Study Title: **Beet the cold:** The effect of inorganic nitrate supplementation on peripheral blood flow and pain in individuals with Raynaud's phenomenon. A pilot, double-blind, placebo controlled, randomised crossover trial.

Internal Reference No: N/A

Ethics Ref: 17/SC/0148

Date and Version No: 15/08/2017 version 1.3

Chief Investigator:	Dr Anthony Shepherd
Investigators:	See protocol development team below
Sponsor:	Portsmouth Hospitals NHS Trust
Funder (if applicable):	Grant under review by Scleroderma and Raynaud's UK

Signatures:

Protocol Development Team:

Chief Investigator	**- Dr Anthony Shepherd**	**(University of Portsmouth)**
Co-investigator 1	**- Dr Clare Eglin**	**(University of Portsmouth)**
Co- investigator 2	**- Dr Joe Costello**	**(University of Portsmouth)**
Co- investigator 3	**- Dr Zoe Saynor**	**(University of Portsmouth)**
Co- investigator 4	**- Dr Paul Gorczynski**	**(University of Portsmouth)**
Co- investigator 5	**- Dr Heather Massey**	**(University of Portsmouth)**
Co- investigator 6	**- Dr Steven Young Min**	**(Queen Alexandra Hospital)**
Co- investigator 7	**- Mrs Jan MacDonald**	**(Patient and public involvement member)**
Co- investigator 8	**- Dr Mark Gilchrist**	**(Royal Devon and Exeter Hospital)**
Co- investigator 9	**- Dr Stephen Bailey**	**(Loughborough University)**

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1. AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
1	1.2		Dr A I Shepherd	Added sodium nitroprusside to the iontophoresis technique.
2	1.3		Dr A I Shepherd	"participants who do not want their GP informed will be provided with the GP letter"

2. SYNOPSIS

Study Title	**Beet the cold:** The effect of inorganic nitrate supplementation on peripheral blood flow and pain in individuals with Raynaud's phenomenon. A pilot, double-blind, placebo controlled, randomised crossover trial.
Internal ref. no.	n/a
Problem statement	Raynaud's Phenomenon (RP) can cause significant discomfort and pain to individuals during a vasospasm. Dietary nitrate appears to offer a simple, low cost means of modifying blood flow to the peripheries and, ultimately, reducing both the discomfort and pain experienced by individuals with RP. This study will also advance our understanding of the aetiology and pathophysiology of RP, specifically the role that the nitrate-nitrite-nitric oxide pathway might play in modulating RP symptoms. An understanding of the effects of concentrated beetroot juice on microvascular blood flow and pain may lead to a range of simple, low cost and effective therapeutic interventions to prevent and treat episodes of RP.
Research question / hypothesis	Primary objectives: Does concentrated beetroot juice (CBJ) increase peripheral blood flow, improve endothelial function and increase skin temperature compared to placebo in people with RP. Secondary objectives: 1) Does CBJ reduce perceived discomfort and pain and, 2) is our protocol acceptable to individuals with RP (i.e. cold water stimulus, CBJ palatability) and feasible to perform in a future multi-centre randomised control trial (RCT) and, 3) to establish recruitment and retention rates. Resulting estimated effect size in relation to their confidence intervals from our primary objective(s) measures will be utilised to identify our primary outcome measure and to power a larger, definitive, multi-centre randomised, control trial.
Study Design	The proposed study will be a pilot, randomised placebo-controlled, double-blind, crossover trial.

Study Participants	Individuals with primary and secondary Raynaud's phenomenon.
Planned Sample Size	Twenty-five individuals with RP would need to be recruited in order to estimate a moderate to large effect size in a pilot study. Therefore, we aim to recruit 30 individuals (at a rate of 1 per week) to account for drop outs.
Follow-up duration	2 weeks
Planned Study Period	1 year
Primary Objective	Primary objectives: Does CBJ increase peripheral blood flow, improve endothelial function and increase skin temperature compared to placebo in people with RP.
Secondary Objectives	Secondary objectives: 1) Does CBJ reduce perceived discomfort and pain and, 2) is our protocol acceptable to individuals with RP (i.e. cold water stimulus, CBJ palatability) and feasible to perform in a future multi-centre randomised control trial (RCT) and, 3) to establish recruitment and retention rates. Resulting estimated effect size in relation to their confidence intervals from our primary objective(s) measures will be utilised to identify our primary outcome measure and to power a larger, definitive, multi-centre randomised, control trial.
Primary Endpoint	N/A: pilot study.
Secondary Endpoints	N/A: pilot study.
Intervention (s)	4 weeks of CBJ supplementation. 2 with nitrate rich CBJ and 2 weeks with nitrate depleted CBJ.

3. ABBREVIATIONS

Ach	= Acetylcholine
CBJ	= Concentrated beetroot juice
CRF	= Clinical records folder
eGFR	= Estimate of glomerular filtration rate
flux/MAP	= Cutaneous vascular conductance
FTE	= Full time equivalent
GTN	= Glyceryl trinitrate
NHS	= National Health Service
NO•	= Nitric oxide
PCPI	= Patient, carer and public involvement
RCT	= Randomised control trial
REC	= Research Ethics Committee
RP	= Raynaud ’s phenomenon
SF-36	= Short Form Health Survey
TBC	= Total blood count

4. BACKGROUND AND RATIONALE

Raynaud's phenomenon (RP) is characterised by a recurrent transient vasospasm of the fingers or toes in response to a cold or stressful stimulus (Wigley 2002). Nitric oxide (NO•) is a known vasodilator and NO• donors, such as Glyceryl Trinitrate (GTN), improve blood flow in patients with RP (Anderson et al., 2002; Teh et al., 1995) and in cold sensitive individuals (Hope, Eglin, Golden, & Tipton, 2014) (Figure 1, see accompanying document). However, individuals develop a tolerance to GTN and show diminishing vasodilatory effects with chronic treatment (Needleman & Johnson, 1973). In addition, the deleterious side effects such as headaches (Hsi, Roshandel, Singh, Szombathy, & Meszaros, 2005; Wigley 2002) means that organic nitrates (i.e. GTN and isosorbide mononitrate) are not optimal long-term therapies for RP. Alternative treatments therefore, warrant further investigation.

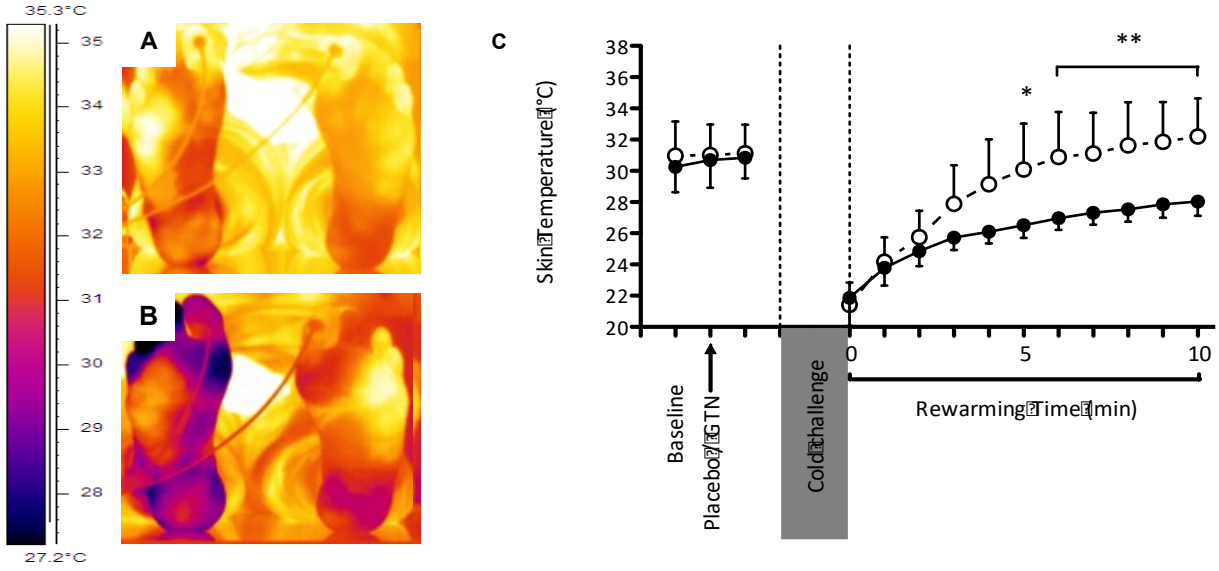


Figure 1. Effect of GTN on foot skin temperature of cold sensitive individuals during a 2 min cold challenge test. (A) individual thermogram taken after 5 min of rewarming following a 2 min immersion in 15°C water with GTN and (B) with placebo (C) depicts mean toe temperature before during and after the cold challenge with placebo (closed circles) and GTN (open circles; n=6) (Hope et al., 2014).

Diets rich in fruit and vegetables have been shown to be effective in reducing blood pressure (Appel et al., 1997; Rouse, Beilin, Armstrong, & Vandongen, 1983). In addition, they lower the risk of morbidity and mortality from cardiovascular disease (Bazzano et al., 2001;

Joshipura et al., 1999; Joshipura et al., 2001) and are thought to be beneficial to cardiovascular health due to their vasodilatory effects (M Gilchrist, Winyard, & Benjamin, 2010). As diet exhibits such tremendous intra- and inter-individual variation, elucidating which components of such a diet are responsible for this effect is difficult. There is a growing weight of evidence from both human and animal studies that nitrate and nitrite derived from the diet can serve as a source for nitric oxide (NO; please see below), particularly where it is deficient (V. Kapil, Weitzberg, Lundberg, & Ahluwalia). Indeed, the greatest protective effect on cardiovascular disease is to be found in those diets with the greatest consumption of green leafy and or cruciferous vegetables (Joshipura et al., 1999; Joshipura et al., 2001) which typically have high nitrate content.

NO is produced in the body in two ways. The first requires the availability the amino acid L-arginine, molecular oxygen, and families of enzymes, the nitric oxide synthases ((NOS); (Moncada & Higgs, 1993; Moncada, Palmer, & Higgs, 1989)); that is the NOS pathway. The second pathway is independent of the NOS pathway and involves the stepwise enzymatic and chemical reduction of inorganic nitrate to nitrite (Benjamin et al., 1994; Björne et al., 2004; Lundberg, Weitzberg, & Gladwin, 2008; Zweier, Wang, Samouilov, & Kuppusamy, 1995). A major source of nitrite in humans is the reduction of dietary nitrate by facultative anaerobic bacteria in the mouth (Duncan et al., 1995). The remaining nitrite is then absorbed into the circulation where it acts as a storage pool for subsequent NO• production, which is expedited in hypoxaemia (Cosby et al., 2003).

Localised hypoxemia such as that observed in the digital vasculature of individuals with RP (Wigley 2002) is a potential therapeutic target for dietary nitrate supplementation. In contrast to organic nitrates (GTN), inorganic nitrate (in the form of beetroot juice) does not cause the same negative side effects (Anthony I. Shepherd et al., 2015) or demonstrate tachyphylaxis (Vanhatalo et al., 2010) whilst it does notably improve skin blood flow (Levitt, Keen, & Wong, 2015), microvascular function (Hobbs et al., 2013; Vikas Kapil, Khambata, Robertson, Caulfield, & Ahluwalia, 2015) and lower blood pressure (BP) in healthy individuals and chronic conditions such as hypertension (Vikas Kapil et al., 2015), peripheral arterial disease, (Kenjale et al., 2011) heart failure (Zamani et al., 2015) and chronic obstructive pulmonary disease (Berry et al., 2014). Thus concentrated beetroot juice (CBJ)

may offer an inexpensive, safe and potentially effective intervention to improve the pain and reduced peripheral blood flow characterising individuals with RP.

RP can cause significant discomfort and pain to individuals during a vasospasm. Dietary nitrate appears to offer a simple, low cost means of modifying blood flow to the peripheries and, ultimately, reducing both the discomfort and pain experienced by individuals with RP. This study will also advance our understanding of the aetiology and pathophysiology of RP, specifically the role that the nitrate-nitrite-nitric oxide pathway might play in modulating RP symptoms. An understanding of the effects of CBJ on microvascular blood flow and pain may lead to a range of simple, low cost and effective therapeutic interventions to prevent and treat episodes of RP.

The proposed study will be a pilot, randomised placebo-controlled, double-blind, crossover trial. The effect of acute (1 day) and chronic (2 weeks) CBJ supplementation on microvascular function (skin blood flow and speed of rewarming of the hand and foot) in people with RP will be compared to a placebo (CBJ with the nitrates removed). A summary of the main risks of this study include; a well-recognised side effect of beetroot consumption, is that it may turn urine and faeces red. This is not harmful and will pass rapidly after discontinuation of the juice. Subjects will be told of this during the consent visit. Intravenous access can cause discomfort associated with insertion of the needle. We will take a blood sample on three occasions. Some discomfort can be caused during iontophoresis (an itchy feeling) and some pain may be felt when immersing the hand and foot in to 15°C water for 2 minutes. Participants will be made aware that they can withdraw at any stage in accordance with GCP and informed consent.

5. PRELIMINARY STUDIES AND EXPERIENCE OF INVESTIGATORS

Dr's Shepherd, Gilchrist and Bailey are leaders in the field of nitrate supplementation (particularly beetroot) and have over 70 peer reviewed publications between them in the field. The Department of Sport and Exercise Science (DSES) at the University of Portsmouth (UoP) has a world leading environmental group. Dr's Eglin and Costello from this group are leaders in this field and will be helping with controlling environmental conditions and the specific tests for this study (i.e. iontophoresis and cold sensitivity tests). Dr Gorczynski has extensive experience of running focus groups and performing qualitative research in patient groups.

Dr's Young Min and Gilchrist are clinicians with extensive experience of running clinical trials. See reference list for publications and attached CV's.

6. AIMS AND OBJECTIVES

The overall aim is to establish if this protocol is acceptable and feasible to perform whilst establishing effect sizes for our primary and secondary outcomes to power a larger definitive trial.

6.1 Primary Objective

Primary objectives: Does CBJ increase peripheral blood flow, improve endothelial function and increase skin temperature compared to placebo in people with RP.

6.2 Secondary Objectives

Secondary objectives: 1) Does CBJ reduce perceived discomfort and pain; 2) is our protocol acceptable to individuals with RP (i.e. cold water stimulus, CBJ palatability) and feasible to perform in a future multi-centre randomised control trial (RCT); and 3) to establish recruitment and retention rates. Resulting estimated effect sizes in relation to their confidence intervals from our primary objective(s) measures will be utilised to identify our primary outcome measure and to power a larger, definitive, multi-centre randomised, control trial.

7. STUDY DESIGN

7.1 Summary of Study Design

The proposed study will be a pilot, randomised placebo-controlled, double-blind, crossover trial. The effect of acute and chronic CBJ supplementation on microvascular function in people with RP will be compared to a placebo (CBJ with the nitrates removed). The placebo is the same in colour, odour, texture and taste as CBJ (Mark Gilchrist et al., 2014) but does not cause an increase in plasma nitrate or nitrite (Shepherd et al., 2016). The volunteers will be given a full briefing about the study and given the participant information sheet before we seek their written consent (see below). The individual's severity of RP symptoms will be established according to the International Consensus Criteria for Diagnosing RP (Maverakis et al., 2014).

Participants and Recruitment: Twenty-five individuals with RP would need to be recruited in order to estimate a moderate to large effect size in a pilot study (Johanson & Brooks, 2009). Therefore, we aim to recruit 30 individuals (at a rate of 1 per week) with RP to account for a 15% drop-out rate (typically seen in our clinical trials). Participants will be recruited from a database of known individuals with RP who attend outpatient clinics at the Queen Alexandra Hospital (Portsmouth) and via posters and word of mouth within the University of Portsmouth. The participants will attend the lab on five occasions during which cold sensitivity and endothelial function (iontophoresis) will be assessed (Spinnaker Building, University of Portsmouth).

Consent and Screening: Visit 1 will act as a screening, consent and baseline assessment (3.5-4 hours).

Participants will be given the opportunity to ask any questions they may have after reading the participant information sheet. A standard medical history and clinical examination will be undertaken by a research nurse or member of the research team, including for example height, mass, electrocardiogram, ankle-brachial pressure index and blood sample collection for lipids and biochemical markers, urea and electrolytes, and liver function tests. Seated resting blood pressure measurements will be performed to calculate systolic, diastolic blood pressure and cutaneous vascular conductance (flux/MAP). The participants will then undertake an endothelial function (iontophoresis) test in an ambient temperature of 23°C followed by a cold challenge in an ambient temperature of 30°C (both described below) as baseline measures. Quality of life will be examined through the provision of the Medical Outcomes Study 36-item Short Form Health Survey (SF-36). |Following this visit, a letter will be sent to their General Practitioner (GP), confirming their participation within the trial. Participants who do not want their GP informed will be provided with the GP letter.

Randomisation: During the first visit the participants will be randomised by a nurse to begin either the CBJ or placebo arm of the study. A computer programme will be utilised to randomly allocate to treatment order, whilst stratifying for disease type and gender. Participants will be provided with instructions and their first acute dose to take away with them.

Visit 2 and 4 (acute supplementation visits ~ 1.5-2 hours). One and a half hours before visits 2 and 4, participants will be instructed to ingest an acute dose of 140 ml of either CBJ (delivers ~ 12.4 mmol·L^1 of inorganic nitrate) or placebo (~ 0.002 mmol·L^1 Beet It, Heartbeet Ltd.). The primary objectives (peripheral blood flow, endothelial function and skin temperature; see below for details) will be assessed ~ 1.5 hours after arrival in the research facility. The timing of the supplement is key to ensure plasma / saliva nitrate and nitrite are elevated throughout the testing period (Webb et al., 2008). On arrival at the laboratory, the participants will have their BP measured and a ~ 20 ml venous blood sample taken. The participants will then undertake an endothelial function (iontophoresis) test in an ambient temperature of 23°C followed by a cold challenge in an ambient temperature of 30°C (both described below). The SF-36 will also be measured here. Visits 3 and 4 (crossover period) will be separated by at least 7 days to allow washout (Wylie et al., 2013) and all visits will be conducted at the same time of day in a counter-balanced order at a time suitable for the participants.

Visit 3 and 5 (chronic supplementation visits ~ 1.5-2 hours). Visits 3 and 5 will be identical in nature to visit 2 and 4, however, following chronic supplementation of 70 ml·day^{-1} of either CBJ or placebo for 14 days (Vanhatalo et al., 2010; Wylie et al., 2013). With consent, participants will be reminded to take the juice via text message or voice mails. Please see the accompanying documents for the participant flow through the study.

The participants diet will be standardised prior to testing and they will be asked to abstain from using antibacterial mouth wash for 7 days prior to each test and throughout the period of testing as this has been shown to reduce the concentration of oral microflora that are responsible for the reduction of nitrate to nitrite (Govoni, Jansson, Weitzberg, & Lundberg, 2008). Participants will also be asked to refrain from using other medicines during the testing period that may affect blood flow or the release of nitrite from the CBJ with such as organic nitrates, proton pump inhibitors, ranitidine and nicorandil.

7.2 Primary and Secondary Endpoints/Outcome Measures

This is a pilot study and as such, primary outcome measures are N/A. See above for our primary and secondary objectives. These will be used to inform what our primary outcomes will be in a future trial.

8. STUDY PARTICIPANTS

8.1 Study Setting

Firstly, the Extreme Environments Laboratory at the University of Portsmouth has a 30 year history of conducting innovative human research trials. The laboratory contains some of the world's leading facilities for conducting environmental research including three bespoke environmental chambers, with temperature, humidity, solar, wind and hypoxia control, and a research group involving over 20 academics and researchers. Two of the applicants (Drs Eglin and Costello) have extensive experience of conducting examinations of cold sensitivity and endothelial function in both healthy and clinical populations (Eglin, Golden, & Tipton, 2013; Hope et al., 2014; Maley, House, Tipton, & Eglin, 2015). Dr. Saynor also has significant experience working with patient groups, including several rheumatic conditions and in assessing endothelial function etc. in current research. Presently, the team are completing similar research on individuals with cold sensitivity and the proposed methodology, including infrastructure, equipment and expertise, is therefore feasible and requires no additional training or time to develop.

8.2 Overall Description of Study Participants

Participants with a clinical diagnosis of Primary or Secondary Raynaud's Phenomenon.

8.3 Eligibility Criteria

Inclusion Criteria

The participants must meet ALL of the following criteria to be considered eligible for the study:

- Male or Female, aged 18 years or above.
- Diagnosed with Raynaud's Phenomenon.
- Participant is willing and able to give informed consent for participation in the study.

Exclusion Criteria

The participant may not enter the study if ANY of the following apply:

- Patients with significant renal impairment (eGFR<30),

- Uncontrolled hypertension,
- Taking regular organic nitrates, nicorandil, or thiazolidinidiones,
- or any medication which may interfere with data interpretation or safety,
- who have had a myocardial infarction or cerebro−vascular event,
- who smoke,
- or any other serious medical condition which would interfere with data interpretation or safety will be excluded from participation.

9. SAMPLING

Twenty-five individuals with RP would need to be recruited in order to estimate a moderate to large effect size in a pilot study (Johanson & Brooks, 2009). Therefore, we aim to recruit 30 individuals (at a rate of 1 per week) with RP to account for a 15% drop-out rate (typically seen in our clinical trials). We will utilise an opportunistic sampling process, from both clinics and posters and word of mouth.

10. STUDY PROCEDURES

Endothelial function test: Following a 30 minute acclimatisation period, acetylcholine (ACh) and sodium nitroprusside will be delivered transdermally using iontophoresis to three sites, the middle phalanx of the middle finger of the left hand, the dorsal aspect of the left foot and the volar aspect of the left forearm. Cutaneous vascular conductance and skin temperature will be measured to determine the dose response to ACh at each site separately (Maley et al., 2015).

Cold challenge test: Following a 15 min acclimatisation period, participants will undertake light exercise on a cycle ergometer for 12 min (maximum of 50 W, to elevate their core temperature by approximately 0.3°C). Prior exercise has been found to increase the reproducibility of the test (Eglin et al. 2005) by removing the effect of varying central vasoconstrictor tone. Participants will then undertake a standardised cold challenge test by immersing their hand and foot separately into 15°C water for 2 minutes followed by spontaneous rewarming for 10 minutes (Eglin et al., 2013; Hope et al., 2014). Skin

temperature (from a thermal imaging camera), peripheral (skin) blood flow (laser Doppler) thermal sensation, comfort and pain will be measured throughout the test.

Quality of life:

Quality of life will be examined through the provision of the Medical Outcomes Study 36-item Short Form Health Survey (SF-36) (Ware Jr & Sherbourne, 1992). The SF-36 measures eight aspects of quality of life: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. The SF-36 also provides a single item score that indicates a perceived change in health. The SF-36 has been shown to be reliable with Cronbach alpha scores ranging from .80 to .92 for various aspects of quality of life (Ruta, Abdalla, Garratt, Coutts, & Russell, 1994). This will be measured at each experimental visit post CBJ or placebo consumption.

Qualitative analysis (acceptability):

Semi-structured interviews will be used to examine the acceptability of CBJ and placebo supplementation. Specifically, interviews will explore research methods / testing procedures and the participant experience of consuming beetroot juice regularly. Approximately 15-20 semi-structured interviews will be necessary to reach a point of data saturation where no new information is provided. Interviews will be conducted by a researcher with experience (co-applicant) in qualitative research methods and methodologies. Interviews will be transcribed verbatim and analysed thematically (Braun & Clarke, 2006).

10.1 Recruitment

Participants will be recruited from a database of known individuals with RP who attend outpatient clinics at the Queen Alexandra Hospital (Portsmouth) and via posters and word of mouth within the University of Portsmouth. If this study is funded, a research nurse will utilised to find potential participants.

10.2 Screening and Enrolment

Consent and Screening: Visit 1 will act as a screening, consent and baseline assessment (3.5-4 hours).

Participants will be given the opportunity to ask any questions they may have after reading the participant information sheet. A standard medical history and clinical examination will be undertaken by a research nurse or member of the research team, including for example height, mass, electrocardiogram, ankle-brachial pressure index and blood sample collection for lipids and biochemical markers, urea and electrolytes, and liver function tests. Seated resting blood pressure measurements will be performed to calculate systolic, diastolic blood pressure and cutaneous vascular conductance (flux/MAP). If this study is funded, a research nurse will utilised to find and screen potential participants.

10.3 Randomisation

During the first visit the participants will be randomised by a nurse to begin either the CBJ or placebo arm of the study. A computer programme will be utilised to randomly allocate to treatment order, whilst stratifying for disease type and gender. Participants will be provided with instructions and their first acute dose to take away with them.

10.4 Study Assessments

Endothelial function test: Following a 30 minute acclimatisation period, acetylcholine (ACh) and sodium nitroprusside will be delivered transdermally using iontophoresis to three sites, the middle phalanx of the middle finger of the left hand, the dorsal aspect of the left foot and the volar aspect of the left forearm. Cutaneous vascular conductance and skin temperature will be measured to determine the dose response to ACh at each site separately (Maley et al., 2015).

Cold challenge test: Following a 15 min acclimatisation period, participants will undertake light exercise on a cycle ergometer for 12 min (maximum of 50 W, to elevate their core temperature by approximately 0.3°C). Prior exercise has been found to increase the reproducibility of the test (Eglin et al. 2005) by removing the effect of varying central vasoconstrictor tone. Participants will then undertake a standardised cold challenge test by immersing their hand and foot separately into 15°C water for 2 minutes followed by spontaneous rewarming for 10 minutes (Eglin et al., 2013; Hope et al., 2014). Skin temperature (from a thermal imaging camera), peripheral (skin) blood flow (laser Doppler) thermal sensation, comfort and pain will be measured throughout the test.

Secondary Objectives

Quality of life:

Quality of life will be examined through the provision of the Medical Outcomes Study 36-item Short Form Health Survey (SF-36) (Ware Jr & Sherbourne, 1992). The SF-36 measures eight aspects of quality of life: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. The SF-36 also provides a single item score that indicates a perceived change in health. The SF-36 has been shown to be reliable with Cronbach alpha scores ranging from .80 to .92 for various aspects of quality of life (Ruta et al., 1994). This will be measured at each experimental visit post CBJ or placebo consumption.

Qualitative analysis (acceptability):

Semi-structured interviews will be used to examine the acceptability of CBJ and placebo supplementation. Specifically, interviews will explore research methods / testing procedures and the participant experience of consuming beetroot juice regularly. Approximately 15-20 semi-structured interviews will be necessary to reach a point of data saturation where no new information is provided. Interviews will be conducted by a researcher with experience (co-applicant) in qualitative research methods and methodologies. Interviews will be transcribed verbatim and analysed thematically (Braun & Clarke, 2006).

Biochemical analysis:

Blood / saliva samples will be taken and assessed prior to testing on each study visit. Blood samples for plasma nitrate/nitrite will be taken in chilled Lithium Heparin tubes and EDTA tubes for assessment of oxidative stress markers. The blood / saliva needs to be placed in a chilled (4ºC) centrifuge and spun at 4000g for 10 minutes immediately on collection. Once spun the plasma or saliva will be pipetted into aliquots. The samples will be placed in a -20ºC freezer prior to transfer to a -80ºC freezer for storage at the end of each day.

Deproteinised samples will be analysed for nitrite and nitrate concentration using a Sievers nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK) and a modification of our previous chemiluminescence technique. Elisa's will be utilised using commercially available

assays for markers of oxidative stress such as glutathione and nitrotyrosine. We will also measure plasma for the quantification of endothelin 1.

10.5 Discontinuation/Withdrawal of Participants from Study Treatment

Participants may be withdrawn from treatment, or from the study as a whole; if they ask to withdraw, if participant safety is in question or if the study is stopped early.

10.6 Definition of End of Study

The end of study will be the date of the last visit of the last participant.

11. INTERVENTIONS

11.1 Description of Study Intervention / Treatment

Visit 2 and 4 (acute supplementation visits ~ 3 hours). One and a half hours before visits 2 and 4, participants will be instructed to ingest an acute dose of 140 ml of either CBJ (delivers ~ 12.4 mmol·L^1 of inorganic nitrate) or placebo (~ 0.002 mmol·L^1 Beet It, Heartbeet Ltd.). The primary objectives (peripheral blood flow, endothelial function and skin temperature; see below for details) will be assessed ~ 1.5 hours after arrival in the research facility. The timing of the supplement is key to ensure plasma nitrate and nitrite are elevated throughout the testing period (Webb et al., 2008). On arrival at the laboratory, the participants will have their BP measured and a ~ 20 ml venous blood sample taken for determination of plasma [nitrate], [nitrite] and nitrotyrosine and protein carbonyl concentrations (which are clinically relevant markers of oxidative stress (Frijhoff et al., 2015)). The participants will then undertake an endothelial function (iontophoresis) test in an ambient temperature of 23°C followed by a cold challenge in an ambient temperature of 30°C (both described below). Visits 3 and 4 (crossover period) will be separated by at least 7 days to allow washout (Wylie et al., 2013) and all visits will be conducted at the same time of day in a counter-balanced order at a time suitable for the participants.

Visit 3 and 5 (chronic supplementation visits ~ 3 hours). Visits 3 and 5 will be identical in nature to visit 2 and 4, however, following chronic supplementation of 70 ml·day^{-1} of either CBJ or placebo for 14 days (Vanhatalo et al., 2010; Wylie et al., 2013). With consent,

participants will be reminded to take the juice via text message or voice mails. Please see the accompanying documents for the participant flow through the study.

The participants diet will be standardised prior to testing and they will be asked to abstain from using antibacterial mouth wash for 7 days prior to each test and during the testing phase as this has been shown to reduce the concentration of oral microflora that are responsible for the reduction of nitrate to nitrite (Govoni et al., 2008). Participants will also be asked to refrain from using other medicines during the testing period that may affect blood flow or the release of nitrite from the CBJ with such as organic nitrates, proton pump inhibitors, ranitidine and nicorandil.

11.2 Adherence to Study Treatment

Venous blood samples will be taken on all visits. Deproteinised samples will be analysed for nitrate and nitrite concentration using a Sievers nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK) with our established technique (S. Bailey, J et al., 2010; S. Bailey, J et al., 2009; S. J. Bailey, Wilkerson, DiMenna, & Jones, 2009; M Gilchrist et al., 2013; Mark Gilchrist et al., 2014; Anthony I Shepherd et al., 2015; Anthony I. Shepherd et al., 2015; Shepherd et al., 2016). This will essentially provide strong evidence of adherence to the supplement.

11.3 Accountability of the Study Treatment

This study is double-blind in nature. The research nurse (if funded) or technicians from the department of Sport and Exercise Science at the University of Portsmouth will dispense the CBJ. At each time the CBJ is dispensed, the juice will be checked for treatment arm and double signed by 2 trained staff members.

11.4 Concomitant Medication / Therapies

Participant involvement is anticipated to be ~6 weeks. Medication will be recorded on enrolment and updated throughout their time on supplementation.

12. ASSESSMENT OF SAFETY (IF APPLICABLE)

12.1 Definitions

A serious adverse event is any untoward medical occurrence that:

- Results in death,
- Is life-threatening,

NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant 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.

- Requires inpatient hospitalisation or prolongation of existing hospitalisation,
- Results in persistent or significant disability/incapacity, or
- Is a congenital anomaly/birth defect.
- Other important medical events*

*Other events that may not result in death, are not life threatening, or do not require hospitalisation, may be considered a serious adverse event when, based upon appropriate medical judgement, the event may jeopardise the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

12.2 Reporting Procedures for Serious Adverse Events

A serious adverse event (SAE) occurring to participant should be reported to the REC that gave a favourable opinion of the study where in the opinion of the Chief Investigator the event was: ‘related’ – that is, it resulted from administration of any of the research procedures; and ‘unexpected’ – that is, the type of event is not listed in the protocol as an expected occurrence. Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES report of serious adverse event form (see NRES website).

12.3 Recording and Reporting Procedures for All Adverse Events

All adverse events will be recorded in participant file notes. Distinction between SAE and AE will be made by an independent medical officer. All SAE will be reported to the sponsor and the REC regardless of if it is related or not.

13. DATA HANDLING AND RECORD KEEPING

13.1 Data Collection Forms

Visit 1 will act as a screening, consent and baseline assessment (4 hours). All measures for this visit will be stored in the clinical records folder (CRF). Separate forms will be prepared for; anthropometrics, medical history, screening (i.e. blood pressures etc) and blood markers. On subsequent visits data forms will be used to collect temperatures of the labs and the rewarming of the hands and feet and also for the iontophoresis test. All paper copies will be stored in the CRF's.

13.2 Data Management

All study data will be entered on paper copies and stored in participant CRF's. All data will be double data entered into excel / access. Macro's will be used to check for anomalies and corrected. The participants will be identified by a study specific participants number and/or code in any database. The name and any other identifying detail will NOT be included in any study data electronic file. The chief investigator (Dr Ant Shepherd) is responsible for database maintenance and management.

14. DATA ANALYSIS

14.1 Description of Analysis Populations

This is a pilot study. All participants who complete all 5 visits will be entered into the analysis as per protocol.

14.2 Analysis of Endpoints

All data will be tested for normality. Where data are not normally distributed a nonparametric test will be performed. Data will be presented as means ± SD. Statistical analyses will be performed on SPSS software version 22.0 (Chicago, IL). Statistical difference will be accepted when $P < 0.05$. Statistical differences will be assessed by repeated-measures

ANOVAs. Where baseline differences are present, analyses of covariance (ANCOVAs) will be used with baseline as a covariate.

14.3 Procedure for Dealing with Missing, Unused and Spurious Data

Where statistic differences are present, post hoc (Bonferroni-corrected) analysis will be performed. Outliers will remain within the data. Within the data analysis software missing data will be coded 9999 and the data point missed during analysis.

14.4 Procedures for Reporting any Deviation(s) from the Original Statistical Analysis Plan

For this pilot a SAP is not required (excluding the above).

14.5 Interim analysis and criteria for early study termination

Given the small samples size and time frame, no interim analysis will be performed. Early termination will only occur if the safety of participants is in question.

15. ETHICS

NHS Local Research Ethics Committee (REC) approval will be obtained prior to commencement of the study. The REC, local NHS, research and development department and all site specific forms and patient identification centre forms will be forwarded to SRUK prior to recruitment of participants. Written informed consent will be obtained from all participants. Insurance indemnity will be provided by the UoP for this study.

Every effort has been made to keep the risks and discomforts to a minimum but there are some risks associated to taking part. Not all volunteers will experience any or all of the risks stated below, but participants will be made aware of them.

The main burden to participants is a significant lifestyle alteration because of a change in dietary practices for two, two week periods. This will involve drinking 70ml of either nitrate rich beetroot juice or nitrate depleted beetroot juice on a daily basis for two, two week periods.

We will also be asking people with RP to put their hand and foot into 15°C water as part of the cold sensitivity test. This may cause some discomfort associated with RP. We will be measuring the rewarming and temperature of the hands and feet. Participants will be reminded that they are free to withdraw at any time point.

Other less burdensome tests will be performed such as:

Blood pressure; participants will feel a cuff squeeze their arm and this can be uncomfortable for a few moments. Blood samples: Intravenous access can cause discomfort associated with insertion of the needle. We will take a blood sample on four occasions. Iontophoresis may cause some local discomfort/itching sensation during the delivery of acetylcholine and sodium nitroprusside, however this is transient occurring only when the current is applied (8 x 20 s).

A well-recognised side effect of beetroot consumption is that it may turn urine and faeces red. This is not harmful and will pass rapidly after discontinuation of the juice. Subjects will be told of this during the consent visit. Subjects will also be asked to refrain from using anti-bacterial mouthwashes for a week before drinking the juice and during the intervention itself. This is critical as it interferes with the biological pathway in which we believe this intervention works.

15.1 Participant Confidentiality

The study staff will ensure that the participants' anonymity is maintained. The participants will be identified only by initials and a participants ID number on the CRF and any electronic database. All documents will be stored securely and only accessible by study staff and authorised personnel. The study will comply with the Data Protection Act which requires data to be anonymised as soon as it is practical to do so.

15.2 Other Ethical Considerations

N/A

15.3 Declaration of Helsinki

Refer to declaration and that the study protocol will be carried out in accordance with it.

15.4 ICH Guidelines for Good Clinical Practice

All staff will be GCP trained and will be monitored by the sponsor to ensure adherence to GCP.

16. PATIENT PUBLIC INVOLVEMENT (PPI)

16.1 Study design

Evidence of patient, carer and public involvement (PCPI):

The chief investigator has a strong track record of PCPI work having been involved in PCPI award winning projects recently from the UK Stroke Forum (October 2016) for another acceptability and feasibility trial. We have a PCPI co-applicant who has be instrumental in the design and development of this pilot RCT and will continue to be involved in the trial steering committee, reporting, dissemination and future NIHR funding bids. Other individuals with RP have been consulted and have stated that they liked the juice and the quantity should not be an issue. Some concerns over the testing schedule (5 visits of 3 hours) for working aged individuals were raised. In response we have made weekend testing available to be as inclusive as possible. The lay summary and patient facing documentation has been reviewed by our PCPI co-applicant.

16.2 Study implementation

This is a pilot study and as such we do not have a TSC or DMC. Issues will be managed by the senior members of the research team. We will endeavor to involve the PCPI member where possible on decisions regarding the study and data.

16.3 Dissemination

Our PCPI member will help disseminate findings through local groups, local/national media, and co-authoring the paper. Our PCPI involvement has developed the protocol to this stage and will continue to play a large role in this project and the larger definitive trial to come.

17. FINANCING AND INSURANCE

17.1 Research Costs

These are the costs that have been requested from Scleroderma & Raynaud's UK. If the study is not funded by SRUK, the UoP has allocated funding for this study (excluding staff costs).

Table 1. Breakdown of budget costs.

Costs		Details	Total
Staff[1,2]	Researcher (grade 31)[1]	0.5 FTE for 1 year	21,889
	Nurse (grade 6)[2]	0.2 FTE for 1 year	7,817
	Staff advertisement	Online advert (researcher only)	400
Participant transport[3]	Maximum £10 per visit	Total 5 visits	1500
Screening bloods[4]	£15 per participant	Lipids, TBC, urea eGRF etc	450
Visit bloods[5]	Nitrate and Nitrite	£3 a sample, pre each visit in duplicate	900
		Nitrothyrosine, 4 kits	918
	Oxidative stress	Protein carbonyl's, 3 kits	807
Beetroot juice[6]	Placebo	17, 70 ml drinks @ £5 x 30 participants	2550
	CBJ	17, 70 ml drinks @ £1 x 30 participants	510
		Total	**£37,741**

Acronyms; FTE (Full time equivalent), TBC (total blood count), eGFR (estimate of glomerular filtration rate), and CBJ (concentrated beetroot juice).

17.2 Service Support Costs

No service support costs are being sort.

17.3 Excess Treatment Costs

No excess treatment costs are being sort.

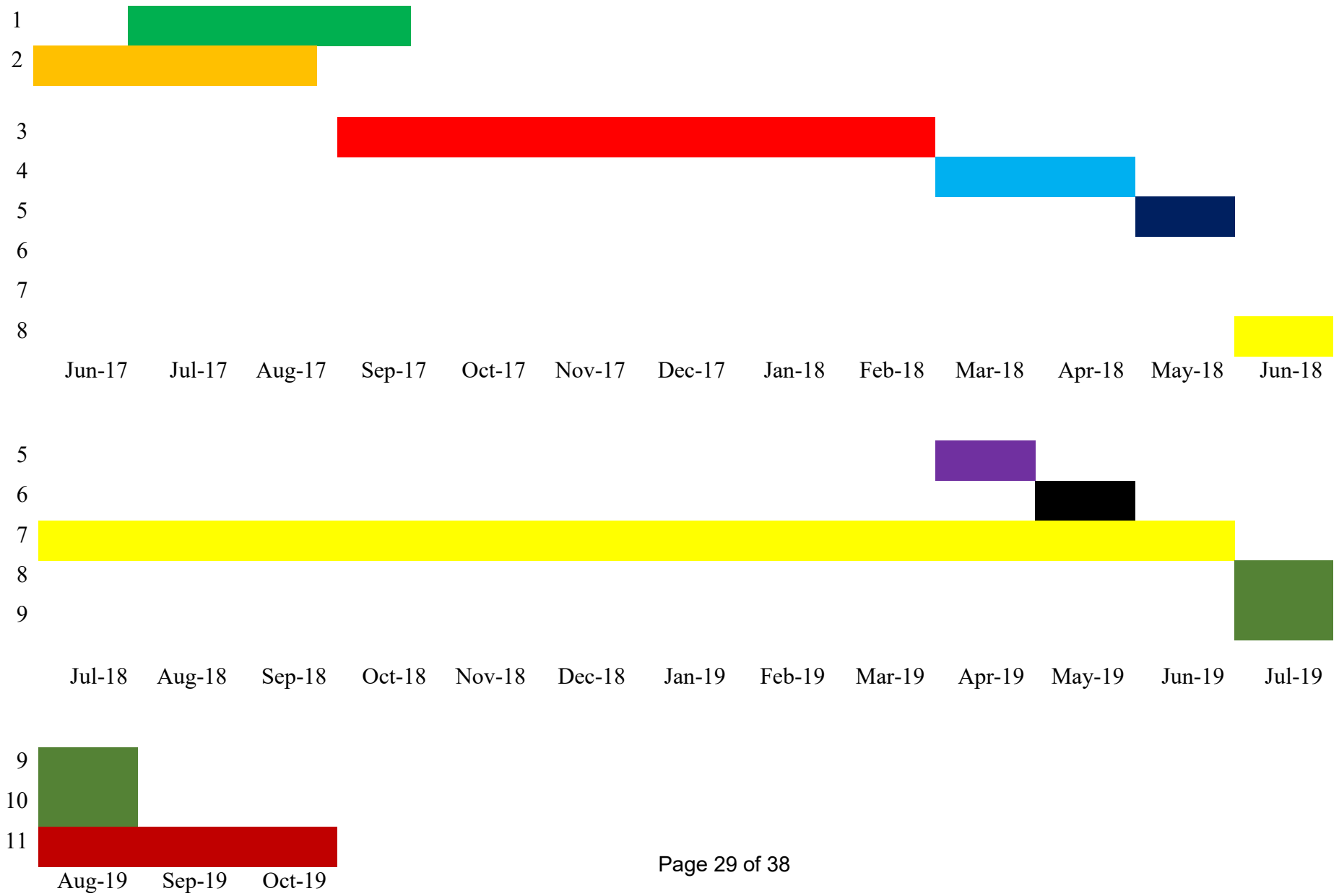
17.4 Study Sponsorship

This study is being sponsored by the UoP.

18. TIMETABLE AND ORGANISATIONAL CHART

See corresponding numbers below in Gantt.

- 1; Setting up collaborations with interested parties and PPI
- 2; Literature search and designing data collection tools
- 3; Piloting of data collection tool and subsequent review
- 4; R+D / ethics milestones
- 5; Data collection period – first inclusions
- 6; Data collection period – last inclusions
- 7; Data collection period – last follow-up visits
- 8; Data entry
- 9; Analysis
- 10; Writing up reports / publications
- 11; Dissemination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19. RESOURCES, EQUIPMENT AND PHYSICAL FACILITIES

In addition to the departmental support, and in order to facilitate the completion of the work, Dr Shepherd will have full access to all of the necessary world-leading extreme environment facilities that are housed within the department estate given that they are pivotal to the success of the project. Specifically, this will enable temperature control within the facilities to ensure reproducibility. In addition, other equipment, including a thermal imaging camera, laser Doppler system and an iontophoresis kit will be made available. Finally, the department will ensure that technical assistance is provided to enable the set-up of the laboratories and testing rooms, and support with randomisation and beetroot juice dispensing to maintain the double-blind nature of this study.

20. DISSEMINATION AND OUTCOME

RP can cause significant discomfort and pain to individuals during a vasospasm. Dietary nitrate appears to offer a simple, low cost means of modifying blood flow to the peripheries and, ultimately, reducing both the discomfort and pain experienced by individuals with RP. This study will also advance our understanding of the aetiology and pathophysiology of RP, specifically the role that the nitrate-nitrite-nitric oxide pathway might play in modulating RP symptoms. An understanding of the effects of concentrated beetroot juice on microvascular blood flow and pain may lead to a range of simple, low cost and effective therapeutic interventions to prevent and treat episodes of RP.

Results (estimates of effect sizes and confidence intervals) from this pilot study will be utilised to define our primary outcome and to power a larger, definitive multi-centre randomised control trial with our co-applicants and collaborators around Europe. Once our final report has been submitted to Scleroderma and Raynaud's UK, we will apply for NIHR funding via a researcher-led Research for Patient Benefit (RfPB) or an Efficacy and Mechanism Evaluation (EME) bid (depending on the effect size and sample size) and or a Horizon 2020 collaboration bid. The results will be disseminated through publication in peer-reviewed scientific and clinical journals and via presentations, local, national and international meetings. A summary of the results will be sent to all the participants. Finally, an open day and presentation will be carried out where the participants will be informed of

the study findings and have the opportunity to ask any pertinent questions and/or provide feedback.

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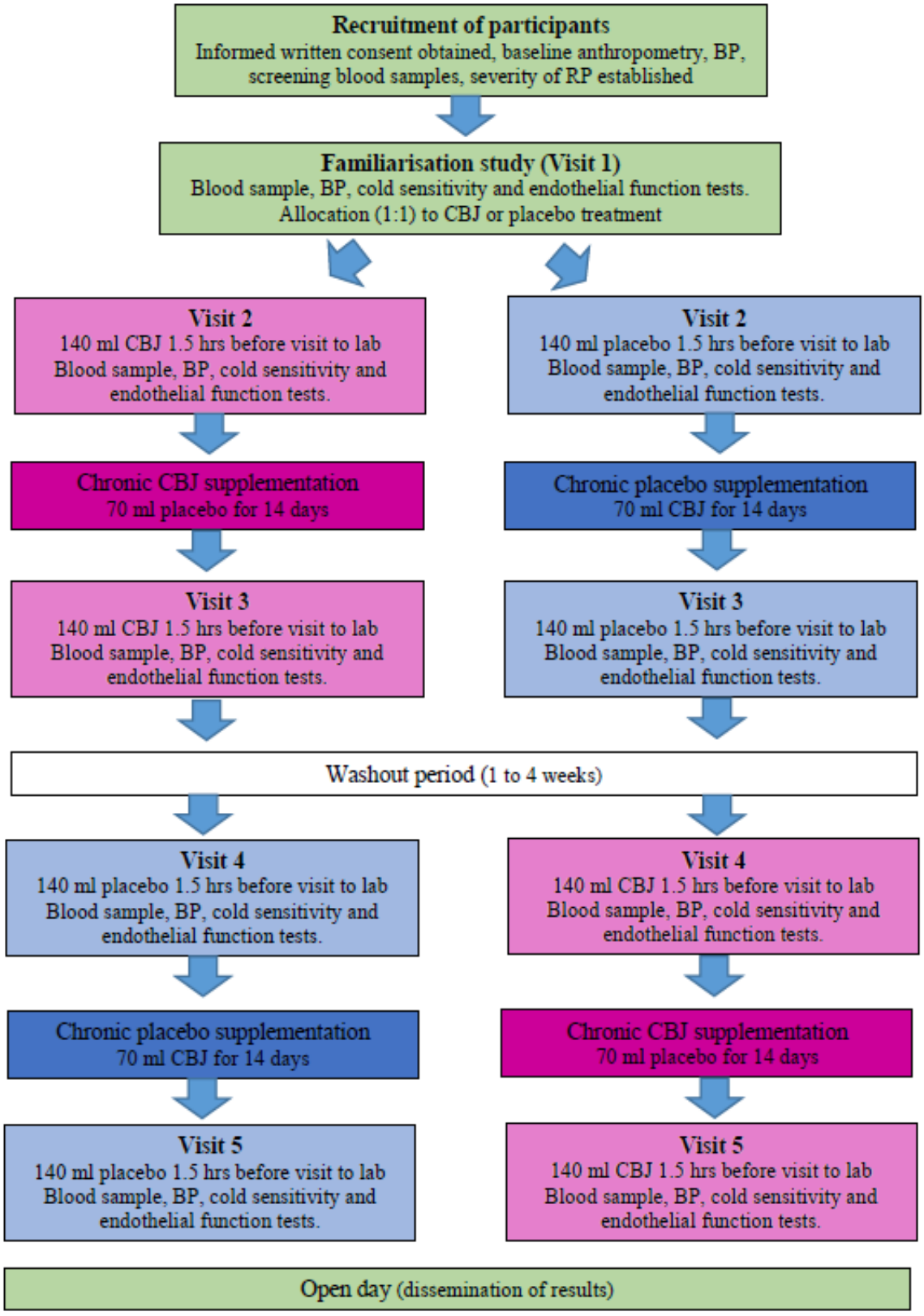
22. APPENDIX 1 SCHEDULE OF PROCEDURES

See Gantt chart in section 19.

23. APPENDIX 2 STUDY FLOW CHART


Recruitment of participants
Informed written consent obtained, baseline anthropometry, BP, screening blood samples, severity of RP established
Familiarisation study (Visit 1)
Blood sample, BP, cold sensitivity and endothelial function tests. Allocation (1:1) to CBJ or placebo treatment
Visit 2
140 ml CBJ 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Visit 2
140 ml placebo 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Chronic CBJ supplementation
70 ml placebo for 14 days
Chronic placebo supplementation
70 ml CBJ for 14 days
Visit 3
140 ml CBJ 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Visit 3
140 ml placebo 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Washout period (1 to 4 weeks)
Visit 4
140 ml placebo 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Visit 4
140 ml CBJ 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Chronic placebo supplementation
70 ml CBJ for 14 days
Chronic CBJ supplementation
70 ml placebo for 14 days
Visit 5
140 ml placebo 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Visit 5
140 ml CBJ 1.5 hrs before visit to lab Blood sample, BP, cold sensitivity and endothelial function tests.
Open day (dissemination of results)

24. APPENDIX 3 PARTICIPANT INFORMATION SHEET

See attachment (booklet format)

25. APPENDIX 4 INFORMED CONSENT FORM

26. APPENDIX 5 SAMPLE QUESTIONNAIRES

27. APPENDIX 6 SAMPLE DATA COLLECTION FORMS

28. APPENDIX 7 DRUG INFORMATION (SUMMARY OF PRODUCT CHARACTERISTICS)

29. APPENDIX 8 MANUFACTURERS BROCHURE FOR NOVEL EQUIPMENT

30. APPENDIX 9 CONTRACTUAL AGREEMENTS WITH OUTSIDE CONSULTANTS / COLLABORATORS / INSTITUTIONS (E.G. INDUSTRY, CONTRACT RESEARCH ORGANISATIONS)