EFFECTS OF COCOA FLAVANOLS ON PERIPHERAL BLOOD FLOW AND SYMPTOMS IN PRIMARY RAYNAUD'S PHENOMENON

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Thesis submitted for the degree of Doctor of Philosophy at the University of Nottingham

November 2020

Abstract

Primary Raynaud's phenomenon (PRP) is characterised by the periodic vasospasm of the digits precipitated by exposure to cold/emotional stimuli. PRP may involve vascular endothelium dysfunction, abnormalities in neural control of vascular tone and/or increases in circulating mediators which promote vasoconstriction. Cocoa products have been shown to promote vasodilation, and therefore could mitigate PRP symptoms. The work described in this thesis investigates the effects of cocoa flavanols on peripheral blood flow, thermoregulation and symptoms in people with PRP.

The acute effects of high flavanol cocoa (HFC) consumption on cardiovascular parameters and peripheral thermoregulation was compared between people with PRP and control participants using a double blind, cross over design. The chronic effects of 3 months daily consumption of HFC was then investigated using a double blind, placebo-controlled, parallel group design in people with PRP. The experiencing of Raynaud's symptoms, their duration and level of pain, and an assessment of Raynaud's Condition Score were documented by participants in a symptoms diary throughout the intervention. Dietary macronutrient composition as well as total flavanol and epicatechin intake, were assessed monthly using diet diaries and Food Frequency Questionnaires, respectively, with Physical and Mental Health status being determined at these time points using the SF-36™. A Finometer was used to measure cardiovascular variables and Laser Doppler Flowmetry used to assess skin blood flow before and after the supplementation period. In pilot studies, FMD was used to determine endothelial function following HFC consumption.

Data from the pilot studies found that acute consumption of a HFC drink induces a greater vasodilatory response to a shear stress stimulus at 1hr compared to a LFC drink. In acute supplementation studies, it was found that consumption of a HFC drink did not impact peripheral thermoregulation in either PRP participants or controls. Meanwhile, consumption of HFC

capsules in a chronic supplementation study reduced total peripheral resistance and increased cardiac output, but these beneficial effects were not accompanied by an increase in skin blood flow, or improvements in Raynaud's symptoms among PRP participants. It was also observed that in this cohort, having PRP did not appear to negatively impact mental or physical wellbeing, compared to a general population. The thesis proposes that it is feasible to recruit, retain, and provide follow-up with the participants for a full randomised control trial involving individuals with PRP in the future.

The findings from acute supplementation studies demonstrated no differential effect of HFC drink on cardiovascular measures and skin blood flow in either PRP participants or controls, while data from chronic supplementation study found no differential effect of HFC capsules on skin blood flow and Raynaud's symptoms among PRP participants. Therefore, it is important that people with PRP are aware that cocoa flavanols probably will not help to reduce their symptoms, despite studies that suggest their consumption improves the compliance of resistance vessels and microvasculature in healthy individuals.

Presentations

Wan Syaheedah Ghazali, Liz Simpson, Ian Macdonald. Pilot Study to investigate the effect of cocoa flavanols on symptoms in Primary Raynaud's Phenomenon; introducing the protocol and preliminary data. Life Sciences Postgraduate Symposium 2019, School of Life Sciences, University of Nottingham UK. **Oral Presentation.**

Wan Syaheedah Ghazali, Liz Simpson, Ian Macdonald. Effects of Cocoa Flavanols on Peripheral Vascular Function in Healthy Subjects. Life Sciences Postgraduate Symposium 2015, School of Life Sciences, University of Nottingham UK. **Poster Presentation.**

Wan Syaheedah Ghazali, Liz Simpson, Ian Macdonald. Pilot Study to investigate the effect of cocoa flavanols on symptoms in Primary Raynaud's Phenomenon; introducing the protocol and preliminary data. Allied Health Postgraduate Research Conference 2019, School of Health Sciences, School of Pharmacy, Division of Rehabilitation Ageing and Wellbeing, University of Nottingham UK. **Poster Presentation.**

ACKNOWLEDGEMENTS

Praise be to Allah

Declaration

I hereby declare that the work presented in this thesis, has not been submitted for any degree or diploma, at this, or any other university, and that all of the experiments, unless otherwise stated, were performed by me.

Wan Syaheedah Wan Ghazali

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Abbreviations

Ach	acetylcholine
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
AVA	arteriovenous anastomoses
BF	blood flow
BMI	body mass index
BP	blood pressure
Ca^{2+}	calcium ion
cAMP	cyclic Adenosine Monophosphate
cGMP	cyclic Guanosine Monophosphate
CGRP	Calcitonin Gene-Related Peptide
CO	cardiac output
CONSORT	Consolidated Standards of Reporting Trials
COX	cyclooxygenase
CPT	cold pressor test
CV	coefficient of variation
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
ED	endothelial dysfunction
EDHF	Endothelium Derived Hyperpolarizing Factor
EETs	Epoxyeicosatrienoic Acids
eNOS	endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FFQs	Food Frequency Questionnaires
FMD	Flow Mediated Dilatation
gm	gram
GTN	glyceryl trinitrate
GTP	Guanosine Triphosphate
HCU	Height Correction Unit
HFC	high flavanol cocoa
HR	heart rate
IU	international units

iNOS	inducible Nitric Oxide Synthase
K^+	potassium ion
kg/m^2	kilogram-metre squared
kJ	kilojoule
LDF	Laser Doppler Flowmetry
LFC	low flavanol cocoa
mg	milligram
mg/d	milligram per day
ml	milliliter
mmHg	millimetre of mercury
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NMMA N^G	Monomethyl-L-Arginine
nNOS	neuronal Nitric Oxide Synthase
NO	nitric oxide
NOS	Nitric Oxide Synthase
PGI_2	prostacyclin
PU	perfusion units
PRP	Primary Raynaud's Phenomenon
RBC	red blood cell
RCS	Raynaud's Condition Score
RCT	Randomised Controlled Trial
RP	Raynaud's Phenomenon
RT	room temperature
SBP	systolic blood pressure
SD	standard deviation
SPSS	Statistical Package for Social Science
SRP	Secondary Raynaud's Phenomenon
SV	stroke volume
TPR	total peripheral resistance
TXA_2	thromboxane
TNF-α	tumour necrosis factor-alpha
UK	United Kingdom
USB	Universal Serial Bus

Chapter 1: General Introduction

1.1 An overview of vascular functions in the healthy state

1.1.1 The structure of the vasculature

(i) The vasculature of hands

The hand has a rich and complicated vascular network. The main blood supply to the hand is developed from the superficial and deep palmar arches, originating from ulnar and radial arteries which are interconnected by various collaterals (Gellman *et al.*, 2001). The digital arteries derive from the superficial palmar arch which then further divides to supply the sides of the digits and finger pulps. The blood vessels in the skin are superficial and the arrangement of the venous network allows heat transfer (Figure 1.1)
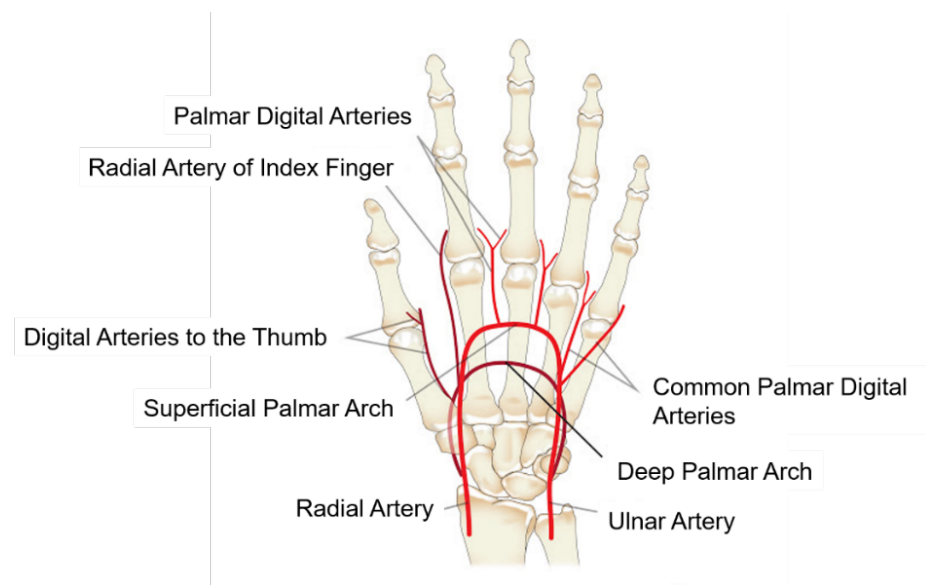
Figure 1.1: The vasculature of hands (American Society for Surgeon of the Hand).

(ii) The cutaneous vasculature

The vasculature of the skin plays an important part in skin surveillance and body thermoregulation as well as delivering important nutritional functions. The human skin is capable of dissipating heat by increasing the blood flow and dilating the superficial vessels (Swerlick, 1997). The cutaneous microvasculature includes a lower horizontal plexus lying at the junction of adipose tissue, and dermis, connected by vascular channels to an upper horizontal plexus lying in the papillary dermis (Figure 1.2). These channels permit transfer between the two plexuses and supply branches to hair bulbs and sweat glands. Capillary loops arise from the upper plexus, with the ascending arm sharing characteristics with the arterioles and the descending with the venules. The cutaneous microvasculature is characterised by extremely thick walls (2-3µm) compared to the normal 0.1 µm thickness in most tissues (Braverman, 1983).

At acral skin sites, increasing numbers of arteriovenous anastomoses (AVA) are found. AVAs are found at acral sites, which include the fingertips, toes, ears and tip of the nose, and allow blood to bypass the superficial plexus of the skin (Rowell, 1986). AVA are situated deeper in skin in the subcutaneous fat, at sweat glands level. AVA allow fluctuations in regional blood flow rates, resulting in transfer of heat to the surroundings. This is due to their inside diameter being up to 100µm compared to the common arteriole which has an internal diameter of 10µm (Grant and Bland, 1931).
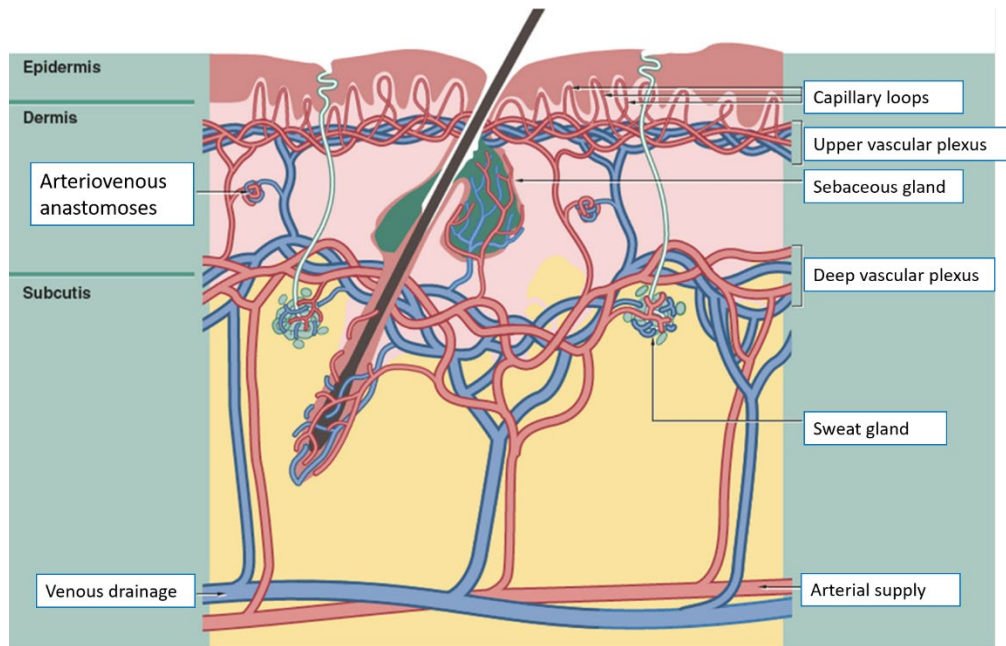
Figure 1.2: The cutaneous vasculature (Morikawa *et al*., 2019).

(iii) The neural regulation of cutaneous blood flow

The thermoregulatory control of human cutaneous blood flow is essential to the maintenance of normal body temperatures during challenges to thermal homeostasis. Human cutaneous blood flow is regulated by 2 types of sympathetic nerves, the sympathetic adrenergic vasoconstrictor and the sympathetic vasodilator nerves. Both the nerves innervate nonglabrous skin (the vast majority of the skin on the body, which contains different types of hairs) areas (Charkoudian, 2003), while only sympathetic vasoconstrictor nerves innervate glabrous skin (non-hairy skin) areas such as lips, palms, soles (Johnson and Proppe, 1996). Apart from that, the presence of AVA, which are low-resistance channels that permit high flow rates directly from arterioles to venules and are thick-walled, is another main difference between glabrous and nonglabrous skin. AVA are abundant and innervated by sympathetic vasoconstrictor nerves; thus, the opening or closing of these AVA may lead to large alterations in cutaneous blood flow (Lossius *et al*., 1993).

Cutaneous blood flow and thermoregulation

In humans, physiological thermoregulation includes the generation of heat by shivering as well as conservation or dissipation of heat by changes in cutaneous blood flow and sweating following different internal and external thermal stimuli. The site for central thermoregulatory control is in the anterior hypothalamus/preoptic area in the brain. Information on skin and core temperatures is relayed and carried to these areas of the brain which then coordinate the relevant efferent response (Boulant, 2000). The anterior hypothalamus/preoptic area is similar to a thermostat, which initiates heat conservation or dissipation, or heat generation. The control of thermoregulation by the anterior hypothalamus/preoptic area is summarized in Figure 1.3.
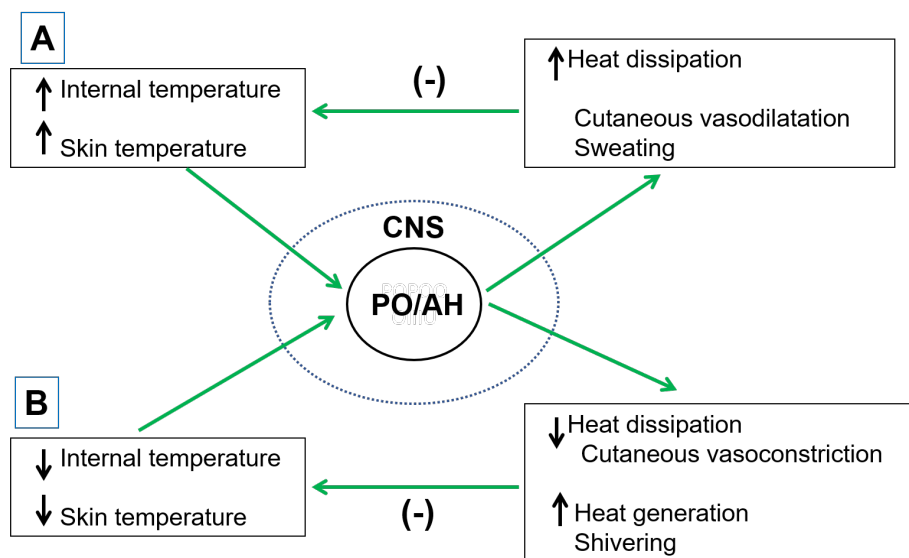
Figure 1.3: Negative feedback loops required in physiologic thermoregulation in humans. Minus sign indicates the correction of the error signals which is changes in the skin/ or internal temperature by the relevant effector response. A, Increases in skin and/or internal temperatures are detected by the preoptic/anterior hypothalamus (PO/AH) causing heat dissipation by cutaneous vasodilatation and sweating, which then corrects the initial increased temperature. B, Decreased skin/or internal temperature result in decreases in heat dissipation by cutaneous vasoconstriction and increased heat generation by shivering to correct the initial decreases in temperature (Adapted from Charkoudian, 2003).

In thermoneutral environments, resting cutaneous blood flow is approximately 250ml/min and results in heat dissipation from the skin surface (Johnson and Proppe, 1996). Following heat exposure or exercise, the core body temperature rises, and cutaneous vasodilation and sweating occurs (Johnson and Proppe, 1996, Johnson, 1992). Cutaneous vasodilation raises blood flow to the skin severalfold, subsequently increasing the convective heat transfer from the core to the periphery. These large increases in cutaneous blood flow often require increased cardiac output to maintain blood pressure and redistribution of blood flow from areas such as the splanchnic region, that demonstrate concurrent vasoconstriction. These adjustments are usually sufficient to address the requirement for raised skin blood flow, such that the provision of oxygen to vital organs such as the heart is not compromised (Johnson and Proppe, 1996).

Together with cutaneous vasodilation, sweat evaporation also reduces skin temperature and results in the blood in the dilated cutaneous vessels cooling before it returns to the core. Overall, to get a stable state at which heat dissipation and heat generation are equivalent to maintain body temperature, cutaneous blood flow and sweating remain elevated in proportion to internal temperature until the body temperature is restored. Cutaneous blood flow decreases towards normal and sweating stops once the internal (core) temperature reduces towards normal (Charkoudian, 2003). In this sense, thermoregulation represents a classic negative feedback loop (Figure 1.3).

Exposure to cold environments can have both local and central thermoregulatory effects to induce a decrease in skin blood flow by cutaneous vasoconstriction. This vasoconstriction reduces the convective transfer of heat from the core to the skin surface and decreases heat dissipation from the skin surface. However, shivering starts if body cooling continues. These muscular contractions increase heat generation, and together with decreased heat dissipation from cutaneous vasoconstriction, assist in maintaining core temperature when exposed to cold (Charkoudian, 2003).

1.1.2 The endothelium

The endothelium, which lines the entire vascular system, is considered as a simple barrier between the blood and vessel wall. Endothelial cell structure and functional integrity are essential in maintaining the vessel wall and circulatory function. The blood vessel wall is formed of three layers: the tunica intima (consisting of endothelial cells and sub endothelial tissue), the tunica media (consisting primarily of smooth muscle cells) and the tunica externa (consisting of fibroblasts) (Kennedy and Touyz, 2019). Endothelial cells form the inner lining of the intima and this lining appears thicker and more continuous in arteries and veins compared to those in capillaries, which are thinner and fenestrated to allow for exchange of metabolites and gases (Rhodin, 2011).The structure of the vascular endothelium is shown in Figure 1.4.
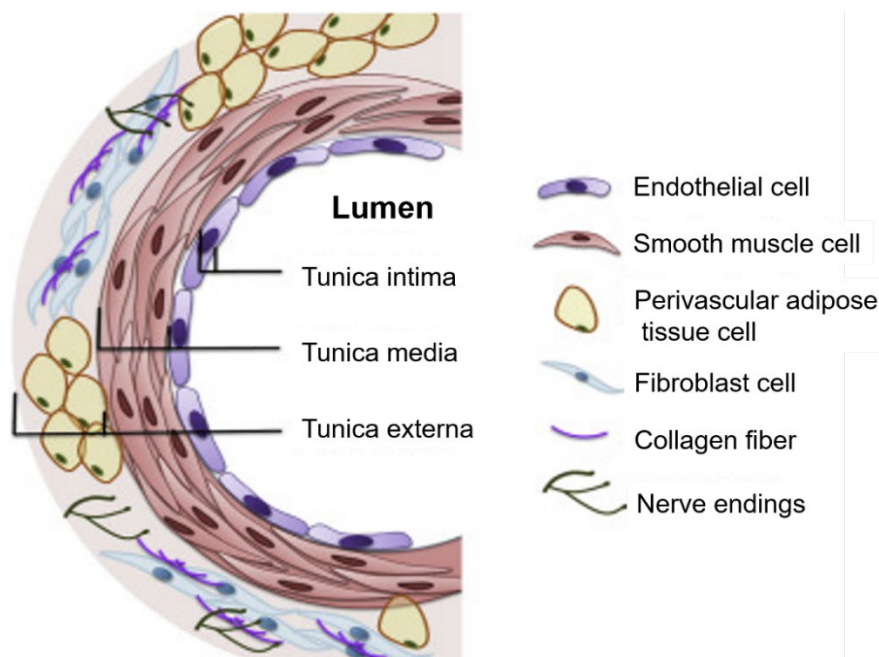
Figure 1.4: Structure of vascular endothelium (Zhao *et al.*, 2015).

(i) Endothelial functions

The endothelium is responsible for the regulation of vascular tone, leucocyte and platelet interaction with the vessel wall, regulation of thrombosis and thrombolysis and growth of blood vessels (Verhamme and Hoylaerts, 2006).

The present discussion will focus on the endothelium's role in regulating vascular tone.

a. Regulation of vascular tone

Endothelial cells release a variety of substances to regulate vascular tone. These can be vasodilatory factors such as nitric oxide, prostacyclin and endothelium derived hyperpolarizing factor, or vasoconstrictive factors such as thromboxane and endothelin-1. These factors are discussed in detail below.

Nitric oxide

Nitric Oxide (NO) is produced by conversion of the amino acid L-arginine to NO and L-citrulline by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS that produce NO. The predominant constitutive NOS isoform within the endothelium is eNOS and its activation leads to reduced vascular tone (Sprague *et al*., 2010). Inactive eNOS is bound to the protein caveolin, located in the structures of the cell membrane called caveolae (Bucci *et al.,* 2000). When intracellular levels of Ca^{2+} increase, eNOS detaches from the caveolin and becomes activated (Bucci *et al*., 2000). NO agonists such as bradykinin, ACh, adenosine tri-phosphate, adenosine di-phosphate, substance P and thrombin (Moncada and Higgs, 2006) can influence eNOS detachment from the caveolin by releasing Ca^{2+} from the endoplasmic reticulum. Once intracellular Ca^{2+} stores are depleted, a signal is sent to the membrane receptors to open Ca^{2+} channels allowing extracellular Ca^{2+} into the cell (Schilling *et al.*, 1992). Ca^{2+} binds to the protein calmodulin in the cell cytoplasm, after which it undergoes structural changes which allows it to bind to eNOS (Fleming and Busse, 1999). eNOS then converts L-arginine into NO (Palmer *et al.,* 1988). A reduction in Ca^{2+} leads to the calcium-calmodulin complex dissociating from eNOS, which in turn binds with caveolin and becomes inactivated (Fleming and Busse, 1999).

The short term increase in NO is dependent on the intracellular Ca^{2+} but once this decreases, additional mechanisms are activated to regulate NO production which is the phosphorylation of eNOS (Butt *et al.*, 2000). This mechanism occurs via protein kinases (Michael and Feron, 1997). Increases of the hemodynamic forces on the vessel wall such as shear stress, causes activation of specialised Ca^{2+}-activated K^+ channels on the endothelial cell surface, leading to K^+ efflux and Ca^{2+} influx into the cell (Moens *et al.*, 2005). The contribution of eNOS phosphorylation and Ca^{2+} to NO production is dependent on the period of the shear stress. For instance, release of intracellular Ca^{2+} is dependent on shear stress of short periods (Kuchan and Frangos, 1994). Meanwhile, shear stress of longer periods (>30 minutes) can reduce intracellular Ca^{2+} stores, and thus, production of NO is dependent on eNOS phosphorylation (Pittner *et al.,* 2005). NO diffuses across the endothelial cell into the adjacent smooth muscle and binds to the enzyme soluble guanylyl cyclase. Activated guanylyl cyclase enzyme raises the conversion rate of guanosine triphosphate (GTP) to cGMP causing decreases in smooth muscle tension (Jones *et al.*, 1999). These actions cause reduction in the contraction of smooth muscle cells and vasodilation. The NO signalling pathway is shown in Figure 1.5.
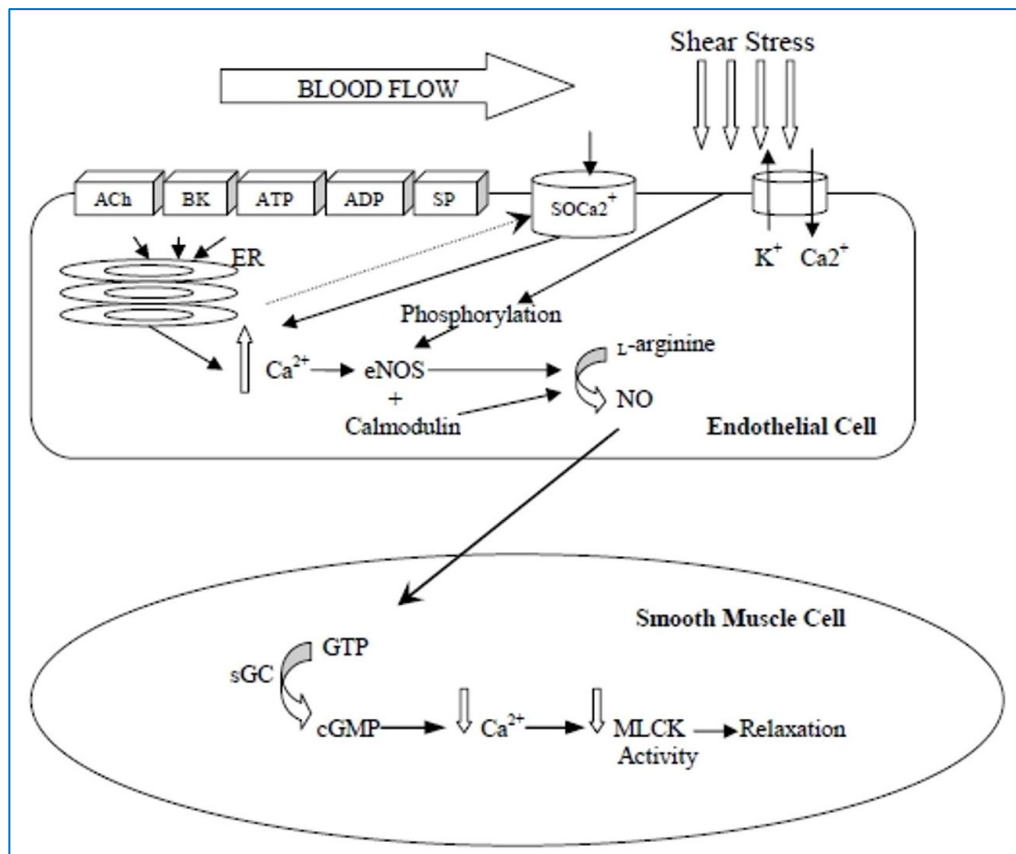
Figure 1.5: Endothelial nitric oxide production and its actions in the vascular smooth muscle cell. ACh= acetylcholine; BK= bradykinin; ATP= adenosine triphosphate; ADP= adenosine diphosphate; SP= substance P; $SOCa^{2+}$= store-operated Ca^{2+} channel; ER= endoplasmic reticulum; NO= nitric oxide; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5-monophosphate; MLCK= myosin light chain kinase. *When Ca^{2+} stores of the endoplasmic reticulum are depleted, a signal is sent to $SOCa^{2+}$ channel which allows extracellular Ca^{2+} into the endothelial cell (Sandoo *et al.*, 2010).

Prostacyclin and Thromboxane A₂

The synergistic actions of two prostanoids, thromboxane (TXA_2) and prostacyclin (PGI_2), also play a role in the regulation of vascular function. Their production is catalysed by cyclooxygenase (COX) enzymes, which are present in two isoforms, namely COX-1 and COX-2 (Flavahan, 2007). COX-1 is released continuously in endothelial cells, while COX-2 is only released

when the endothelium is exposed to inflammatory cytokines (Needleman and Isakson, 1997).

COX-2 can cause conversion of arachidonic acid to prostaglandin H_2 which is then synthesised into PGI_2 by prostacyclin synthase (McAdam *et al.*, 1999). PGI_2 then binds to the prostacyclin receptors (Coleman *et al.*, 1994), which are found on vascular smooth muscle cells and platelets (Chow *et al.*, 2003). Binding of PGI_2 to smooth muscle cell prostacyclin receptors activates adenylate cyclase which stimulates the synthesis of cyclic adenosine monophosphate (cAMP) (Fetalvero *et al.,* 2007). cAMP can activate protein kinase A leading to relaxation of the smooth muscle (Fetalvero *et al.*, 2007).

On the other hand, TXA_2 which is synthesised by thromboxane synthase (Bunting *et al.,* 1983) causes vasoconstriction and platelet aggregation (Thomas *et al.,* 1998). TXA_2 produces its effects by acting on thromboxane-prostanoid receptors located on platelets, and their activation results in platelet aggregation (Heptinstall *et al.*, 2006). The balance in the PGI_2 and TxA_2 activity in healthy vessels helps in maintaining homeostasis.

Endothelin-1

Endothelin-1 (ET-1), which is released from the endothelial cells, is produced by converting Big-ET-1 to ET-1 by endothelin converting enzyme (Yanagisawa *et al.*, 1988). Regulation of ET-1 production and release of ET-1 is stimulated by cytokines released from inflammatory cells, which include TNF-α and interleukin, and decreased by PGI_2 and NO (Alonso and Radomski, 2003).

ET-1 receptors have been found on endothelial cells (ET_{B1}) and smooth muscle cells (ET_A and ET_{B2}) (Davenport *et al.,* 1995). The activation of ET_{B1} receptors on the endothelium results in vasodilatation by inducing the release of PGI_2 and NO (Cardillo *et al.*, 2000). Binding of ET-1 to ET_A or ET_{B2} receptors causes opening of smooth muscle Ca^{2+} channels allowing extracellular Ca^{2+} into the cell. This leads to vasoconstriction in the same

way as TxA_2. In the phenomenon of endothelial dysfunction, ET_{B2} receptors on smooth muscle cells are upregulated while ET_{B1} receptors on the endothelial cells are downregulated, therefore leading to vasoconstriction predominating (Bohm *et al.*, 2002).

Endothelium-Derived Hyperpolarising Factor

The endothelium-derived hyperpolarising factor (EDHF), which is currently an unidentified vasodilator substance, is released when there is activation of endothelial cells by agonists which include ACh and bradykinin (Cohen and Vanhoutte, 1995). EDHF causes the hyperpolarisation of underlying smooth muscle by making the membrane potential of the cell more negative (Feletou and Vanhoutte, 2006). In addition, PGI_2 and NO can also cause dilatation of the vessel by hyperpolarising the smooth muscle cells. However, when there is inhibition of NO and PGI_2, hyperpolarisation still occurs, indicating the involvement of a third hyperpolarising factor (Scotland et al., 2005). Various pathways have been suggested as causing this hyperpolarisation. Although the exact mechanism is not known, three factors in particular have been suggested.

The activation of endothelial receptors and the subsequent increase in Ca^{2+} levels lead to efflux of K^+ from the cell (Edwards and Weston, 2004). The smooth muscle cell reacts to changes in the extracellular K^+ levels by releasing K^+, resulting in hyperpolarisation (Edwards *et al.*, 1998). Changes in the membrane potential of the smooth muscle cell then reduces the levels of Ca^{2+}, leading to relaxation (Edwards and Weston, 2004).

Epoxyeicosatrienoic acids (EET), which are synthesised in the endothelial cell, are products of arachidonic acid metabolism (Quilley and McGiff, 2000). EET play their role by raising the efflux of K^+ from the smooth muscle cells leading to hyperpolarisation and relaxation (Gauthier *et al.,* 2002). However, when there is inhibition of EET activity in the vessel, hyperpolarisation still occurs (Petersson *et al.*, 1997) indicating that other mechanisms must be responsible in the hyperpolarisation of smooth muscle cells.

Gap junctions are intercellular channels involved in transferring signals from the endothelial cells to the smooth muscle cells (Sandow and Hill, 2000). It is suggested that gap junctions in particular can transfer K^+ ions from the smooth muscle cells into the endothelial cell (Bryan *et al.,* 2005). However, the exact mechanism is not known since previous studies have only transferred artificial dye between the two cells.

1. 2 An overview of Raynaud's Phenomenon

1.2.1 Introduction to Raynaud's Phenomenon

Raynaud's phenomenon (RP), named after the French physician Maurice Raynaud, is a disorder of the microvasculature that generally affects the fingers and toes but can present on other sites which include the nose, ears and nipples (Block and Sequeira, 2001, Goundry *et al.*, 2012). Raynaud first described the disease in his 1862 thesis, believing his patients' symptoms resulted from the deregulated constriction of precapillary arterioles caused by an overactive neurological reflex (Wigley, 2002). Overall, RP is a transient and peripheral vasoconstrictive response to cold temperatures or emotional stress (Musa and Qurie, 2019).

 Clinically, RP is divided into Primary Raynaud's Phenomenon (PRP) and Secondary Raynaud's Phenomenon (SRP). PRP is the presence of symptoms without any known underlying cause (Herrick, 2005) and clusters in certain families (Maricq *et al.,* 1986), consistent with a genetic dimension to the disorder. On the other hand, SRP symptoms are thought to develop as a consequence of a systemic autoimmune disease, particularly mixed connective tissue disease (Grader-Beck and Wigley, 2005) or systemic sclerosis (Guiducci *et al.*, 2007).

PRP has an earlier age of onset than SRP and large epidemiological studies show the prevalence of PRP is higher in women. Moreover, in the United States, PRP has an estimated incidence of 10% with both the Black and White population being affected (Maricq *et al.,* 1986). Differentiating between primary and secondary RP is important as SRP can be

complicated by digital ischemia, ulceration and gangrene, whereas PRP is generally a benign condition. PRP attacks commonly have a symmetrical or bilateral involvement of the fingers and are accompanied by minimal pain. In contrast, asymmetrical or unilateral involvement of fingers as well as intense pain is reported in SRP, which may be due to the underlying pathology associated with autoimmune disease (Bakst *et al.*, 2008). Table 1.1 represents the summary of the differences between PRP and SRP.

Primary Raynaud's Phenomenon	Secondary Raynaud's Phenomenon
Younger age (30, but can be any age) Median age of onset is 14 years	Older age (>30, but can be any age)
Common in female Family history	Less common (10-20%)
No symptoms/signs of underlying disease	Symptoms and signs of underlying disease
Symmetrical/ bilateral involvement of fingers	Asymmetrical/ unilateral involvement of fingers
Less pain	Tightness of finger skin; more severe pain
No tissue necrosis or gangrene	Digital ischaemia (digital pitting scars, ulceration, or gangrene)
Normal nail fold capillaries	Abnormal nail fold capillaries
Normal erythrocyte sedimentation rate	Raised erythrocyte sedimentation rate
Negative antinuclear antibodies	Positive antinuclear antibodies or anti-extractable nuclear antigen antibodies

Table 1.1: Distinguishing between Primary and Secondary Raynaud's Phenomenon (Goundry *et al.*, 2012).

Regardless of the subtype, RP is characterised by ischemia of the digits, usually in response to a cold stimulus, which causes three phases of typical 'triphasic' colour changes, as well as numbness and swelling (Block and Sequeira, 2001). The first phase is characterised by pallor of the digits due to excessive vasoconstriction and cessation of regional blood flow. This phase is followed by a cyanotic phase (blue deoxygenation), as the residual blood in the finger desaturates. The red phase (reperfusion) is due to hyperaemia as the attack subsides and blood flow is restored (Kuryliszyn-Moskal *et al.,* 2015) (Figure 1.6).

Exposure to cold is reported to be the most common trigger to RP. Other triggers include emotional stress, forcible trauma or injury following vibrations, drugs such as beta-blockers as well as prolonged use of the digits as seen in an extended duration of typing (Brand *et al.*, 1997).

Figure 1.6: Three phases of typical 'triphasic' colour changes which include pallor (figure i), cyanotic (figure ii) and reactive hyperaemia with redness of the digits (figure iii) (Taken from Scleroderma and Raynaud's UK website).

1.2.2 Pathogenesis of Raynaud's Phenomenon

The main factor in the pathogenesis of RP is thought to be dysfunction in the control mechanisms (i.e., neural, endothelial, and intravascular) of vascular motility, causing an imbalance between vasodilatation and vasoconstriction (Herrick, 2005). The present discussion will be broadly considered under the headings of 'vascular', 'neural and 'intravascular'.

(i) Vascular abnormalities

a. Impaired vasodilatation

It is also reported that decreased endogenous vasodilator concentrations acting on the endothelium, or injury to the endothelium, may result in impaired vasodilatation in the peripheries (Herrick, 2005). In SRP and possibly also in PRP, it has been suggested that endothelial-independent vasodilation is compromised at a later stage of disease compared to endothelial-dependent vasodilation (vasodilation requiring an intact endothelium). This field of study has been simplified by the use of non-invasive approaches to measuring the vasodilatation in small and large vessels. A technique known as iontophoresis can be used to assess the microcirculation. It involves applying a small electric current at the skin surface to drive vasoactive chemicals through the epidermis and simultaneously measuring the blood flow responses using a laser Doppler. The 'endothelial-independent' component of vascular dilation can be assessed by using iontophoresis with sodium nitroprusside, as the nitroprusside acts as a NO donor bypassing the endothelium. On the other hand, 'endothelial-dependent' components can be assessed using iontophoresis of acetylcholine chloride, as its action involves the production of NO by eNOS within the endothelium. In PRP patients, Khan *et al*., (1997) demonstrated impairment of both endothelial independent and endothelial dependent vasodilatory responses (Khan *et al.,* 1997), while some studies have observed effects on endothelial-dependent vasodilatation alone in this patient group (Smith *et al.*, 1999).

b. Reduced production of vasodilators

The endothelium releases an abundance of vasodilator substances, including NO. These substances are produced in lower amounts when the endothelial function is impaired. A previous study reported that low availability of NO at the endothelium may lead to the development of RP symptoms, as topical use of topical glyceryl trinitrate (GTN) was shown to enhance localised blood flow in individuals who report RP symptoms. In this study, 2% GTN ointment or placebo ointment was rubbed on the dorsum of the first and second finger for 1 minute, respectively, while the third finger

remained untreated. Laser Doppler scanning of these three fingers was conducted immediately, 10 and 20 minutes after ointment application. It was found that an exogenous supply of NO by topical GTN ointment caused local endothelial-independent vasodilatory responses in PRP, SRP and control participants (Anderson *et al.*, 2002). This finding may indicate the role of NO in the pathogenesis of RP.

c. Increased vasoconstriction

Apart from vasodilator substances, the endothelium also releases vasoconstrictors such as ET-1, which is a potent vasoconstrictor and important mediator of vascular remodelling (Kirchengast and Munter, 1999). A previous study observed that in reaction to cold challenge, the levels of ET-1 increased more in PRP compared to controls, as well as being elevated at the baseline (Zamora *et al.*, 1990). On the other hand, Smyth et al., (2000) reported no difference in baseline ET-1 levels between PRP patients and healthy controls, but levels rose after whole-body cooling in the PRP but not in the control group (Smyth *et al.*, 2000).

(ii) Neural abnormalities

a. Impaired vasodilation

Calcitonin gene-related peptide (CGRP), an alternative product of the calcitonin gene, is released by nerves that supply blood vessels and is a very potent vasodilator of the microvasculature. CGRP triggers the intracellular synthesis of adenylate cyclase in smooth muscle cells, which results in increased intracellular cyclic adenosine monophosphate (cAMP). This in turn activates both endothelial dependent and independent mechanisms to cause smooth muscle cell relaxation (Russell *et al.,* 2014). In an immunohistochemical study, the number of CGRP-immunoreactive nerve fibres in biopsies of finger skin from SRP and PRP patients was significantly reduced compared to healthy controls (Bunker *et al.,* 1990).

b. Increased vasoconstriction

Noradrenaline is released by the depolarization of the sympathetic nerve fibre at the neuroeffector junction. Noradrenaline stimulates postjunctional α_1 and α_2-adrenoreceptors on the vascular smooth muscle cells, resulting in vasoconstriction. However, the α_2-adrenoreceptors are thought to be more important in the regulation of digital vascular tone than α_1 (Freedman *et al.,* 1995). The balance between α_1- and α_2-adrenoreceptors differs between proximal and distal arteries. Proximal and distal blood vessels were studied from each limb. It was found that α_1 and α_2-adrenoceptors were present on the vascular smooth muscle of arteries of human limbs, and that α_2-adrenoceptors responsiveness was more prominent on distal arteries compared with more proximal vessels (Flavahan *et al.*, 1987). This further supports the hypothesis that increased peripheral expression of α_2-adrenoreceptors could be involved in RP.

c. Central mechanism

Many patients with RP experience stress-induced vasospasm which may be due to the involvement of a central nervous component. Previous work by Edwards *et al.,* (1999) reported that patients with PRP do not habituate in the same way as healthy controls to the components of the 'alerting response' evoked by acute emotional stress. The effect of repetition of stimuli was studied in patients with PRP and in sex- and age matched control participants. A pure sound delivered by headphones was used as the stressor was delivered five times at randomized intervals on day 1 and again on days 3 and 5. For each sound, the participants were asked to score their level of discomfort on a rising scale of 0–10. In response to the first sound and repetition of the sound on day 1, both groups showed the pattern of similar alerting response, including vasodilation in forearm muscle and vasoconstriction in the finger. However, on each of days 3 and 5, the control participants showed habituation such that the individual components of the alerting response decreased from the first to the fifth stimulus, as well as over the three sessions in which the similar response had virtually

disappeared by day 5. Thus, the control participants showed short- and medium-term habituation. By contrast, the PRP patients show no habituation at all. The alerting response evoked by the last sound on day 3 were comparable in magnitude with those evoked by the first sound on day 1. By contrast, both groups awarded similar scores to the level of discomfort produced by the first sound on day 1 and showed habituation of their perception of discomfort over the three experimental sessions. Therefore, the interpretation of these results is that PRP patients show impairment of the central neural process of habituation, rather than a simple hyper-reactivity of the sympathetic nervous system as originally proposed by Raynaud (Edward *et al.,* 1998). Central mechanisms may be responsible for causing vasospasm but in most situations, peripheral neural mechanisms are the more important.

(iii) Intravascular Abnormalities

Below are discussed some of the intravascular factors involved in the pathogenesis of RP.

a. Platelet activation

Increased platelet aggregation and activation have been found in both PRP and SRP (Kallenberg *et al.,* 1982). Platelet activation can be demonstrated by increased circulating levels of β-thromboglobulin and thromboxane released from platelet α-granules. In a previous study, it was found that the levels of beta-thromboglobulin, a platelet specific protein released during platelet activation was elevated in 41 patients with PRP and 73 with SRP (Kallenberg *et al.,* 1982).

b. Impaired fibrinolysis

Fibrinolysis is considered to be normal in patients with PRP (Herrick *et al.*, 1996), but in 26 patients with systemic sclerosis, the defective release of tissue plasminogen activator has been described (Ames *et al.*, 1997).

c. Oxidative stress

A previous study was conducted to determine the level of serum oxidative stress parameters which included serum hydroperoxide level and total antioxidant capacity in 15 patients with PRP, 19 patients with SRP and 14 healthy control participants. It was found that the serum hydroperoxide concentrations were significantly higher and serum total antioxidant capacity level was significantly lower in PRP and SRP patients compared to the control participants. No significant differences in both the parameters were detected between the groups of RP patients (Biondi *et al.*, 2008).

d. Reduced red blood cell deformability

Reduced red blood cell deformability may reflect damage to the erythrocyte membrane by free radicals. In a study by Solans *et al.,* (2000), lipid peroxidation (marker for oxidative injury) and erythrocyte membrane fluidity was measured in patients with systemic sclerosis. Fluidity was assessed using fluorescence anisotropy measurements and lipid peroxidation products were determined as thiobarbituric acid–reactive substances. It was found that erythrocyte membrane fluidity was significantly lower and lipid peroxidation was significantly higher in patients with systemic sclerosis compared to patients with PRP and controls. These observations support the idea that oxidative injury occurs in systemic sclerosis and that, via lipid peroxidation, it induces structural and functional changes of the erythrocyte membrane that may lead to the development of the microvascular abnormalities (Solans *et al.,* 2000).

e. Increased blood viscosity

Increased blood viscosity has been reported in patients with RP. In a study by Picart *et al.*, (1998), it was found that the whole blood viscosity measured by a scanning capillary viscometer increased in patients with systemic sclerosis; however, the blood viscosity did not differ between patients with PRP and healthy controls (Picart *et al.,* 1998).

f. Leucocyte activation

Activated leucocytes may physically obstruct blood flow in the microvasculature and has been reported in patients with SRP and PRP (Lau *et al.,* 1992). In this study, leucocyte activity in 38 patients with SRP and 22 patients with PRP was measured using a whole blood leucocyte aggregation assay. It was found that leucocyte activity was increased in patients with PRP as well as those with SRP, which the authors suggested may be related to digital vasospasm (Lau *et al.,* 1992). Mechanisms contributing to RP are shown in Figure 1.7
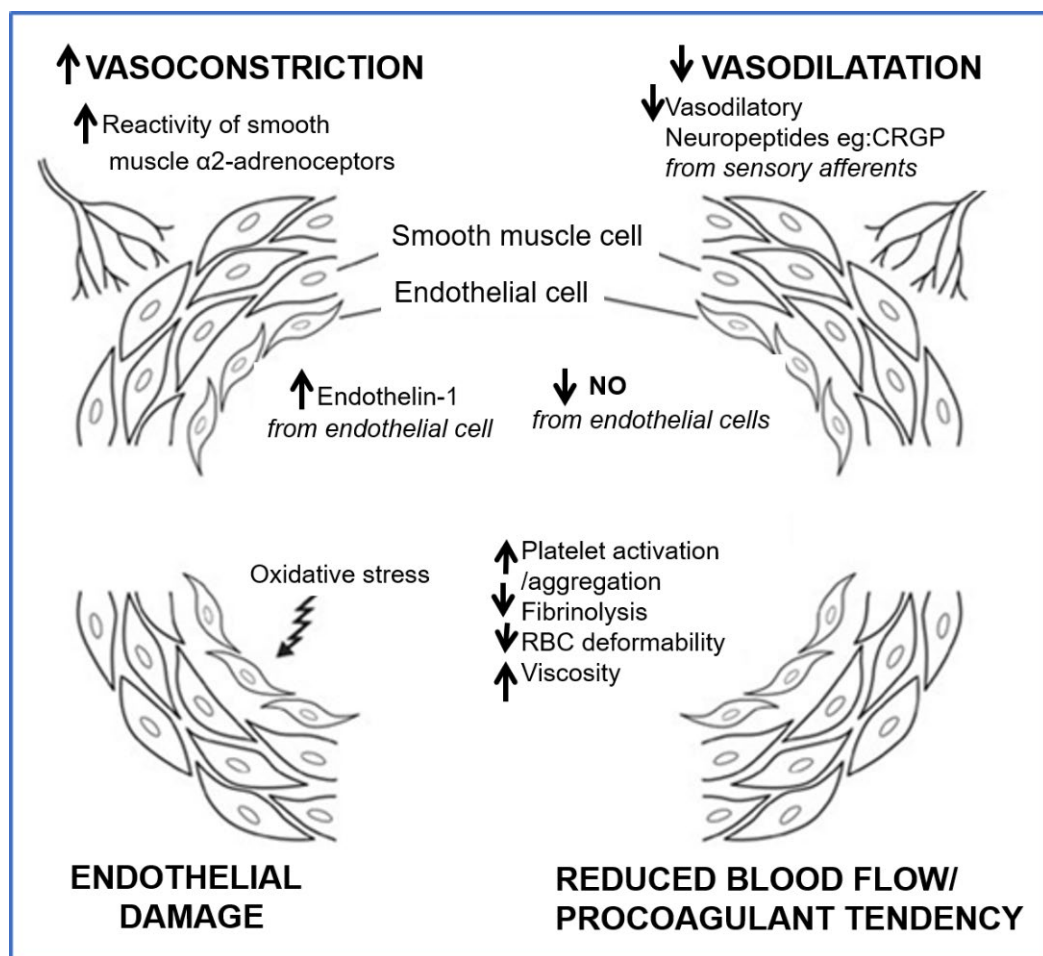
Figure 1.7 Mechanisms contributing to Raynaud's Phenomenon (Herrick, 2005).

1.2.3 Effect of Oestrogen in Raynaud's Phenomenon

Previous studies have reported that the incidence of RP is significantly higher in females *versus* age-matched males (Garner *et al.*, 2015). Seventy percent of American patients suffering from RP are estimated to be females. This clearly indicates a sex-based factor in the prevalence of the disease and therefore, suggests a potential role of sex hormones in its onset or pathology (Maricq *et al.,* 1993). Specifically, the incidence is higher in premenopausal *versus* post-menopausal women, with an interesting association between the menstrual cycle and cold-modulated digital blood flow (Greenstein *et al.*, 1996). In their study, 24 patients with PRP and 24 controls were exposed to environmental heating and cooling at three stages of the menstrual cycle (menstruation, periovulation, midluteal) to coincide with peaks and troughs in sex-hormone levels. It was found that cold sensitivity was altered during the menstrual cycle in both groups with the fastest finger rewarming pattern during menstruation suggesting that the menstrual cycle is associated with changes in the effect of cold on digital blood flow (Greenstein *et al.,* 1996)

Another study found that post-menopausal females receiving unopposed oestrogen replacement therapy (i.e. oestrogen replacement alone) were more likely to suffer from the disease than post-menopausal women that were not receiving oestrogen replacement therapy (Mayes, 1999). Therefore, these findings suggest that oestrogen may explain the higher incidence in premenopausal women (Figure 1.8). Chan *et al*., (2001) demonstrated that in premenopausal females, noradrenaline-mediated vasoconstriction is higher at the mid-point of the menstrual cycle, which is characterized by relatively high oestrogen levels, compared to those seen during the early stage of the cycle (Chan *et al.,* 2001).

Raynaud's + Oestrogen

- More α_{2c}-adrenoreceptor in females
- Oestrogen increases α_{2c}-adrenoreceptor expression
- High prevalence in premenopausal women
- High vasoconstriction in midmenstrual cycle
- High incidence in postmenopausal women on ERT
- Other factors?

Figure 1.8. Evidence of positive association between oestrogen and Raynaud's phenomenon. ERT; Oestrogen Replacement Therapy (Fardoun *et al.,* 2016).

Another study found that in vascular smooth muscle cells from rat tail arteries, 17β-oestradiol increased the α_{2C}-adrenoreceptor expression and selectively increased the cold-induced amplification of α_2-adrenoreceptor constriction, which is mediated by α_2-adrenoreceptor. Therefore, an oestrogen-dependent increase in the expression of cold-sensitive α_{2C}-adrenoreceptors may contribute to the increased activity of cold-induced vasoconstriction seen in pre-menopausal females (Eid *et al.,* 2007). Interestingly, among the α_2- adrenoreceptors, only the α_{2C}-adrenoreceptor subtype is differentially expressed in rat tail arteries, with a greater expression in females tail arteries (McNeill *et al.*, 1999).

1.3   An overview of local cooling

1.3.1   Local cooling of the skin

Local cooling of the skin generates a potent localised vasoconstriction that can reduce cutaneous blood flow to zero. A previous study showed–that vasoconstriction induced by local cooling is achieved by virtue of its ability

to selectively cause amplification of the α_2-adrenoreceptor mediated vasoconstrictor effects (Eid *et al.,* 2008). This selective amplification of α_2-adrenoreceptor allows cold induced constrictive effects to overcome any local drivers of vasodilation (Freedman *et al.*, 1993).The main components involved are the role of the vasoconstrictor via postsynaptic upregulation of α_{2c}-adrenoreceptor and inhibition of the NO system (Johnson and Kellogg, 2010).

During whole body or localised cooling, blood flow to the skin is reduced to avoid excessive heat loss. This effect is mediated by a reflex action of the sympathetic nervous system and appears to be abnormally augmented in RP. Moreover, it has been reported that following exposure to cold, the expression of α_2-adrenoreceptors on smooth muscle cells of cutaneous arterioles is markedly increased, causing potent vasoconstriction (Flavahan *et al.*, 2000).

A previous study reported that local cooling stimulates an increase in reactive oxygen species (ROS) activity from the mitochondria of vascular smooth muscle. Increased ROS activity causes activation of the Rho-kinase system and subsequently, leads to the translocation of α_{2c}-adrenoreceptors from the Golgi apparatus to the plasma membrane, which in turn induces vasoconstriction. Therefore, the cold-induced activation of the Rho-kinase system can mediate cold-induced constriction in cutaneous arteries by enabling the translocation of α_{2c}-adrenoreceptors to the plasma membrane (Bailey *et al.*, 2004). Another study reported that intradermal administration of a Rho-Rho kinase inhibitor (fasudil) to forearm skin via microdialysis, caused a reduced and delayed vasoconstrictor response to local cooling, suggesting that Rho kinase mediates cold-induced vasoconstriction (Thompson-Torgerson *et al.*, 2007).

1.3.2 Effect of local cooling on cardiovascular response

(i) In healthy people

The human thermoregulatory system is sensitive to thermal information both within the body core and at the skin surface. Detection of decreased body temperature leads to peripheral vasoconstriction which reduces peripheral blood flow and thus, reduces the heat transfer from the core to skin. Conversely, as core temperature rises, peripheral vasodilation and increased blood flow at the skin's surface enables heat to pass from the core to the skin and subsequently, to the atmosphere (Nadel, 1977).

The basic outcome of exposure to the cold is a rapid and generalised decrease in skin temperature causing cutaneous vasoconstriction (Webb-Peploe and Shepherd, 1968), with increased tone in the capacitance vessels, resulting in a reduction in the blood volume at the periphery. The heat flux from the body core to skin involves convective and conductive pathways. In the convective pathway, the heat is carried by the blood via a variable resistance that includes skin vessels, where the tone is under autonomic control. In the conductive pathway, the heat is transferred across a fixed resistance that is determined by the thickness of subcutaneous body fat (Nadel, 1977).

During cold exposure, the reduction in peripheral blood flow as a consequence of cold exposure causes delays in the cooling of deeper peripheral tissues and as a result, the predominance of conductive pathways for heat loss. A combination of these factors, together with increased thermogenesis during cooling of the skin, can result in a paradoxical increase in core temperature, which can be seen during the early phase of cold exposure (Hardy, 1953).The duration of increased core temperature is dependent on the muscle mass and thickness of subcutaneous fat.

The cold-induced increase in peripheral arterial resistance is elicited via activation of β-adrenergic receptors (Granberg *et al.,* 1971), through direct action by increased plasma noradrenaline concentration, resulting in vasoconstriction and reduction in peripheral blood flow (Iatridis, 1987).

These changes in the skin blood vessels cause displacement of blood into deeper veins from cutaneous vessels and into the core from the periphery (Rowell, 1974), generating an increase in mean arterial pressure, stroke volume and cardiac output. All of these processes lead, in healthy individuals, to a decrease in heart rate (Granberg *et al.*, 1971).

(ii) In people with Raynaud's Phenomenon

Freedman *et al.,* (1993) investigated the effects of local cooling on α-adrenergic responses in the fingers of patients with PRP. In their study, Clonidine HCl (an α_2- adrenergic agonist) and phenylephrine HCl (an α_1-adrenergic agonist) were administered through a brachial artery catheter while blood flow was measured by plethysmography in cooled and uncooled fingers. It was found that cooling potentiated α_2-adrenergic vasoconstriction in the patients but not in the control group, suggesting that the cold-induced sensitization of peripheral vascular α_2-adrenoceptors may be involved in the mechanism by which cooling triggers the vasospastic attacks of RP (Freedman *et al.*, 1993).

Another study found that vasospastic attacks can be induced by cooling, despite the blockade of the sympathetic nervous pathway, in RP. In this study, two fingers on one hand were anesthetized by local injection of lidocaine of 10 patients with PRP and 10 patients with scleroderma. The frequency of vasospastic attacks in nerve-blocked fingers was not significantly different from that in the corresponding intact fingers on the contralateral hand, suggesting that the vasospastic attacks of RP can occur without the involvement of efferent digital nerves (Freedman *et al.,* 1989).

It was also found that people with PRP have greater vasoconstrictor responses to α_1- and α_2- adrenergic agonists in uncooled fingers. Some attacks of RP are triggered by emotional stress (Freedman and Ianni, 1983), which is normally accompanied by catecholamine release (Hjemdahl *et al.*, 1986). Although RP patients do not have consistently higher plasma catecholamine levels than controls (Surwit *et al.*, 1983), catecholamine elevations induced by normal stress would cause greater digital

vasoconstriction in the patients when acting upon hypersensitive α_1- and α_2-adrenoceptors.

1.4   An overview of Cocoa Polyphenols

1.4.1   Composition

Cocoa beans consist of more than 300 identifiable chemical compounds (Araujo *et al.*, 2016). Many other substances are present in the cocoa matrix during fermentation, roasting and processing of the cocoa beans (Petyaev and Bashmakov, 2016). Previous studies have found at least three groups of substances in cocoa beans with potential health effects which include flavonoids (epicatechins catechin and procyanidin), methylxanthines (theobromine, caffeine) and minerals (magnesium, iron, and zinc). Procyanidins provide the majority of antioxidant activity in cocoa products (Ramiro-Puig and Castell, 2009) (Figure 1.9).

Flavonoids are a large class of dietary polyphenol compounds present in a range of fruits and vegetables. Flavonoids comprise about 12–18% by dry weight of the cocoa beans (Hii *et al.*, 2009) and confer a bitter taste to cocoa beans causing them to be unpalatable in their unprocessed form to most people. However, the amount of polyphenols in cocoa beans may become significantly reduced during manufacturing, thus affecting the antioxidant properties of the final cocoa products, but bringing about an improvement in the taste (Oracz *et al.,* 2015).

Xanthine derivatives caffeine, theobromine and theophylline are widely found in the human diet. These compounds naturally occur in food products such as tea, coffee and cocoa beans, with caffeine and theobromine being the two most abundant xanthines in chocolate. Caffeine is a central nervous system cardiac and respiratory stimulant, while theophylline and theobromine are clinically used as smooth muscle relaxants. All three of these compounds can cause diuresis (Srdjenovic *et al.,* 2008). Mean concentrations of methylxanthine determined in cocoa, coffee and tea are listed in Table 1.2.
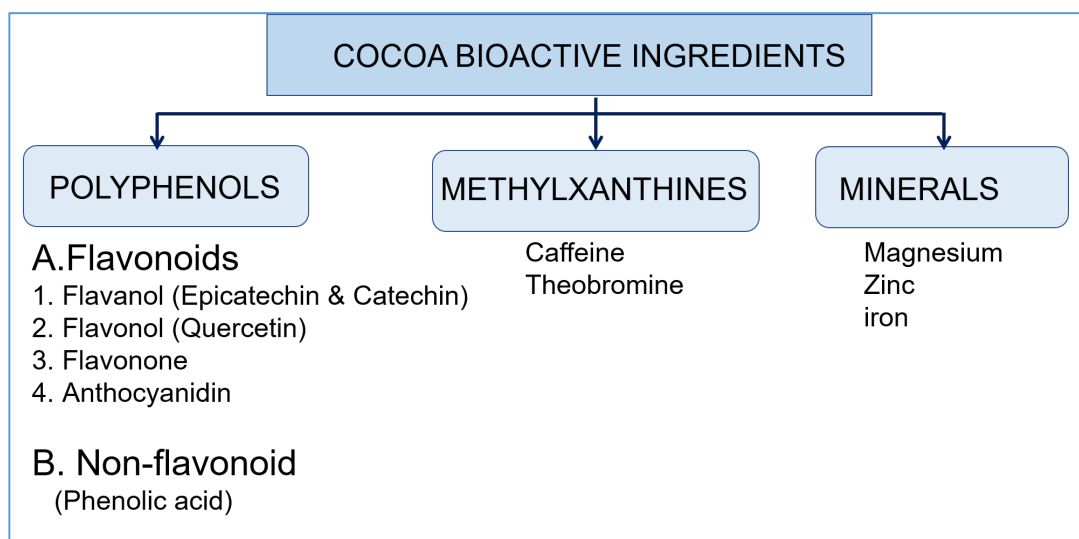
Figure 1.9: Cocoa bioactive ingredients (Petyaev and Bashmakov, 2017).

	Caffeine (µg/mL)	Theobromine (µg/mL)	Theophylline(µg/mL)
Cocoa	4	17.0	$< 10^{-7}$
Coffee	350	17.0	$< 10^{-7}$
Tea	217	12.0	$< 10^{-7}$

Table 1.2: Mean concentrations of methylxanthines in cocoa, coffee and tea (Bispo *et al.*, 2002).

1.4.2 Molecular structure

In 2012, Landete *et al.,* (2012) described polyphenols as substances with more than 1 phenol unit, the aromatic carbon ring with a hydroxide group (Landete, 2012). Polyphenols are a complex group of secondary plant metabolites and the most common group of phenolic compounds in the diet are flavonoids and phenolic acids (Manach *et al.*, 2004). Flavonoids can be further classified into subclasses based on the functional groups in the C ring which include flavanols (found in cocoa, tea, wine, fruits), flavonols (found in broccoli, tomato, onion) isoflavones (a component of soyabeans), flavones (found in herbs), anthocyanidins (rich sources include wine and berries) and flavanones (found in citrus juices and citrus fruits) (Manach *et al.*,2004).

The main categories of cocoa polyphenols are:

(i)   Flavanols, or flavan-3-ols, which constitute up to 38% of total polyphenols and are present as monomers and polymers. The monomers include (−)-epicatechin, (+)-catechin, (−)-epigallocatechin and (+)-gallocatechin (Figure 1.11). The most abundant polyphenol both in cocoa and cocoa derived products is epicatechin which represents up to 35% of total polyphenols (Aprotosoaie *et al.*, 2016).

(ii)   Proanthocyanidins. Chemically, these are oligomers- or polymers of monomeric flavanols, which constitute up to 65% of total polyphenols in cocoa (Aprotosoaie *et al.*, 2016).

(iii)   Anthocyanins, which constitute up to 4% of total polyphenols in cocoa (Aprotosoaie *et al.*, 2016).

Previous studies investigating refined cocoa products have demonstrated that (+) catechin, (-) epicatechin monomers and procyanidin dimers are significant to cardiovascular health (Fraga *et al.*, 2010). Chemical structure of flavanol monomers is shown in Figure 1.10.

(+)-Catechin   (-)-Epicatechin

(+)-Gallocatechin   (-)-Epigallocatechin
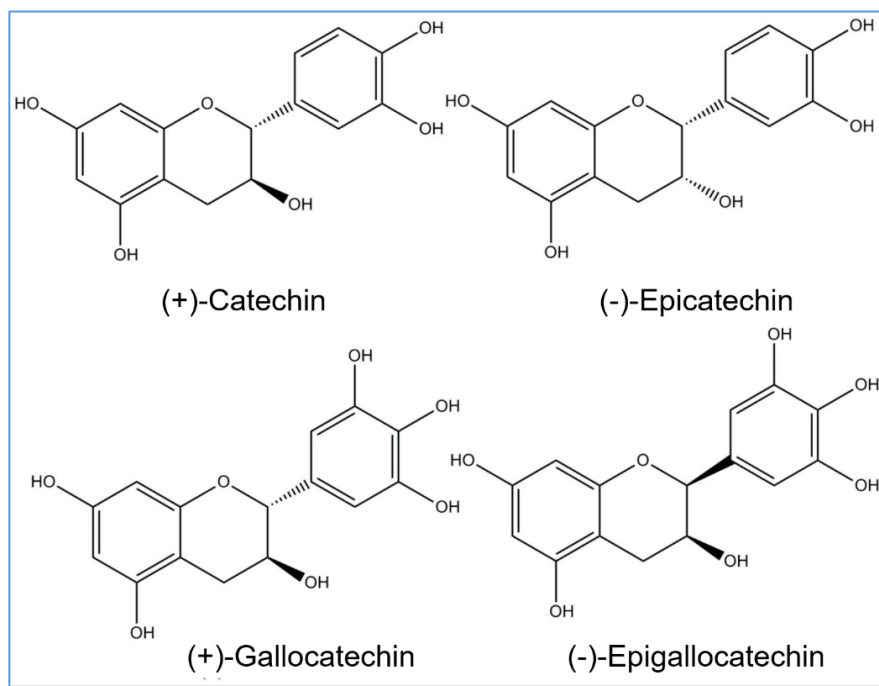
Figure 1.10: Chemical structure of flavanol monomers (Aprotosoaie *et al.,* 2016).

1.4.3 Bioavailability

A compound has to be absorbed, distributed and taken into a tissue, within an adequate time period, in order to produce its biological effect on that tissue. Cocoa polyphenols have a relatively short plasma half-life, with rapid excretion and comparatively low bioavailability (Cooper *et al.,* 2008). Polyphenolic compounds with low molecular weight, like flavanol monomers, can produce greater concentrations in the blood and can reach the target organs in the body, as bioavailability is strongly influenced by molecular size (Cooper *et al.*, 2008). Monomers, and dimeric or trimeric proanthocyanidins, are quite stable in the gastric environment and travel to the small intestine for absorption (Fraga *et al.*, 2010). Monomers are absorbed in the small intestine and distributed to the liver as well as lymphoid organs which include spleen, thymus and mesenteric lymph nodes (Goya *et al.,* 2016). The mammalian metabolism of simple cocoa flavanols occurs in small intestine and liver via glucuronidation, sulfation and O-methylation (Schroeter et al., 2010), and the circulating flavanols are predominantly the conjugated forms (Field and Newton, 2013). Apart from that, flavonoid metabolism can occur in the vascular endothelial cells. In human umbilical vein endothelial cells (HUVEC), epicatechin is converted via catechol-O-methyltransferase to 3'-O-methyl-epicatechin and 4'-O-methyl-epicatechin metabolites. Although the endothelial concentrations of epicatechin are not known so far, it is reported that they are higher than the plasma concentrations (Schewe *et al.*, 2008).

Flavanols produce peak concentrations in the plasma commonly in the nanomolar or low micromolar range, 2 to 3hrs after ingestion of cocoa depending on the administered dosage (Goya *et al.*, 2016). Epicatechin has a higher bioavailability compared to other cocoa flavanols such as catechins (Steffen *et al.,* 2008). This is due to the different stereochemistry of epicatechin allowing higher intestinal permeation of this flavanol (Field and Newton, 2013).

In the acidic environment of the stomach, some dimers can be degraded to epicatechin by interflavan bond conversion. Polymeric flavanols and

oligomeric proanthocyanidins are not absorbed in native forms (Khan *et al*., 2012). Within 48hrs, they are metabolized into various low-molecular weight phenolic compounds in the colon (Khan *et al*., 2012). These metabolites can be absorbed and reach the liver as well as produce significant biological properties (Khan *et al*., 2012, Goya *et al*., 2016).

It has been demonstrated that flavanol monomers have short-half lives in the body and undergo rapid excretion in the urine or bile as conjugates (Keen *et al*., 2002). Previous study reported a half-live of 1.9hr for epicatechin after ingestion of 40gm chocolate bars (Field and Newton, 2013). Meanwhile, the ingestion of carbohydrates has been shown to significantly enhance the absorption of polyphenols (Rimbach *et al*., 2009), and the presence of lipids in cocoa preparations have been found to stimulate flavanols digestion in the duodenum (Ortega *et al.,* 2009). A summary of the bioavailability of cocoa polyphenols is shown in Figure 1.11.
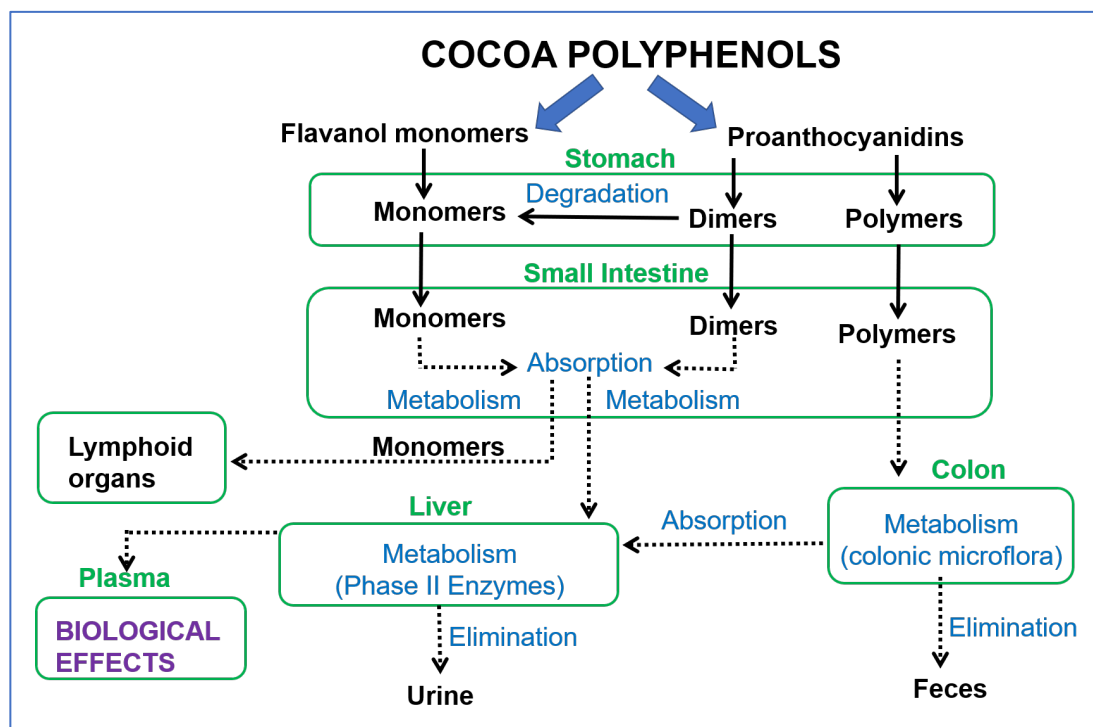
Figure 1.11: The metabolism of cocoa polyphenols (Adapted from Aprotosoaie *et al*., 2016).

Previous studies reported that plasma concentration of the free monomer is less than generally reported (Yang *et al*., 1998, Lee *et al.,* 2002). However, it is possible that the metabolites of the free monomer may also be bioactive. Previous studies structurally described major flavanol metabolites in human plasma (Hackett *et al.,* 1983, Schroeter *et al.,* 2003), the direct proof that, the absorbed flavanols and their metabolites are causally related to the observed cardiovascular effects in humans. However, the mechanisms has yet to be determined. Importantly, most data observed from *in vitro* studies are based on the use of flavanol forms as present in foods and not as they exist in circulation, possibly limiting the validity of a subsequent data analysis in the context of human physiology (Schroeter *et al.,* 2006).

1.4.4   Epidemiological evidence for cardiovascular benefits

Epidemiological studies indicate that regular consumption of flavanol rich foods and beverages decreases the risk of strokes (Keli *et al.,* 1996) and coronary heart disease, and is inversely associated with the risk of developing cardiovascular diseases (Joshipura *et al.,* 2001). Risk factors for these cardiovascular diseases appear to be related to an attenuation of endothelium-dependent vasodilation. Previous published data (involving 2,000 or more patients) have demonstrated the value of endothelial vasomotor dysfunction with predicting atherosclerosis (Widlansky *et al.,* 2003). In this context, flow mediated dilatation (FMD) served as the common gold standard for the non-invasive measurement of endothelial function in humans. Regarding dietary interventions, many studies have demonstrated increases in FMD following the acute and chronic consumption of flavonoid rich foods and beverages which include cocoa (Fisher *et al*., 2003, Heiss *et al*., 2003), tea (Duffy *et al.,* 2001) and red wine (Agewall *et al.,* 2000). Among all these foods, cocoa might be an important mediator of cardiovascular health (Corti *et al.,* 2009).

In a previous study, 34 healthy elderly subjects were asked to consume either high flavanol cocoa (HFC; 900 mg of cocoa flavanols per day) or low flavanol cocoa (LFC; 36 mg of cocoa flavanols per day) for one week in a

randomized, double-blind, placebo-controlled, parallel-arm study. It was observed that mean blood flow velocity in the middle cerebral artery assessed by Transcranial Doppler ultrasound was significantly higher in the HFC group as compared with the LFC group (Sorond *et al.*, 2008), indicating the potential of cocoa flavanols for the treatment of vascular impairments associated with, or preceding dementia and strokes and therefore, maintaining cardiovascular health.

1.4.5 Possible mechanism of action of the protective effects of cocoa polyphenols on endothelial function and NO

The endothelium in its healthy state shows a highly selective permeability. When there are pharmacologic or physical stimuli, which include acetyl choline (Ach), serotonin, bradykinin, substance P, methacholine, and shear stress, NO will be released by endothelial cells as previously described. Below is an overview of possible mechanisms of action of the protective effects of cocoa polyphenols on endothelial function and NO bioavailability.

(i) Increased bioavailability of NO

In a randomized double-blind crossover study involving 11 smokers, the effects of flavanol-rich cocoa on circulating NO species in plasma were measured by reductive gas-phase chemiluminescence. Participants were instructed to consume 100 ml cocoa drink with high (176 to 185 mg) or low (<11 mg) flavanol content. There were significant increases in circulating NO species in plasma at 2hrs after ingestion of the cocoa drink with high flavanol and the changes correlated with increases in flavanol metabolites (Heiss *et al.*, 2005).

(ii) Increased prostacyclin synthesis

In a randomised, blinded, crossover design, plasma samples were collected before as well as 2 and 6hrs after consuming low-procyanidin and high

procyanidin chocolate in 10 healthy participants. High-procyanidin chocolate induced increases in plasma prostacyclin and decreases in plasma leukotrienes. Furthermore, cultured human aortic endothelial cells were treated *in vitro* with procyanidins to determine whether the effects of procyanidin *in vivo* were associated with procyanidin-induced alterations in endothelial cell eicosanoid synthesis. After the *in vitro* procyanidin treatments, aortic endothelial cells synthesized twice as much prostaglandin and 16% less leukotriene as did control cells. These *in vitro* and *in vivo* findings suggesting that flavonoids can alter eicosanoid synthesis in humans (Schramm *et al.*, 2001).

(iii) Inhibit endothelin-1 synthesis

A randomised, placebo-controlled, crossover trial in 12 healthy men was conducted to compare the acute effects of the oral administration of 200mg quercetin and epicatechin on indices of NO and ET-1 production. Consumption of quercetin and epicatechin resulted in a significant increase in plasma *S*-nitrosothiols, plasma nitrite and urinary nitrate concentrations and a significant reduction in plasma ET-1 concentration. Quercetin also significantly decreased urinary ET-1 concentration. These findings suggest that dietary flavonoids can augment NO status and reduce ET-1 concentrations and may thereby improve endothelial function (Loke *et al.,* 2008). In another study, 20 healthy participants were assigned to receive either five treatments with daily intake of 10gm cocoa (0, 80, 200, 500 and 800 mg cocoa flavonoids/day) for 1 week each in a randomized, double-blind, controlled, cross-over design. It was found that cocoa dose-dependently decreased ET-1 level suggesting cocoa, might representing a consistent tool in cardiovascular prevention (Grassi *et al.*, 2015).

(iv) Inhibit platelet aggregation

In a study by Guerrero *et al.*, (2005), the effect of several types of flavonoids on platelet aggregation, and thromboxane A_2 (TxA_2) generation was

investigated. Competitive radioligand binding assays were used to screen for affinity of these compounds to TxA$_2$ receptors. It was shown that flavones (apigenin and luteolin) and isoflavones (genistein) abolished arachidonic acid (which is the substrate for TXA$_2$ synthesis) and collagen-induced platelet responses such as aggregation and secretion, with a lesser effect on TxA$_2$ synthesis. The inhibition of *in vitro* platelet responses induced by collagen or arachidonic acid by specific flavonoids seems to be associated with their ability to compete for binding to the TxA$_2$ receptor. Therefore, antagonism of this TxA$_2$ receptor may represent an additional mechanism for the inhibitory effect of these compounds in platelet function (Guerrero *et al.*, 2005).

(v) Inhibit leucocyte activation

Hepstintall *et al.*, (2006) investigated the effects of cocoa flavanols on platelet and leucocyte function in vitro. In this study, several cocoa flavanols and their metabolites were shown to inhibit monocyte and neutrophil activation. There was also inhibition of platelet aggregation and platelet activation. It was found that epicatechin was consistently effective as an inhibitor of platelet and leucocyte activation (Heptinstall *et al.*, 2006).

(vi) Reduced oxidative stress

Peripheral blood was obtained from 8 healthy participants before and 1, 2, 4 and 8hrs after consuming a flavonoid-rich cocoa beverage (epicatechin and catechin). Erythrocyte haemolysis was evaluated using a controlled peroxidation reaction. Epicatechin and catechin were detected in the plasma within 1hr after the consumption of the beverage. It was found that erythrocytes from participants consuming flavonoid-rich cocoa show reduced susceptibility to free radical-induced haemolysis (Zhu *et al.*, 2005).

A summary of the mechanism of action of the protective effects of cocoa polyphenols on endothelial function and NO is shown in Figure 1.12.
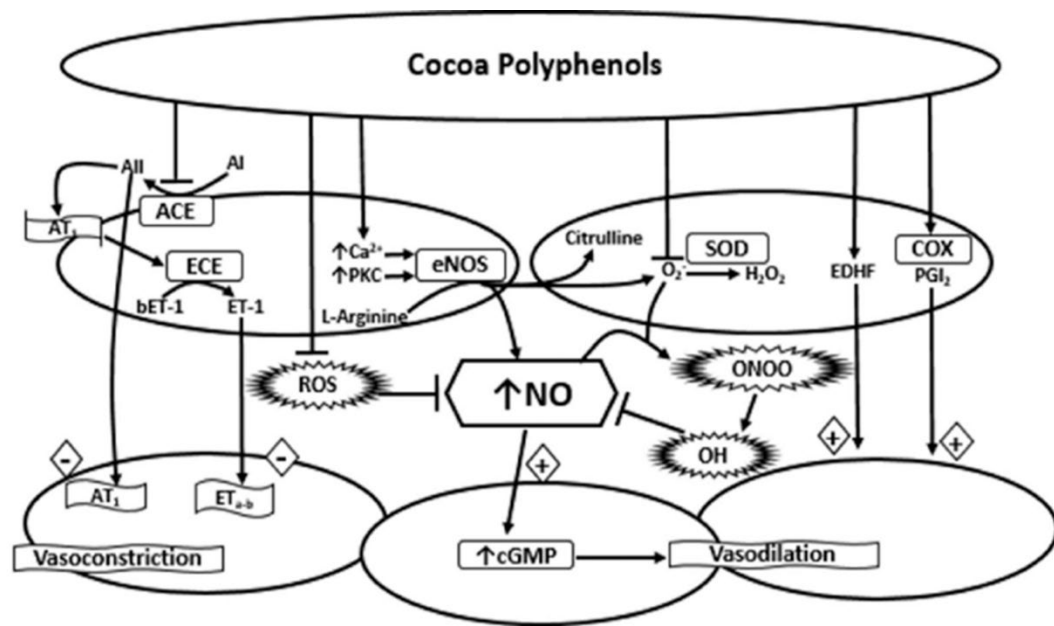
Figure 1.12: Mechanism of action of the protective effects of cocoa polyphenols on endothelial function and NO. AII indicates angiotensin II; AI, angiotensin I; PKC, protein kinase C; SOD, superoxide dismutase; PGI_2, prostacyclin; ACE, angiotensin-converting enzyme; ECE, endothelin-converting enzyme; AT_1, angiotensin receptor; ET-1, endothelin 1; bET-1, big endothelin 1; $ET_{a/b}$, endothelin receptor a and b; cGMP, cyclic guanosine monophosphate; and ROS, reactive oxygen species (Sudano *et al.,* 2012).

(i) Cocoa polyphenols and acute response of Flow Mediated Dilatation

Previous studies have reported that HFC consumption produces an acute increase in FMD. In a randomised, double-blind, cross-over design study with 3-7 days wash out between treatments, 21 overweight or obese participants were instructed to consume either 200ml of a HFC drink (701 mg total flavanols) or a LFC drink (22mg cocoa total flavanols). It was demonstrated that consumption of the HFC significantly enhanced FMD response at 2hrs compared to LFC (Berry *et al.,* 2010). In another study, Monahan *et al.,* (2011) conducted a randomised, placebo-controlled study in five experimental sessions, separated by at least 3 days on healthy older

adults. They were asked to consume five experimental drinks which were 250ml of cocoa drink containing 0gm (0 flavanols/placebo), 5gm (26mg flavanols), 13gm (67mg flavanols) and 26gm (146mg flavanols) of cocoa. It was observed that the three highest levels of cocoa consumption (5, 13, and 26gm cocoa) significantly increased the FMD at both 1 and 2hrs after consumption compared to the placebo. The greatest increase in FMD was demonstrated after consumption of 26gm cocoa, suggesting that the acute increase in FMD following cocoa ingestion was dose dependent (Monahan *et al.,* 2011). Based on these studies, cocoa consumption has been demonstrated to improve artery reactivity following a shear stress stimulus.

1.4.6 Clinical research on antihypertensive effects of cocoa polyphenols

Cocoa polyphenols are able to alter specific pathways in order to increase production of NO, inhibiting vasoconstriction and stimulating vasodilatation resulting in an overall BP reduction within the endothelium. The mechanisms through which flavanols modulate BP are discussed in 1.4.6

The first evidence of the antihypertensive effects of cocoa was noted in native Kuna Indians who live on the San Blas islands off the coast of Panama. Interestingly, this population is one of the few cultures that appear to be protected against age-related progression of arterial hypertension and increases in BP. Island dwelling Kunas consume large amounts of cocoa every day (Hollenberg *et al.,* 1997) and it was observed that these individuals had lower BP (Hollenberg *et al.,* 1997) and no age-dependent deterioration in kidney function (Hollenberg *et al.,* 1999). Meanwhile, when compared with Kuna Indians living in Panama City, it was found that mortality resulting from cardiovascular events was markedly lower in the Island population. In this study, diagnoses on death certificates were used to compare cause-specific death rates from the year 2000 to 2004 for the islands and the mainland. On the mainland (Panama), cardiovascular disease was the leading cause of death. In contrast, the rate of these diseases among island-dwelling Kuna was much lower. The comparatively

lower risk among Kuna in the San Blas from developing cardiovascular diseases was attributed to a large intake of flavanol and sustained activation of NO synthesis (Bayard *et al.,* 2007). When these islanders migrate to urban Panama City, other food with a lower flavanol content was found to have replaced the home-prepared cocoa and this protection was lost. This observation indicated that the protective factors concerned were environmental rather than genetic (Hollenberg *et al.,* 1997).

More data supporting the potential antihypertensive effects of cocoa comes from interventional studies. In a randomised, controlled, double blind, parallel-group dietary intervention trial, it was found that twice daily consumption of a cocoa drink (450mg of total flavanols) compared to a nutrient-matched cocoa-free control drink for 1 month significantly increased FMD and decreased BP among 100 healthy, middle-aged men and women. These findings suggested that the regular consumption of cocoa flavanol had the potential to maintain the cardiovascular health in healthy individuals (Sansone *et al.*, 2015).

1.4.7 Cocoa polyphenols and Raynaud's phenomenon

The pathogenesis of RP is thought to be due to increased vasoconstriction, perhaps through the reduced production of NO, increased platelet aggregation and activation and/or presence of leucocyte activation and oxidative stress. Previous studies have demonstrated that cocoa polyphenols may increase the bioavailability of NO, increase prostacyclin release, inhibit ET-1 synthesis, inhibit platelet and leucocyte activation as well as reduce the production of free radicals (as discussed earlier). Table 1.3 represents the summary of the possible mechanisms of action of the protective effects of cocoa polyphenols on endothelial function and NO in RP.

Pathogenesis of RP	Protective effects of cocoa polyphenols
(i) Reduced production of NO/prostacyclin	-increased synthesis of NO and prostacyclin (Heiss *et al.,* 2005, Schramm *et al.,* 2001a)
(ii) Increased vasoconstriction due to increased released of ET-1	- acutely lowers plasma ET-1 levels and enhances bioavailability of NO (Loke *et al*., 2008, Grassi *et al.,* 2015).
(iii) Increased platelet aggregation and activation	- inhibits TXA_2 synthesis and antagonism at TXA_2 receptors (Guerrero *et al.,* 2005).
(iv) Leucocyte activation	-inhibits platelet and leucocyte activation (Heptinstall *et al.,* 2006).
(v) Oxidative stress	-reduces free-radical-induced erythrocyte haemolysis (Zhu *et al.,* 2005).

Table 1.3: Summary of protective effects of cocoa polyphenols on endothelial function and NO in RP.

1.4.8 Summary

PRP is characterised by periodic vasospasm of the digits precipitated by exposure to cold or emotional stimuli. PRP may involve vascular endothelial dysfunction, abnormalities in neural control of vascular tone and/or increases in circulating mediators which promote vasoconstriction. Cocoa flavanols have been demonstrated to induce NO dependent vasodilation possibly by reducing vascular arginase activity and activating eNOS. In addition, cocoa flavanols have been reported to inhibit ET-1 synthesis, activate EDHF as well as inhibit platelet aggregation and adhesion. It is hypothesised that these beneficial effects of cocoa flavanols could address several of the pathologies seen in RP and alleviate the prevalence and/or severity of symptoms associated with this condition. Below are the aims and hypotheses of the present PhD project.

a) Chapter 3

FMD Study 1: To validate vasoactive properties of cocoa drink in   healthy participants

    i.   To determine the acute vasoactive properties of a high flavanol cocoa (HFC) drink (Chococru™) and compare it to a low flavanol cocoa (LFC) drink (Sainsbury's cocoa)

    ii. To determine the optimal time point for scheduling measurements in future acute protocols using HFC and LFC drinks.

Hypothesis: The HFC drink would induce a greater flow-mediated dilatory (FMD) response compared to the LFC drink

b) Chapter 4

FMD Study 2: To validate vasoactive properties and the suitability of delivering cocoa in capsules in healthy participants

    i.   To determine the time frame for the acute vasodilatory effect of the HFC delivered in a capsule (Chococru™) to occur and to compare any effect with a non-cocoa placebo (coconut flour).

    ii.   To determine the optimal time point for scheduling measurements in future acute protocols using HFC and placebo capsules.

Hypothesis: The HFC capsules would induce a greater flow-mediated dilatory (FMD) response compared to the placebo capsules

c) Chapter 5

    i.   To determine the effect of consuming a HFC drink (at 1hr) in healthy participants on:

➢   the core temperature and cardiovascular response to localised cooling;

➢   skin BF and skin temperature responses of the finger during localised cooling and at a room temperature (RT) of 25°C during rewarming after localised cooling;

➢   the time taken for skin temperature of the finger to stabilise during localised cooling and at RT.

Hypothesis: Although HFC drink ingestion may have effects on cardiovascular measures, counter-regulatory responses to localised cold exposure would not be compromised.

d)   Chapter 6

    i.   To determine the effect of consuming a HFC drink (at 1 and 3 hrs) in healthy controls *versus* participants with PRP on:

➢   the core temperature and cardiovascular response to localised cooling;

➢   skin BF and skin temperature responses of the finger during localised cooling and at a RT of 25°C during rewarming after localised cooling;

➢   the time taken for skin temperature of the finger to stabilise to localised cooling and at RT.

Hypothesis:

- HFC ingestion would induce vasodilation and increase skin BF in participants with PRP when compared to LFC and the difference in response would be more pronounced in participants with PRP than healthy controls.

- The time taken for skin temperature to stabilise in the cold, and at RT following cold exposure, would be longer in those with PRP compared to healthy controls.

e)   Chapter 7

    i.   To determine the effects of supplementing the diet with cocoa flavanols on Raynaud's symptoms in those with PRP.

    ii.   To determine the effects of supplementing the diet with cocoa flavanols on quality of life measures.

    iii.   To determine the feasibility of recruitment for a full randomised controlled trial.

    iv.   To determine adverse events and attrition rates in a PRP cohort asked to supplement their diet with HFC extract.

Hypothesis:

- The prevalence and/or duration, and/or severity of symptoms associated with PRP would be reduced in those taking cocoa flavanols capsules compared to placebo.

- Quality of life measures in participants with PRP would be improved in those taking cocoa flavanols capsules compared to placebo.

Therefore, the overall thesis hypothesis is:

HFC ingestion would induce vasodilation and increase skin BF in participants with PRP and therefore, the prevalence and/or duration, and/or severity of symptoms associated with PRP would be reduced in those taking cocoa flavanols capsules compared to placebo.

Chapter 2: Methods and Materials

2.1 Finometer

2.1.1 Introduction

The Finometer is a non-invasive beat-by-beat haemodynamic monitor that generates a continuous pulse waveform by means of a finger cuff. It uses the Penaz volume-clamp method (Penaz, 1973) and the Physiocal (physiological calibrating) criteria (Wesseling, 1995) to obtain a continuous pulse waveform recording. The Finometer is composed of:

(i) The main unit

The main unit of the Finometer has the central monitor which has been programmed to show instantaneous values for CO, HR, SBP, DBP, TPR, total arterial compliance, tabs for navigation between its windows, socket outlets (for electric cable and air hosepipe), an event marker and tabs to turn the machine on and off. It also has an integral air pump which produces air for inflation of the cuffs. The upper arm (brachial) cuff is connected with the machine's air producing system via the air pipe. The main unit is connected with the small sub-unit which is placed on the participant's wrist during the experiment (Figure 2.1).

Figure 2.1: The main unit of the Finometer

(ii)The sub-unit

The sub-unit is connected with the main unit via an electrical cable and an air-line. This unit has a photoplethysmograph, a pressure transducer and a servo-controller system. It also has two vertical socket inlets which allow the air tube and electric cable of the finger cuff to be attached to the sub-unit.

(iii) The finger cuff

The finger cuff is the sensory component of the Finometer (Figure 2.2). It is in contact with the participant's finger to detect the smallest changes in finger artery size at high speed and to control cuff pressure to oppose even subtle changes in arterial pressure dynamically. There are 3 sizes which can be attached on the ring, middle (usually middle) or index finger. On the inside of the finger cuff, there is a light emitting diode and a separate light detector, each of which should be positioned symmetrically on either side of the finger.

Figure 2.2: Finger cuffs are available in three sizes: large, medium and small (Taken from smartmedical.co.uk).

2.2.2 Principles of the technique

The volume-clamp method of Penaz: the non-invasive measurement of the finger arterial pressure utilises the volume-clamp method which is based on the dynamic unloading of the finger's arterial wall. This is designed to continuously maintain the diameter of the artery constant. A finger inflatable cuff is used, to which an infrared photo-plethysmograph is attached that detects any changes in the arterial diameter. In response to any change in diameter, an electro pneumatic system acts by changing the cuff pressure in response to that change. Accordingly, when the arterial diameter

increases following a rise in blood pressure, the cuff will inflate and when the diameter decreases following a drop in blood pressure, the cuff will deflate, thus keeping the arterial diameter constant. The cuff pressure is a reflection of the intra-arterial pressure waveform and is measured by an electronic gauge (Penaz, 1973) [Figure 2.3].
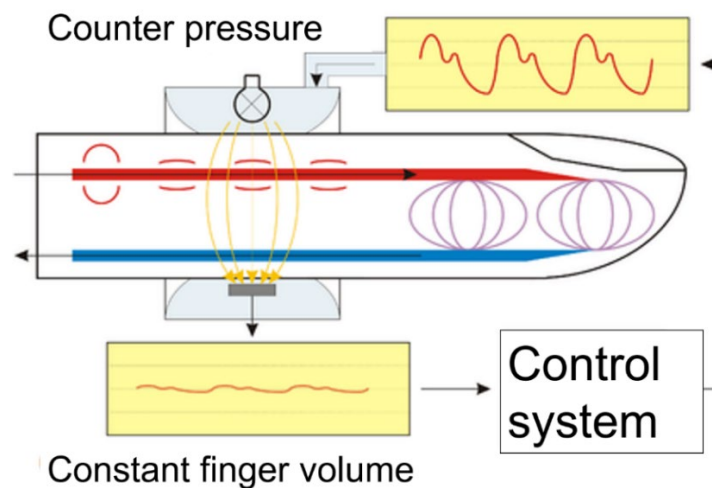
Figure 2.3: Principle of volume clamp method (Fortin, 2018).

The Physiocal (physiological calibration) criteria of Wesseling: The Physiocal criteria of Wesseling states that the intra-arterial pressure will be equal to the recorded finger cuff pressure only in the unloaded finger artery, where the transmural pressure is zero. Therefore, in order to obtain the accurate arterial blood pressure, an unloaded finger artery must be obtained, and the diameter used as the volume-clamp set point. Physiocal calibrations are made at regular intervals by temporary interruptions to the measuring procedure. The volume-clamp servo loop is opened and the pressure in the cuff is kept midway between the systolic and diastolic blood pressure readings of the previous pulsation. Pressure volume relations are recorded by the plethysmograph and analysed by a computer algorithm that determines the volume-clamp finger artery diameter set point (Wesseling, 1995). Ultimately, a pulse waveform is obtained, which is further analysed by the Modelflow algorithm to generate a continuous estimate of the cardiac output.

(i) Modelflow technique

The Modelflow technique is a computer-based algorithm that analyses the blood pressure waveform by using the non-linear three element model, which represents the three characteristics of aortic input impedance, arterial compliance and the peripheral vascular resistance (Harms *et al.,* 1999).

Aortic input impedance (Z^o)

The aortic input impedance is the behaviour of the aorta in opposing the ejection of blood from the left ventricle, i.e., the relationship of aortic pressure offered by the blood already present in the aorta to aortic inflow from the left ventricle (Bogert and van Lieshout, 2005).

Arterial compliance (Cw)

Compliance is defined as a change in volume divided by a change in pressure (Harms *et al.,* 1999) and therefore, explains the rise in aortic pressure for a given volume of blood. When blood enters the aorta, the aorta expands elastically and its pressure increases, offering opposition to further entry of blood from the left ventricle, until there is a further increase in left ventricular pressure. A compliant (elastic) aorta expands quickly with little rise in aortic pressure; compliance decreases with age (Bogert and van Lieshout, 2005).

Peripheral resistance

Peripheral resistance reflects the resistance to blood flowing from the aorta to the periphery (Wesseling *et al.,* 1993). There are various factors which increase or decrease systemic vascular resistance. Mechanisms causing vasoconstriction include sympathetic stimulation, the action of endothelin I or angiotensin II. By contrast, mechanisms causing vasodilatation include nitric oxide and tissue metabolites such as adenosine or hydrogen ions (Wesseling *et al.,* 1993). Vasoconstrictor mechanisms are essential in maintaining systemic vascular resistance and arterial pressure, whereas vasodilator mechanisms primarily regulate blood flow within organs.

Model flow computes the Z^o and Cw from the intra-arterial pressure waveform and uses the constants derived from the participant's age, sex, height and weight. Instantaneous values of Z^o and Cw are utilized in the model simulation to produce the aortic flow waveform. The Finometer, analysing the arterial wave-form computes and determines the aortic pressure wave and aortic flow wave. Integrating the aortic flow wave-form gives instantaneous stroke volume (SV) (Harms *et al.,* 1999). Meanwhile, HR is determined from the beat to beat arterial pressure wave. CO is then calculated by multiplying the SV by the HR.

(ii) Reconstruction of brachial artery pressure

The calibration for finger arterial pressure is achieved by the 'return-to-flow' method. In this method, the cuff on the upper arm on the same side as the finger cuff is inflated to above systolic pressure and therefore, finger flow ceases. As the brachial artery cuff deflates, it falls to systolic pressure, at which point finger flow returns. With further deflation, full flow is restored in the finger and the pressure in the brachial cuff at which this occurs corresponds to diastolic blood pressure. The systolic and diastolic pressures are then included in the reconstruction of the brachial artery wave form using a general inverse anti resonance model (Jellema *et al.,* 1999).

Reconstructed brachial arterial pressure waveform is obtained to correct for arterial blood pressure differences that naturally occur between central and peripheral arteries. SBP tends to be higher peripherally, which is mainly due to the propagating pulse wave amplifications which depend on heart rate (Bos *et al.,* 1995). There is also a delay of several dozen milliseconds between finger blood pressure pulsations and intrabrachial pulsations since the former travel further. In order to overcome this site related blood pressure difference, a waveform filtering is used in the Finometer to change a finger to a brachial artery blood pressure waveform. This includes amplifying frequencies below 2.5 Hz by a factor slightly greater than one and attenuating frequencies near the 8 Hz resonance peak that occurs in

the transmission path between brachial and finger artery sites. Waveform filtering is done in real-time.

2.2.3   Details of the methodology as used in this study

- The brachial cuff is attached to the participant's left arm followed by attaching the finger cuff to the middle finger of the same arm. The correct size of finger cuff must be chosen as loosely fitting cuffs will provide inaccurate results (Jones *et al.,* 1993) and will damage the cuff (Figure 2.4).

- The Finometer is then switched on and patient data including age, sex, weight and height are entered.

- The height correction unit (HCU) is made of a transparent thin tube containing water with two ends: a terminal that attaches to the finger and a terminal that is attached to the brachial cuff at heart level during measurement. The HCU is connected to the Finometer by a thin cable. Both the finger and the heart level ends of the HCU are initially brought together. This provides the 'zero' height difference value. This process is to correct for hand movements to correspond with the heart level (Imholz *et al.*, 1993).

- When started, the Finometer delivers an un-calibrated finger arterial pressure waveform. Calibration with the brachial BP takes approximately 2 minutes. Reliable beat-by-beat continuous haemodynamic monitoring is provided thereafter.

- The [mark] button is pressed to annotate the Finometer's readings at specific times such as the start and end of a procedure. This allows the researchers to review the Finometer's readings which correspond to those events after having retrieved the data from the Finometer.

- The Finometer is then switched off after data have been collected from the participants and the brachial and finger cuffs are then removed.
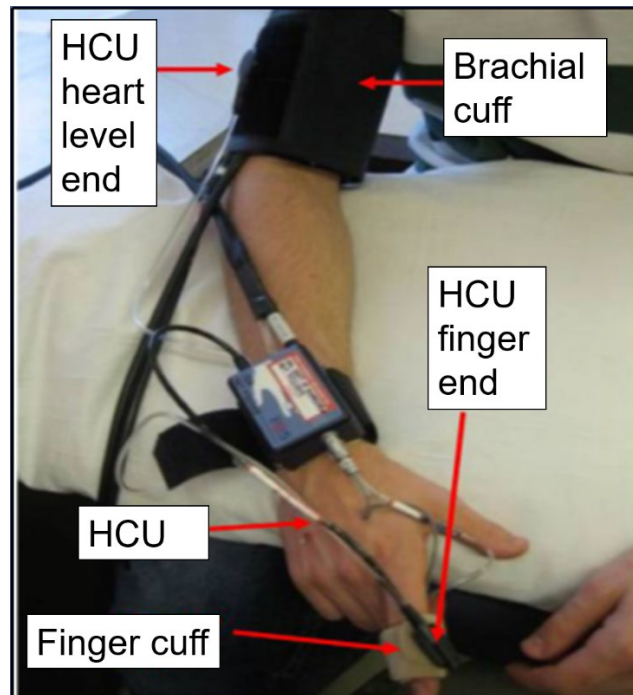
Figure 2.4: Finometer attached to the participant's arm

(i) Power Lab

The Power Lab is a data acquisition system developed by ADInstruments comprising hardware and software elements (Manual, 2004) and consists of an input device connected to a computer using a Microsoft Windows computer using a Universal Serial Bus (USB) cable and LabChart software which provides the recording, display and analysis functions. The system is used to record and analyse physiological signals. In this thesis, the data were recorded by the Finometer and simultaneously shown on the Lab Chart display. Data were subsequently saved as a Labchart file. After each experiment, the data were analysed and exported into an Excel spreadsheet, saved and further analysed. The Power lab has the ability to record the data at speeds up to 500,000 samples per second.

2.2.4 Limitations

(i) Finger arterial vasoconstriction

The presence of finger arterial vasoconstriction and cold fingers might affect the integrity of photo plethysmographic readings (Bos *et al.*, 1995). Nevertheless, it is usually possible to obtain readings in cases where fingers are cold and in cases of vasoconstriction, only when it is severe does it impede the plethysmographic measurements (Wesseling *et al.,* 1985). In the present study, there were no severe cases of vasoconstriction as the Finometer was placed on the control finger (in RT) and the baseline measurements were taken only when the skin temperature was stable in a room-controlled temperature of 25°C.

(ii) Size of the finger cuff

The size of the finger cuff significantly affects the data obtained; thus, choosing an appropriate size is crucial. It has been demonstrated that using a finger cuff that is too tight or too loose decreases accuracy (Sprafka *et al.,* 1991).

2.2   Laser Doppler Flowmetry

2.2.1 Introduction

The Laser Doppler Flowmetry (LDF) is a non-invasive measure of capillary blood perfusion. It uses a monochromatic low-energy laser beam. This beam penetrates the tissue and, depending on individual tissue penetration, it is reflected, recorded by a sensitive sensor and subsequently analyzed using the Doppler (Sarnik *et al.*, 2007). The technique is based on the principle of the Doppler shift, the relative shift in frequency of an energy wave-form that is reflected or emitted by moving objects (Figure 2.5). This selective change in frequency is used to differentiate between the reflected light from the moving and static components in a tissue, thus, can be used to estimate blood flow in tissues such as the dermal circulation.

Figure 2.5: Laser Doppler

2.2.2 Principles of the technique

(i) Doppler shift

In 1842, Johann Christian Doppler reported his first observation of the observed shift in wave frequency from moving objects in relation to the colour of light he observed from the stars and realised the principle was also valid for sound waves. His first experimental demonstration of the change in observed frequency from a moving object used sound. Two musicians with perfect pitch were employed to determine the frequency of the note. The first musician was a trumpeter who played the same pitched note continuously whilst standing on a flat carriage of a train. The second musician observed that the pitch of the note was higher as the train approached him at the train station and lower as it went away (Shepherd, 1990).

The Doppler principle permits calculation of increased frequency of energy waves encountered when an object is moving towards the receiver, and the reduced frequency in waves when the object is moving away from the receiver.

If both the source of light and the receiver are stationary, then the receiver encounters

$$CT/\lambda \text{ waves}$$

C is the speed of light, T is over time and λ is the wavelength.

If the receiver is moving towards the source of light at a velocity, V_L, then an additional

$$V_L T/\lambda \text{ waves}$$

are received over time. The frequency of waves is the number of waves/ unit time, for the stationary receiver

$$f_s = (C/\lambda)$$

and for the moving object

$$f_o = (CT/\lambda + V_L T/\lambda) / T$$
$$= (C+V_L) / \lambda$$

The differences between these two frequencies is the Doppler shift (f_d)

$$f_d = f_o - f_s$$
$$= f (1+V_L / C) - F_s$$
$$= V_L . f_s / C$$

(ii) LASER light (Light Amplification by the Stimulated Emission of Radiation)

The laser is a monochromatic light source produced by the emission of identical electromagnetic waves by excited molecules, usually helium-neon in LDF. The unique properties of the laser are the purity of the wave form which comprises of the same amplitude, frequency, phase and direction of light waves (coherent). The uniform frequency of the light makes it appropriate to detect a Doppler shift in frequency.

Previous studies have used the Doppler effect and lasers to measure velocity. These have utilised the light beating effect to determine the change

in frequency and therefore, the speed of the moving component in a fluid undergoing laminar flow (Yeh and Cummins, 1964). In contrast to moving object, light reflected from a stationary object undergoes no change in frequency. The difference in frequency between these two waves which are close in frequency can be perceived by the beat effect. This is the changing magnitude of the sum of the two wave forms and can be used to determine the magnitude in the shift in frequency, and hence the velocity.

Principle of Laser Doppler Flowmetry

The basic principle of Laser Doppler Flowmetry (LDF) is to analyse changes in the spectrum of light reflected from living tissues as a response to the beam of monochromatic laser light emitted (Figure 2.6). It reflects the total local microcirculatory blood perfusion including perfusion in the arterioles, venules and capillaries with a cross-sectional area of 0.002 mm^2 – 0.008 mm^2 (Hofirek *et al.*, 2004) with basic depth penetration which is about 1.5 mm. LDF perceives the movement of cells (especially erythrocytes, sporadically leucocytes) at a velocity of 0.01–10 mm/s.

One of the earliest studies concerning this issue was reported by Stern (Stern, 1975). Stern performed an experiment to determine the feasibility of the method of coherent light scattering, in this case from a fingertip. He realised that when he occluded blood flow through his finger with an elastic band, a speckled pattern occurred. This pattern then disappeared on releasing the elastic band due to the return of blood flow to the finger. The coherent light entering the tissue had undergone Doppler broadening to an extent that was related to the range of flows in the small area of tissue.

When a beam of light, carried by the fibre-optic probe to the skin surface penetrates the tissues and hits moving blood cells in a random order, it undergoes changes in wavelength and produces a Doppler shift (Doppler, 1842); the wavelength of light hitting static tissue structures is unchanged. The magnitude and frequency distribution of these changes in wavelength are directly related to the number of moving blood cells, but unrelated to their direction of movement.

The tissue volume occupied by moving blood cells is small with an average capillary density of about 50 capillaries per mm^2 mucosal area and most photons are backscattered or absorbed and do not undergo a frequency shift (Hoff *et al.,* 2009). The backscattered and Doppler broadened light carries information about the speed and concentration of blood cells traversing the scattering volume (Leahy *et al.,* 1999).

The quantity that is measured in laser Doppler is referred to as Perfusion Units which are arbitrarily chosen, as tissue blood perfusion cannot be expressed in absolute physical units (e.g. as ml/min/100g of tissue). Perfusion is defined as the product of local velocity and concentration of blood cells. Speed refers only to the magnitude (mm/s) of the velocity vector, and even though the majority (99%) of blood cells in the undisturbed microcirculation are red cells, LDF does not selectively measure red cells (Leahy *et al.,* 1999).

Tissue blood supply (perfusion) = n_{ke} X $v_{ke;}$

n_{ke} is the number of moving blood elements in the measured volume;

v_{ke} is the mean velocity of blood elements in the measured volume.

Meanwhile, the depth of measurement is determined by wavelength, tissue characteristics and probe configuration (Sarnik *et al.,* 2007).
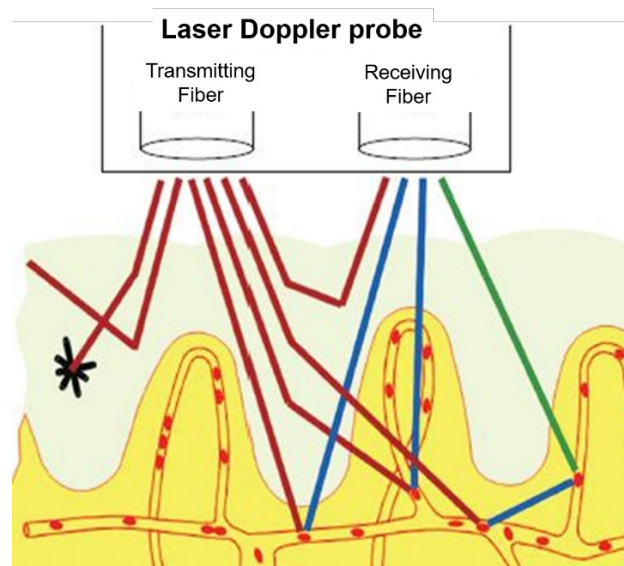
Figure 2.6: A schematic depiction of Laser Doppler perfusion monitoring showing the probe with its emitting fibre bundle which applies monochromatic laser light to the tissue, and its receiving fibre bundle which returns reflected light for analysis. The light that has undergone a Doppler shift due to moving blood cells in the tissues, reflects the microcirculatory perfusion at a given time (Hoff *et al.*, 2009).

2.2.3 Details of the methodology as used in this study

(i)The following were used to maintain the consistency of recordings

Calibration

Calibration of the probe was performed at regular intervals using a standard solution of polystyrene microspheres in water using Brownian motion of the particle to produce reference signals. The probe was suspended in a fresh solution in a room temperature between 20-22ºC and allowed to stabilise before calibration.

Biological zero

The biological zero was recorded from the dorsal surface of the experimental and control finger (index finger). Firstly, the blood pressure

cuff was attached to the lower arm. Then, the baseline flux was recorded before the cuff was inflated to 200mmHg for 3 minutes, with continued recording of flux. Finally, the cuff was deflated and the maximum flux during reactive hyperaemia was recorded. Biological zero was performed on every participant to ensure the measurement was consistent between the participants.

(ii) Method of use of Laser Doppler in this study

The Laser Doppler monitor used during this study was the Laser Blood Flow Monitor (MBF2), (Motor Instruments, Axminister, Devon, UK). This laser uses a 780nm helium-neon laser source. The recordings were made from the dorsum of the index fingers of each hand. The laser Doppler probe was attached to the participant using a probe holder. This holder was then secured to the skin by double-sided adhesive tape and further fastened by adhesive tape, which was carefully applied so as not to occlude the flow to the finger, or to apply any pressure to the skin. The Laser Doppler probe was positioned at 90^0 to the skin. The fibre optic cable was then lightly secured to maintain this angle and ensure it did not move during recordings.

(iii) Data collection

Once the data were recorded using the programme LabChart (AD Instruments), it was shown on the Lab Chart display and saved on the computer. After each experiment the data were exported into an Excel spreadsheet, saved and analyzed.

(iv) Biological variation

In order to achieve consistency in measurement, a number of biological variables were controlled to minimise variability.

- ***Age of the participant***

There is no evidence that age influences skin flux except in neonates (Bircher *et al.,* 1994). All participants in these experiments were between 18 to 60 years old.

- ***Ambient temperature***

The ambient temperature will change skin BF. However, there is a range of skin temperatures within which there is little variation in skin BF. A previous study found that skin BF was stable at the fingertip between 17°C and 28 ^{o}C while in the great toe, this range was between 24 ^{o}C to 30 ^{o}C (Hatanaka, 1984). Large increases in flux were seen above 30 ^{o}C (Winsor *et al.*, 1989). In the present study, all the experiments were done in a room-controlled temperature of 23 ^{o}C -25 ^{o}C and local skin temperature was purposefully altered as part of the protocol. In addition, participants were encouraged to wear light, unrestricted clothing.

- ***Sex of participants***

Women have been shown to have lower resting finger and hand cutaneous blood flow than men, a factor thought to reflect increased sympathetic tone (Schabauer and Rooke, 1994). The increased tone may explain the greater prevalence of Raynaud's phenomenon in women. In addition, Cooke *et al.,* (1990) observed that there were differences in skin thickness and skin BF between the sexes (Cooke *et al.*, 1990). Thus, all the experiments were done on female participants except for the long-term supplementation study; 9% of the participants in the latter study were male due to recruitment limitations.

- ***Ethnic differences***

Melanin reduces the penetration of laser light via the stratum corneum and epidermis as well as having significant absorption characteristics above 800nm. However, the pigment is confined to a thin layer superficial to the microvasculature and the light passing via this layer is attenuated by nearly a constant fraction (Johnson, 1990). Increased melanin leads to a decrease in the total signal, although the volume which is sampled and the

pathlengths are unaffected, thus leaving the relationship between the static and Doppler shift unchanged (Nossal *et al.*, 1988). Thus, melanin levels in the superficial layers of the skin have not been reported to alter recorded skin flux, despite ethnic differences in skin pigmentation (Bircher *et al.,* 1994). The majority of control participants in the present study were Caucasian and Asian, but for the experiments involving people with Primary Raynaud's, all the participants were Caucasian.

- ***Physical and mental activity***

Exercise is well known to cause an alteration to skin BF and skin temperature (Bircher *et al.,* 1994). Apart from that, mental stress, which includes performing mathematical calculations, can alter the skin BF blood flow via arteriovenous anastomoses (Bircher *et al.,* 1994). In order to minimise such variability, all participants were discouraged from undertaking exercise 24 hrs prior to and on the day of their laboratory visit and were encouraged to relax throughout the laboratory visit. A minimum initial resting period of 30 minutes was used to allow them to equilibrate to the laboratory temperature.

- ***Drugs and nicotine***

Agents such as alcohol intake (Vatsalya *et al.*, 2019) and smoking (Waeber *et al.*, 1984) have been reported to alter skin BF. Participants were asked to remain caffeine free for 18hrs prior to the laboratory visit. A drug history was obtained and those who were taking regular vasoactive medication were excluded.

- ***Blood components***

Even though theoretically variation in the serum parameters and haemoglobin level might cause alteration in the skin BF, there is no evidence that these may influence Laser Doppler recordings (Bircher *et al.,* 1994). As an alteration in blood fluidity has been reported in SRP, those who had been diagnosed with SRP were not recruited.

- *Menstrual cycle*

Variation on flux has been observed throughout the menstrual cycle (Tooke *et al.*, 1981). In the present study, participants were examined during the follicular phase of the cycle or during the first two weeks of oral contraceptive pills administration.

(iv) Variation in the recorded flux caused by differences in flow

- *Anatomical variation in the cutaneous vasculature*

A previous study reported that there was a considerable difference in the flux recorded from different skin sites, with higher flux found in the arms compared to the legs (Tur *et al.,* 1983) whereas the flux at contralateral sites showed symmetry. A significant difference in the flux was observed between proximal and distal forearm in 27 healthy participants (Bezemer and Bruynzeel, 1989). In the present study, the choice of site was consistent between participants.

- *Intra participant variability*

A previous study observed that flux varied by 100% at different skin sites as little as 2mm apart. On skin biopsy, sites which were directly over an arteriole demonstrated high flux levels with prominent vasomotion. In contrast, there was lower flux with loss of vasomotion at sites without an arteriole. This indicates that the positional variation in LDF reflects local anatomical variation. In view of difficulties in precisely repositioning the probe, the method is easiest to use when comparing changes in flux at a single site, rather than comparing changes in flux at different sites. These problems can be overcome by using multiple lasers over a wider area to observe mean flow from a range of vessels within the cutaneous microvasculature (Braverman *et al.,* 1992).

The variation in flux with posture (Tsai *et al.,* 2012) has been reported, and is easily observed during experimentation. In the present study, a consistent posture was adopted by participants. Participants were examined while

lying semi-supine on a couch with their forearms and hands supported by a pillow at approximately the level of right atrium. Meanwhile, for the experiments controlling the local skin temperature, the temperature regulated box was arranged at a height so that the forearm and hand inside the box were at the level of right atrium with the participants lying in a semi-supine position. As the forearm and hand entered the box, care was taken not to restrict the vascular supply of the arm.

2.3   Flow Mediated Dilatation

2.3.1 Principles of the technique

Flow-mediated dilatation (FMD) is a non-invasive vascular function test that assesses the change in artery diameter in response to reactive hyperaemia. In a typical FMD protocol, baseline artery diameter and blood flow velocity are calculated using duplex ultrasound (Figure 2.7). Then, an occlusion cuff is inflated to stop blood flow to the lower arm for approximately 5 minutes. Ischemia in the tissue distal to the cuff causes the distal vessels to dilate, lowering vascular resistance. When the occlusion cuff is released, this reduction in downstream resistance dramatically increases blood flow to the arm. The endothelium responds to the resulting increase in shear stress by releasing vasodilators such as nitric oxide, which results in dilatation in a healthy artery (Green *et al.,* 2014). The dilatation is reduced or absent in people with vascular dysfunction. FMD is the difference between the baseline diameter and the maximum diameter reached after cuff release.
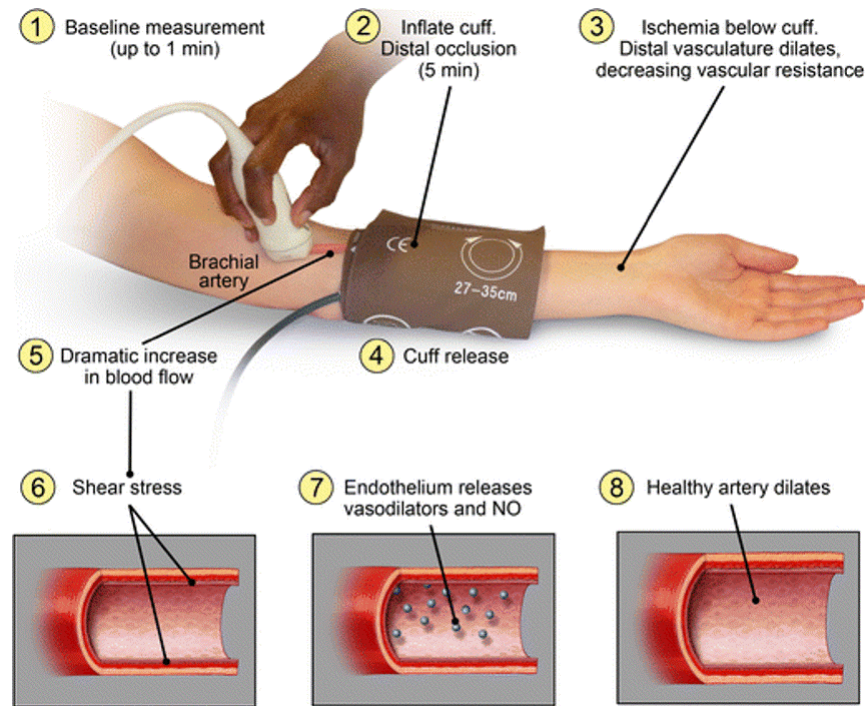
Figure 2.7: Principle of FMD (Weissgerber, 2014).

2.3.2 Details of the methodology as used in this study

In the present study, the FMD was assessed using ultrasound (Toshiba Aplio). The assessment of vascular function was done in a dimly lit and quiet room at an ambient temperature of 25°C. The Ultrasound system was set up with spectral Doppler, and vascular software for two-dimensional (2D) imaging, and used a high frequency (12-14MHz) linear array transducer, as well as an integral electrocardiogram (ECG) monitor.

The FMD measurements were made according to the method outlined by Coretti *et al*., (2002). In short, the brachial artery was imaged above the antecubital fossa in the longitudinal plane and a cine clip over 4 complete cardiac cycles was recorded for diameter analysis at a later date. Blood velocity through the brachial artery was then assessed using Doppler with an angle of incidence of <60°. An occlusion cuff was then placed around the forearm and inflated to at least 60mmHg above systolic pressure for 5 minutes. A blood velocity measurement was made 15 seconds after the occlusion was released, and cine clips for diameter assessment were captured 50, 60, 70 and 80 seconds after cuff deflation. Vessel diameter

measures were made by the selection of a segment with clear anterior and posterior intimal surfaces. Images were gated using R-wave detection so that diameter could always be assessed at the end diastole (Corretti *et al.,* 2002).

Changes in the brachial artery diameter were determined as the average diameter recorded at 60, 70 and 80 seconds after cuff deflation, minus the pre-occlusion diameter. FMD was calculated by expressing this change in vessel diameter as a percentage of the pre-occlusion diameter. Figure 2.8 represents a FMD image showing blood flow during vascular occlusion and reactive hyperaemia.
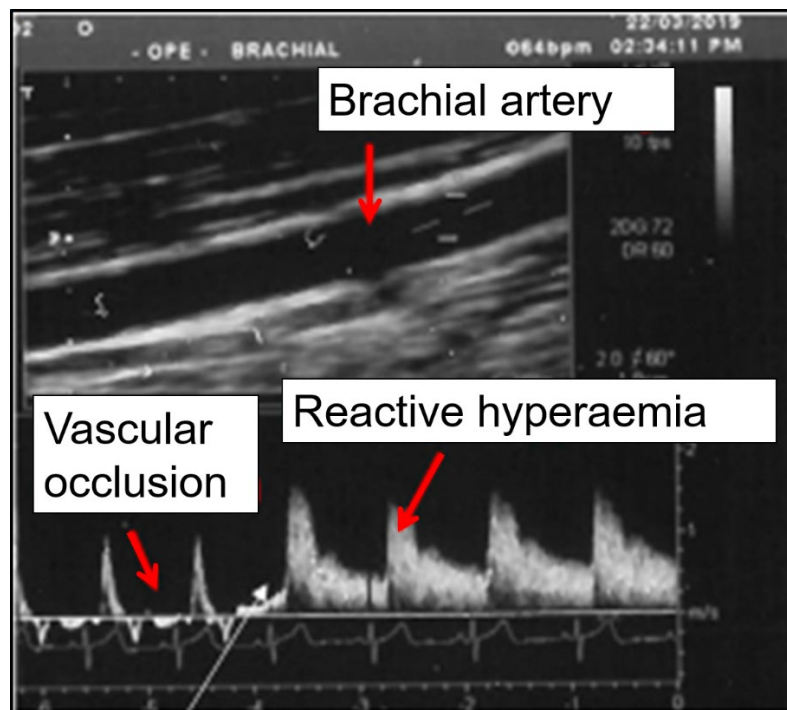
Figure 2.8: FMD image showing blood flow during vascular occlusion and reactive hyperaemia

2.4   Thermocouples

Thermocouples are temperature measurement sensors that produce a voltage that changes over temperature. Thermocouples are made from two wire leads made from different metals. The wire leads are welded together to create a junction. As the temperature changes from the junction to the ends of the wire leads, a voltage develops across the junction.

In 1820, Thomas Johann Seebeck observed that when a metal bar is heated on one end, a voltage (known as the Seebeck voltage) develops across the length of the bar. This voltage varies with temperature and is different depending on the type of metal used in the bar. By joining dissimilar metals that have different Seebeck voltages at a temperature sensing junction (T_{TC}), a thermocouple voltage (V_{TC}) is developed. The dissimilar metals are joined at a temperature sensing junction to form a thermocouple. The voltage is measured at a reference temperature (T_{CJ}) through the two metals. The leads of the thermocouple are required to be at the same temperature and are often connected to the ADC (analog-to-digital converters) through an isothermal block. Figure 2.9 represents a thermocouple constructed from two dissimilar metals with the thermocouple leads connected to an isothermal block.
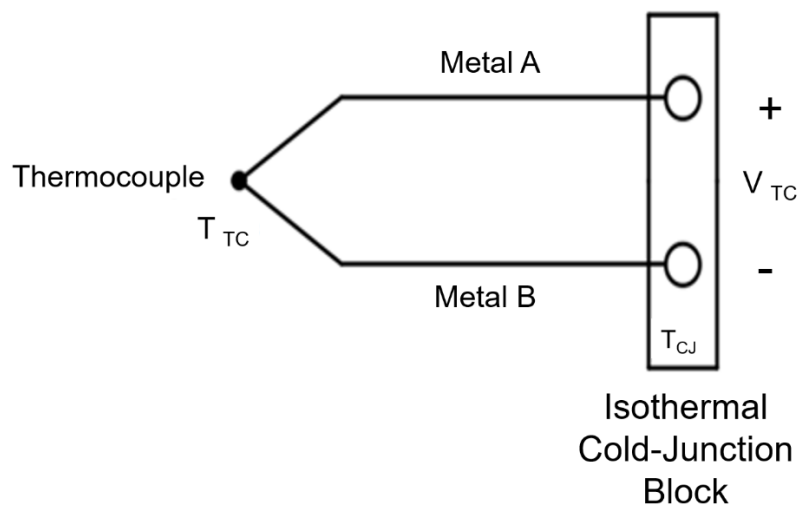
Figure 2.9: Thermocouple voltage (Wu, 2018).

The connection of the thermocouple to an isothermal block is essential for the temperature measurement. For an accurate thermocouple measurement, the return leads of different metals must be at the same known temperature. Any connection between two different metals creates a thermocouple junction (Wu, 2018).

In the present study, the thermocouples were attached directly to the dorsum surface of both index fingers (experimental and control) using adhesive tape. These thermocouples were attached to the picoLog recorder

which was then connected to the laptop via a USB port. The actual values of skin temperature were recorded using a PicoLog recorder programme. After each experiment, the data were exported into an Excel spreadsheet, saved and analyzed (Figure 2.10).
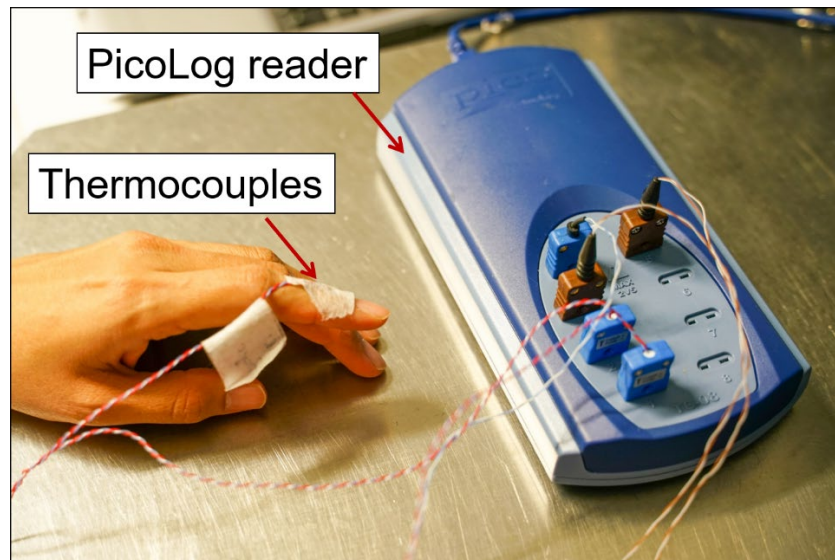
Figure 2.10: The thermocouples attached to the dorsum surface of index finger and PicoLog recorder.

2.5   Temperature regulation

Local hand skin temperature was regulated during experiments by means of a hand cooling box.

2.5.1 Hand cooling unit

A small standard commercial -20^0C freezer was connected to the hand cooling unit and adapted as a cold box. Ports were created on one side of the fridge for the participants to put in their right hand. Meanwhile, the fridge door was replaced with a double-glazed glass screen and another two ports were created on the front of the fridge to allow the experimenter to access the participant's hand inside the box (Figure 2.11).

The cooling system was adapted (Medical Physics Department QMC) to allow much more sensitive control of the environment in the box. In addition, the box temperature could be programmed within 1 degree of the range from -20^0C to room temperature. The choice of a freezer unit is in view of

rapid temperature modulation that allows the changing of temperatures to be used throughout the experimental protocol.

In the present study, the temperature of the cold box was set by adjusting the hand cooling unit to 0°C at 30 minutes prior to the start of study. The skin temperature of the experimental finger was then monitored and recorded via thermocouples. The thermocouple was also placed inside the cooling box to monitor the box temperature.
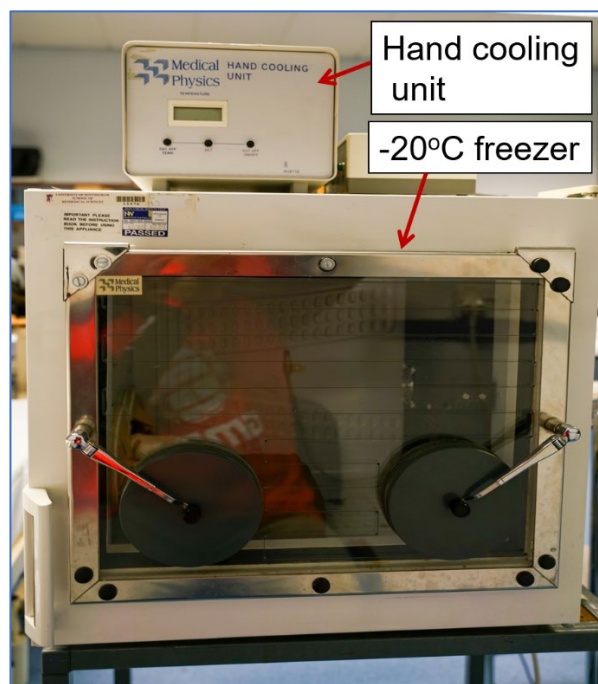
Figure 2.11: Hand cooling unit

2.6   Cocoa used in this study

2.6.1 Cocoa drinks

The cocoa powders used in this study were a high flavanol cocoa powder, containing 830mg of flavanols per 10gm (Chococru™) containing 99.6mg epicatechin, and a low flavanol 'alkalised' cocoa powder (Sainsbury's) which was assumed to contain <50mg of flavanols per 10gm (Miller *et al.*, 2008) (Figure 2.12). Compositional details for high flavanol and low flavanol cocoa powder per 10 gm are given in Table 2.1. The cocoa was consumed as a drink by mixing 10 gm of cocoa powder with 50ml of warm water (70°), and 50ml of skimmed milk. For the high flavanol drink, 3 tablets of saccharin

sweetener ('Hermasetas') were added and for the low flavanol cocoa drink, 1 tablet of saccharin sweetener was added. This was to offset the different bitterness of the 2 cocoas. Compositional details for the high flavanol and low flavanol cocoa drinks are given in Table 2.2.

High Flavanol Cocoa (Chococru)   Low Flavanol Cocoa (Sainsbury's)
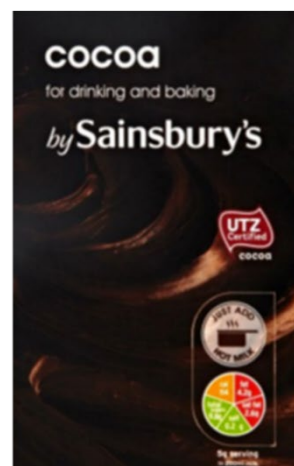
Figure 2.12: The cocoa powders used in this study.

Content	High flavanol (per 10gm)	Low flavanol (per 10gm)
Energy (kJ)	108.4	149.4
Total Fat (gm)	1.10	2.07
Saturated fat (gm)	0.69	1.25
Carbohydrates (gm)	1.42	0.87
Fibre (gm)	1.60	2.82
Sugar (gm)	0.03	<0.05
Protein (gm)	2.36	1.98
Sodium (gm)	0.002	0.018
Caffeine (gm)	0.04	0.023
Theobromine (gm)	0.25	0.263

Table 2.1: Compositional details for high flavanol and low flavanol cocoa powder per 10 gm.

Nutritional content of the drinks	High flavanol Drink	Low flavanol drink
Energy (kJ)	177.4	218.4
Total Fat (gm)	1.20	2.17
Saturated fat (gm)	0.69	1.35
Carbohydrates (gm)	3.62	3.07
Fibre (gm)	1.60	2.82
Sugar (gm)	2.23	2.25
Protein (gm)	4.06	3.68
Sodium (mg)	6.65	19.55

Table 2.2: Compositional details for the high flavanol and low flavanol cocoa drinks.

2.6.2 Cocoa capsules

(i) Cocoa capsules used in the acute study

Ten capsules (cellulose) used in the pilot study, providing either 500mg of high-flavanol cocoa (www.chococru.com) or coconut flour (Sevenhills wholefoods organic raw coconut flour mixed with brown artificial food colour) as placebo. The capsules were tested on each study day, depending on the randomisation (Figure 2.13). Capsules were filled in a food preparation kitchen in the David Greenfield Human Physiology Unit, using a manual capsule filler (https://www.youtube.com/watch?v=xo92dGCt1Rc). Quality control of this manufacturing process indicated a coefficient of variation (CV) of 2.7% in the weight of unfilled capsules and a variability of 2.9% for the weight of filled capsules. Ten cocoa capsules provided the equivalent of 415mg total flavanols (50mg epicatechin) when filled with Chococru and 63kJ of energy in both types of capsule.

Figure 2.13: High-flavanol cocoa or organic raw coconut flour mixed with brown artificial food colour (placebo) within cellulose capsules.

(ii) **Cocoa capsules used in chronic study**

The cocoa capsules used in the long-term supplementation study were kindly provided and 'blinded' by Mars Wrigley Confectionery (Figure 2.14). These capsules were provided in 2 different codes, either containing high flavanol cocoa extract (CocoaVia™) or identical placebo capsules containing alkalized cocoa powder delivered daily in 3 vegetarian capsules. The capsules were allocated at the point of entry into the trial. Compositional details for high flavanol cocoa extract and placebo are given in Table 2.3.
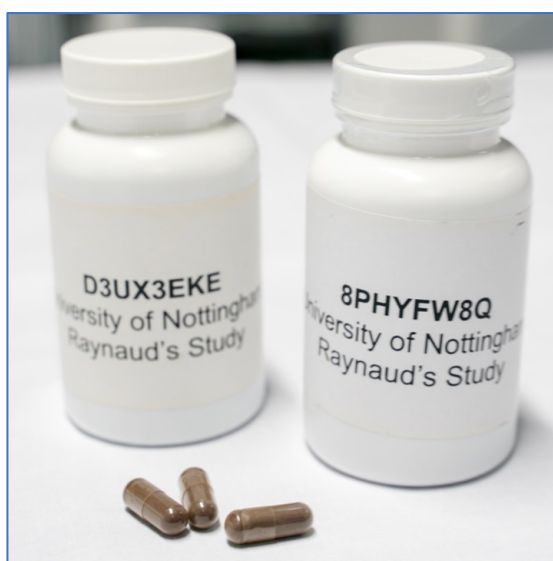
Figure 2.14: Cocoa capsules supplied by Mars Wrigley Confectionery.

Cocoa capsules supplied by Mars Wrigley Confectionery.	High Flavanol Cocoa Extract Per 3 capsules (mg)	Placebo capsules Per 3 capsules (mg)
Cocoa Flavanols	836	0
(-)-Epicatechin	115	0
Caffeine	21	13
Theobromine	109	113

Table 2.3: Compositional details for capsules with high flavanol cocoa extract and placebo capsules

Estimated mean daily total flavanol intake was 158 mg/day among U.S. adults (Chun *et al.,* 2007) and mean daily epicatechin intake was 17.3 mg/day among UK adults (Plumb *et al.,* 2017). In the current study, the amount of total flavanol given in the chronic study (836 mg) was 5.2 times more and the amount of epicatechin (115mg) was 6.6 times more compared to the daily intake of the UK and US population, respectively.

2.7 Statistical analysis

The data were analysed using the Statistical Package for Social Science (SPSS version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. Comparison of mean data before and after intervention within the same group were made using paired *t*-test for normally distributed data or Wilcoxon's signed ranks test for not normally distributed data; comparison between the group were analysed using independent *t*-test for normally distributed data and Mann-Whitney test for not normally distributed data.

For comparison of mean data within group and analysing the change over time, One-Way ANOVA was employed for normally distributed data or Friedman's test for not normally distributed data. In addition, for comparison of mean data within and between groups and analysing the change over time, Two-Way ANOVA was employed. All data are presented as mean (SD) for normally distributed data or displayed as the median (25th and 75th

percentile) for non-parametric data. The normally distributed data in the figures are presented as mean (SEM). A value of p<0.05 is considered to be statistically significant.

Chapter 3: Developmental Study

FMD study 1: To validate vasoactive properties of cocoa drink in   healthy participants

3.1. Introduction

Cocoa derived products, rich in the phytonutrients 'flavanols', have been shown to be efficient in promoting vasodilation (Ried *et al.,* 2012). It is hypothesised that some of the effects of cocoa within the body could address several of the pathologies seen in RP and alleviate the prevalence and/or severity of symptoms associated with this condition. However, before looking at any potential benefits of dietary supplementation with high flavanol cocoa on ameliorating vasospasm, it was necessary to identify a source of suitable high and low flavanol cocoa products. An internet search revealed a supplier of flavanol-rich cocoa who specified the polyphenol content and nutritional information of their product (Chococru™), and the literature provided information regarding the average flavanol contents of baking cocoa available from supermarkets. The known vasoactive properties of cocoa flavanols were then exploited in the current pilot study to determine the relative effectiveness of each cocoa product and their suitability to be used as the active and control product in future protocols. A previous study found that FMD increased from pre consumption levels 1hr and 2hrs after ingestion of (5, 13, and 26gm) cocoa flavanols (compared with 0gm placebo) in healthy older adults, and enhanced FMD measures correlated with an increase in total plasma epicatechin (Monahan *et al.*, 2011). These findings suggesting that in the current study, it would be appropriate to make measurements at 1hr and 2hrs post-cocoa consumption.

3.1.1 Study aims

The aim of this pilot study was

- to validate the acute vasoactive properties of a HFC and compare it to a standard supermarket brand cocoa, by using brachial artery FMD in healthy female participants. This was to ensure that the chosen cocoa products were suitable for use in future studies.

- To determine the optimal time point for scheduling measurements in future acute protocols using HFC and LFC drinks.

Hypothesis

Consumption of the cocoa with known high flavanol content would induce a greater FMD response to that seen following consumption of the cocoa which was assumed to have a lower flavanol content.

3.2 Methods and Materials

3.2.1 Participants

The study protocol was approved by the Medical School Research Ethics Committee, Faculty of Medicine & Health Sciences, Queen's Medical Centre, Nottingham. Written informed consent was obtained from all the participants. The present study was a randomised, double-blind, cross-over design involving 6 healthy women. The number of participants recruited in both the pilot studies were based on the previous pilot studies conducted in the research group found that at least 6 participants were enough to obtain a significant difference between HFC and LFC intake.

For both the developmental studies, female participants were recruited. A single sex cohort was chosen because men and women exhibit different cardiovascular (BP and FMD) profiles and a mixed sex cohort would therefore increase the variability in the sample, thereby reducing the statistical power of the measurement.

Inclusion criteria: female, aged 18-40years, Body Mass Index (BMI) of <27 kg/m^2.

Exclusion criteria: history or current diagnosis of cardiovascular or circulatory diseases, diabetes mellitus, and hypertension.

The recruitment for female control participants was done via posters placed around the university campus and Medical School

3.2.2 Materials

Cocoa drinks

Cocoa powders used in this study are described in Chapter 2 (2.6.1)

3.2.3 Experimental outline

Six healthy female participants who successfully fulfilled the criteria, attended a screening visit (visit 1). They were briefed concerning the nature of the study and informed consent was obtained. Randomisation was computer generated (www.randomization.com) and participants were then allocated into 2 groups, namely group A and group B. Group A was given cocoa drink with high flavanol content during visit 2, followed by cocoa drink with low flavanol content during visit 3 and vice versa for group B. Experimental visits were carried out during the follicular stage of the participants' menstrual cycle with a wash-out period of at least ≥1 week between visits. For participants with regular menstrual cycle, visit 1 was conducted in between day 1 and 3 of menses day while visit 2 was conducted between day 9 and day 11 of menses day following 1 week of washout period. Meanwhile, for participants with irregular menstrual cycle, visit 2 was conducted in subsequent menstrual cycle (washout period for ± 1 month between visits). See Figure 3.1.

Figure 3.1: The study protocol for group A and group B

Participants were asked to fast between 8 to 12 hours (hrs), and to have the same meal the evening before each study visit. Participants were instructed to avoid eating and drinking foods rich in polyphenolics (cocoa/chocolate, tea, wine, coloured fruits), avoid taking alcohol as well as refraining from consuming caffeine and high fat foods for 24hrs prior to each study visit. Participants were also asked to avoid strenuous exercise for 24hrs before the study visit.

Immediately on arrival, participants were given a glass of water and asked to lie down on a hospital bed. Then, they were asked to rest quietly on this bed for the duration of the 3-hr protocol. After lying down for 45 minutes, the BP and HR recording was made, then endothelium-dependent FMD was measured. The FMD assessment was made according to Coretti *et al*., (2002) as described in Chapter 2 (2.3.2).

After these measurements, participants were given a cocoa drink with high flavanol content or with low flavanol content, depending on the randomisation. At 1 and 2hrs after cocoa drink consumption, the BP and HR as well as the measurements of endothelium-dependent FMD were performed again, as described above. The participants were able to read or listen to music between measurements but needed to remain resting quietly on the bed during this time. After the 2-hr time point, all equipment was removed, a snack was provided and they were free to leave.

On the second laboratory visit, they were given the alternative cocoa drink to the one they received on visit 2, and the protocol described above was

repeated. All tests were conducted between 09.00 and 12.00 hours. The study protocol for visits 2 and 3 is shown in Figure 3.2:
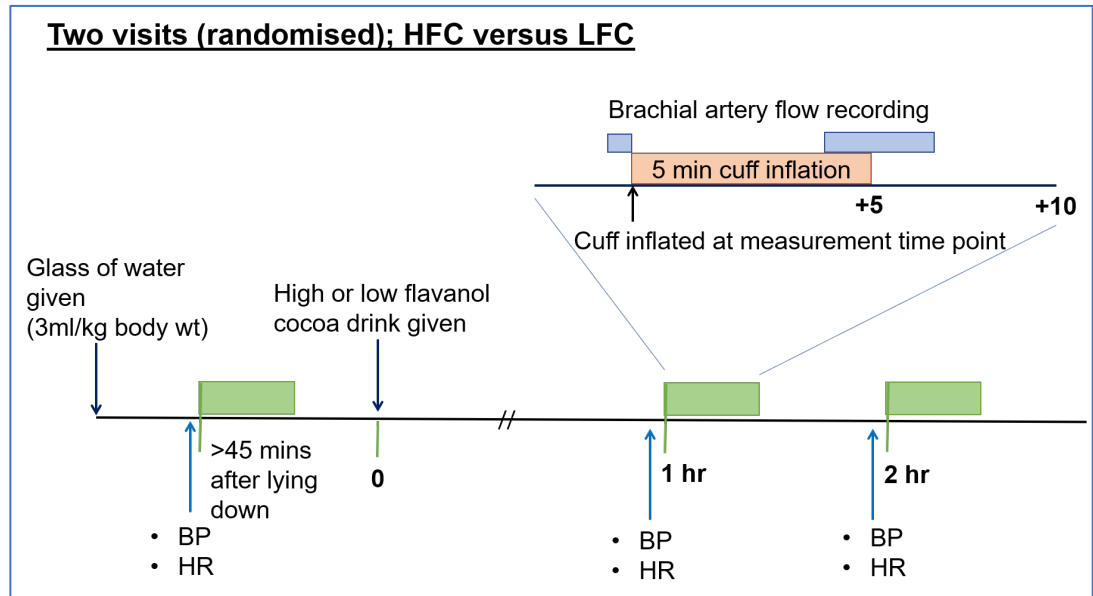
Figure 3.2: The study protocol for visits 2 and 3

3.2.4 Statistical analysis

The data were analysed using the Statistical Package for Social Science (SPSS version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. Repeated measures across the 2 visits were analysed using 2-way ANOVA e.g. to compare the difference in the FMD response seen after consumption between the HFC drink and the LFC drink. The difference in FMD at 1hr and 2 hrs time point measurement between the group were analysed using Independent *t*-test for normally distributed data and Mann-Whitney test for not normally distributed data. Meanwhile, the pre-cocoa FMD readings and the incremental FMD at each time point after cocoa ingestion within group were further assessed using paired *t*-test and presented as mean (SD) for normally distributed data, or analysed using Wilcoxon signed rank test and displayed as the median (25th and 75th percentile) for non-parametric data. A value of $p<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Characteristics of participants

A total of six healthy women [mean age 28(6.88) years; BMI 21.5(2.9) kg/m^2] completed the study.

3.3.2 Flow Mediated Dilatation

(i) To compare the acute vasoactive properties of a HFC with LFC.

Figure 3.3 shows the FMD response to the HFC and LFC drink, expressed as mean difference in FMD from pre-cocoa values (Δ%). The change in FMD response seen after consumption of the HFC drink was significantly different from the response seen after the LFC drink ($p<0.05$). The FMD response was significantly greater at 1hr following consumption of the HFC drink, compared to that seen 1hr following the LFC drink ($p<0.005$). However, the FMD response at 2hrs after cocoa consumption was not significantly different between drinks, although a trend for FMD to be higher following HFC consumption was observed ($p=0.075$).
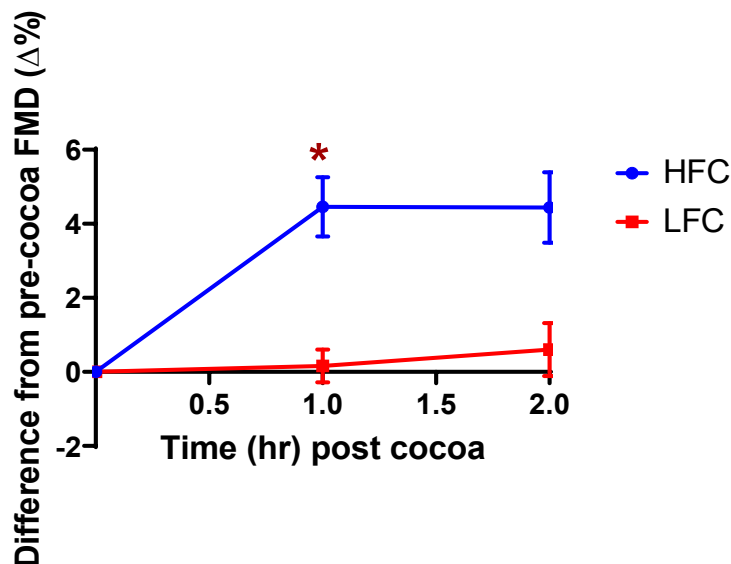
Figure 3.3: Mean difference in the FMD from pre-cocoa values following HFC and LFC drink consumption. *$p<0.005$ *versus* 1hr post LFC drink consumption

(ii) To determine the optimal time point for scheduling measurements in future acute protocols using HFC and LFC drinks.

Figure 3.4 shows the FMD values pre-cocoa consumption and post 1 and 2 hrs consumption of HFC and LFC drink, respectively. Each line represents a single participant. Mean FMD pre-cocoa consumption was different on the 2 study visits (HFC 4.4 (3.4)%; LFC 8.2 (2.7)%; $p < 0.05$). Following HFC consumption, the mean FMD response increased over the measurement period ($p < 0.005$), the mean FMD being higher ($p < 0.005$) than pre-cocoa values at both 1hr (9.1(4.5)%) and 2hrs (9.5 (5.4)%). Meanwhile, following LFC consumption, the mean FMD response did not increase over the measurement period ($p = 0.688$), mean FMD being 8.3 (2.7)% at 1hr and 8.8 (2.6)% at 2hrs. Comparison of the FMD responses to cocoa consumption observed on each study day showed a difference ($p < 0.05$).
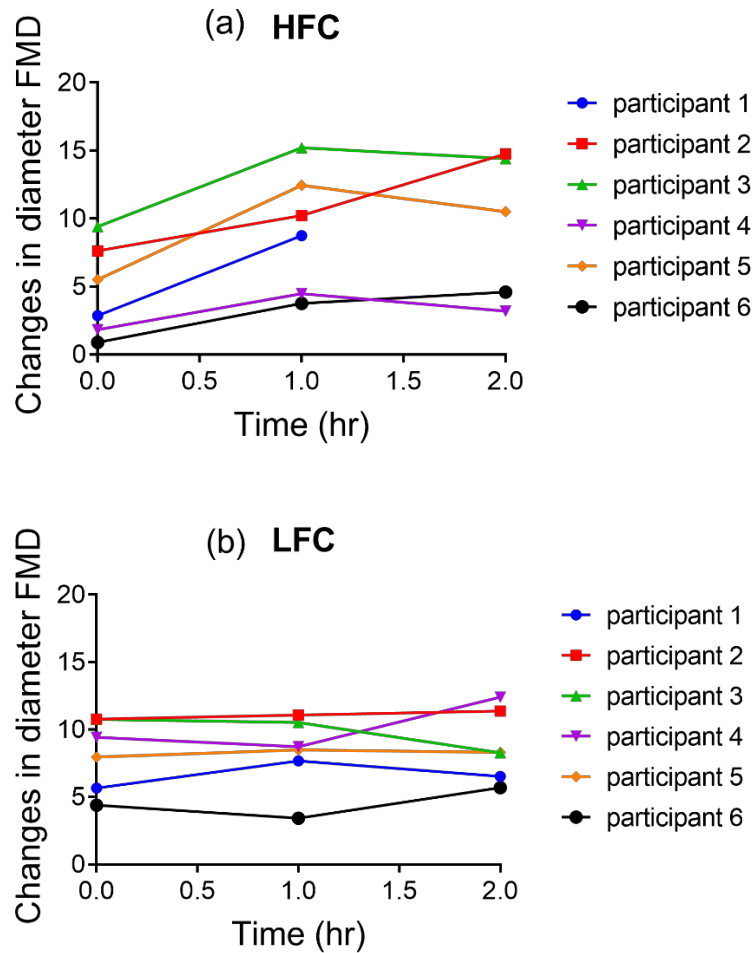
Figure 3.4: FMD values at baseline and after 1 and 2 hours consumption of HFC drink (a) and LFC drink (b).

3.4 Discussion

The present pilot study demonstrated the vasodilator effects of high flavanol cocoa compared to a cocoa assumed to have a lower flavanol content, in healthy humans. In this study, it was found that the FMD response after HFC was significantly greater compared to LFC at 1hr post consumption. Manufacturers indicated that the HFC used in the current study was made using a method which limited flavanol degradation during processing so that the end product was rich in flavanols content. Flavanols in cocoa seeds are labile. Handling the cocoa after harvesting and common cocoa processing practices often significantly reduce these compounds (Hurst *et al.*, 2011). Therefore, a significant flavanol content reduction can occur by the time

cocoa has been transformed into most commercially available cocoa for mass consumption.

The present study found that the FMD response after HFC was significantly increased compared with the supermarket cocoa drink at 1hr post consumption. In agreement with this finding, Heiss *et al*., (2015) reported that consumption of cocoa drink containing 450mg total flavanols (64mg epicatechin) significantly increased FMD at 1hr post consumption compared to a nutrient-matched cocoa flavanol-free drink, in both young and elderly (Heiss *et al.,* 2015). Another study reported that consumption of 300ml of a HFC drink containing 917mg total flavanols significantly increased FMD response at 2hrs after consumption compared to 100ml cocoa drink with < 10mg of flavanols, in patients with at least one cardiovascular risk factor (Heiss *et al.*, 2003). These results are also in line with a study which showed that consumption of 300ml of a HFC drink containing 917mg total flavanols by healthy participants significantly increased the FMD at 2hrs after consumption, compared to a LFC drink containing 37mg total flavanols (Schroeter *et al.*, 2006). Meanwhile, in healthy smokers, the consumption of 100ml of a HFC drink containing 306 mg of total flavanols significantly increased FMD at 2hrs post consumption compared to 100ml of a LFC drink containing 12mg of flavanols (Heiss *et al.,* 2007). The mechanisms causing the vasodilator response to cocoa are probably complex. In the present study, the fact that vasodilation was evident within 60 minutes after consumption of cocoa indicates an acute effect.

In the present study, FMD responses showed variation at both 1 and 2hrs following LFC consumption. Some previous studies have seen FMD improvements with very small cocoa doses. Grassi *et al*.,(2015) reported that consumption of 10gm of cocoa containing only 80mg flavonoids per day for 1 week with a very low energy (38 kcal) content by healthy participants, significantly increased FMD compared with the control containing 0mg flavanol (Grassi *et al.*, 2015). Indeed, in the present study, some of the participants did show an increase in FMD at the 2hrs mark following LFC consumption, which is probably the reason why the difference between the high and low flavanol cocoa at this point is not significant.

It has also been reported that FMD response can be affected by other factors such as antioxidant therapy (Engler *et al.*, 2003), sleep deprivation (Takase *et al.,* 2004), smoking (Lekakis *et al.*, 1997) and caffeine intake (Papamichael *et al.*, 2005). Engler *et al.*, (2003) evaluated the effects of antioxidant vitamins C (500 mg/d) and E (400 IU/d) for 6 weeks in children with familial hypercholesterolemia or the phenotype of familial combined hyperlipidemia. It was observed that vitamins C and E significantly improved FMD, suggesting that these vitamins restore endothelial function in hyperlipidemic children (Engler *et al.,* 2003). In another study, FMD was measured in healthy male college students who were under great stress to pass the examination and had chronic sleep deprivation for 4 weeks, during which sleep lasted <80% of that on ordinary days. It was found that chronic stress caused significantly decreased FMD (Takase *et al.*, 2004). It was also found that short-term smoking led to a significant decrease in FMD in healthy participants (Lekakis *et al.*, 1997). Another study evaluated the acute effect of coffee ingestion on endothelial function and the potential role of caffeine in healthy participants. It was found that caffeinated coffee led to a significant decrease of FMD lasting for at least 1hr after intake but no significant effect on FMD was found with the decaffeinated coffee, suggesting that this effect might be attributed to caffeine (Papamichael *et al.*, 2005). Therefore, it is very important to control these factors which have been shown to influence FMD.

The present study demonstrated benefits in endothelial function after acute consumption of HFC containing 830mg of flavanols per serving; the improvement observed with the consumption of HFC was significantly greater than the supermarket cocoa which was assumed to contain <50mg of flavanols per serving. Thus, the significantly increased FMD response at 1hr after consumption of HFC could be explained by the presence of higher levels of flavanols compared to LFC. Based on this finding, another study was then conducted to look at the acute effects of cocoa drink at 1hr on peripheral blood flow in healthy participants.

Chapter 4: Developmental Study

FMD study 2: To validate vasoactive properties and the suitability of delivering cocoa in capsules in healthy participants

4.1   Introduction

Cross-sectional and prospective studies carried out in recent years suggest that regular dietary intake of plant-derived foods and beverages could reduce the risk of cardiovascular disease (Corti *et al.,* 2009). Cocoa can be a rich source of 'flavanols', which are often referred to as 'antioxidants', and are thought to confer cardiovascular benefits by improving arterial endothelial function (Schroeter *et al.,* 2006). Studies have demonstrated an improvement in blood flow and BP following the consumption of cocoa flavanols in healthy people, as well as in patients at high risk of cardiovascular disease (Grassi *et al.,* 2005b, Sansone *et* al., 2015). Previous studies in the last 15 years have investigated the properties of cocoa when consumed as a drink or in chocolate bars. However, the present study would like to investigate whether the same properties can be seen when the cocoa is consumed in capsules, and whether any effects are delayed due to the time required for the contents to be released following capsule dissolution. The protocol used in this validation study duplicates the protocol used in the previous pilot study investigating the effect of a supermarket brand cocoa and Chococru™ on FMD but extends the measurement period to 3hrs.

4.1.1   Study aims

The aim of this pilot study was

- To determine the time frame for the acute vasodilatory effect of the HFC delivered in a capsule (Chococru™) to occur and to compare any effect with a non-cocoa placebo (coconut flour).

- To determine the optimal time point for scheduling measurements in future acute protocols using HFC and placebo capsules.

Hypothesis

The HFC ingestion would induce a greater vasodilatory response to a shear stress stimulus than a non-cocoa control.

4.2   Methods and Materials

4.2.1   Participants

The study design, selection of the participants were the same as discussed in Chapter 3 [3.2 (i)].

4.2.2   Materials

Cocoa capsules

As this proposed study was not being carried out in conjunction with a cocoa manufacturer who could provide a high and low flavanol cocoa product, suitable cocoa with a high flavanol content (which would be palatable for long term supplementation studies) as well as an appropriate placebo which participants could be effectively blinded to, needed to be sourced. HFC had greater bitterness and is paler than cocoa which has lower flavanol content (i.e. that is processed using alkali, the Dutch method). It was therefore difficult to blind participants to the high and low flavanol cocoa products available to purchase in the United Kingdom. Moreover, the HFC used in the previous study was not generally liked by participants due to its

bitterness, making compliance in chronic supplementation studies difficult if cocoa drinks were used. An European Union approved health claim had been given to the company Barry Callebaut for their HFC when consumed as cocoa, chocolate or within capsules (Actocoa™). This HFC can be purchased in the UK as 'Chococru™' and the present study aimed to determine its cardiovascular effects (SBP, DBP and FMD) when it was consumed within cellulose capsules (suitable for vegetarians). Delivery of the cocoa in these opaque capsules would enable the present study to produce a placebo capsule which contained no flavanols and would mask the bitterness of the HFC product. Fat-reduced coconut flour was chosen as the placebo for this study as it had a similar macronutrient profile to the Chococru™ cocoa powder. Details on cocoa capsules used in this study was described in Chapter 2 [2.6.2 (i)].

4.2.3 Experimental outline

Nine healthy female participants who fulfilled the inclusion criteria attended a screening visit (visit 1). They were briefed concerning the nature of the study and informed consent was obtained. Participants were then allocated into 2 groups - namely group A and group B, based on the randomisation (www.randomization.com). Group A was given cocoa flavanol capsules during their visit 2 followed by placebo capsules during their visit 3 and vice versa for group B. Experimental visits were carried out during the follicular stage of the participants' menstrual cycle with a wash-out period of at least ≥1 week between visits.   See Figure 4.1.
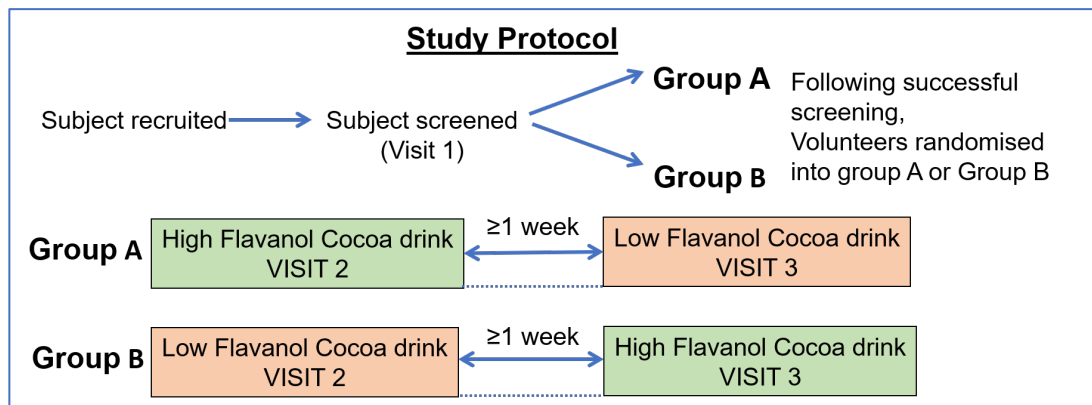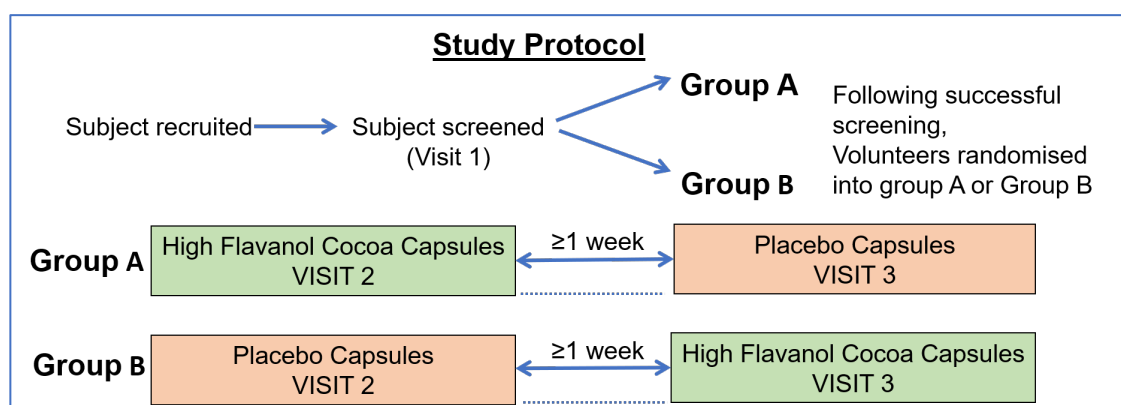

Figure 4.1: The study protocol for group A and group B

Participants were asked to fast between 8 to 12hrs and to have the same meal the evening before each study visit. They were given similar instructions as in the pilot study described in Chapter 3 [3.2 (iii)]. Immediately on arrival, participants were asked to empty the bladder. Then, a glass of water was given and they were asked to rest quietly on a bed for the 4-hr protocol. BP and HR were measured in triplicate every 15 minutes throughout. After lying down for 45 minutes, the BP and HR were recorded. Endothelium-dependent FMD was measured using the same technique as described in Chapter 2 (2.3.2). After this measurement, participants were given ten capsules containing either 415mg of HFC or fat-reduced coconut flour (placebo) cocoa, depending on the randomisation. At 1, 2 and 3hrs after capsule consumption, measurements of endothelium- dependent FMD were performed. They were able to read or listen to music between measurements but needed to remain resting quietly on the bed during this time. After the 3-hr time point, all the equipment was removed, a snack was provided and they were free to leave. The study protocol for visits 2 and 3 is shown in Figure 4.2:
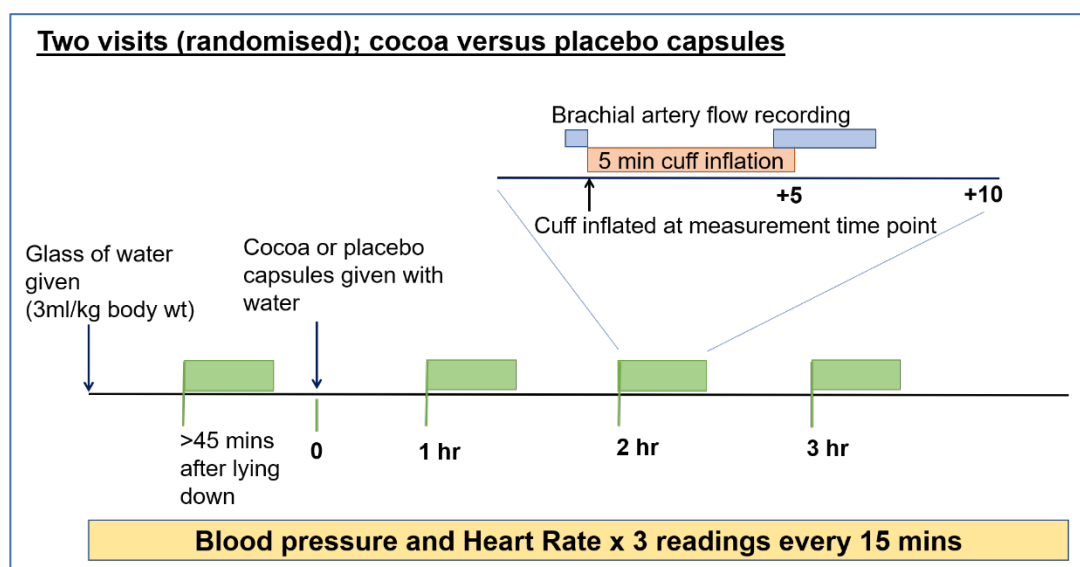
Figure 4.2: The study protocol for visits 2 and 3

On the second laboratory visit (visit 3), they were again given 10 capsules, the alternative capsules to those received on visit 2, and the protocol described above was repeated.

4.2.4 Statistical analysis

The data were analysed using the SPSS (version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. Repeated measures across the 2 visits were analysed using Repeated Measure 2-way ANOVA e.g. to compare the differences in the FMD, SBP, DBP and HR responses seen 1, 2 and 3hrs after capsules consumption between HFC or coconut flour (placebo). The decrease in SBP and HR at 45 minutes before cocoa ingestion within group was assessed by using paired t-test and presented as mean (SD) for normally distributed data, or analysed using Wilcoxon signed rank test and displayed as the median (25th and 75th percentile) for non-parametric data. The results indicated that the data for SBP, DBP and HR did not show a normal distribution. Therefore, these parameters were presented as the median (25th and 75th percentile). A value of $p < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Characteristics of participants

A total of nine healthy women (mean age 28 (5.6) years; BMI 22.5 (2.6) kg/m^2) completed the study.

4.3.2 Flow Mediated Dilatation

(i) To compare the acute vasodilatory effect of the HFC delivered in a capsule (Chococru™) to occur with a non-cocoa placebo (coconut flour).

Figure 4.3 shows the difference in the FMD response to the HFC and placebo capsules, expressed as a difference in FMD from pre-cocoa values (Δ%). The change in FMD response seen over the 3hrs after consumption of the HFC capsules was similar from the response seen after the placebo capsules (p>0.05).
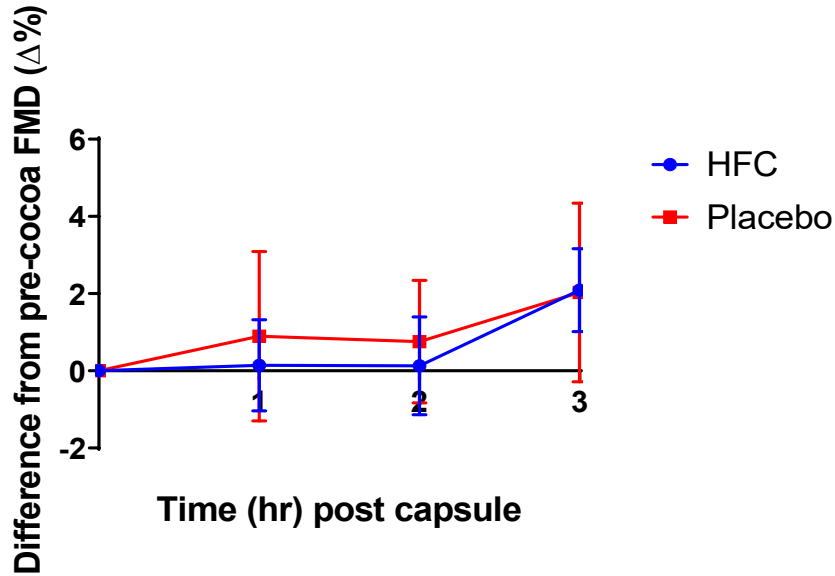
Figure 4.3: Mean difference in FMD from pre-cocoa values following consumption of HFC and placebo capsules over the 3 hours was the same (p=0.987).

(ii) To determine the optimal time point for scheduling measurements in future acute protocols using HFC and placebo capsules.

Figure 4.4 (a) and (b) show FMD values at baseline and 1, 2 and 3hrs after consumption of cocoa and placebo capsules, respectively. Each line represents a single participant. Mean FMD values pre-cocoa consumption on the HFC and placebo visits were similar (HFC 5.2 (2.4)%; placebo 6.9 (3.7%); p>0.05). Following consumption of HFC capsules, the mean FMD response did not change over the measurement period (p>0.05); mean FMD being [5.3 (3.8)% at 1hr, 5.3 (4.4)% at 2hrs and 6.9 (2.3)% at 3hrs]. Similarly, no changes were observed in the mean FMD response over the measurement period following the consumption of placebo capsules (p>0.05); mean FMD was 7.8 (5.1)% at 1hr, 7.6 (1.7)% at 2hrs and 8.9 (4.5)% at 3hrs). Comparison of the FMD responses between the 2 visits showed no significant difference (p>0.05).
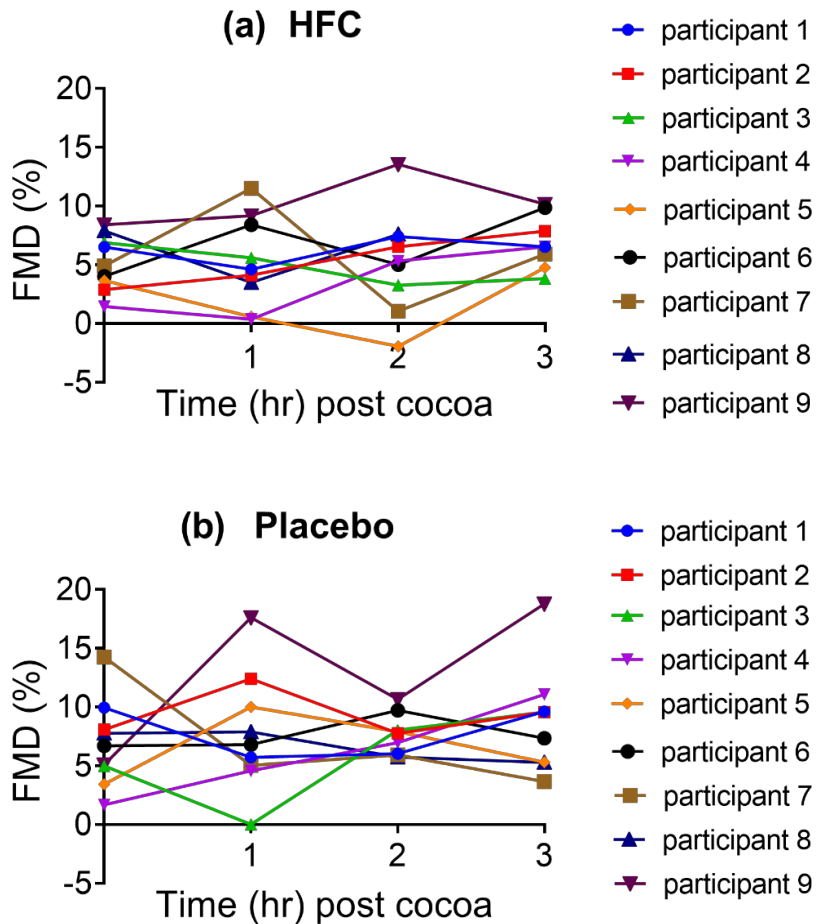
Figure 4.4: FMD values at baseline and after 1, 2 and 3 hours consumption of HFC capsules (a) and placebo capsules (b).

4.3.3 Blood pressure

Before capsule consumption, SBP significantly decreased over the 45 minutes resting period during each visit, $p < 0.005$. However, the response in SBP over this period was the same on each visit ($p > 0.05$). Similarly, after capsule consumption, the response over the 3hrs was similar on each visit ($p > 0.05$) (Figure 4.5).

On the other hand, before capsules consumption, DBP did not change over the resting period during each visit ($p > 0.05$). The response in DBP over this period and after capsule consumption over 3hrs, was the same on each visit, ($p > 0.05$) (Figure 4.6).
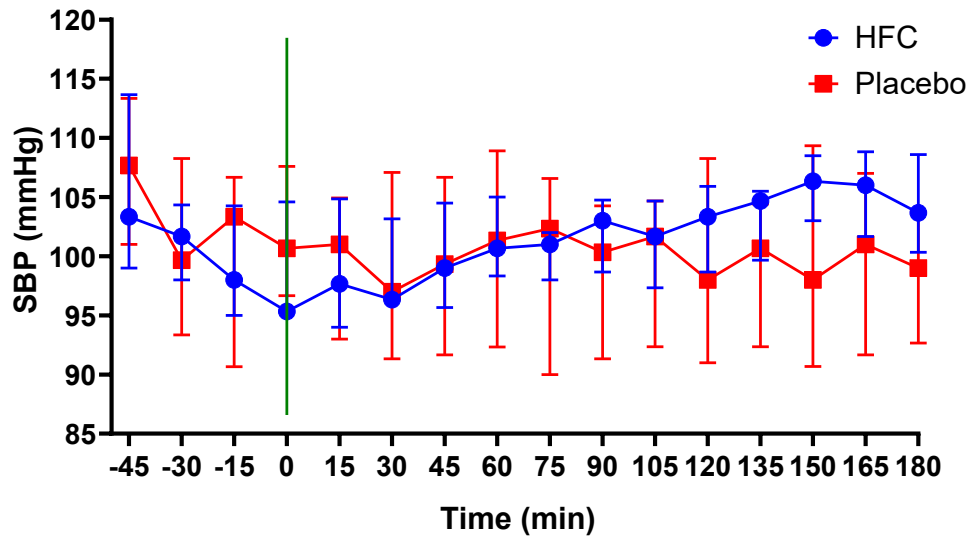
Figure 4.5: Systolic blood pressure (SBP) pre and post capsules consumption. Data are presented as medians with error bars indicating the 25th and 75th percentile value. The vertical line indicates when the capsules were consumed. There was a significant decrease over the initial rest period during each visit, $p < 0.005$.
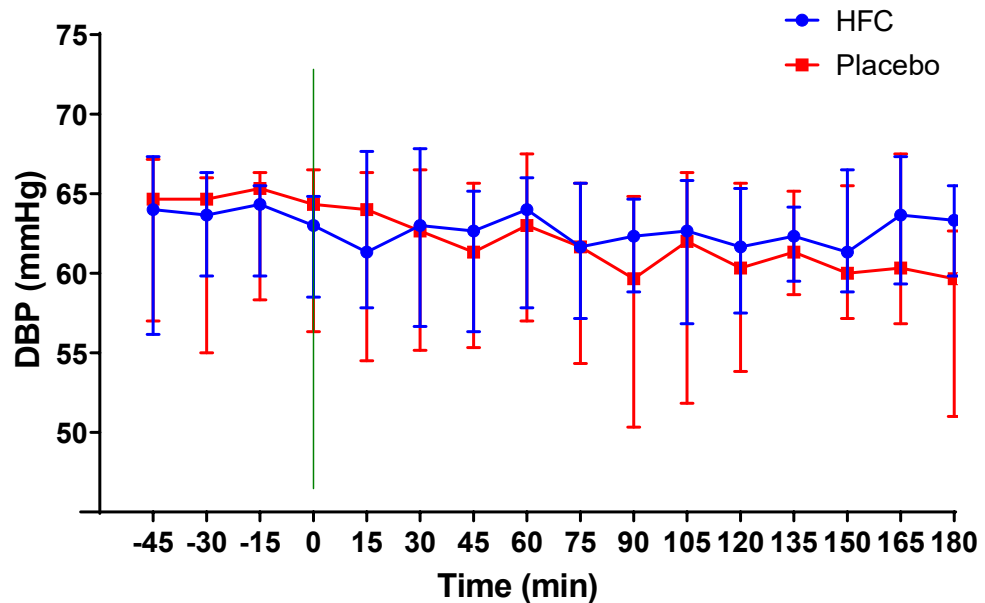
Figure 4.6: Diastolic blood pressure (DBP) pre and post capsules consumption. Data are presented as medians with error bars indicating the 25th and 75th percentile value. The vertical line indicates when the capsules were consumed. No statistical differences were observed.

4.3.4 Heart rate

Before capsule consumption, HR significantly decreased over the initial rest period during each visit, $p < 0.005$. However, the response in SBP over this 45 minute period was the same on each visit ($p > 0.05$). Similarly, after capsule consumption, the response in HR over the 3hrs was the same during each visit ($p > 0.05$) (Figure 4.7).
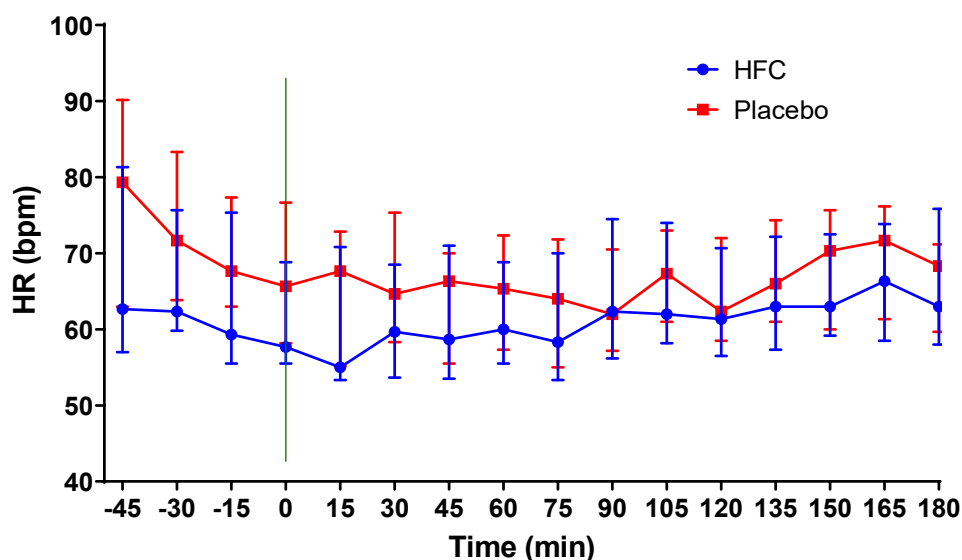
Figure 4.7: Heart rate (HR) pre and post capsules consumption. Data are presented as medians with error bars indicating the 25th and 75th percentile value. The vertical line indicates when the capsules were consumed. There was a significant decrease over the baseline period during each visit, $p < 0.005$.

4.4 Discussion

The present study demonstrated no effect on FMD, 1, 2 and 3hrs after acute consumption of HFC capsules, or on other cardiovascular parameters (SBP, DBP and HR). However, there was a significant decrease in the SBP and HR over the baseline period during both the visits. In line with these findings, Sala *et al.,*(2005) found a significant decrease in SBP during a 16 minutes rest in the chair-seated position in 55 patients with untreated essential hypertension as a result of systemic vasodilation (Sala *et al.,* 2006). It was reported that the sympathetic activation induced by the gravitational pooling

of blood in the dependent veins during active standing is known to cause an arteriolar and venous constriction (Wallin and Fagius, 1988). In the present study, when the participants were instructed to lie down, the progressive decrease in vascular resistances observed was likely due to the sympathetic withdrawal from the resistance vessels following reduced hydrostatic pressure on the capacitance vessels, compared with the active standing position before lying down (Morganti *et al.,* 1985). Meanwhile, the significant decrease in the HR during both the visits in response to the fall in SBP over the baseline period (during lying down) could be consistent with sympathetic withdrawal.

Apart from that, no effect on FMD and cardiovascular parameters (SBP, DBP and HR) of acute consumption of ten HFC capsules after 1, 2 and 3hrs may be because the flavanol content was too low. The amount of flavanol content given in the current study was 415mg of flavanols (50mg epicatechin) which was half of the dose given in the previous FMD pilot study. In that study, the HFC was given as a beverage containing 830mg of flavanols (99.6mg of epicatechin) per serving and this amount of epicatechin significantly increased the FMD response at 1hr after consumption compared to the LFC which was assumed to contain <50mg of flavanols per serving. The European Food Safety Authority (EFSA) approved a cardiovascular health benefit claim for consuming dark chocolate, cocoa extract and cocoa based beverages containing 200mg total flavanols (46mg of epicatechin /day), and therefore, the amount of epicatechin given in the present study met this recommendation. However, this is a recommendation based on chronic intake for cardiovascular health, rather than on the acute effects of cocoa flavanols on vascular function. A previous meta-analyses subgrouped the dose of epicatechin into <50mg/day, 50-100mg/day and > 100mg/day to systematically review the effects of acute and chronic ingestion of flavanols on major cardiovascular risk factors. Although authors conclude that, acutely, improvements to FMD were related to epicatechin dose, and that FMD improvements could be demonstrated after chronic ingestion of doses <50mg/day, they did not identify any studies which had tested the acute effects of cocoa consumption at epicatechin doses <50mg,

to be able to assess FMD response to these levels of intake (Hooper *et al.*, 2012). Therefore, the lack of effect of acute consumption of HFC observed in the present study is likely to have been due to the the lower dose of flavanols given to the participants.

Meanwhile, the HFC in the present study was given in the form of capsules. Capsule shell materials come in two different types, either gelatin- or cellulose-based materials. The type of capsule used in this study was cellulose-based. A previous study reported that an unwanted interaction of cellulose-based material may occur with polyphenol-containing extracts. Polyphenols and cellulose have a strong hydrophobic interaction, which may significantly reduce the bioavailability of the polyphenol (Phan *et al.,* 2015). Another study observed that hydroxypropyl methylcellulose (cellulose-based) capsule shell materials, commonly used in human trials with plant extracts may cause an unfavourable interaction with green tea catechins. Compared with gelatin-based capsules, cellulose-based capsules adversely influence the disintegration and the dissolution characteristics of the green tea catechins, thus compromising the rate and extent of absorption. Dissolution may be hampered in both the fasting and fed states, with up to one-half of the material still undissolved after 2hrs (Glube *et al.,* 2013). Thus, the use of cellulose-based capsule shell material might be an additional factor contributing to the apparent absence of significant effects of HFC in the present study.

Moreover, it was found that delivering cocoa in capsules to the participants might not be suitable for longer term supplementation studies, especially if the number of capsules required to be taken daily needed to be increased to meet target epicatechin intakes. It has been reported that the prevalence of discomfort when swallowing pills ranges between 26% and 50%, depending on the population studied. In a nationwide study of the general population, 40% of an adult population in the United States reported to have difficulties with swallowing tablets or capsules at some point, even though most had no problems swallowing food or liquid (Llorca, 2011). This observation can be due to the anatomical organization of the oropharynx,

which is not well adapted to swallowing tablets but is well suited to swallowing food of greater size (Andersen *et al.,* 1995).

Interestingly, difficulty with swallowing pills was demonstrated more frequently in patients aged 18–64 years than in patients aged ≥65 years (44 vs 26%, respectively) (Llorca, 2011), despite the general perception that such problems would increase with age (Spieker, 2000). Perhaps in the general population, older individuals use tablets more frequently than younger individuals and are, thus, more practiced at swallowing them. In addition, it was also found that the prevalence of experiencing discomfort with pill swallowing is higher in women compared with men (51 vs 27%, respectively) (Llorca, 2011). In the long-term cocoa supplementation study among participants with PRP, the age range of participants (18-60 years) would be almost similar with the reported cases and the recruitment would involve more females, as PRP is more common among women compared to men. In addition, of the respondents who report discomfort when swallowing pills, 14% delayed doses, 8% missed doses and 4% discontinued treatment (Llorca, 2011). These observations highlight the potential problem of reduced participant compliance and persistence with the long-term supplementation study as a result of pill-swallowing issues.

As a conclusion, the present study demonstrated no effect of acute consumption of ten HFC capsules containing 415mg of flavanols (50mg epicatechin) on FMD and cardiovascular parameters (SBP, DBP and HR) after 1, 2 and 3hrs. The reason for not being able to demonstrate an acute cardiovascular effect could be because the flavanol content may have been low. It also seems that delivering cocoa in ten or more capsules per serving for 3 months to the participants in the long-term supplementation study to participants with PRP would be unpleasant and impractical; thus, there was a need to find other alternatives.

Chapter 5: Acute effects of cocoa drink on peripheral blood flow in healthy participants

5.1　Introduction

Cocoa flavanols have been demonstrated to promote vasodilation in conducting arteries, as well as in the microvasculature (Heiss *et al.*, 2015). However, there is the possibility that the acute effects of cocoa on promoting vasodilation, and increasing skin BF (Neukam *et al.*, 2007) could affect temperature regulation, and therefore exacerbate symptoms in those with PRP. Therefore, before looking at any potential benefits of dietary supplementation with HFC on ameliorating vasospasm in those with PRP, it was important to characterise any peripheral and central effects of cocoa on temperature regulation in those without PRP.

5.1.1 Study aim

The aim of this study was to determine the effect of a HFC drink (at 1 hr) on:

- The core temperature and cardiovascular response (BP, HR, CO, TPR) to localised cooling.

- Skin BF and skin temperature responses of the finger during localised cooling and at a RT of 25°C, during rewarming after localised cooling (experimental and control finger).

- The time taken for skin temperature of the finger to stabilise during localised cooling (in an air temperature of 15°C) and at a RT of 25°C during rewarming after localised cooling.

Hypothesis

Although HFC ingestion may have effects on cardiovascular measures (reduction in BP and decreased peripheral resistance), counter-regulatory responses to localised cold exposure would not be compromised.

5.2   Methods and Materials

5.2.1 Sample size calculation

This study's primary aim was to characterise the effect of HFC on the peripheral skin temperature response to cold exposure, but the degree of any effect was not known and had not been characterised previously in the literature.

Previous studies have suggested that a 70% increase in skin BF could be induced by acute consumption of HFC (Neukam *et al.,* 2007) and 5 participants would be sufficient to power the study for this variable at the level of 80% (Lenth, 2006). Moreover, acute consumption of HFC induced a difference in SBP (compared with LFC) of 5.3mmHg and in DBP of 4.0 mmHg (Faridi *et al.,* 2008). To power these observations at the level of 80% would require 11 and 18 participants, respectively. It was therefore proposed that 12 participants be recruited onto the current study to power the skin BF and SBP observations, with temperature regulation data obtained from this study being used to power subsequent studies.

5.2.2 Participants

The study protocol was approved by the Medical School Research Ethics Committee, Faculty of Medicine & Health Sciences, Queen's Medical Centre, Nottingham. Written informed consent was obtained from all the participants. The present study was a randomised, double-blind, cross-over design involving twelve healthy women, aged between 18-40 years. Selection of the participants was based on the inclusion and exclusion criteria as discussed in Chapter 3 [3.2 (i)].

5.2.3 Materials

Cocoa drinks

The cocoa powders used in this study were a high flavanol cocoa powder, containing 830mg of flavanols per 10gm (Chococru™), and an alkalised

cocoa powder (Sainsbury's) which was assumed to contain <50mg of flavanols per 10gm (Miller *et al.*, 2008). Compositional details for high flavanol and low flavanol cocoa powder are described in Chapter 2 (2.6.1)

5.2.4 Experimental outline

Participants who were successful in fulfilling the criteria attended a screening visit (visit 1), were briefed concerning the nature of the study and informed consent was obtained. Randomisation was computer generated (www.randomization.com) and participants were then allocated into 2 groups, namely group A and group B. Group A was given a cocoa drink with high flavanol content during their visit 2, followed by the cocoa drink with low flavanol content during their visit 3 and vice versa for group B. Experimental visits were carried out during the follicular stage of the participants' menstrual cycle with a wash-out period of at least ≥1 week between visits. See Figure 5.1.

Study Protocol

Subject recruited → Subject screened (Visit 1) → Group A / Group B

Following successful screening, Volunteers randomised into group A or Group B

| Group A | High Flavanol Cocoa drink VISIT 2 | ≥1 week | Low Flavanol Cocoa drink VISIT 3 |

| Group B | Low Flavanol Cocoa drink VISIT 2 | ≥1 week | High Flavanol Cocoa drink VISIT 3 |
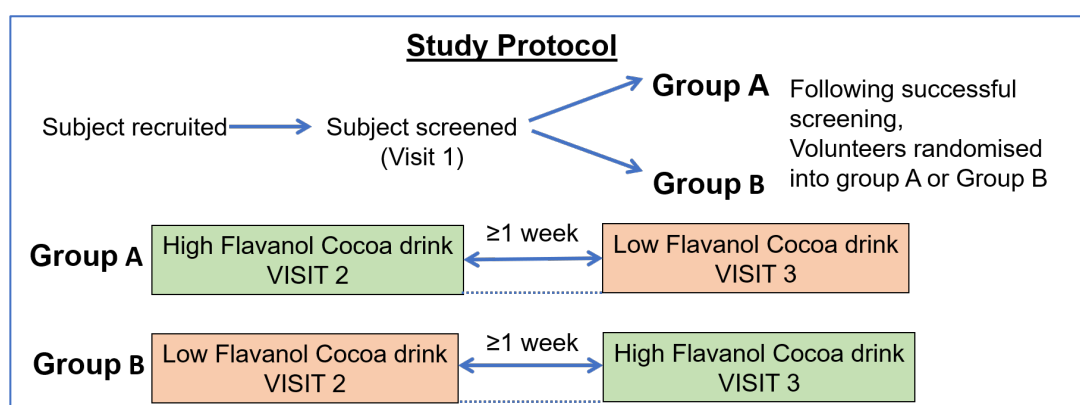
Figure 5.1: The study protocol for group A and group B

Participants were asked to fast between 8 to 12hrs and to have the same meal the evening before each study visit. They were given similar instructions as in the pilot study described in Chapter 3 [3.1.2 (iii)]. Immediately on arrival, they were asked to lie semi-supine on a hospital bed and skin temperature was recorded by thermocouples, attached using adhesive tape to 3 sites on the skin surface (index finger of both hands, back of right hand). Temperature was recorded from these sites every 2 minutes throughout the experiment. The 'core' temperature was recorded at

certain time points throughout the study using an infrared tympanic membrane thermometer, and the RT was monitored. Outside temperature was recorded based on the temperature reported for Nottingham (Watnall Weather Station) from the Meteorological Office website on the same day. A summary of the study protocol during the experimental visit is shown in Figure 5.2.
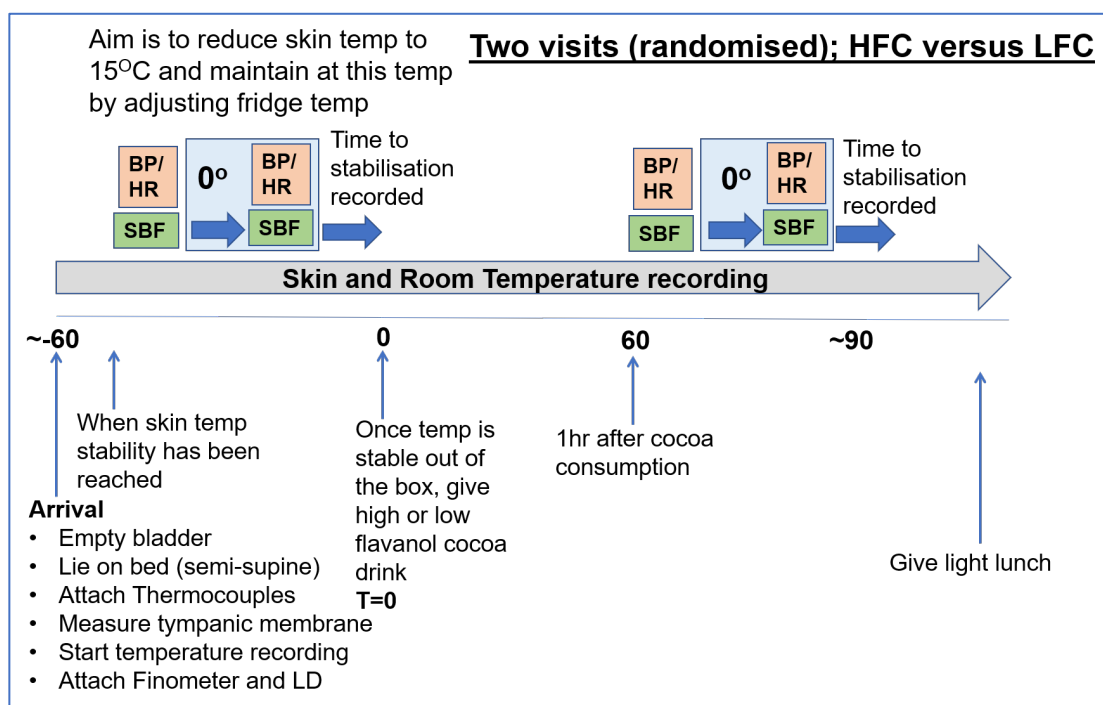
Figure 5.2: The study protocol during experimental visit. LD:Laser Doppler; SBF: Skin blood flow; LFC: low flavanol cocoa; HFC: high flavanol cocoa

The Finometer was attached to the left middle finger to record cardiovascular parameters (CO, SBP, DBP, HR) via a small finger cuff. Then, a Laser Doppler (LD) probe was attached to the dorsum of the index finger at the distal phalanx of both hands, to record blood flow in the microcirculation. The LD probe was positioned at 90° to the skin and held gently in place by surgical tape, so as not to compress the capillary bed and connected to a Doppler monitor. LD flow was measured in arbitrary units. This perfusion value was recorded by a laptop computer for subsequent analyses. In the present study, the LD probes attached to the fingers of both hands were different. Therefore, to compare the skin BF of left and right fingers, LD data obtained from both fingers were standardised by expressing values as the % of the value seen pre-cocoa, at RT.

In RT (set at 25°C), once the skin temperature of the right finger (experimental hand) had remained stable for 6 minutes (constant ± 0.3°C for a minimum of 6 minutes after arrival in the laboratory), the baseline Finometer and LD measurements were recorded and the skin and 'core' temperature were noted. Then, the right hand was placed in a temperature regulated box which was set at 0°C. The hand was cooled to a finger skin temperature of 15°C; then the box temperature was modified to maintain the skin temperature at 15°C. The time that it took for the skin temperature on the fingers to reach 15°C was recorded. With the finger skin temperature stabilised at 15°C, Finometer and LD measurements were recorded from the left hand and the 'core' temperature at this point was noted. Then, the hand was removed from the chamber, and allowed to re-equilibrate at RT. The time taken for the skin temperature to reach stability and the skin temperature at this point, were recorded. Once the skin temperature had been stable for 6 minutes, the cocoa drink was given to the participants. At 1hr after consuming the drink, Finometer, LD, skin and 'core' temperature measurements were recorded. Then, the right hand was placed in the temperature regulated box again and the same protocol was repeated, as described above. The left hand acted as a control site, remaining at RT throughout, and was used for the Finometer measurements.

5.2.5 Statistical analyses

The data were analysed using the SPSS (version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. The pre-cocoa consumption for core temperature, SBP, DBP, TPR, CO, HR, time taken to stabilise in RT and in the cold box, skin BF and skin temperature of the experimental finger and control finger readings were compared with data that was recorded 1 hr after cocoa ingestion, within the same group, using paired *t*-test for normally distributed data, or analysed using Wilcoxon signed rank test for non-parametric data; comparison between the visits were analysed using independent *t*-test for normally distributed data and Mann-Whitney test for not normally distributed data. The change over time in the skin BF of the experimental and control finger across the study during either visit was compared using One Way ANOVA. In addition, the change over time in the skin BF of the experimental and control finger between the visits was compared using Two-Way ANOVA.The results indicated that most of the data did not show a normal distribution. Therefore, all parameters were presented as the median (25th and 75th percentile). A value of p<0.05 was considered to be statistically significant.

5.3. Results

5.3.1 Characteristics of participants

A total of twelve healthy women completed the study. The majority of the participants were from Asian countries (70%) and the remaining 30% from the UK. The baseline characteristics of participants are shown in Table 5.1.

Characteristics	Mean	SD
Age (years)	26.42	4.48
Weight (kg)	57.84	10.69
Height (m)	1.62	0.07
BMI (kg/m^2)	22.06	2.74

Table 5.1: Characteristics of participants. BMI, body mass index;

SD, standard deviation

5.3.2 Effect of cocoa on core temperature and cardiovascular measures (SBP, DBP, CO, HR, TPR) to localised cooling

(i) Core temperature

After stabilising in RT, the core body temperature recorded on both the visits was similar. When the experimental hand was exposed to localised cooling before cocoa consumption, core temperature showed a trend to increase over this time period, during both the visits (HFC p=0.072, LFC p=0.073). One hour after cocoa consumption, when the experimental hand was at RT, core temperature after HFC consumption had returned to the pre-cooling values seen at the start of the study. However, core temperature 1hr after LFC consumption was significantly warmer compared to pre-cooling values, although this was not significantly different from the values recorded after HFC. When the experimental hand was exposed to localised cooling, after cocoa consumption, core temperature was significantly warmer on the HFC visit whereas this response was not seen during the LFC visit. Meanwhile, comparison of the core temperature between the visits at each time point measurements showed no significant difference (p>0.05) (Table 5.2).

Core temperature	HFC visit	LFC visit
Pre cocoa in RT	36.7 [36.5-37.0]	36.6 [36.5-36.8]
Pre cocoa in cold box	36.7 [36.7-36.9]	36.7 [36.5-36.8]
Post cocoa in RT	36.7 [36.6-36.9]	36.8 [36.5-37.0]*
Post cocoa in cold box	36.9 [36.8-37.0]#	36.7 [36.5-36.9]

Table 5.2: Core temperature pre and post cocoa consumption at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). *p<0.05 vs pre-cocoa RT, #p<0.005 vs post-cocoa RT.

(ii) Cardiovascular measures

At the start of the study, all the cardiovascular variables were similar on both the visits. When the experimental hand was exposed to localised cooling,

before cocoa consumption, SBP, DBP and TPR were significantly higher and this response was the same between both the visits. By contrast, CO during both visits and HR during the HFC visit were significantly lower. After consumption of both the drinks, when the experimental hand was exposed to localised cooling, SBP and DBP were significantly higher whereas no significant changes were observed in CO, HR and TPR during either visit. SBP and DBP during both visits as well as TPR and CO during the HFC visit did not appear to be affected by cocoa consumption, with these measurements returning to pre-cooling values after the experimental hand had been returned to RT 1hr after cocoa consumption. TPR demonstrated a trend to be higher (p=0.059) and CO demonstrated a trend to be lower (p=0.060) after LFC consumption.

Meanwhile, during localised cooling, HR significantly increased 1hr after LFC consumption, and showed a trend of increasing (p=0.060) after HFC consumption, in comparison with exposure to the cold pre-cocoa consumption. In addition, comparison of the cardiovascular parameters between the visits at each time point measurements were similar (p>0.05) (Table 5.3).

	HFC visit	LFC visit
SBP (mmHg)		
Pre cocoa in RT	112.1 [108.83 -117.71]	108.7 [104.86-113.94]
Pre cocoa in cold	116.1 [113.50 -121.70]*	115.0 [112.79 -122.45]**
Post cocoa in RT	117.5 [110.61-121.46]	108.4 [105.69-114.20]
Post cocoa in cold	119.6 [114.48 -128.61]#	114.8 [110.27-121.55]#
DBP (mmHg)		
Pre cocoa in RT	65.6 [62.59-67.72]	64.1 [60.53-66.65]
Pre cocoa in cold	68.2 [65.63-73.68]*	68.3 [66.41-72.01]***
Post cocoa in RT	66.1 [63.58 -69.12]	63.0 [61.94-66.15]
Post cocoa in cold	69.3 [65.89-71.53]#	66.0 [62.04-70.15]#
Total Peripheral Resistance (PRU)		
Pre cocoa in RT	16.3 [15.03-18.42]	16.4 [13.07-19.39]
Pre cocoa in cold	17.7 [16.38-21.04]*	18.9 [16.42-21.54]*
Post cocoa in RT	18.8 [16.79-20.86]	19.8 [17.57-22.44]
Post cocoa in cold	19.6 [17.29-22.12]	20.2 [18.56-22.29]
Cardiac output (L/min)		
Pre cocoa in RT	4.9 [4.51-5.29]	4.8 [4.29 -5.63]
Pre cocoa in cold	4.5 [4.14-5.23]*	4.7 [4.01-5.09]*
Post cocoa in RT	4.3 [3.85-5.08]	4.0 [3.62 -4.59]
Post cocoa in cold	4.2 [3.79-5.32]	4.1 [3.72-4.63]
Heart rate (bpm)		
Pre cocoa in RT	59.5 [57.31-67.13]	58.2 [50.58-65.52]
Pre cocoa in cold	58.9 [54.04-65.33]*	59.5 [52.12-62.06]
Post cocoa in RT	60.3 [57.60-67.01]	60.5 [57.43-63.64]
Post cocoa in cold	60.0 [56.52-70.24]	60.5 [55.96-65.80]ε

Table 5.3: Comparison of cardiovascular parameters pre and post cocoa consumption in healthy participants (n=12). Data are presented as the median (25th and 75th percentile).*p<0.05 vs pre cocoa RT, **p<0.005 vs pre cocoa RT, ***p<0.001 vs pre cocoa RT, # p<0.05 vs postcocoa RT, ##p<0.005 vs post cocoa RT, εp<0.05 vs pre cocoa in cold

5.3.3 Effect of cocoa on the skin BF and skin temperature responses during localised cooling and at RT following localised cooling

Outside temperature

The mean outside (environmental) temperature recorded from the Nottingham (Watnall) Weather Station from the Meteorological Office website on HFC visits was 13.98 (2.75)^{0}C and on the LFC visits was 14.63 (2.17) ^{0}C. The outside temperature was similar between the visits.

(i) Skin BF and skin temperature response of the experimental finger

After stabilising in RT, the skin BF (Figure 5.3) and skin temperature (Figure 5.4) of the experimental finger on both the visits was similar. When the experimental hand was placed into the cold box before cocoa consumption, both measurements were significantly lower and the response was similar between both visits. These responses were replicated when the experimental hand was placed into the cold box after consumption of both the drinks. One hour after the experimental hand had been returned to RT, the skin BF and skin temperature after both cocoa consumptions were at pre-cooling values. Meanwhile, comparison of the skin BF and skin temperature of the experimental finger between the visits at each time point measurements showed no significant difference ($p > 0.05$).
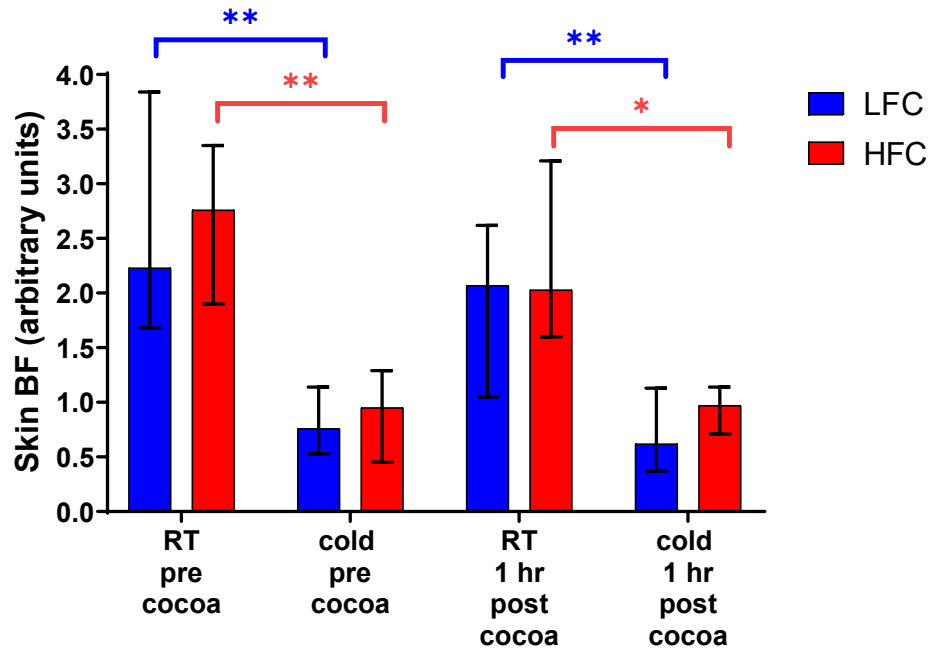
Figure 5.3: Skin blood flow of the experimental finger pre and post cocoa consumption at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). *p<0.05, **p<0.005.
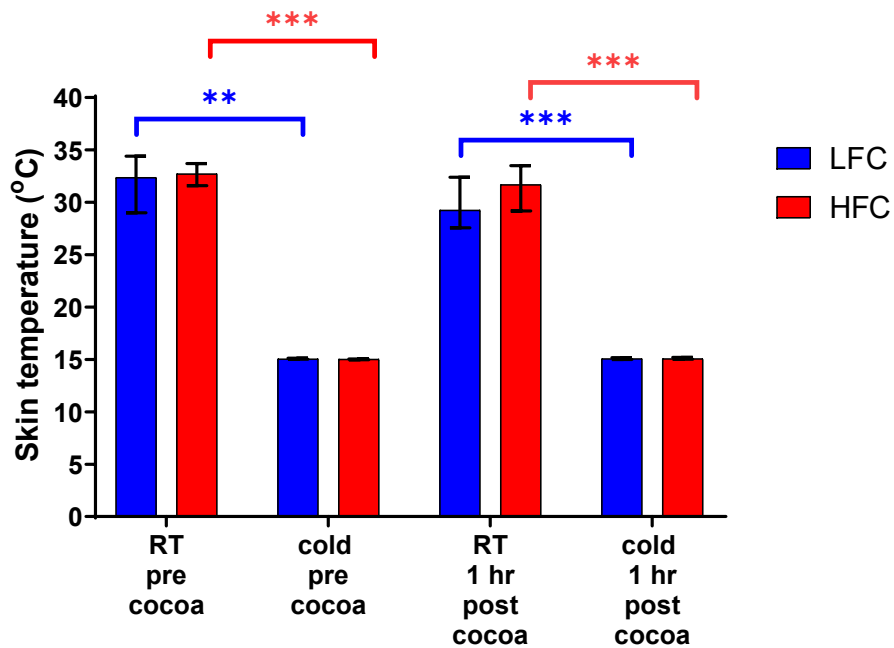
Figure 5.4: Skin temperature of the experimental finger pre and post cocoa consumption at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). **p<0.005; ***p<0.001.

(ii) Skin BF and skin temperature response of the control finger

Figure 5.5 shows skin temperature of the control finger during both visits. At the beginning of the study, skin BF and skin temperature of the control finger on both visits was similar. When the experimental hand was placed into the cold box before cocoa consumption, skin temperature of the control finger was significantly lower during both visits. When the experimental hand was placed into the cold box after cocoa consumption, a significant decrease in the skin temperature of the control finger was seen after LFC consumption but no changes were seen after HFC consumption. The skin temperature of the control finger showed a trend of being lower after consumption of both HFC (p=0.058) and LFC (p=0.073) drinks during localised cooling. When the experimental hand was returned to RT, and 1hr after cocoa consumption, the skin temperature of the control finger after HFC consumption was significantly lower and skin temperature of the control finger after LFC consumption demonstrated a strong trend of being lower (P=0.051). Despite changes observed in the skin temperature, no changes were demonstrated in the skin BF (Figure 5.6). Meanwhile, comparison of the skin BF and skin temperature of the control finger between the visits at each time point measurements were similar (p>0.05).
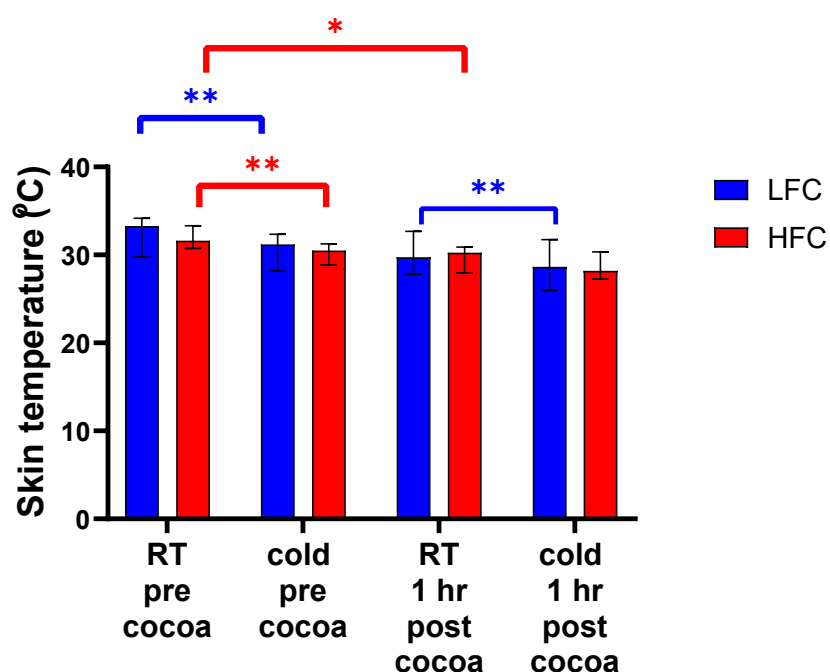
Figure 5.5: Skin temperature of the control finger pre and post cocoa consumption at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). *p<0.05; **p<0.005
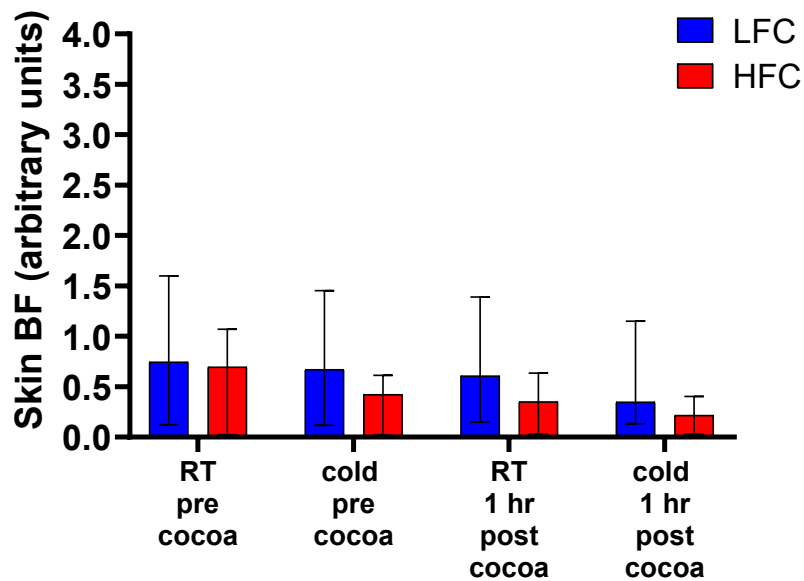
Figure 5.6: Skin BF of the control finger pre and post cocoa consumption at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). No statistical differences were observed.

(iii) Skin BF and skin temperature response of the experimental finger compared with the control finger during localised cooling and at RT during rewarming after localised cooling

Figure 5.7 shows skin BF of the experimental finger and the control finger during both visits. There were no significant changes in skin BF of the control finger across either study visits (LFC p=0.671; HFC p=0.504). As previously described, skin BF on the experimental hand was lower during cooling, but cocoa consumption did not appear to affect this response.
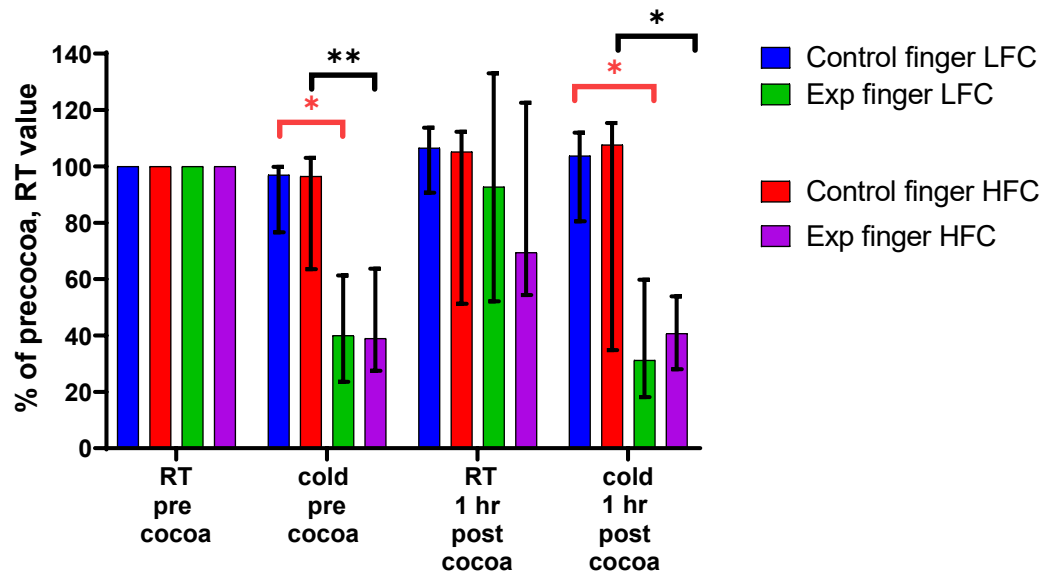
Figure 5.7: Changes in the skin blood flow of the experimental and control finger (compared to pre-cocoa room temperature values), pre and post cocoa consumption when at room temperature (RT) and during localised cooling in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). *p<0.05, **p<0.005

Table 5.4 represents the skin temperature of the experimental finger *versus* control finger during both visits. During the HFC visit, after stabilising in RT, the skin temperature of the control finger was significantly colder than the experimental finger. When the experimental hand was exposed to localised cooling before and after cocoa consumption, the skin temperature of the experimental finger was significantly colder compared to the control finger. However, when the experimental hand was removed from the cold environment, the skin temperature of the control finger remained significantly colder than the experimental finger.

On the other hand, during the LFC visit, the skin temperature of both fingers was similar at the beginning of the study. When the experimental hand was exposed to localised cooling before and after cocoa consumption, the skin temperature of the experimental finger was significantly colder compared to the control finger. Meanwhile, when the experimental hand was removed from the cold environment, there was no significant difference between the skin temperatures of both fingers.

Skin temperature (°C)	HFC visit		LFC visit	
	Control finger	**Experimental finger**	**Control finger**	**Experimental finger**
Pre-cocoa in RT	31.63 [30.74-33.32]	32.73 [31.60-33.70]**	33.28 [29.78-34.15]	32.37 [29.00-34.40]
Pre-cocoa in cold	30.50 [28.86-31.27]	15.03 [14.96-15.06]**	31.20 [28.17-32.35]	15.05 [15.01-15.13][###]
1hr post-cocoa in RT	30.25 [27.92-30.90]	31.69 [29.16-33.50]*	29.74 [27.82-32.67]	29.25 [27.56-32.39]
1hr post-cocoa in cold	28.20 [27.30-30.34]	15.08 [14.99-15.20]**	28.67 [25.97-31.74]	15.06 [14.99-15.17][###]

Table 5.4: Comparison of skin temperature measurements between experimental and control finger pre-cocoa and 1hr post-cocoa in room temperature and during localised cooling for both the visits in healthy participants (n=12). Data are presented as the median (25th and 75th percentile) *p<0.05 vs control finger HFC visit, **p<0.005 vs control finger HFC visit, ###p<0.001 vs control finger LFC visit

(iv) Changes in the skin temperature of the experimental finger moving from RT to localised cooling

Figure 5.8 shows changes in the skin temperature of the experimental finger going from RT into the cold box during the HFC and LFC visits. During both the visits, before cooling (in RT), the skin temperature was similar before and 1hr after consumption. Similarly, once the experimental hand was placed into the cold box, there was no difference in the skin temperature response to the cold before and after consumption.
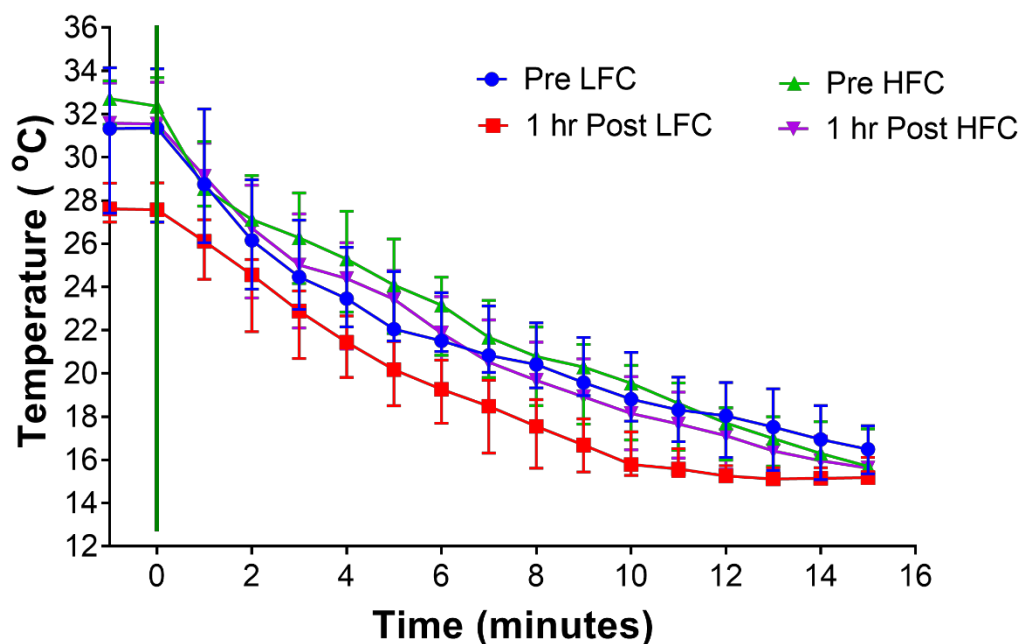
Figure 5.8: Changes in the skin temperature of the experimental finger when moved from RT into a cold box between pre and post consumption of both the drinks in healthy participants (n=12). The vertical line indicates when the experimental hand was placed into the cold box. Medians with 25th and 75th percentile shown as error bar.

(v) Changes in the skin temperature of the experimental finger moving from localised cooling to RT.

Figure 5.9 shows changes in the skin temperature of the experimental finger when taken from the cold box into RT during the HFC and LFC visits. During LFC visit, when the experimental hand was exposed to localised cooling,

the skin temperature of the experimental finger was similar before and after consumption. However, once the experimental hand was removed from the cold box, changes in the skin temperature were significantly slower ($p<0.05$) after consumption, compared to before consumption.

Meanwhile, during the HFC visit, when the experimental hand was in the cold box, the skin temperature of the experimental finger was similar before and after consumption. Once the experimental hand was removed from the cold box, there were no differences in the skin temperature response to the RT.
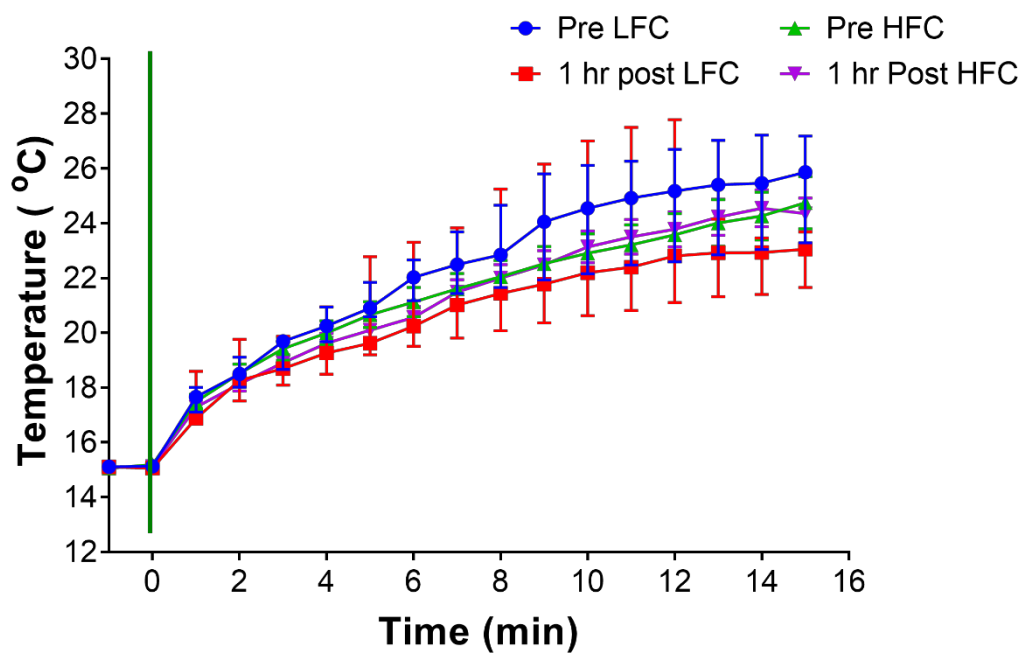
Figure 5.9: Changes in the skin temperature of the experimental finger when moved from a cold box into RT between pre and post consumption of both the drinks in healthy participants (n=12). The vertical line indicates when the experimental hand was placed in RT. Medians with 25th and 75th percentile shown as error bars.

5.3.4 Effect of cocoa on the time taken for the skin temperature of the experimental finger to stabilise to localised cooling and at RT after localised cooling

Table 5.5 represents the time taken for the skin temperature of the experimental finger to stabilise during localised cooling, and in RT after localised cooling. Time taken before cocoa consumption to stabilise during localised cooling and in RT was similar during both the visits. However, after consuming the LFC drink, the time taken to stabilise to localised cooling was significantly shorter and to stabilise to RT was significantly longer, compared to before consumption.

Time taken to stabilise in:	HFC visit	LFC visit
cold pre-cocoa	15.36 [12.51-19.42]	16.10 [11.92-22.71]
RT pre-cocoa	16.91 [11.66-32.38]	10.44 [7.96-24.67]
cold 1hr post-cocoa	15.73 [12.09-17.22]	9.97 [7.35-15.89]*
RT 1hr post-cocoa	19.39 [11.55-25.35]	19.87 [8.13-38.27]#

Table 5.5: Time taken (minutes) for the skin temperature of the experimental finger to stabilise in the cold box and at room temperature (RT) during both the visits in healthy participants (n=12). Data are presented as the median (25th and 75th percentile). *$p < 0.05$ vs time taken to stabilise in cold pre-cocoa, # $p < 0.05$ vs time taken to stabilise in RT pre-cocoa.

Summary of the results:

Core temperature and Cardiovascular changes between HFC and LFC visits in healthy participants (n=12).

	Healthy participants	
Core temperature	**HFC visit**	**LFC visit**
Pre-cocoa in cold vs pre-cocoa in RT	↑ trend	↑ trend
1 hr post-cocoa in RT vs pre-cocoa in RT	-	↑
1 hr post-cocoa in cold vs pre-cocoa in cold	-	-
1 hr post-cocoa in cold vs1 hr post-cocoa in RT	↑	-
SBP		
Pre-cocoa in cold vs pre-cocoa in RT	↑	↑
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	↑	↑
DBP		
Pre-cocoa in cold vs pre-cocoa in RT	↑	↑
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	↑	↑
TPR		
Pre-cocoa in cold vs pre-cocoa in RT	↑	↑
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	-	-
CO		
Pre-cocoa in cold vs pre-cocoa in RT	↓	↓
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	-	-
HR		
Pre-cocoa in cold vs pre-cocoa in RT	↓	-
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	-	-
1 hr post-cocoa in cold vs pre-cocoa in cold	-	↑

Changes in the skin BF and skin temperature of the experimental and control finger between HFC and LFC visits in healthy participants (n=12).

	Healthy	
Skin BF experimental finger	**HFC visit**	**LFC visit**
Pre-cocoa in cold vs pre-cocoa in RT	↓	↓
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	↓	↓
Skin temperature experimental finger		
Pre-cocoa in cold vs pre-cocoa in RT	↓	↓
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	↓	↓
Skin temperature control finger		
Pre-cocoa in cold vs pre-cocoa in RT	↓	↓
1 hr post-cocoa in cold vs 1 hr post-cocoa in RT	-	↓
1 hr post-cocoa in RT vs pre-cocoa in RT	↓	-

Time taken to stabilise in cold and RT between HFC and LFC visits in healthy participants (n=12).

	Healthy	
Time taken to stabilise in cold and RT	**HFC visit**	**LFC visit**
Time taken to stabilise in cold: 1hr post-cocoa vs pre-cocoa	-	Shorter
Time taken to stabilise in RT: 1 hr post-cocoa vs pre-cocoa	-	Longer

Changes in the skin temperature of the experimental versus control finger during HFC and LFC visits in healthy participants (n=12).

	Healthy participants	
	HFC visit	**LFC visit**
Skin temperature	**Experimental vs control finger**	**Experimental vs control finger**
Pre-cocoa in RT	Warmer in exp finger	-
Pre-cocoa in cold	Colder in exp finger	Colder in exp finger
1hr post-cocoa in RT	Warmer in exp finger	-
1hr post-cocoa in cold	Colder in exp finger	Colder in exp finger

5.4 Discussion

The present study demonstrated no effect of acute consumption of the HFC drink on cardiovascular measures (SBP, DBP, CO, HR and TPR) and peripheral skin BF, measured 1hr after consumption. However, the technique of localised cooling used in this study resulted in significant changes in cardiovascular responses, core temperature, skin temperature and skin BF. Thus, the discussion below, which is divided into sections based on the study objectives, will focus more on the effect of localised cooling on cardiovascular responses and peripheral skin BF.

Effect of localised cooling on cardiovascular responses and peripheral skin BF

(i)   Core temperature

In the present study, it was found that the core temperature was significantly warmer when the experimental hand was exposed to localised cooling after consumption of HFC drink. These findings are in agreement with a previous study that found an increase in core temperature when local cooling was applied to a single body part in a warm environment (Huizenga *et al*., 2004). In that study, air sleeves were used to cool the head, hand and pelvis of 109 subjects in a controlled environmental chamber at 28°C. It was found that the core temperature increased around 3 minutes after applying local cooling to these sites (Huizenga *et al*., 2004). Decreases in peripheral blood flow, as a result of cold exposure, delays the cooling of the deeper peripheral tissues, and results in conductive pathways being the major route for heat loss. These factors, combined with increased thermogenesis during cooling of the skin, lead to a paradoxical increase in core temperature which can be seen during the early phase of cold exposure.

The core temperature after HFC drink consumption returned to pre-cooling values when the experimental hand was returned to RT after the period of localised cooling. However, core temperature after LFC consumption was significantly higher compared to pre-cooling values. The difference in core

temperature during the HFC and LFC visits is difficult to explain. Analysis of these data was hampered by the fact that there was a great variability in responses among individual participants. Since experimental conditions were kept strictly constant, it is felt that the variability could possibly be due to different emotional responses to the conditions of the experiment of individual participants and different previous thermal history (Jansky *et al.*, 2003).

(ii)   SBP, DBP and TPR

In this experiment, it was found that local cooling triggered a significant increase in BP. The increase in both SBP and DBP was demonstrated before and after both cocoa drinks were consumed. These results are in line with a previous study by Victor *et al.*, (1987) which reported that the BP increased significantly during a cold pressor test (CPT). In that study, the arterial pressure and HR were monitored during immersion of the hand in ice water for 2 minutes. It was found that arterial pressure increased steadily during the first and second minutes of the CPT (Victor *et al.*, 1987). These results were also in line with a study which showed that immersion of a hand in cold water at 5^0C for two minutes significantly increased both SBP and DBP in 20 healthy participants (Jauregui-Renaud *et al.*, 2001). In another study, within the first 8 minutes of immersion of the lower legs in water at 12^oC, both SBP and DBP significantly increased in 6 healthy participants (Jansky *et al.,* 2006). These findings were due to global sympathetic activation during the CPT or limb cooling, which results in a significant arteriolar vasoconstriction with a subsequent increase in BP (Seals, 1990). In the present study, SBP and DBP after consumption of both of the cocoa drinks were not affected by local cooling in the longer term and returned to pre-cooling values after the experimental hand had been returned to RT.

In the current study, it was found that local cooling significantly initiated an increase in the TPR before consumption of both cocoa drinks. These results are in agreement with a study that also observed a significant increase in TPR during the second and third minutes of immersion of the left hand to

the wrist into a 0-1⁰C water bath for 3 minutes in 40 healthy male participants (Mourot *et al.*, 2009). Similarly, another study conducted on 10 healthy men found significantly increased TPR upon immersing the left hand up to the wrist in 4°C iced water for 2 minutes (Yamamoto and Iwase, 1992). These observations suggest that the cold stimulus may induce α-adrenergic mediated vasoconstriction, resulting in increased TPR (Pickering and Gerin, 1990). Meanwhile, no significant changes in TPR were observed after both cocoa drinks were consumed. This was an interesting finding, as it may suggest that consumption of the cocoa prevented an increase in TPR during localised cooling. In addition, TPR was not affected by localised cooling in the longer term and returned to pre-cooling values when the experimental hand was returned to RT. These findings indicate that the technique used in the present study was adequate to produce significant and expected cardiovascular responses to localised cooling.

(iii)   CO and HR

In the present study, it was demonstrated that local cooling significantly decreased the CO before both cocoa drinks were consumed. This could be due to decreased HR observed under similar conditions. The decreased in HR was significant during the HFC visit. These results were in agreement with a study that reported that HR was initially increased and then decreased thereafter during a CPT (Mourot *et al.,* 2009). In that study, participants were asked to immerse their left hand to the wrist into 0-1°C water bath for a period of 3 minutes. It was found that HR increased during the second minute of the CPT and decreased thereafter, to reach the baseline value at the end of the CPT. Decreases to HR after an initial increase indicates the involvement of both sympathetic and vagal outflow. The increment of vagal outflow during CPT is possibly a baroreflex correction to the sustained BP increase in the latter part of the CPT. Therefore, the baroreflex is capable of modulating HR during CPT appropriately (Mourot *et al.*, 2009).

In contrast, Jansky *et al*., (2006) reported a significant increase in HR within the first 8 minutes following immersion of the lower legs in 12 °C cold water for 45 minutes (Jansky *et al*., 2006). These results indicate a decrease in cardiac vagal outflow and an increase in sympathetic activity (Mourot *et al*., 2009). In the present study, the decrease in HR following localised cooling could be possibly due to a distinct autonomic nervous activity, such as enhanced sensitivity of the baroreflex or higher vagal outflow among the participants. However, this hypothesis could not be confirmed as this was not evaluated in the present study.

On the other hand, after consumption of both of the cocoa drinks, no significant changes were seen in CO during exposure of the experimental finger to local cooling. This finding may be because no changes in HR were observed after both cocoa consumptions. Both HR and CO returned to pre-cooling values after the experimental hand was returned to RT. Meanwhile, during local cooling, it was found that the HR after consumption of the LFC drink was significantly higher while after consumption of the HFC drink, it showed a trend of being higher compared to before consumption. This finding was in agreement with a randomized double-blind placebo controlled, crossover design (Rull *et al.*, 2015). In this study, 32 patients with untreated mild hypertension were asked to consume high flavanol dark chocolate containing 1064mg of total flavanols or lower flavanol dark chocolate, containing 88mg of total flavanols, daily for 6 weeks. It was found that the HR was increased by the low flavanol dark chocolate but not after the high flavanol dark chocolate compared with baseline values, even though they had similar methylxanthine contents. This finding could be due to the higher flavanol content modulating sympathetic activation (Rull *et al.*, 2015). Therefore, in the present study, the significantly increased HR seen after consumption of the LFC drink (and not seen after the HFC drink) could be explained by a flavanol-dependent increase in parasympathetic tone and suppression of methylxanthine-induced sympathetic activation.

(iv) Skin temperature and skin BF of the experimental and control finger

In the present study, it was observed that local cooling significantly reduced the skin temperature and skin BF of the experimental finger before and after consumption of both cocoa drinks. These results are also in line with a study of 64 men which showed a significant decrease in skin temperature and skin BF of the experimental finger after immersing the hand for 2 minutes in 0–4°C cold water, indicating a vasoconstrictor response (Naidu and Sachdeva, 1993). In the present study, skin temperature and skin BF of the experimental finger before and after consumption of both cocoa drinks was not affected in the longer term by the cold exposure and returned to pre-cooling values when the experimental hand had been returned to RT. This suggests that the cold stimulus applied in this experiment was adequate to produce a significant response in skin temperature and skin BF of the experimental finger.

Interestingly, apart from reduction in the skin temperature of the experimental finger seen following local cooling, it was also found that exposure of the experimental hand to local cooling significantly reduced the skin temperature of the control finger before consumption of both cocoa drinks and after consumption of the LFC drink. These results are in line with a study that showed decreases in skin temperatures of non-cooled areas following local cooling. In that study, 6 men were asked to immerse up to the knees in 12°C water in a standing position for 45 minutes. Based on infrared thermographic recordings, it was observed that the skin temperature of cooled and some non-cooled areas of the body (fingers, palms and thighs) decreased significantly within the first 5 minutes of cold exposure. The average skin temperature in the centre of the body (trunk, forehead) tended to decrease slightly. In that study, decreases in the skin temperatures of the control finger could have been due to the permanent and generalised activation of the sympathetic nervous system which occurs during localised cooling (Jansky *et al.,* 2003). When the experimental hand was removed from the cold environment, the skin temperature of the control finger after HFC consumption was significantly colder than pre-cooling

values. Jansky *et al*,. (2003) found that the average skin temperatures in all areas of the body returned to the pre-cooling level after 10 minutes of cooling and further fluctuated at about 10–15 minutes intervals (Jansky *et al*., 2003). In the present study, this could be due to high variability between participants.

When a comparison was made between skin temperature of the experimental finger and the control finger, there were no significant differences between these fingers after stabilising in RT during the LFC visit. However, skin temperature of the control finger was significantly colder than the experimental finger during the HFC visit. This may be due to individual variability and different previous thermal history among participants. Furthermore, when the experimental hand was exposed to the cold environment before and after cocoa consumption, the skin temperature of the experimental finger was significantly colder than the control finger during both the visits. This finding was expected on the basis of direct exposure of the experimental hand to local cooling. When the experimental hand was returned to RT, the skin temperature of both fingers returned to pre-cooling values and was similar during the LFC visit. However, the control finger was significantly colder than the experimental finger during the HFC visit. This could be due to the temperature of the control finger at the start of the study being already colder (significant) than the experimental finger.

On the other hand, when a comparison was made between skin BF of the experimental finger and the control finger, the skin BF of the experimental finger in the cold was significantly lower compared to the control finger before and after cocoa consumption on both visits. These findings were due to direct exposure of the experimental finger to the cold environment.

Effect of cocoa on cardiovascular measures and peripheral skin BF

The present study has demonstrated no effect of acute consumption of the HFC drink after 1hr on cardiovascular measures and peripheral skin BF responses of the experimental and control finger. The reason could be due to the effects of the cocoa at 1hr not being adequate to elicit a detectable

vasodilatory response, or the flavanol content may have been too low as mentioned earlier. The measurement at 1hr after cocoa consumption was chosen based on the previous pilot study (Chapter 3) which demonstrated that acute consumption of HFC significantly increased FMD response 1hr after consumption. In agreement with this finding, Heiss *et al*., (2015) reported that consumption of cocoa drink containing 450mg total flavanols (64mg epicatechin) significantly increased FMD at 1hr post ingestion in both young and elderly as discussed in Chapter 3. Indeed, the epicatechin dose given was lower compared to the present study. However, the discrepancies between the studies could be due to different assessment methods in the study protocol; FMD measurement used in pilot study (Chapter 3) reflects the endothelial function of large conduit arteries whereas, in the present study, LD measurement reflects the microvascular blood perfusion. Therefore, there was a possibility that the beneficial effects of acute consumption of HFC was not associated with an improvement in resistance vessels. However, a previous study reported that plasma levels of epicatechin were significantly increased 1hr after ingestion of a single dose of a HFC drink containing 329 mg flavanols (61.1mg epicatechin) and that the skin BF was significantly increased at 2hrs after ingestion in healthy women (Neukam *et al.,* 2007). These findings suggest that it should have been possible to detect changes to the peripheral blood flow in the current study.

In regard to the level of flavanol, the EFSA have approved a cardiovascular health benefit claim for dark chocolate, cocoa extract and cocoa based beverages containing 200mg total flavanols (46mg of epicatechin), although previous studies suggest that 100mg/day of epicatechin is required to elicit a long term cardiovascular benefit (Heiss *et al.*, 2015, Sansone *et al.*, 2015). In the present study, the participants were given HFC drink containing in total, 830mg of flavanols (99.6mg epicatechin) which met both recommended intakes and thus, unlikely to be the reason.

Changes in the skin temperature of the experimental finger moving between RT and cold box

In the present study, it was found that the changes in the skin temperature of the experimental finger in RT were similar before and after consumption of both the drinks. Similarly, once the experimental hand was exposed to localised cooling, no significant changes were observed before or after consumption of both cocoa drinks. This finding is important as it indicates that cocoa consumption does not affect temperature regulation in healthy participants.

On the other hand, during localised cooling, there were no significant differences in the skin temperature of the experimental finger before or after consumption of both cocoa drinks. These findings were expected as in this experiment, the cold box temperature was modified to maintain the skin temperature at 15°C. When the experimental hand was removed from the cold environment, it was observed that changes of the skin temperature after consumption of the LFC drink were significantly slower than before consumption, but no significant difference was observed before compared to after consumption of the HFC drink. These findings could be due to higher flavanol content in the HFC drink causing increased vasodilatation in RT and thus, the skin temperature of the experimental finger recovers faster after localised cooling.

Time taken for the skin temperature of the experimental finger to stabilise to localised cooling and at RT after localised cooling

In the present study, the time taken before cocoa consumption to stabilise to localised cooling and when returned to RT, was similar during both visits, indicating a similar response at baseline to localised cooling and return to RT before cocoa consumption. After LFC consumption, it was found that the time taken to stabilise to localised cooling was significantly shorter and the time taken to stabilise to RT was significantly longer, compared to before consumption. The significantly shorter duration taken to stabilise in localised cooling following the LFC consumption could be due to rapid heat loss

occurring during exposure to the cold environment. However, the significantly longer duration taken to stabilise on the return to RT following LFC consumption, is difficult to explain. This observation was not seen after taking the HFC drink which could be due to the higher flavanol content in HFC drink modulating the heat loss appropriately in the cold environment and RT and thus, preventing rapid heat loss in the cold environment and stable recovery in RT after localised cooling.

In conclusion, the present study failed to find an acute effect of consuming cocoa with a high flavanol content on cardiovascular measures and peripheral skin blood flow, and it was observed that peripheral thermoregulation following periods of cooling and rewarming was not affected. In addition, it was also observed that the local cooling stimulus caused a significant change in cardiovascular responses, core temperature, skin temperature and skin BF, indicating that the technique used was appropriate to produce significant responses.

Chapter 6: Acute effects of cocoa drink on peripheral blood flow in control participants *versus* participants with Primary Raynaud's Phenomenon

6.1 Introduction

It is known that cocoa, which is rich in flavanols, causes arterial vasodilation proposed to occur through the primary mechanism of increasing NO bioavailability by both stimulating the NO synthase activity (Fraga *et al.*, 2010) and increasing the availability of L-arginine (Schnorr *et al.*, 2008). Indeed, Chapter 3 (3.1) reported an increase in FMD in healthy young women, 1 hr after the consumption of a cocoa drink with a high flavanol content. In the condition of PRP, symptoms are associated with the vasoconstriction of the microvascular bed, resulting in a period of ischaemia. It has been suggested that this vasoconstriction could be attenuated by increasing NO bioavailability and some alleviation of symptoms has been reported in people with PRP with the use NO donors such as GTN (Teh *et al.,* 1995). It was therefore hypothesised that regular consumption of cocoa flavanols could be beneficial for those with PRP. However, it is also possible that regular consumption of cocoa flavanols could result in a vasodilation in the microvasculature which could increase heat loss from the periphery and consequently make PRP individuals more likely to have symptoms triggered. In 12 healthy women who reported no circulatory problems, it was observed that high flavanol cocoa (HFC) consumption did not affect cardiovascular variables or peripheral thermoregulation following periods of cooling and rewarming (Chapter 4). From these initial data it was concluded that there was no convincing evidence to suggest that cocoa flavanol consumption impacted on peripheral thermoregulation and that it would be appropriate to extend this investigation of acute responses following cocoa flavanol consumption to people with PRP.

Therefore, the present study aimed to look at the acute effects of HFC consumption (at 1 hr and 3 hrs) on cardiovascular variables and peripheral thermoregulation in women with PRP.

6.1.1 Study aim

The aim of this study was to characterize the acute effect of cocoa with a high flavanol content (consumed as beverage) at 1hr and 3hrs in women with PRP compared to those without PRP, with the following parameters:

- The core temperature and cardiovascular response (BP, HR, CO, TPR) to localised cooling.

- Skin BF and skin temperature responses of the finger during localised cooling and at a RT of 25°C during rewarming after localised cooling (experimental and control finger).

- The time taken for skin temperature of the finger to stabilise during localised cooling (in an air temperature of 15°C) and at a RT of 25°C during rewarming after localised cooling.

Hypothesis

HFC ingestion would induce vasodilation and increase skin BF in participants with PRP when compared to LFC and the difference in response would be more pronounced in participants with PRP than healthy controls.

The time taken for skin temperature to stabilise in the cold and at RT following cold exposure, would be longer in those with PRP compared to healthy controls.

6.2 Methods and Materials

6.2.1 Sample size calculation

The primary aim of this study was to characterise the effect of HFC on the peripheral skin temperature response to cold exposure, but the degree of any effect in those with PRP was not known and had not been characterised previously in the literature.

A sample size of 10 should allow a 40% difference in skin BF to be detected between the 2 groups (Lenth, 2006). Previous reports have suggested that a 70% increase in skin BF could be induced by acute consumption of HFC in those without PRP (Neukam *et al.*, 2007). Moreover, acute consumption of HFC induced a difference in SBP (compared with LFC) of 5.3mmHg and in DBP of 2.8mmHg (Faridi *et al.,* 2008), and to power these observations at the level of 80% would require 11 and 18 participants, respectively.

It was therefore proposed that 10 participants be recruited onto the current study to power the skin BF and SBP observations, with temperature regulation data obtained from this study being used to power subsequent studies.

6.2.2 Participants

The study protocol was approved by the Medical School Research Ethics Committee, Faculty of Medicine & Health Sciences, Queen's Medical Centre, Nottingham (Appendix page *xxii*). Written informed consent was obtained from all the participants. The present study was a randomised, double-blind, cross-over design involving 10 women with PRP and 10 healthy women without PRP. Selection of participants with PRP were based on the criteria of Allen and Brown (1932). Those who had been diagnosed with PRP by medical practitioner were included in the study.

Inclusion criteria: Male or female, aged 18-60yrs, BMI of <27 kg/m^2, daily consumption of caffeine containing foods/drinks. More than 1 attack a week through the winter months.

Exclusion criteria: pregnant or breast feeding (women only), clinically significant metabolic or endocrine abnormalities, random glucose >7.8mmol/l; taking Bosentan™, aspirin, dipyridamole or transdermal nitrates; herbal supplement use; food allergies related to the investigational product (cocoa, milk); sensitivity to methylxanthines (e.g. caffeine, theobromine); presence or history of digital ulceration; blood parameters suggesting SRP and history of migraines.

The recruitment of participants with PRP was done via local newspapers, British Broadcasting Corporation Radio Nottingham, 'Call for Participants', Facebook and posters placed around the university campus and Medical School.

6.2.3 Cocoa drink

The cocoa powders used in this study were a high flavanol cocoa powder, containing 830mg of flavanols per 10gm (Chococru™) and a lower flavanol alkalized cocoa powder (Sainsbury's) which was assumed to contain <50mg of flavanols per 10gm (Miller *et al.,* 2008). Compositional details for high flavanol and low flavanol cocoa powder were described in Chapter 2 (2.6.1).

6.2.4 Experimental outline

Ten women with PRP and 10 control women attended a screening visit (visit 1). They were briefed concerning the nature of the study and informed consent was obtained. Participants in each group were then randomised into 2 groups, namely group A and group B. Group A was given a cocoa drink with high flavanol content during visit 2, followed by a cocoa drink with low flavanol content during visit 3, and *vice versa* for group B. Experimental visits were carried out during the follicular stage of the participants' menstrual cycle with a wash-out period of ≥1 week between visits. See Figure 6.1.
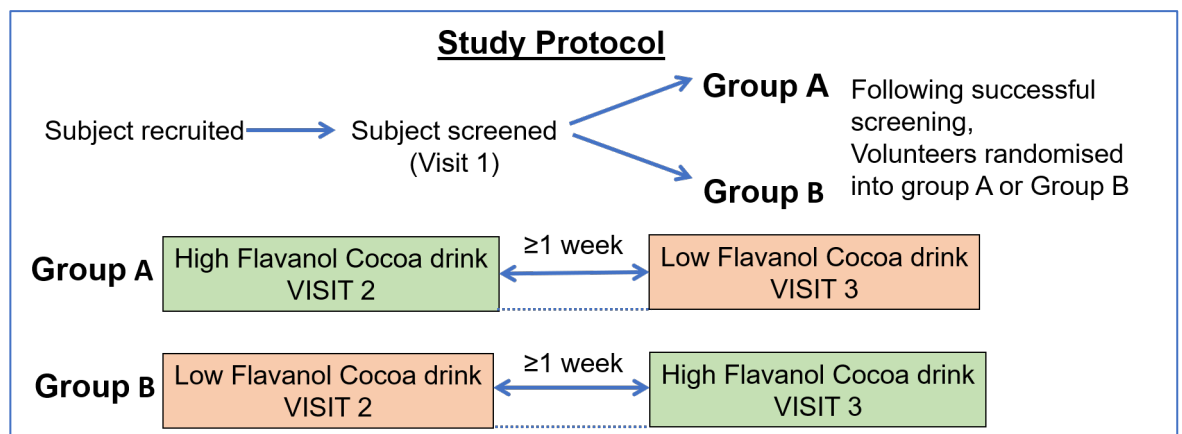
Figure 6.1: The study protocol for group A and group B

The protocol schematic for study days was the same as described in Chapter 5 (5.2.4) and illustrated in Figure 6.2.
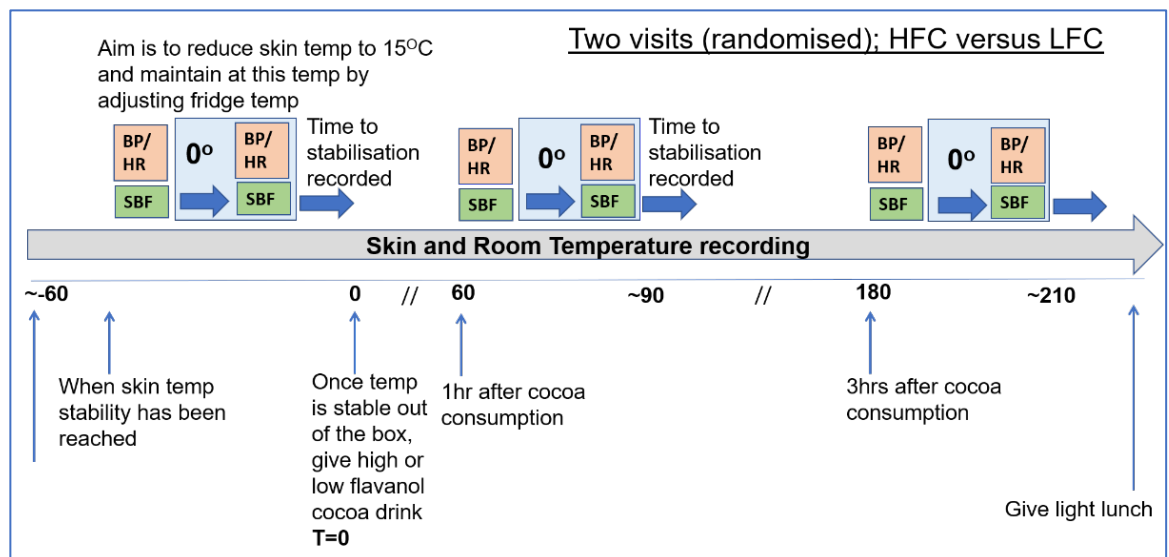
Figure 6.2: Protocol schematic for study days

6.2.5 Statistical analyses

The data were analysed using the SPSS (version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. The pre-cocoa consumption values for cardiovascular parameters (SBP, DBP, TPR, CO, HR), core temperature, skin BF and skin temperature of the experimental finger and control finger, changes in the skin temperature of the experimental finger moving in between the RT and a cold box, were

compared with post-cocoa consumption at 1 and 3hrs within and between groups, using Two-Way ANOVA.

Meanwhile, the pre-cocoa and post-cocoa at 1 and 3hrs comparison within the same group for the environmental (outside) temperature, time taken to stabilise in RT and in the cold box were made using paired t-test for normally distributed data or Wilcoxon's signed ranks test for not normally distributed data; comparisons between the groups were analysed using independent t-test for normally distributed data and Mann-Whitney test for not normally distributed data. All data were presented as mean (SD) for normally distributed data or displayed as the median (25th and 75th percentile) for non-parametric data. A value of $p<0.05$ was considered to be statistically significant.

6.3. Results

6.3.1 Characteristics of participants

10 control women and 10 women with PRP completed the study. The baseline characteristics are shown in Table 6.1.

Characteristics	Control (n=10)	PRP (n=10)
Age	29.20 (6.16)	37.30 (14.70)
Weight (kg)	54.71 (8.60)	59.00 (5.97)
Height (m)	1.61 (0.05)	1.65 (0.05)
BMI (kg/m^2)	20.98 (2.45)	21.74 (1.94)

Table 6.1: Characteristics of participants. BMI, body mass index. Data are presented as the mean (SD). No statistical differences were observed.

6.3.2 Effect of cocoa on core temperature and cardiovascular measures (SBP, DBP, CO, HR, and TPR) to localised cooling

(i) Core temperature

Table 6.2 shows the effect of localised cooling on core temperature before and after cocoa, during both (high and low flavanol cocoa) visits and between the participant groups. At the beginning of each study day (pre-cocoa), within the groups, the core temperature at RT and during LC were similar between the visits. In the control group, the mean core temperature in RT was warmer (+0.18 ^{o}C at 1hr and +0.26 ^{o}C at 3hrs) ($p<0.05$) after consumption of the LFC drink compared to before consumption. During localised cooling at 3hrs, the mean core temperature was warmer (+0.16 ^{o}C) after taking the HFC drink ($p<0.05$) and showed a trend to be warmer after taking the LFC drink ($p=0.057$) compared to before consumption.

In the PRP group, the mean core temperature at RT was warmer (+0.12^{o}C 1hr post HFC drink and +0.10 ^{o}C 3hrs post LFC drink; $p<0.05$) compared to before consumption. During localised cooling, there was a trend towards a small rise in the core temperature at 1hr and at 3hrs after taking the LFC drink ($p=0.058$ for both) compared to before consumption. No differences were observed in the responses of the core temperature throughout the study between the visits within each group.

When the comparison was made between the groups, the responses in core temperature throughout the study during both visits were not different.

	Control		PRP	
	HFC visit	**LFC visit**	**HFC visit**	**LFC visit**
Pre-cocoa in RT	36.9 [36.73-37.08]	36.8 [36.65-36.88]	37.0 [35.75-37.08]	36.8 [36.73-37.00]
Pre-cocoa in cold	37.1 [36.90-37.18]	37.0 [36.90-37.10]	37.0 [36.83-37.08]	36.9 [36.80-37.08]
Post-cocoa 1 hr in RT	37.1 [36.90-37.28]	37.0 [37.00-37.18]*	37.1 [36.93-37.18]**	37.0 [36.83-37.00]
Post-cocoa 1 hr in cold	37.1 [37.00-37.20]	37.1 [36.93-37.25]	37.1 [36.85-37.25]	37.1 [36.90-37.10]
Post-cocoa 3hr in RT	37.2 [36.93-37.30]	37.1 [36.93-37.28]**	37.0 [36.85-37.10]	37.0 [36.90-37.10]*
Post-cocoa 3hr in cold	37.3 [37.20-37.30]##	37.1 [37.00-37.35]	37.1 [36.93-37.18]	37.1 [36.85-37.25]

Table 6.2: Comparison of core temperature between HFC and LFC visits pre-cocoa consumption, 1 hr and 3hr after cocoa consumption at room temperature (RT) and during localised cooling in control group (n=10) and PRP group (n=10), respectively. Data are presented as the median (25th and 75th percentile). *p<0.05 vs pre-cocoa in RT, **p<0.005 vs pre-cocoa in RT, ##p<0.005 vs pre-cocoa in cold

(ii)   Cardiovascular measures

At the start of each study day (pre-cocoa), in the control group, all the cardiovascular measurements in RT and cold box were similar between the visits. After consumption of the HFC drink, in RT, the mean SBP increased by 6.6 mmHg at 1hr (p<0.05), with mean TPR increasing by (22% at 1hr and 24% at 3hrs; p<0.05) from the pre-cocoa measurements. These findings were accompanied by a decrement in the mean CO by (-14% at 1hr and -16% at 3hrs; p<0.05), from the pre-cocoa measurements (Table 6.3).

Similarly, after consumption of the LFC drink, mean TPR increased from the pre-cocoa values by 42% at 1hr and 39% at 3hrs (p<0.05), and this was accompanied by a decrease in the mean CO by (-28% at 1hr and -26% at 3hrs; p<0.05) from the pre-cocoa measurements. In addition, HR showed a trend to be lower at 1hr (p=0.063) from the pre-cocoa values (Table 6.4).

SBP	Control		PRP	
	HFC visit	LFC visit	HFC visit	LFC visit
pre-cocoa in RT	104.3 [98.44-109.89]	104.2 [102.40-111.75]	113.6 [105.45-120.42]	119.4 [110.56-125.70]
pre-cocoa in cold	111.7 [106.62-114.21]	114.8 [109.45-118.22]	122.5 [114.54-129.30]	128.1 [118.52-130.22]
1 hr post-cocoa in RT	112.0 [106.98-116.77]*	108.6 [105.22-109.41]	122.4 [114.33-131.71]	122.7 [112.46-124.95]
1 hr post-cocoa in cold	117.5 [115.94-120.66]#	113.6 [111.01-115.74]	130.3 [117.81-133.95]	127.5 [113.24-127.97]
3 hr post-cocoa in RT	110.7 [109.24-113.96]	112.8 [105.07-118.26]	126.3 [116.55-129.51]*	125.7 [113.24-127.97]
3 hr post-cocoa in cold	113.9 [111.40-116.16]	116.6 [110.07-121.27]	129.0 [113.58-133.87]	125.3 [116.39-131.07]
TPR				
pre-cocoa in RT	14.2 [13.39-15.79]	12.9 [11.46-14.15]	13.5 [11.61-19.54]	16.1 [13.53-17.14]
pre-cocoa in cold	16.8 [15.14-17.73]	13.8 [12.89-17.33]	15.2 [13.65-18.87]	15.8 [14.49-19.74]
1 hr post-cocoa in RT	18.0 [16.49-19.12]**	18.2 [15.98-19.41]**	18.9 [17.28-21.28]	19.9 [18.07-20.89]*
1 hr post-cocoa in cold	19.4 [18.89-20.24]#	18.4 [17.08-20.71]#	20.9 [18.13-22.16]##	21.6 [18.31-23.48]#
3 hr post-cocoa in RT	17.5 [16.54-20.47]**	17.7 [16.61-18.75]**	20.2 [18.58-22.00]*	19.9 [16.94-20.86]
3 hr post-cocoa in cold	18.1 [16.97-19.77]	17.7 [17.06-19.38]#	19.7 [18.76-21.55]#	19.9 [15.90-23.37]
CO				
pre-cocoa in RT	5.4 [4.91-5.82]	5.8 [5.29-7.09]	5.4 [4.06-6.65]	5.8 [4.58-6.42]
pre-cocoa in cold	5.0 [4.68-5.20]	6.0 [4.73-6.49]	5.4 [4.23-6.03]	5.8 [4.68-6.12]
1 hr post-cocoa in RT	4.4 [4.34-4.81]*	4.3 [4.16-4.61]*	4.3 [3.96-5.27]*	4.2 [3.84-4.74]*
1 hr post-cocoa in cold	4.3 [4.23-4.70]	4.3 [4.18-4.71]#	4.3 [3.77-4.94]#	4.2 [3.65-4.66]#
3 hr post-cocoa in RT	4.5 [3.96-4.93]*	4.5 [4.46-4.98]*	4.1 [3.91-4.81]*	4.3 [4.14-4.91]
3 hr post-cocoa in cold	4.7 [4.25-4.87]	4.7 [4.46-4.99]#	4.1 [3.88-4.86]#	4.5 [3.64-5.62]

Table 6.3: Comparison of cardiovascular parameters (SBP, TPR, CO) pre and post cocoa consumption in RT and in cold in control group (n=10) and PRP group (n=10), respectively. Data are presented as the median (25th and 75th percentile).*p<0.05 vs pre cocoa RT, **p<0.005 vs pre cocoa RT, ** p<0.05 vs pre-cocoa RT, #p<0.05 vs pre-cocoa in cold, ##<0.005 vs pre cocoa in cold.

	Control		PRP	
	HFC visit	**LFC visit**	**HFC visit**	**LFC visit**
DBP				
pre-cocoa in RT	63.6 [61.02-65.91]	64.7 [61.01-65.39]	61.5 [57.99-67.04]	65.2 [58.62-70.49]
pre-cocoa in cold	65.6 [64.23-67.33]	67.4 [65.72-68.85]	66.3 [60.62-72.03]	73.0 [65.61-74.18]
1 hr post-cocoa in RT	64.9 [63.67-68.50]	63.7 [61.05-68.08]	67.5 [64.64-74.87]	67.6 [64.27-70.62]
1 hr post-cocoa in cold	68.3 [66.94-70.56]	66.8 [60.55-69.71]	71.4 [65.35-76.57]	71.5 [65.70-72.85]
3 hr post-cocoa in RT	66.5 [61.30-67.79]	64.4 [59.88-72.43]	68.4 [62.90-70.73]	66.4 [64.56-68.47]
3 hr post-cocoa in cold	68.7 [65.36-69.79]	68.7 [61.64-72.81]	69.0 [60.36-71.82]	68.6 [64.31-74.42]
HR				
pre-cocoa in RT	65.5 [60.36-70.37]	66.0 [61.71-71.43]	58.6 [55.31-64.15]	61.5 [55.30-64.79]
pre-cocoa in cold	61.3 [58.07-67.91]	61.1 [58.06-70.75]	57.7 [56.40-62.02]	58.4 [55.59-64.08]
1 hr post-cocoa in RT	64.0 [60.44-70.75]	62.4 [60.01-67.72]	61.0 [57.04-65.95]	60.5 [57.71-61.04]
1 hr post-cocoa in cold	61.8 [55.33-71.92]	60.7 [59.81-64.72]	59.3 [57.27-60.82]	59.7 [58.30-61.15]
3 hr post-cocoa in RT	62.1 [58.53-67.54]	61.9 [58.89-70.69]	60.6 [56.53-63.17]	60.2 [55.31-61.00]
3 hr post-cocoa in cold	62.3 [58.23-64.94]	62.7 [58.63-72.58]	58.2 [55.81-63.04]	61.0 [57.63-63.69]

Table 6.4: Comparison of cardiovascular parameters (DBP, HR) pre and post cocoa consumption in RT and in cold in control group (n=10) and PRP group (n=10), respectively. Data are presented as the median (25th and 75th percentile).*$p<0.05$ vs pre cocoa RT, **$p<0.005$ vs pre cocoa RT, ** $p<0.05$ vs pre-cocoa RT, #$p<0.05$ vs pre-cocoa in

During localised cooling at 1hr after HFC consumption, the mean SBP increased by 6.5 mmHg (p<0.05) and mean TPR increased by 17% (p<0.05) from the pre-cocoa values. Meanwhile, following LFC drink consumption at 1hr and 3hrs, an increment in mean TPR (p<0.05) was accompanied by a decrement in mean CO (p<0.05), both by 20% from the pre-cocoa values, during localised cooling.

Despite the changes observed between the visits, the responses in SBP, CO and TPR either in RT or during localised cooling were similar. No significant changes were observed in DBP or HR within each visit (Table 6.4).

In the PRP group, before cocoa consumption, all the cardiovascular measurements in RT and during localised cooling were similar between the visits. After consumption of HFC at RT, mean CO was significantly decreased by (-16% at 1hr and -21% at 3hrs), accompanied by a trend showing an increment in the mean TPR at 1hr (p=0.056) and a significant increment of 26% at 3hrs from pre-cocoa values. At the same time, mean SBP showed a trend to increase at 1hr (p=0.052) and increased by 7.2mmHg at 3hrs (p<0.05) from pre-cocoa measurements.

Similarly, at 1hr after consuming the LFC drink RT, mean CO decreased by 20% (p<0.05) accompanied by an increase in the mean TPR by 25% (p<0.05) from pre-cocoa measurement.

During localised cooling, after consumption of the HFC drink, mean CO decreased by (-18% both at 1hr and 3hrs; p<0.05) accompanied by an increase in the mean TPR (25% at 1hr and 20% at 3hrs; p<0.05) from pre-cocoa measurement. No statistical differences were observed in DBP or HR (Table 6.4).

Despite the changes observed between the visits, the responses demonstrated in SBP, CO and TPR either in RT or during localised cooling were similar. No differences were found in the responses of all the cardiovascular parameters across the study within each group and between the visits.

When the control group was compared with the PRP group (figure 6.3 and 6.4), the responses throughout the intervention in all the cardiovascular measurements were similar in both the visits. However, SBP in the PRP group showed a trend to be higher (p=0.054) during the HFC visit and was significantly higher (p<0.050) during the LFC visit compared to the control group.

At the start of each study day (pre-cocoa) in RT, SBP demonstrated a strong trend to be higher in the PRP group compared to the control group during both the visits (HFC visit, p=0.054; LFC visit, p=0.053; both are power to the level of 48%; 80% would require 20 people in each group). At the same time, during the LFC visit, mean TPR was 20% higher in the PRP group compared to the control group (p<0.05). After taking the HFC drink in RT, mean SBP showed a trend to be higher at 1hr (p=0.053) and was 11mmHg higher at 3hrs in the PRP group compared to the control group (p<0.05). Similarly, after taking the LFC drink, mean SBP was 11mmHg higher at 1hr (p<0.05) and showed a trend to be higher at 3hrs in the PRP group compared to the control group (p=0.054).
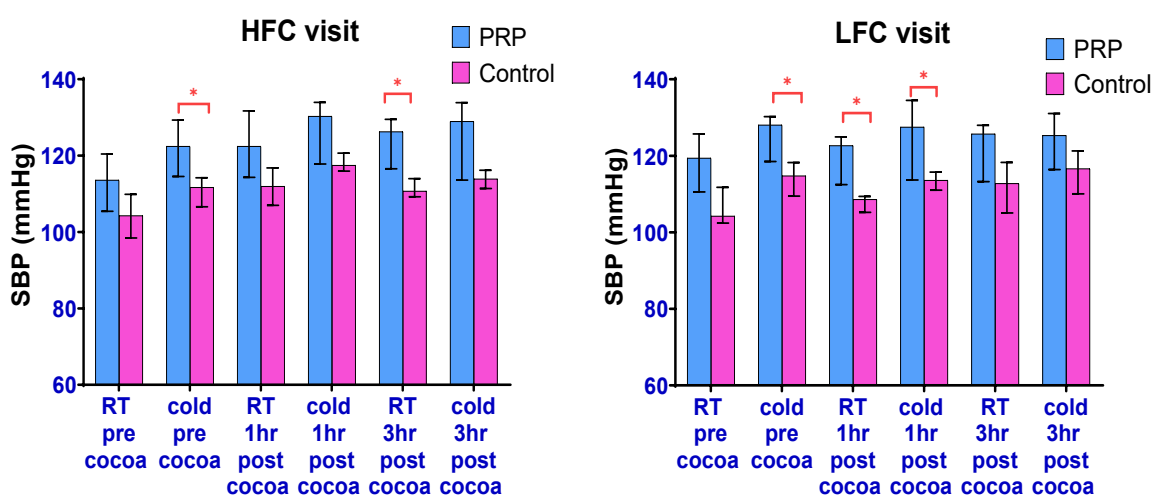
Figure 6.3: Systolic blood pressure (SBP) in the PRP group (n=10) *versus* control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits. Data are presented as the median (25th and 75th percentile) *p<0.05

Figure 6.4: Total peripheral resistance (TPR) in the PRP group (n=10) *versus* control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits. Data are presented as the median (25th and 75th percentile). *p<0.05

When the experimental hand was placed in the cold box (pre-cocoa) during both the visits, mean SBP was 10mmHg higher in the PRP group compared to the control group (p<0.05). Meanwhile, 1 hr after taking the LFC drink, mean SBP was 12mmHg higher in the PRP group compared to the control group (p<0.05).

6.3.3 Effect of a cocoa on the skin BF and skin temperature responses of the finger during localised cooling and at RT during rewarming after localised cooling, in control *versus* PRP

Outside temperature

During the HFC visit, the mean 8am outside (environmental) temperature recorded from the Nottingham (Watnall) Station from the Meteorological Office was 4.2°C colder in the PRP group compared to the control group (p<0.05). However, during the LFC visit, there was no difference between the groups (Figure 6.5).
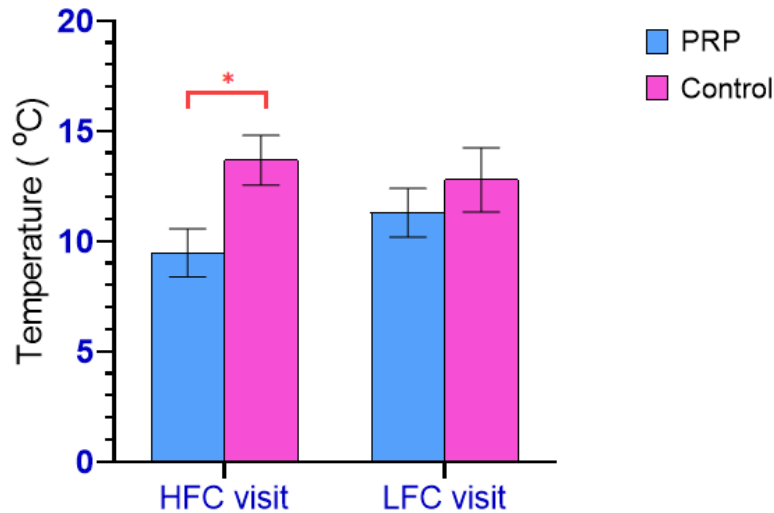
Figure 6.5: Outside temperature during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits in the PRP group (n=10) *versus* control group (n=10). Data are presented as the mean (SEM). *p<0.05

(i)   Skin BF and skin temperature response of the experimental finger during localised cooling and at RT during rewarming after localised cooling.

Figures 6.6 and 6.7 show the skin BF and skin temperature responses of the experimental finger during both (HFC and LFC) the visits in the control group. At the beginning of each study day (pre-cocoa), the skin BF and skin temperature of the experimental finger in RT and after localised cooling were similar between the visits.

After taking the LFC drink in RT, there was a decrement in the mean skin BF by (-30% at 1hr and -37% at 3hrs; p<0.05), accompanied by a decrease in the mean skin temperature (-3.6°C at 1hr and -2.2 $^{\circ}$C at 3hrs; p<0.05) from the pre-cocoa measurement. Similarly, 1hr after taking the HFC drink, the mean skin temperature at RT decreased by 2.6°C from the pre-cocoa measurement (p<0.05). Three hours after taking the same drink, there was a decrement in mean skin BF in RT by 54% (p<0.05) and was accompanied by a significant decrease in mean skin temperature by 4.4 $^{\circ}$C (p<0.05) from the pre-cocoa values.

During localised cooling, 3hrs after consuming the HFC drink, the mean skin temperature decreased by 1.2°C from the pre-cocoa measurement (p<0.05).
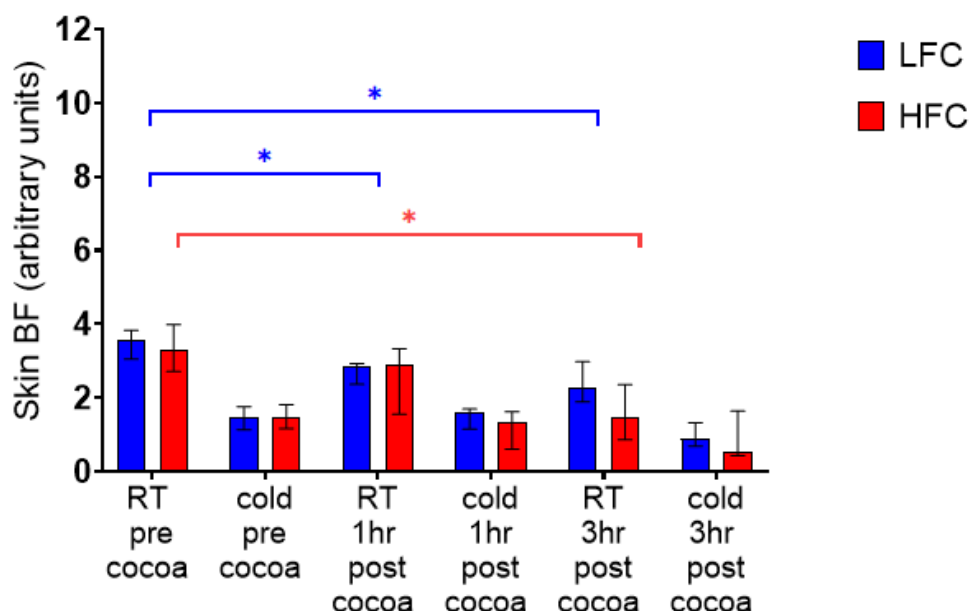
Figure 6.6: Skin BF (blood flow) of the experimental finger in the control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visit at room temperature (RT) and during localised cooling. Data are presented as the median (25th and 75th percentile). *p<0.05
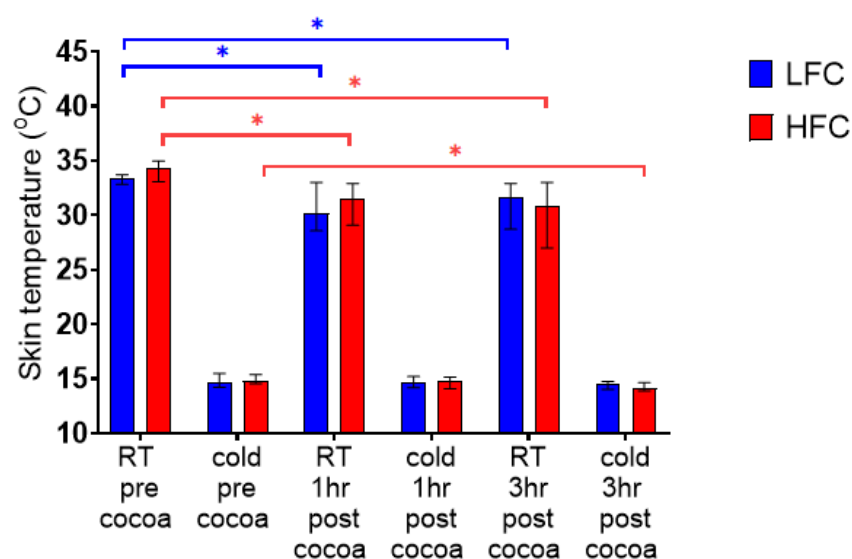
Figure 6.7: Skin temperature of the experimental finger in the control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling. Data are presented as the median (25th and 75th percentile). *p<0.05

In the PRP group, before cocoa consumption, the skin BF and skin temperature of the experimental finger in RT and cold box were similar between both visits (Figure 6.8 and 6.9). After consumption of the HFC drink, the mean skin BF decreased by (-33% at 1hr and -40% at 3hrs; $p<0.05$) at RT from pre-cocoa measurements. At the same time, the skin temperature showed a trend to decrease ($p=0.062$) at 1hr but no changes were observed at 3hrs compared to before consumption.

During localised cooling at 3hrs, the mean skin BF decreased by 32% after consuming the HFC drink ($p<0.05$) and by 34% after consuming the LFC drink, compared to pre-cocoa values ($p<0.05$). Although changes were observed, the responses in the skin BF and skin temperature either in RT or cold box, as well as across the study, were similar between the visits within each group.
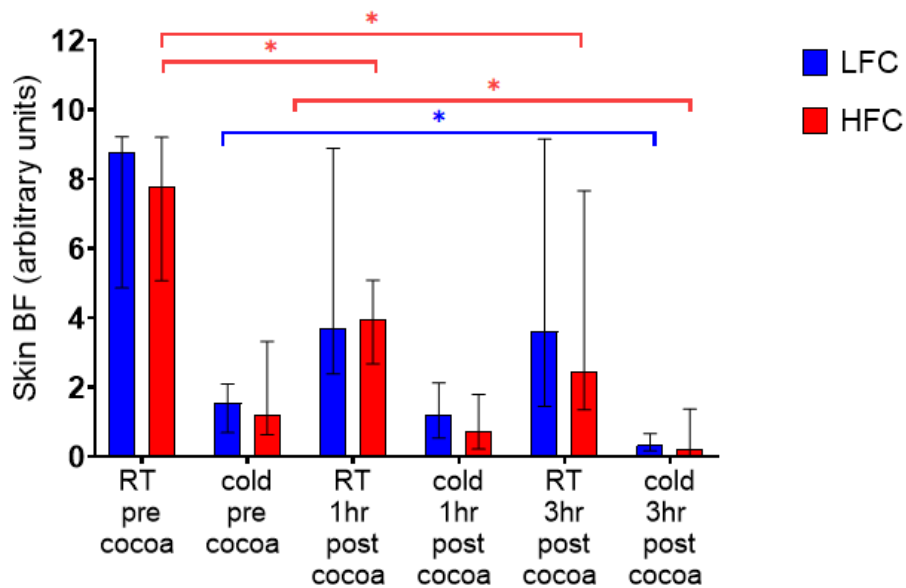
Figure 6.8: Skin BF (blood flow) of the experimental finger in the PRP group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling. Data are presented as the median (25th and 75th percentile). *$p<0.05$

Figure 6.9: Skin temperature of the experimental finger in the PRP group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling. Data are presented as the median (25th and 75th percentile). No statistical differences were observed.

When comparisons were made between the groups (Figure 6.10 and 6.11), the responses in the skin BF of the experimental finger across the study were significantly different between the groups: the skin BF of the experimental finger was significantly higher in the PRP group compared to the control group during both visits (HFC visit, $p<0.005$; LFC visit, $p<0.05$; between groups: $p<0.05$ for both the visits).

At the beginning of each study day (pre-cocoa) in RT, the mean skin BF of the experimental finger in the PRP group was higher by (47% during HFC visit and 50% during LFC visit; $p<0.05$) compared to the control group. However, the mean skin temperature (pre-cocoa) was colder in the PRP group compared to the control group and the difference was 1.9°C during the HFC visit ($p<0.05$). Three hours after consumption of both the drinks in RT, the skin BF of the experimental finger in the PRP group showed a trend to be higher compared to the control group (HFC drink, $p=0.060$; LFC drink, $p=0.058$).
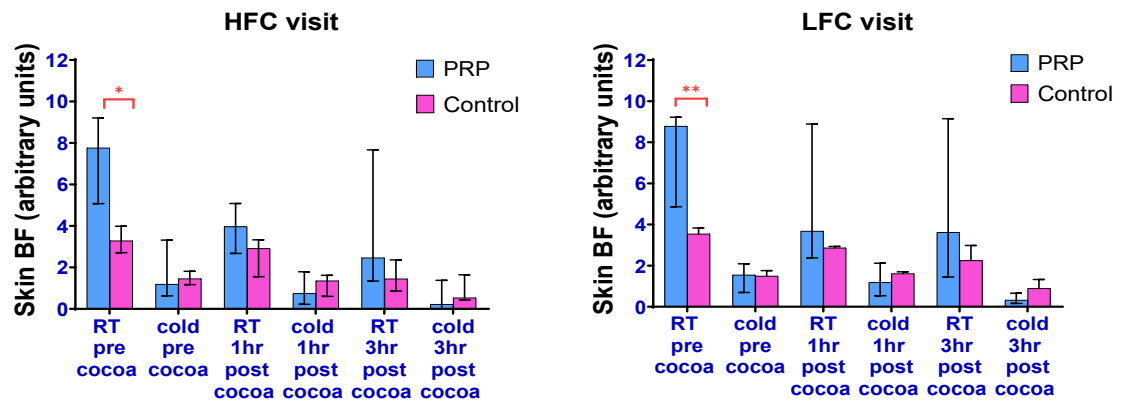
Figure 6.10: Skin BF (blood flow) of the experimental finger in the PRP group (n=10) *versus* control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits. Data are presented as the median (25th and 75th percentile). *p<0.05, **p<0.005
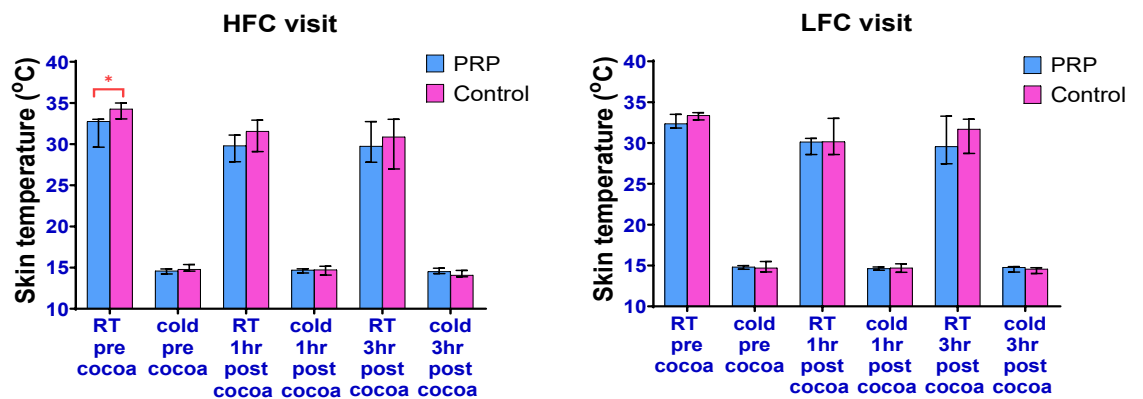
Figure 6.11: Skin temperature of the experimental finger in the PRP group (n=10) *versus* control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visit. Data are presented as the median (25th and 75th percentile) *p<0.05

(ii) Skin BF and skin temperature response of the control finger when the experimental finger was exposed to the cold and then at RT during rewarming after cold exposure

Figures 6.12 and 6.13 show the skin BF and skin temperature responses of the control finger during both the visits in the control group. At the start of each study day (pre-cocoa), the skin BF and skin temperature of the control finger in RT and when the experimental finger was exposed to the cold box,

were similar between visits. After taking the HFC drink, the mean skin BF of the control finger in RT significantly decreased by (-40% at 1hr and -31% at 3hrs), accompanied by a decrease in the mean skin temperature by (-3°C at 1hr; p<0.05) and a trend to decrease at 3hrs (p=0.059) from the pre-cocoa values.

Similarly, after consumption of the LFC drink in RT, the skin BF showed a trend to decrease at 1hr (p=0.059) and was accompanied by a significant decrease in the mean skin temperature by (-4 °C) at 1hr (p<0.05) from the pre-cocoa measurement. In addition, there was also a decrement in the mean skin temperature by (-3 °C) at 3hrs (p<0.05) from the pre-cocoa measurement after taking the same drink.
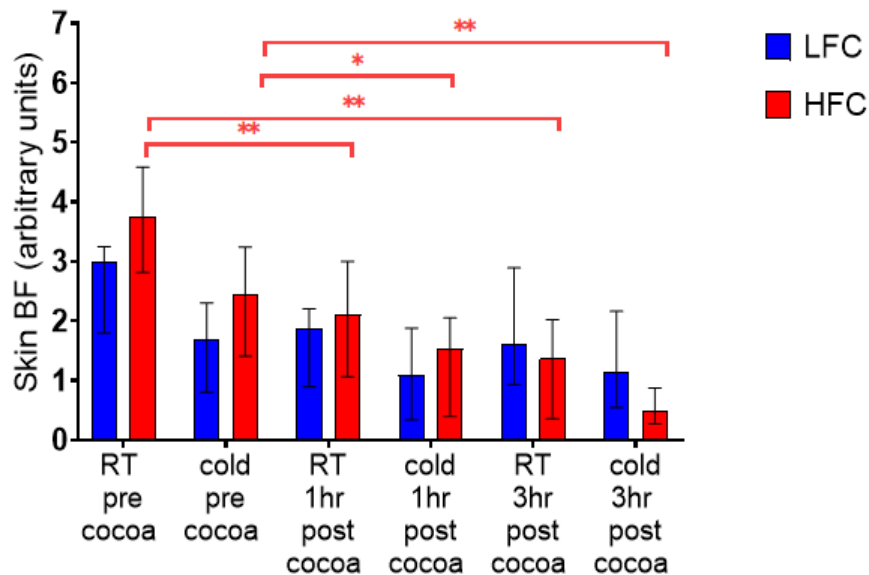
Figure 6.12: Skin BF (blood flow) of the control finger in the control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling of the experimental finger. Data are presented as the median (25th and 75th percentile). *p<0.05, **p<0.005.
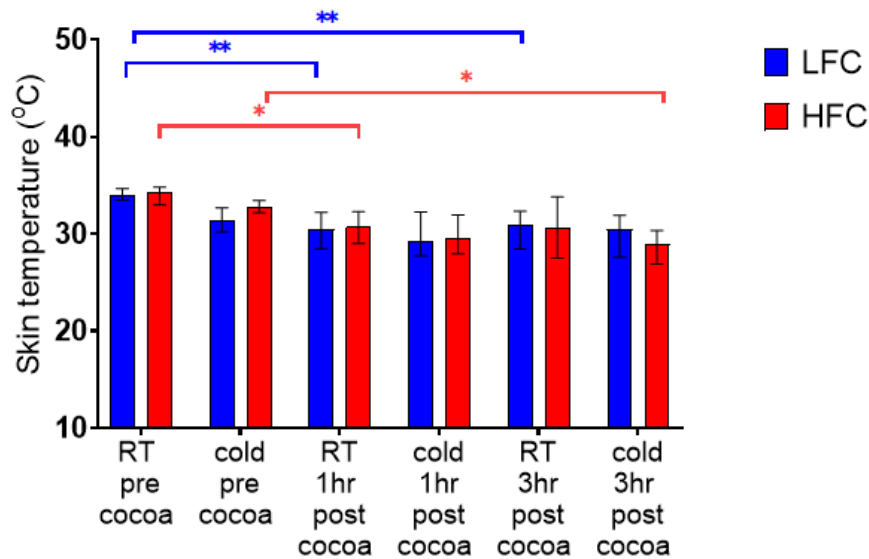
Figure 6.13: Skin temperature of the control finger in the control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling of the experimental finger. Data are presented as the median (25th and 75th percentile). *p<0.05, **p<0.005

During localised cooling of the experimental finger, after taking the HFC drink, the mean skin BF of the control finger decreased by (-43% at 1hr and -72% at 3hrs; p<0.05)   with a concomitant decrease in the mean skin temperature at 1hr (p=0.059) and at 3hrs (-3°C; p<0.05)   from the pre-cocoa measurement. Similarly, 1hr after consumption of the LFC drink, the skin temperature of the control finger was colder (p=0.054) compared to before consumption. In addition, the responses of the skin BF in the RT and during localised cooling of the experimental finger were different between visits (p<0.05 for both responses).

In the PRP group (Figures 6.14 and 6.15), before cocoa consumption, the skin BF and skin temperature of the control finger in RT and cold box were similar between visits. During the HFC visit, 3hrs after taking the drink in RT, the mean skin BF of the control finger decreased by 58% from the pre-cocoa measurement (p<0.05). At the same hour, when the experimental hand was placed in the cold box, the mean skin BF of the control finger also decreased by 72% from the pre-cocoa measurement (p<0.05).

During localised cooling at 1hr after consumption of HFC drink, the mean skin temperature decreased by 1.7°C from the pre-cocoa measurement. (p<0.05). However, no differences were observed in the skin BF and skin temperature responses across the study between the visits.
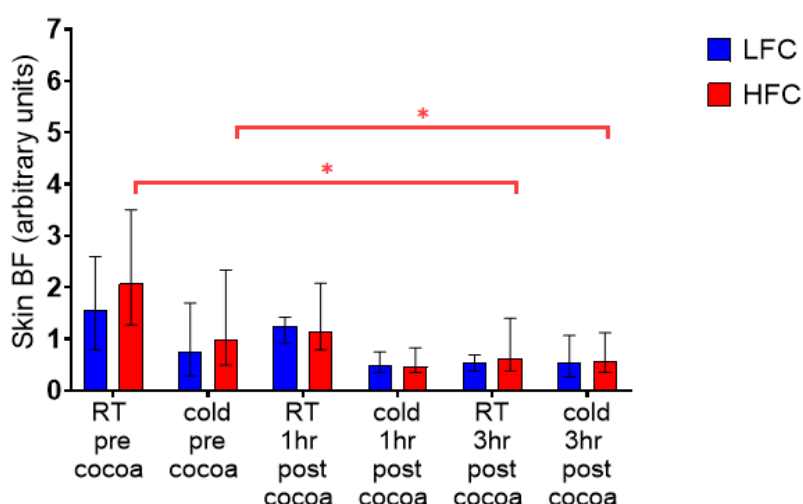
Figure 6.14: Skin BF (blood flow) of the control finger in the PRP group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling of the experimental finger. Data are presented as the median (25th and 75th percentile). *p<0.05
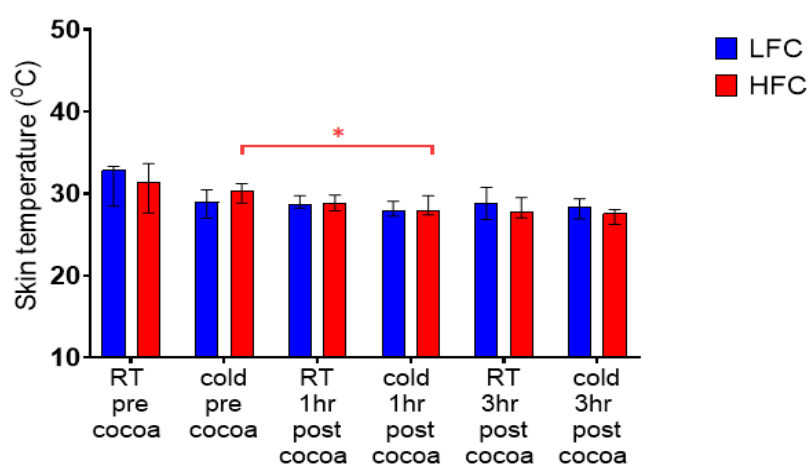
Figure 6.15: Skin temperature of the control finger in the PRP group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits at room temperature (RT) and during localised cooling of the experimental finger. Data are presented as the median (25th and 75th percentile). *p<0.05

When the control group was compared with the PRP group, the responses in the skin BF and skin temperature of the control finger across the study were similar during both visits. However, the skin temperature in the PRP group was colder than the control group during both visits ($p < 0.05$ for both).

At the beginning of each study day (pre-cocoa), in the PRP group compared with the control group, the mean skin temperature of the control finger in RT showed a trend to be colder ($p = 0.059$) during the HFC visit and was colder by 3°C ($p < 0.05$) during the LFC visit. Similarly, during both the visits, when the experimental hand was placed in the cold box (pre-cocoa), the skin temperature of the control finger was colder by 2°C ($p < 0.05$) in the PRP group compared to the control group (Figure 6.16). No changes were observed in the skin BF of the control finger between groups in both the visits.
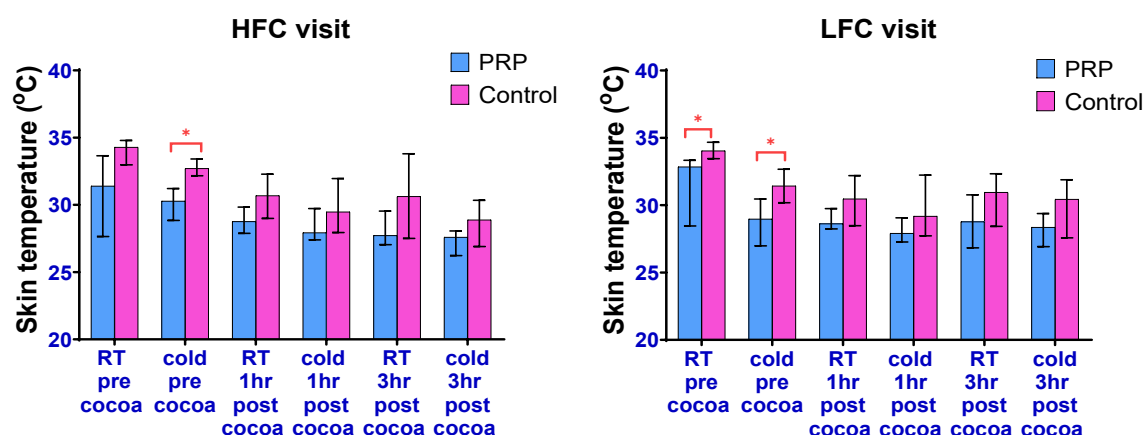
Figure 6.16: Skin temperature of the control finger in the PRP group (n=10) *versus* Control group (n=10) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits. Data are presented as the median (25th and 75th percentile). *$p < 0.05$
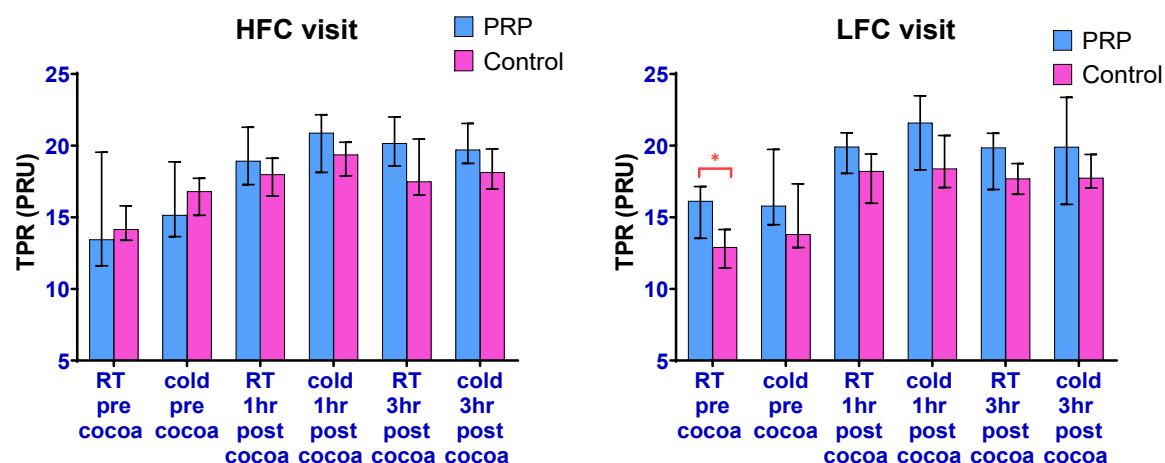
(iii)   Skin BF and skin temperature response of the experimental finger compared with the control finger during localised cooling and at RT during rewarming after localised cooling

Figure 6.17 shows changes in the skin BF of the experimental compared with the control finger in the control group. In the control group, 1hr after consumption of the HFC drink in RT, the mean skin BF was higher by 32% ($p < 0.05$) in the experimental finger compared to the control finger.
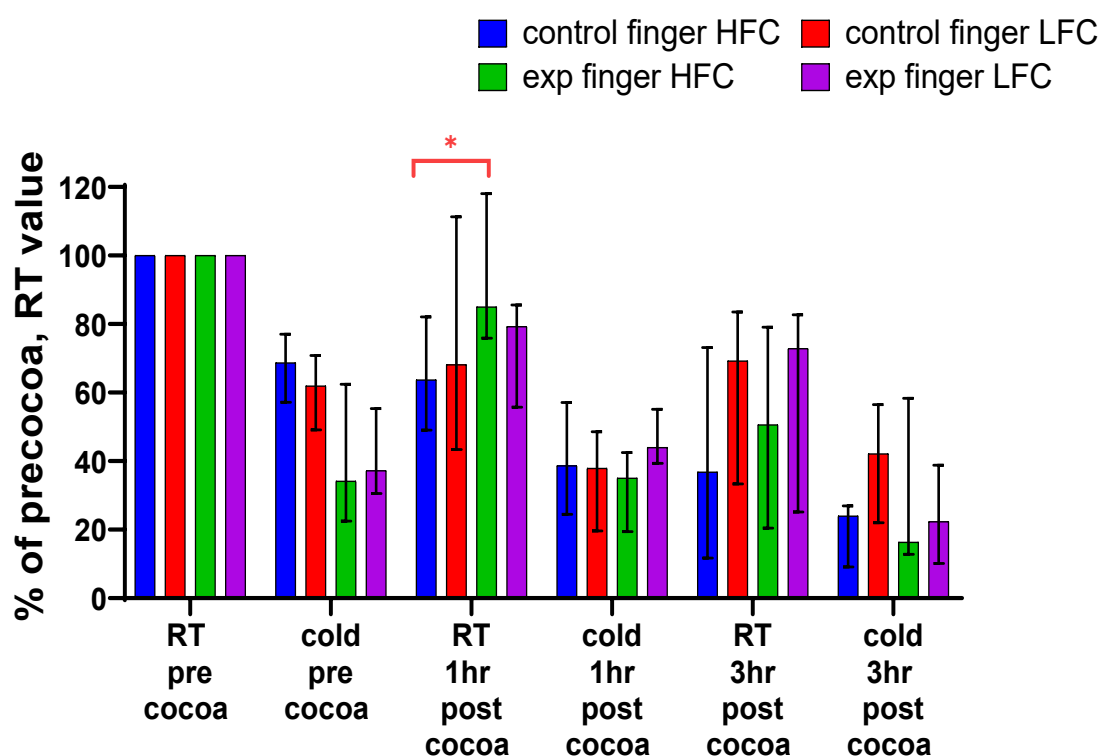
Figure 6.17: Changes in the skin blood flow of the experimental and control finger (compared to pre-cocoa room temperature values), pre, 1hr and 3hr post both cocoa consumption at room temperature (RT) and during localised cooling in the control group (n=10). Data are presented as the median (25th and 75th percentile). *$p < 0.05$

In the PRP group (Figure 6.18), during the HFC visit, when the experimental hand was placed in the cold box, the mean skin BF of the experimental finger compared to the control finger was lower by (63% pre-cocoa and 83% at 3hrs post-cocoa) ($p < 0.05$).

Meanwhile,1hr after taking the HFC drink in RT, the skin BF of the experimental finger tended to be higher than the control finger (p=0.051) but this measure was lower than the pre-cocoa baseline. In addition, the responses in the skin BF of the experimental finger compared to the control finger throughout the intervention were similar in each visit for both the groups.
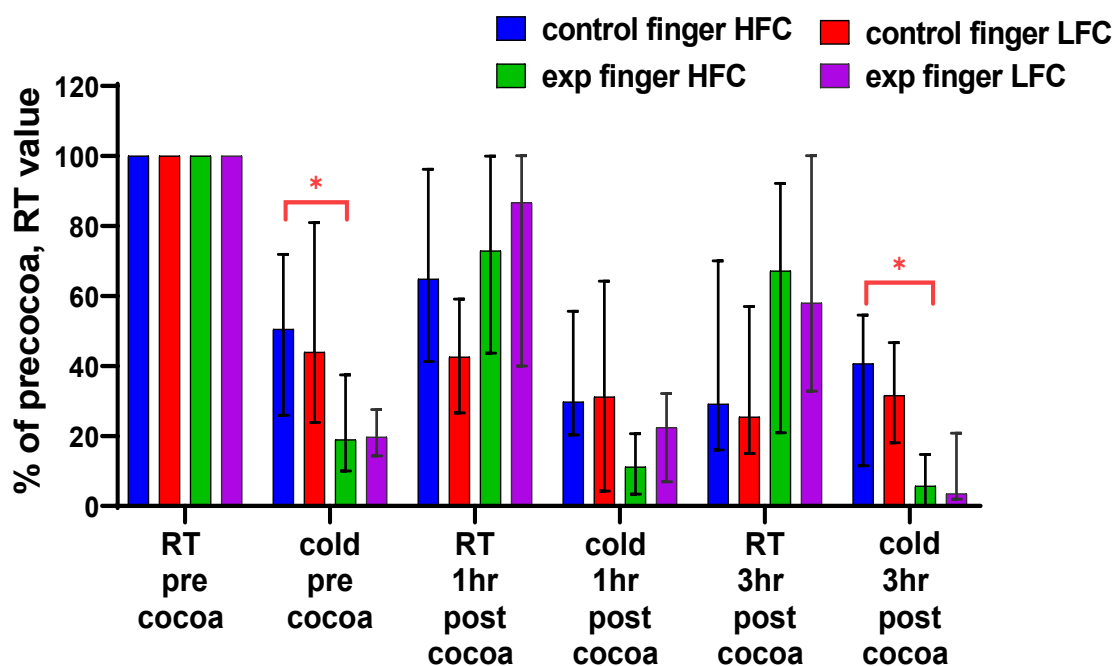
Figure 6.18: Changes in the skin blood flow of the experimental and control finger (compared to pre-cocoa room temperature values), pre-, 1hr and 3hr post both cocoa consumption at room temperature (RT) and during localised cooling in the PRP group (n=10). Data are presented as the median (25th and 75th percentile). *p<0.05

Comparisons between the skin temperature of the experimental finger *versus* control finger are shown in Table 6.5. In the control group, during both the visits, when the experimental hand was placed in the cold box, the mean skin temperature of the experimental finger compared to the control finger was colder before as well as 1hr and 3hrs after consuming the drinks (p<0.05). Similarly, in the PRP group, during both the visits, the mean skin temperature of the experimental finger compared to the control finger was

colder each time when the experimental hand was placed in the cold box (p<0.05).

In the PRP group, the mean skin temperature of the experimental finger compared to the control finger was significantly warmer by 1.2°C at 1hr post LFC drink and 1.7 °C at 3hrs post HFC drink in RT (p<0.05). However, these measures were lower than the pre-cocoa baseline in RT. In addition, the responses between the skin temperature of the experimental finger *versus* control finger throughout the study were different in each visit within groups (each visit for both the groups; p<0.005).

Skin temperature (°C)	HFC visit		LFC visit	
	Control finger	Experimental finger	Control finger	Experimental finger
Control group (n=10)				
Pre-cocoa in RT	34.29 [32.98-34.79]	34.27 [33.07-35.00]	34.04 [33.46-34.67]	33.37 [32.83-33.72]
Pre-cocoa in cold	32.71 [32.17-33.42]	14.80 [14.56-15.38]*	31.44 [30.18-32.67]	14.71 [14.21-15.49] ##
1hr post-cocoa in RT	30.69 [28.99-32.29]	31.56 [29.09-32.91]	30.47 [28.48-32.19]	30.16 [28.59-33.03]
1hr post-cocoa in cold	29.48 [27.94-31.95	14.74 [14.09-15.16]*	29.18 [27.73-32.23]	14.71 [14.18-15.21] ##
3hr post-cocoa in RT	30.63 [27.50-33.80]	30.89 [26.99-33.01]	30.95 [28.43-32.33]	31.70 [28.72-32.92]
3hr post-cocoa in cold	28.89 [26.90-30.34]	14.08 [13.89-14.67]**	30.44 [27.57-31.89]	14.57 [14.02-14.73] ##
PRP group (n=10)				
Pre-cocoa in RT	31.40 [27.65-33.65]	32.77 [29.63-33.02]	32.85 [28.46-33.33]	32.37 [31.83-33.51]
Pre-cocoa in cold	30.28 [28.85-31.22]	14.61 [14.23-14.84]**	28.98 [26.99-30.47]	14.82 [14.54-14.98] ##
1hr post-cocoa in RT	28.77 [27.89-29.83]	29.81 [27.86-31.10]	28.63 [28.24-29.75]	30.13 [28.59-30.57] #
1 hr post-cocoa in cold	27.93 [27.40-29.73]	14.70 [14.35-14.86]**	27.92 [27.27-29.06]	14.63 [14.43-14.83] ##
3hr post-cocoa in RT	27.72 [27.04-29.53]	29.74 [27.80-32.73]*	28.77 [26.83-30.76]	29.56 [27.46-33.28]
3hr post-cocoa in cold	27.60 [26.23-28.07]	14.52 [14.28-14.93]*	28.36 [29.92-29.39]	14.78 [14.20-14.87] ##

Table 6.5: Comparison of skin temperature measurements between experimental and control finger of the control and PRP group during pre-cocoa, 1hr and 3hr post both cocoa consumption at room temperature (RT) and during localised cooling. Data are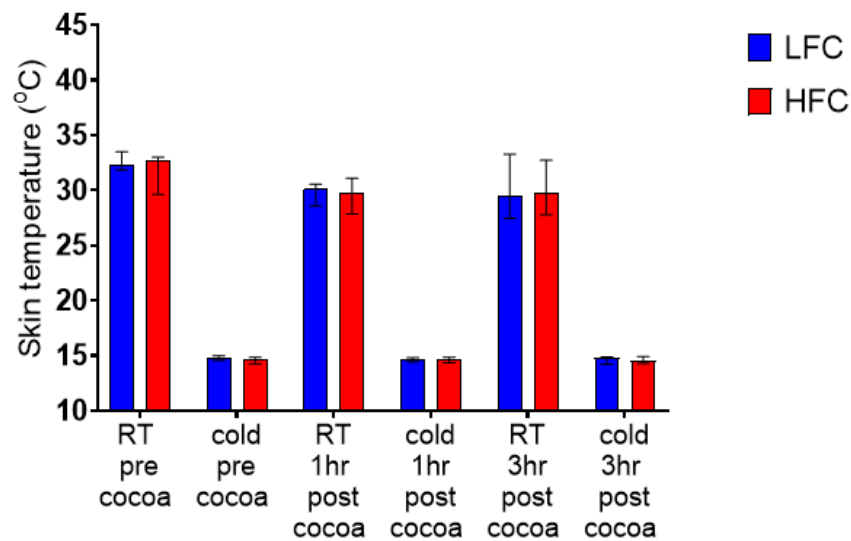 presented as the median (25th and 75th percentile). *p<0.05 vs control finger HFC, **p<0.005 vs control finger HFC, #p<0.05 vs control finger LFC, ##p<0.005 vs control finger LFC

(iv)   Changes in the skin temperature of the experimental finger moving from RT into the cold box.

Figures 6.19 and 6.20 represent the changes in the skin temperature of the experimental finger, moving from RT into the cold box, before and after cocoa consumption between the visits in the control and the PRP groups, respectively. At minute zero, the skin temperature prior to cooling before, 1hr and 3hrs after cocoa consumption, were similar within both the groups. Once the experimental hand was placed into the cold box, there were no differences in skin temperature response to the cold before and after both cocoa consumptions between visits within both the groups.

When the comparison was made between the PRP and control groups during both the visits (graph not shown), no differences were observed.
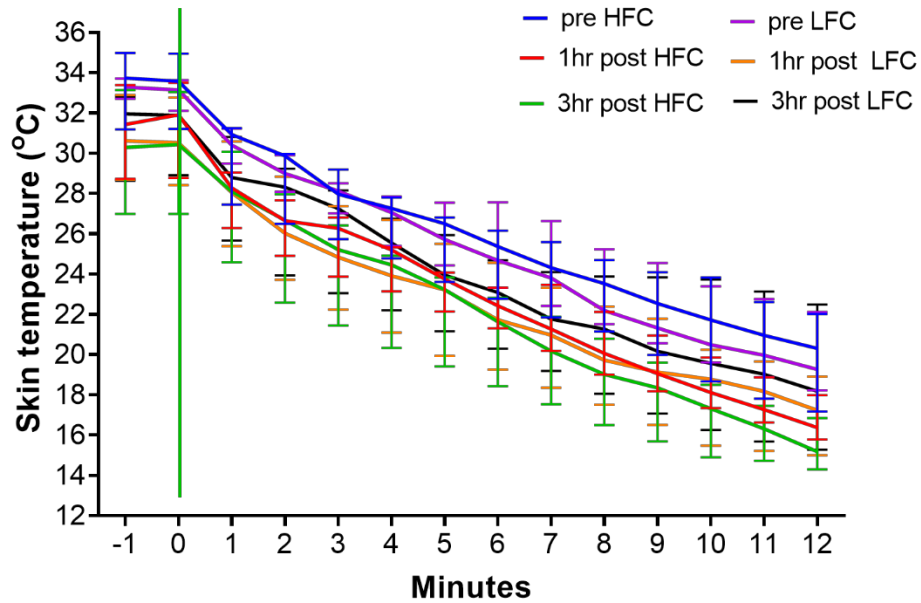
Figure 6.19: Changes in the skin temperature of the experimental hand moving from RT into the cold box before cocoa, and 1hr and 3hr post-cocoa consumption in the control group (n=10). The vertical line indicates when the experimental hand was placed into the cold box. Medians with 25th and 75th percentile shown as error bars. No significant changes were observed.
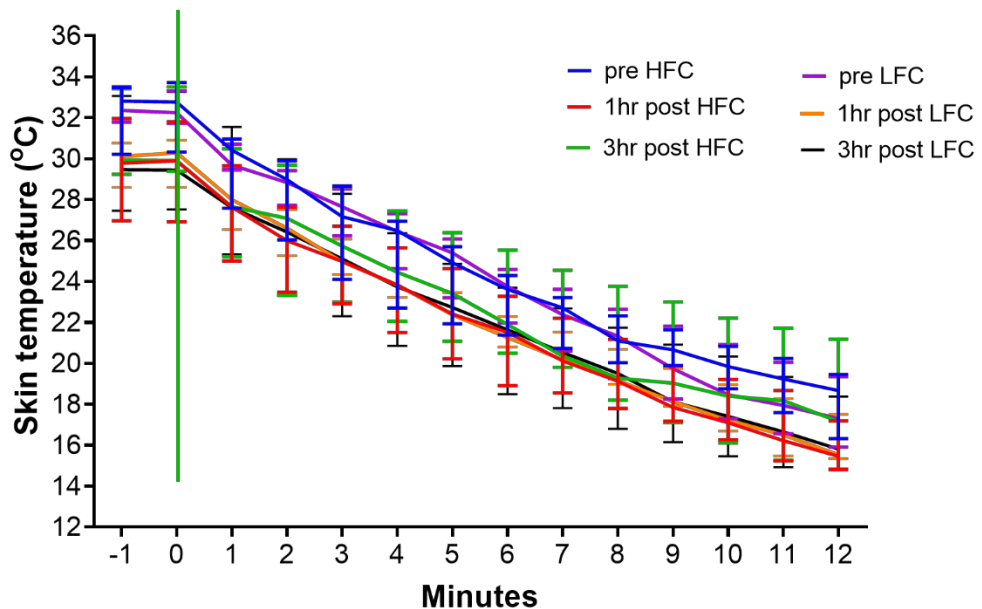
Figure 6.20: Changes in the skin temperature of the experimental hand moving from RT into the cold box before cocoa, and 1hr and 3hr post-cocoa consumption in the PRP group (n=10). The vertical line indicates when the experimental hand was placed into the cold box. Medians with 25th and 75th percentile shown as error bars. No significant changes were observed.

(v) Changes in the skin temperature of the experimental finger moving from the cold box into RT

Figures 6.21 and 6.22 show changes in the skin temperature of the experimental finger moving from the cold box back into RT before and after cocoa consumption between the visits, in the control and the PRP groups, respectively. At minute zero, the skin temperature values in the cold box before, 1hr and 3hrs after both cocoa consumptions were similar between the visits within both the groups. Once the experimental hand was placed in the RT, there were no differences in skin temperature response to the RT before and after both cocoa consumptions, between the visits within both the groups.

Similarly, no differences were observed when the comparison was made between the PRP and control groups during both the visits (graph not shown).
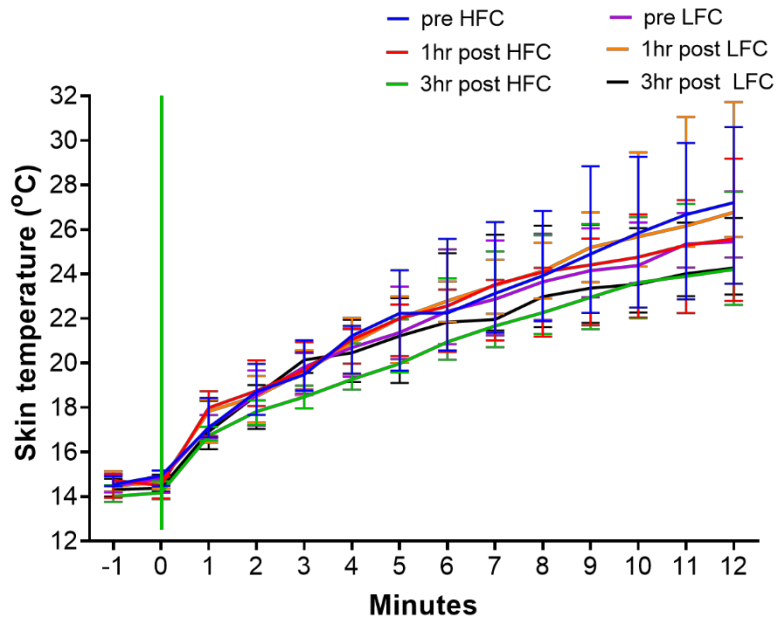
Figure 6.21: Changes in the skin temperature of the experimental hand moving from the cold box to RT before cocoa, and 1hr and 3hrs post-cocoa consumption in the control group (n=10). The vertical line indicates when the experimental hand was placed into RT. Medians with 25th and 75th percentile shown as error bars. No significant changes were observed.
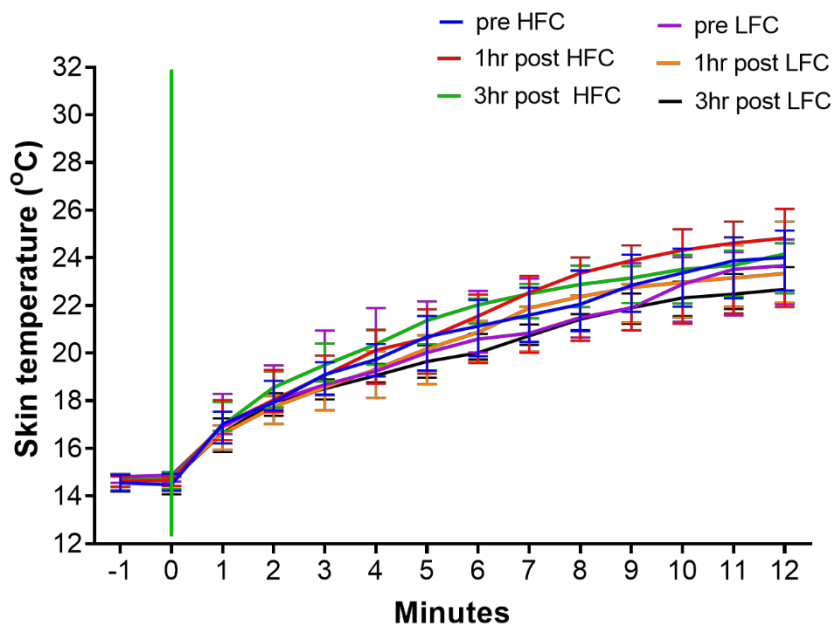
Figure 6.22: Changes in the skin temperature of the experimental hand moving from the cold box to RT before cocoa, and 1hr and 3hrs post-cocoa consumption in the PRP group (n=10). The vertical line indicates when the experimental hand was placed into RT. Medians with 25th and 75th percentile shown as error bars. No significant changes were observed.

6.3.4 Effect of cocoa on the time taken for the skin temperature of the experimental finger to stabilise to localised cooling and to RT during rewarming after localised cooling

Table 6.6 shows the time taken (minutes) for the skin temperature of the experimental finger to stabilise in the cold box and at RT before and after cocoa consumption. At the beginning of each study day (pre-cocoa), the time taken for the skin temperature to stabilise in the cold box and at RT between the visits was similar within both the groups. In the control group, during localised cooling, 1hr after consuming both the drinks, the time taken for skin temperature to get cold was faster compared to pre-cocoa measurements ($p < 0.05$). Similarly, at 3hrs after consuming the HFC drink, the time taken for skin temperature to get cold was faster compared to pre-cocoa measurements ($p < 0.05$).

In the PRP group, no changes were observed between the visits. The responses in the time taken for the skin temperature of the experimental finger to stabilise in the cold and at RT across the study was similar between the visits within each group.

When the control group was compared with the PRP group, during the LFC visit at RT (pre-cocoa), the mean skin temperature of the experimental finger in the PRP group rewarmed slower compared to the control group. The response across the study during the LFC visit was different ($p < 0.05$).

Time taken	Control group (n=10)		PRP group (n=10)	
	HFC visit	LFC visit	HFC visit	LFC visit
Time taken to stabilise in cold (pre-cocoa)	21.1 [13.9-23.9]	16.9 [15.6-21.9]	15.0 [11.3-17.0]	12.53 [12.1-15.9]
Time taken to stabilise in RT (pre-cocoa)	6.4 [5.2-17.4]	10.4 [4.5-12.3]	16.0 [11.2-22.8]	17.3 [12.5-20.4]$^{\#}$
Time taken to stabilise in cold (1 hr post-cocoa)	12.2 [11.5-14.4]*	13.4 [9.4-18.2]*	11.1 [9.6-13.9]	11.3 [9.5-13.8]
Time taken to stabilise in RT (1hr post-cocoa)	10.0 [8.4-20.2]	9.3 [6.9-19.4]	12.3 [9.4-24.9]	18.8 [11.6-24.4]
Time taken to stabilise in cold (3hr post-cocoa)	10.7 [8.5-12.4]*	15.8 [10.4-18.2]	11.3 [9.4-19.0]	11.3 [8.8-15.3]
Time taken to stabilise in RT (3hr post-cocoa)	14.3 [7.9-18.3]	10.8 [7.8-23.2]	14.3 [12.0-16.1]	19.7 [18.3-26.9]

Table 6.6: Time taken (minutes) for the skin temperature of the experimental finger to stabilise in the cold box and room temperature (RT) during High Flavanol Cocoa (HFC) and Low Flavanol Cocoa (LFC) visits in both the groups. Data are presented as the median (25th and 75th percentile). *p<0.05 vs time taken to stabilise in the cold pre-cocoa, $^{\#}$ p<0.05 vs time taken to stabilise in RT, pre-cocoa in control group

Summary of the results:

Core temperature and cardiovascular changes between HFC and LFC visits in both groups

Core temperature	Control		PRP	
	HFC visit	LFC visit	HFC visit	LFC visit
1 hr post-cocoa in RT vs pre-cocoa in RT	-	↑	↑	-
1 hr post-cocoa in cold vs pre-cocoa in cold	-	-	-	↑ trend
3 hr post-cocoa in RT vs pre-cocoa in RT	-	↑	-	↑
3 hr post-cocoa in cold vs pre-cocoa in cold	↑	-	-	↑ trend
SBP				
1 hr post-cocoa in RT vs pre-cocoa in RT	↑	-	↑ trend	-
1 hr post-cocoa in cold vs pre-cocoa in cold	↑	-	-	-
3 hr post-cocoa in RT vs pre-cocoa in RT	-	-	↑	-
3 hr post-cocoa in cold vs pre-cocoa in cold	-	-	-	-
CO				
1 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	↓	↓
1 hr post-cocoa in cold vs pre-cocoa in cold	-	↓	↓	↓
3 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	↓	-
3 hr post-cocoa in cold vs pre-cocoa in cold	-	↓	↓	-
TPR				
1 hr post-cocoa in RT vs pre-cocoa in RT	↑	↑	↑ trend	↑
1 hr post-cocoa in cold vs pre-cocoa in cold	↑	↑	↑	↑
3 hr post-cocoa in RT vs pre-cocoa in RT	↑	↑	↑	↑ trend
3 hr post-cocoa in cold vs pre-cocoa in cold	-	↑	↑	-

Changes in the skin BF and skin temperature of the experimental and control finger between HFC and LFC visits in both groups

	Control		PRP	
Skin BF experimental finger	**HFC visit**	**LFC visit**	**HFC visit**	**LFC visit**
1 hr post-cocoa in RT vs pre-cocoa in RT	-	↓	↓	-
1 hr post-cocoa in cold vs pre-cocoa in cold	-	-	-	-
3 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	↓	-
3 hr post-cocoa in cold vs pre-cocoa in cold	-	-	↓	↓
Skin temperature experimental finger				
1 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	↓ trend	-
1 hr post-cocoa in cold vs pre-cocoa in cold	-	-	-	-
3 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	-	-
3 hr post-cocoa in cold vs pre-cocoa in cold	↓	-	-	-
Skin BF control finger				
1 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓ trend	-	-
1 hr post-cocoa in cold vs pre-cocoa in cold	↓	-	-	-
3 hr post-cocoa in RT vs pre-cocoa in RT	↓	-	↓	-
3 hr post-cocoa in cold vs pre-cocoa in cold	↓	-	↓	-
Skin temperature control finger				
1 hr post-cocoa in RT vs pre-cocoa in RT	↓	↓	-	-
1 hr post-cocoa in cold vs pre-cocoa in cold	↓ trend	↓ trend	↓	-
3 hr post-cocoa in RT vs pre-cocoa in RT	↓ trend	↓	-	-
3 hr post-cocoa in cold vs pre-cocoa in cold	↓	-	-	-

Time taken to stabilise in cold and RT between HFC and LFC visits in both groups

Time taken to stabilise in cold and RT	Control		PRP	
	HFC visit	LFC visit	HFC visit	LFC visit
Time taken to stabilise in cold: 1 hr post-cocoa vs pre-cocoa	↓	↓	-	-
Time taken to stabilise in cold: 3 hr post-cocoa vs pre-cocoa	↓	-	-	-
Time taken to stabilise in RT: 1 hr post-cocoa vs pre-cocoa	-	-	-	-
Time taken to stabilise in RT: 3 hr post-cocoa vs pre-cocoa	-	-	-	-

Changes in the cardiovascular parameters between PRP and control groups in both visits.

Cardiovascular parameters	HFC visit	LFC visit
SBP	**PRP versus Control**	**PRP versus Control**
Pre-cocoa in RT	Higher in PRP (trend)	Higher in PRP (trend)
Pre-cocoa in cold	Higher in PRP	Higher in PRP
1 hr post-cocoa in RT	Higher in PRP (trend)	Higher in PRP
1 hr post-cocoa in cold	-	Higher in PRP
3 hr post-cocoa in RT	Higher in PRP	Higher in PRP (trend)
3 hr post-cocoa in cold	-	-
TPR		
Pre-cocoa in RT	-	Higher in PRP
Pre-cocoa in cold	-	-
1 hr post-cocoa in RT	-	-
1 hr post-cocoa in cold	-	-
3 hr post-cocoa in RT	-	-
3 hr post-cocoa in cold	-	-

Changes in the skin BF and skin temperature of the experimental and control finger between PRP and control groups in both visits.

	HFC visit	LFC visit
Skin BF experimental finger	**PRP versus Control**	**PRP versus Control**
Pre-cocoa in RT	Higher in PRP	Higher in PRP
Pre-cocoa in cold	-	-
1 hr post-cocoa in RT	-	-
1 hr post-cocoa in cold	-	-
3 hr post-cocoa in RT	Higher in PRP (trend)	Higher in PRP (trend)
3 hr post-cocoa in cold	-	-
Skin temperature experimental finger		
Pre-cocoa in RT	Colder in PRP	-
Pre-cocoa in cold	-	-
1 hr post-cocoa in RT	-	-
1 hr post-cocoa in cold	-	-
3 hr post-cocoa in RT	-	-
3 hr post-cocoa in cold	-	-
Skin temperature control finger		
Pre-cocoa in RT	Colder in PRP (trend)	Colder in PRP
Pre-cocoa in cold	Colder in PRP	Colder in PRP
1 hr post-cocoa in RT	-	-
1 hr post-cocoa in cold	-	-
3 hr post-cocoa in RT	-	-
3 hr post-cocoa in cold	-	-

Changes in the skin BF and skin temperature of the experimental *versus* control finger between HFC and LFC visits in both groups

	Control		PRP	
	HFC visit	**LFC visit**	**HFC visit**	**LFC visit**
Skin BF response	**Exp vs control finger**	**Exp vs control finger**	**Exp vs control finger**	**Exp vs control finger**
Pre-cocoa in RT	-	-	-	-
Pre-cocoa in cold	-	-	Lower in exp finger	-
1hr post-cocoa in RT	Higher in exp finger	-	-	-
1hr post-cocoa in cold	-	-	-	-
3hr post-cocoa in RT	-	-	-	-
3hr post-cocoa in cold	-	-	Lower in exp finger	-
Skin temperature response				
Pre-cocoa in RT	-	-	-	-
Pre-cocoa in cold	Colder in exp finger	Colder in exp finger	Colder in exp finger	Colder in exp finger
1hr post-cocoa in RT	-	-		Warmer in exp finger
1 hr post-cocoa in cold	Colder in exp finger	Colder in exp finger	Colder in exp finger	Colder in exp finger
3hr post-cocoa in RT	-	-	Warmer in exp finger	-
3hr post-cocoa in cold	Colder in exp finger	Colder in exp finger	Colder in exp finger	Colder in exp finger

Changes in the time taken to stabilise in cold and RT between PRP and control groups in both visits.

Time taken to stabilise in cold and RT	HFC visit PRP versus Control	LFC visit PRP versus Control
Time taken to stabilise in cold pre-cocoa	-	-
Time taken to stabilise in RT pre-cocoa	-	Longer in PRP
Time taken to stabilise in cold 1 hr post-cocoa	-	-
Time taken to stabilise in RT 1hr post-cocoa	-	-
Time taken to stabilise in cold 3hr post-cocoa	-	-
Time taken to stabilise in RT 3hr post-cocoa	-	-

6.4 Discussion

The present study demonstrated no differential effect of acute consumption of the HFC drink 1hr and 3hrs after consumption, compared to the LFC drink, on cardiovascular parameters (SBP, DBP, CO, HR and TPR), and peripheral thermoregulation (skin temperature and blood flow) in the control and PRP groups.

Effect of cocoa on core temperature and cardiovascular measures (BP, CO, HR, TPR) to localised cooling

Core temperature

In the present study, during localised cooling in the control group, it was found that the core temperature was significantly warmer at 3hrs after consuming HFC drink. This finding was in agreement with a previous study (Huizenga *et al.,* 2004) that found an increase in core temperature when local cooling was applied to a single body part in a warm environment as discussed in Chapter 5.

When the experimental hand was returned to RT after the period of localised cooling, the core temperature in the control group after 1hr and 3hrs consumption of LFC drink was significantly warmer compared to pre-cooling values. Similar findings were observed in the PRP group 1hr after consumption of the HFC drink and 3hrs after consumption of the LFC drink. An increase in core temperature characteristically indicates that the body is producing more heat than it is dissipating. The gradual rise in the core temperature seen over the 3hrs study period was observed after consumption of both HFC and LFC drinks in both participant groups suggesting that this observation was independent of the type of drink and group. It has been reported that the ingestion of nutrients causes an increase in heat production by the body known as dietary thermogenesis (meal-induced thermogenesis) as a result of the metabolic costs of digestion, absorption, metabolism and storage of nutrients (Case *et al.,* 2010). In a previous animal study, it was found that the heat produced is not

useful to an animal that is living in a thermal neutral environment but will contribute to the maintenance of body temperature when an animal is exposed to a cold environment. The magnitude of this heat production appears to be influenced by the energy and nutrient composition of the diet and by the nutritional state of the animal (Case *et al.,* 2010). The cocoa drink contains a small amount of energy (177.4 kJ in the HFC drink and 218.4 kJ in the LFC drink) and in the present study, the increase in the core temperature throughout the 3hrs of the study protocol might suggest the presence of dietary thermogenesis following consumption of both the cocoa drinks. However, it is unlikely that the energy provided by the drinks would be sufficient to induce a substantial thermogenic response. Another possibility to consider is that the cocoa drink was served warm. It has been previously reported that adding energy to the food by heating it ought to have a relatively straightforward additive effect on the food's thermic value (Herman, 1993). However, the volume of the drink is small and the exact temperature of the drink was not measured, thus, the relative addition of heat to the body might be minimal and likely to be transient. Therefore, the increase in the core temperature detected after 1hr and 3hrs post consumption is unlikely to be due to the temperature of both the drinks.

Cardiovascular parameters

In both the groups, there was a gradual increase in the SBP which could be due to an increase in the TPR throughout the study protocol. In the control group, the increment in the SBP was significant 1hr after consumption of the HFC drink in the RT and during localised cooling. Meanwhile, in the PRP group, the increment was significant 3hrs after consumption of the HFC drink in RT. On the other hand, the increment in SBP was not observed following consumption of the LFC drink in both the groups. These contradictory data could be due to compensatory reduction in CO (significant) throughout the study protocol shown during the LFC visit which led to a maintenance of SBP.

Moreover, there was a significant increase in the TPR observed in most of the time point measurements in both the groups which could be due to the presence of increased vasoconstriction throughout the study protocol. These assumptions were made based on the decrease in the skin temperature and skin BF of the experimental finger and control finger observed during localised cooling, as well as in the RT after localised cooling at 1hr and 3hrs. The cold stimulus may induce α-adrenergic vasoconstriction resulting in increased TPR (Pickering and Gerin, 1990). Meanwhile, the compensatory decrease in the CO in order to control the SBP was significant in most of the time point measurements in both the groups. The TPR and CO did not return to pre-cooling values after the experimental hand was returned to RT, which could be due to the continuous vasoconstriction of both the hands throughout the study protocol as mentioned earlier.

When the control group was compared with the PRP group, the SBP in the PRP group was significantly higher at most time points compared to the control group. Similarly, TPR in RT before LFC consumption in the PRP group was significantly higher compared to the control group. This finding could be due to the participants from the PRP group being 8 years older than participants from the control group. In line with these findings, data from a representative sample of the United States adult population reported that older adults (aged 55-74 years) had a twofold greater prevalence of high BP than younger adults (25—54 years) (Harlan *et al.*, 1984).However, the mean age of the PRP group (37years old) was within younger adults age group.

Although the present findings may contrast with observations from previous studies, in which a reduction in BP after cocoa flavanols intake was observed (Davison *et al.,* 2010), the participants in the latter study were individuals who had either been diagnosed with CVD or who exhibited a high risk of CVD. Davison *et al.,* (2010) reported that chronic consumption of 1052mg of cocoa flavanols per day for 6 weeks led to significant reductions in SBP and DBP in 32 men and 20 postmenopausal women with untreated mild hypertension. In contrast to the above, the present study

conducted to determine the acute effects of cocoa flavanols and population recruited consisted of a healthy cohort who exhibited a very low CVD risk. Furthermore, previous studies have suggested that the effect of dietary flavanol intake can be observed when cocoa flavanol supplementation is provided alongside a diet with very low in flavanols (Grassi *et al.*, 2005a, Grassi *et al.*, 2005b). Some study protocols which have reported a beneficial effect of cocoa-containing foods have an initial phase where the participants were taking a low flavanol diet which was maintained throughout the supplementation period; the beneficial effect of flavanol supplementation was then compared during the phase of a low flavanol diet. Moreover, some of these studies investigated the effects of either dark or white chocolate bars on BP in healthy participants (Grassi *et al.*, 2005a) and patients with essential hypertension (Grassi *et al.,* 2005b). In both of those studies, the participants were carefully instructed to maintain their diet and to refrain from flavonoid-rich foods and beverages, including wine and other alcoholic beverages for a 7 days cocoa free run-in phase. In addition, a list of these foods and beverages was given to all healthy participants (Grassi *et al.,* 2005a). It was found that consumption of dark chocolate bars for 15 days significantly reduced the SBP and DBP in patients with essential hypertension and SBP in healthy participants. In contrast, the present study did not modify the diet and was investigating the acute effect of adding 830mg flavanols on top of their habitual diet. Therefore, it was possible that the supplementation given in the present study was not able to demonstrate a vasodilator response when given alongside the diet already rich in flavanols (the usual diet in the UK).

Similar outcomes were observed in studies with quercetin supplements, in which participants with hypertension showed improvements after the intake of a quercetin supplement, whereas healthy individuals showed no detectable change in BP over the course of the trial (Edwards *et al.*, 2007, Larson *et al.,* 2012). Although quercetin and epicatechin have a different chemical structure, their cardioprotective effect is presumed to be similar as both are in the same subclass of flavonoids. In a study by Edward *et al.,* (2007), men and women with prehypertension and stage 1 hypertension

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were enrolled in a study to test the efficacy of 730mg quercetin daily for 28 days *versus* placebo. BP was not altered in prehypertensive patients after quercetin supplementation. However, a significant reduction in BP was observed in stage 1 hypertensive patients after quercetin treatment. In another study, 1095mg quercetin or placebo was administered to men with normal BP or with stage 1 hypertension. It was found that ingesting quercetin did not affect BP in normotensive men at 10hrs but in stage 1 hypertensive men, the mean BP was significantly decreased (Larson *et al.,* 2012).

Previous studies reported that a certain degree of hypertension might be required for quercetin to exert a blood pressure-lowering effect (Edwards *et al.*, 2007, Conquer *et al.*, 1998). Conquer *et al.*, (1998) reported no changes in BP when 27 normotensive individuals (120mmHg systolic/, 80mmHg diastolic) were supplemented with 4 capsules daily of either 1000mg quercetin supplement or rice flour placebo for 28 days in a double-blind study. Moreover, another study was conducted in patients with prehypertension and stage 1 hypertension to test the efficacy of 730mg quercetin daily for 28 days. They found that the BP was significantly reduced in stage 1 hypertensive patients but not in prehypertensive patients and therefore, concluded that the threshold appears to occur at approximately 130-139mmHg SBP, which was the upper range of pre-hypertension (Edwards *et al.,* 2007). In the present study, the mean SBP in the healthy group was 105mmHg while in PRP group was between 115-117mmHg during both the visits which were below the threshold level.

Effect of a cocoa on the skin BF and skin temperature response of the finger during localised cooling and at RT during rewarming after localised cooling, in control *versus* PRP.

Skin BF and skin temperature

The present study has demonstrated that 1hr and 3hrs after acute consumption of the HFC drink, there was no change in skin temperature and skin BF in the control and PRP groups. This could be due to the cold

temperature stimulus of 15°C being too great to demonstrate an effect of cocoa in increasing the skin temperature and skin BF as mentioned earlier. When there is whole-body or local exposure of the extremities to cold, the initial response is a strong vasoconstriction which causes a rapid decrease in hand and foot temperature. At the same time, the blood flow to the extremities of the hands and feet responds rapidly with a sympathetically-mediated vasoconstriction to reduce the blood flow to the peripheries in favour of a central pooling of blood in the centre of the body. The skin temperature of the fingers and toes tends to rapidly and exponentially decrease to a level approaching that of the ambient environment due to this vasoconstriction (Cheung, 2015).

In the control group, there was a reduction in the skin temperature and skin BF of the experimental finger in the cold and at RT following localised cooling at 1hr and 3hrs in both the visits when compared to pre-cooling values. Similar findings were observed in the PRP group, although the reduction in the skin temperature and skin BF of the experimental finger appeared to be less pronounced compared to the control group. These findings suggest the presence of vasoconstriction in both the groups throughout the study protocol.

Interestingly, as well as the reduction in the skin temperature and skin BF of the experimental finger seen during the study protocol, these reductions were also observed in the control finger in both groups. The reduction in the skin temperature and skin BF of the control finger when the experimental hand was exposed to cold suggests the presence of sympathetically-mediated vasoconstriction as mentioned earlier. However, a gradual reduction in skin temperature and skin BF were also observed in both fingers when at RT across the study visit (i.e. at each measurement time point before localised cooling) and both the parameters in both the groups did not return to precooling values after exposure to localised cooling. These observations suggest that a RT of 25°C may not be a thermoneutral environment to maintain the skin temperature and skin BF of the participants, and that the body was counter-regulating to conserve heat. The

gradual rise in the core temperature observed in both the groups after both the drinks supports this assumption

The whole-body thermal state affects the rate at which hand skin temperature recovers after exposure to local cooling. If the body is warm, the hand quickly vasodilates and the hand skin temperature recovers quickly. When the body is neutral or cool, after hand cooling is removed, there is no need to pump the blood to the hand to release heat, so the hand skin temperature recovers very slowly (Huizenga *et al.*, 2004).

When the comparison was made between the groups, before cocoa consumption at both the visits, the skin temperatures of the experimental finger in PRP group was lower in both the groups and was significant during HFC visit compared to the control group. In line with this observation, Stoyneva *et al.,* (2016) found that the initial superficial skin temperatures of the fingers in 30 female and male participants with PRP were significantly lower compared to the control group. This finding was also reported by O'Reilly *et al.,* (1992), who found that basal skin temperature measurements were significantly lower in patients with PRP and SRP compared with controls. The lower basal temperatures in people with PRP may reflect some permanent vascular compromise and thus implies that RP is not completely reversible (O'Reilly *et al.,* 1992). However, in the current study, the outside environmental temperature was colder during the PRP group's HFC study visit compared to the control group study visit which might have been a contributing factor.

In the present study, despite having lower skin temperature of the experimental finger, the skin BF of the same finger in the PRP group before cocoa consumption was significantly higher compared to the control group. Conversely, other studies have reported that the superficial skin blood flow values of the fingers in PRP participants were significantly lower compared to the control group (Stoyneva, 2016, Droste *et al.*, 1990). For example, Droste *et al.,* (1990) reported that baseline laser Doppler flux was lower in 12 patients with PRP in comparison to 12 healthy control participants. However, this is not a consistent observation as Khan and Coffman reported

that baseline skin BF was similar between 10 control participants and 10 patients with RP (Khan and Coffman, 1994). It is difficult to fit the finding of increased skin BF into a theory of generalised vasospasm. People with RP often have excess vasodilation responses (hyperaemia) after their vasospastic attacks (Khan and Coffman, 1994). The present study was conducted between March to June and the weather was cold (temperature between 9-11°C during PRP group study visit and 12-13°C during control group study visit). It is possible that there was a degree of vasoconstriction induced prior to entering the laboratory and that the higher blood flow recorded after being in the laboratory for a period of time reflected a reactive vasodilation. However, none of the participants reported symptoms prior to arriving for the study, and the higher skin BF demonstrated by PRP individuals in this study was therefore unlikely to be due to the reactive hyperaemia which characteristically accompanies a symptomatic event. In this study protocol, it was confirmed that the skin temperature of experimental and control fingers was stable before any baseline measurements were recorded. However, the skin BF of these fingers was not monitored immediately upon arrival as the equipment needed to be set up before monitoring could commence, and therefore, it is difficult to make any assumptions around the presence of vasoconstriction on arrival. However, these findings might suggest an uncoupling between skin BF and skin temperature, perhaps indicating the presence of underlying thermoregulatory dysfunction in the PRP group.

Effect of cocoa on the time taken for skin temperature of the experimental finger to stabilise to localised cooling and at RT after localised cooling.

In the present study, time taken (before cocoa consumption) to stabilise in response to localised cooling, and when returned to RT, were similar during HFC and LFC visits in the PRP and control group suggesting that in PRP participants responses to localised cooling in the absence of vasospasm, are not impaired.

It is interesting to note that in the control group, the time taken to stabilise in the cold 1hr and 3hrs after consumption of both cocoas was shorter compared to before consumption; it was significant 1hr after taking both the drinks and 3hrs after taking the HFC drink. These findings suggest that a greater heat loss was occurring in the hand during exposure to the cold environment, or that a greater vasoconstriction was present. However, in view of the reduction in the blood flow observed over the study period, the shorter time taken to stabilise in the cold could have been due to the presence of greater vasoconstriction.

In a study by Young *et al.,* (1986) the effects of repeated cold water immersion on thermoregulatory responses to cold air were studied in 7 men. A whole body cold air stress test was performed before and after completion of an acclimation program consisting of daily 90-min cold (18^oC) water immersion, repeated 5 times/week for 5 consecutive weeks. Following the acclimation program, it was observed that the rectal temperature and skin temperature were lower compared with before acclimation. The reduction in skin temperature may reflect a greater degree of cutaneous vasoconstriction. These findings suggest that repeated cold water immersion stimulates the development of true cold acclimation in humans which appears to be of the insulative type. This finding demonstrates that insulative adaptations in the physiological responses to cold air can be produced by acclimation procedures accomplished in a relatively short period of time (Young *et al.,* 1986). Although acclimation to repeated cold challenge tests has been demonstrated following a short period of time, this observation appears to require a reduction in the core temperature. In the present study, the cold challenge test was localised and did not result in any heat loss and therefore, was unlikely to be due to insulative acclimation. The increased vasoconstriction observed could be due to the room not being thermoneutral, as discussed earlier. No differences were observed in the PRP group.

When the comparison was made between PRP and control groups, the time taken to stabilise to RT (during rewarming after localised cooling) before and 1hr and 3 hrs after both cocoa consumptions were longer in the PRP

group compared to the control group; the difference was significant before consumption of the LFC drink. A previous study investigated the rewarming time of fingers after hand cooling to 10°C during ischaemia between patients with RP secondary to occupational origin compared with healthy participants and patients with PRP. It was found that two-thirds of the patients classified as having PRP showed a completely normal rewarming time. A prolonged rewarming time was observed in women with pronounced PRP (Juul and Nielsen, 1981). In the PRP group, a slower recovery over a prolonged period of time following cold exposure could indicate the presence of thermoregulatory impairment.

As a conclusion, the present study has demonstrated no effect of acute consumption of a drink rich in cocoa flavanols measured 1hr and 3hrs after consumption and it is possible that the cold stimulus of 15°C might have been too great to demonstrate an effect of cocoa in reducing the BP and improving the thermoregulatory response to cold. This study also seems to suggest that increases in the sympathetic tone (manifested by increased vasoconstriction) previously demonstrated following repeated local cooling might be occurring in the participants.

Chapter 7: Chronic effects of cocoa capsules on peripheral blood flow and symptoms in participants with Primary Raynaud's Phenomenon

7.1 Introduction

It is known that there is no definitive cure for RP, although several drug treatment strategies are available to improve vasodilatation. Indeed, the majority of sufferers appear to self-manage their condition by trying to avoid trigger situations, and wearing protective clothing (Howard, 2016). However, there is also an interest in diet and nutritional interventions, with ginger, oranges and cayenne pepper consumption recommended by some to alleviate the condition (Fleming, 2017). Few controlled trials have been carried out to assess the effectiveness of such strategies, although there is some evidence that the herbal supplement Ginkgo Biloba could reduce the incidence of vasospasm (Muir *et al.*, 2002). As this herb has been found to contain high levels of the flavonoid, quercetin, which appears, *in vitro*, to be protective against endothelial dysfunction (Lodi *et al.*, 2009), investigation of other high flavonoid foods appears warranted.

Cocoa flavanols, in particular epicatechin, have been shown to induce NO-dependent vasodilatation (Fisher *et al.,* 2003) by several mechanisms, as discussed earlier. It is hypothesised that the varied actions of cocoa flavanols could address several of the pathologies described in PRP and ameliorate the prevalence and/or severity of symptoms associated with this condition. There has been interest in the role of functional foods to improve the health and wellbeing of the population (Agricultural Research, 2010). However, much nutritional advice relating to RP is not supported by research evidence. Preliminary results from the Ginkgo trial suggest that flavonoid-rich foods might confer a benefit in conditions where there is vascular dysfunction and that cocoa would be an ideal candidate to be investigated as the vascular benefits of cocoa are well characterised. However, a previous study among healthy volunteers indicated that high flavanol cocoa could cause headaches in 6% of volunteers (unpublished

data). These were usually reported in the first week of dosing and settled down after this time. However, headaches are frequently co-reported by individuals with Raynaud's (Palmer *et al.*, 2000) and it is feasible that the cocoa could exacerbate this. As compliance issues and high attrition rate can potentially introduce bias into the data, it is important that these are quantified before a full randomised control trial is carried out. Moreover, before a full trial can be instigated, information regarding the feasibility of recruitment for a more extensive trial, and effect size, is also required.

In this study, the effects of supplementing the diet with cocoa flavanols on Raynaud's symptoms in those with PRP were determined. In addition, the effects of supplementing the diet with cocoa flavanols on quality of life measures, the feasibility of recruitment for a full randomised controlled trial (RCT), as well as the adverse events and attrition rate in a PRP cohort asked to supplement their diet with high flavanol cocoa extract were also determined.

7.1.1 Study aims

Primary aim:

•   To determine the effects of supplementing the diet with cocoa flavanols on Raynaud's symptoms in those with PRP using a parallel group, double-blinded, randomised design

Secondary aims:

•   To determine the effects of supplementing the diet with cocoa flavanols on quality of life measures.

•   To determine the feasibility of recruitment for a full RCT.

•   To determine adverse events and attrition rate in a PRP cohort asked to supplement their diet with high flavanol cocoa extract, to assess whether supplementing the diet with cocoa flavanols for 3 months affects the cardiovascular responses seen on cold exposure.

Hypotheses

The prevalence and/or severity of symptoms associated with PRP would be reduced in those taking cocoa flavanols capsules compared to placebo.

Quality of life measures in participants with PRP would be improved in those taking cocoa flavanols capsules compared to placebo.

7.2 Methods and Materials

7.2.1 Sample size calculations

No previous studies had been carried out to assess the effect that cocoa polyphenols have on symptom frequency in patients with Raynaud's. However, a placebo-controlled trial using the polyphenol-rich herbal supplement Ginkgo Biloba has been executed (Muir *et al.*, 2002). If a similar magnitude of effect was achieved by supplementing with high flavanol cocoa, as that seen in the Ginkgo Biloba trial, then the study would require 73 participants per group in a parallel group design to power at the level of 80% (Lenth, 2006).

Mean symptom frequency after placebo intervention = 10.7 (SD 12.3) attacks per week.

Mean symptom frequency after Ginkgo intervention = 5.8 (SD 8.3) attacks per week.

However, the current study aimed to provide data to enable a more accurate power calculation for any future RCT to be carried out.

7.2.2 Participants

The study protocol was approved by the Medical School Research Ethics Committee, Faculty of Medicine & Health Sciences, Queen's Medical Centre, Nottingham (Appendix page *xxiii*). Written informed consent was obtained from all the participants. The present study was a randomised, double-blind, parallel design study involving 22 healthy participants (20

female and 2 male), fulfilling the criteria of Allen and Brown (1932) for the diagnosis of PRP. Selection of the participants was based on the inclusion and exclusion criteria as discussed in Chapter 5 [5.2.2].

7.2.3 Cocoa capsules

The cocoa capsules used in this study were kindly provided and 'blinded' by Mars Wrigley Confectionery. These vegetarian capsules contained either high flavanol cocoa (HFC) extract (CocoaVia™) or identical placebo capsules containing alkalized cocoa powder, and 3 were consumed daily, providing 836mg or 0mg of total cocoa flavanols for cocoa (HFC) and placebo capsules, respectively. The capsule type to be consumed was allocated to participants at the point of entry into the trial. Compositional details for high flavanol cocoa extract and placebo were described in Chapter 2 [2.6.2 (ii)].

7.2.4 Symptoms diaries

Participants with Raynaud's symptoms were asked to complete a daily symptom diary. This diary consisted of a series of questions to document in more detail any episodes of vasospasm the participants encountered, which included date and time of the attack, symptoms experienced, possible trigger factors, duration, severity, as well as what they did to relieve the symptoms. At the end of each day, the participants were asked to complete the Raynaud's Condition Score (RCS) whether they experienced symptoms that day or not. The RCS is a validated Likert scale (1-10) used to assess the level of difficulty experienced due to Raynaud's symptoms each day (anchored from "no difficulty" to "extreme difficulty"). This score rates how much difficulty the participants experienced with their Raynaud's symptoms considering the number and duration of attacks, the level of pain, numbness, other Raynaud's symptoms and how much the Raynaud's alone affected the use of their hands on that day. This variable enabled the participant's Raynaud's symptoms to be characterised during the intervention period and allowed analysis of any changes over time and any

differences between the 2 intervention groups to be investigated (Appendix page *x*). In addition, a space in the diary was provided to document any medical conditions or symptoms that participants had experienced using free text. The latter data allowed possible adverse effects of the cocoa to be determined and quantified. The number of symptoms, duration of pain in minutes and level of pain (which was expressed as a visual analogue scale score) were determined for each individual by calculating the mean for each month, with individual means being combined to form group data. On the other hand, the RCS results for each individual were assessed by calculating the median reported monthly for 3 months. Meanwhile, proportion of days with symptoms for each month was expressed as a percentage, obtained by calculating the days with symptoms in a month divided by days in that month and multiplied by 100. As before, individual data were combined to derive group data.

7.2.5 SF-36[TM]

The British adaptation of the SF-36 Health Survey (version 1) (Jenkinson *et al.*, 1999, Ware Jr and Gandek, 1998) was used to evaluate mental and physical health attributes of participants every month for the 3 months duration. The SF-36 [TM] consists of 33 self-administered questions which can be divided into 8 subdivisions or scales, comprising Physical Functioning, Role Physical, Role Emotional, Social Functioning, Bodily pain, Vitality, Mental Health, General Health. These 8 scales represent measures which have been reported to be relevant across different ages, diseases and medical interventions (Ware et al., 1993,2000).

All questions were included in the questionnaires that were given to study participants (Appendix page *xiv*). Responses and missing data were coded and scored, based on Quality Metric guidelines (Ware and Kosinski, 2001, Ware et al., 1993,2000). Scores were normalised with respect to the 1998 general US population data (Ware Jr and Gandek, 1998) and aggregated into Physical and Mental Health category scores. Aggregation takes into account the positive and negative effects of component scales (Ware and Kosinski, 2001). Data were explored to determine whether experiencing

Raynaud's symptoms had an effect on mental and physical health category scores and whether these altered during the supplementation period.

(i) Physical Functioning

The 10-question Physical Functioning scale determined physical functioning over a range of activities from low to high intensity (Appendix page *xix*). This scale assesses 'function' in terms of the degree of limitation or disability, with the highest score being achieved when no limitations are reported.

(ii) Role Physical

Participants were asked to determine whether during the previous 4 weeks, they had had any problems with their work or other regular activities as a result of their physical health, by answering 'yes' or 'no' to four questions. A 'Don't know' option was included to classify individuals who felt unable to give a response, rather than those who overlooked the question. The 'Don't know' response was classified as missing data.

Questions 3a to 3d consist of the Role Physical scale and define health as the absence of limitation or disability with the highest score being achieved when no limitations were reported.

(iii) Role Emotional

Questions 4a to 4c comprise of three dichotomous questions ('Yes' or 'No' responses) and were used to determine whether the individual's work or daily living activities had been limited over the previous 4 weeks as a result of any emotional problems. The health status was defined with regard to the absence of limitation or disability, with the highest score being achieved when no limitations were reported. A 'don't know' option was included as with the Role Physical scale.

(iv) Social Functioning

The 2 questions in the Social Functioning scale assessed the impact that health related issues (both physical and mental) had on social activities. The questions (Question 5 and 9j), asked participants to determine to what

extent their physical and emotional health had interfered with their normal social activities over the previous 4 weeks. The responses ranged from not at all (none of the time) or extremely (all of the time), with the highest score achieved when no limitations were reported.

(v) Bodily pain

Questions 6 and 7 assessed the intensity of any bodily pain (from 'none' to very 'severe') and the extent pain interfered with the participant's normal work and daily activities over the previous 4 weeks (from 'not at all' to 'extremely'). Health status was defined with respect to the absence of limitation or disability, with the highest score being achieved when no limitations were reported.

(vi) Vitality

The vitality scale is a subjective wellbeing item determined from the energy levels and fatigue. The 4 questions (Question 9a, 9e,9g,9i) are 'bipolar' in nature, with the maximum score demonstrating the reporting of positive or favourable states and a minimum score demonstrating negative states with respect to vitality.

(vii) Mental Health

The 5 questions comprising this scale determined the anxiety, depression, loss of behavioural or mental wellbeing, emotional control or psychological wellbeing. The questions (Questions 9b, 9c, 9d, 9f,9h) are 'bipolar' in nature, with the maximum score indicating the reporting of positive or favourable states and a minimum score indicating negative states with respect to mental wellbeing.

(viii) General Health

The participant's perception of their general health was evaluated with the single item General Health Rating question. Participants assessed their general health using a single factor in a five-category scale either 'excellent, very good, good, fair or poor' (Question 1 of the General Health section). Each category was given a score to improve the linearity of the scale. For 4 questions (Questions 10a, 10b, 10c, 10d), participants were asked to

assess to what extent the 4 statements were 'true' or 'false'. A maximum score indicated the reporting of positive or favourable states and a minimum score indicated negative states with respect to general health.

7.2.6 Assessment of food intake

Adequate assessment of food intake and changes in dietary habits plays a major role in research on health and nutrition (Gibson, 2005). The Food Frequency Questionnaire (FFQs) and the diet diary are 2 of the most common methods in dietary research (Gibson, 2005).

The diet diary is a prospective method, independent of the respondent's memory and usually covers several consecutive days (Gibson, 2005). The limitations of this method include possible changes in dietary habits, not taking into consideration the long-term variety of consumption, and simplification of menus due to significant burden on the respondents (Gibson, 2005). The size of error depends on characteristics of the respondent (e.g., underestimation of intake is more common in persons after a slimming diet) and time of examination (Gibson, 2005). Period of examination for more than seven consecutive days is not recommended as this method excessively involves the respondent and leads to poor compliance. Although the diet diary usually produces sufficiently accurate data concerning intake of food to assess macronutrients, it is known that for some micronutrients, a diet diary does not adequately capture intake (Basiotis *et al.*, 1987).

Meanwhile, FFQs can focus on dietary exposures associated with a certain disease, consumption of specific nutrients or determine different nutrients (Thompson and Subar, 2017). It is a retrospective method, based on the memory of the respondent, and most FFQs generally concern a longer period of time, e.g., one month or year, and thus represents a habitual diet (Gibson, 2005). Possible errors include inadequate assessment of the frequency and amount of consumed products or omission or addition of food (Gibson, 2005).

The important components of FFQs are the food list, the frequency of consumption in time units and the portion size consumed of each item. The food list can be newly developed specifically for a study or can be altered from an existing instrument, but in that situation, it must be adapted for the specific study population. The foods listed should be foods commonly consumed in the study population (Thompson and Subar, 2017) and reflect the food habits and routine practices in that particular group. The length of the food list can range from about 20 to 200 items and take 20-30 minutes to complete; it can be self-administered or completed during an interview. FFQs should be designed specifically for each study group and research purposes, as diet may be influenced by culture, ethnicity, economic status as well as an individual's preferences (Cade *et al.*, 2004). FFQs require validation, adjustment to the purpose of research, region, or age of respondents and constant updating of the food list (Gibson, 2005).

In the present study, the diet diary was used to assess macronutrient intake while the FFQs was designed specifically for this study to assess the level of total flavanols and epicatechin intake by the participants during the intervention.

(i)   Diet diaries

Participants were asked to recall all food intake (including snacks and drinks) in a diet diary, by using household measures to estimate portion size (Appendix page *xx*). They were asked to record over three weekdays and one weekend day at each time point. Each participant was given a diet diary in advance before the study visit and was asked to fill in the diary a week before each visit. Participants were instructed to fill in a total of 4 diaries over the study, which included 1 diary before the intervention began and 3 subsequent diaries, 1 each month during the 3 months intervention. The diaries were then analysed using nutrition analysis software (Nutritics, Eire).

To determine daily dietary intake for each participant, the intake of each variable was calculated over each 4 days of recording, then divided by 4. These variables included total energy consumed, macronutrients (protein,

fat, carbohydrate), alcohol and caffeine intake. The nutrient composition of the habitual diet was determined by expressing the mean of the daily nutrient intake collected prior to the first visit, with any change in dietary intake occurring as a result of study participation assessed by comparing with pre-intervention intake. The mean values for each visit were also compared between those taking active capsules and placebo.

(ii)   Food Frequency Questionnaires

The purpose of the FFQs developed in this study was to estimate the daily intake of total flavanols and epicatechin before as well as throughout the 3 months intervention. A list of foods with higher flavanol content was obtained from the Phenol-Explorer version 3.6 (an on-line Database of polyphenol content in foods). The list was then amended to remove the food items that were not commonly consumed by the general population in the United Kingdom. In the resultant list, a total of 164 food and beverages were listed and categorised into fruits, vegetables, nuts and seeds, alcoholic beverages, non-alcoholic beverages and miscellaneous (appendix *xxiv*). The total flavanol and epicatechin content from each food (expressed as mg/100gm of the food) was obtained from the Phenol explorer version 3.6 and supplemented with data from the United States Department of Agriculture Database for the Flavonoid Content of Selected Foods (David B. Haytowitz et al., 2018). The total flavanol content for each food (mg/100gm) was calculated by summing the amount of catechin, gallocatechin, epicatechin, epigallocatechin, theaflavin and thearubigins present in that food.

Pre-intervention (Visit 1), participants were asked to recall their intake of the listed foods over the previous 1 year. During the intervention (Visits 2, 3 and 4) participants were asked to recall when they had eaten the listed foods in the past month. In the 'portion size' column, the participants were asked to describe their portion size in terms of household measures or the weight for each food item they had consumed, if they knew this. During the study protocol, participants were asked to maintain their normal diet.

In the FFQs completed on visit 1, the frequency of taking each food was classified into daily, once/week, >once/week, once/month, 2-3 times/month, 7-11 times/year, 1-6 times/year and never (National Health and Nutrition Examination Survey Food Questionnaires). Participants were instructed to report the frequency of taking each food by placing a cross in the appropriate frequency category listed. In the FFQs completed at subsequent visits, the frequency categories were daily, once/week, >once/week, once/month, 2-3 times/month and never.

Analysis of Food Frequency Questionnaires

For each frequency category, the median of the reported range was used in subsequent calculations (Table 7.1).

Frequency reported by the participants	Estimation
1-6 times/year	3 times/year
7-11 times/year	9 times/year
2-3 times/month	2.5 times/month
once/month	once/month
>once/week	3.5 times/week
once/week	1/week
daily	daily for 365

Table 7.1: Estimation for frequency of food intake reported by participants

The weight of each food consumed over the month / year was based on the portion size that had been reported by the participant. However, if the participants did not describe a specific portion size for a food, then an estimation of amount consumed was based on the weight for a medium portion size, with this average weight (in gram) being taken from the Nutritics (Nutrition analysis software) or the 'Food Portion Sizes' (Ministry of Agriculture, Fisheries and Food, 1993) (Crawley, 1993). The total weight of food or beverages being consumed over a year (or the previous month as appropriate) was obtained by multiplying the portion size in grams with the frequency of each food intake reported by the participants. The weight

obtained was then divided by 365 (or 31 days) to get a daily weight of food or beverages consumed by the participants during the assessment period. Daily intake of total flavanol was subsequently calculated by dividing the total flavanol content of each food by 100gm, then multiplying by weight (in grams) of each food that was consumed. Similarly, the daily intake of epicatechin was calculated by dividing the total epicatechin content or each food by 100gm, then multiplying this figure by the weight of that food consumed per day. The participants' daily intake of total flavanol and epicatechin were then estimated by adding up the data from the individual foods.

7.2.7 Experimental outline

Twenty-two individuals appeared to meet the inclusion criteria and attended a screening visit (Visit 1). The volunteers were briefed concerning the nature of the study and informed consent was obtained. They were asked to complete a medical screening questionnaire (Appendix page *i)*, a Raynaud's symptom questionnaire (Appendix *viii*), the SF-36™ (health-related quality of life) questionnaire and the FFQs. Their height and weight measurements were taken and if they were suitable to participate with regards to their health and Raynaud's assessment, a 15ml blood sample for Full Blood Count, Liver Function Tests, Thyroid Function Tests, urea and electrolytes, antinuclear antibodies, extractable nuclear antigen, erythrocyte sedimentation rate and random blood sugar assessment was taken by venepuncture from a forearm vein.

PRP was confirmed by fulfilment of the following criteria;

• Periodic vasospastic attacks of pallor or cyanosis for at least 2 years

• Negative antinuclear antibody test

• Normal erythrocyte sedimentation rate

• Absence of pitting scars or ulcers of the skin, or gangrene in the affected digits.

If the individual was suitable to participate, they were asked to complete a 4-day diet diary and attend the laboratory on a convenient morning before the start of the intervention. This visit occurred in the winter months (Nov/Dec/Jan) and in women, it was carried out in the first 10 days after the start of menses (follicular phase).

Participants were asked to fast between 8 to 12hrs and to have the same meal the evening before each study visit. They were given similar instructions as in the pilot study described in Chapter 3 [3.1.2 (iii)]. Immediately on arrival, they were asked to empty the bladder before lying semi-supine on a hospital bed. The core and skin temperatures were recorded. After resting semi supine for 5 minutes, a baseline BP using an arm cuff was measured. The Finometer, was then attached to the left middle finger and a laser Doppler probe was attached to the dorsum of both index fingers. Once the finger skin temperature had remained stable for 6 minutes in RT, recordings of baseline Finometer, Laser Doppler, tympanic and skin temperature measurements were made. Recordings were repeated when the hand was in the cold box. After recording, the hand was removed from the chamber, and allowed to equilibrate at RT. The time taken for the skin temperature to reach stability was recorded. Finally, an assessment of Finometer, Laser Doppler and tympanic temperature variables was carried out. All equipment was then removed (Figure 6.2).

Participants were provided with a drink and snack. They were then asked to complete the SF-36™ (to obtain a second baseline assessment), and their weight was then measured. Participants were given sufficient capsules to provide 3 capsules per day for a month (the capsule code being allocated at randomisation), a diet diary to complete in the week before the next study visit and a daily symptom diary to complete.

Weekly telephone or eMail contact with the study participants was maintained over the dosing period to improve compliance and identify any problems. The study protocol is illustrated in Figure 7.1:
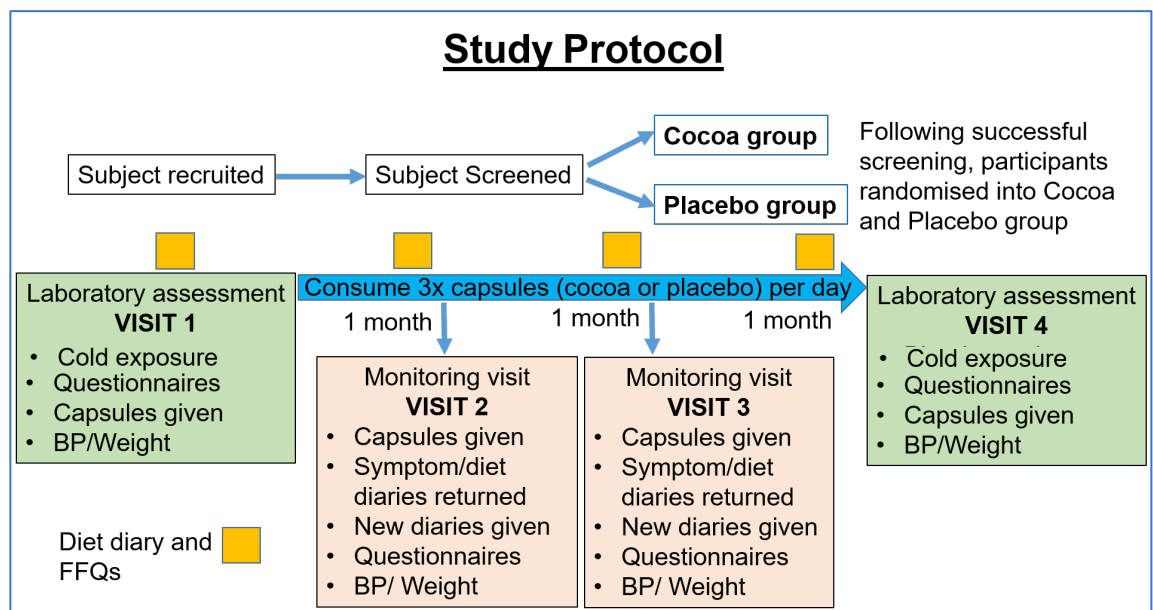
Figure 7.1: Study protocol for all the visits
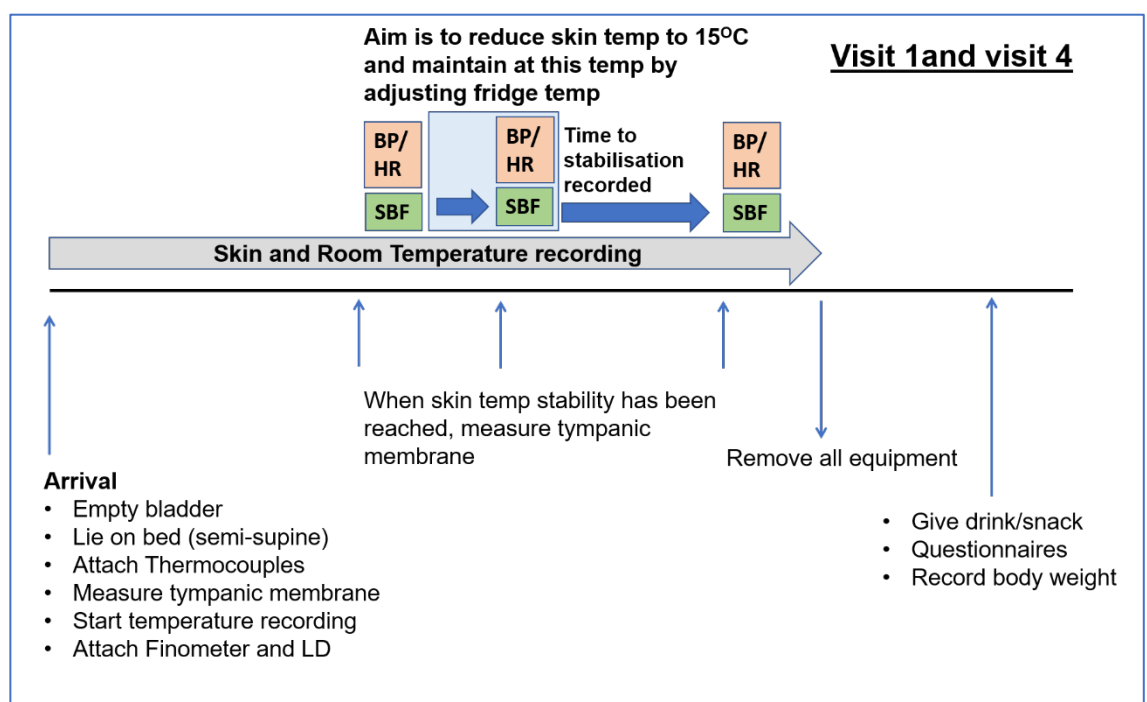
Figure 7.2: Protocol schematic for study days.

Participants came into the laboratory at the end of months 1 and 2 to collect their next month's supply of capsules and to return their diary data (Visits 2 and 3). They were also given a FFQs via email to complete a day before each visit. Their body weight was recorded and a BP measurement was made after resting for 5 minutes. The SF-36™ was also administered. On the final visit (Visit 4), participants were asked to return their diary data and

complete the FFQs and SF-36™ as before, and the protocol described for visit 1 was repeated. Any adverse events reported at laboratory visits or weekly contact was documented. In addition, a short feedback questionnaire was provided at visit 4 to be returned anonymously at a later date. This questionnaire explored the participants' subjective views regarding the acceptability of the intervention and whether they felt that their health had been affected (either positively or negatively) over the course of the study (appendix *xi*).

The total supplementation period was 3 months over the winter months between December 2018 to April 2019, with dosing completed between March and April 2019. During the intervention period, average daily air temperatures for the Nottingham area were acquired from the UK Meteorological Office (Watnall Weather Station, Nottinghamshire).

7.2.8 Statistical analyses

The data were analysed using the SPSS (version 23, 2015). All numerical data were checked for normal distribution and homogenous variance. The pre-capsule consumption values for cardiovascular parameters (SBP, DBP, TPR, CO, HR), core temperature, skin BF and skin temperature of the experimental finger and control finger, changes in the skin temperature of the experimental finger moving in between the RT and a cold box, were compared with data recorded 3 months after capsules ingestion within and between groups, using Two-Way ANOVA. The data from Raynaud's symptoms diary (number of symptoms reported, RCS, proportion of days with symptoms monthly (%), duration of symptoms, level of pain), FFQs (total flavanol and epicatechin intake), diet diary (energy, protein, total fat, carbohydrate, sugars, alcohol and caffeine) and SF-36™ (physical and mental well-being) collected during all visits were compared within groups (using One-Way ANOVA for normally distributed data or Friedman's test for not normally distributed data) and between groups (using Two-Way ANOVA). For data collected from diet diary, FFQs and SF-36 ™, the differences between groups before intervention were compared using

independent *t*-test for normally distributed data and the Mann-Whitney test for not normally distributed data.

Meanwhile, the pre-capsule and post-capsule comparison within the same group for the environmental (outside) temperature, time taken to stabilise in RT and in the cold box were made using paired *t*-test for normally distributed data or Wilcoxon's signed ranks test for not normally distributed data; comparisons between the groups were analysed using independent *t*-test for normally distributed data and Mann-Whitney test for not normally distributed data. All data were presented as mean (SD) for normally distributed data or displayed as the median (25th and 75th percentile) for non-parametric data. A value of *p*<0.05 was considered to be statistically significant.

7.3 Results

7.3.1 CONSORT diagram

Figure 7.3 represents the Consolidated Standards of Reporting Trials (CONSORT) diagram for the study, illustrating the number of participants recruited at the start of study and drop-out throughout the intervention to the completion of study. Thirty two out of the 420 people with Raynaud's interested in the study came for the screening. After the screening visit, 30 people met the inclusion criteria. They were then randomised into 2 groups (15 people in each group). However, there was attrition throughout the intervention period, leaving 12 and 10 participants completing the protocol in the placebo and cocoa group, respectively.
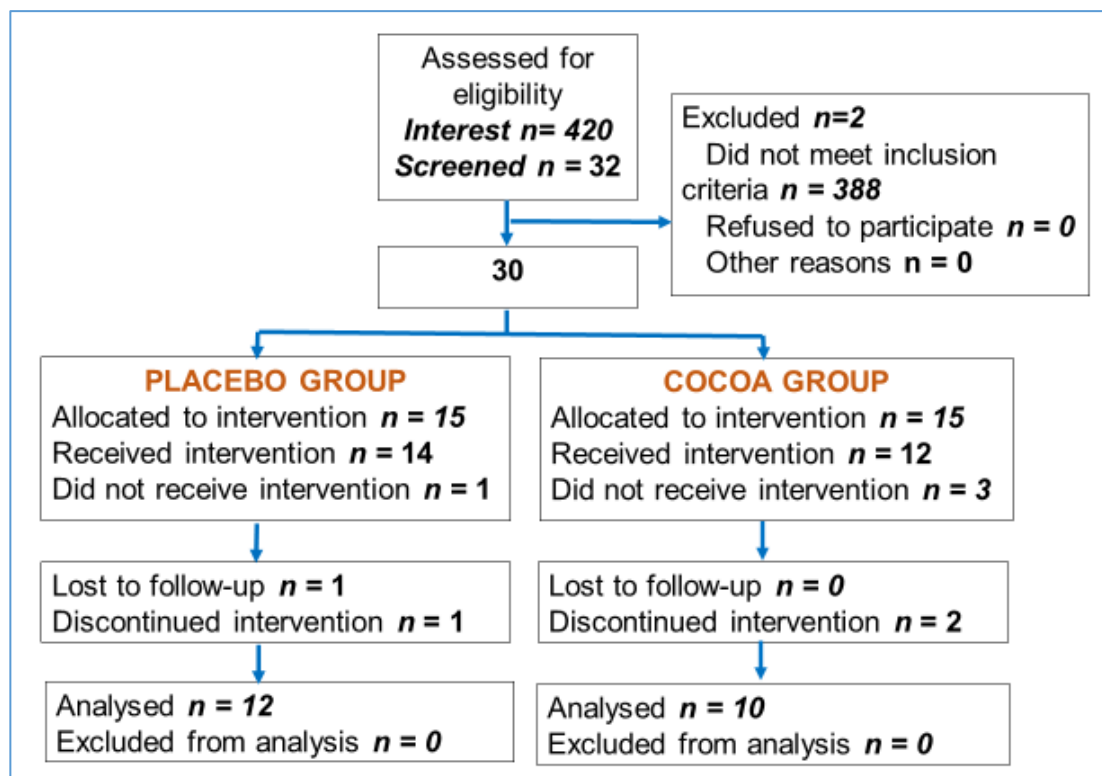
Figure 7.3: CONSORT diagram

7.3.2 Characteristics of participants

A total of twenty-two participants involving 10 participants in the cocoa group (9 females: 1 male) and 12 participants in the placebo group (11 females:1 male) completed the study. The baseline characteristics are shown in Table 7.2. The groups taking cocoa and placebo capsules were matched for age and BMI with weight and height being similar between the groups.

Characteristics	Cocoa group	Placebo group
Age	44.3 (11.8)	45.3 (13.4)
Weight (kg)	60.6 (7.6)	59.0 (8.3)
Height (m)	1.66 (0.1)	1.64 (0.1)
BMI (kg/m^2)	22.1 (2.7)	21.8 (2.3)

Table 7.2: Characteristics of participants. BMI, body mass index. Data are presented as the mean (SD). No statistical differences were observed.

7.3.3 Effects of cocoa capsules on core temperature and cardiovascular measures (SBP, DBP, CO, HR and TPR) to localised cooling

(i) Core temperature

At the start of study, before the consumption of either capsule, the response in core temperature across the study was similar between groups. In the cocoa group, core temperature throughout the assessment visit, before capsule supplementation, demonstrated a trend to be warmer compared to after the supplementation period (p=0.075). However, the core temperature within the placebo group was similar before and after the supplementation period (Figure 7.4).
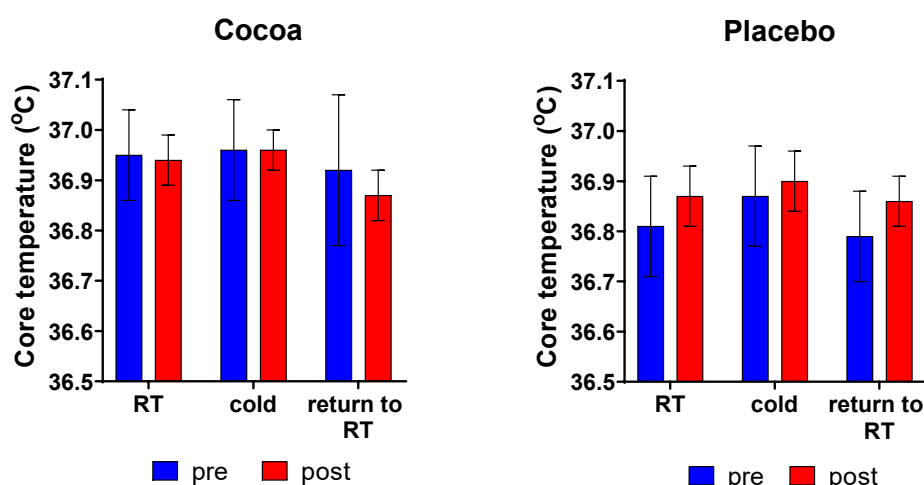
Figure 7.4: Core temperature pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.

(ii) Cardiovascular measures (SBP, DBP, CO, HR, and TPR)

At the start of study, before daily capsule consumption started, SBP, DBP, TPR, CO and HR were similar in between the groups (Figures 7.5-7.9).

In the cocoa group, after 3 months of capsule consumption (Visit 4), no significant difference was observed in SBP compared to the start of the

study (visit 1). However, DBP demonstrated a weak trend to be lower (p=0.100) and TPR decreased significantly (p<0.05) in RT on visit 4 compared to visit 1. In addition, the reduction in TPR on visit 4 was also significant when the experimental hand was placed into the cold environment (p<0.05). Meanwhile, CO within the cocoa group demonstrated a trend to be higher at each measurement timepoint during visit 4 ('post') (p=0.074), compared to before the intervention ('pre'). Moreover, the increase in CO observed on visit 4 was also significant in RT (p<0.005) and when the experimental hand was placed into the cold environment (p<0.05) compared to visit 1. The response in HR was similar throughout the intervention as well as during pre- and post- capsule consumption.

Cardiovascular parameters did not appear to be affected in the placebo group with measures being similar on visit 4 compared with visit 1.
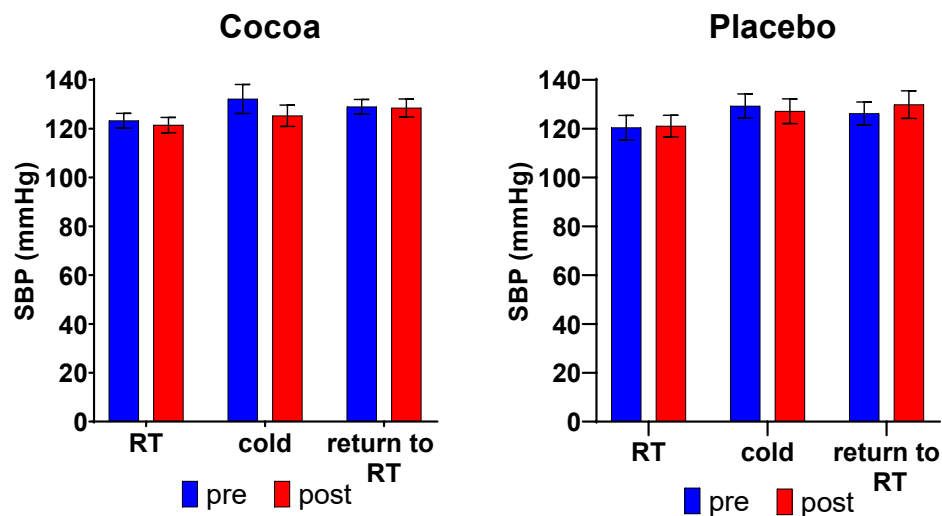
Figure 7.5: Systolic blood pressure pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.
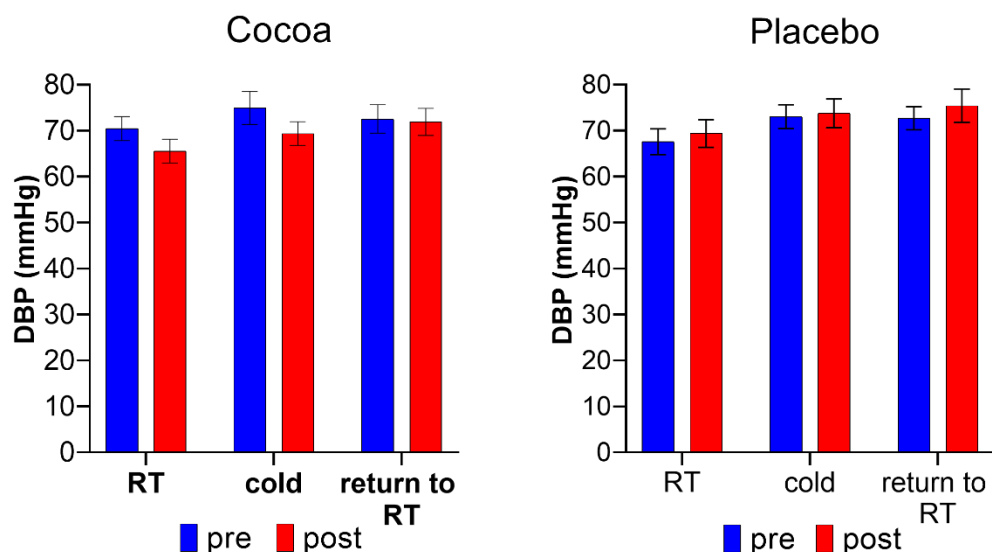
Figure 7.6: Diastolic blood pressure pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.
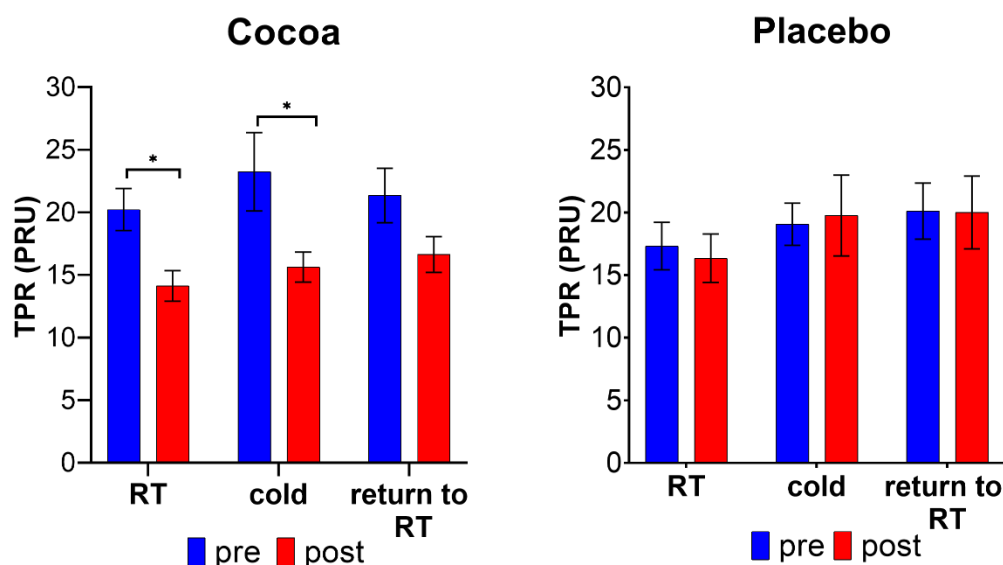
Figure 7.7: Total peripheral resistance (TPR) pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Medians with 25th and 75th percentile shown as bars *p< 0.05.
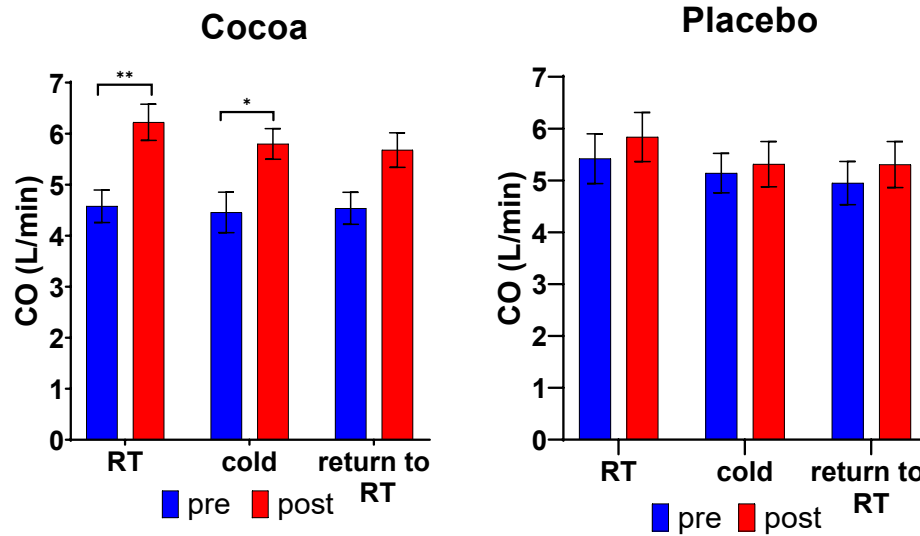
Figure 7.8: Cardiac output (CO) pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). *$p<0.05$, **$p<0.005$.
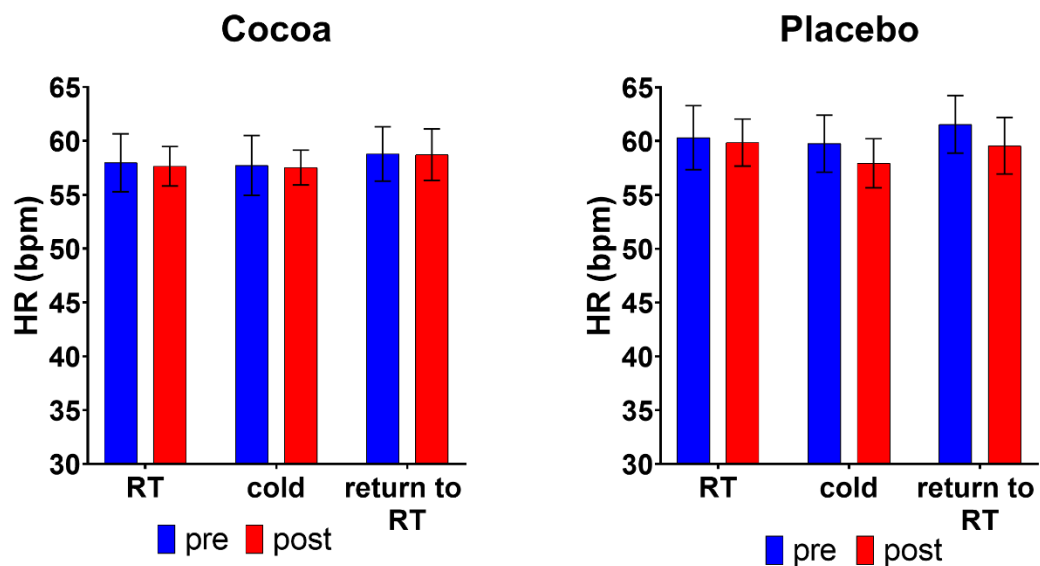
Figure 7.9: Heart rate (HR) pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.

7.3.4 Effects of cocoa capsules on the skin BF and skin temperature response of the experimental and control finger

Outside temperature

The mean outside (environmental) temperature recorded at 8am from the Nottingham (Watnall) Meteorological Office Station on visit 1 was significantly colder than on visit 4 for both groups (p<0.005). However, there was no significance difference between the groups on visit 1 or visit 4 (Figure 7.10).
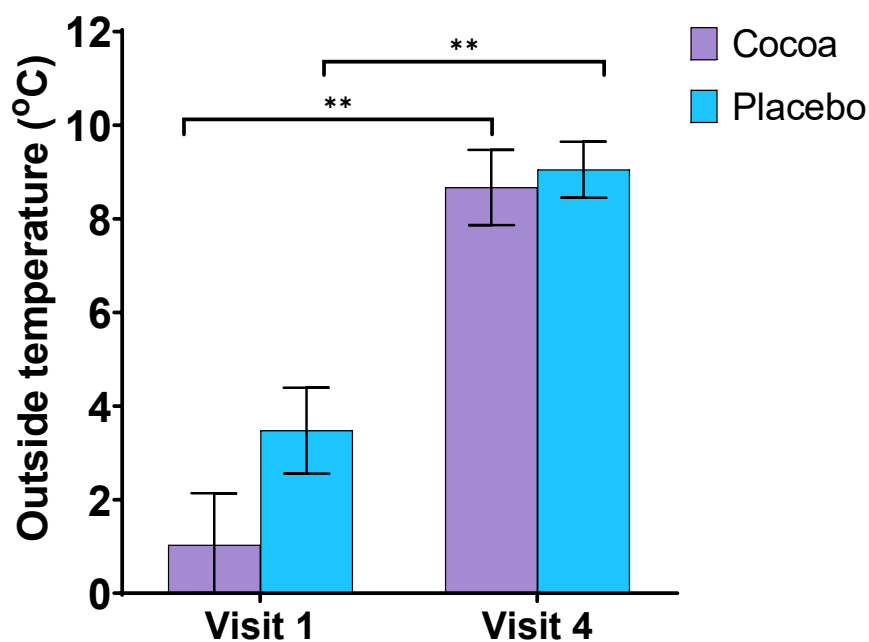
Figure 7.10: Outside temperature during visits 1 and 4 for groups cocoa (n=10) and placebo (n=12). Data are presented as the mean (SEM) **p<0.005.

(i) Skin BF and skin temperature response of the experimental finger

On visit 1, before either treatment, the responses in skin BF of the experimental finger when in room temperature (RT), placed in the cold box and returned to RT showed a weak trend to be higher (p=0.098) in the placebo group compared to control group. However, the response in skin

temperature of the experimental finger on visit 1 across measurement timepoints between groups were not different (p>0.05).

In addition, no significant differences in the response of skin BF and skin temperature of the experimental finger over measurement timepoints were observed within each group on visit 4 (after consumption of capsules) when compared with visit 1 (before consumption of capsules commenced) (Figures 7.11 and 7.12).
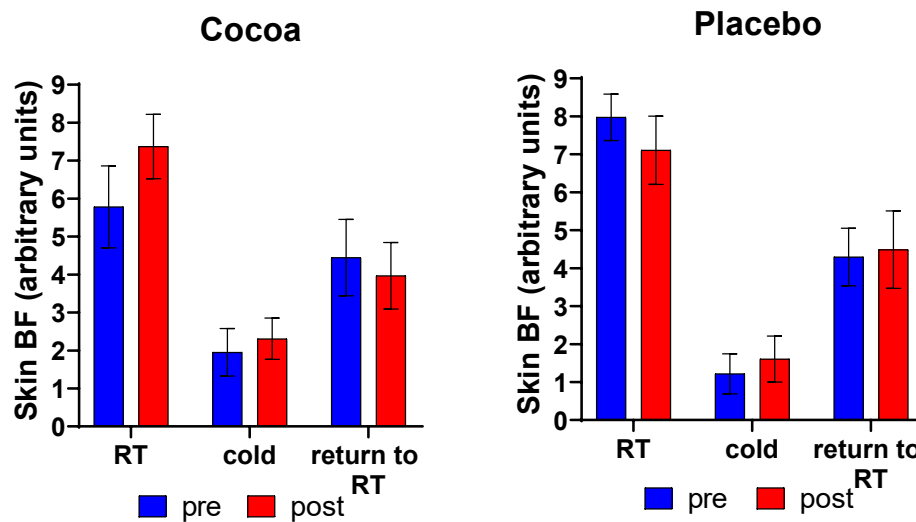
Figure 7.11: Skin blood flow of experimental finger pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.
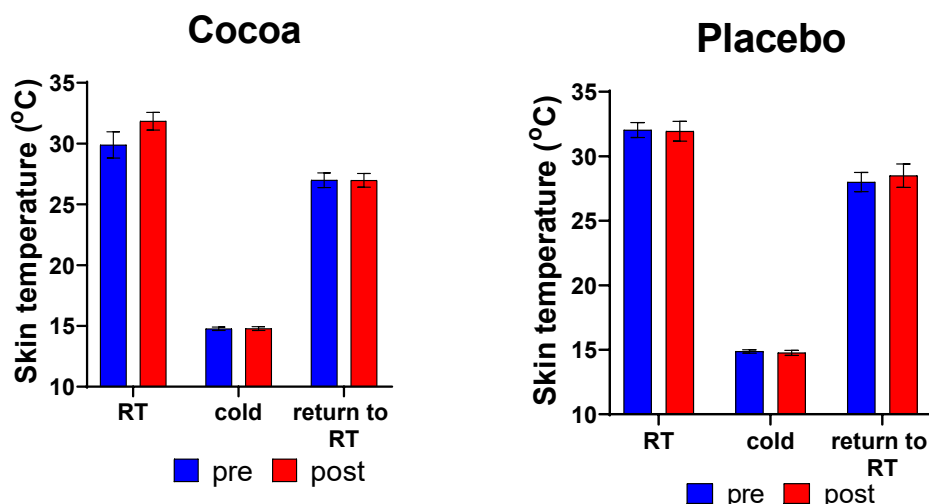
Figure 7.12: Skin temperature of experimental finger pre- (visit 1) and post-treatment (visit 4) when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT, in the cocoa (n=10) and placebo (n=12) group. Data are presented as the mean (SEM). No statistical differences were observed.

(ii) Skin BF and skin temperature response of the control finger

Figures 7.13 and 7.14 show the skin BF and skin temperature responses of the control finger, respectively. On visit 1, there were no differences in the response of the skin BF and skin temperature of the control finger across the study day between the groups. Similarly, in RT, the skin BF and skin temperature of the control finger were similar between the groups.

In the cocoa group, after 3 months of capsule consumption, the skin temperature of the control finger was significantly warmer in RT ($p<0.05$) and during localised cooling of the experimental finger ($p<0.05$) compared to before consumption (visit 1). However, no significant changes were observed in the placebo group.

The responses of skin BF and skin temperature of the control finger were similar within each group across the study before and after consumption of the capsules (Figures 7.13 and 7.14).
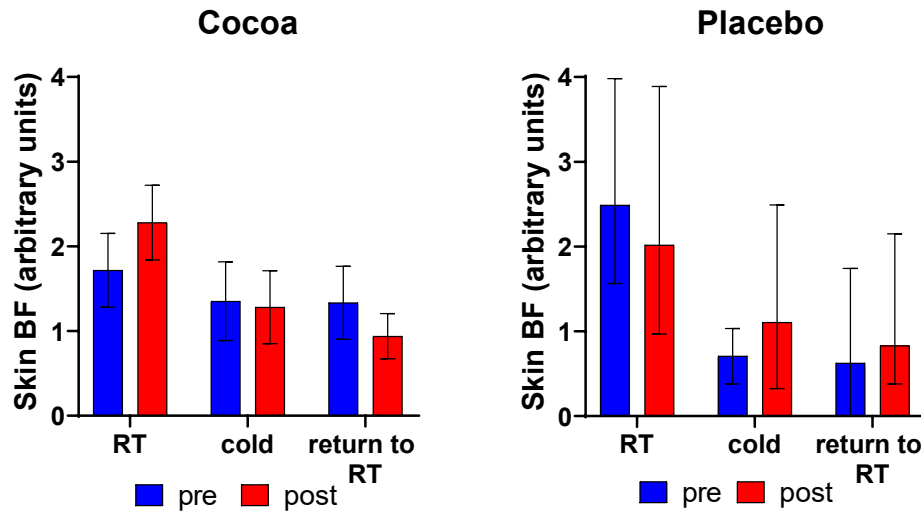
Figure 7.13: Skin blood flow of the control finger pre- and post-treatment when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT. Data for cocoa group (n=10) are presented as the mean (SEM) and for the placebo group (n=12) are presented as the median (25th and 75th percentile). No statistical differences were observed.
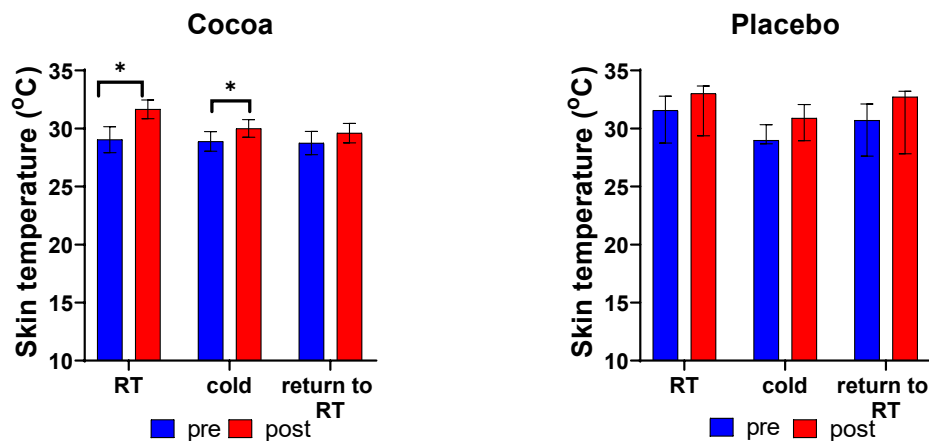
Figure 7.14: Skin temperature of the control finger pre- and post-treatment when the experimental hand was in room temperature (RT), placed in a cold box and returned to RT. Data for the cocoa group (n=10) are presented as the mean (SEM) and for the placebo group (n=12)   are   presented   as the median (25th and 75th percentile). *p<0.05.

(iii) Skin BF and skin temperature response of the experimental finger vs control finger

Before and after the intervention period in both groups, during cooling of the experimental finger, the skin BF of the same finger was significantly lower compared to the control finger ($p < 0.05$). Meanwhile, the response throughout the study day between skin BF of the experimental and control finger within group were similar on visits 1 and 4. Table 7.3 shows the Laser Doppler measurements obtained from both fingers, standardised by expressing values as the % of the value seen on visit 1 when in RT.

On the other hand, the skin temperature responses throughout the study visit before and after the 3-month period of daily consumption of either capsule (within the group) were significantly colder in the experimental finger compared to the control finger ($p < 0.005$; for both groups). Similarly, during cooling of the experimental finger at pre and post capsule supplementation period, the skin temperature of the same finger was significantly colder compared to the control finger ($p < 0.005$; for both groups) (Table 7.3).

	Cocoa group (n=10)		Placebo group (n=12)	
	Experimental finger	Control finger	Experimental finger	Control finger
Skin BF				
(% of pre-capsule, RT value)				
visit 1 RT	100	100	100	100
visit 1 in cold box	31.2 [14.6-50.0]	61.4 [37.8-115.7]*	16.9[4.0-30.2]	39.3 [21.8-53.4]*
visit 1 return to RT	79.7 [51.4-81.3]	73.9 [50.3-83.0]	49.1 [30.4-74.3]	40.6 [13.3-91.6]
visit 4 RT	121.5 [108.5-339.7]	156.1 [70.5-891.0]	98.3 [69.4-112.7]	117.9 [46.6-167.1]
visit 4 in cold box	39.6 [17.3-65.3]	99.8 [46.1-214.5]*	21.8 [3.7-32.2]	50.3 [17.1-141.5]*
visit 4 return to RT	118.0 [38.3-169.2]	95.1 [39.8-139.6]	56.7 [28.1-89.9]	47.0 [16.5-104.1]
Skin temperature (ºC)				
visit 1 RT	29.9 ± 1.1	29.0 ± 1.1	32.0 ± 0.6	30.9 ± 0.7
visit 1 in cold box	14.8 ± 0.1	28.9 ± 0.9**	14.9 ± 0.1	29.5 ± 0.4**
visit 1 return to RT	27.0 ± 0.6	28.8 ± 1.0	28.0 ± 0.7	30.2 ± 0.8
visit 4 RT	31.8 ± 0.7	31.7 ± 0.8	31.9 ± 0.8	31.6 ± 0.9
visit 4 in cold box	14.8 ± 0.2	30.0 ± 0.8**	14.8 ± 0.2	30.4 ± 0.7**
visit 4 return to RT	27.0 ± 0.6	29.6 ± 0.8	28.5 ± 0.9	30.9 ±1.0

Table 7.3: Comparison of skin BF and skin temperature measurements between experimental and control fingers of both groups during pre- (visit 1) and post-treatment (visit 4) when in room temperature (RT), during localised cooling and returned to RT. Data for skin BF are presented as the median (25th and 75th percentile) and for skin temperature are presented as mean (SEM). * $p < 0.05$ compared with experimental finger, **$p < 0.005$ compared with experimental finger.

(iv) Changes in the skin temperature of the experimental finger moving from RT to localised cooling

Figure 7.15 shows changes in the skin temperature of the experimental finger going from RT into the cooling box before and after supplementation with the capsules. Before capsule supplementation (visit 1), there were no differences in the response to the cold between the groups. In the cocoa group, before cooling (in RT), the skin temperatures at visits 1 and 4 were similar. Once the experimental hand was placed into cold environment, there was no differences in skin temperature response to the cold before and after consumption within group.

In the placebo group, the skin temperature in RT prior to cooling was similar at visits 1 and 4**.** Similarly, within group there was no difference in the skin temperature response observed between visits 1 and 4 when the experimental hand was placed into the cold environment.
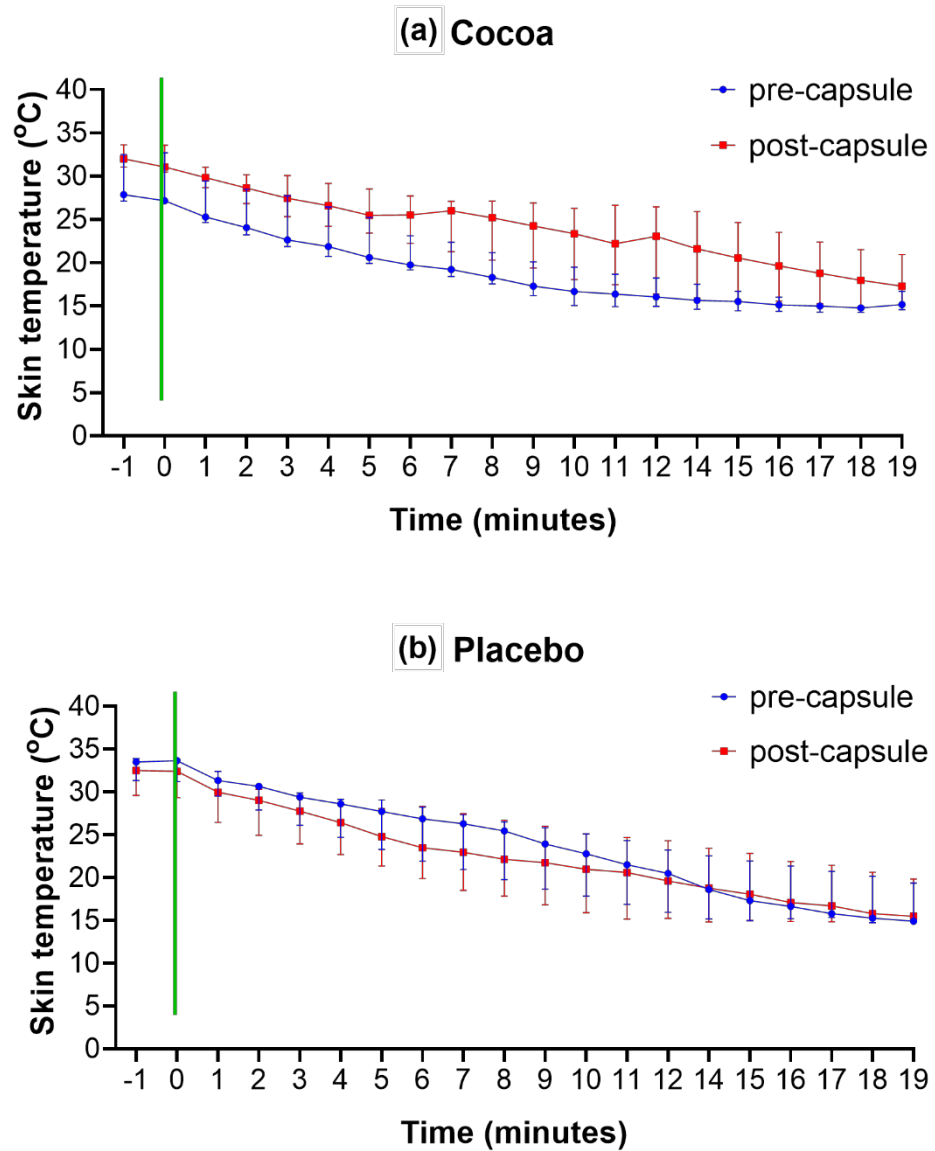
Figure 7.15: Changes in the skin temperature of the experimental finger when placed into a cold box pre- and post-treatment in the cocoa group, n=10 (a) and placebo group, n=12 (b). The vertical line indicates when the experimental hand was placed into the cold box. Medians with 25th and 75th percentile shown as bars. No statistical differences were observed.

(v) Changes in the skin temperature of the experimental finger moving from localised cooling to RT

Figure 7.16 shows changes in the skin temperature of the experimental finger when taken from the cold box into RT. Before capsule supplementation (visit 1), there was no differences in the response between the groups. In the group consuming cocoa capsules for 3 months, the skin temperature of the experimental finger during localised cooling, was similar before and after the consumption period. Moreover, once the experimental hand was removed from the cold environment, changes to the skin temperature were similar on visits 1 and 4 within the group.

Similarly, in the group consuming placebo capsules, when the experimental hand was in the cold environment, the skin temperature of this finger was similar on the 2 assessment visits, and once the experimental hand was removed from the cold environment, there were no differences in the skin temperature responses between visits within the group.
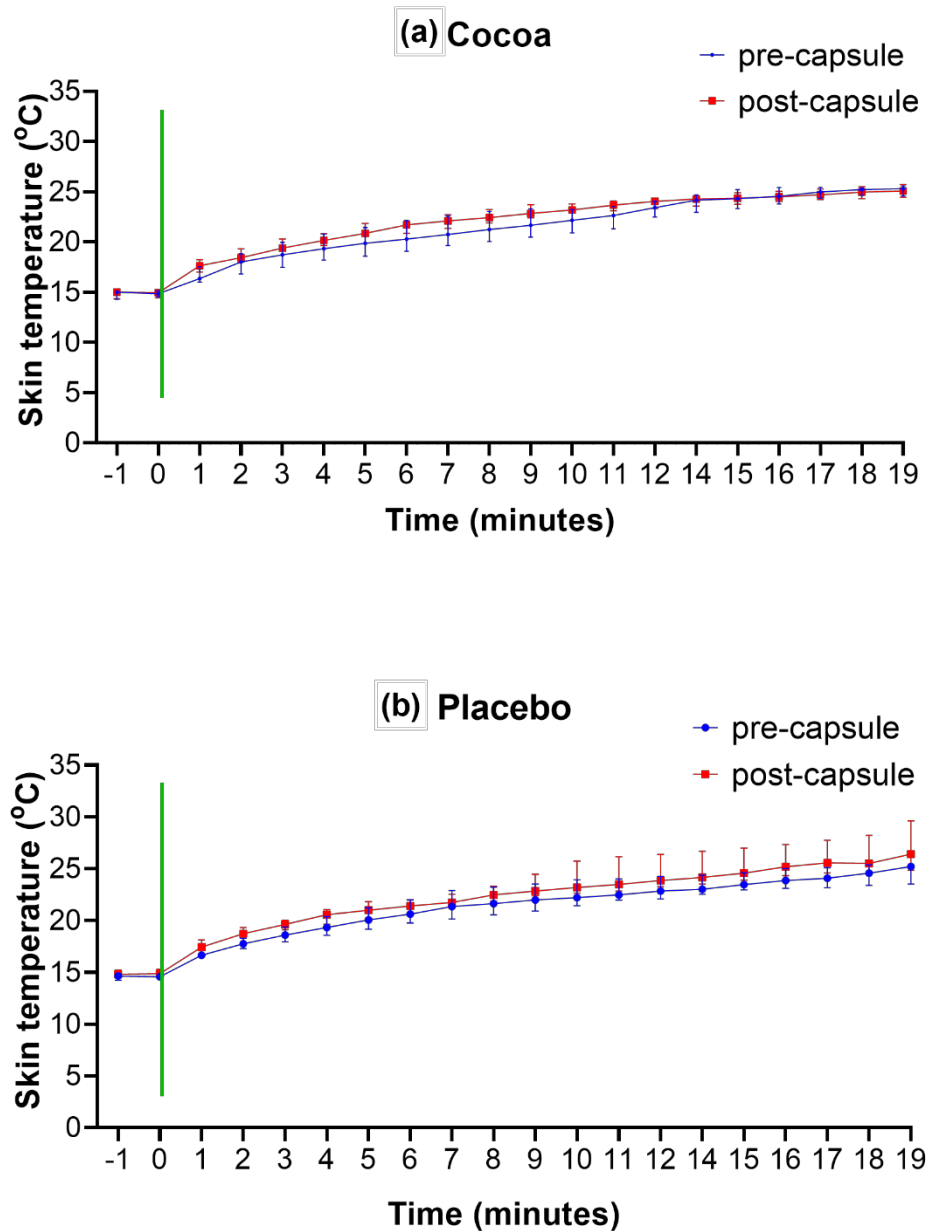
Figure 7.16: Changes in the skin temperature of the experimental finger when moved from a cold box into RT, pre- and post-treatment in cocoa group, n=10 (a) and placebo group, n=12 (b). The vertical line indicates when the experimental hand was placed into RT. Medians with 25th and 75th percentile are shown as bars. No statistical differences were observed.

7.3.5 Effects of cocoa capsules on the time taken for skin temperature of experimental finger to stabilise to localised cooling and at RT after localised cooling

Figure 7.17 reports the time taken for the skin temperature of the experimental finger to stabilise during localised cooling and when returned to RT after localised cooling. On visit 1, there were no differences between the groups in the time taken for skin temperature to stabilise when exposed to localised cooling or when returned to RT. Similarly, no statistical differences were observed in the time taken to stabilise during localised cooling, and when returned to RT after localised cooling, before and after the capsule supplementation period, within each group.
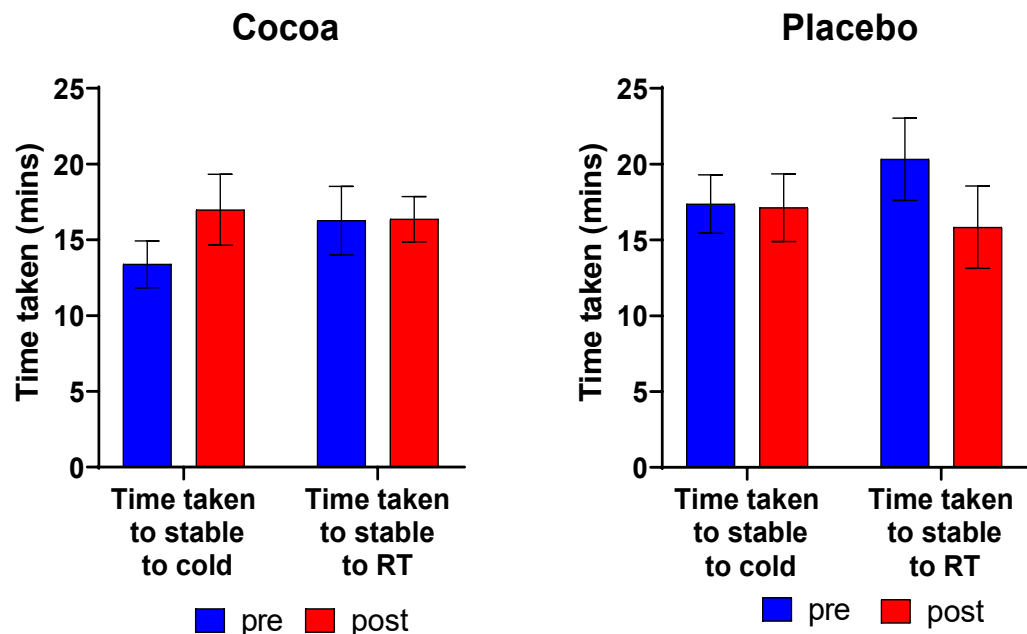
Figure 7.17: Time taken for the skin temperature of the experimental finger to stabilise when placed in a cold box and when returned back into room temperature (RT), in the cocoa group (n=10) and placebo group (n=12). Data are presented as the mean (SEM). No statistical differences were observed.

7.3.6 Effects of cocoa capsules on Raynaud's symptoms (number of symptoms reported, Raynaud's Condition Score, proportion of days with symptoms monthly (%), duration of symptoms and level of pain)

Trigger factors for Raynaud's symptoms

Over the 3 month intervention, 1021 symptomatic events were recorded in 22 participants. The most commonly reported self-perceived trigger for Raynaud's symptoms in this cohort was cold exposure (65%). This trigger was further classified into exposure to cold air/environment or cold physical touch. Examples of the cold physical touch was handling food or drink from the fridge or touching cold metal. Changes in temperature was the next highest reported trigger and this category was described as occurring when they moved either from cold to warm, or warm to cold environment, or during cooling down after exercise. The third most commonly reported trigger was being sedentary, which included sitting in public transport or in the office, reading or writing. Other triggers included 'Gripping' (3%), e.g. when lifting heavy bags, and vibration (1%) e.g. when using a hair dryer or during hoovering (Figure 7.18).
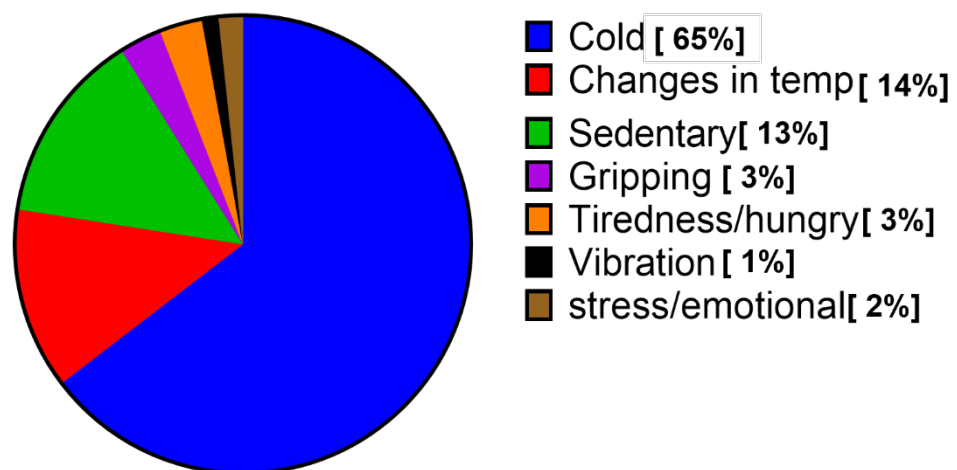
Figure 7.18: Self-reported trigger factors for Raynaud's symptoms

(i) Number of symptoms reported

Figure 7.19 shows the number of symptoms reported by participants from both the groups during the 3 month intervention. The number of symptoms across the 3 month study was similar within and between the groups.
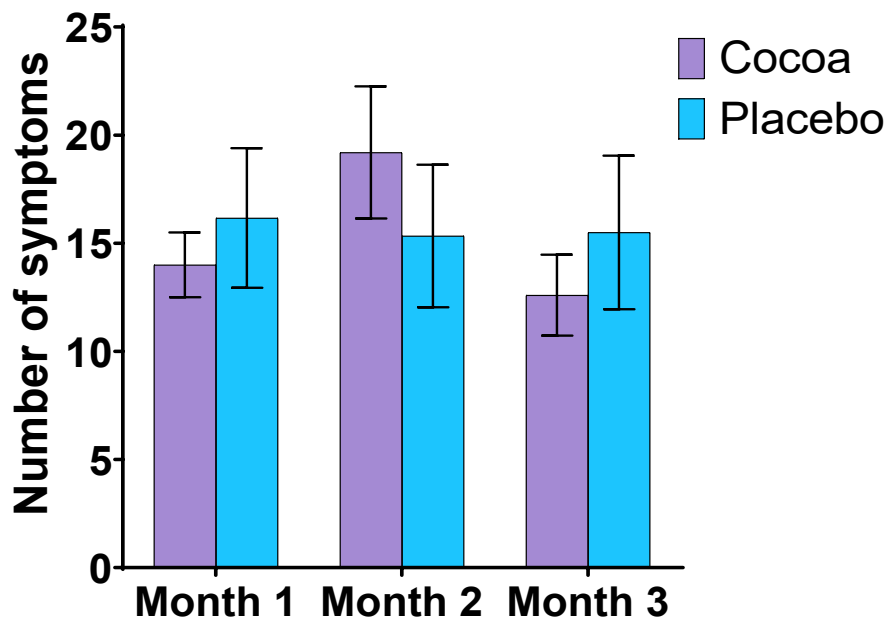
Figure 7.19: Number of symptoms reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Data are presented as the mean (SEM). No statistical differences were observed.

(ii) Raynaud's Condition Score (RCS)

Figure 7.20 shows the RCS obtained from the Likert scale in the symptom diary. The responses in RCS across the 3 months study were similar within and between the groups.
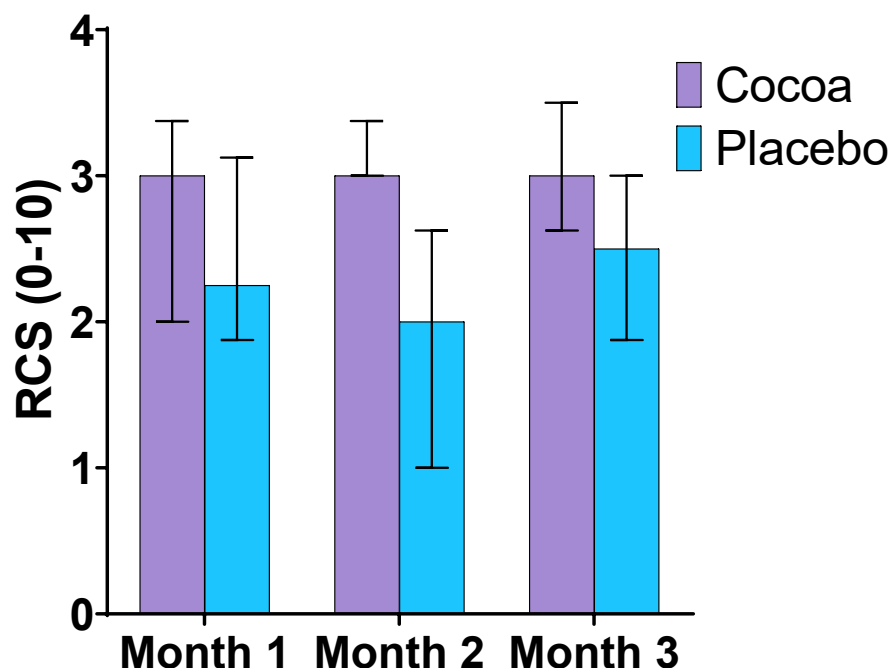
Figure 7.20: Raynaud's Condition Score reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Medians with 25th and 75th percentile are shown as bars. No statistical differences were observed.

(iii) Proportion of days with symptoms monthly (%)

The proportion of symptomatic days reported by participants each month from both the groups across the 3 months was significantly different (p<0.05). In addition, the proportion of days with symptoms reported by the participants taking cocoa capsules was significantly higher in month 2 compared to participants taking the placebo capsule (p<0.05). However, within each group there were no significant differences in the proportion of days with symptoms (Figure 7.21).
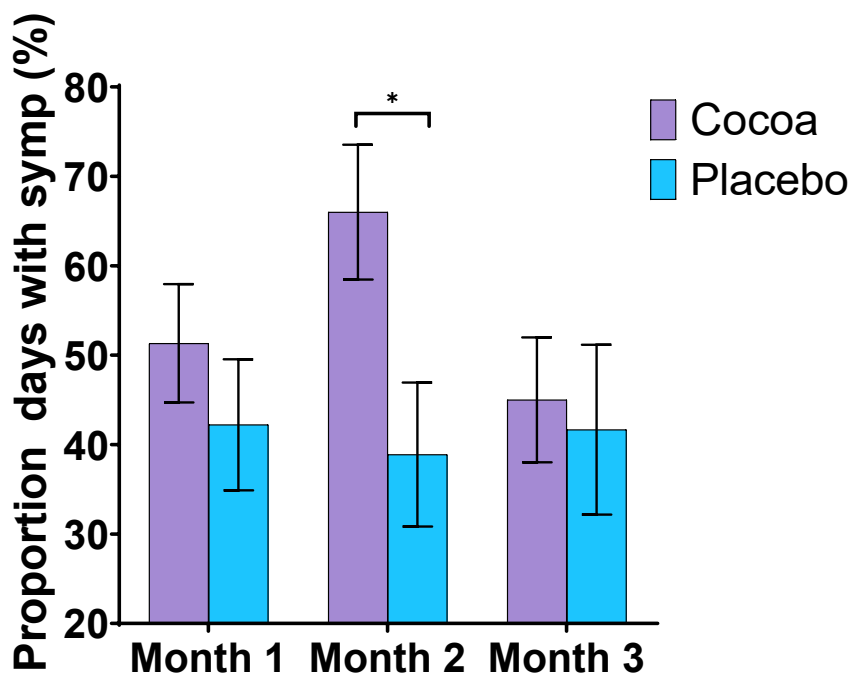
Figure 7.21: Proportion of days with symptoms reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Data are presented as the mean (SEM). *p<0.05.

(iv) Duration of symptoms

Figures 7.22 shows the percentage of symptoms with duration classified into less than 20 minutes, between 20-60 minutes and more than 60 minutes, as reported by participants from both groups. No statistical differences were observed over the 3-month intervention between or within the groups across all the classified symptom durations.
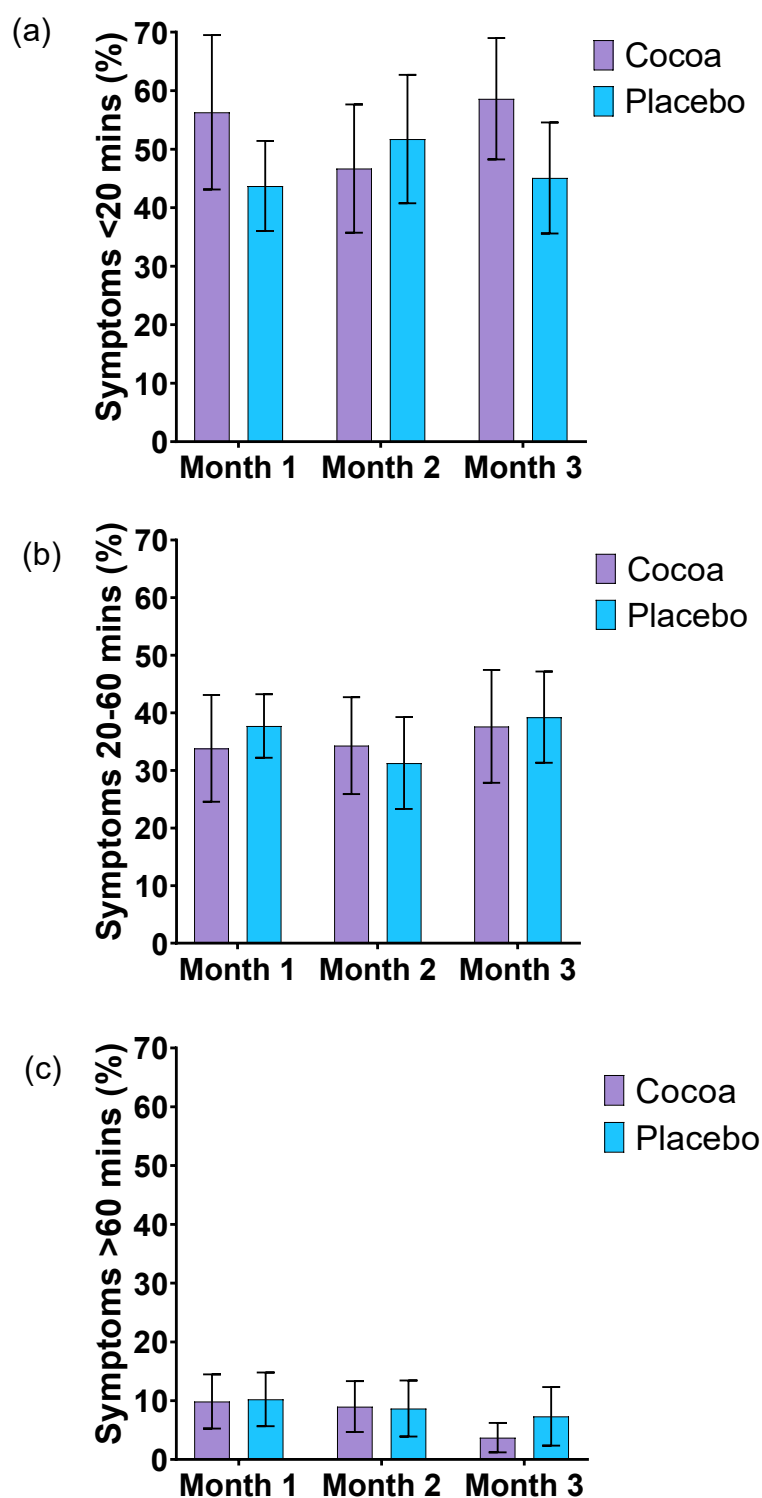
Figure 7.22: Percentage of Raynaud's symptoms having a duration of < 20 mins (a), 20-60 mins (b) and >60 mins (c) reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Data are presented as the mean (SEM). No statistical differences were observed.

(v) Level of pain

The response in the level of pain throughout the intervention between the groups was significantly different (p<0.05). In addition, the level of pain reported by the cocoa group showed a trend to be higher compared to the placebo group (p=0.070) in month 2. However, the response in the level of pain within each group was similar across the study (Figure 7.23).
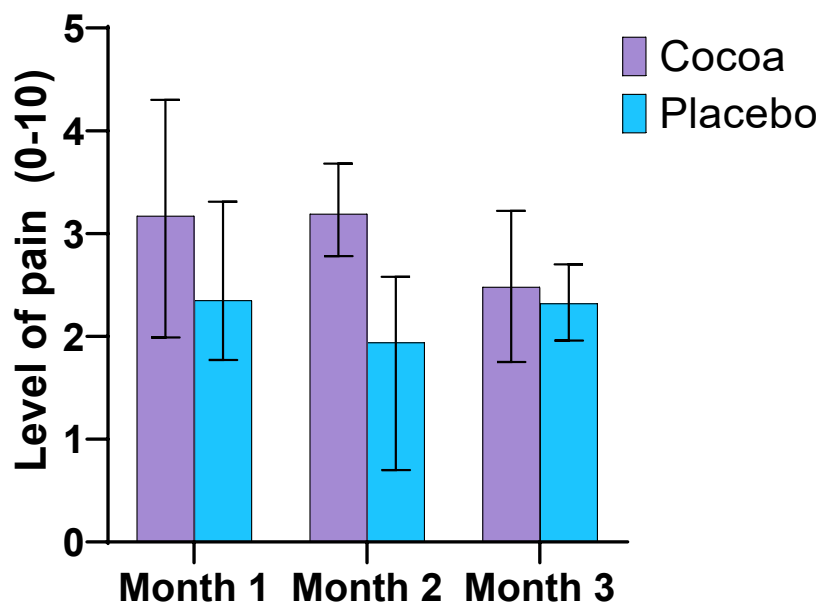
Figure 7.23: Level of pain by Visual Analog Score reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Medians with 25th and 75th percentile are shown as error bars. No statistical differences were observed.

7.3.7 Effects of cocoa capsules on the Physical and Mental Wellbeing of participants

Figures 7.24 shows the scores for the physical and mental wellbeing, respectively, obtained from the SF 36[TM] questionnaires. At pre-intervention (during screening visit and visit 1), both the groups had similar physical and mental wellbeing. In the cocoa group, the normalised score for physical and mental wellbeing during screening visit and visit 1 indicated a coefficient of

variation of 10.1% and 9.9%, respectively. Meanwhile, in the placebo group, the variability for physical and mental wellbeing were 7.2% and 9.8%, respectively. The response in score in both the physical and mental wellbeing across the intervention between and within the group was similar.

(a)

Physical Wellbeing

(b)
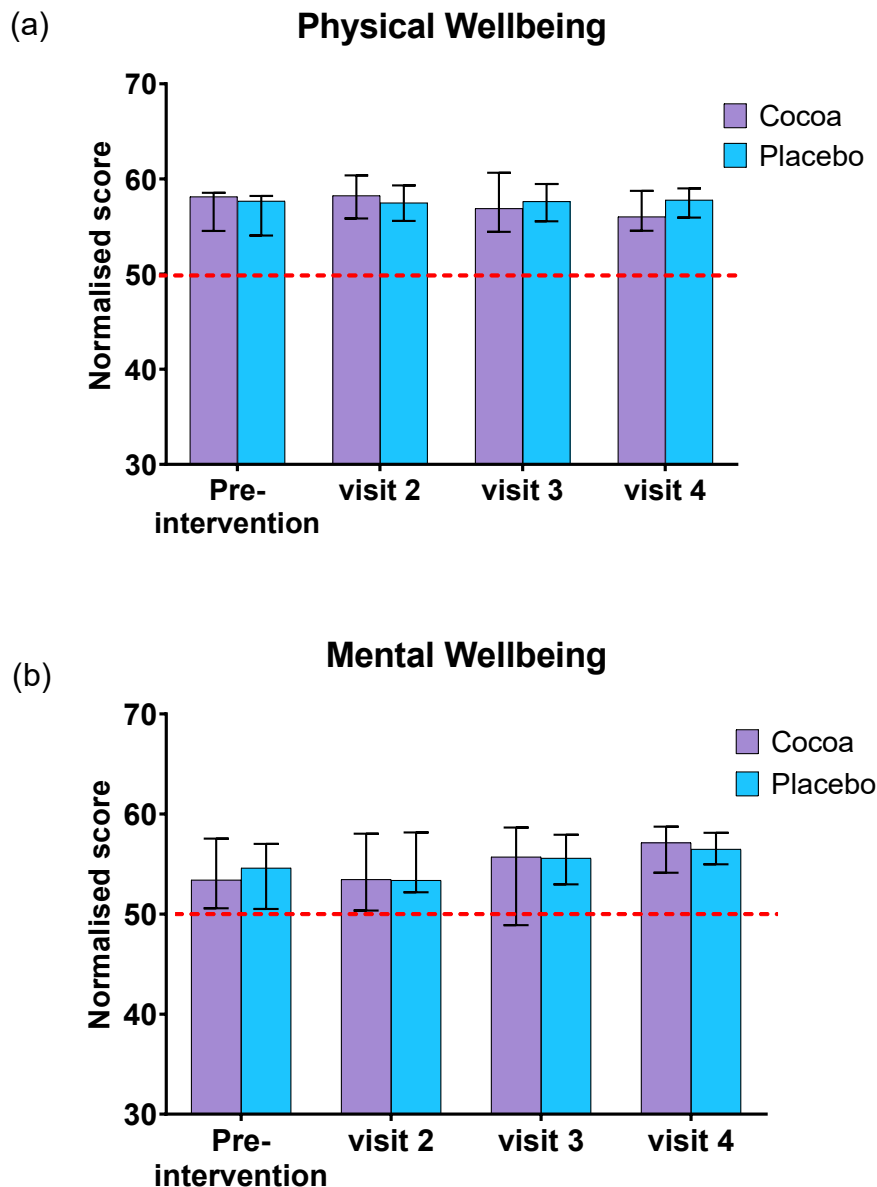
Mental Wellbeing

Figure 7.24: Aggregated SF-36[TM] Physical Wellbeing (a) and Mental Wellbeing (b) component scores reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Medians with 25th and 75th percentile are shown as bars. The dotted line indicates the average score for the 1998 general US population. No statistical differences were observed.

7.3.8 Effects of cocoa capsules on level of total flavanol and epicatechin intake in participants

Figures 7.25 and 7.26 show the average intake of total flavanol and epicatechin per month across the 3 months of supplementation. The FFQs-derived data for the total flavanol and epicatechin level were corrected for supplementation in the cocoa group by adding 836mg of total flavanols and 115mg of epicatechin daily, on top of the intake from their habitual diet.

Before the intervention, total flavanol and epicatechin intakes were similar between the groups. The response in the total flavanol and epicatechin intake across the study were also similar between and within the groups. However, the intake of total flavanol and epicatechin was significantly higher in the cocoa group compared to the placebo group at each assessment point (p≤0.005).
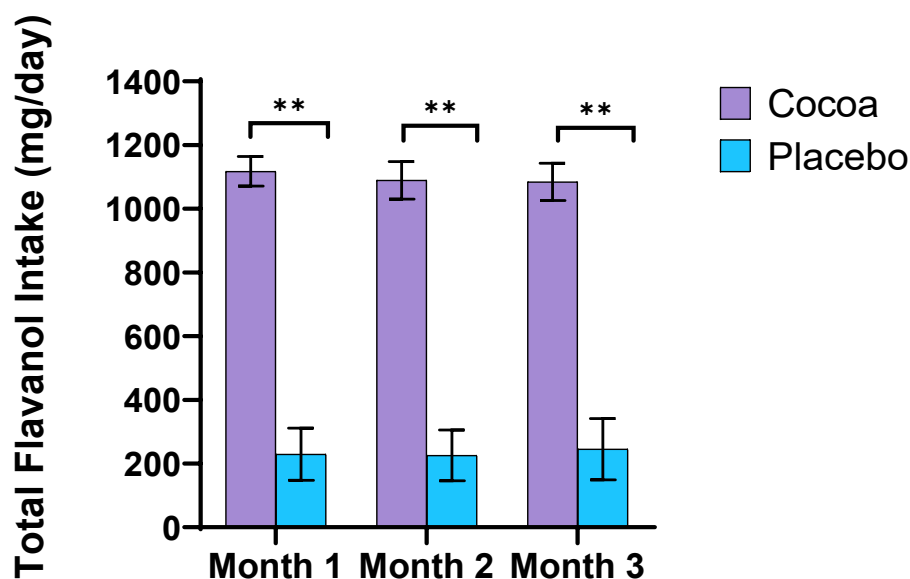
Figure 7.25: Daily calculated total flavanol intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules throughout the study. Data are presented as the mean (SEM). **p≤0.005.
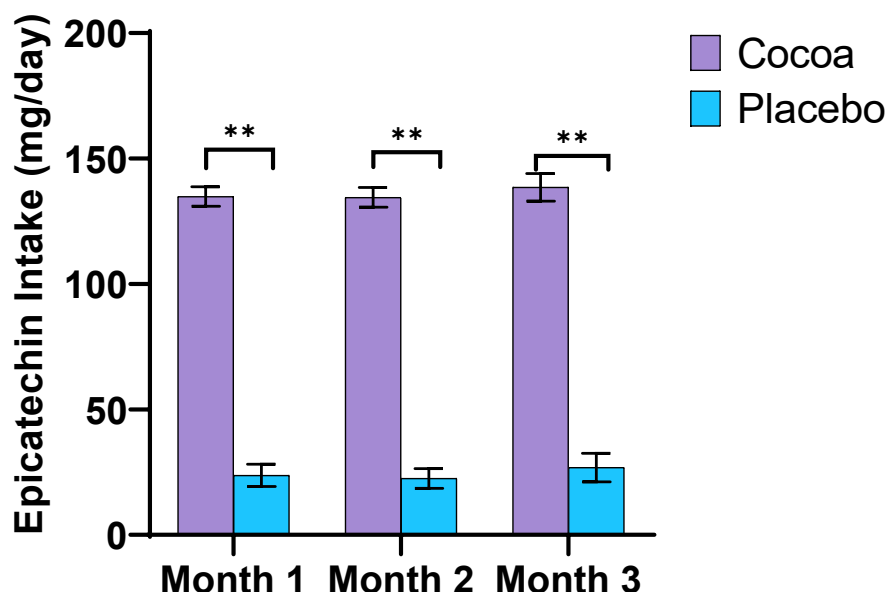
Figure 7.26: Daily calculated epicatechin intake in those taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). **p≤0.005.

7.3.9 Effects of cocoa capsules on dietary intake

All the participants completed and returned the diet diaries on each study visit. The diary returned on visit 1 was recorded before the intervention. Four days of recording monthly were obtained from 21 participants, with 1 participant recording for only 3 days during visits 3 and 4.

Before the intervention (visit 1) and over the recording period across the study, the total energy, protein, total fat, carbohydrate, sugars, alcohol and caffeine daily intake were similar between the groups. Similarly, there were no statistical differences in the total energy, protein, total fat, carbohydrate, sugars, alcohol and caffeine daily intake within and between the groups (Figures 7.27-7.33).
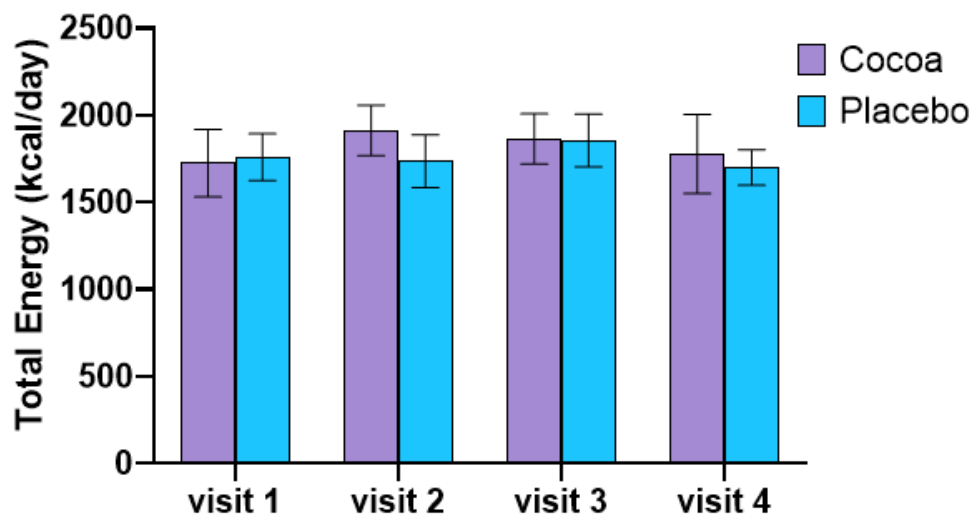
Figure 7.27: Daily total energy intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). No statistical differences were observed.
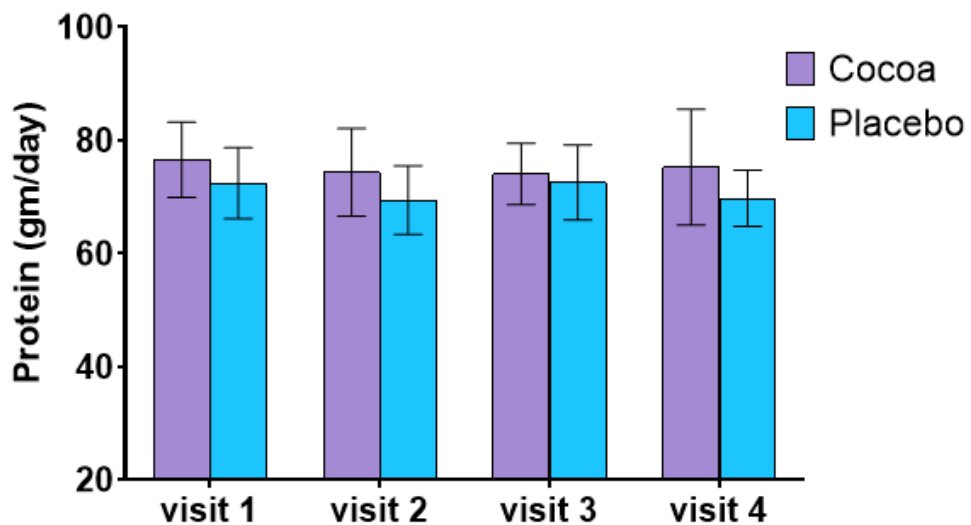
Figure 7.28: Daily protein intake reported by participants taking either cocoa or placebo capsules during the study. Data are presented as the mean (SEM). No statistical differences were observed.
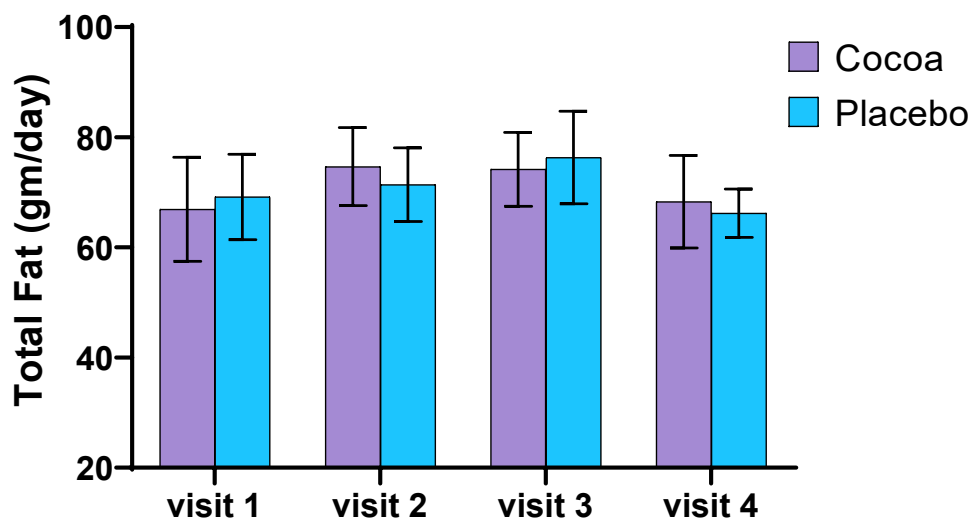
Figure 7.29: Daily total fat intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). No statistical differences were observed.
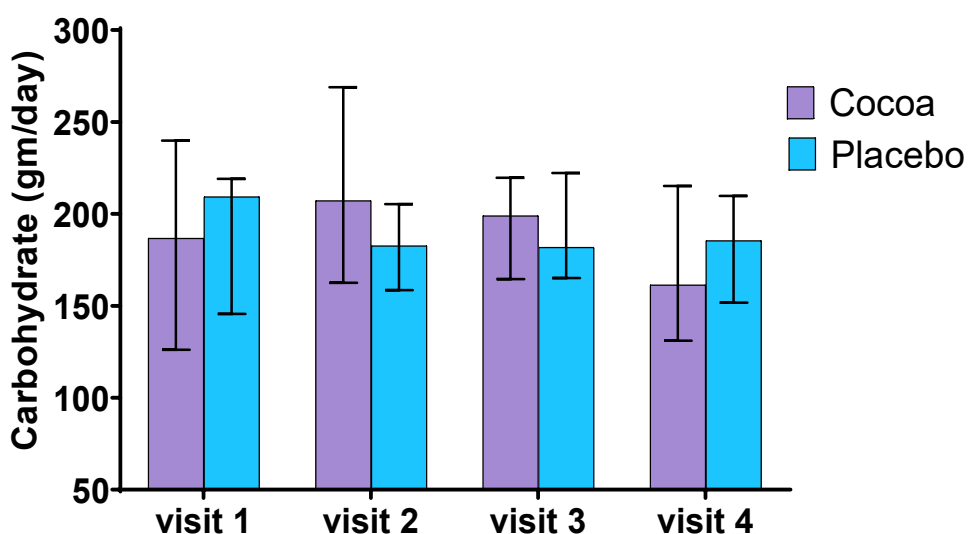
Figure 7.30: Daily carbohydrate intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the median (upper and lower bar indicate 25th and 75th percentile, respectively). No statistical differences were observed.
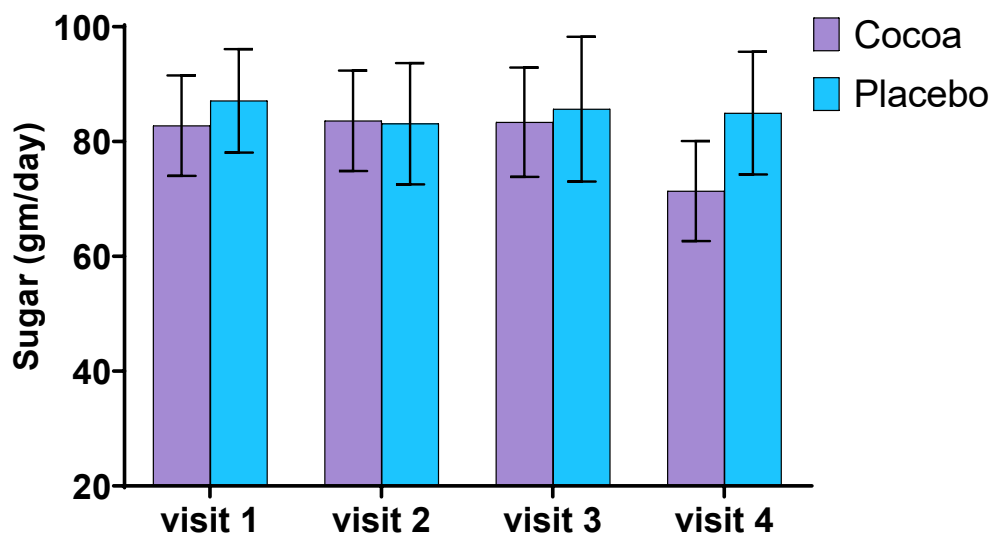
Figure 7.31: Daily sugar intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). No statistical differences observed.
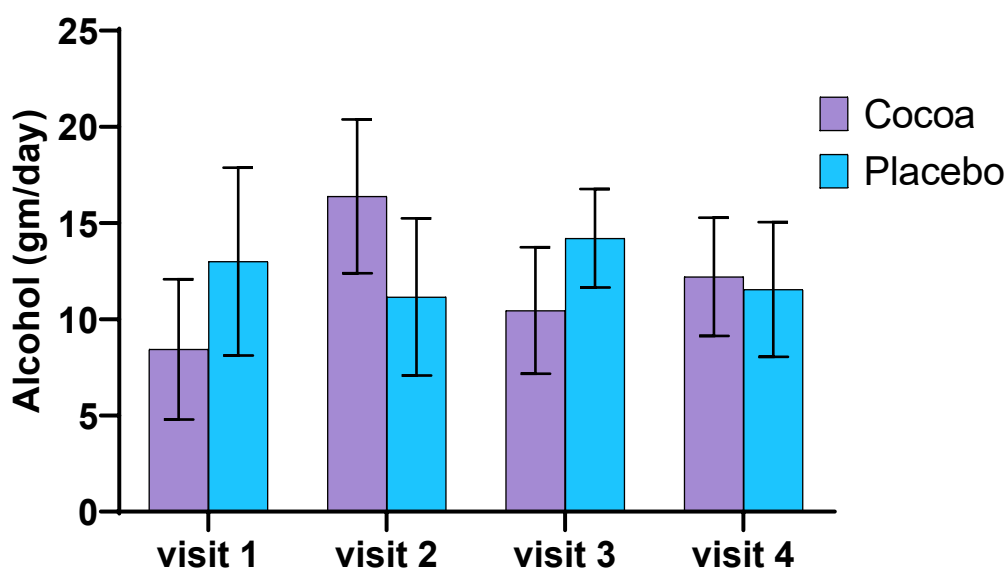
Figure 7.32: Daily alcohol intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). No statistical differences observed.
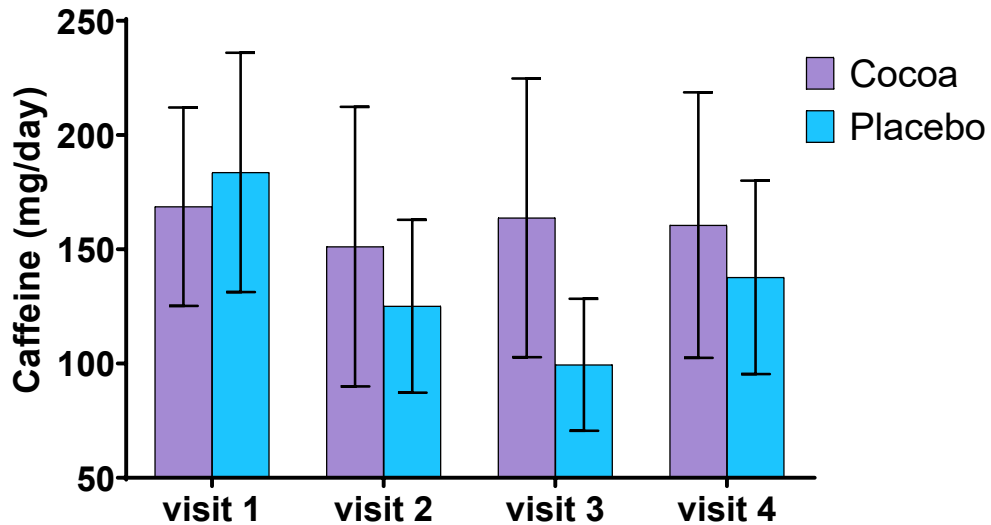
Figure 7.33: Daily caffeine intake reported by participants taking either cocoa (n=10) or placebo (n=12) capsules during the study. Data are presented as the mean (SEM). No statistical differences observed.

7.4 Discussion

The present study demonstrated that 3 months of supplementation with cocoa capsules containing 836mg total flavanols (115mg epicatechin) caused a significant reduction in the TPR as well as a significant increase in the CO and skin temperature when resting in RT. However, no changes in the skin BF or Raynaud's symptoms were observed.

The duration of 3 months was chosen based on a previous controlled randomised, double-blind study which demonstrated that 3 months of supplementation with HFC (902mg total flavanols) compared with LFC (36 mg total flavanols) among overweight and obese adults significantly increased FMD acutely (2hrs post dose) and chronically over 12 weeks, reduced DBP and mean arterial BP, suggesting that HFC improves endothelial function (Davison *et al.*, 2008).

Effect of capsules on core temperature and cardiovascular measurements (SBP, DBP, CO, HR and TPR)

(i) Core temperature

In the present study, it was found that the core temperature was not affected by supplementation with either of the capsules, although the core temperature on visit 1 (before cocoa capsule consumption) demonstrated a trend to be warmer compared to visit 4 (post consumption). The stable core temperature suggests that the body was able to maintain a constant internal temperature despite the localised cold stimulus.

(ii) Cardiovascular measurements (SBP, DBP, CO, HR, and TPR)

It was demonstrated that 3 months consumption of cocoa capsules caused a significant reduction in the TPR and a significant increase in CO in RT and during localised cooling. In addition, DBP showed a trend to be reduced after stabilising in RT. Reduction in the TPR and DBP after 3 months consumption of cocoa capsules suggests that flavanol-induced peripheral vasodilatation was occurring, and the increase in CO is consistent with a compensation mechanism in healthy individuals in order to maintain SBP. The participants in the present study represent a healthy group (no history of cardiovascular diseases), although it is assumed that they were suffering from microvascular dysfunction. In a previous pilot study conducted among 24 patients with chronic heart failure, participants were randomised to consume 50gm daily of high-flavanol dark chocolate (1064mg of flavanols/day) or low-flavanol dark chocolate (88mg of flavanols/day) for 4 weeks and then crossed over to consume the alternative dark chocolate for a further 4 weeks. The results demonstrated a significant decrease in DBP indicating a decrease in peripheral vascular resistance with a consequent reduction in cardiac afterload and therefore, suggesting a potential improvement in cardiac function after consumption of flavanols (De Palma *et al.,* 2016).

In the present study, it is proposed that daily supplementation with cocoa capsules improved the compliance of resistance vessels evident by a reduction in the TPR and DBP. However, these beneficial effects were not associated with an increase in microcirculatory perfusion. As the present study did not find any microcirculatory effects in the fingers, it suggests that the predominant mechanisms controlling blood flow in the microvasculature are not impacted by regular cocoa consumption. However, Heiss *et al*., (2015) found that cocoa flavanol intake improved the endothelial function in large conduit arteries as well as increases the conductance of small arteries and in microcirculation perfusion. As resistance to blood flow is principally mediated by the arteriolar diameter, which was modified by arteriolar vasoconstriction and dilation, respectively, in that study, it was found that the maximal forearm blood flow and cutaneous perfusion during reactive hyperaemia were significantly increased. These findings suggest that cocoa flavanol intake might increase not only the dilatory function of conduit arteries but also that of the arterioles. It was also found that the red blood cell (RBC) deformability was increased in that study. These factors contribute to the increase in the microcirculation perfusion observed following cocoa flavanol intake (Heiss *et al.,* 2015). Mechanistically, cocoa flavanol-related vascular effects have been linked to an increase in eNOS activity (Del Rio *et al.,* 2013). RBCs have been reported to express a functional eNOS that may modulate RBC deformability independent of vascular tone (Cortese-Krott *et al*., 2012, Horn *et al*., 2011). Therefore, the work by Heiss *et al*., (2015) supports the hypothesis that cocoa flavanol intake improves tissue perfusion by increasing RBC deformability and increasing dilator capacity of the arterioles. Similarly, another study also found that the ingestion of flavanol-rich cocoa was associated with an enhanced FMD response of conduit arteries, acute elevations in levels of circulating NO and an augmented microcirculation (Schroeter *et al.,* 2006).

In addition, the assumption of the increase in plasma NO following 3 months consumption of cocoa capsules might be inhibited from having an effect on the microvasculature by free radicals, which are increased in patients with PRP (Lau *et al.,* 1992). The pathophysiology of vasospasm in

PRP has been related to increased sympathetic nervous activity and local factors, such as increased sensitivity to α-adrenoceptors and 5-hydroxytryptamine receptors (Coffman, 1990), as well as increases in plasma endothelin levels. These factors might inhibit the activity of NO and vasodilator function and may explain why cocoa did not improve microcirculation in this patient group.

Effect of capsules on the skin BF and skin temperature responses of the experimental and control finger

(i) Outside temperature

The mean outside temperature recorded from the Nottingham Meteorological Office Station on visit 1 was significantly colder than on visit 4 for both groups. These findings were expected as Visit 1 (pre-intervention) was conducted between November and December and visit 4 (post-intervention) was conducted between March and April.

(ii) Skin BF and skin temperature response of the experimental finger

In the present study, consumption of cocoa capsules for 3 months did not appear to confer any effect on the skin temperature and skin BF of the experimental finger. These findings could be due to either the cocoa capsules not having a chronic effect on the skin temperature and skin BF in the Raynaud's microvasculature, or the study might have been underpowered to be able to detect any effect. A crossover design study, in 10 healthy women, reported a significant increase in dermal blood flow 2 hrs after ingestion of HFC drink (329 mg flavanols; 61mg epicatechin), changes were not seen following LFC drink (27mg flavanol; 6.6 mg epicatechin) (Neukam *et al.,* 2007). The level of epicatechin in that study (61mg) was much lower when compared with the level of epicatechin in present study (115mg). However, that study was looking at the acute effects (at 2hrs) of cocoa flavanol ingestion among healthy women, while the present study did not assess acute effects, but looked at the skin BF /

temperature effects of chronic (3 months) ingestion among people with PRP. Measurements in the current study were taken >12hrs after the last dose of cocoa flavanols and if the beneficial effects of cocoa flavanols on vascular function are predominantly through their acute effects, then the present protocol design would have missed these effects.

(iii) Skin BF and skin temperature response of the control finger

Interestingly, in the cocoa group, after 3 months of capsule consumption, it was found that the skin temperature of the control finger was significantly warmer in RT as well as during localised cooling of the experimental hand compared to before consumption commenced, which might suggest that cocoa capsules had improved skin BF. However, an increase in skin BF of the control finger was not observed, suggesting the presence of uncoupling between skin BF and skin temperature in people with PRP, as discussed in Chapter 5.

(iv) Skin BF and skin temperature response of the experimental finger vs control finger

During cooling of the experimental finger in the cold box, before and after 3 months of taking both the capsules, the skin BF and skin temperature responses of the experimental finger were significantly lower compared to the control finger. These findings were expected as the temperature in the cold box was set at 0°C to induce vasoconstriction in the experimental hand. No significant changes were observed in the skin BF or skin temperature between the fingers before and after taking both the capsules in the RT. However, the skin temperature during study days 1 and 4 (before and after consumption of either the capsule), within the group, were significantly colder in the experimental finger compared to the control finger, which could be due to the direct exposure of the experimental finger to localised cooling during the study visit .

(v) Changes in the skin temperature of the experimental finger moving from RT to localised cooling and *vice versa*

In the present study, it was found that changes in the skin temperature of the experimental finger when moved from RT into the cold box or from the cold box into RT, were similar before and after consumption of both the capsules.

Moreover, during localised cooling, there were no significant differences in the skin temperature of the experimental finger between visits 1 and 4. These findings were expected, as in this experiment, the cold box temperature was modified to maintain the skin temperature at 15°C (as discussed in the previous study in Chapter 5). No changes in the skin temperature responses observed in either condition indicated that cocoa and placebo capsules consumption did not affect temperature regulation in people with PRP.

Effect of capsules on the time taken for skin temperature of experimental finger to stabilise to localised cooling and at RT after localised cooling

In the present study, the time taken for the skin temperature of the experimental finger to stabilise in the cold box and at RT after localised cooling was similar following consumption of cocoa and placebo capsules. These findings suggest that daily supplementation with the cocoa capsules had no effect on responses to the cold or during rewarming when compared to the placebo capsules. In a previous study (Chapter 6), when this comparison was made between the healthy group and PRP group, it was found that the PRP group took longer to stabilise during recovery in the RT compared to the healthy group. However, in the present study, the participants from both of the groups who were suffering from PRP, had a similar response between the groups indicating that the cocoa capsules were not having an effect.

Effect of capsules on Raynaud's symptoms (number of symptoms reported, RCS, proportion of days with symptoms monthly (%), duration of symptoms and level of pain)

(i) Trigger factors for Raynaud's symptoms

In the present study, 1021 symptomatic events were reported by 22 participants over the 3 month intervention. The most commonly reported self-perceived trigger for Raynaud's symptoms in this cohort was cold exposure, which supports the viewpoint of the literature. However, a wider range of triggers were reported by this cohort, suggesting that the classic definitions may be too restrictive.

(ii) Raynaud's symptoms

The present study found that Raynaud's symptoms did not improve after cocoa and placebo capsules administration. The number of symptoms and the RCS reported by the participants from both the groups were similar throughout the intervention, indicating that cocoa capsules did not help in reducing the number of symptoms and RCS. The median RCS reported was between 2-3 out of 10, which is considered to be low and might suggest that the severity of Raynaud's symptoms in both the groups was low. Therefore, an improvement in RCS might not be detected within people with mild symptoms. The mild symptoms reported in this cohort could be because of a relatively warm winter during the intervention period. Although the number of reported symptomatic events over the 3 month study was high (1021), these events might represent only mild Raynaud's events as evident by the low reported RCS.

In month 2, the proportion of days when symptoms were experienced was significantly higher and the level of pain showed a trend to be higher in the participants taking cocoa capsules compared to participants taking placebo capsules suggesting that cocoa might have worsened the symptoms. It is also possible that regular consumption of cocoa flavanols could result in a vasodilation in the microvasculature, which could increase heat loss from

the periphery and consequently make PRP individuals more likely to have symptoms triggered. However, other variables measured for Raynaud's symptoms in this study were not worsened, thus unlikely to support these findings. There was also a possibility that the participants in the cocoa group were exposed to more factors that might have triggered the symptoms in month 2. However, if the cocoa was having a beneficial effect on microvasculature, then the proportion of days with symptoms and the level of pain would not have increased even if they were exposed to more trigger factors.

Effects of capsules on Physical and Mental Wellbeing of participants

The present study demonstrated no effect of the cocoa or placebo capsules on the physical and mental wellbeing of participants throughout the intervention. In contrast, a previous study assessed health-related quality of life in 81 patients with PRP, and the scores were compared with data from the control participants. It was found that the normalised score for physical wellbeing and mental wellbeing in PRP patients were significantly lower compared to the control participants (normalised score physical wellbeing: 48 in PRP *versus* 52 in control; normalised score mental well-being: 42 in PRP *versus* 46 in control). However, in that study, 14% of the PRP patients and 2% of the control participants described themselves as having extreme anxiety/depression. Therefore, these findings could be because of the anxiety and depression reported by the PRP group (De Angelis *et al.,* 2008).

In the current study, none of the participants reported having anxiety or depression on any of the study visits. The normalised score for physical and mental wellbeing in cocoa and placebo groups indicated that both physical and mental wellbeing were more positive than for a general United States population. These findings could be related to the mild symptoms experienced by both the groups and indicate that PRP symptoms in these individuals does not appear to have a negative impact on mental and physical wellbeing compared to a general population.

Effect of capsules on level of flavanol and epicatechin intake in participants

The habitual diet did not appear to be modified during the intervention period in either group, and total flavanols provided by the diet was similar between the groups. However, when the flavanol content of the capsules was taken into account, the level of total flavanol and epicatechin intake reported by the cocoa group each month was significantly higher compared to the placebo group. Therefore, the intervention achieved its goal of elevating cocoa flavanol intake.

The EFSA has approved the health claim that total flavanol intake of 200mg (46mg epicatechin) daily has cardiovascular benefits but subsequent studies recommend that to be able to reduce the BP, 100mg of epicatechin daily is required (Heiss *et al.,* 2015, Sansone *et al.*, 2015). In the present study, the average daily epicatechin intake in the cocoa group, after adding 115mg/day of epicatechin on top of their habitual diet, was 136mg. Thus, daily intake in the cocoa group achieved the optimum level suggested. The beneficial effect of this increase in epicatechin intake was evidenced by a significant reduction in TPR and increase in CO observed in the cocoa group. In addition, these changes suggest that the participants in the cocoa group were compliant in taking the capsules daily during the intervention.

Diet diaries

The average daily total energy, macronutrients (protein, total fat, carbohydrate, sugars), alcohol and caffeine intake over the recording period were similar throughout the study within and between the groups. These findings suggest that the habitual diet of the participants was not influenced by study participation.

Adverse events

During the intervention, 1 participant from the placebo group reported having sleeplessness during the first month of the intervention, but this symptom subsided in the following month. Although the amount of caffeine per 3 capsules ingested by the participants in the placebo group is lower (13mg daily) compared to the amount found in coffee, tea or cola in individuals who regularly consume tea, coffee or chocolate, is unlikely to have resulted in sleeplessness; in those with low habitual caffeine intake, it is possible that this adverse event could be linked to capsule consumption. No other adverse events reported.

Attrition rate

Interestingly, only 32 out of the 420 people with Raynaud's initially expressing interest in participating in the study were eligible or willing to come for the screening. The main reason for not being able to come for the screening visit was because they lived outside of Nottingham. Others were unsuitable for the study due to not fulfilling inclusion criteria. Reasons for exclusion included the presence of a medical illness (such as hypertension, diabetes and hypothyroidism), reporting having SRP, or being above the age limit. Based on the blood test taken during the screening visit, 2 participants were found to have abnormal thyroid function tests, leaving 30 participants who met the inclusion criteria. These were then randomised into 2 groups (15 people in each group), namely a cocoa and a placebo group for the intervention, respectively.

For the cocoa group, a total of 5 participants dropped out (33%); 3 participants decided to withdraw before the intervention started due to the long distance for them to travel; 1 participant dropped out during the first month because of work commitments; and another 1 participant dropped out during the second month as they claimed to have observed no improvement in the symptoms. Meanwhile, for the placebo group, a total of 3 participants dropped out (20%); 1 participant decided to withdraw before

the intervention because of family commitments; 1 participant claimed to have observed no improvements in the symptoms during the first month; and another 1 participant dropped out during the second month due to work commitments. Therefore, the final number of participants was 10 for the cocoa group and 12 for the placebo group.

In the cocoa group, the subject who dropped out during the second month because of a lack of improvement in the symptoms observed, might support the findings that cocoa capsules did not help in improving the symptoms. Meanwhile, the overall attrition rates of 27% in the present study were higher than generally experienced in shorter term healthy volunteer studies (~10%) but are similar to other longer duration patient studies. A previous study carried out within the research group reported that the attrition rates among overweight and obese participants with increased risk of Type-2 diabetes was 26% within 0-3 months of a lifestyle intervention (unpublished data from PREVIEW: Prevention of diabetes through lifestyle intervention and population studies in Europe and around the World. EU FP7 Grant 312057). This finding indicates that patient groups may be more likely to leave the study compared to the control group in intervention studies or that attrition is greater in protocols of longer duration.

Feasibility of recruitment for a full RCT investigating vasoactive compounds in PRP participants

Although the findings of the current study do not support a role for cocoa flavanol supplementation as a treatment for PRP symptoms, it is possible that future studies in PRP participants may be proposed to test other vasoactive compounds. The *a priori* sample size, calculated from previous studies supplementing with Ginkgo, indicate that 73 participants per group would be required to be able to detect a difference in frequency of Raynaud's symptoms. In the current study it was observed that only 32 out of the 420 people with Raynaud's that were interested in the study came for the screening. With this 8% recruitment rate, almost 2000 people would need to express an interest in the study to be able to meet the recruitment requirements for a future RCT, assuming a predicted efficacy comparable

to that seen with Ginkgo supplementation. Moreover, although both men and women were targeted in the current study to maximise recruitment opportunities, males were under represented in this cohort with a ratio of male: female of 1:10. The prevalence of PRP is higher in females compared to males (10:8) (Brand *et al.,* 1997) which may have impacted on recruitment numbers, but equally, men may be less interested in participating in nutrition studies (Kollajtis-Dolowy and Zamojcin, 2016). Therefore, to achieve a sex distribution in future studies that more accurately reflects PRP prevalence rates in the general population, researchers may need to consider recruitment strategies used to target males.

Although the 8% recruitment rate is low, the study was advertised on some nationwide forums and the total of 420 people who were interested with the study came from all over the UK. As the protocol required that individuals attend early morning study visits, it was difficult for those living a distance away from Nottingham to participate in the study and were therefore not suitable for recruitment. Future studies should target local populations and the use of more than one study site would enable a wider proportion of the UK population to be accessed to achieve recruitment numbers. Meanwhile, it is important that volunteers are correctly characterised during the screening to ensure that they have PRP, and not SRP or just cold hands without experiencing the classic symptoms of PRP. In the present study, none of the participants had blood tests that suggested that they had SRP. In addition, most of the participants who attended the study had been diagnosed as having PRP by a doctor but were not on medication.

A follow-up with the participants was done weekly via eMail or telephone contact, to check compliance and to identify any problems. Ninety-five percent of the participants preferred to have eMail contact compared to telephone contact, as they could reply at their convenience. Apart from that, a follow up eMail enables a written record of the correspondence to be obtained. These findings indicate that it should be feasible to recruit, retain, and provide follow-up with participants in a full RCT in the future, but that

more than one recruitment centre may be needed to access sufficient people with PRP.

In conclusion, the present study has shown that 3 months supplementation of cocoa capsules containing 836mg flavanols (115mg epicatechin) resulted in an improvement in the compliance of resistance vessels, as evidenced by a reduction in the TPR and increment in the CO. However, these beneficial effects were not associated with an increase in microcirculatory perfusion or improvements in Raynaud's symptoms. These findings could be either because cocoa capsules do not have any effect on the Raynaud's microvasculature, or cocoa capsules may be inhibited from having an effect on the microvasculature by free radicals which are increased in people with PRP, or the study might have been underpowered of being able to detect the effect. It was also found that having PRP in this cohort did not appear to have a negative impact on mental or physical wellbeing when compared to a general population.

Chapter 8.0: General Discussion

The experimental work which is presented in this thesis is concerned with determining the effects of cocoa flavanols on peripheral blood flow and symptoms in PRP. PRP is characterised by periodic vasospasm of the fingers and toes precipitated by exposure to cold or emotional stimuli. Previous studies have demonstrated that underlying this condition can be vascular endothelium dysfunction, abnormalities in neural control of vascular tone and/or increases in circulating mediators which promote vasoconstriction. Cocoa derived products, rich in the phytonutrients 'flavanols', in particular epicatechin, have been shown to increase the bioavailability of NO at the vascular endothelium and to promote vasodilation, which may address an underlying cause of PRP and mitigate the symptoms.

A summary of the objectives of this experimental study is provided in Chapter 1 (1.4.8).

8.1 HFC ingestion would induce a greater vasodilatory response to a shear stress stimulus than a lower or non-flavanol placebo

Findings were not consistent across the two FMD studies, so it has not been possible to confirm or refute this hypothesis. In FMD study 1 (Chapter 3), it was observed that acute consumption of HFC drink (Chococru™) containing 830mg of flavanols per serving significantly increased FMD response at 1hr compared to an alkalised supermarket cocoa, which was assumed to contain <50mg of flavanols per serving. However, in FMD study 2 (Chapter 4), this effect was not observed 1hr or 3hrs after consuming this cocoa (containing 415mg of flavanols per serving) delivered in capsules compared to the placebo capsules. The inconsistencies between the results could be due to differences in delivery methods. Following capsule consumption, the absorption rate tends to be slower due to the time required for capsule dissolution and contents to be released and available for uptake, which could result in greater variability in response,

compared to consuming the contents as a drink. For example, a study comparing 2 oral formulations of itraconazole, 100mg capsules with 10ml of solution (10mg/ml), report that absorption of the itraconazole capsule in humans was associated with more variability as compared to the solution (De Beule and Van Gestel, 2001). Oral absorption is dependent on the granules inside the capsules dissolving in the stomach and intestinal fluids. This process can be altered by stomach emptying time, the pH of the stomach, and the presence of a meal, particularly the fat content of a meal, whereas the itraconazole solution was better absorbed, with less variability because the drug is already in a solution and does not depend on other factors such as food or acid production for dissolution (Barone *et al.*, 1998). In human studies, overall drug exposures are significantly higher following the administration of a solution as compared to a capsule, and this difference can approach 60% (Barone *et al.*, 1998, De Beule and Van Gestel, 2001). Although the absorption kinetics of cocoa flavanols might be different from itraconazole, based on these findings, it is concluded that the absorption rate of bioactive compounds in cocoa is faster following administration as a drink compared to capsules. If using capsules delayed the absorption of the cocoa flavanols, it is possible that the 3hr protocol was not long enough to measure acute changes to brachial artery function. In any future studies, blood epicatechin measurement before and after consumption would improve the ability to optimise measurement timepoints and would explain the difference between delivery methods.

The discrepancies in findings between the two FMD studies could also be due to the placebo choice. In FMD study 1 (Chapter 3) and Chapter 5, the high flavanol cocoa drink was compared with a cocoa drink with lower cocoa flavanol content, and both the drinks contained small amounts of caffeine and theobromine, whereas in FMD study 2 (Chapter 4), the HFC capsule was compared with a placebo capsule containing coconut flour, which contained no caffeine, theobromine or flavonoids, but did contain L-arginine (Lima *et al.*, 2015, Thaiphanit and Anprung, 2014). L-arginine is the precursor for NO production by eNOS and theoretically, could increase NO species within the blood vessel and improve blood vessel reactivity

(Zhao *et al.*, 2015), resulting in improved FMD values. However, the acute administration of higher quantities of L-arginine than would be found in the placebo capsules did not result in elevated NO or NO metabolites in the blood of healthy individuals (Alvares *et al.*, 2012). Therefore, the type of placebo chosen is not thought to impact the study findings.

It is possible that the amount of epicatechin in the cocoa capsules (FMD study 2) was not sufficient to elicit a detectable improvement in FMD. Although the EFSA has approved a cardiovascular health benefit claim for consuming dark chocolate, cocoa extract and cocoa based beverages containing 200mg total flavanols (46mg of epicatechin /day), Heiss *et al*., (2015) suggest that a higher amount (100mg/day of epicatechin) is required to elicit cardiovascular benefit in the longer term. In the current study, the participants were given 10 capsules containing, in total, 415mg of flavanols (50mg epicatechin), which met the EFSA recommended intake, but contained half the amount of epicatechin recommended by Heiss *et al*.

Experimental limitations in these studies include the use of FMD to determine endothelial function, which inherently can produce highly variable results. Although FMD has become the most widely used technique to measure endothelial function (Flammer *et al.*, 2012), this technique requires a skilled sonographer and an appropriate training period, is difficult to carry out and the 'error' of the method may preclude serial studies of endothelial function for individuals (Raitakari and Celermajer, 2000). In the present research, the FMD measurements in FMD study 1 and 2 was conducted by a different scan operator due to the logistic issues. The standard measurement timepoints were set according to standard methods (Corretti *et al.*, 2002), but in recent years, there has been recognition that time to peak in vasodilation is variable between individuals and it is possible that the standard 90 seconds collection period may miss this peak. In addition, the degree of vasodilation can be impacted by the magnitude of the hyperaemic response. The brachial artery blood flow was measured at 15 seconds after releasing the cuff, but this 'snap shot' of the hyperaemic response may not provide a complete picture of the shear stress stimulus. More recent recommendations propose that

brachial artery blood flow should be measured simultaneously with diameter assessments (Tortoli *et al.,* 2011). However, existing ultrasound equipment in the research group does not have the capability to make these simultaneous measurements and would not be possible at the time of writing.

8.2 HFC ingestion would affect cardiovascular measures (SBP, DBP, CO, HR, TPR) and skin BF at RT

The findings from Chapters 5 and 6 would not accept this hypothesis but findings in Chapter 7 would accept. Chapters 5 and 6 demonstrated no differential effect of acute consumption of HFC drink on the cardiovascular parameters (SBP, DBP, CO, HR, TPR) and skin BF in people without PRP (Chapter 5) nor in either PRP or controls groups (Chapter 6). However, findings from Chapter 7 demonstrated a significant reduction in the TPR and increment in the CO but no changes in the skin BF after chronic consumption of HFC capsules in participants with PRP. The study objective in Chapter 7 was to determine the chronic (3 months) effects of HFC delivered in capsules whereas the studies objectives in Chapters 5 and 6 were to determine the acute effects of HFC given as a beverage.

We recognise that the oral formulations between the studies is different; the absorption rate tends to be slower following capsule consumption compared to when given as beverage (as discussed earlier). However, this factor is unlikely to be the reason for the different findings between studies since supplementation during the chronic study was with capsules. The participants were instructed not to take their capsules on the day of the final laboratory (post intervention) visit, so that only the chronic effect of capsules was measured. The inconsistencies between the findings could therefore be because of differences between the cardiovascular effects of acute vs. chronic consumption of flavanol-rich cocoa. With regards to acute consumption, previous studies suggest that plasma epicatechin might partially contribute to an improvement in vascular function by increasing NO bioavailability, and the response peaks about 2hrs

after cocoa consumption (Fisher *et al.*, 2003, Heiss et al., 2005). Although it is proposed that the beneficial cardiovascular effects of longer term cocoa consumption may be due to repeated, acutely augmented, NO synthesis (Schroeter *et al.*, 2006), it is also possible that other mechanisms are primarily responsible for the improvements in BP seen in the current participants in the longer term. Previous studies have demonstrated the acute antihypertensive mechanisms of cocoa flavanols may include modulation of eNOS (Schnorr *et al.*, 2008, Ramirez-Sanchez *et al.*, 2010), direct inhibition of NADPH oxidase (Steffen *et al.*, 2007), reduction in plasma ET-1 levels (Loke *et al.*, 2008) and inhibition of angiotensin-converting enzyme activity (Actis-Goretta *et al.*, 2006). The alteration of specific pathways by cocoa flavanols would result acutely in the increased production of endothelial NO, inhibition of vasoconstriction and stimulation of vasodilatation, leading to an overall BP reduction via effects within the endothelium (as discussed in Chapter 1). However, longer term effects of cocoa flavanol ingestion on vascular endothelial health include more chronically mediated mechanisms such as reducing platelet aggregation, leucocyte activation, oxidative stress and inflammation (Goya *et al.,* 2016), and these effects might explain the differences in responses observed between acute and chronic study.

In regard to skin BF, no changes were observed after acute consumption of HFC in participants without PRP (Chapter 5) control or PRP participants (Chapter 6), as well as after chronic consumption in PRP participants (Chapter 7), suggesting that HFC was not having any acute effect on the microvasculature in either of the groups, or a chronic effect in PRP participants. In contrast, previous studies have observed an improvement in the skin BF of healthy participants following acute consumption (Phillips *et al.*, 2016, Neukam *et al.*, 2007) and 3 months chronic consumption (Brickman *et al.,* 2014, Heinrich *et al.,* 2006) of cocoa flavanols. Interestingly, the work by Heinrich *et al.*, (2006) was similar to the present study (Chapter 7) in terms of the sample size, participant's age group, HFC product (provided by Mars Wrigley Confectionery) and duration of intervention. It is possible that the current study was underpowered to

detect a difference in blood flow following 3-months supplementation of HFC. However, if data from Heinrich *et al.*, (2006), which represents the healthy individual, were used in a power calculation, the recruitment numbers achieved in the present study should provide a statistical power of 78%. However, retrospective interrogation of data from Chapter 6 indicates that only a power of 33% was achieved, with the number of participants required in each group to reach a statistical power of 80% being proposed as 26. Therefore, the chronic effect of cocoa flavanols on skin blood flow of PRP participants, appears to be blunted when compared to that seen in healthy controls and it is possible that the beneficial effect of cocoa capsules on the microvasculature is being inhibited by free radicals which were increased in people with PRP (Lau *et al.,* 1992).

With regards to acute consumption studies, it may be that taking measurements at 1hr (Chapter 5) as well as 1hr and 3hrs (Chapter 6) was not long enough to elicit measurable vasodilatory responses following HFC consumption. However, the duration of 1hr (Chapter 5) was chosen based on data from the first pilot study in FMD study 1 (Chapter 3) which found that acute consumption of HFC drink significantly increased FMD response at 1 hr and was supported by results from other group (Heiss *et al.,* 2015). Meanwhile, the failure of HFC to induce a vasodilatory effect after 1hr and 3hrs (Chapter 6) is proposed to be due to the cold stimulus (when skin temperature is at 15°C) being too great to be able to demonstrate any effect of cocoa in improving the skin BF in PRP and control groups. This observation could also be a reason why the HFC drink was not able to demonstrate a vasodilatory response among healthy people observed in Chapter 5. However, the design of this research project had been chosen based on a series of studies previously conducted in the research group looking at peripheral temperature regulation in those with PRP, which found that at a skin temperature of 15°C, microvascular dysfunction could be detected without triggering vasospasm. As the stimulus used is considered to be appropriate, the failure to demonstrate the vasodilatory responses following repeated cold exposure could be due to the RT (25°C) being too low such that it was not a thermoneutral environment and a degree of

peripheral vasoconstriction was occurring in the participants. Future studies would benefit from prior assessment of an appropriate RT to ensure a thermoneutral environment.

As the current study found that the beneficial effects of cocoa were not associated with an increase in microcirculatory perfusion (Chapter 7), the use of LDF, accompanied by iontophoresis of acetylcholine and sodium nitroprusside, could be considered in any future supplementation studies to confirm the findings. This technique is increasingly being adopted as a clinical measure of endothelial function in the microcirculation, rather than focussing solely on the large conduit arteries. Iontophoresis allows delivery of vasodilators across the skin using a weak current, with increased blood perfusion measured by the Laser Doppler technique (Turner *et al.*, 2008). As with all techniques that assess endothelial function in the peripheral vessels, the assumption is made that measurements made here are representative of generalized endothelial function.

Other methods such as nailfold microscopy and digital thermography have been used to evaluate distal digital vascularity and to assess the microvascular damage in Secondary Raynaud's (Keberle *et al.*, 2000). However, as PRP is not associated with microvascular abnormalities (Goundry *et al.,* 2012), nailfold microscopy is not considered as suitable for use in interventional studies involving people with PRP.

8.3 Counter-regulatory responses to localised cold exposure would not be compromised by the ingestion of HFC

The findings from this research project would accept this hypothesis, in view of having observed no difference in the time taken to stabilise in cold box before and after acute HFC ingestion. In addition, there were no changes in the skin temperature of the experimental finger moving from RT to localised cooling and *vice versa*, suggesting that acute HFC consumption did not affect temperature regulation in the control or PRP groups. Chapter 6 reported a shorter time taken to reach a skin temperature of 15°C in the control group following consumption of both the drinks, which

could be due to the presence of greater vasoconstriction, as a result of the room not being warm enough (as discussed earlier) rather than due to the effect of the cocoa drinks. Similarly, no effect of 3 months consumption of HFC capsules was observed on time taken to stabilise in the cold box (Chapter 7), suggesting that products containing cocoa flavanols can be consumed by people with PRP without compromising their thermoregulation.

Based on these findings, it is concluded that HFC capsules and HFC drink would be safe to be given as a supplement in any future studies in those with PRP.

8.4 Regular HFC ingestion would improve Raynaud's symptoms in those with PRP

The findings from this research reported in Chapter 7 have demonstrated that 3 months supplementation of HFC capsules did not improve Raynaud's symptoms (number of symptoms reported, RCS, proportion of days with symptoms monthly (%), duration of symptoms and level of pain) and therefore, would reject this hypothesis. Therefore, it is important that people with PRP are aware that cocoa flavanols probably will not help to reduce their symptoms despite it having been shown to improve the compliance of resistance vessels and microvasculature in healthy individuals.

People with PRP should also be made aware that currently, the evidence suggests that pharmacological treatments with oral vasodilators apart from CCB (drug of choice) also do not appear to improve Raynaud's symptoms (Stewart and Morling, 2012). However, authors of this review conclude that the methodological quality of most trials was poor, the sample sizes were small and the data available were limited, resulting in low precision of the statistical results. In addition, ACE inhibitors, such as lisinopril, have been used in the treatment of RP, owing to their relative tolerability and inhibition of vasoconstriction. There is, however, only limited evidence for their efficacy and no evidence to suggest that they are more effective than CCB.

Results of one randomised controlled trial demonstrated no effect of the ACE inhibitor quinapril on either the incidence of digital ulcers or on the frequency or severity of Raynaud's episodes when compared with placebo (Gliddon *et al*., 2007).Therefore, although the overall results suggest that there is no evidence for vasodilator drugs having an effect on PRP, it is still possible that a proportion of individuals may derive benefit from these treatments.

Apart from that, a possible new approach to therapy of RP is antagonism of the α_{2C}-adrenoceptor. Altered adrenergic function contributes to the pathogenesis of RP, especially α_{2c}-adrenoceptor dysfunction, which is thought to be more essential than α_1-adrenoceptor function in the control of digital vascular tone (Coffman and Cohen, 1988). A previous randomized, double-blind, crossover, placebo-controlled investigating the α_{2C}-adrenoceptor antagonist (ORM-12741) for prevention of cold-induced vasospasm in 12 patients with systemic sclerosis. It was observed that α_{2C}-adrenoceptor antagonist did not expedite recovery from a cold challenge in the fingers of patients with SSc. It is possible that ORM-12741 increases sympathetic tone by blocking α_{2C}-adrenoceptor in the central nervous system. Increased sympathetic tone would cause release of noradrenaline at sympathetic nerve endings that acts on α_1-adrenoceptors to produce constriction. Thus, despite all the convincing evidence implicating this possible target, studies with a suitable selective antagonist in a small group of patients yielded no evidence to support its use (Herrick *et al*., 2014).

The present study has demonstrated no effect of HFC capsules on physical or mental wellbeing throughout the intervention, which would contradict a related hypothesis proposing that the quality of life measures in participants with PRP could be improved in those taking HFC capsules compared to those taking the placebo. However, this absence of change in wellbeing was not unexpected as improvement in symptoms (which was the anticipated stimulus for improvements in wellbeing) was not observed. The similar SF-36^{TM} scores recorded across the supplementation period could also be related to the cohort reporting relatively mild symptoms of

Raynaud's (as evidenced by the low RCS), such that the participants' wellbeing was not being overly impaired by their PRP. Indeed, the physical and mental wellbeing scores for participants in this PRP cohort indicated that both mental and physical wellbeing were more positive than for a general population.

In the field of RP, more research is still required in view of available studies being criticized for one of the following design limitations that: participants were not homogenous, sample sizes were too small, studies were not placebo-controlled and only the acute effects of the treatment were tested. Future studies targeting foods or food components that have effects on specific receptors involved in the manifestation of RP may be warranted. For example, future studies might target food components that affect peripheral alpha-2 receptors, which are suggested to be hypersensitive in RP (Freedman *et al.,* 1995) and may be of use in attenuating vascular constriction to cold. Thus, in line with the increasing public interest in the health benefits of supplements, better-designed studies need to be conducted and more dietary components could be assessed.

8.5 Undertaking further intervention studies in those with PRP would be feasible

The findings reported in Chapter 7 indicate that it should be feasible to recruit, retain and provide follow-up with the participants for a full RCT involving individuals with PRP in the future and therefore, the findings from the present study would accept this hypothesis. It was found that the overall attrition rate of 27% over the 3 month intervention was similar to other longer duration UK patient studies. In regard to adverse events, 1 participant from the placebo group reported having sleeplessness during the first month intervention which could be the adverse event, although the amount of caffeine given was low (placebo capsules: 13mg daily; cocoa capsules: 21mg daily). No other adverse events were reported suggesting that these capsules are safe to be given as supplements in the future.

Although future intervention studies are feasible, the main limitation of the whole study was the recruitment for the PRP group. The participants need to fulfil the criteria of Allen and Brown (1932) for the diagnosis of PRP and thus, in the supplementation study (Chapter 7), both men and women were targeted in the PRP group to maximise recruitment opportunities. Only those who live in Nottingham or the nearby area were able to come for the study visits, so to address this limitation, more than one recruitment site should be considered in future to maximise access to PRP individuals. If more people were able to come for the study visits, then the sample size would be improved. However, it is difficult to scale up the sample size to more than the number of people that were recruited (see Chapter 7) without increasing resources at this site, having the study run across multiple sites, or extending the study over more than one winter. This would also help to address the problem that participants who dropped out in the middle of intervention could not be replaced, as the period of the intervention should be within the winter months. Finally, the laboratory equipment used to assess cardiovascular parameters and thermoregulation in this PhD project (e.g. the cooling box, Laser Doppler and Finometer) are not widely available in hospitals or research institutions and require that only one participant be assessed at a time. Therefore, any future study designs with larger cohorts would need to consider measurement techniques which would be more appropriate.

In summary, the findings from the current PhD project found that 3 months supplementation of HFC capsules did not improve Raynaud's symptoms (Chapter 6). However, a second cohort of participants has been recruited to the supplementation study (Winter 2019-2020) and if the findings from this second phase is able to demonstrate an improvement in the symptoms by increasing the statistical power of measurements, then an extension of this pilot study to a full RCT may be warranted to evaluate the potential effect of cocoa flavanols as a treatment for people with PRP.

Publication Bias

The findings from this study might create a publication bias as scientific journals are much more likely to accept for publication a study which reports some positive than a study with negative findings. Such conduct may produce long-term consequences to the entire scientific community and produces false impression in the literature. In addition, if negative results would not have so many problems to get published, other scientists would not unnecessarily waste their financial resources and time by conducting the same experiments.

Journal editors are the most liable for this phenomenon. Ideally, a study should have the same chance to be published regardless of the nature of its findings, if designed in an appropriate way, with valid scientific assumptions, well performed trials and sufficient data analysis, presentation and conclusions. Nevertheless, in reality, this is not the case. To facilitate publication of studies reporting negative findings, several journals have already been launched, which include Journal of Negative Results in Biomedicine, Journal of Pharmaceutical Negative Results and some other. The objective of such journals is to compensate the ever-increasing pressure in the scientific literature to publish only positive findings (Simundic, 2013).

One type of publication bias is the so called *funding bias* which happens caused by the prevailing number of studies funded by the same company, linked to the similar scientific question and supporting the interests of the sponsoring company. In the current project, the cocoa capsules used in chronic supplementation study were provided by Mars Wrigley Confectionery. It is absolutely acceptable to accept funding from a company to perform a research, as long as the study is not being influenced in any way by the sponsoring company and run independently and provided that the funding source is declared as a potential conflict of interest to the journal editors, reviewers and readers (Simundic, 2013).

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APPENDICES

Faculty of Medicine and Health Sciences

Screening Form

School of Life Sciences
Medical School
Queen's Medical Centre
Nottingham
NG7 2UH

PERSONAL INFORMATION

Volunteer Number _____   Date ___/____/___

1. **Name**
    ………………………………………………………………..

2. **Contact Address**
    ……………………………………………………….………………
    …………………………………………………………………..

3. **Contact Telephone number**
    ....……………………………………………...

4**.** **Email address**
    …………………………………………………………..

5. **Date of Birth**   ……./……./ 19…….

6. **Workplace/ Department/ School (if applicable):** ……..................

GENERAL HEALTH

1.   In general would you say your health is;
       Please tick one response

       a.☐ *Excellent*
       b.☐ *Very good*
       c.☐ *Good*
       d ☐ *Fair*
       e.☐ *Poor*

2.   Compared to one year ago, how would you rate your health in
       general now?
       Please tick one response

       a.☐ *Much better than a year ago*
       b.☐ *Somewhat better than a year ago*
       c.☐ *About the same*
       d.☐ *Somewhat worse than a year ago*
       e.☐ *Much worse than a year ago*

3. The following questions are concerned with any medical conditions you might have.

Do you suffer from any of the following?

Please tick one box on each line

	No	*Yes*	*Don't Know*
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems *			
Indigestion / heartburn			
Irritable bowel Syndrome			
Any other medical condition not listed above (please			

* other than asthma
Please detail any other medical condition/s

……………………………………………………………………………………………………
……………………………………………………………………………………

If you answered '*yes*' to any of the above, when did you first start having the problem and what medication (if any) do you use?

……………………………………………………………………………………………………
……………………………………………………………………………………………………
……………………………………………………………………………………………………

4. Have you had any operations in the last year?…………

If '*Yes*, please detail;

………………………………..………………………………….………..…

..…………………………………………………………………………………

5. **Do you have a tendency to faint in certain situations?**

☐ *Yes*   ☐ *No*   ☐ *Don't Know*

eg. on standing, in a warm room, after fasting

6. Are you currently taking contraception pills?

☐ *Yes*   ☐ *No*

If you are a female,

<u>Menstrual History</u>

a. Is your menses regular every month? _____

b. When is your expected next menses?(estimated) _____

c. Are you taking hormonal pills or any other form of contraception (please indicate)? _____

<u>Diet and Health</u>

a. Do you regularly (at least once a week) take **vitamin** and / or **mineral** supplements

☐ **Yes** ☐ **No** ☐ **Don't Know**

If '*yes*', which supplements do you take?

…………………………………………………………………………..……
…………………………………………………………………………..……

b. Do you regularly (at least once a week) take any other supplements for your health? Eg. Creatine, Carnitine, Echinacea, St John's Wort, etc

☐ **Yes** ☐ **No** ☐ **Don't Know**

If '*yes*', which supplements do you take?

…………………………………………………………………………..………
…………………………………………………………………………..……

Have you taken part, or do you intend to take part in any other trials within the three months prior to or following this particular study?
No

☐ ☐
Yes

PHYSIOLOGICAL MEASUREMENTS

1.   **Height**   **………cm**

2.   **Weight**   **………kg**

3.   **BP**   **……/…….**   **Lying**   **HR:**   **………..bpm**

                                   ……/…….   **Standing (1min)**   **HR:**   **………..bpm**

                                   ……/…….   **Standing (3 min)**   **HR:**   **………..bpm**

4.   **BMI:**   **…………..kg/m^2**

Recruited?   ☐   ☐

                            Yes   **No**

Raynaud's Questionnaires

We would like to understand a little bit about the symptom of Raynaud's that you experience. Please answer the questions below as fully as possible and please let us know if you have any questions

1. Describe your symptoms

   _____
   _____
   _____
   _____
   _____
   _____

2. Which part of your body is affected?

   _____
   _____
   _____

3. What triggers your symptoms?

   _____
   _____
   _____

4. What do you do to relieve your symptoms?

   _____
   _____
   _____

5. How often do you experience symptoms?

   _____
   _____

6. Do you ever experience migraine or ulceration to your fingers?

   _____
   _____
   _____

Consent form

Title of Study:

REC ref:

Name of Researcher:

Name of Participant: **Please initial box**

1. I confirm that I have read and understand the information sheet version number …………dated..................................... for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis.

3. I understand that relevant sections of data collected in the study may be looked at by authorise individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential.

4. I understand that the tests or procedures are carried out for research only and not for clinical diagnostic purposes. However, if the study investigator should feel it necessary to inform my GP of my participation in the study, or of an adverse event or abnormal test result, I understand I am giving my consent to do so.

5. I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation.

6. I voluntarily agree to take part in the above study.

_____   _____   _____
Name of Participant   Date   Signature

_____   _____   _____
 Name of Person taking consent   Date   Signature
 (if different from Principal Investigator)

_____   _____   _____
Name of Principal Investigator   Date   Signature

2 copies: 1 for participant, 1 for the project notes.

Symptoms diary

Date:…/…../……   Time:……………

What symptoms did you experience?

………………………………………………………………………………

What were you doing before you developed symptoms?

………………………………………………………………………………..

What do you think triggered your symptoms?

………………………………………………………………………………

What did you do to relieve your symptoms?

………………………………………………………………………………

How long did symptoms last?

………………………………………………………………………………

Please indicate the maximum pain/minimum pain/ discomfort you experienced.

No pain   **Severe pain**
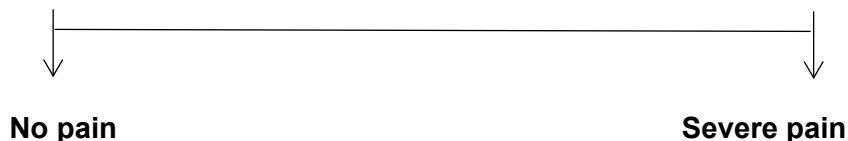
Raynaud's Condition Score

The Raynaud's Condition score is your rating of how much difficulty you had with your Raynaud's TODAY. Consider how many attacks you had and how long they lasted. Consider how much pain, numbness, or other symptoms the Raynaud's caused in your fingers (including painful sores) and how much the Raynaud's ALONE affected the use of your hands today.

SELECT the number that best indicates the difficulty you had today with your Raynaud's condition by marking an "X" in the appropriate box:

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

No Difficulty

Extreme Difficulty

University of Nottingham

School of Life Sciences

Pilot Study to investigate the effect of cocoa flavanols on symptoms in Primary Raynaud's phenomenon

To help us assess the techniques we carry out and to ensure that we are maintaining a good standard of care within the Medical School, we would like you to complete the following questionnaire. Please be as honest as possible.

1.   STUDY ORGANISATION & PREPARATION

a)   Where did you hear about the study?

………………………………………………………………………………………………….

b)   Why did you choose to participate in this study?

…..

....

c)   What did you hope to get from volunteering in this study?

…………………………………………………………………………………………………

…………………………………………………………………………………………………

……………………………………………………………………………………………..

d)   Once you expressed an interest in the trial, did you feel pressured into taking part?
Y / N

e)   Was the organisation of study visits satisfactory?   **Y / N**

If 'No', how could this be improved?

…………………………………………………………………………………………………

…………………………………………………………………………………………………

f)   Was the protocol and techniques explained adequately?   **Y / N**

If 'No', how could this be improved?

…………………………………………………………………………………………………

…………………………………………………………………………………………………

2.   STUDY PROCEDURES

a)   The total experimental time was 3 months, did you find this too long?   **Y / N**

If 'Yes' why did it feel too long

…………………………………………………………………………………………..

…………………………………………………………………………………………

The study involved consuming either capsules containing cocoa extract or alkalized cocoa powder (placebo);

b)   Did you experience any side effects that you attribute to this product? **Y / N**

If 'Yes', on when did you notice this

……………………………………………………………………………………………

…………………………………………………………………………………………….

c)   Would you be happy to take part in similar studies again? **Y / N**

If 'No', please explain

..…………………………………………………………………………………………..

…………………………………………………………………………………….…………

.

3.   FOLLOW-UP

a)   Did you experience any other problems after the study finished? **Y / N**

If 'Yes', please detail

…………………………………………………………….……………………….

…………………………………………………………………………………………………

…………………………………………………………………………………………

b)   Did you contact the experimenter during / after the study concerning any problems? **Y/ N**

c)   What advice / follow up was given?

    …………………………………………………………………………………………

    …………………………………………………………………………….…………………

d) Was this satisfactory?   **Y / N**

If 'No', how could this be improved?

……

……

e) Do you think that you experienced any benefits from participating in this study? **Y / N**

If 'yes', please detail

……

……

Finally...

If there is anything else you would like to feedback to us, other comments or ways we can improve, please detail below;

……

…….

...........…………………………………………………………………………………………………

Thank you for answering this questionnaire

Effects of Cocoa Flavanols on Peripheral Vascular Function and Symptoms in Raynaud's phenomenon

University of Nottingham
School of Life Sciences

This survey is concerned with the health and wellbeing of women with symptoms of Primary Raynaud's phenomenon. We would like you to answer the questionnaire as fully as possible. However, if you have any questions or concerns, please do not hesitate to contact Syaheedah on tel number 07864961545/ email address: mbxww@nottingham.ac.uk

Your questionnaire is identified with a subject number, which is unique for you. This will enable the information that you provide to remain completely confidential and will only be used for the purposes of this research.

Subject Number _____

Date ___/____/___

GENERAL HEALTH

The following questions are about your general health and wellbeing

1. In general would you say your health is;
   Please tick one response

   a. ☐ Excellent
   b. ☐ Very good
   c. ☐ Good
   d ☐ Fair
   e. ☐ Poor

2. Compared to one year ago, how would you rate your health in general now?
   Please tick one response

   a. ☐ Much better than a year ago
   b. ☐ Somewhat better than a year ago
   c. ☐ About the same
   d. ☐ Somewhat worse than a year ago
   e. ☐ Much worse than a year ago

3. During the past **4 weeks** have you had any of the following problems with your work or other regular daily activities **as a result of your physical health**?

   Please tick one of the boxes for each question.

   a. Cut down on the **amount of time** you spent on work or other activities?

       ☐ Yes   ☐ No   ☐ Don't know

   b. **Accomplished less** than you would like?

       ☐ Yes   ☐ No   ☐ Don't know

   c. Were limited in the **kind** of work or other activities?

       ☐ Yes   ☐ No   ☐ Don't know

   d. Had **difficulty** performing the work or other activities (eg. it took extra effort)

       ☐ Yes   ☐ No   ☐ Don't know

4. During the past **4 weeks** have you had any of the following problems with your work or other regular daily activities **as a result of any emotional problems,** such as feeling depressed or anxious ?

   Please tick one of the boxes for each question.

   a. Cut down on the **amount of time** you spent on work or other activities?

       ☐ Yes   ☐ No   ☐ Don't know

   b. **Accomplished less** than you would like?

       ☐ Yes   ☐ No   ☐ Don't know

   c. Didn't do work or other activities as **carefully** as usual

       ☐ Yes   ☐ No   ☐ Don't know

5. During the **past 4 weeks**, to what extent has your physical or emotional health interfered with your normal social activities with family, friends, neighbours or groups?

Please tick one response

a. ☐ *Not at all*
b. ☐ *Slightly*
c. ☐ *Moderately*
d ☐ *Quite a bit*
e. ☐ *Extremely*

6. How much **bodily** pain have you had during the past **4 weeks**?

Please tick one response

a. ☐ *None*
b. ☐ *Very mild*
c. ☐ *Mild*
d ☐ *Moderate*
e. ☐ *Severe*
f. ☐ *Very severe*

7. During the past **4 weeks,** how much did pain interfere with your normal work (including work both outside the home and housework)

Please tick one response

a. ☐ *Not at all*
b. ☐ *A little bit*
c. ☐ *Moderately*
d ☐ *Quite a bit*
e. ☐ *Extremely*

8. During the past **4 weeks,** how happy, satisfied, or pleased have you been with your personal life?

Please tick one response

a. ☐ *Much better than a year ago*
b. ☐ *Somewhat better than a year ago*
c. ☐ *About the same*
d. ☐ *Somewhat worse than a year ago*
e. ☐ *Much worse than a year ago*

9. These questions are about how you feel and how things have been with you **during the past 4 weeks.**

How much time during the past 4 weeks:	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of life?						
b. Have you been a nervous person?						
c. Have you felt so down in the dumps that nothing could cheer you up?						
d. Have you felt calm and peaceful?						
e. Did you have a lot of energy?						
f. Have you felt downhearted and low?						
g. Did you feel worn out?						
h. Have you been a happy person?						
i. Did you feel tired?						
j. Has your health limited your social activities (like visiting friends or close relatives)?						

10.   Please choose the answer that best describes how **true** or **false** each of the following statements is for you.

	Definitely true	Mostly true	Not sure	Mostly false	Definitely false
a. I seem to get ill more easily than other people					
b. I am as healthy as anybody I know					
c. I expect my health to get worse					
d. My health is excellent					

This questionnaire was developed at RAND Corporation as part of the Medical Outcomes Study

Physical Functioning

The following questions are about activities you might do during a typical day. Does your health limit you in these activities? If so, how much?

Please tick one box on each line

	My health does not limit these activities	*My health limits these activities a little*	*My health limits these activities a lot*	*I do not take part in these activities*
Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports				
Moderate activities, such as housework, gardening or playing with children				
Lifting/carrying groceries				
Climbing SEVERAL flights of stairs				
Climbing ONE flights of stairs				
Bending, kneeling or stooping				
Walking more than one mile (eg: more than 20 min)				
Walking half a mile (eg: more than 10 min)				
Walking a 100 yards				
Bathing/dressing yourself				

Pilot study to investigate the effect of cocoa flavanols on symptoms in Primary Raynaud's Phenomenon

Food Diary
(Household Measures)

Participant │ __ │ __ │ __ │ __ │

Month 1 / Month 2/ Month 3   (please circle)

Please complete your diary on the following days:

Day 1………………………….. (eg Monday)   Date………………

Day 2………………………..   Date………………………………

Day 3………………………..   Date………………………………….

Day 4………………………..   Date…………………………………

Please return the diary when you come for your next visit

Subject ID -............................		Day –No......... ....Date...			

Time	Type of food or drink with brand names and cooking method	Amount	Food or drink left over and amount	Office use only: code and weight

Subject ID…………………………………………………………

Recipe no: 1	Dates used:	Serves: Please also indicate on food diary the proportion of recipe you had on each occaision	Name of recipe:	
Ingredient (with brand names and cooking method)		Amount	Comments	

**Faculty of Medicine & Health Sciences
Research Ethics Committee**

c/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

17 April 2018

Dr Liz Simpson
Senior Research Fellow
Room E43,
E Floor, Medical School
School of Life Sciences (SoLS)
University of Nottingham
QMC Campus
Nottingham University Hospitals
Nottingham
NG7 2UH

Dear Dr Simpson

Ethics Reference No: 228-802 – please always quote	
Study Title: Effects of Cocoa Flavanols on Peripheral Vascular Function and Symptoms in Raynaud's Phenomenon. **Short Title:** Raynaud's Acute Study	
Chief Investigator/Supervisor: Professor Ian Macdonald, Professor of Metabolic Physiology, SoLS	
Lead Investigators/student: Syaheedah Wan Ghazali, PhD student, SoLS	
Other Key Investigators: Dr Liz Simpson, Senior Research Fellow, SoLS	
Type of Study: PhD Student project, Basic science study involving procedures	
Proposed Start Date: 01/04/2018	**Proposed End Date:** 30/09/2019 18mths
No of Subjects: 24	**Age:** 18-40 years

Thank you for responding to the comments made and the following documents were received:

- FMHS REC Application form and supporting documents version 1.1: 27.11.2017

These have been reviewed and are satisfactory and the study has been given a favourable opinion.

A favourable opinion has been given on the understanding that:

1. The protocol agreed is followed and the Committee is informed of any changes using a notice of amendment form (please request a form).
2. The Chair is informed of any serious or unexpected event.
3. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

**Faculty of Medicine & Health Sciences
Research Ethics Committee**

c/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

16 October 2018

Dr Liz Simpson
Senior Research Fellow
Room E43, Metabolic Physiology
School of Life Sciences
E Floor, Medical School
QMC Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

Dear Dr Simpson

Ethics Reference No: 112-1809 – please always quote	
Study Title: Pilot Study to investigate the effect of cocoa flavanols on symptoms in Primary Raynaud's phenomenon.	
Short Title: Raynaud's Supplementation Study.	
Chief Investigator/Supervisor: Professor Ian Macdonald, Professor of Metabolic Physiology, School of Life Sciences.	
Lead Investigators/student: Syaheedah Wan Ghazali, PhD Student, School of Life Sciences	
Other Key Investigators: Dr Liz Simpson, Senior Research Fellow, School of Life Sciences, Dr P Lanyon, Consultant Rheumatologist, Department of Rheumatology, QMC, Nottingham University Hospitals NHS Trust.	
Type of Study: PhD, Basic science study involving procedures, medicinal or food products.	
Proposed Start Date: 01/09/2018	**Proposed End Date:** 30/09/2020 24mths
No of Subjects: 30	**Age:** 18+years

Thank you for responding to the comments made and the following documents were received:

- FMHS REC Application form and supporting documents version 1.1: 27.11.2017

These have been reviewed and are satisfactory and the study has been given a favourable opinion.

A favourable opinion has been given on the understanding that:

1. The protocol agreed is followed and the Committee is informed of any changes using a notice of amendment form (please request a form).
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Yours sincerely

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

FOOD FREQUENCY QUESTIONNAIRES

Name of participant:

Date:

Note: Pls mark the correspondence box (x), you can justify 'your portion size' by number or weight (estimated)

Over the last year, how often did you eat the following?

FOOD Fruits;	Your Portion size	Frequency							
		NEVER	1-6 times/yr	7-11 times/yr	2-3 times/mth	once/mth	>once/wk	once/wk	daily
Apples, with skin									
Apples without skin									
Apple Juice, unsweetened, without added vitamin C									
Apricot Jams									
Avocados									
Bananas									
Blackberries									
Blueberries									
Cherries									
Cherry Jams									
Cranberries, fresh									
Cranberries, dried, sweetened									
Cranberry sauce, sweetened									
Cranberry Juice									
Blackcurrants									
White Currants									
Red Currants									

Blackcurrant Juice									
Currants, dried									
Dates, dried									
Figs, fresh									
Gooseberries									
Grapefruit, ink and red									
Grapefruit, white									
Grapefruit Juice, white, unsweetened									
grapefruit Juice, pink									
Red Grapes,									
White Grapes									
Grape Juices, unsweetened without added vitamin C									
Kiwifruit, green									
Lemon Juice (not cordial)									
Mangos									
Melons, cantaloupe									
Melons, honeydew									
Nectarines									
Olives, pickled, canned or bottled									
Oranges									
Orange Juice, fresh									
Orange Juice from concentrate									
Tangerines, mandarins, satsumas									
Marmalade									
Papaya									
Peaches, fresh									
Peaches, canned									
Peach Jams									

			1-6 times/yr	7-11 times/yr	2-3 times/mth				
Pears									
Pineapple, fresh									
Pineapple juice, unsweetened, without added vitamin C									
Plums									
Plum Jams									
Pomegranates									
prunes, dried, uncooked									
Pummelo									
Raisins, seedless									
Raspberries, fresh									
Raspberries, frozen									
Rhubarb stalks, cooked									
Strawberries, fresh									
Strawberries, frozen									
Strawberry / raspberry Jams									
Watermelon									
Vegetables;	**Your Portion**	**NEVER**	**1-6 times/yr**	**7-11 times/yr**	**2-3 times/mth**	**once/mth**	**>once/wk**	**once/wk**	**daily**
Artichokes, (globe or french)									
Asparagus									
Beans, kidney									
Broadbeans (fava beans)									
Broccoli, cooked									
Brussels sprouts, cooked									
Cabbage, white, raw									
Cabbage, white, cooked									
Cabbage, red, raw									

Cabbage, red, cooked									
Cabbage, savoy/ green, raw									
Cabbage, savoy, cooked									
Cabbage, chinese (pak-choi), raw									
Cabbage, chinese (pak-choi), cooked									
Carrots, raw									
Carrots, cooked									
Carrots, canned									
Cauliflower, raw									
Cauliflower, cooked									
Cauliflower, frozen, cooked,									
Celeriac, raw									
Celery, raw									
Chard, swiss, raw									
Chives, raw									
Corn, sweet, yellow, raw									
Courgette, raw									
Courgette, cooked									
Cucumber, with peel, raw									
Endive, raw									
Garlic, raw									
Horseradish root									
Kale, raw									
Leeks, (bulb and lower leaf-portion), raw									
Lettuce, cos or romaine, raw									
Lettuce, iceberg (includes crisphead types), raw									
Lettuce, green leaf, raw									
Lettuce, red leaf, raw									

Mushrooms, white, raw								
Mushrooms, tinned								
Okra								
Onions, raw								
Onions, cooked								
Onions, spring or scallions (includes tops and bulb), raw								
Parsnips								
Peas, green, raw								
Peas, green , canned								
Peas, green, frozen, unprepared								
Peas, green, frozen, cooked								
Peppers, sweet, raw								
Potatoes, white, cooked								
Peas, green, raw								
Peas, green , canned								
Peas, green, frozen, unprepared								
Peas, green, frozen, cooked								
Peppers, sweet, raw								
Potatoes, white, cooked								
Potatoes, red, cooked								
Radishes, raw								
Spinach, raw								
Spinach, frozen								
Sweet potato, cooked								
Tomatoes, raw								
Tomatoes, fresh, cooked								
Tomatoes, tinned								
Tomato puree								
Tomato Soup, tinned								

Tomato-based pasta Sauce									
Watercress, raw									
Radicchio, raw									
Fennel bulb									
Rocket, raw									
Nuts and seeds	**size**	**NEVER**	**1-6 times/yr**	**7-11 times/yr**	**2-3 times/mth**	**once/mth**	**>once/wk**	**once/wk**	**daily**
almonds									
brazilnuts									
cashew nuts,roasted									
chestnuts,									
coconut water									
hazelnuts									
macadamias									
pecans									
pine nuts									
pistachios									
walnuts									
Peanuts									
Alcoholic beverages	**size**	**NEVER**	**1-6 times/yr**	**7-11 times/yr**	**2-3 times/mth**	**once/mth**	**>once/wk**	**once/wk**	**daily**
Beer									
Sweet dessert wine									
Red table wine									
White table wine									
Cider									
Non-Alcoholic beverages									
Cocoa, dry powder, unsweetened,									
Hot Choc powder mix									
Coffee, brewed from grounds									

	size	NEVER	1-6 times/yr	7-11 times/yr	2-3 times/mth	once/mth	>once/wk	once/wk	daily
Coffee, instant									
Tea, black, brewed, decaffeinated									
Tea, black, brewed									
Tea, instant powder, prepared									
Tea, green, brewed									
Tea, green, brewed, decaffeinated									
Tea, oolong, brewed									
Miscellaneous	size	NEVER	1-6 times/yr	7-11 times/yr	2-3 times/mth	once/mth	>once/wk	once/wk	daily
Tofu, firm									
Soya-based non-dairy milks									
Soya-based non-dairy yoghurts									
dark chocolate, unsweetened									
milk chocolate									
Honey									
Buckwheat									
Bread, whole-wheat									
White wine Vinegar									
Red wine Vinegar									

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