors to declare whether they had assistance with study design, data collection, data analysis, or manuscript preparation. Authors should therefore disclose in the Acknowledgements section the identity of any individuals who provided this assistance and any entities that supported the work in the published article
- financial support should also be acknowledged and, if appropriate, the grant identified
- material or logistical support, in particular giving recognition to support provided in developing countries, should always be acknowledged

7. Order of authorship

- the authors shall decide the order of authorship together. Contributors should discuss authorship issues frankly at the start of the work for each anticipated publication and not wait to raise concerns at submission time
- authors shall specify in their manuscript a description of the contributions of each author and how they have assigned the order in which they are listed so that readers can interpret their roles correctly
- the corresponding author or guarantor shall prepare a concise, written description of how the order of authorship was decided
- examples of authorship order include:
    - descending order of contribution
    - placing the person who took the lead in writing the manuscript or doing the research first and the most experienced contributor in the field last

- alphabetical
- random order

8. If an individual leaves the project the question of contribution to publications and authorship should be discussed in advance of their departure to minimise misunderstandings and to agree how this will be managed.

19. QUALITY CONTROL AND QUALITY ASSURANCE

19.1 Case report forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF will be recorded. All missing data will be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, "N/D" will be inserted. If the item is not applicable to the individual case, "N/A" will be inserted. All entries will be printed legibly in black ink. If any entry errors are made, to correct such an error, a single straight line will be drawn through the incorrect entry and the correct data entered above it. All such changes will be initialled and dated. A random sample of 20% of CRFs will be checked against the computerised data base for quality purposes. This percentage will be increased if a significant error rate is found.

20. FINANCE AND INSURANCE

20.1 Finance

We have small grant from the University of Bristol (£10,000) to complete this study (ECH Return Carers Scheme).

20.2 Reimbursement to participants

We will reimburse participants for their travel to the Bristol Royal Infirmary and Clinical Research Facility, Bristol. Participants will receive up to £20 per visit.

20.3 Insurance

This study will be sponsored by the University of Bristol. The University has Public Liability insurance to cover the liability of the University to research participants. In the event that something goes wrong, and a participant is harmed during the research study there are no special compensation arrangements. If a participant is harmed and this is due to someone's negligence then they may have grounds for a legal action for compensation against Bristol University or the NHS Trust or one of the other parties to the research, but they may have to pay their own legal costs.

21. STUDY SPONSOR, NHS ETHICS, R&D APPROVAL

The study sponsor is the University of Bristol. Full NHS REC and HRA approval will be required. This study is not a CTIMP, so MHRA approval is not required. Confirmation of capacity and capability will be sought from University Hospitals Bristol and Weston NHS Foundation Trust

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