EFFECT OF COCOA FLAVANOLS ON MICROVASCULAR FUNCTION: A COMPARISON IN SOUTH ASIANS AND WHITE EUROPEANS

By

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Abstract

South Asians (SAs) are an ethnic group with increased cardiovascular disease (CVD) risk, and SA women are at particular risk of coronary artery disease (CAD), which is attributed to microvascular endothelial dysfunction. There is also evidence of impaired endothelium-dependent dilator (EDD) responses in young SA men and women relative to White Europeans (WEs), which is considered predictive of future CVD. It has already been established that EDD responses in arterial vessels can be improved by cocoa flavanols (CFs). Thus, this project focussed on current evidence of whether dietary CFs can improve microvascular function and tested experimentally whether microvascular responses differ between young WE and SA women, and the effect of an acute CF intervention on these responses.

A systematic review of the literature indicated that consumption of CFs improved microvascular responses in several different tissues, but these studies were mostly performed in men or mixed gender groups with wide age ranges. Experimental recordings using venous occlusion plethysmography and near-infrared spectroscopy (NIRS) to monitor forearm blood flow (FBF) showed that reactive hyperaemia (RH) and exercise hyperaemia (EH), the vasodilator responses evoked following release of arterial occlusion and by rhythmic handgrip contractions respectively, were impaired in young SA, compared to WE women. In addition, NIRS recordings suggested that microvascular regulation of tissue oxygenation was impaired in SA women. However, there was no effect of CFs on RH, or EH in either WE or SA women. This suggests that CFs are less effective in women than in men at augmenting EDD responses.

In addition, mental stress induced by a mental arithmetic task, evoked forearm vasodilation in half of women from each ethnic group, but vasoconstriction in the rest, suggesting that

the muscle vasodilation was blunted in a similar proportion of WE and SA women. When grouped according to the direction of their response to mental stress, CFs increased FBF in vasoconstrictors, but not vasodilators, consistent with CFs augmenting nitric oxide-dependent vasodilation, which is impaired in vasoconstrictors. Given that exaggerated vasoconstrictor responses to mental stress predict future hypertension and CVD, the novel finding that CFs attenuated vasoconstriction in some women raises the possibility that dietary CFs may help to limit CVD risk in this group.

Finally, a comparison of diet and lifestyle between a larger population of WE and SA men and women showed that SAs had lower physical activity levels, and consumed fewer portions of foods associated with improving vascular function, including flavonoids, than WEs. This suggests that lifestyle factors, including diet, may at least partly explain the impaired EDD responses of young SA relative to WE women, and are likely to increase their risk of future CVD.

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List of abbreviations

ABP	Arterial blood pressure
ACh	Acetylcholine
Akt	Protein kinase B
ANOVA	Analysis of Variance
AU	Arbitrary units
BA	Black African
BH_3	Trihydrobiopterin
BH_4	Tetrahydrobiopterin
BMI	Body mass index
Ca^{2+}	Calcium
CAD	Coronary artery disease
CaM	Calmodulin
CF	Cocoa flavanol
cGMP	Cyclic guanine monophosphate
COX	Cyclooxygenase
CVC	Cutaneous vascular conductance
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
deoxyHb	Deoxygenated haemoglobin
ECG	Electrocardiogram
EDCF	Endothelium-derived constricting factor
EDD	Endothelium-dependent dilation
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EET	Epoxyeicosatrionic acid
EFSA	European Food Safety Authority
EH	Exercise hyperaemia
ET-1	Endothelin-1
FBF	Forearm blood flow
FFQ	Food Frequency Questionnaire
FLV	Flavonoid
FMD	Flow-mediated dilatation
FVC	Forearm vascular conductance
GPER	G-protein oestrogen receptor
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HR	Heart rate
IPAQ	International Physical Activity Questionnaire
IQR	Interquartile range
K^+	Potassium
LDPI	Laser Doppler perfusion imaging
L-NMMA	N-monomethyl-L-arginine
MABP	Mean arterial blood pressure
Mb	Myoglobin
MET	Metabolic equivalent of task
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MSNA	Muscle sympathetic nerve activity
MVC	Maximum voluntary contraction
MW7	McCance and Widdowson's The Composition of Foods
NADPH	Nicotinamide adenine dinucleotide phosphate
NIRS	Near-infrared spectroscopy
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide anion
OCT-A	Optical coherence tomography angiography
$ONOO^-$	Peroxynitrite
oxyHb	Oxygenated haemoglobin
PA	Physical activity
PAD	Peripheral artery disease
PASAT	Paced Auditory Serial Addition Task
$PG(H_2)$	Prostaglandin (H_2)
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PK(A/G)	Protein kinase A/G
PRISMA	Preferred Reporting System for Systematic Reviews and Meta-Analyses
RCT	Randomised controlled trial
RH	Reactive hyperaemia
ROI	Region of Interest
SA	South Asian
SBP	Systolic blood pressure
SD	Standard deviation
SERCA	Sarco/endoplasmic reticulum Ca^{2+}/ATPase
SO_2	Oxygen saturation
T2DM	Type-2 diabetes mellitus
totalHb	Total haemoglobin
TXA_2	Thromboxane A_2
USF	User-added food
VOP	Venous occlusion plethysmography
VSM	Vascular smooth muscle
WE	White European

Chapter 1:

General Introduction

1.1 Overview

Cardiovascular disease (CVD) is a leading cause of death worldwide, but the incidence is particularly high in ethnic groups such as Black Africans (BAs) and South Asians (SAs) (Cappuccio, 1997). The prevalence of risk factors such as diabetes, metabolic syndrome and hypertension, is also higher amongst SAs compared to White Europeans (WEs) (Tziomalos et al., 2008), with SAs showing an earlier onset and more severe outcomes (Cappuccio, 1997, Yusuf et al., 2001). SA women are particularly at risk of early coronary artery disease (CAD), and in women CAD commonly manifests at the microcirculatory level (Mishra and Monica, 2019, Reis et al., 2001) . There is almost certainly a genetic component to CVD and CAD risk, though environmental factors, for example diet and lifestyle, are also likely to contribute (Goyal and Sanghera, 2021).

Individuals at risk of CVD present endothelial dysfunction even before the onset of symptoms, and endothelium-dependent dilator (EDD) responses can be a useful predictor of future CVD (Anderson et al., 2011, Philpott et al., 2009). Thus, comparison of EDD responses between groups can provide insight into their endothelial function, and can be used to assess how interventions, may influence this. This project builds on current knowledge of differences between EDD responses in WEs and SAs, focussing on young women, and explores how cocoa flavanols (CFs), which have a proposed cardioprotective role (Aprotosoaie et al., 2016b), may improve microvascular endothelial function within these groups.

1.2 Role of the endothelium in vascular regulation

The endothelium, the single layer of cells lining all blood and lymphatic vessels, has various vascular functions, including forming a barrier between the vessel lumen and surrounding tissue, blood clotting, inflammation and angiogenesis (Rajendran et al., 2013). Another major

role is the maintenance of vascular tone, as controlled by the balanced release of various factors, termed endothelium derived- relaxing (EDRF) or -constricting factors (EDCF); in a healthy endothelium the balance favours release of relaxing factors, promoting vasodilation (Rubanyi, 1991).

The first evidence for the existence of EDRF came in 1980 when Furchgott and Zawadski demonstrated the requirement of an intact endothelium for acetylcholine (ACh)-induced vasodilation in isolated rabbit aorta. They used subsequent sandwich preparations to demonstrate that this phenomenon was due to diffusion of a powerful vasodilator substance from the endothelium to the underlying vascular smooth muscle (VSM) (Furchgott and Zawadzki, 1980). Superfusion-bioassay studies aimed at identifying the nature of EDRF provided mounting evidence of its short half-life and scavenging by superoxide anions (O_2^-), findings which were consistent with the known properties of nitric oxide (NO), leading to the conclusion that EDRF was indeed NO (Ignarro et al., 1987, Vanhoutte, 2009). These findings were supported by the indistinguishable bioactivity, stability and response to inhibitors and potentiators (Palmer et al., 1987). However, it later became apparent that vascular responses persisted even when NO was inhibited, leading to the observation that other EDRFs must also exist (Hellsten et al., 2012, Mortensen and Saltin, 2014). Two major groups of these have been characterised; prostaglandins (PGs), predominantly prostacyclin (PGI_2), and a group of factors which cause VSM hyperpolarisation directly, termed endothelium-derived hyperpolarising factors (EDHFs) (Mortensen and Saltin, 2014).

1.2.1 Nitric oxide

1.2.1.1 Synthesis

NO biosynthesis is mediated by nitric oxide synthase (NOS) enzymes, of which there are three isoforms; endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Vallance and

Chan, 2001). eNOS is constitutively active, producing low levels of NO due to mechanical stimulation of shear stress caused by constant blood flow. This role could be inferred from early evidence of vasoconstriction evoked by removal of the endothelium (Collier and Vallance, 1989) or from the elevation in arterial blood pressure following NOS inhibition by N-monomethyl-Larginine (L-NMMA) in experimental animals (Rees et al., 1989b), and in man, in whom basal forearm blood flow was dose-dependently decreased by infusion of L-NMMA (Vallance et al., 1989). Tonic NO release is responsible for maintaining peripheral vascular tone, with loss of basal NO production, promoting an atherosclerotic-prone endothelium (Cayatte et al., 1994). NO release can also be upregulated by physical stimuli including shear stress and pulsatile stress, and by pharmacological stimuli involving receptor activation by agents such as ACh, bradykinin, serotonin or substance P (Vallance and Chan, 2001).

eNOS activation can be initiated by calcium (Ca^{2+})-dependent and independent pathways (Figure 1.1). Firstly, binding of agonists such as ACh and bradykinin to cell surface receptors triggers increases in intracellular Ca^{2+}, which activates calmodulin (CaM) and causes this to couple with eNOS. Inactive eNOS is bound to the intracellular membrane, therefore the binding of CaM causes this to be released and thus activated (Fleming and Busse, 1999). The eNOS dimer is activated by phosphorylation at Ser1177 and Ser633 activation sites, whilst Thr495 is an inhibitory site (Yang et al., 2001). Phosphorylation at the activation sites occurs via the phosphoinositide 3-kinase (PI3K) pathway and is initiated by protein kinase A (PKA) and protein kinase B (Akt) pathways (Schmitt and Dirsch, 2009).

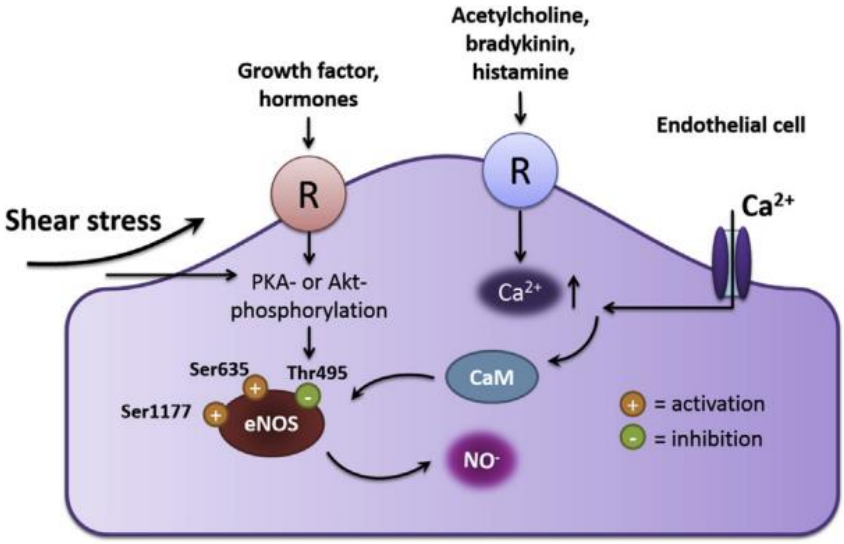

Figure 1.1: Signalling pathways involved in the activation of endothelial nitric oxide synthase (eNOS)*. These can be calcium (Ca^{2+}) -dependent; receptor (R) binding of acetylcholine, bradykinin and histamine causes increases in intracellular Ca^{2+} which bind to calmodulin (CaM) for eNOS activation. Activation of eNOS also occurs via phosphorylation by PKA and Akt at the activation sites, via hormones and shear stress. Figure from: (Zhao et al., 2015)*

The process of NO synthesis by active eNOS requires an abundance of the precursor L-arginine (Figure 1.2); several studies document reversal of L-NMMA-induced inhibition of dilator responses by administering excess of L-arginine, an effect which is not conferred by D-arginine (Palmer et al., 1988, Rees et al., 1989a). NO synthesis also requires the presence of several co-factors, for example tetrahydrobiopterin (BH_4) binding facilitates formation of eNOS dimers, which are required for its activation (Moncada and Higgs, 1993).

1.2.1.2 Mechanism of action

Following synthesis, NO diffuses through endothelial cells to the underlying VSM where it activates guanylate cyclase through binding haem moieties and stimulates production of cyclic guanine monophosphate (cGMP) which evokes relaxation by several key mechanisms (Arnold et al., 1977, Denninger and Marletta, 1999). cGMP acts as a secondary messenger, activating protein kinase (PK)G, which reduces intracellular Ca^{2+} concentration in VSM by

promoting re-uptake into the sarcoplasmic reticulum, and closing membrane channels which allow Ca^{2+} influx. As myosin light chain kinase (MLCK) activity is Ca^{2+}-dependent, falling intracellular Ca^{2+} reduces myosin phosphorylation, hence preventing contraction (Carvajal et al., 2000, Mizuno et al., 2008) (Figure 1.2). Furthermore, the elevated cGMP levels cause opening of potassium (K^{+}) channels, promoting hyperpolarisation of the VSM and limiting further Ca^{2+} influx, and cGMP can indirectly activate myosin light chain phosphatase (MLCP) to promote vasorelaxation (Lee et al., 1997).

Independently of its cGMP-mediated activity NO can also stimulate the sarco/endoplasmic reticulum Ca^{2+}/ ATPase (SERCA) to promote Ca^{2+} uptake, thus further decreasing intracellular Ca^{2+} to reduce myosin phosphorylation for contraction (Adachi et al., 2004). A combination of these mechanisms promotes relaxation rather than contraction of the VSM, hence facilitating vasodilation (Zhao et al., 2015).

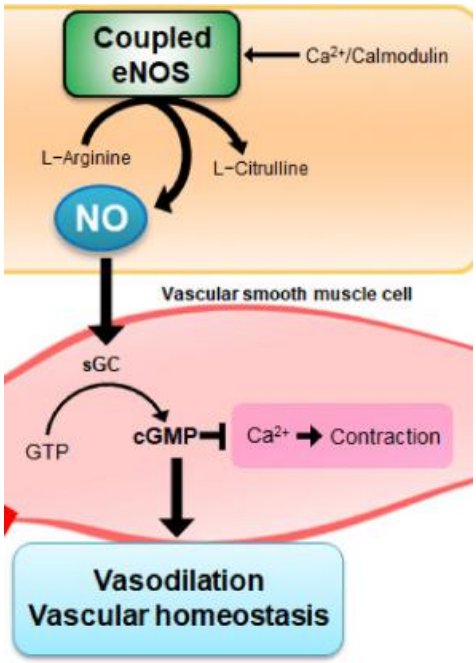

Figure 1.2: Nitric oxide (NO) synthesised within endothelial cell diffuses to the vascular smooth muscle and causes relaxation, and thus vasodilation *via soluble guanylate cyclase (sGC), stimulating to production of cGMP which reduces intracellular $Ca^{2+}$and therefore prevents contraction. Adapted from (Lee and Im, 2021)*

1.2.2 Cyclooxygenase products

Cyclooxygenase (COX) enzymes, predominantly the COX-1 isoform in the endothelium, are responsible for the production of several important mediators of vascular tone (Feletou et al., 2011). Arachidonic acid is first liberated from the cell membrane by phospholipase A2 and can then follow several potential metabolic pathways. These each involve a series of steps involving enzymes from the cytochrome P450 superfamily. Firstly, arachidonic acid undergoes a two-stage reaction catalysed by COX-1 to produce prostaglandin (PG)H_2. This intermediate product can subsequently be metabolised by various cell-specific PG-synthase enzymes, most commonly prostacyclin synthase in the endothelium to produce PGI_2, a key vasodilator. Alternatively, PGH_2 can be metabolised by thromboxane synthase to produce thromboxane A_2 (TXA_2), a potent vasoconstrictor (Mitchell et al., 2008) (Figure 1.3).

These products all signal via binding G-protein coupled receptors on the VSM cells, of which there are several major classes, each targeted by different ligands; IP receptors are the most abundant on VSM, binding mainly PGI_2 and eliciting vasodilation (Figure 1.3) via increasing cyclic AMP which activates PKA signal cascade to decrease intracellular Ca^{2+} and thus promote myosin dephosphorylation for VSM relaxation (Narumiya et al., 1999). Another major receptor class is the TP receptor, for which TXA_2 is the preferred ligand, but under pathological conditions of excessive PGH_2 and/or PGI_2, they may also act on TP receptors, thereby contributing to enhanced vasoconstriction (Vanhoutte and Tang, 2008).

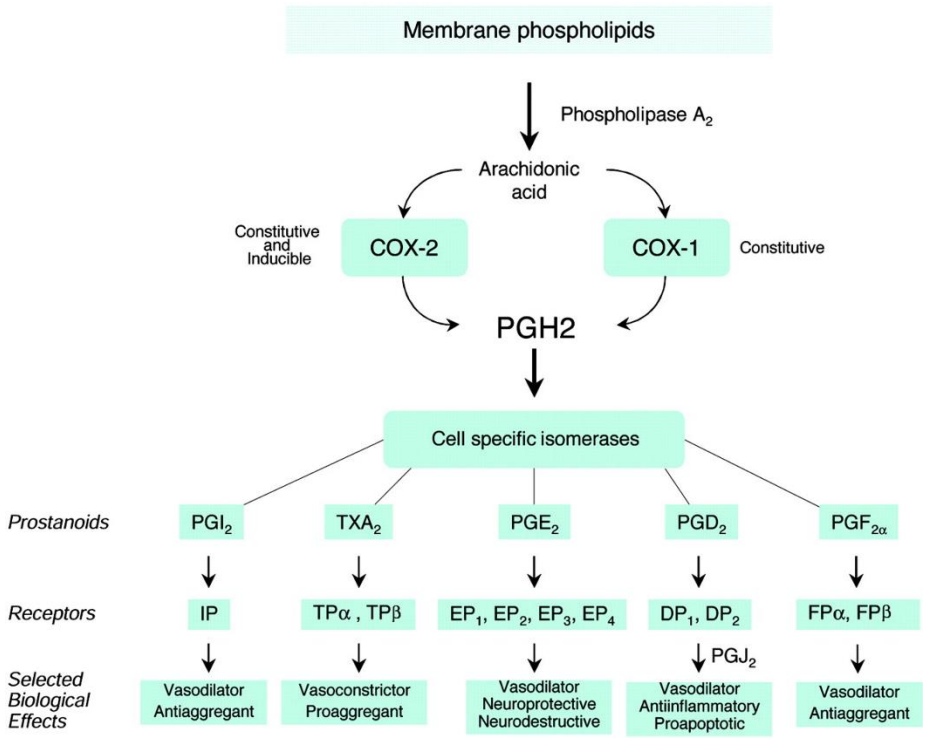

Figure 1.3: Prostanoid formation from arachidonic acid, liberated from the phospholipid membrane, *by COX-1 and COX-2 enzymes, to produce prostaglandin H_2, which is then converted to various prostanoids by cell specific isomerases, and these act on specific receptors. Adapted from (Iadecola and Gorelick, 2005)*

1.2.3 EDHFs

Following the observation that endothelium-dependent relaxations persisted in the presence of dual NOS and COX inhibition, it was proposed that there must be some other factor involved (Bolton et al., 1984, Chen et al., 1988). When it was shown that the persistent EDD involved hyperpolarisation, the factor was termed 'EDHF', although EDHF is now thought to comprise a collection of substances rather than one single entity (Feletou and Vanhoutte, 2009). A major group of these substances, the epoxyeicosatrionic acids (EETs), are also products of arachidonic acid metabolism, via cytochrome P450 monooxygenase enzymes, such as CYP C29 (Busse et al., 2002). Other EDHFs released from the endothelium which act upon VSM cells to elicit hyperpolarisation include hydrogen peroxide (H_2O_2), peptides such as C-type natriuretic peptide, and gaseous mediators such as carbon monoxide (Feletou and Vanhoutte, 2009). Alternatively, endothelium-derived K^+ ions may facilitate the transfer of

endothelial hyperpolarisation to the underlying VSM via myo-endothelial gap junctions or the activation of inwardly-rectifying K^+ channels or Na^+/K^+-ATPase, or a combination of these mechanisms (Edwards et al., 2010). The common pathway of all of these factors is the hyperpolarisation of VSM cells to reduce intracellular Ca^{2+} and hence promote vasorelaxation (Busse et al., 2002).

1.2.4 EDCFs

The most potent vasoconstrictor substance is endothelin-1 (ET-1), which interacts antagonistically with NO. Thus, under physiological conditions, the constitutive release of NO antagonises ET-1 synthesis from the pro-peptide big-ET. Any ET-1 that is produced acts on endothelial ET_B receptors, which are coupled to eNOS, in an inhibitory feedback mechanism to stimulate NO synthesis and prevent excessive ET-1 signalling via ET_A receptors on the VSM, which would lead to vasoconstriction (Bourque, Davidge and Adams, 2011). Other endothelium-derived constrictor factors include TXA_2, PGH_2, and O_2^- (Rubanyi, 1991).

1.3 Endothelial dysfunction

The term 'endothelial dysfunction' defines the functional and reversible alteration of the endothelium associated with the dysregulation of dilator mechanisms disrupting the balance between dilator and constrictor influences (Taddei and Salvetti, 2002). Decreased NO bioavailability is an important hallmark of endothelial dysfunction and represents an early stage in the progression of CVD (Vita and Keaney, 2002). This commonly results from eNOS uncoupling, which arises due to limited availability of the BH_4 co-factor and leads to enhanced production of O_2^- rather than NO (Cosentino and Luscher, 1999, Vasquez-Vivar et al., 1998) (Figure 1.4). The importance of BH_4 bioavailability for maintaining normal eNOS

function is strongly supported by *in vitro* evidence of BH_4 inhibition abolishing NO production (Schmidt et al., 1992), and BH_4 supplementation increasing NO and reducing O_2^- production (Bevers et al., 2006, Wever et al., 1997). Furthermore, impaired EDD associated with various CVD risk factors was shown to be ameliorated by BH_4 supplementation (Maier et al., 2000, Stroes et al., 1997).

The ensuing endothelial dysfunction is augmented by the reaction of NO with O_2^-, generated both by uncoupled eNOS and other enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase- or xanthine-oxidase, to generate peroxynitrite ($ONOO^-$) (Figure 1.4) (Wink and Mitchell, 1998, Darley-Usmar et al., 1995, Cai and Harrison, 2000). Besides $ONOO^-$ itself being a potent ROS which can cause dramatic cellular damage, it also reduces BH_4 to inactive trihydrobiopterin (BH_3), thus establishing a positive feedback loop by further limiting the availability of the essential co-factor and resulting in further eNOS uncoupling (Forstermann and Li, 2011).

Furthermore, reduced NO bioavailability leads to an exaggerated role of ET-1. Since the tonic antagonistic effect of NO is reduced, ET-1 synthesis becomes unmitigated, and the stimulatory effect on the ET_A receptors of the VSM dominates, leading to increased intracellular Ca^{2+} and hence promoting vasoconstriction (Bourque, Davidge and Adams, 2011). Thus, the imbalance of NO and ET-1 is characteristic of endothelial dysfunction and contributes to the progression of CVD.

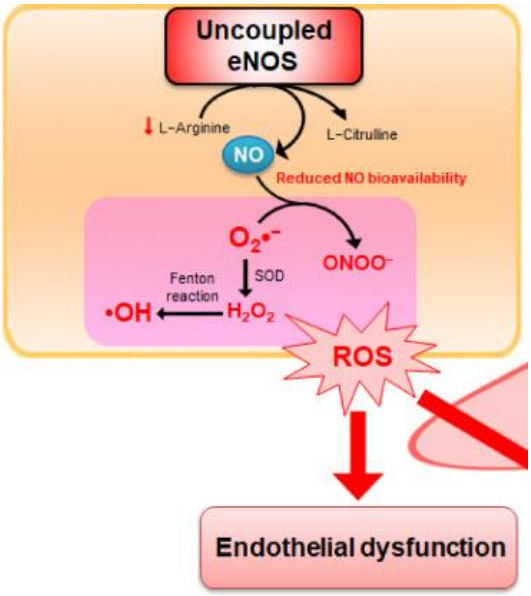

Figure 1.4: eNOS uncoupling leads to production of reactive oxygen species and thereby endothelial dysfunction. *Adapted from (Lee and Im, 2021)*

Endothelial dysfunction and enhanced ROS production due to eNOS uncoupling is associated with CVD risk and progression and is characterised by impaired EDD responses as shown with ageing (Taddei et al., 2001), hypertension (Linder et al., 1990, Panza et al., 1990), and even in the normotensive offspring of hypertensive parents (Taddei et al., 1996).

1.4 Tools for assessing endothelial function

The endothelium lines all blood vessels, from large conduit arteries to capillaries; Figure 1.5 shows the basic components of the cardiovascular system and highlights key measures of vascular function across this network. EDD responses are commonly used for assessing endothelial function; these will be described in more detail in the following section.

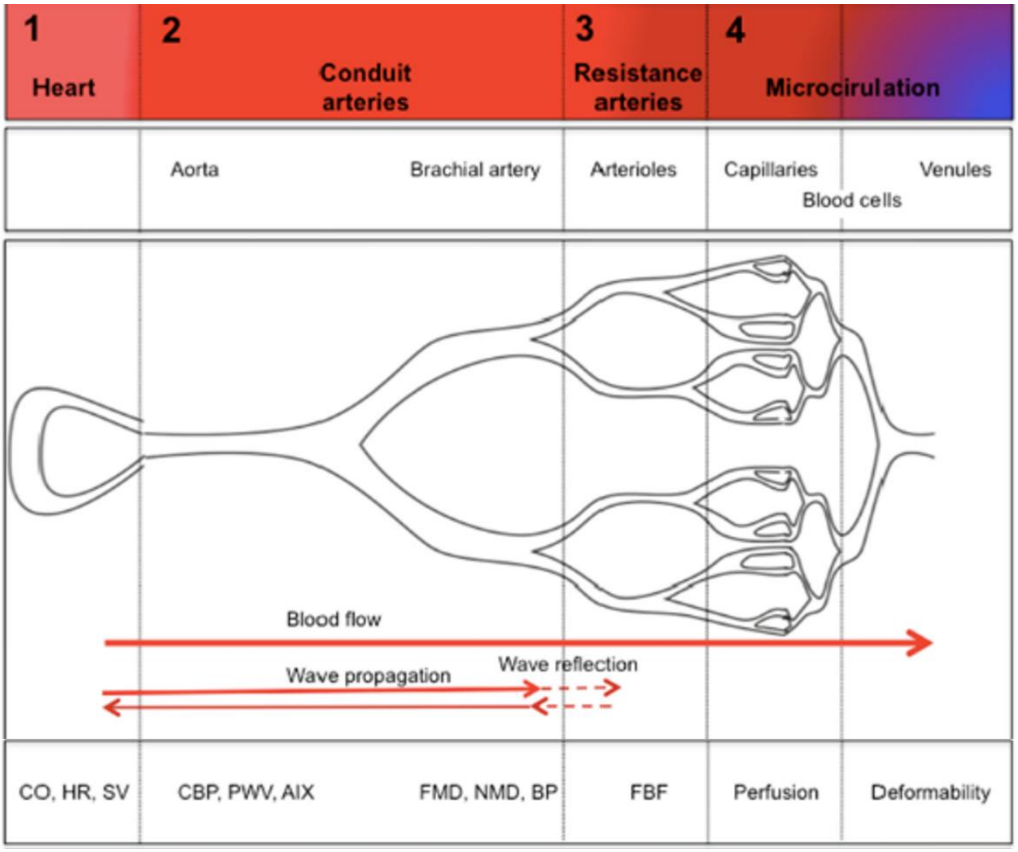

Figure 1.5: Schematic of the cardiovascular system and the basic physiological measuers. *Conduit artery function is determined by flow-mediated dilatation (FMD) and peripheral blood pressure. Forearm blood flow (FBF) represents arteriolar conductance, and capillary perfusion is commonly measured by laser Doppler perfusion imaging. Adapted from (Heiss et al., 2015)*

1.4.1 Flow mediated dilatation

Flow-mediated dilatation (FMD) is a non-invasive technique, which is widely used for assessing endothelial function within conduit arteries, commonly the brachial or femoral artery (Ras et al., 2013). A cuff around the limb is inflated to suprasystolic pressures in order to temporarily occlude arterial inflow, and upon release the rapid increase in blood flow and hence shear stress, results in vasodilatation. This increase in vessel diameter is quantified from ultrasound imaging of the conduit artery to obtain a measure of FMD (Celermajer et al., 1992). Importantly, impaired FMD is closely linked to CVD risk (Ras et al., 2013), with evidence of blunted FMD in many at-risk populations such as type-2 diabetics (Meyer et al., 2008), older adults (Königstein et al., 2022), hypertensives (Muiesan et al., 2008), BAs

(Campia et al., 2002) and SA men (Murphy et al., 2007). FMD is also considered to be of prognostic value in relation to CVD, with a 1% reduction in FMD corresponding to a 13% increase in risk of future cardiovascular events (Inaba et al., 2010).

In terms of the mechanisms underpinning FMD, a strong role for NO has been postulated, and meta-analyses of studies comparing FMD with and without NO-inhibition (by infusion of L-NMMA), have proposed that at least half of the FMD response is mediated by NO (Green et al., 2011, Green et al., 2014), but that the contribution of NO may depend placement of the cuff relative to the imaged artery, with distal placement resulting in a more NO-dependent response than proximal placement (Green et al., 2011). Nonetheless, whilst a role of NO in FMD clear, the response cannot fully be attributed to NO, and other potential contributory factors must also be considered. Possible candidates include PGI_2, which is known to be released from the endothelium due to increased shear stress (Mitchell et al., 2004), and EDHF, for which evidence of attenuated vasodilator responses to a greater extent when EDHF is inhibited alongside NO suggests an interplay of these two systems, and a likely role of EDHF in the FMD response (Bellien et al., 2006). Vasoconstrictors such as endothelin-1 and angiotensin II may also be implicated with FMD; for example, ET_A receptor blockade has been shown to improve FMD in heart failure patients (Berger et al., 2001). Furthermore, increased sympathetic activity has also been shown to impair FMD responses (Dyson et al., 2006, Hijmering et al., 2002). Taken together, these findings suggest that , whilst FMD is predominantly NO-mediated, the response is likely to be explained by the interplay between various vasodilator and vasoconstrictor stimuli, and it is possible that the contribution of each factor differs between healthy individuals and those with increased CVD risk.

1.4.2 Microvascular vasodilator responses

The microvasculature, which comprises resistance vessels (<150μm diameter), terminal arterioles (<15μm diameter), capillaries and venules, is essential for regulating perfusion of all tissues and organs according to local metabolic demand (Levy et al., 2001, Roustit and Cracowski, 2013). Importantly, changes in vascular function within microvessels are thought to precede macrovascular endothelial dysfunction in many cases, therefore the ability to measure microvascular function is of clinical importance (Sena et al., 2013, Bonetti et al., 2003, Minson, 2010). Commonly used techniques include venous occlusion plethysmography (VOP), laser Doppler perfusion imaging (LPDI), capillary microscopy and near-infrared spectroscopy (NIRS), which can be used to measures blood flow across vascular beds such as cerebral, skeletal muscle and cutaneous circulations (Rosenberry and Nelson, 2020, Eriksson et al., 2014, Holowatz et al., 2008). EDD responses to various stimuli can be measured within the microvasculature, for example by reactive hyperaemia (RH), exercise hyperaemia (EH), ACh infusion or mental stress; these will be described in more detail below.

1.4.2.1 Reactive hyperaemia

Reactive hyperaemia (RH) represents the magnitude of limb reperfusion following release of arterial occlusion, providing a non-invasive measure of peripheral microvascular function (Rosenberry and Nelson, 2020). RH is thought to be regulated by a combination of physical myogenic and local metabolic factors, with a strong endothelial contribution (Sparks and Belloni, 1978). In order to ascertain the contribution of key endothelial vasodilator NO, various studies have tested the effect of NOS inhibition, by L-NMMA, on RH; some showed that NOS inhibition blunted total hyperaemic flow but with no effect on peak forearm blood flow (FBF), measured by VOP (Bank et al., 2000, Tagawa et al., 1994), whilst others found no effect of NOS inhibition on peak or total RH (Nugent et al., 1999, Engelke et al., 1996,

Crecelius et al., 2013b). These findings suggest that NO plays some role in RH but does not itself contribute to peak RH. The role of PGs in RH has also been considered, with COX inhibition using various agents showing differing effects on RH; COX inhibition with ketorolac, alongside NOS inhibition, had no effect on peak or total FBF measured with VOP (Crecelius et al., 2013b) whilst COX inhibition by ibuprofen attenuated peak, but not total RH (Engelke et al., 1996). Other studies have shown that inhibition of prostaglandin synthesis blunted both peak and total FBF (Kilbom and Wennmalm, 1976, Carlsson and Wennmalm, 1983).

Adenosine is another vasodilator substance which is directly related to tissue ischaemia (Hellsten and Frandsen, 1997). A study investigating the effect of adenosine receptor blockade, by intravenous theophylline, found this to blunt the total, but not peak RH, whilst dipyridamole, which inhibits adenosine reuptake and thereby prolongs its effects, enhanced total, but not peak FBF (Carlsson et al., 1987). In contrast, dipyridamole has also been shown to enhance peak, as well as total hyperaemic FBF measured by VOP, this effect being prevented with the addition of caffeine (an adenosine receptor antagonist), suggesting that adenosine does indeed contribute to RH (Meijer et al., 2008). Furthermore, K^+ is an important ion for the regulation of vascular tone, through its ability to alter membrane potential of VSM and endothelial cells (Edwards et al., 2010), thus its contribution to RH cannot be ruled out. Whilst it has been shown that blockade of ATP-sensitive K^+ channels, by glibenclamide, has no effect on peak or total hyperaemic FBF, as measured by VOP (Farouque and Meredith, 2003), inhibition of the same channels by tolbutamide resulted in reduction of total but not peak RH (Banitt et al., 1996). Interestingly, combined blockade of inwardly-rectifying K^+ channels and Na^+/K^+ ATPase resulted in a 90% reduction in total RH, suggesting that the action of K^+ directly on the VSM is a key mechanism by which K^+ induces vasodilation in RH (Crecelius et al., 2013b).

Taken together, it appears that no single factor is solely responsible for RH, rather that an interplay of the pathways together likely contributes to the response, and that these exact interactions may differ between individuals and populations. Peak RH can be used as an important predictor of future cardiovascular events in healthy (Anderson et al., 2011) and at-risk populations (Huang et al., 2007), and impaired responses are indicative of endothelial dysfunction, for example in elderly individuals (Rosenberry et al., 2018b), ethnic groups such as SAs (Ormshaw et al., 2018, Petrofsky et al., 2012) and even offspring of hypertensive parents (Hirst and Marshall, 2018).

1.4.2.2 Exercise hyperaemia

During exercise, skeletal muscle blood flow increases proportionally to the increasing metabolic demand of the tissue, a phenomenon known as exercise hyperaemia (EH) (Delp and Laughlin, 1998). This is mediated primarily by local controls, such as metabolic, endothelial, myogenic and muscle pump mechanisms, with some additional central regulation (Joyner and Casey, 2015).

Prostaglandins, particularly PGI_2 and PGE_2, released from endothelial and skeletal muscle cells respectively (Feletou et al., 2011, Testa et al., 2007, Trappe and Liu, 2013), are proposed to play an important role in EH. COX inhibition is shown to attenuate EH by 20-40% at medium-heavy workloads (Cowley et al., 1985, Wilson and Kapoor, 1993, Duffy et al., 1999, Win and Marshall, 2005) and to attenuate PGE_2 and PGI_2 efflux, assessed by immunoassay (Junejo et al., 2020b) . Further, breathing 40% O_2 attenuated EH and PG release to the same extent as COX inhibition, while the combination of COX inhibition and breathing 40% O_2 had no greater effect than either independently, suggesting that the release of PGs is dependent on the fall in O_2 within exercising muscle (Junejo et al., 2020b). In contrast, studies using lower intensity exercise show no effect of COX inhibition on EH (Mortensen et

al., 2007, Shoemaker et al., 1996), implying that the effect of prostaglandins may be greater in higher intensity exercise when O_2 levels fall further (Aiku and Marshall, 2019, Boushel et al., 2002). This may also be related to the interplay between COX products and other vasodilator pathways, with some suggesting that prostaglandins are involved in EH, but when their effect is removed that other vasodilators, such as NO and ATP/adenosine are able to compensate (Schrage et al., 2004). This is supported by evidence that COX inhibition alone had no effect on EH, whereas dual NOS and COX inhibition attenuated EH by 30% following knee extensor exercise at 20% maximum workload (Mortensen et al., 2007). The synergistic activity of NO and prostaglandins is evident, with NO thought to facilitate COX activity via its stimulation of cGMP, which inhibits cAMP catabolism, thereby enhancing dilator responses evoked by mediators such as PGI_2 which act via cAMP (Salvemini et al., 2013, de Wit et al., 1994). The direct contribution of NO to EH is unclear, with NO blockade shown to attenuate peak EH in some studies at various intensities (Dyke et al., 1995, Schrage et al., 2004, Gilligan et al., 1994b), but others show no effect of NO inhibition beyond that on baseline haemodynamics (Rådegran and Saltin, 1999, Frandsenn et al., 2001, Shoemaker et al., 1997).

PGI_2 is also involved in release of ATP from erythrocytes, a process which occurs proportionately to O_2 unloading from haemoglobin (Hammer et al., 2001). There is also evidence of ATP release from skeletal muscle fibres during contraction (Hellsten, 1999, Hellsten and Frandsen, 1997), by lactic acid and O_2 dependent mechanisms (Tu et al., 2010, Marshall and Ray, 2012), and from the endothelium when O_2 levels are low (Lim To et al., 2015). ATP is thus proposed to play a role in EH, with intraarterial ATP infusion shown to evoke muscle vasodilation, and this being attenuated by NOS/ COX inhibition, suggesting that ATP acts via luminal receptors to stimulate NO and PGI_2 release (Ray et al., 2002, Mortensen et al., 2009). A similar effect is apparent with intraarterial adenosine infusion (Ray

et al., 2002, Mortensen et al., 2009), and interstitial adenosine is shown to increase proportionately to exercise intensity (Hellsten et al., 1998). This may be partly due to increased ectonucleotidase activity, which generates adenosine from ATP, in hypoxia such as occurs due to increased O_2 consumption in exercise, resulting in accumulation of adenosine (Hellsten, 1999). Importantly, adenosine-receptor inhibition has been shown to cause a 15-20% reduction in EH (Rådegran and Hellsten, 2000).

Microdialysis has also shown increased interstitial K^+ levels proportional to exercise intensity in humans, with evidence indicating this is released through voltage-gated K^+ channels on the sarcolemma, as occurs during the propagation of skeletal muscle contractions (Juel et al., 2000, Armstrong et al., 2007). This interstitial K^+ is thought to contribute to EH, since inhibition of voltage-gated, as well as Na^+/K^+ ATPase and inwardly-rectifying K^+ channels, has been shown to attenuate arteriolar vasodilator responses to stimulated contractions in hamster cremaster muscle (Armstrong et al., 2007) and to attenuate EH in humans (Crecelius et al., 2013a). These findings support the involvement of K^+ in EH and, since the opening of K^+ channels on endothelial cells is EDHF-dependent, allows the possibility that EDHFs also contribute to EH. Accordingly, there is also evidence that CYP 2CP, which generates EETs, is present in microvascular endothelial cells, and that inhibition of CYP C29 by sulfaphenazole attenuated EH in thigh muscle when given together with L-NMMA, but not alone; this suggests that EETs contribute to EH when the influence of NO is compromised (Hillig et al., 2003).

Taken together, this evidence suggests that EH involves a complex interaction of mechanisms such as NO, prostaglandins, ATP, adenosine and EDHFs, with the endothelium clearly playing a large role in this response. Importantly, there is evidence of impaired EH in populations with increased CVD risk, and associated with endothelial dysfunction, such as in

BAs (Aiku and Marshall, 2019, Barbosa et al., 2018), type-2 diabetics (Senefeld et al., 2019), and older adults (Kirby et al., 2009).

1.4.2.3 Mental stress

Tasks such as the Stroop test, word-identification test, or time-pressured mental arithmetic are commonly used to represent acute mental stress, evoking arousal and autonomic changes which are characteristic of the alerting phase of the defence response (Hilton, 1982). During mental stress, enhanced sympathetic activation and parasympathetic withdrawal evoke an increase in heart rate (HR) which, together with the increased sympathetic noradrenergic activity to most vascular beds, evokes increased arterial blood pressure (ABP) (Mestanik et al., 2015, Wasmund et al., 2002, Carter and Ray, 2009, Kuipers et al., 2008). There is also evidence of muscle vasodilatation in the forearm during mental stress (Butt et al., 1999, Rusch et al., 1981, Freyschuss et al., 1990, Lindqvist et al., 1996b), approximately a third of which is thought to be attributable to increased circulating adrenaline, released from the adrenal glands due to sympathetic activation (Lindqvist et al., 1996b). It was initially thought that withdrawal of muscle sympathetic nerve activity (MSNA), which is associated with vasoconstriction, contributes to this response (Halliwill et al., 1997). However, there is also evidence to the contrary; MSNA, measured directly in the radial nerve, was shown to be unchanged or increased during forearm vasodilator response to mental stress (Anderson et al., 1987, Carter et al., 2005) and forearm vasodilation persists with sympathetic blockade (Halliwill et al., 1997, Lindqvist et al., 1996a). Taken together, these findings suggest neural control alone is not responsible for the forearm response to mental stress, and thus other vasodilator factors should also be considered (Dampney, 2015).

There is mounting evidence for the involvement of NO, derived from both eNOS and nNOS, with L-NMMA infusion shown to attenuate forearm vasodilator responses to mental stress

(Seddon et al., 2008, Cardillo et al., 1998a, Dietz et al., 1994). In healthy individuals, the influence of NO is thought to overcome the increase in MSNA, resulting in forearm vasodilation (Donadio et al., 2012), whereas overall dilator responses are smaller, and constrictor responses are more common in at-risk groups such as SAs (Ormshaw et al., 2018). Importantly, as well as impaired dilator responses, the contribution of NO is also shown to be attenuated in groups with endothelial dysfunction, such as hypertensives (Khan et al., 2015, Cardillo et al., 1998a) and BAs (Cardillo et al., 1998b). This is of clinical relevance as vasoconstriction and accentuated pressor responses to mental stressors are associated with future CVD risk (Chida and Steptoe, 2010).

1.5 The Role of Cocoa Flavanols

Flavonoids, polyphenolic molecules derived from plants, are a large group of bioactive substances accountable for the various beneficial effects of consuming foods such as fruit, vegetables, tea, wine and cocoa (Kozlowska and Szostak-Wegierek, 2014). There are six main subclasses of flavonoids, into which they are classified according to their chemical structure; these are: flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanins (Panche et al., 2016). With the growing interest in the health benefits of flavonoids, in particular flavanols, cocoa is an ideal source from which these can be extracted for research, given the high content of the main types of flavanols, which can be used to determine the benefit of other foods (Bussy et al., 2022, Schroeter et al., 2010). Flavanols comprise 29-38% of total polyphenols in cocoa, and exist as monomers including (-)-epicatechin, (+)-catechin, (+)-gallocatechin or (−)-epigallocatechin: (-)-epicatechin is the most abundant polyphenol in cocoa products, comprising 35% of total polyphenols (Fraga et al., 2019, Aprotosoaie et al., 2016a). Proanthocyanidins are polymers of (-)-epicatechin and (+)-catechin molecules and make up 58-65% of total polyphenols (Aprotosoaie et al., 2016a). Together these polyphenols, and traces of others, represent 12-18% of cocoa bean dry weight, and 460-

610mg/kg of dark chocolate (Jalil and Ismail, 2008). Despite this, the bioavailability of cocoa flavanols (CFs) is relatively low and they have a relatively short half-life, being rapidly excreted (Cooper et al., 2008), but with peak concentrations being reached in the plasma around 2hrs after ingestion and diminishing within the next couple of hours (Rimbach et al., 2009, Goya et al., 2016). The proposed benefits of CFs are widespread, with evidence of anti-inflammation, chemoprotection and prevention of age-associated neurodegenerative diseases (Kozlowska and Szostak-Wegierek, 2014, Aprotosoaie et al., 2016a). Their potential cardioprotective properties are of particular interest, including anti-hypertensive, anti-inflammatory and anti-atherogenic effects, as well as ability to control platelet reactivity, and attenuate endothelial dysfunction (Aprotosoaie et al., 2016b).

1.5.1 Epidemiological evidence

The first evidence surrounding the cardioprotective effects of cocoa arose from observations in Kuna Indians; natives demonstrated a relatively low incidence of hypertension with ageing and a reduction in cardiovascular mortality, though this protection was not conferred in those who had migrated to Panama City, suggesting that the protection was related to environmental rather than genetic factors (Hollenberg et al., 1997). It was also noted that natives living on Kuna Island have some of the highest intake of cocoa worldwide, drinking up to five cups of flavanol-rich cocoa (approximately 900mg) per day; hence their low incidence of cardiovascular events is thought to be associated with this high CF consumption (McCullough et al., 2006).

This proposal has since been supported by evidence from several other epidemiological studies in various other populations. Firstly, the Zutphen Elderly Study involving 470 elderly men highlighted the correlation between cocoa intake and systolic blood pressure (SBP), this being 3.7mmHg lower in participants in the highest compared to the lowest tertiles of cocoa

consumption (Buijsse et al., 2006). The relative risk for cardiovascular mortality was similarly correlated with cocoa-intake, as supported by a study by the same group involving 19357 middle aged participants (Buijsse et al., 2010). Furthermore, the Iowa Women's Health Study of 34,489 post-menopausal women demonstrated that chocolate consumption was inversely correlated with stroke mortality, and that following multivariate adjustment, the risk of CVD was reduced in women with a higher intake (Mink et al., 2007). In the Stockholm Heart Epidemiological Programme, cardiovascular mortality after myocardial infarction was reduced by chocolate consumption in a dose-dependent manner as hazard ratios were lowest at the highest levels of consumption (Janszky et al., 2009). The National Heart, Lung and Blood Institute Family Heart Study on participants ranging in age from 25 to 93 years supported this finding, the age-adjusted risk of CVD being 57% lower in those who consumed chocolate more than five times per week (Djousse et al., 2011). There is also evidence of lower risk of hypertension in participants in the highest quintiles of flavan-3-ol consumption, particularly in those less than 60 years of age (Cassidy et al., 2011). By contrast, in healthy university graduates there appeared to be no association between chocolate intake and hypertension incidence (Alonso et al., 2005); the authors explain the disparity with other studies by crediting it to chocolate consumption being linked to snacking and hence to a higher sugar intake in this population group .

1.5.2 Randomized Controlled Intervention Trials

There are clear limitations of using epidemiological studies as evidence, such as the inability to control flavanol intake relative to the rest of the diet, and reliance on participants accurately reporting their food habits. As such, intervention studies, whereby participants are administered known amounts of flavanol-containing cocoa products are more appropriate for assessing their cardiovascular impact. These are considered below.

1.5.2.1 Arterial blood pressure

Slight reductions in ABP can have marked effects on cardiovascular health, with even a 2mmHg fall in SBP conferring a 10% reduction in stroke mortality, and 7% lower mortality from ischaemic heart disease or other vascular diseases (Lewington et al., 2002), and a 10mmHg decline in ABP dramatically reducing risk of major cardiovascular events, thus contributing to a 13% reduction in all-cause mortality (Ettehad et al., 2016).

Initial studies conducted in spontaneously hypertensive rats showed that an (-)-epicatechin supplemented diet decreased ABP by 23mmHg after six days (Galleano et al., 2013). Furthermore, reductions have been shown at several doses; the maximal effect, obtained at the middle dose of 300mg, was similar to the reduction in ABP achieved with captopril, an angiotensin-converting enzyme inhibitor widely used as an anti-hypertensive drug (Cienfuegos-Jovellanos et al., 2009). However, ABP was shown to increase upon withdrawal of cocoa intake, suggesting a short duration of effects conferred by cocoa consumption (Sanchez et al., 2010).

Following on from this evidence, various randomised controlled trials (RCTs) have been conducted in humans, aiming to determine the potential of CFs for reducing blood pressure across many populations, the results of which have been collated in several reviews, the findings of which are outlined below.

A Cochrane Review of 35 RCTs showed significant reductions in both SBP and diastolic blood pressure (DBP; approximately -1.76mmHg each) in a total of 1804 participants from trials ranging 2 to 18 weeks. Notably, differences were apparent depending on baseline blood pressure, with hypertensives and pre-hypertensives showing larger effects than normotensives (Ried et al., 2017). This theme was common in previous reviews conducted by the same group, they also showed overall reductions in SBP and DBP in the entire population, but

retaining statistical significance only in studies involving hypertensive and pre-hypertensive patients (Ried et al., 2010, Ried et al., 2012). Other meta-analyses incorporating findings from healthy or stage-1 hypertensive individuals suggested larger mean reductions in SBP compared to DBP; these being 4.5mmHg and 2.5mmHg (Desch et al., 2010), and 4.7mmHg and 2.8mmHg (Taubert et al., 2007); a limitation of both these studies was the small datasets, incorporating 297 and 173 individuals respectively. Nonetheless, variability between effects on SBP and DBP was also apparent in a larger study which showed show a 1.63mmHg fall in SBP but no significant effect on DBP, in their meta-analyses of studies on 1106 participants (Shrime et al., 2011).

Overall, the evidence from these studies implies an ABP-lowering effect of CFs both in healthy individuals and patients with increased CVD risk. Despite this, the techniques used to measure ABP may influence findings; ambulatory monitoring is typically much more accurate than the use of single office measurements which may be elevated to differing extents in anxious patients (Muntner et al., 2019). Furthermore, it is difficult to decipher the cause and mechanism of these differences, since blood pressure is determined by integration of local, neural, renal-endocrine, and hormonal mechanisms (Chopra et al., 2011).

1.5.2.2 Vascular function

As well as the apparent role of CFs in regulating blood pressure, whether they can directly impact vascular function has also been a widely studied topic. A meta-analysis of 9 studies reporting effects of chronic CF supplementation, ranging from 2-12 weeks, showed an overall 1.53% improvement in FMD, despite significant heterogeneity among studies (Shrime et al., 2011). Furthermore, a more recent review of 15 RCTs, including data from 730 participants also found improvements in FMD following daily consumption of CFs (1-12 weeks) in the form of dark chocolate (0.84% increase in FMD) and cocoa drinks (1.13% increase in FMD)

(Sun et al., 2019). This study also highlighted that the maximum improvement in FMD was shown in studies using a daily dose of 900mg total flavonoids, and 50-150mg (-)-epicatechin, with the (-)-epicatechin dose proposed to drive the biological effect (Sun et al., 2019). The disparity in the magnitude of effect in studies using dark chocolate compared to cocoa flavanol interventions was also demonstrated in another review of chronic studies spanning 2-84 days (Ebaditabar et al., 2020). Across 17 studies, including 615 participants, there was a 0.69% improvement in FMD with dark chocolate supplementation, meanwhile in 6 studies, including 179 participants, a 1.16% increase in FMD was shown with flavonoid supplementation, and a significant improvement (0.61% increase) shown after pooling of all studies (Ebaditabar et al., 2020). A non-linear dose response relationship was also apparent, with the greatest improvement in FMD at doses around 400-600mg/day. The same review also considered the effect of acute CF supplementation, which was assessed in 8 studies, and a 0.41% improvement in FMD was found (Ebaditabar et al., 2020). The magnitude of improvement in FMD shown by these meta-analyses, particularly chronically, is physiologically relevant; a 1% difference in FMD corresponds to a 13% change in CVD risk, thus these small improvements in FMD could dramatically reduce an individuals' CVD risk (Inaba et al., 2010).

Importantly, these analyses were conducted across a mixture of healthy and clinical populations, and across a range of doses and duration, with significant heterogeneity amongst studies. Sub-group analysis, for example comparing effects between groups stratified by age, body mass index (BMI) or dose ranges, could not account for this (Shrime et al., 2011, Sun et al., 2019, Ebaditabar et al., 2020), suggesting reasonable consistency of results across these groups.

There is also evidence of CFs improving microvascular function in a recent review, which proposed the contribution of CFs in the microvasculature may be greater in healthy

individuals compared to those with increased CVD risk (Woodward et al., 2018). The role of CFs within the microvasculature is further explored in Chapter 3.

1.5.3 Underlying mechanisms of action

Following on from mounting evidence of CF's beneficial effects on cardiovascular function, focus turned to investigating the mechanism by which these effects are exerted. Early evidence for the involvement of NO in CF-mediated effects came from findings that peak epicatechin levels, and elevated circulating plasma nitro-adducts, coincided with the improvement in FMD following intake of high flavanol cocoa drink (Heiss et al., 2003). Mechanistic studies have mainly focussed on the contribution of (-)-epicatechin, this being the main constituent of CFs (Aprotosoaie et al., 2016a), though other CFs are likely to act via similar pathways but to a smaller extent, with (+)-catechin treatment shown to elicit only ~25% of the maximal effects of (-)-epicatechin (Ramirez-Sanchez et al., 2011).

In broad terms, NO bioavailability can be increased either by enhancing NO production, or by inhibiting NO degradation. It was initially proposed that the main mechanism whereby CFs increased NO bioavailability was by acting as antioxidants, and this was supported by *in vitro* evidence showing a correlation between total polyphenol content and oxygen radical absorbance capacity (Keen et al., 2002), as well as cell culture studies demonstrating increased plasma antioxidant capacity and reductions in low-density lipoprotein oxidation following flavanol treatment (Waterhouse et al., 1996, Vinson et al., 1999, Lotito et al., 2000). However, it seems more likely that any antioxidant activity observed is indirect and occurs via interference with prooxidant enzymes such as NADPH oxidase or lipoxygenase, because the poor bioavailability and low plasma concentrations of CFs means that they do not reach sufficient levels to exert antioxidant effects (Schewe et al., 2008). Regulation of NADPH oxidase activity by CFs was originally postulated following observations that (-)-

epicatechin pre-treatment suppressed NADPH oxidase-mediated O_2^- production, as determined by ferricytochrome-c reduction assay, effects which were replicated by apocynin, a known NADPH oxidase inhibitor (Steffen et al., 2007a). In a subsequent study, steady-state NO levels measured with an NO-sensitive fluorophore, were found to be similarly elevated by (-)-epicatechin and apocynin both at baseline, and following exposure to angiotensin II (which elicits oxidative stress via activation of NAPDH oxidase). (Steffen et al., 2007b). Thus, there is clear evidence that one of the ways in which flavanols improve EDD is by blunting the activity of NADPH oxidase, which is a major enzyme contributing to ROS accumulation (Cai and Harrison, 2000).

In terms of increasing NO bioavailability, a role for CFs in increasing NO production via eNOS has also been postulated. Immunoblots measuring relative eNOS phosphorylation at activation residues (ser-1177 and ser-633) and inactivation residues (thr-495) showed significant changes in phosphorylation statuses of these residues in favour of activation of eNOS following (-)-epicatechin treatment (Ramirez-Sanchez et al., 2010). The study also demonstrated that inhibition of PKA or Akt, by H89 or SH5 respectively, partially blocked eNOS phosphorylation, thereby reducing NO synthesis, thus implicating the PI3K pathway in (-)-epicatechin-mediated NO synthesis (Ramirez-Sanchez et al., 2010). Furthermore, the eNOS phosphorylation effects were also blocked by U73122 and KN-93, inhibitors of PLC and CaMKII activation respectively, suggesting that the actions of (-)-epicatechin are also mediated via the calcium-dependent PLC pathway (Ramirez-Sanchez et al., 2010).

These pathways are thought to be activated via binding of (-)-epicatechin to a cell surface receptor, since membrane-impermeable (-)-epicatechin was shown to induce phosphorylation of PI3K, AKT and eNOS, to an even greater extent than membrane-permeable (-)-epicatechin (Moreno-Ulloa et al., 2014). The proposed entity of this receptor was the G-protein coupled oestrogen receptor (GPER), due to its cardioprotective effects and activation of intracellular

signalling pathways similar to those implicated with (-)-epicatechin (Prossnitz and Barton, 2014). This role was consolidated by evidence of favourable binding between (-)-epicatechin and GPER, comparable to that by the GPER agonist, G1, and selective blockers demonstrated activation of the ERK1/2 pathway (Moreno-Ulloa et al., 2015). Furthermore, epicatechin was shown to elicit vasodilation of pre-constricted aortic rings through GPER activation (Moreno-Ulloa et al., 2015). The involvement of the GPER in mediating the effects of CFs raises the question as to whether these effects would be greater in women, compared to men, due to the enhanced role of oestrogen. Despite this, the majority of studies assessing the effect of CFs on cardiovascular health are conducted in male or mixed populations.

1.6 General aims and hypotheses

The general aim of the current project was to elucidate the potential of CFs for improving endothelial function within young, healthy women. We aimed to compare microvascular vasodilator responses, and the contribution of CFs to these in young WE and SA women, since SAs are a group with increased CVD risk. Further, we aimed to compare diet and physical activity between these ethnic groups, in order to ascertain whether lifestyle differences may contribute to SA's elevated CVD risk. Unfortunately, the experimental elements of the project were limited due to the COVID-19 pandemic. The study comparing vasodilator responses in young women was interrupted by lockdown meaning that no participants could attend the laboratory for over a year, and thus there was not time to conduct a follow-up study once this had been completed. Instead, the systematic review and questionnaire studies were begun during the pandemic and were expanded to provide detailed insight into the diet and lifestyle of ethnic groups studied.

Briefly, the aims and hypotheses of the studies conducted are as follows (these are properly introduced in the relevant chapters):

- Chapter 3:
    - Aim: conduct a systematic review of existing evidence for the role of CFs in the microvasculature
    - Hypothesis: CFs will improve endothelial function and vasodilator responses across microvascular beds
- Chapter 4:
    - Aims: compare RH measured with VOP and with NIRS to determine whether NIRS-derived ΔtotalHb can provide a useful index of FBF, and to compare responses between young WE and SA women
    - Hypothesis: NIRS-derived ΔtotalHb will be well correlated with FBF measured by VOP during RH, and RH will be greater in WE than SA women
- Chapter 5:
    - Aims: assess the effect of an acute CF intervention on vasodilator responses to RH, EH and mental stress in young WE and SA women using NIRS
    - Hypothesis: peak RH, EH, and vasodilator response to stress will be greater following high-flavanol cocoa compared to control
- Chapter 6:
    - Aim: compare diet and physical activity between young WE and SA men and women
    - Hypothesis: WEs will be more physically active than SAs and will have higher intake of foods/nutrients that are considered cardioprotective

Chapter 2:

General Methods

2.1 Study Participants

All participants involved were young (aged 18-26 years), South Asians (SAs) or White Europeans (WEs). Ethnicity was self-reported, and participants were required to have both parents of the same ethnic origin as advised by the Office for National Statistics (ONS, 2018). Participants for all studies were briefed on what the study would involve, how their data would be used and their option to withdraw from the study at any time, then were asked to sign a consent form (Appendix 1). All studies were conducted in accordance with approval granted by the University of Birmingham Ethics Committee (ERN17_1755).

For the laboratory study (Chapters 4 and 5), participants were recruited from the University of Birmingham by word of mouth and poster advertisements. Prior to the first visit, participants were asked to complete a General Health Questionnaire (Appendix 5), in order to ascertain their eligibility for the study. Inclusion criteria were that subjects must be female; non-smokers; not consume more than 21 units of alcohol per week; have no history of cardiovascular, respiratory, metabolic, liver or inflammatory disease; not have any food intolerances or allergies; not be on a weight reducing dietary regime, or taking any dietary supplements; not be on any long-term medication, or have been on antibiotics in the last 3 months; and no known medical disorder at the time of visit. The questionnaire study (Chapter 6) included WE and SA participants of any gender recruited from across the UK.

2.2 Experimental Conditions

For the laboratory study (Chapters 4 and 5), participants attended the temperature-controlled laboratory (20-22ºC) on two occasions. They were seated on a couch reclined at ~45º, with their legs outstretched and both arms supported at approximately heart level by cushions to their sides (Figure 2.1A). The recording equipment and trace displays was arranged behind

the participant, in order to avoid potential distractions. Before the protocol began, participants were habituated with the equipment and expectations of them during the study, often by attending a familiarisation visit.

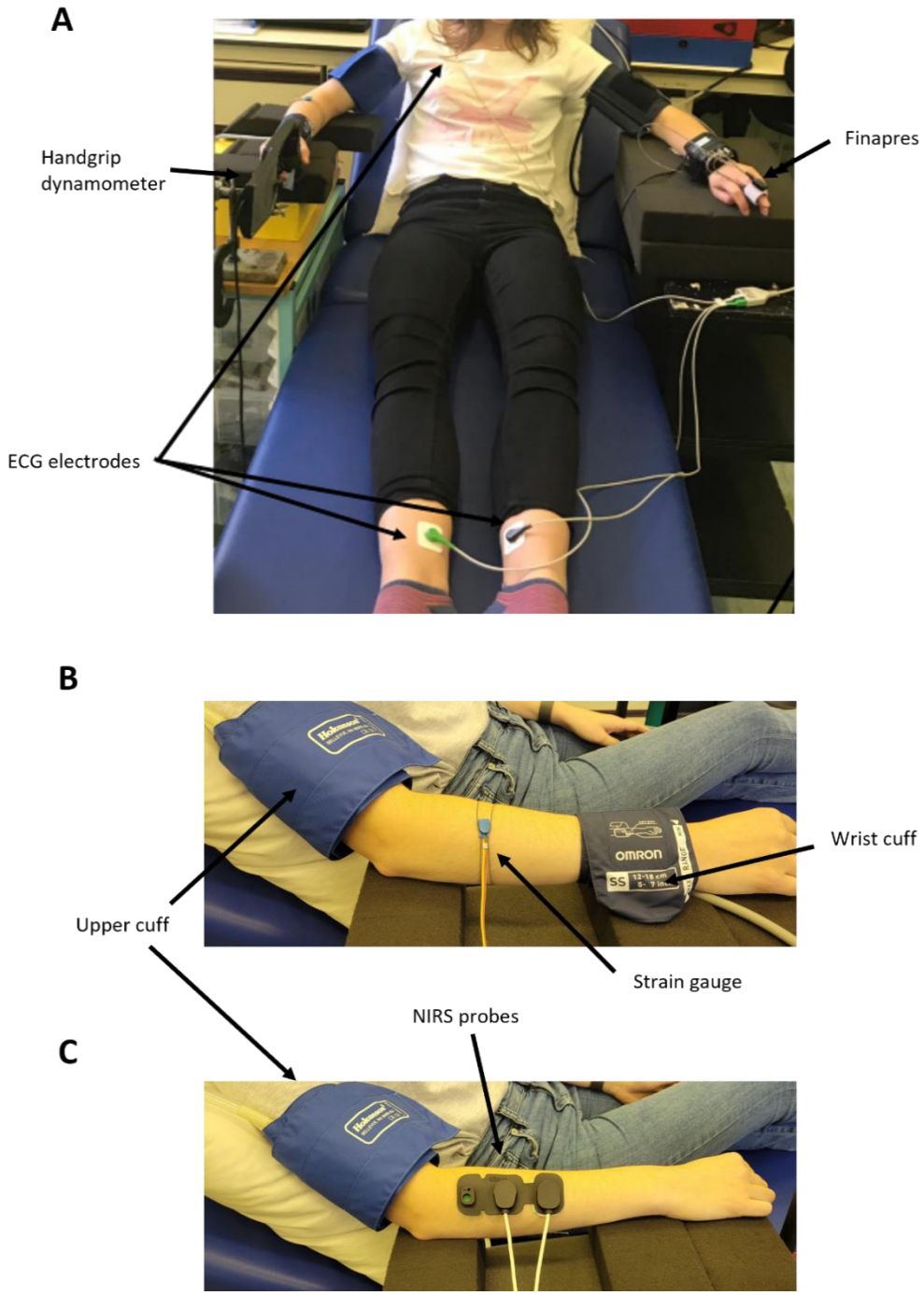

Figure 2.1: Participant set-up for venous occlusion plethysmography (VOP) and near- infrared spectroscopy (NIRS) recording*. (A) shows the participant reclined on the couch with arms supported with cushions to either side. The set-up of equipment for recording on the dominant forearm is shown for (B) VOP and (C) NIRS*

2.3 Measurements

2.3.1 Electrocardiogram (ECG)

Cardiovascular variables were monitored continuously throughout the laboratory protocol. Electrocardiogram (ECG) recordings were made from three electrodes placed on the skin, these were positioned proximal to the left and right malleolus on each ankle and below the right clavicle, areas which required minimal movement throughout the protocol, thus limiting noise on recordings. The ECG was used to determine changes in heart rate (HR), represented by the reciprocal of the R-R interval, automatically calculated by the LabChart8 programme to which the ECG pre-amp was connected via PowerLab 4/35 (AD Instruments).

2.3.2 Arterial blood pressure (ABP)

Pulsatile ABP was monitored non-invasively using the Finometer® MIDI (Finapres Medical Systems), via a finger cuff of the appropriate size secured around the middle finger of the non-dominant hand. The control unit was fastened around the participant's wrist, and the arm was arranged on a cushion at heart level. The Finapres was connected to the PowerLab 4/30 (AD Instruments), and the output displayed using LabChart8. This enabled real-time visualisation and beat-by-beat measures of ABP and HR throughout the protocol.

2.3.3 Forearm blood flow (FBF)

Strain gauge venous occlusion plethysmography (VOP) was also used to measure the changes in forearm blood flow (FBF) evoked by RH. VOP is recognised as a reliable method for recording blood flow in the forearm (Joyner et al., 2001, Wilkinson and Webb, 2001) and works on the principle that the initial rate of increasing forearm circumference during venous occlusion corresponds to the rate of arterial inflow (Greenfield et al., 1963). A rapid inflation cuff (AG101 Cuff Inflator Air Source; E20 Rapid Cuff Inflator Hokanson Inc.) was wrapped

around the upper part of the dominant arm and a second manually inflatable paediatric sphygmomanometer cuff positioned around the wrist of the same limb. The strain gauge and gadolinium-filled silastic tubing connected to the plethysmograph (EC6, Hokanson Inc) was fastened around the widest part of the forearm, which was elevated by supports under the elbow and wrist (Figure 2.1B). The length of strain gauge was selected on the basis that it should be 2cm smaller than the widest circumference of the subject's forearm (Joyner et al., 2001). Strain gauge output was connected with the other variables to a computer via PowerLab 4/35 (AD Instruments) and outputs displayed via LabChart8.

Each measurement of FBF was made by inflating the upper cuff to 50mmHg to occlude venous drainage from the forearm whilst maintaining inflow through the brachial artery, hence expanding forearm circumference and increasing stretch on the strain gauge. It was important that the wrist cuff was inflated to 200mmHg prior to, and maintained throughout, each measurement of FBF as this occludes blood flow to the hand so that hand circulation is not included in the measurement (Higashi, 2015). Each VOP cycle was at least 15s, allowing for 8s venous occlusion and 7s relaxation, and measurements of FBF were taken at baseline and for 2 mins following arterial occlusion.

2.3.4 Near-infrared spectroscopy (NIRS)

NIRS is a widely used technique for non-invasively monitoring perfusion and oxygenation, such as of muscle and cerebral tissues, via probes placed on the skin. The principle behind the technique relates to the Beer-Lambert law, whereby light is absorbed as it passes through a compound, thereby decreasing intensity of detected light. Infrared light, at wavelengths 700 to 1100nm, is delivered into the tissue via an emitting probe placed on the skin, and the intensity of the exiting light is measured by a receiving probe; the difference between these represents the amount of light absorbed by the tissue, and hence the chromophore

concentration (Barstow, 2019). The main chromophores within skeletal muscle are haemoglobin (Hb) and myoglobin (Mb), which absorb light differently depending on whether oxygen is bound to the iron core, enabling continuous monitoring of oxygen saturation (Mozina and Podbregar, 2011). The exact proportional contributions of Hb, from the muscle microvasculature, and intracellular Mb, from the interrogated muscle, to the total NIRS signal strength remain unknown (Marcinek et al., 2007, Mancini et al., 1994). Nonetheless, since intracellular Mb will remain constant during acute interventions such as exercise or RH, changes in NIRS signals can largely be attributed to the tissue Hb (Davis and Barstow, 2013). Indeed, many NIRS systems, including the one used, provide outputs of oxygenated (oxy-) and deoxygenated (deoxy-) Hb only, assuming the contribution of Mb is minimal. Importantly, the Hb signal is thought to originate in blood vessels less than 1mm in diameter, hence is useful for monitoring changes in the tissue microvasculature (Cui et al., 1991).

The NIRS probe, comprising emitter and detector (MoorVMS-NIRS Near Infrared Oxygen Monitor, Moor Instruments) was placed on the muscle belly of the forearm (Figure 2.1C). A probe separation of 40mm was used; as NIRS light penetrates approximately half the distance between the probes, this allowed a penetration depth of 2cm, which is recommended for monitoring the forearm skeletal muscle (Homma et al., 1996b). The same separation was used for all participants tested. The monitoring arm was positioned comfortably on raised cushions next to the couch and covered with a black cloth in order to minimise effects of background light.

Each set of probes provides simultaneous recordings of tissue oxyHb and deoxyHb. These are combined to give totalHb, an index of microvascular blood flow, and oxygen saturation (SO_2, oxyHb/totalHb) gives a measure of tissue oxygenation, expressed as a percentage.

2.4 Vasodilator Stimuli

2.4.1 Reactive hyperaemia

RH was induced by inflation of the upper cuff to 200mmHg for 2mins. Participants were informed what sensations they could expect in their arm during the occlusion and recovery period and were instructed to keep their arm as still as possible throughout, as any movement would affect the recording devices. For measurement with VOP, the wrist cuff was inflated 10s before the end of the 2 mins occlusion period, so as to exclude the hand circulation prior to the first measurement. At the end of the 2 mins, the upper cuff was deflated from 200mmHg and then reinflated as quickly as possible (within <5s) to 50mmHg to obtain the first measure of FBF. FBF was measured over 15s cycles for the first min and 30s cycles for the second min after occlusion. The wrist cuff remained inflated throughout this 2min recovery period. When measured with NIRS, the upper cuff was inflated for the same 2min duration, but no other action was required as data was later extracted from traces offline.

2.4.2 Exercise hyperaemia

Exercise hyperaemia (EH) was induced by submaximal rhythmic handgrip for 2mins. At the start of the protocol, Maximum Voluntary Contraction (MVC) was measured. The handgrip dynamometer (Lafayette Instrument Company; Loughborough, UK) was positioned for comfortable use by the dominant hand, and the subject was asked to grip with maximum strength for a couple of seconds; this was repeated three times and the highest measurement deemed to represent their MVC. The output of the dynamometer was displayed via the transducer so that MVC was set as 1.0; this was positioned so that the subject could visualise the force exerted on each contraction. The subject performed rhythmic handgrip in time with a metronome (1s contraction, 1s relaxation) for 2 min, aiming for each contraction to reach

60% MVC (displayed as 0.6 on transducer). Participants were instructed to maintain this level of force as best they could, and they were encouraged to continue with the rhythm of the metronome even if they were not able to reach the target force. Following exercise, the subject retained this position of the dominant arm for a further 3 min and NIRS monitoring was continued during this recovery period.

2.4.3 Mental stress

Mental stress was elicited by the 8min Paced Auditory Serial Addition Task (PASAT). This has previously been shown to induce a physiological stress response with repeatable and reliable results (Baynham et al., 2021, Paine et al., 2013, Ginty et al., 2013). The PASAT is an 8min pre-recorded audio sequence of numbers 1-9, which progressively increases in speed, and participants were required to add sequential numbers on the recording, giving their answers verbally for the experimenter to mark. A buzzer was sounded for incorrect answers or hesitation, or otherwise at the end of every 10-answer block. Before beginning the task, the subject listened to a pre-recorded set of instructions and was given the opportunity to ask questions before undertaking a short practice test. The NIRS equipment remained in place on the dominant arm throughout the task, and participants were told to remain still in order not to impact the recording.

2.5 Data acquisition and analysis

2.5.1 Venous occlusion plethysmography (VOP)

FBF (ml per min per 100g tissue) was calculated from the VOP recordings using the equation below; maximum average slope was taken across the first two cardiac cycles following venous occlusion, and average calibration value was taken as the mean of the three 1%

calibrations (Figure 2.2). Baseline FBF was calculated from a mean of four 15s cycles of VOP (8s occlusion, 7s relaxation) prior to RH. Peak RH was taken as the first measure of FBF made following deflation of the upper cuff at the end of arterial occlusion, and reinflation for VOP.

$$Forearm\ Blood\ Flow\ (ml.min^{-1}(100g\ tissue)^{-1}) = \frac{2\ x\ average\ slope}{average\ calibration}\ x\ 60$$

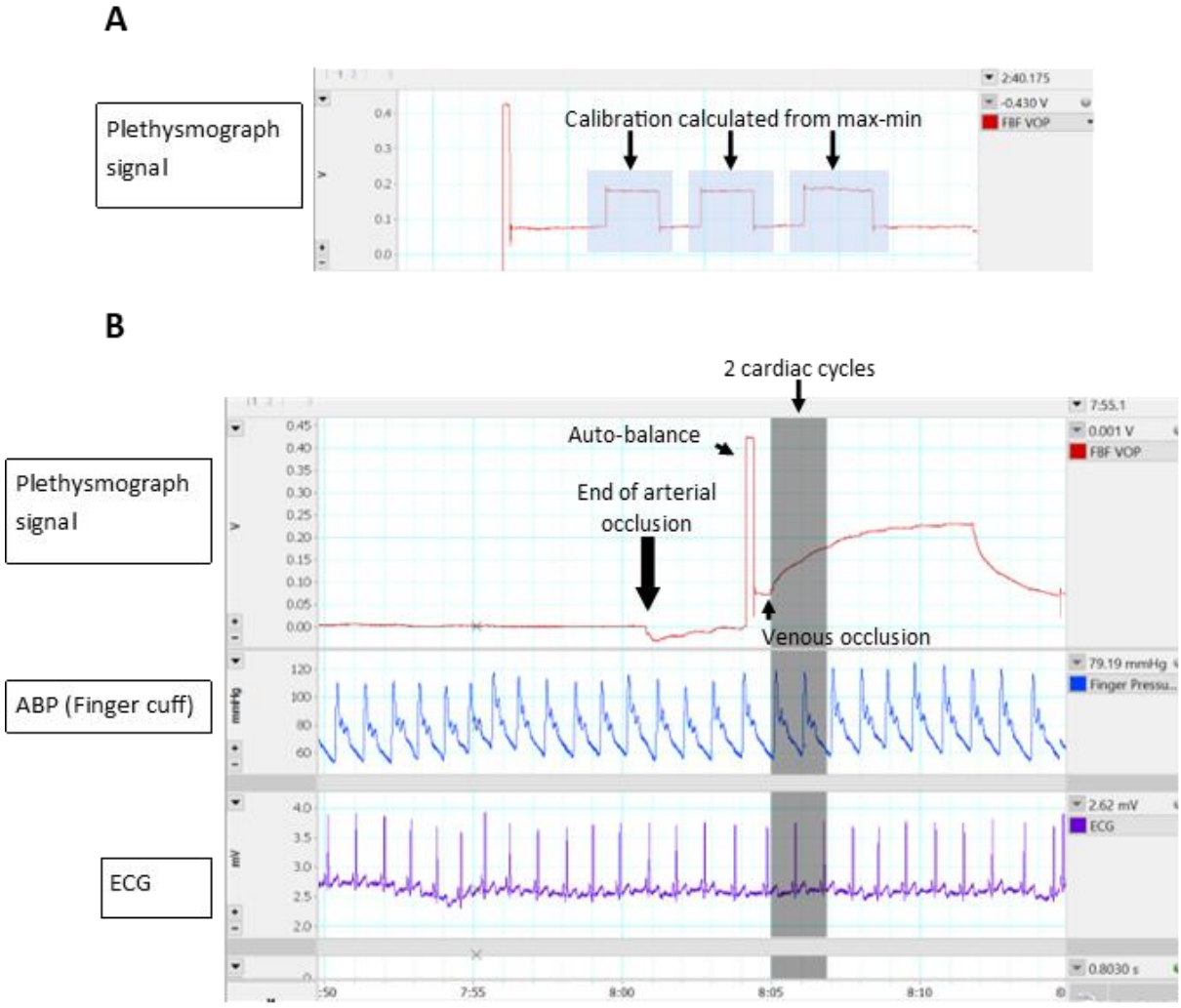

Figure 2.2: Data extraction from Labchart traces for venous occlusion plethysmography (VOP). *(A) Average calibration calculated as the mean from 3 calibrations (maximum- minimum) at the start of VOP recording. (B) Slope of plethysmograph signal extracted across the first 2 cardiac cycles after venous occlusion*

2.5.2 Near-Infrared Spectroscopy (NIRS)

NIRS outputs were collected separately on a Windows laptop supplied with Moor Instruments software (Figure 2.3). To enable accurate cross-referencing with the information stored in LabChart8, each intervention was labelled on both datasets and time-points noted. Data was extracted from the NIRS traces by highlighting regions of interest (ROI) at regular intervals, with time-points being selected to enable the closest possible representation of the time course of responses. A ROI was selected prior to onset of each stimulus to represent baseline. For RH, ROIs were selected at 0s, 5s, 10s, 15s, 30s, 45s, 60s, 90s and 120s. During forearm contractions, ROIs were at 5s intervals for the first 20s, then 20s intervals for the remainder of the 2 min period and at the same intervals during the 3 mins post-exercise. During mental stress, ROIs were selected every 15s for the first min, then every 30s for the remaining 7 mins. Following visualisation of the stress response throughout the 8min period, it was apparent that not all these time-points were required in order to capture the time-course of the response, and only ROIs at 2,4, 6 and 8mins were used for statistical analysis. All ROIs, excluding those made during exercise, represented the mean of a 0.5s period, allowing flat portions to be selected and avoiding any movement artefacts. During exercise, each value extracted was averaged over 2s spanning a contraction and relaxation period (Figure 2.3C). All data extracted were stored in Excel for easy collation of information from all participants.

Continuous NIRS monitoring does not provide absolute chromophore concentrations due to the actual light path length being unknown (Barstow, 2019), rather is useful for observing changes relative to initial arbitrary baseline. As such, the baseline was deducted from each time-point for all Hb measures, and values presented in arbitrary units (AU). On the basis that totalHb provides an index of FBF, forearm vascular conductance (FVC) index was calculated by dividing ΔtotalHb by MABP at the same time-point; this was included only during exercise and mental stress during which changes in MABP could be expected.

Meanwhile, SO_2 is expressed as the percentage of oxyHb/totalHb. An example SO_2 schematic during and following occlusion release is shown in Figure 2.4, alongside the measures calculated from this. Slope 1 represents the desaturation rate during cuff occlusion; as arterial occlusion prevents oxygen-rich blood from flowing into the forearm, the reduction in oxygen saturation during this period is directly related to tissue oxygen consumption and can be considered an indirect measure of skeletal muscle metabolism (Doerschug et al., 2007). The minimum SO_2 at the end of occlusion represents the stimulus to vasodilate. Meanwhile Slope 2 represents the rate of reperfusion in the first 10 seconds following occlusion release, thereby providing an indication of microvascular reactivity (Soares et al., 2020). SO_2 max and hyperaemic reserve (SO_2 hypr, difference between baseline and peak SO_2) are measures of the peak hyperaemic response.

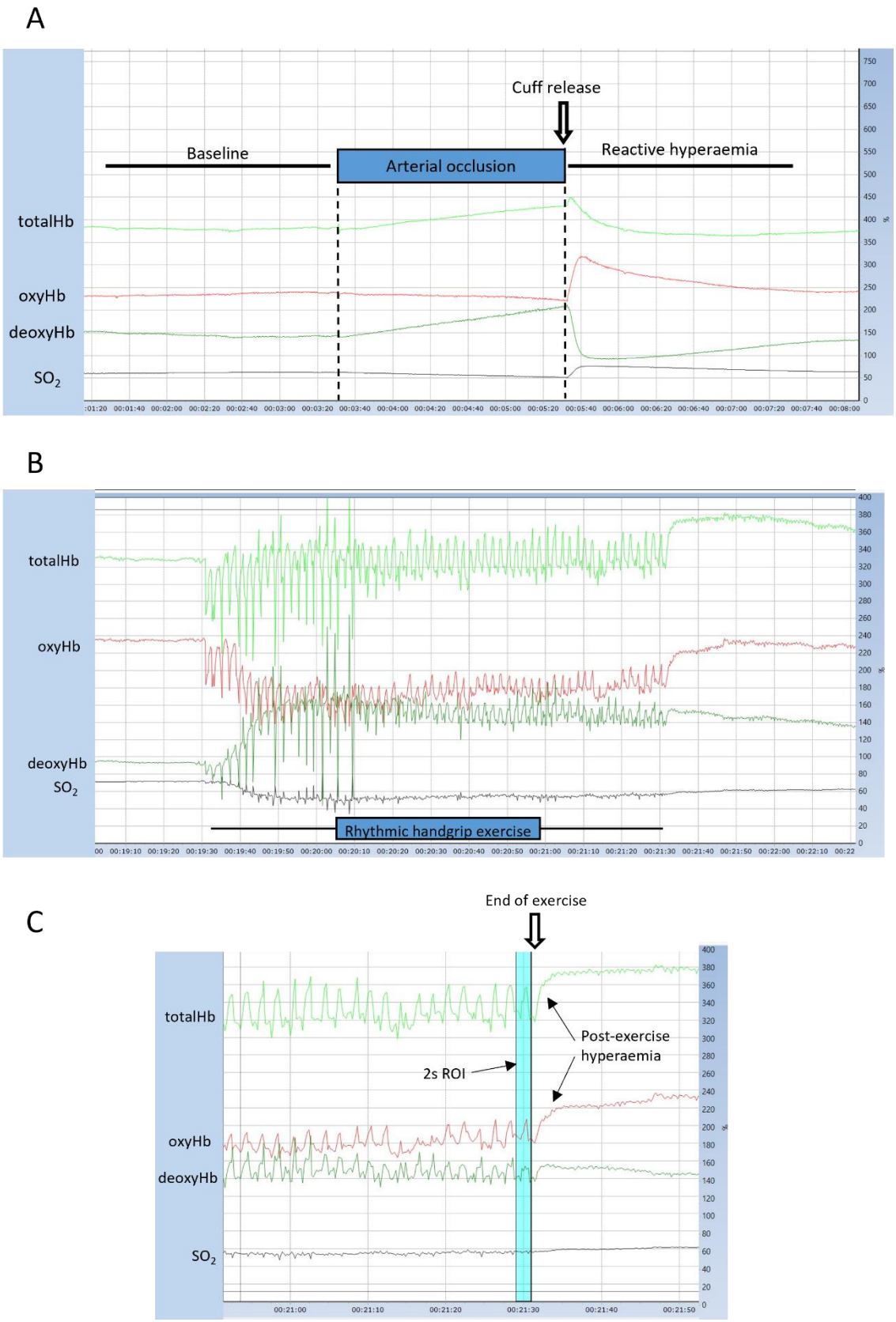

Figure 2.3: Near-infrared spectroscopy (NIRS) trace showing (A) reactive hyperaemia, (B) rhythmic handgrip and exercise hyperaemia, and (C) data extraction from exercise trace by selecting region of interest (ROI). *Continuous traces shown for oxygen saturation (SO_2), oxygenated (oxy-), deoxygenated (deoxy-) and total haemoglobin (totalHb)*

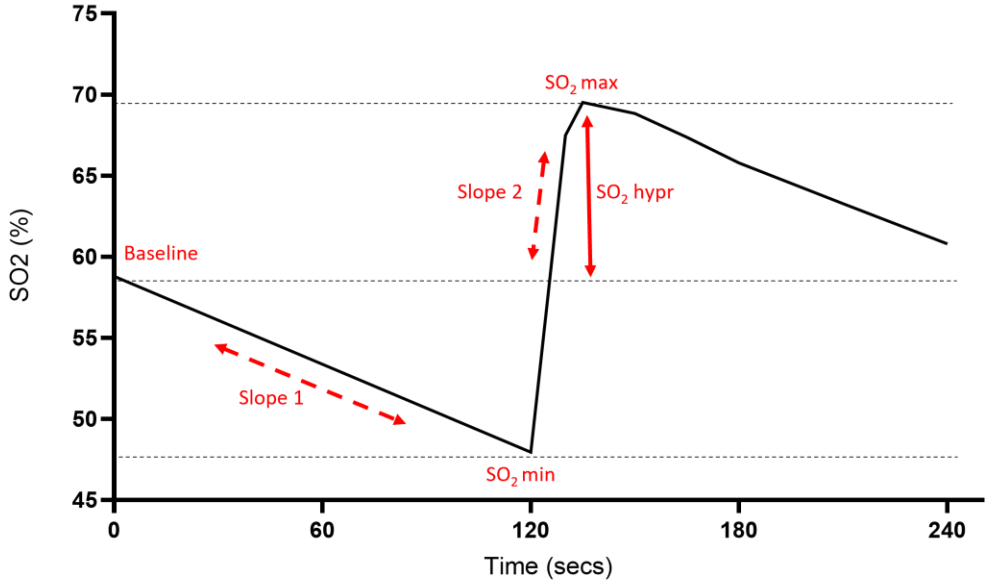

Figure 2.4: Tissue oxygen saturation (SO_2) profile during and after arterial occlusion. *Slope 1: desaturation slope, SO_2 min: mimumum SO_2; SO_2 max: maximum SO_2; slope 2: 10s reperfusion slope; SO_2 hypr: hyperaemic reserve (SO_2 max- baseline)*

2.6 Questionnaires

Information on participants' diet and lifestyle were collected as part of the experimental and questionnaire studies (Chapters 4-6). This included a General Health Questionnaire (Appendix 5) and 3-day food diary (Appendix 6) as well as the widely available EPIC-Norfolk Food Frequency Questionnaire, and International Physical Activity Questionnaire (short form).

2.6.1 EPIC Food Frequency Questionnaire

The EPIC- Food Frequency Questionnaire (FFQ) is widely used to assess participants' average weekly consumption of various foods during the last year in order to estimate their nutrient intake (Mulligan et al., 2014); this was used to gain an insight into participants' habitual diet as part of the laboratory and questionnaire studies. The main part of the questionnaire comprises a list of 130 food items, for which participants were asked to indicate their usual frequency of consumption from 9 categories ranging from 'never or less

than once per month' to '6+ times per day'. For many items the serving size is specified in terms of common portion size (e.g., one slice) or household measures (e.g., glass, spoon) and a common serving size is assigned to each question which is used for all participants. The second section of the questionnaire asks if there are any items outside of the list that participants consume regularly. It also asks about the type and quantity of milk that they consume, as well as the type of fats they cook with and how much visible fat on meat they eat; these questions relate to the relevant items in the previous part of the questionnaire in estimating participants' total fat consumption.

The FFQ data was interpreted using FETA software (Mulligan et al., 2014), which uses comma-separated values input files to process participant answers and generate output estimations of each respondents' consumption of various dietary components, including macronutrients, vitamins and flavanols. Each frequency category is converted to a proportion multiplier (e.g. once per week= 1/7; multiplier: 0.14), which is multiplied by the designated portion size to obtain an average daily food weight for each item on the list. This is then multiplied by the known nutrient composition per gram of each food item to calculate the nutrient composition of the actual amount of each food eaten, and all items are summed for each participant to generate an average daily intake of each nutrient.

2.6.2 Three-day Food Diary

The three-day food diary was used to provide a snapshot of participants' daily diet as part of the questionnaire study only (Chapter 6). In order to obtain the most in-depth depiction possible of food consumed throughout the day, participants were asked to provide quantities and preparation methods. Food-diary responses were manually inputted to Dietplan6 software to provide total nutrient consumption during the three-day period for each participant which was used to calculate daily intake. Where possible, portions used were exactly as stated in the

food diary, however where this was not possible estimates were based on average portion sizes from the British Food Standards Agency (FSA, 2002). A list was kept of assumptions made when selecting items on Dietplan to ensure consistency in data inputting (Appendix 7). To facilitate analysis of flavanol intake as well as nutrients, flavanol-containing food items were selected from both the McCance and Widdowson's The Composition of Foods (MW7) and Flavonoid (FLV) databases, with these then collated separately to ensure no duplicated values were included in calculation of overall nutrient intake. Where no item was available on the MW7 database a close alternative was used at the discretion of the inputter; if none was available then a new user-added food (USF) was added using items selected from the Sainsburys website (Sainsburys). Participants also had the option to submit their meals on the food-diary in the format of recipes, which some found more convenient and helped with accuracy of portion sizes; recipes were inputted to Dietplan by their ingredients and added to the daily intake diaries as portions, with FLV items added separately.

2.6.3 International Physical Activity Questionnaire

The International Physical Activity Questionnaire (IPAQ) asks questions relating to activity levels and sitting time during a seven-day period and responses are used to quantify respondents' physical activity across a range of intensity levels. It is suitable for adults aged 15-69 years and has high test-retest reliability (Craig et al., 2003). We used the short-form of the questionnaire which comprises seven open-ended questions asking about participants' involvement in activities across a range of intensities, with examples given for each. Results from the IPAQ were interpreted manually using Excel, using instructions provided to estimate energy expenditure (TrinityCollegeDublin). MET (metabolic equivalent of task) minutes represent energy expenditure during physical activity; one MET represents resting expenditure, therefore walking is considered 3.3 METs, moderate activity is 4 METs, and vigorous activity 8 METs. Participants' responses to each question were used to calculate

their MET per week at each intensity, bouts of exercise less than 10 minutes duration were not included, and those longer than 3 hours duration were truncated. Durations of activity reported in each question of the IPAQ were converted to minutes and multiplied by the number of days carried out, and by the MET value given to calculate MET minutes per week in each category. These were then combined to give total expenditure and participants categorised according to total MET minutes per week; over 3000 MET minutes per week was categorised as 'high' physical activity, 'moderate' was over 600 MET minutes per week, and anything below this was categorised as 'low' physical activity.

2.7 Statistical analysis

All statistical analysis was performed, and figures were created using Graphpad Prism Version 9.2.0. Analysis techniques utilised in each study are detailed in their relevant chapters; oftentimes this comprised an Analysis of Variance (ANOVA), with appropriate post-hoc tests to detect the exact point of the difference where appropriate. A significance level of $p<0.05$ was used for all statistical analyses, and all values reported are mean±SD.

Chapter 3:

The role of cocoa flavanols in modulating peripheral and cerebral microvascular function in healthy individuals and populations at-risk of cardiovascular disease

3.1 Introduction

The European Food Safety Authority (EFSA) recommend consumption of cocoa flavanols (CFs), in the context of a balanced diet, in order to "help maintaining the elasticity of blood vessels, which contributes to normal blood flow" (Ludovici et al., 2017). This is supported by extensive evidence that CFs can improve endothelium-dependent dilatation (EDD), as measured by flow-mediated dilatation (FMD) of the brachial artery, which is a predictive measure of future cardiovascular disease (CVD) risk (Ras et al., 2013).

The proposed cardioprotective effects were originally observed in Kuna Indians, who demonstrated a relatively low incidence of hypertension with ageing, and a significant reduction in cardiovascular mortality, considering their high salt consumption. This protection was no longer apparent in individuals who had migrated to Panama City, suggesting that the difference was attributable to environmental rather than genetic factors (Hollenberg et al., 1997). Although various environmental components were considered, raw cocoa consumption emerged as the most important, given that natives living on Kuna Island had the highest intake worldwide, drinking up to five cups of flavanol-rich cocoa (approximately 900mg) per day (McCullough et al., 2006).

Early epidemiological studies supported the cardiovascular benefits of CFs, demonstrating reduction in blood pressure and CV mortality in populations with high cocoa consumption (Buijsse et al., 2006, Buijsse et al., 2010). However, interpretations drawn from epidemiological studies are limited as they rely on participants providing an accurate account of their diet, and largely do not account for other potential sources of flavanols within the diet. On the other hand, reductions in blood pressure have been observed after CF supplementation ranging from 2 to 18 weeks, greater effects being observed in individuals with hypertension in comparison to normotensives (Ried et al., 2012, Ried et al., 2017).

Furthermore, randomised controlled trials demonstrate improvements in FMD following both acute and chronic ingestion of CFs in healthy young (Vlachopoulos et al., 2005, Grone et al., 2019, Schroeter et al., 2006) and elderly (Monahan et al., 2011, Grone et al., 2019) populations, as well as in individuals with increased CVD risk such as smokers (Heiss et al., 2005, Loffredo et al., 2018), those who are overweight or obese (Davison et al., 2008, West et al., 2014, Njike et al., 2011), and individuals with hypertension (Grassi et al., 2005), type-2 diabetes (Balzer et al., 2008) and peripheral artery disease (Loffredo et al., 2014). The effects identified in such studies have been reviewed extensively (Hooper et al., 2012, Ebaditabar et al., 2020) and a non-linear dose-response relationship has been observed in chronic studies, with maximal FMD improvements obtained at 500-700mg flavonoids per day (Sun et al., 2019, Shrime et al., 2011).

By contrast, studies concerning the effect of CFs on the microvasculature are more limited. A single systematic review has investigated effects of polyphenols, including CFs, on microvascular function, and reported that they improved microvascular function across a range of tissues (cutaneous, skeletal muscle and cerebral) in healthy individuals, but not in patient populations (Woodward et al., 2018). However, this review does not consider potential bias of the included studies and, as information regarding CFs made up only a small part of this review, limited detail is provided with no formal synthesis of the study findings. This greatly limits the interpretations that can be made. Thus, the aim of the present systematic review was to address these limitations by focussing only on CFs and to conduct a more comprehensive search and analysis of the findings.

Techniques used to non-invasively monitor blood flow within the resistance vessels and microvessels include venous occlusion plethysmography (VOP), Laser Doppler and near-infrared spectroscopy (NIRS), which are typically used in skeletal muscle, cutaneous and cerebral vascular beds (Johnson et al., 2014). VOP is a widely used technique for measuring

whole limb (muscle and skin) blood flow by detecting changes in limb circumference which correspond to arterial inflow when venous drainage is occluded (Joyner et al., 2001); this is described in more detail in Section 2.3.3. Meanwhile, Laser Doppler measures changes in skin microvascular perfusion based upon detection of laser shifted photons in moving red blood cells (Cracowski and Roustit, 2016). NIRS is used to detect changes in muscle tissue oxygenation at the microcirculatory level by continuous monitoring of oxy- and deoxyHb (Rosenberry et al., 2018a), as described in Section 2.3.4. These techniques can be used to observe resting vascular function and EDD, such as RH and responses to local heating, exercise, or mental stress and altogether they can provide a robust way of assessing endothelial function across different microvascular beds.

Similarly to the macrovasculature, changes in EDD in the microvasculature can also be predictive of CVD progression (Green et al., 2011). Indeed, some studies suggest that peak hyperaemic flow in smaller vessels following release of arterial occlusion (i.e. peak RH), measured for example by VOP, provides a more accurate prediction of cardiovascular health than brachial FMD (Philpott et al., 2009, Anderson et al., 2011). Microvascular dysfunction, which is evident in CVD, ageing and other associated risk factors (Wiernsperger, 2000, Sprague and Ellsworth, 2010, Jaap et al., 1994, Nguyen et al., 2007), is closely linked with impaired endothelial function and leads to structural and functional changes in the microvasculature (De Boer et al., 2012, Houben et al., 2012). Importantly, functional declines within the microvasculature often precede macrovascular complications and can lead to development of pathological interactions and disease in both small and large vessels, across multiple organs (Sena et al., 2013, Bonetti et al., 2003, Minson, 2010). Hence changes in endothelial function within the microvasculature may be useful in the early prediction of the progression of disease within the macrovasculature, and the ensuing CVD. It is therefore important to understand the effect of CFs within the microvasculature and how this may

differ from the effects previously demonstrated in conduit vessels, as well as how this may vary between healthy individuals, and those with increased CVD risk.

The aim of the present study was therefore to conduct a systematic review of the literature relating to CFs and the microvasculature. We aimed to synthesise and compare results between studies in order to establish whether CF intervention leads to improvements in microvascular vasodilator responses in healthy populations and those at risk of CVD.

3.2 Methods

This systematic review was conducted following the Preferred Reporting System for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009). The review has been registered with PROSPERO (registration number: CRD42023483814); this includes full details on sample search query, criteria for inclusion and exclusion, data extraction, and analysis as recommended according to the PRISMA-P guide (Moher et al., 2009, Moher et al., 2015).

3.2.1 Search Strategy

Search terms were selected based on the PICO format (Moher et al., 2009, Needleman, 2002) and are detailed in Table 3.1. As recommended by this model, terms were chosen according to Population (healthy adults, and those with increased CVD risk), Intervention (CF supplementation, in the form of dark chocolate, cocoa drink or pure CFs), Comparison (studies with a control group), and Outcome (reporting microvascular function). Systematic searches were conducted up to September 2023, using Medline (Ovid, 1946 onwards), Embase (Ovid, 1980 onwards), PubMed (1996 onwards), Web of Science Core Collection (Clarivate Analytics, 1900 onwards) and Cochrane Central Register of Controlled Trials (Wiley Interface, current issues).

***Table 3.1: Systematic review search terms organised by the PICO format**. Words within each column were listed with 'OR' and 'AND' function was used to combine columns into search list.*

Population	Intervention	Control	Outcome
Human	Chocolate	Placebo	Microcirc*
Subjects	Cocoa	Control	Microvasc*
Volunteers	Cacao		Microvessels
Patients			Capillar*
Males			Arteriol*
Females			Venul*
Overweight			Iontophoresis
Obes*			Blood flow
Diabet*			Perfusion
Hypertens*			Vasodila*
Elder*			Laser doppler
Young			Video capillaroscopy
Menopaus*			Laser speckle
			NIRS
			Plethysmography

3.2.2 Selection Criteria and Screening

Screening was conducted using the Rayyan online screening tool (Ouzzani et al., 2016). Full citations were collated and screened initially by title and abstract to determine those for which the full text should be accessed. This was conducted separately by two independent reviewers, and any conflicts were settled by discussion with a third reviewer, as recommended by the Cochrane guidelines (Shamseer et al., 2015). Reviews and meta-analyses identified during literature searches were manually evaluated for any additional studies not returned by the initial database searches. During full-text screening, studies were included according to the following criteria; (i) randomised studies with placebo control arm, or cross-over studies with sufficient washout time and appropriate blinding, (ii) CFs administered orally including within dark chocolate, or pure individual flavanols, (iii) healthy, young adults or clinical populations with elevated CVD risk, (iv) studies reporting relevant outcome measures of microvascular function, such as cerebrovascular or peripheral blood flow, with sufficient data for comparison of control and treatment groups. Exclusion

criteria included: (i) non-human studies, (ii) articles that did not report the dose of cocoa intervention, (iii) studies with no placebo group, (iv) studies investigating the effects of cocoa on other measures, such as macrovascular responses, or where the contribution of the microcirculation to the measure was unclear. Full details of the inclusion/ exclusion criteria can be found in the PROSPERO submission (registration number: CRD42023483814).

3.2.3 Data Extraction and Synthesis

Data extraction was conducted by a single reviewer using a pre-prepared table including the following information; study characteristics (author, year of publication, journal), study design (including details on randomisation, placebo), population details (sample size, gender, age, health status), type of intervention (cocoa drink, capsule, dark chocolate), dosage and duration of intervention, and all available data on pre- and post- intervention for CFs and placebo. Where necessary, vascular measures were extracted from graphs if not given in the text. In studies where multiple doses of a single supplement, or multiple interventions or outcome measures were compared, all the available measures were initially extracted and then the most relevant selected to report so as to ensure consistency between studies wherever possible. For example, only the 1-2hr time-points were reported for acute studies as this is when blood CF concentrations are at their peak (Francis et al., 2006, Neukam et al., 2007, Schramm et al., 2003). For NIRS studies, data is shown as oxyHb or SO_2, as these were the most widely reported measures and provide insight into microvascular vasodilator behaviour (McLay et al., 2016a, Soares et al., 2019a).

Tables of findings are arranged according to vascular bed studied and ordered by risk of bias. Study outcomes are presented for placebo and CFs from each study; this is shown as pre- and post- intervention or change from pre- intervention wherever possible. Each study is represented throughout by a letter (as shown in Figure 3.2) and subgroups within a study are

allocated numbers (i.e. for Heiss et al (b_1) represents the young group, and (b_2) the elderly group). We had aimed to conduct some form of meta-analysis, however this requires at least two studies reporting the same outcome measures (Sataloff et al., 2021), and was not possible due to the heterogeneity of techniques, vasodilator stimuli and statistical methods across studies without enough information being provided to reduce these to the same format (Ioannidis, 2008). Instead, we synthesised findings from studies by 'vote counting', as recommended in the Cochrane Handbook for reviews where the limited availability of data does not allow for summarising effect estimates or combining p values as would be preferable alternatives to meta-analysis (Higgins, 2023). This involves assigning each outcome a 'vote' depending on the direction (regardless of statistical significance) of the percentage change calculated between placebo and CFs; '1' for a positive percentage change (i.e. higher with CFs) or '0' for a negative change (i.e. higher with placebo) (McKenzie, 2023). The assigned votes are shown in Tables 3.4 and 3.5 and were used to determine groupings used in the Harvest plot (Figure 3.3), which presents the direction of effect and risk of bias for each outcome.

3.2.4 Quality assessment

Cochrane Collaboration recommendations were used when assessing the quality of studies, this is based on five criteria covering random sequence generation; (1) selection bias (allocation concealment), (2) performance bias (blinding of participants and researchers), (3) detection bias (blinding of outcome assessment), (4) attrition bias (incomplete outcome data), and (5) reporting bias (selective reporting) (Higgins et al., 2011). Studies were given a bias rating for each domain based on a set of signalling questions, these being combined according to Cochrane recommendations to give an overall assessment of bias for the study. When less than 95% of original participants were included in the analysis, studies were considered to have 'missing data'. Studies were judged overall 'low risk' if they were 'low

risk' across all domains, 'some concerns' if concerns were raised for at least one domain, or 'high risk' either if any domain was judged 'high risk' or if there were multiple domains with concerns deemed sufficient to lower confidence in result. Overall bias ratings were important for considering bias as a potential cause for heterogeneity within the results, as recommended by established guidelines (Needleman, 2002).

3.2.5 Statistical analysis

Due to the lack of studies reporting similar enough outcomes for synthesis of findings by meta-analysis, alternative tools were used for analysis of our results. From the 'votes' assigned to each outcome, we calculated the proportion of studies which showed a beneficial effect of CFs, which is reported alongside a 95% confidence interval estimated by Wilson intervals method (Brown, 2001). Binominal tests were also used to calculate the probability of the overall direction of effect being true ($p<0.05$ considered significant). This was conducted in Microsoft Excel using the formula '=2*BINOM.DIST(x,y,z, TRUE), where x is the smaller of the number of effects favouring the intervention or control, y is the total number of effects, and z is the null value (true proportion of effects favouring intervention=0.5). Sensitivity analysis was also conducted, in which only studies judged overall as 'low risk of bias' were included in the analysis.

3.3 Results

3.3.1 Search results

The process of search, screening and selection of eligible studies is shown in Figure 3.1. A total of 846 papers were identified through searches of Pubmed, Embase, Medline and Web of Science, of which 511 unique articles remained once duplicates had been removed. 468 records were removed during screening by title and abstract due to not meeting the inclusion

criteria; for example animal studies, studies using interventions other than CFs, or lack of clear microvascular outcome measures. Full texts for the remaining 43 articles were then assessed for eligibility, of which 24 were excluded due to the following reasons: incorrect publication type (abstract only or methods paper, n=10); primary outcomes not reported, or without sufficient detail (n=10), lack of detail regarding the intervention dosage (n=2), or no suitable control group (n=2).

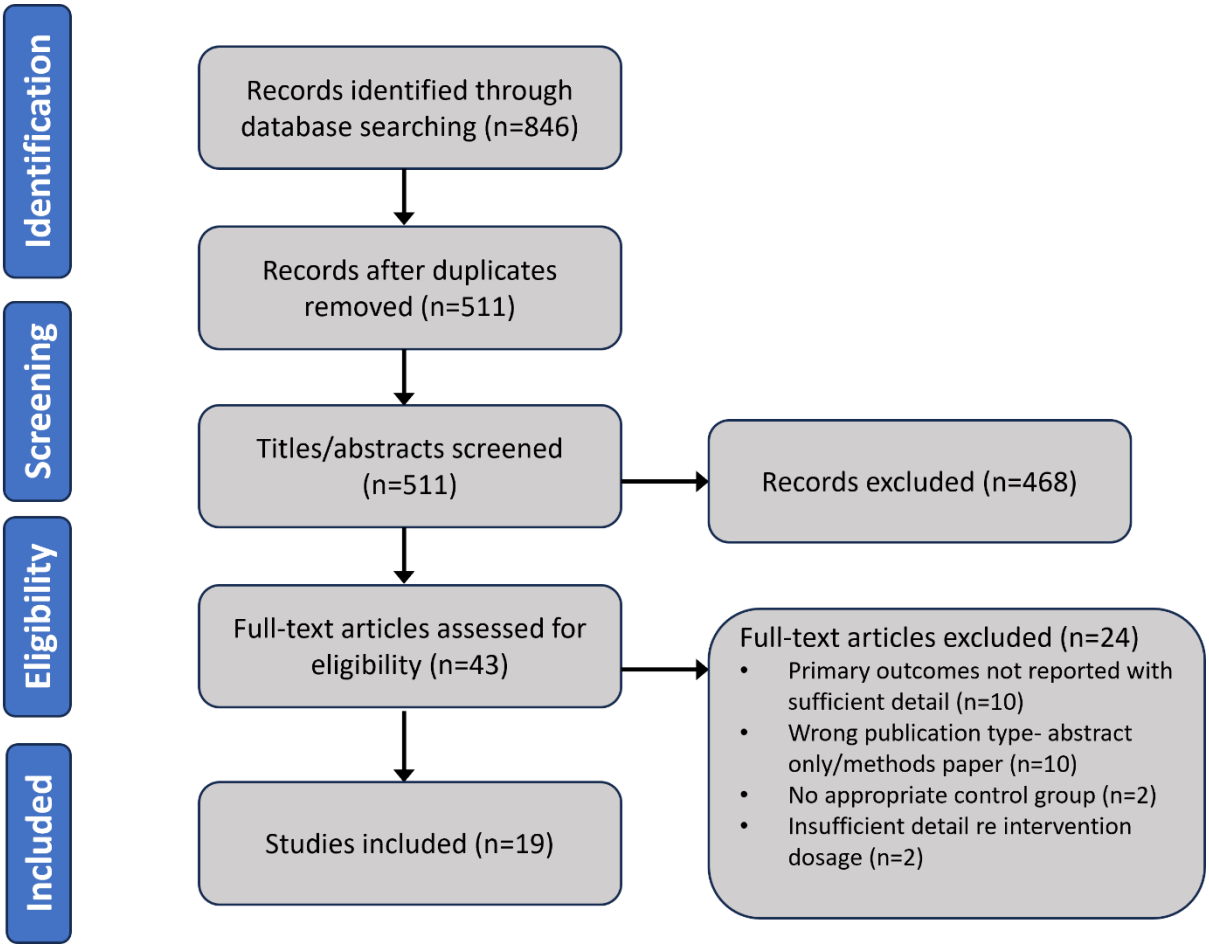

Figure 3.1: PRISMA flow diagram *showing the stages from identification to selection of studies to include, as well as reasons for exclusion of full-text articles*

3.3.2 Risk of bias of included studies

Each study included was assessed for Risk of Bias using the Cochrane Risk of Bias assessment guidelines. The reviewer's judgements for each domain, alongside the overall

bias, for each study are shown in Figure 3.2. 8 of the included studies were considered ‘low risk of bias’, 10 were classified as ‘some concerns’ and only one study was deemed ‘high risk of bias’. Potential sources of concern for bias were largely due to missing data (n=4), uncertainties surrounding the randomisation process (n=5) or blinding issues, for example, use of white chocolate placebo (n=4).

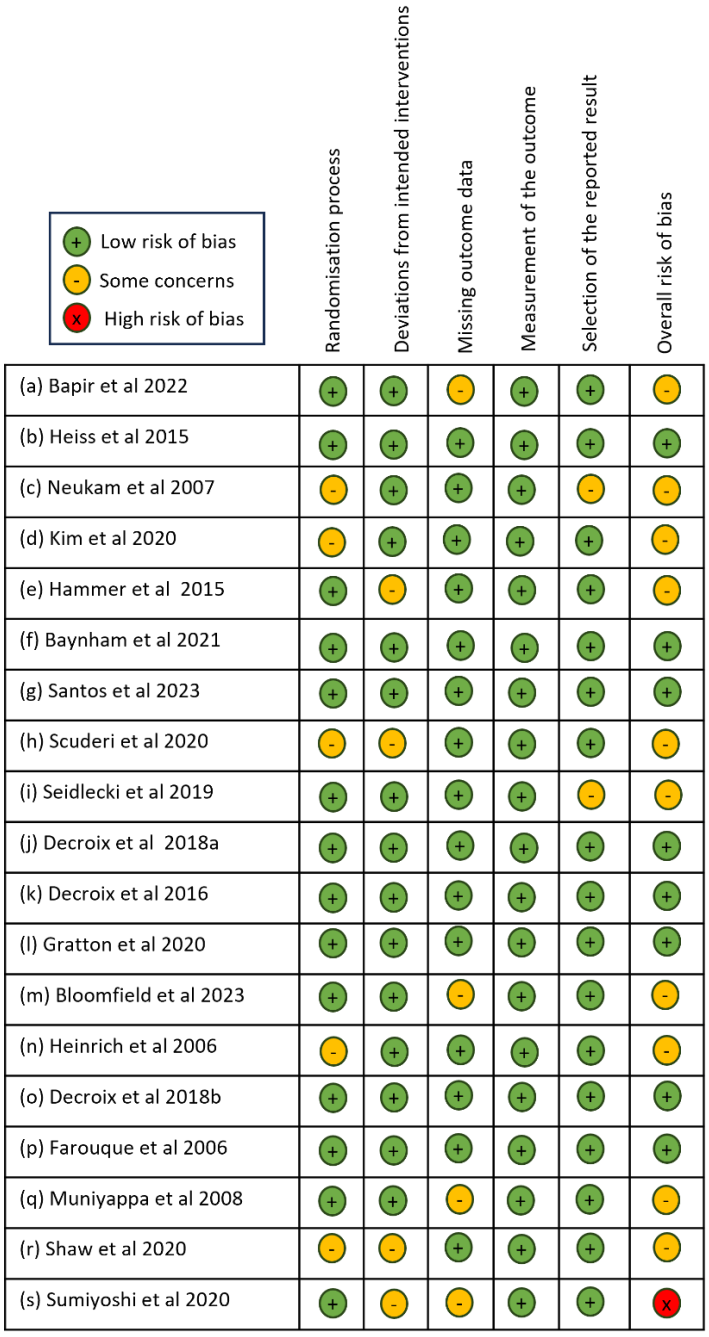

Figure 3.2: Risk of bias assessment according to the Cochrane recommendations

3.3.3 Study characteristics

Of the 19 studies that met the inclusion criteria, some used a single CF dose (n=12) (Baynham et al., 2021, Bapir et al., 2022, Bloomfield et al., 2023, Decroix et al., 2016, Decroix et al., 2018a, Gratton et al., 2020, Hammer et al., 2015, Kim and Brothers, 2020, Neukam et al., 2007, Santos et al., 2023, Scuderi et al., 2020, Siedlecki et al., 2019), whilst others investigated effects of daily CF intervention over a longer period (n=6) (Decroix et al., 2018b, Farouque et al., 2006, Heinrich et al., 2006, Muniyappa et al., 2008, Shaw et al., 2020, Sumiyoshi et al., 2019) and one study included both acute and chronic effects (Heiss et al., 2015). The characteristics of all included studies are displayed in Tables 3.2 and 3.3 for acute and chronic studies respectively; in each case they are arranged by vascular bed studied.

Table 3.2: Characteristics of included acute studies, including information on population studied and interventions used. *Data shown is mean±SD. T2DM= type 2 diabetes mellitus. PAD= peripheral artery disease. WE= White European, BA= Black African*

	Study design (washout period)	Participants' health	Number of participants	Age (yrs)	% female	Cocoa intervention type/dose	Control intervention type	Microcirculation studied
(a)	Crossover	Healthy	n= 11	51±10	72.7%	6 capsules- *Total flavs: 1350mg* *Epicatechin: 255mg*	6 capsules containing 3.9g brown sugar- *Total flavs: 0mg*	Cutaneous (hands/feet)
		T2DM	n=11	59±10	27.3%			
(c)	Crossover (14 days)	Healthy	n=10	18-65	100%	Cocoa beverage (18g cocoa powder in 100ml water) *Total flavs: 329mg* *Epicatechin: 61.1mg*	Matched beverage (18g cocoa powder in 100ml water) *Total flavs: 27mg* *Epicatechin: 6.6mg*	Cutaneous
(d)	Crossover (min 7days)	(d_1) Healthy WEs	n=7	22±4	42.9%	Cocoa beverage (in 250ml water)- *Total flavs: 528mg* *Epicatechin: 47mg*	Matched placebo beverage (in 250ml water) *Total flavs: 0mg*	Cutaneous (forearm)
		(d_2) Healthy BAs	n=7	22±4	42.9%			
(e)	Crossover (7 days)	PAD patients	n=21	66.9±7.41	28.6%	50g dark choc *Total flavs: 780mg* *Epicatechin: 45mg*	50g white choc *Total flavs: 0mg*	Cutaneous (forearm)
(b)	Parallel	(b_1) Healthy young (18-30yrs)	n=22	26±4.69	0%	Cocoa beverage (7g cocoa powder in ~500ml water) *Total flavs: 450mg* *Epicatechin: 64mg*	Cocoa beverage (7g matched powder in ~500ml water) *Total flavs: 0mg*	Cutaneous/ muscle (forearm)
		(b_2) Healthy elderly (50-80yrs)	n=20	60±8.94	0%			
(f)	Crossover (7 days)	Healthy (all White European)	n=30	23±4.3	0%	8.3g cocoa powder (dissolved in 300ml Buxton water) *Total flavs: 681.4mg*	8.3g matched cocoa powder (dissolved in 300ml Buxton water) *Total flavs: 4.1mg*	Skeletal muscle (forearm)

						Epicatechin: 150mg	*Epicatechin: <4mg*	
(g)	Crossover (1 month)	Healthy	n=12	25±4	53.8%	25mg microencapsulated cocoa powder (dissolved in 250ml water) *Total flavs: 80mg*	7g microencapsulated Ovaltine *Total flavs: 9mg*	Skeletal muscle (forearm)
(h)	Crossover (72 hrs)	Healthy	n=18 (36 eyes)	26.3±1.5	44.4%	100g dark chocolate *Epicatechin: 447mg*	100g white chocolate *Total flavs: trace*	Retinal
(i)	Crossover (7 days)	Healthy	n=22	27.3±11.1	59.1%	20g dark chocolate *Total flavs: 400mg*	7.5g milk chocolate *Total flavs: 5mg*	Retinal
(j)	Crossover (7 days)	Healthy	n=20	23.2±4.3		4 capsules (1765mg cocoa extract) *Total flavs: 530mg* *Epicatechin: 100mg*	4 matched capsules *Total flavs: 0mg*	Cerebral
(k)	Crossover (7days)	Trained males	n=12	30±3	0%	Cocoa powder dissolved in 300ml semi-skimmed milk *Total flavs: 903mg* *Epicatechin: 185mg*	Matched placebo powder dissolved in 300ml semi-skimmed milk *Total flavs: 15mg* *Epicatechin 0mg*	Cerebral
(l)	Crossover (2 weeks)	Healthy	n=18	23.9±7.3	0%	8.3g cocoa powder (dissolved in 300ml Buxton water) *Total flavs: 681.4mg* *Epicatechin: 150mg*	8.3g matched cocoa powder (dissolved in 300ml Buxton water) *Total flavs: 4.1mg* *Epicatechin: <4mg*	Cerebral
(m)	Crossover (2 weeks)	Healthy	n=12	26.1±6.2	41.7%	Encapsulated cocoa powder (15mg/kg body weight) *Mean total flavs: 1031mg* *Mean epicatechin: 145mg*	Encapsulated placebo cocoa powder *Mean total flavs: 0.82mg* *Mean epicatechin: n/a*	Cerebral

Table 3.3: Characteristics of included chronic studies, including information on population studied and interventions used. *Data shown is mean±SD. CAD= coronary artery disease, WE=White European, BA= Black African*

	Study design (washout period)	Participants' health	Number of participants	Age (yrs)	% female	Study duration	Daily cocoa intervention type/dose	Daily control intervention type	Microcirculation studied
(n)	Parallel	Healthy females	n=24 (12 per group)	18-65	100%	12 weeks (tested also at 6wks)	18g powder in 100ml water *Total flavs: 329mg* *Epicatechin: 61.1mg*	18g powder in 100ml water *Total flavs: 26.8g* *Epicatechin: 1.6mg*	Cutaneous
(b)	Parallel	(b_1) Healthy young (18-30yrs)	n=22	26±4.69	0%	14 days	Cocoa beverage (7g cocoa powder in ~500ml water) *Total flavs: 450mg* *Epicatechin: 64mg*	Cocoa beverage (7g matched powder in ~500ml water) *Total flavs: 0mg*	Cutaneous/ skeletal muscle (forearm)
		(b_2) Healthy elderly (50-80yrs)	n=20	60±8.94	0%				
(p)	Parallel	CAD patients	CFs: n=20 Control: n=20	CFs:61±9 Control: 61±8	CFs: 35% Control: 15%	6 weeks	Chocolate bar and cocoa beverage; *Total flavs: 444mg* *Epicatechin: 107mg*	Matched placebo bar and beverage *Total flavs: 19.6mg* *Epicatechin: 4.7mg*	Skeletal muscle (forearm)
(q)	Crossover (1 week)	Mild-moderate hypertensives	n=20 (13 WE, 7 BA)	51± 6.71	60%	2 weeks	2*31g cocoa powder with 150ml water *Total flavs: 902mg* *Epicatechin: 174mg*	2*31g matched placebo powder with 150ml water *Total flavs: 28mg* *Daily epicatechin: 2mg*	Skeletal muscle (forearm)
(o)	Crossover (1 week)	Trained cyclists	n=14	30.7±3.1	0%	1 week	4 capsules *Total flavs: 1765mg* *Epicatechin: 100mg*	4 matched capsules *Total flavs: 0mg*	Skeletal muscle (leg)/ cerebral
(r)	Crossover (2 weeks)	Trained cyclists	n=12	35±12	16.7%	2 weeks	120g (60g*2) per day 72% dark choc *Total flavs: 1788mg* *Epicatechin: 37.4mg*	120g (60g*2) per day non-choc placebo *Total flavs: 0mg*	Skeletal muscle (leg)/ cerebral
(s)	Parallel	Healthy	CFs: n=10 Control: n=8	20-31	27.8%	30 days	24g 70% dark chocolate *Total flavs: 540mg* *Epicatechin: 34.8mg*	24.5g white chocolate *Total flavs: ND*	Cerebral

3.3.3.1 Acute studies

Studies using a single dose of CFs tested effects across the cutaneous (n=5, (Bapir et al., 2022, Hammer et al., 2015, Heiss et al., 2015, Kim and Brothers, 2020, Neukam et al., 2007)), skeletal muscle (n=3, (Baynham et al., 2021, Heiss et al., 2015, Santos et al., 2023)), retinal (n=2, (Scuderi et al., 2020, Siedlecki et al., 2019)) and cerebral microcirculations (n=4, (Bloomfield et al., 2023, Decroix et al., 2016, Decroix et al., 2018a, Gratton et al., 2020)) and their characteristics are shown in Table 3.2. Of the 13 studies, 12 had a cross-over design (with wash-out periods ranging from 72 hours to 2 weeks), whilst one was run with parallel groups (Heiss et al., 2015). Sample sizes of the included studies ranged between 7 and 22 individuals per group. The majority of studies were conducted in healthy populations (age range: 18-65 years), with many focussed on young adults (< 30 years), whilst others included clinical populations, such as type 2 diabetes (Bapir et al., 2022) or PAD (Hammer et al., 2015). One study drew comparisons between ethnic groups with differing CVD risk (Kim and Brothers, 2020), and only one other study reported the ethnicity of participants (Baynham et al., 2021). Most populations also included a mixture of males and females (Bapir et al., 2022, Bloomfield et al., 2023, Hammer et al., 2015, Kim and Brothers, 2020, Santos et al., 2023, Scuderi et al., 2020, Siedlecki et al., 2019); 4 studies included only males (Baynham et al., 2021, Decroix et al., 2016, Gratton et al., 2020, Heiss et al., 2015) and just one study was female only (Neukam et al., 2007).

The source of CFs in these acute studies was dark chocolate (n=3, (Siedlecki et al., 2019, Scuderi et al., 2020, Hammer et al., 2015)), capsules (n=4, (Bapir et al., 2022, Bloomfield et al., 2023, Decroix et al., 2018a, Santos et al., 2023)) or cocoa drink (n=6, (Baynham et al., 2021, Bloomfield et al., 2023, Decroix et al., 2016, Heiss et al., 2015, Kim and Brothers, 2020, Neukam et al., 2007, Siedlecki et al., 2019, Scuderi et al., 2020, Hammer et al., 2015)), with total flavanol doses ranging from 80-1350 mg. Epicatechin dosage was also reported in

11 studies, with this ranging from 45-447 mg. Acute effects were assessed within 60-120 minutes in all studies, aligning with the peak CF concentration in the blood at this time (Francis et al., 2006, Neukam et al., 2007), with some also following effects for up to 6 hours post-intervention. Whereas most studies asked participants to fast for a minimum of 8 hours prior to intervention, 4 studies administered the CFs alongside a meal or carbohydrate-rich drink (Bapir et al., 2022, Bloomfield et al., 2023, Decroix et al., 2018a, Decroix et al., 2016), which they reported to increase CF absorption (Schramm et al., 2003). 8 studies compared effects of CF on resting blood flow, meanwhile others looked at the effects of CFs on microvascular vasodilator responses to stimuli such as RH (n=3, (Bapir et al., 2022, Hammer et al., 2015, Heiss et al., 2015)), mental stress (n= 3, (Baynham et al., 2021, Decroix et al., 2016, Decroix et al., 2018a)), local heating (n=1, (Kim and Brothers, 2020)), exercise (n=1, (Decroix et al., 2016)), hypercapnia (n=1, (Gratton et al., 2020)) or hypoxia (n=1, (Bloomfield et al., 2023)).

3.3.3.2 Chronic studies

Seven studies used a longer-term CF intervention and compared effects across a range of 7 days to 12 weeks; their characteristics are shown in Table 3.3. Similar to the acute studies they incorporated a range of vascular beds from cutaneous (n=2, (Heinrich et al., 2006, Heiss et al., 2015)), skeletal muscle (n=5, (Decroix et al., 2018b, Farouque et al., 2006, Heiss et al., 2015, Muniyappa et al., 2008, Shaw et al., 2020)) and cerebral microcirculation (n=3, (Decroix et al., 2018b, Shaw et al., 2020, Sumiyoshi et al., 2019, Heinrich et al., 2006, Heiss et al., 2015)). 3 of the 7 studies used a cross-over design, with wash-out periods of 1-2 weeks, and the remaining 4 studies used parallel groups. Sample sizes ranged from 10 to 20 per group. Healthy individuals made up the majority of volunteers, with some focussing on

trained individuals and others including the effects within coronary artery disease patients (Farouque et al., 2006) and hypertensives (Muniyappa et al., 2008). Studies were conducted mainly in mixed gender (n=4) groups, with two male only (Decroix et al., 2018b, Heiss et al., 2015) and one female only study (Heinrich et al., 2006). Only one study reported the ethnicities of participants: a mixture of WEs and BAs (Muniyappa et al., 2008).

Daily CF doses were obtained from dark chocolate (n=2, (Shaw et al., 2020, Sumiyoshi et al., 2019)), cocoa beverages (n=3, (Heinrich et al., 2006, Heiss et al., 2015, Muniyappa et al., 2008)), capsules (n=1, (Decroix et al., 2018b, Shaw et al., 2020, Sumiyoshi et al., 2019)) or a combination of chocolate and beverages (n=1, (Farouque et al., 2006)). Total daily flavanol intake ranged from 329-1788 mg and daily epicatechin dosage was 35-174 mg. Studies compared effects of CFs on resting values (n=4, (Farouque et al., 2006, Heinrich et al., 2006, Heiss et al., 2015, Muniyappa et al., 2008)) or after vasodilator stimuli such as RH (n=2, (Farouque et al., 2006, Heiss et al., 2015)), exercise (n=3, (Decroix et al., 2018b, Farouque et al., 2006, Shaw et al., 2020, Heinrich et al., 2006, Heiss et al., 2015, Muniyappa et al., 2008)), acetylcholine infusion (n=1, (Farouque et al., 2006)), insulin infusion (n=1, (Muniyappa et al., 2008)) or mental stress (n=1, (Sumiyoshi et al., 2019)).

3.3.4 Study findings

3.3.4.1 Acute CF intervention

Table 3.4 summarises the findings in relation to key outcome measures for all acute studies, grouped according to the vascular bed investigated and risk of bias. Analysis by vote counting using subgroups from Harvest plot (Figure 3.3) suggests there is evidence of a beneficial effect of CFs on vasodilator responses, with 12 out of 14 subgroups showing enhanced vasodilator responses following CF intervention (85.7% (80.4-91.0%), p=0.0129). Importantly, all of the 9 groups which were ‘low risk of bias’ showed a positive effect of CFs

on vasodilator responses (100%, p=0.00391). In comparison, the effect at rest was less profound with benefits shown in 8 out of 13 subgroups (61.5% (52.2-70.9%), p=0.581), an effect which was not statistically significant. A total of 11 acute studies included effects in healthy, young populations; within these, a beneficial effect of CFs was shown at rest in 6 out of 9 subgroups, though this was not statistically significant (66.7% (54.1-79.2%), p=0.508), while an improvement in vasodilator responses was evident in 9 out of 11 subgroups (81.8% (74.2-89.4%), p=0.0654). Indeed, considering only studies with 'low risk of bias', all 8 subgroups showed improvement in vasodilator responses with CFs (100%, p=0.00781). Further statistical analysis within each microcirculation studied, or within the subgroups with increased CVD risk was not possible due to the small number of comparable studies.

Within the cutaneous circulation, three studies found no significant effect of CFs on resting blood flow (Hammer et al., 2015, Heiss et al., 2015, Kim and Brothers, 2020), whereas Neukam et al showed a 1.7-fold increase in resting cutaneous blood flow in the forearm of healthy females (Neukam et al., 2007) and Bapir et al found that CFs increased baseline microvascular diameter in the skin of the feet but not hands (Bapir et al., 2022). The latter study was conducted in healthy and type-2 diabetes patients; diabetic patients showed blunted peak RH relative to healthy individuals, but there was no effect of CFs on peak RH in the hands or feet of the combined population (Bapir et al., 2022). Similarly, smaller cutaneous vasodilator responses were demonstrated in elderly compared to young males, but peak RH was increased by CFs in both groups (Heiss et al., 2015). By contrast, no effect of CFs was found on peak cutaneous RH in peripheral artery disease patients (Hammer et al., 2015).

In the forearm muscle, smaller peak FBF measured by VOP were also observed in elderly versus young males, with peak RH being increased by CFs in both groups (Heiss et al., 2015). Furthermore, there was evidence of increased FBF measured by VOP at rest and during mental stress in a group of young males (Baynham et al., 2021). On the other hand,

Santos et al found no significant effect of CFs on reperfusion rate or peak SO_2 measured by NIRS following a post-exercise vascular occlusion test (Santos et al., 2023).

Within the retinal microvasculature, both studies showed no effect of dark, compared to white chocolate, in young healthy populations of mixed gender (Scuderi et al., 2020, Siedlecki et al., 2019). Finally, in the cerebral cortical microvasculature, there was evidence using NIRS of greater increases in oxyHb following CF supplementation in response to a cognitive task, as well as to hypoxia and hypercapnia, but no significant effect during exercise, in young mixed or male populations (Bloomfield et al., 2023, Decroix et al., 2016, Gratton et al., 2020).

Table 3.4: Findings from acute studies, including reported outcome measures for placebo and cocoa interventions and the direction of effect. *Table is ordered by risk of bias within sub-sections for each vascular bed. Outcome measures are shown as mean±SD for pre/post intervention unless otherwise stated, and data shown in italics has been estimated from graphs. OCT-A= optical coherence tomography angiography. CVC= cutaneous vascular conductance. IQR= interquartile range. VOP= venous occlusion plethysmograph. (f)NIRS= (functional) near-infrared spectroscopy*

	Monitoring technique/ outcome measure	Rest/ vasodilator stimulus	Outcome measures (placebo)	Outcome measures (cocoa flavanols)	P values	Vote counting (direction of effect)	Risk of bias
			Cutaneous				
(b)	Laser doppler perfusion imaging (LDPI)	Rest	(b1) 42±4.69/42.1±4.69 PU (b2) 41±4.47/44±0.44 PU	(b1) 38±4.69/40±4.69 PU (b2) 38±4.47/39±4.47 PU		1 0	Low risk
		RH peak	(b1) 259±70.4/270±79.7 PU (b2) 186±35.8/186±35.8 PU	(b1) 257±65.7/292±70.4 PU (b2) 184±67.1/200±62.6 PU	**p<0.05** **p<0.05**	1 1	
(c)	Laser Doppler (cutaneous blood flow)	Rest	*22±15/22±10 AU*	*30±10/50±8 AU*	**p<0.05**	1	Some concerns
(a)	OCT (hands/ feet, mean vessel diameters)	Resting	Foot: 46±4/43±4 µm Hand: 57±4/49±9 µm	Foot: 44±4/44±4 µm Hand: 48±4/48±4 µm	**p<0.001** p=0.371	1 1	Some concerns
		RH peak	Foot: 49±4/47±4 µm Hand: 57±4/55±4 µm	Foot: 51±9/50±4 µm Hand: 57±4/57±4 µm	p=0.751 p=0.120	1 1	
(d)	Laser Doppler (forearm)	Rest (%CVC max)	(d_1) 11.15±1.44 (d_2) 8.16±2.56	(d_1) 9.73±1.3 (d_2) 9.99±2.31		0 1	Some concerns
		Local heating (flux/mmHg)	(d_1) 3.21±0.43 (d_2) 2.85±0.17	(d_1) 3.03±0.25 (d_2) 3.04±0.26	p=0.4 **p<0.01**	0 1	
(e)	Laser Doppler (forearm) Median (IQR)	Rest	0.22(0.13-0.47)/0.41(0.24-0.51) AU	0.32(0.18-0.60)/0.31(0.25-0.55) AU	p=0.78	0	Some concerns
		RH peak	0.89(0.58-1.49)/1.24(0.85-1.79) AU	1.22(0.84-1.87)/1.18(0.7-2.27) AU	p=0.69	0	
			Skeletal				
(f)	VOP (forearm, % change pre-post)	Rest	-0.38±0.28%	0.34±0.83%	**p<0.001**	1	Low risk
		Stress	0.26±0.66%	1.46±1.81%	**P=0.002**	1	

(b)	VOP (forearm)	Rest	(b_1) 1.5±0.47/1.5±0.47 (b_2) 1.1±0.45/0.9±0.45	(b_1) 1.7±0.47/1.6±0.47 (b_2) 1.5±0.45/1.2±0.45		0 0	Low risk
		RH peak	(b_1) 13.7±7.50/14.3±8.44 (b_2) 11.3±5.36/10.8±6.71 (all ml/100ml*min)	(b_1) 13.2±2.81/16.2±4.69 (b_2) 10.9±5.36/12.3±6.71 (all ml/100ml*min)	**p<0.05** **p<0.05**	1 1	
(g)	NIRS-derived SO_2 (forearm)	Vascular occlusion test post-exercise	Reperfusion rate (%/s): 2.03±0.46/ 2.02±0.59	Reperfusion rate (%/s): 2.14±0.58/ 2.35±0.92	p=0.488	1	Low risk
			Retinal				
(h)	OCT-A (change from baseline @2hrs, SCP whole density)	Rest	0.586±2.67%	0.739±1.816%	p=0.317	1	Some concerns
(i)	OCT-A (superficial retinal plexus)	Rest (vessel density)	47.5±2.6%	48.0±2.7%	p=0.56	1	Some concerns
			Cerebral				
(j)	fNIRS (oxyHb, right PFC)	Stroop task	*1.2±1.5 AU*	*1±1.5 AU*		0	Low risk
(k)	fNIRS (oxyHb)	Cognitive task	*1.5±0.3 AU*	*2±0.3 AU*	**p=0.02**	1	Low risk
		Exercise	*12±1.5 AU*	*12.5±1.5 AU*		1	
(l)	fNIRS (change in oxyHb, at 3-4)	Hypercapnia	*18±41.2/11±24.7 AU*	*17±33.0/36±33.0 AU*	**p=0.03**	1	Low risk
(m)	fNIRS (oxyHb, normalised to normoxic baseline)	Rest	0.8±1.0 µmol	1.7±2.3 µmol	**p=0.005**	1	Some concerns
		Hypoxia	-4.0±3.6 µmol	-1.3±2.7 µmol		1	

3.3.4.2 Chronic CF intervention

Table 3.5 summarises the direction of effects for all chronic studies. Overall, by vote counting subgroups shown in Figure 3.3, 7 out of 9 subgroups favoured a beneficial CF effect at rest (77.7% (67.5-88.0%), p=0.180) and 8 out of 11 showed a beneficial effect on vasodilator responses (72.7% (63.4-82.0%), p=0.227), though these effects did not reach statistical significance. Considering only subgroups from studies with 'low risk of bias', 6 out of 7 showed a beneficial effect at rest (85.7% (75.1-96.3%), p=0.125), and 5 out of 7 showed enhanced vasodilator responses following CF supplementation (71.4% (56.5-86.4%), p=0.453), though neither of these effects were statistically significant. There were 5 chronic studies which included young, healthy populations; of these, 7 subgroups tested effects of CF supplementation at rest and all showed improvement (100%, p=0.0156), and 7 out of 9 subgroups testing vasodilator responses showed improvement with chronic CF supplementation (77.8% (71.2-84.4%), p=0.180). Considering only the 6 subgroups from studies with 'low risk of bias', there was evidence of microvascular improvements at rest in all 6 (100%, p=0.0313), and 5 out of 6 showed improved vasodilator responses (83.3% (77.6-89.0%), p=0.219), though this effect was not statistically significant. The remaining subgroups included populations with increased risk of CVD, though there were not enough of these to conduct statistical analysis, nor were there enough studies considering the same microcirculation for analysis to be made within these.

Studies of the cutaneous microvasculature show mixed findings for the effects of chronic CF on resting blood flow; Heinrich et al found increases at rest in the forearms of healthy women after 6 and 12 weeks of supplementation (Heinrich et al., 2006), whereas Heiss et al showed no difference at rest in young or elderly males after 14 days (Heiss et al., 2015). The latter study also found no difference in resting FBF measured by VOP, although peak cutaneous

and whole-limb RH was enhanced by CFs as measured by Laser Doppler and VOP respectively (Heiss et al., 2015). In contrast, no effect of 6 weeks CF supplementation on FBF measured by VOP was found at rest or at peak RH or exercise in CAD patients (Farouque et al., 2006); indeed, responses to acetylcholine infusion were smaller following CFs versus control in this group (Farouque et al., 2006). Furthermore, in a group of hypertensive patients of mixed gender and ethnicity there was no effect of CFs after 2 weeks at rest or following insulin infusion on capillary blood flow (Muniyappa et al., 2008). Two studies investigated the effects of CFs on leg muscle and cerebral oxygenation measured by NIRS during exercise in trained cyclists; Shaw et al found significant increases in leg, but not cerebral oxyHb, during exercise in a hypoxic chamber after two week's CF supplementation (Shaw et al., 2020), whereas Decroix et al found that one week of CFs increased SO_2 response to hypoxia and exercise in the cerebral microvascular but not leg muscle, despite increases in resting SO_2 in both tissues (Decroix et al., 2018b). Finally, Sumiyoshi found no significant effect of 30 days CFs on cerebral oxyHb during cognitive tasks (Sumiyoshi et al., 2019), although this result should be viewed with caution given the study was judged 'high risk of bias'.

***Table 3.5: Findings from chronic studies, including reported outcome measures for placebo and cocoa inteventions and the direction of effect**. Table is ordered by risk of bias within sub-sections for each vascular bed. Outcome measures are shown as mean±SD for pre/post intervention unless otherwise stated, and data shown in italics has been estimated from graphs. VOP= venous occlusion plethysmography. NIRS= near-infrared spectroscopy*

	Monitoring technique/ outcome measure (units)	**Rest/ vasodilator stimulus**	**Outcome measures (control)** Mean±SD pre/post, unless otherwise stated	**Outcome measures (cocoa flavanols)** Mean±SD pre/post, unless otherwise stated	**P values**	**Vote counting (direction of effect)**	**Risk of bias**
			Cutaneous				
(b)	Laser doppler perfusion imaging (LDPI, forearm)	Rest	(b_1) 42±4.7/39±4.7 PU (b_2) 39±4.5/40±4.5 PU	(b_1) 39±4.7/40±4.7 PU (b_2) 40±4.5/39±4.5 PU		1 1	Low risk
		RH	(b_1) 260±103/258±79.7 PU (b_2) 178±22.4/180±22.4 PU	(b_1) 307±65.7/ 296±79.7 PU (b_2) 214±53.6/213±53.6 PU	**p<0.05** **p<0.05**	1 1	
(n)	Laser Doppler (blood flow)	Rest	6 weeks: 17±9/17±6 AU 12 weeks: 17±9/16±6 AU	6 weeks:16±7/24±12 AU 12 weeks: 16±7/32±16 AU	**p<0.05** **p<0.05**	1 1	Some concerns
			Skeletal muscle				
(b)	VOP (forearm)	Rest	(b_1) 1.5±0.47/1.4±0.47 (b_2) 0.8±0.045/0.9±0.045	(b_1) 1.6±0.47/1.6±0.47 (b_2) 1.1±0.45/1.1±0.045		1 1	Low risk
		RH	(b_1) 13.7±7.97/13.8±7.04 (b_2) 11.6±7.60/11±6.71 (All ml/100ml*min)	(b_1) 16.9±2.81/16.9±5.16 (b_2) 14.0±6.26/13.9±4.92 (All ml/100ml*min)	**p<0.05** **p<0.05**	1 1	
(o)	NIRS (SO_2)	Rest	*59.2±2%*	*60.2±2%*		1	Low risk
		Hypoxia	*60.5±1.5%*	*58.5±1%*		0	
		Exercise	*57±1.5%*	*56.8±1.5%*		0	
(p)	VOP (forearm)	Rest	2.49±1.12/2.8±1.57	2.82±01.57/2.47±0.98		0	Low risk
		ACh infusion	*5±0.2/8±1*	*8±1/8.2±1.5*	**p<0.05**	0	
		RH	28.09±8.23/28.05±7.83	31.15±1.62/30.41±7.38		0	
		Exercise	24.08±9.48/23.06±8.18 (all ml/100ml/min)	22.72±2.1/24.88±9.84 (all ml/100ml/min)		0	
(q)	Doppler (capillary blood flow)	Rest	0.68±1.03 AU	0.51±0.31 AU		0	Some concerns
		Insulin infusion	0.67±0.49 AU	0.74±0.40 AU	p=0.31	1	

(r)	NIRS (average oxyHb, change from baseline)	Exercise at simulated altitude	Sub max: -5.67±5.34 AU Time trial: -5.50±5.31 AU	Sub max: -0.84±3.56 AU Time trial: -1.02±3.41 AU		1 1	Some concerns
Cerebral							
(o)	NIRS (SO_2)	Rest	*61.5±1%*	*64±1%*	**p<0.05**	1	Low risk
		Hypoxia	*59±1%*	*61.5±1%*	**p<0.05**	1	
		Exercise	*62±0.5%*	*64±1%*	**p=0.004**	1	
(r)	NIRS (average oxyHb- change from baseline)	Exercise at simulated altitude	Sub max: 10.7±5.06 AU Time trial: 3.99±8.34 AU	Sub max: 24.4±20.9 AU Time trial: 10.8±17.6 AU		0	Some concerns
(s)	NIRS (oxyHb, at end of word tests)	Cognitive tasks	*45±15/40±15 AU*	*60±10/45±5 AU*		1	High risk

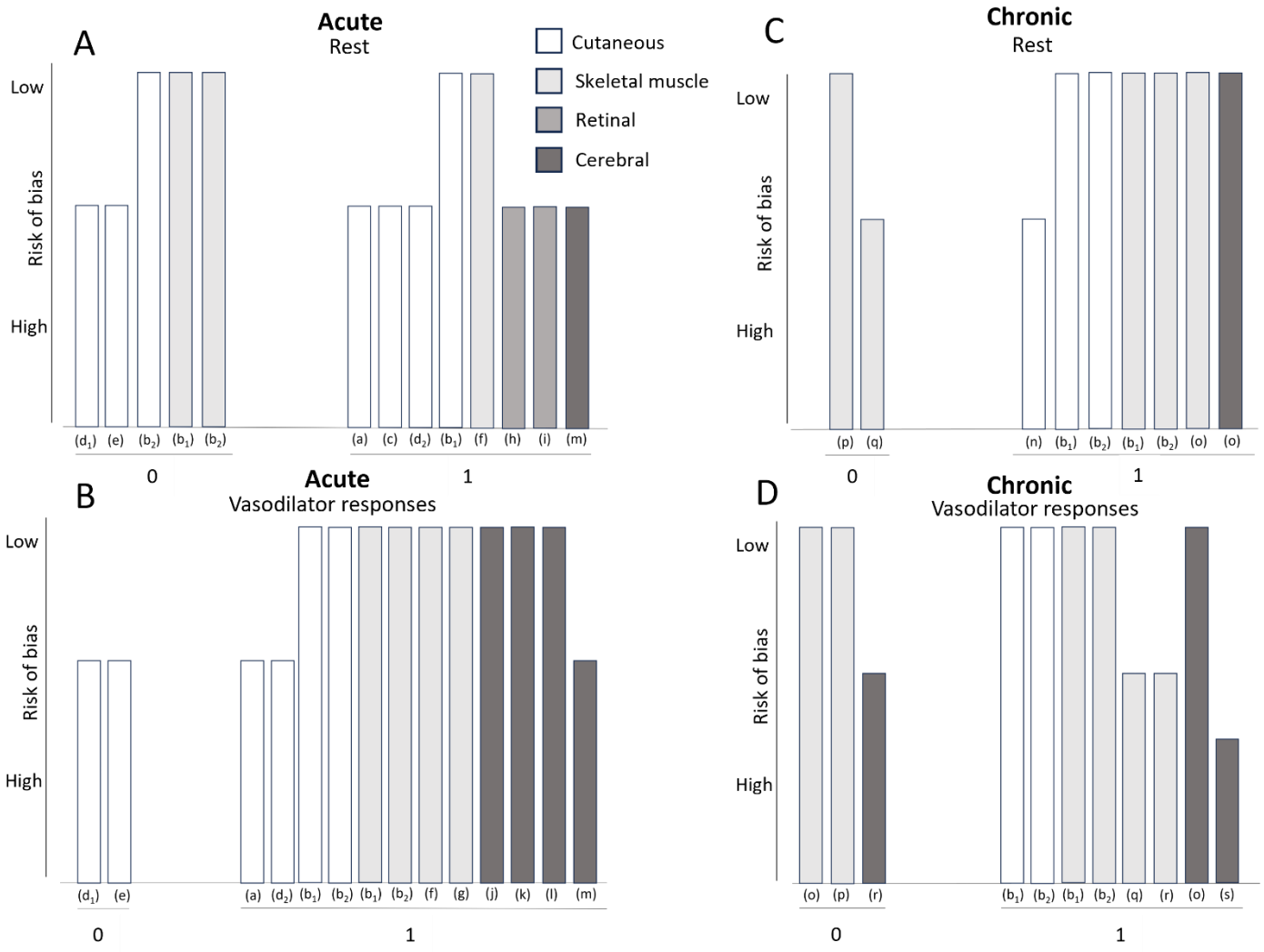

Figure 3.3: Harvest plots presenting direction of effect for subgroups of all studies*, showing acute effects (A) at rest and (B) on vasodilator responses, and chronic effects (C) at rest, and (D) on vasodilator responses. The colours of the bars represent the microvascular bed studied. Tallest bars= 'low risk of bias', shortest bars= 'high risk of bias'. '1' represents a positive effect of cocoa flavanols (CFs), and '0' represents increased outcomes with placebo vs CFs.*

3.4 Discussion

This systematic review explored the role of CFs on microvascular function, a topic which has not been reviewed in depth until now. Summarising the findings, despite the limited studies and heterogeneity of the results which prevented a definitive outcome from being reached, there is evidence to suggest that CFs may improve microvascular function, particularly in young, healthy populations. We noted statistically significant benefits of acute CFs on vasodilator responses from collation of all subgroups, as well as just young, healthy populations, importantly also when only studies with low risk of bias were selected. It also appears that the potential benefits are greater following an acute dose of CFs rather than supplementation over a period of days or weeks; for chronic studies a statistically significant effect was apparent only at rest in young, healthy groups. However, it should be emphasised that the current review necessarily incorporates findings across a range of microvascular vascular beds, and studies comparing effects of CFs at rest and in response to various vasodilator stimuli and using many different measurement techniques. These issues are discussed in more detail below.

We found evidence of a beneficial effect of CFs on vascular tone in 8 subgroups of acute (Bapir et al., 2022, Heiss et al., 2015, Neukam et al., 2007, Kim and Brothers, 2020, Baynham et al., 2021, Scuderi et al., 2020, Siedlecki et al., 2019, Bloomfield et al., 2023) and 7 subgroups of chronic studies (Heinrich et al., 2006, Heiss et al., 2015, Decroix et al., 2018b). Though neither of these effects reached statistical significance when considered all together, we found a significant effect of CFs on resting vascular tone in chronic studies including only young, healthy subgroups (Heinrich et al., 2006, Heiss et al., 2015, Decroix et al., 2018a). As resting vascular tone is largely controlled by tonic NO release (Cayatte et al., 1994), it seems likely that any effects of CFs at rest would be exerted by influences on this pathway. This is also supported

by evidence of CFs facilitation of the NO pathway (Ramirez-Sanchez et al., 2010, Ramirez-Sanchez et al., 2018, Schroeter et al., 2006). Nonetheless, since vascular tone is regulated by the balance of dilator and constrictor factors, an influence of CFs on vasoconstrictor actions cannot be ruled out. Indeed, a role for CFs in reducing circulating endothelin-1 has also been proposed (Loke et al., 2008), which would contribute to reduced resting microvascular tone and hence more dilated vessels at rest.

Considering that the predominant mechanism of CFs action is thought to be by increasing NO bioavailability, (Fisher et al., 2003b, Ramirez-Sanchez et al., 2010, Schroeter et al., 2006), the fact that improvement in dilator responses with CFs on larger vessels has been widely documented (Shrime et al., 2011, Sun et al., 2019), is not surprising since FMD is known to be predominantly NO-mediated (Green et al., 2014). The exact mechanisms underpinning this are largely unknown, though there is evidence to suggest calcium-mediated activation of signalling pathways by CFs leading to eNOS activation (Moreno-Ulloa et al., 2015, Moreno-Ulloa et al., 2014). Furthermore, CFs are proposed to act by modulating antioxidant pathways, reducing levels of circulating reactive oxygen species (ROS), thereby increasing NO bioavailability (Aprotosoaie et al., 2016b, Keen et al., 2002)

With regards to vasodilator responses in the present review, CFs were found to enhance vasodilator responses in 12 subgroups of acute studies (Bapir et al., 2022, Heiss et al., 2015, Kim and Brothers, 2020, Baynham et al., 2021, Santos et al., 2023, Decroix et al., 2018a, Decroix et al., 2016, Bloomfield et al., 2023), an effect which was statistically significant, suggesting that acute CF supplementation may enhance vasodilator responses. Although some studies included effects at rest, as well as on vasodilator responses, many did not account for differences at rest when considering peak vasodilator responses. Therefore, it is difficult to discern whether the

magnitude of dilatation was actually increased, or whether this was just confounded by the differences observed at rest. Importantly all acute studies which showed a positive effect of CFs at rest also found an increase in vasodilator responses (Kim and Brothers, 2020, Baynham et al., 2021, Bloomfield et al., 2023, Bapir et al., 2022), with others showing no benefit of CFs at rest or on vasodilator responses (Kim and Brothers, 2020, Hammer et al., 2015), and only one study showing that CFs increase FBF at peak RH but not at rest (Heiss et al., 2015). Notably, if the size of the effect at rest was similar to that on the dilator response, we could deduce that there was no additional benefit of CFs in vasodilation, however this is not possible to decipher with the evidence from current studies.

It is important to consider the complexity of factors involved in determining vasodilator responses, with differing contributions of metabolic, endothelial and myogenic factors according to the tissue and stimuli (Joyner and Casey, 2015, Clifford and Hellsten, 2004). For example, the vasodilator response to mental stress is thought to be largely mediated by NO (Halliwill et al., 1997, Cardillo et al., 1997, Seddon et al., 2008), and we noted enhanced responses by CFs in the skeletal muscle (Baynham et al., 2021) and cerebral (Decroix et al., 2016) microcirculations. On the other hand, the effect of CFs on peak RH, which is not thought to be NO-mediated (Rosenberry and Nelson, 2020), are mixed; some studies show increased RH following acute and chronic CFs (Heiss et al., 2015, Bapir et al., 2022, Santos et al., 2023), whilst others do not (Hammer et al., 2015, Farouque et al., 2006). Furthermore, exercise responses, for which the contribution of NO is controversial (Rådegran and Hellsten, 2000), are also improved by CFs according to some studies in the cerebral (Decroix et al., 2016, Decroix et al., 2018b, Sumiyoshi et al., 2019) and skeletal muscle (Shaw et al., 2020) microvasculature, but not others (Farouque et al., 2006, Decroix et al., 2018b, Shaw et al., 2020). Other factors such as prostaglandins and EDHFs are proposed to play a key role in these vasodilator responses (Clifford and Hellsten,

2004, Carlsson et al., 1987), and whether CFs also influence these other vasodilator factors has been less explored. Since effects of CFs are apparent even on responses that are not NO-dependent, it is likely that CFs are also able to influence other vasodilator pathways in some way. Nonetheless, there is evidence of interactions between vasodilator pathways (Hellsten et al., 2012, Mortensen and Saltin, 2014), hence it is possible that CFs may act on this indirectly via its effects on NO, and thus benefits may still be NO-dependent.

It has been suggested that the higher prevalence of free radicals and oxidative stress in individuals with elevated CVD risk may result in them being more susceptible to the benefits of CF supplementation (Aprotosoaie et al., 2016b). Considering findings from outside of the microvasculature, systematic reviews have demonstrated greater blood-pressure lowering effects of CFs in hypertensives compared to normotensives (Ried et al., 2017). This idea is supported by a study within the present review, in that responses evoked in cutaneous microcirculation by local heating were increased only in AAs but not in CAs (Kim and Brothers, 2020), for AAs are a group with increased CVD risk compared to CAs (Howard et al., 2017, Geronimus et al., 2007). However, other studies within the present review found no difference in the effects of CFs on RH evoked in the cutaneous or skeletal muscle between healthy young populations, and type-2 diabetics (Bapir et al., 2022) or elderly individuals (Heiss et al., 2015). These apparent disparities involving evidence from individual studies must be treated with some caution, particularly given the small sample sizes and potential concerns of bias. On the other hand, it should be noted that as a result of their systematic review, Woodward et al suggested that benefits of CFs in the microvasculature were greater in healthy individuals than those with high CVD-risk (Woodward et al., 2018). It would therefore be beneficial to conduct future studies directly comparing the role of CFs within the microvasculature in populations of differing CVD risk, in order to determine individuals for whom CF supplementation would be most beneficial.

In regard to the length of CF supplementation, it appears that CFs are more efficacious in modifying microvascular responses in the hours immediately following an acute dose, rather than by prolonged supplementation over a period of days or weeks. Despite enhanced vasodilator responses being observed after chronic CF supplementation in 8 subgroups (Heiss et al., 2015, Muniyappa et al., 2008, Shaw et al., 2020, Decroix et al., 2018b), this effect did not reach statistical significance, possibly due to the smaller number of studies which tested effect of CFs on dilator responses following chronic compared to acute supplementation. This contrasts to findings for FMD, which is modified by CFs both acutely and chronically (Ebaditabar et al., 2020, Hooper et al., 2012). Importantly, the effects on FMD have been seen across studies ranging from 7 days to 6 weeks duration (Sun et al., 2019, Shrime et al., 2011), whereas microvascular effects from studies across the same time period and using similar doses are less consistent, suggesting that the microvasculature may be less susceptible to the effect of CFs than conduit arteries. Contrastingly to in conduit arteries, it appears that the benefits of CFs within the microvasculature are short-lasting and coincide with the peak increases in flavanol metabolites and plasma nitroso species 1-3hrs after supplementation (Schroeter et al., 2006), but the effect does not persist as circulating metabolites diminish. There is evidence that high levels of habitual CF intake result in elevated urinary flavanol metabolites consistent with the intake quantities, whereas the extent of the accompanying increase in urinary nitrite/nitrate levels is much smaller (Schroeter et al., 2006). Whilst the extent of this increase may be sufficient to exert long-term effects on NO-mediated responses within conduit arteries, this does not seem to translate to effects within the microvasculature, where responses may be NO-dependent, or mediated by other factors. It is possible that CF supplementation is required over a longer time period in order to exert microvascular compared to macrovascular effects.

The lack of clear effects in chronic studies may be attributed to the variability in responses measured over time, which makes it difficult to detect changes outside of individual fluctuations, without comparison to a time control. This is likely to be accentuated by the small sample populations included in each study, meaning that they are less powered to detect small effects of cocoa. Additionally, it is difficult to control participants overall diet during a longer time-period as they are likely to be still be consuming flavonoids from other sources such as fruit and vegetables. For this reason, chronic studies should take into account background diet, since the influence of supplementary CFs may be greater in those with otherwise low dietary flavonoid intake as has been shown for memory improvement (Brickman et al., 2023). Nonetheless, these issues also apply to chronic FMD studies which still highlight beneficial effects of CFs; which implies that CFs may exert effects in the conduit vessels to a greater extent than they do in the microvasculature.

Limitations

The outcomes of the present systematic review are limited by the small number of studies available and the heterogeneity of these. There was not sufficient data for any single measure of microvascular function to allow a meta-analysis to be performed, which would have provided a better, more reliable analysis of the efficacy of CFs on human microvasculature. As an alternative to meta-analysis, the limited available data from all studies led us to conduct analysis by vote-counting (Higgins, 2023). However, this is the lowest recommended level of synthesis and it provides no information on the magnitude of effect. Also, in order to categorise all studies in a particular direction, even studies where there was no clear effect had to be categorised, meaning that some of the effects included were very small. Furthermore, vote counting does not account for relative differences in effect sizes between studies and is less powerful than other

methods used to combine p values (Borenstein, 2009). As such, by using vote counting, we were only able to answer whether there is evidence of an effect, rather than provide information on the magnitude of any effect.

There were also limitations within the design of the studies themselves, which further complicates the interpretation of their findings. Firstly, many studies report findings for mixed populations without incorporating any gender comparisons, despite evidence that vasodilator responses differ between males and females (Aiku and Marshall, 2019, Parker et al., 2007, Hashimoto et al., 1995). Furthermore, it is well established that some ethnic groups are at higher risk of CVD (such as SAs and BAs) and exhibit blunted vasodilator responses (Howard et al., 2017, Jain et al., 2017, Ormshaw et al., 2018), but most studies do not report the ethnicity of participants. Indeed, there is evidence to suggest that differences in the effect of CFs can be detected between different ethnicities, particularly CAs and AAs (Kim and Brothers, 2020). Advancing age is another common CVD risk factor that is often overlooked, with some studies reporting data from subjects from a very wide age range, despite extensive evidence of impaired microvascular vasodilator responses in elderly, compared to young populations (Heiss et al., 2015, Rosenberry et al., 2018b). As such, the variability of individual responses within a small sample group of mixed gender, ethnicity or age may mask any potential effect of CFs within the population.

Future directions

A key issue which should be addressed is the heterogeneity of study populations. Future studies of CFs in the microvasculature should stratify by gender, age, ethnicity, and health status in order to gain more valuable insight within targeted populations, without confounding the effects of variable vascular function. They should also assess the habitual diet of participants, and take into

account background flavonoid intake, which may influence the magnitude of effects induced by supplementary CFs, as has been demonstrated for hippocampal-dependent memory (Brickman et al., 2023). It would be of interest to determine whether there is a correlation between habitual flavanol intake and microvascular effects of CFs, in order to identify the individuals who may benefit the most from CF supplementation.

Future research should establish the optimal dose for CFs benefit on the microcirculation, by conducting more controlled dose-effect intervention studies. For example, there is evidence of a dose-dependent response relationship between CFs intake and beneficial effects on FMD and on blood pressure (Shrime et al., 2011). This seems likely to be the case for microcirculatory function as well, but the minimum efficacious dose might be different for macro and microvasculature. As mentioned above, this may be particularly relevant for chronic studies, where the effects of CF on microvascular responses are less profound. In order to address this, long-term supplementation using a range of doses and with assessment of vascular function and monitoring of circulating flavanols at regular intervals would provide insight into the level of CFs required to exert effects. Such information would help inform how the intake of flavanols might be translated into to daily consumption levels and it may guide future dietary recommendations.

Finally, it would be key to establish the mechanisms of action underpinning the effects of CFs within the microvasculature and how this relates to the more established effects in larger vessels. A role for NO has been postulated (Ramirez-Sanchez et al., 2010, Schroeter et al., 2006), but the complexity of the other vasodilator factors involved in microvascular responses to RH, exercise, hypoxia and stress where effects of CFs are less consistently observed, suggests that CFs may also influence other factors, whether this be directly or due to their interplay with NO (Engelke

et al., 1996, Boushel et al., 2002). In that regard, it would be useful to establish whether CFs are still effective within the microvasculature when NO activity is inhibited (for example using L-NAME) both in isolation and alongside inhibition of other pathways (such as prostaglandin synthesis inhibition by cyclooxygenase inhibitors), in order to elucidate whether CFs are acting via other pathways in this instance or whether effects are still NO-dependent.

Conclusion

Overall, having reviewed the available literature, there is evidence to suggest that supplementary CFs may reduce resting microvascular tone and improve vasodilator responses across skeletal muscle, skin and cerebral circulation, particularly when administered acutely. Nonetheless, the variability and heterogeneity between studies limits our ability to go further. No major differences were detected in efficacy of CFs between healthy and at-risk populations, but there is clearly a need to conduct studies that formally compare these. Effects were detected in microvascular responses mediated not just by NO, implying that not all effects of CFs are due to direct action via NO, though they may still be NO-dependent due to the interaction of vasodilator pathways; this is something which also warrants further exploration.

Chapter 4:

Comparison of Near-Infrared Spectroscopy and Venous Occlusion Plethysmography for monitoring Forearm Blood Flow in young White European and South Asian women

4.1 Introduction

Post-occlusion reactive hyperaemia (RH) in the forearm can be an important predictor of cardiovascular health. RH can be observed by using a variety of techniques such as venous occlusion plethysmography (VOP) and near-infrared spectroscopy (NIRS), which provide different insights into tissue blood flow and haemodynamics (Rosenberry and Nelson, 2020). The present study compared RH measured using VOP and NIRS and formed the control phase of the protocol designed to test the effects of ethnicity and cocoa across a range of stimuli (see Chapter 5). Importantly, blunted RH has been observed in populations with increased cardiovascular disease (CVD) risk, for example SAs compared to WEs (Ormshaw et al., 2018) . Thus, this study also compared changes in FBF measured with VOP, as well as changes in NIRS variables, during occlusion and RH, between young WE and SA women.

Reactive hyperaemia (RH) refers to the rapid transient increase in blood flow following release of arterial occlusion and provides a measure of microvascular vasodilatation (Rosenberry and Nelson, 2020). The mechanisms mediating this response are outlined in more detail in Section 1.4.2.1. Briefly, a combination of the loss of basal myogenic tone due to reduced stretch of the vascular smooth muscle, and release of vasodilator substances secondary to tissue ischaemia, contributes to relaxation of the vascular smooth muscle and hence local vasodilatation (Sparks and Belloni, 1978, Carlsson et al., 1987, Bayliss, 1902). The peak increase in microvascular blood flow is considered an accurate predictor of future CVD both in healthy (Anderson et al., 2011) and at risk populations (Huang et al., 2007).

Venous occlusion plethysmography (VOP) is the ‘gold standard’ technique widely used for measuring FBF during RH (Joyner et al., 2001, Rosenberry and Nelson, 2020). Despite this, a key limitation of the technique is the requirement for cuff inflation to obtain measurements of

FBF meaning that continuous monitoring of FBF is not possible; the shortest possible interval for consecutive measurements is approximately 7 seconds, and hence the peak of a hyperaemic response may not be detected if this occurs between the time-points at which FBF is measured (Joyner et al., 2001, Junejo et al., 2019).

Comparatively, NIRS provides continuous recordings of oxygenated (oxy-) and deoxygenated haemoglobin (deoxyHb), allowing limb muscle tissue oxygenation to be non-invasively monitored at the microcirculatory level (Rosenberry et al., 2018a). NIRS-derived totalHb (the sum of oxy- and deoxyHb) has been suggested to provide a useful index of blood flow, since it represents the total volume of haemoglobin in the tissue microcirculation at a given time (Alvares et al., 2020, Barstow, 2019). Indeed, totalHb recorded in the forearm following successive releases of venous occlusion was closely correlated with FBF measured by VOP at the same time-points during RH (Harel et al., 2008). Others have argued that NIRS is not an appropriate alternative to VOP at rest or during reactive hyperaemia (Gomez et al., 2022). However, they used SO_2 reperfusion slopes measured with NIRS following release of arterial occlusion rather than totalHb and therefore were not directly comparing indices of blood flow. On the other hand, continuously monitored totalHb was shown to correlate closely with brachial artery blood flow calculated from blood velocity recorded continuously and brachial artery diameter measured at intervals by Doppler ultrasound (Bopp et al., 2014). However, when using ultrasound, the assessment of FBF is dependent on the accuracy of two separate measurements, which are independently affected by movement artefact and angle of insonation (Joyner et al., 2001), whereas VOP holds the advantage that it directly measures FBF. To our knowledge, there has been no attempt to establish whether totalHb continuously recorded with NIRS correlates closely with FBF recorded at intervals with VOP.

NIRS has also been widely used to study changes in SO_2 (computed from oxyHb/ oxyHb+deoxyHb) during arterial occlusion and RH in order to assess tissue oxygenation and its regulation by microvascular reactivity in the forearm (Soares et al., 2019a, Soares et al., 2018, Rosenberry et al., 2018b, Rogers et al., 2023) and in the leg (McLay et al., 2016b, Iannetta et al., 2019, McLay et al., 2016a). For example, there is evidence in the forearm of lower rates of skeletal muscle oxygen consumption at rest, slower reperfusion rates and smaller peak hyperaemic responses in older compared to younger populations (Rosenberry et al., 2018b, Rogers et al., 2023). This highlights the suitability of NIRS for identifying differences in forearm microvascular reactivity between populations with differing CVD risk; the present study aimed to build on this by exploring these differences between young WE and SA women.

It is well documented that SA ethnicity is a major risk factor for the development of CVD (Rana et al., 2014, Aambo and Klemsdal, 2017, Jain et al., 2017). Attenuated endothelium-dependent vasodilator responses have also been documented in SAs relative to WEs, although there is some heterogeneity in findings. For example, attenuated FMD responses have been documented in SA relative to WE males in young adult (Murphy et al., 2007) and older (Chambers et al., 1999) populations, whereas others have found no ethnic difference (mean age ~30 years (Pusalavidyasagar et al., 2016)). Furthermore, impaired forearm vasodilator responses to acetylcholine infusion (Murphy et al., 2007) and peak RH following arterial occlusion (Ormshaw et al., 2018) were reported in young SA males using VOP. There is also evidence of blunted cutaneous vasodilator responses to acetylcholine in young SA males (Hirst and Marshall, 2018) and females (Ali et al., 2022) relative to WEs. In addition, SAs have been shown to have smaller cutaneous RH responses than WEs in mixed gender (Petrofsky et al., 2012) and female (Ali et al., 2022) groups, but no significant difference in cutaneous RH responses was found between young SA and WE males (Hirst and Marshall, 2018). It is likely that differences in techniques

and vascular beds used contribute to the variability in findings between studies. Furthermore, the majority of existing studies have been conducted in male or mixed populations with wide age ranges, despite evidence of differing vasodilator responses between male and female groups (Aiku and Marshall, 2019, Parker et al., 2007). The present study includes young females only, following on from the hypothesis that endothelial dysfunction may already be present in young SA women who are at greater risk of early CVD (Ahmed and El-Menyar, 2015, Ali et al., 2022).

4.1.1 Aims and hypotheses

The first aim of the present study was to compare RH following arterial occlusion when measured with venous VOP and with NIRS, in order to assess whether NIRS-derived totalHb can provide a useful index of FBF. We aimed to compare this, and other NIRS variables and indices of tissue oxygenation, during occlusion and RH between young WE and SA women. Importantly, this study formed the control phase of the protocol designed to test the effects of cocoa on microvascular function across a range of stimuli.

We hypothesise that:

(i) Changes in FBF measured by VOP and totalHb measured with NIRS will be well-correlated during reactive hyperaemia, indicative of NIRS-derived totalHb providing a useful index of FBF

(ii) WEs will have higher peak FBF and ΔtotalHb than SAs following release of arterial occlusion

(iii) Relative to SAs, WEs will have greater rate of SO_2 decline during arterial occlusion, representing greater rate of resting skeletal muscle oxygen consumption, and higher peak SO_2 following release

4.2 Methods

4.2.1 Subjects

23 healthy women aged 18-26 years participated in this study; all were students at the University of Birmingham and of either SA *(n=12)* or WE *(n=11)* ethnicity, with both parents of the same ethnic origin. Participants were recruited according to the inclusion criteria outlined in Chapter 2. Participants gave informed consent to their participation in the study and completed questionnaires regarding their general health and lifestyle as well as a food frequency questionnaire as described in Chapter 2.

Participants were asked to eliminate certain foods from their diet (full list in Appendix 4) for 24 hours prior to each laboratory visit. They were also required to refrain from vigorous exercise, and not consume any alcohol or caffeine during this time. They were also required to fast for 12 hours prior to each laboratory visit.

All study days were arranged within the first 8 days of subject's menstrual cycle, with the majority (n=40 out of 46 visits) conducted within the first 5 days, when oestrogen levels are lowest, thus minimising the cardiovascular effects of oestrogen and eliminating any cumulative contributions of oestrogen fluctuations (Hashimoto et al., 1995).

4.2.2 Experimental procedure

Each subject attended the temperature-controlled laboratory (20-22ºC) on two occasions to complete the study. Prior to, or at the start of the first visit, the subject was familiarised with the equipment and briefed on the protocol to ensure it was acceptable to them. They were then asked to relax on the couch, reclined at ~45º. ECG leads and Finometer were set-up to enable continuous monitoring of cardiovascular variables, as described in Chapter 2.

Firstly, FBF was recorded by VOP (see Section 2.3.3) before and at intervals during RH (see below). The strain gauge and wrist cuff were then removed, and the NIRS probes fixed on the participant's forearm. Photographs were taken of the positioning of the probes so that these could be replaced at the same site during each visit. Following 5mins rest, RH was measured by NIRS (see Section 2.3.4) so that the recordings of RH made by VOP and NIRS could be compared at the same time points.

4.2.3 Reactive hyperaemia

The upper cuff was inflated to 200mmHg to exceed SBP and hence occlude arterial inflow for 2 mins and then released; the subject was required to resist any temptation to move their arm for the subsequent 2 min during which recordings were made.

For RH recorded by NIRS, no further action was required during this 2 min period; data were extracted off-line. For RH measured by VOP, the first FBF measurement was made as soon as possible following release of arterial occlusion and then at 15s intervals during the first minute, and 30s intervals during the second minute of recovery. The RH procedure is outlined in more detail in Section 2.4.1.

4.2.4 Data acquisition and analysis

All information on subject characteristics and questionnaire responses were stored in an Excel spreadsheet. Outputs from the plethysmograph, Finometer and ECG leads were displayed in LabChart8 from which numerical data was extracted off-line and stored in Excel. FBF was calculated from the plethysmograph traces as described in Section 2.5.1, Figure 2.2.

NIRS outputs were collected separately on a Windows laptop supplied with Moor Instruments software and data was also extracted offline as described in Section 2.5.2, Figure 2.3. All Hb

measures are presented as change from baseline in arbitrary units (AU) and SO_2 as the percentage; the measures taken from SO_2 traces are detailed in Section 2.5.2, Figure 2.4.

4.2.5 Statistical analysis

All statistical analysis were performed and figures were created using Graphpad Prism Version 9.2.0. For anthropometric characteristics and dietary intakes, comparisons between WE and SAs were made using unpaired Student's t-tests. Parental cardiovascular health and physical activity were compared between ethnic groups by Fisher's exact tests. Two-way repeated measures analysis of variance (ANOVA) was used to identify main effects of ethnicity and visit day on cardiovascular variables, baseline and peak FBF, and NIRS variables. Since there was no significant effect of visit day (see Results (Section 4.3.3)), mean values calculated for each variable in each participant across the two visit days were used for graphical representation and unpaired Student's t-tests used for direct ethnic comparison. Pearson's correlation coefficients were used to assess the relationship between change in FBF and totalHb in WEs and SAs.

A significance level of $p<0.05$ was used for all statistical analyses, and all values reported are mean±SD.

4.3 Results

4.3.1 Population characteristics

Anthropometric characteristics of participants are shown in Table 4.1 (WE: n=11, SA: n=12). Unpaired Student's t-tests revealed ethnic differences in height ($p=0.0044$) and weight ($p=0.0142$), WEs being taller and heavier than SAs; there were no differences in BMI ($p=0.457$).

Table 4.1: Anthropometric characteristics of White European (WE) and South Asian (SA) groups. Data shown as mean±SD alongside p values from unpaired students' t-tests: *p<0.05, **p<0.01

	White European (WE, n=11)	South Asian (SA, n=12)	p value
Age (yrs)	20.8±1.88	20.1±1.41	0.325
Height (m)	1.66±0.0441	1.60±0.0553	0.0044 **
Weight (kg)	62.0±6.77	55.3±5.31	0.0142 *
BMI (kg/m^2)	22.4±2.42	21.7±2.10	0.457

As expected from the inclusion criteria, all participants were non-smokers and did not have any recognised cardiovascular or respiratory conditions. The prevalence of parental cardiovascular conditions was higher in SAs than in WEs; 75.0% of SAs reported at least one parent with a cardiovascular condition (hypertension, type 2 diabetes, high cholesterol), compared to 27.3% of WEs (p=0.0391). Hypertension was the most commonly reported condition in both ethnic groups; though the prevalence of parental hypertension was not significantly different between WEs and SAs (SA: 50.0%, WE: 18.2%, p=0.193).

4.3.1.1 Lifestyle habits

Physical inactivity was significantly more prevalent in SAs than WEs; 41.7% of SAs reported less than one day per week of physical activity at any intensity (compared to 0% of WEs, p=0.0373). Furthermore, the proportion of participants partaking in physical activity at least four times per week tended to be higher in WEs compared to SAs (WE: 72.7%, SA: 33.3%, p=0.0995).

On the other hand, the consumption of alcohol (units per week, WE: 12.7±1.76, SA: 1.17±0.833, p<0.0001) and caffeinated beverages (cups per day, WE: 2.36±1.96, SA: 0.964±1.14, p=0.0467) was significantly higher in WEs than amongst SAs.

Table 4.2 presents nutrient intakes as estimated from the FFQ and represents habitual diet over the last year. There were no ethnic differences in the majority of nutrients, although WEs had significantly higher intake of fibre ($p=0.0269$) and vitamin C ($p=0.0241$) than SAs. According to the FFQ, WEs also consumed more fruit than SAs (WE: 7.91±0.680 times/week, SA: 4.50±0.657 times/week, $p=0.0017$), though no ethnic difference was found in weekly consumption of any other foods ($p>0.164$)

Table 4.2: Habitual daily nutrient intake estimated from the Food Frequency Questionnaire for White European (WE) and South Asian (SA) groups. *Data shown as mean±SD, with p values from unpaired students' t-tests (*: p<0.05, #: p<0.1)*

	White European (WE, n=11)	South Asian (SA, n=12)	p value
Energy (kcal)	1820±614	1740±515	0.729
Carbohydrate (g)	218±91.7	221±59.8	0.922
Cholesterol (g)	241±80.6	242±93.3	0.991
Protein (g)	73.4±23.1	68.6±24.5	0.638
Fat (g)	71.1±24.0	69.3±26.5	0.866
Saturated fat (g)	25.7±9.72	25.1±10.3	0.897
Fibre (g)	19.7±5.67	14.4±5.05	0.0269 *
Folate (mcg)	305±74.9	254±97.0	0.176
Sugar (g)	103±46.5	94.0±31.0	0.608
Sodium (mg)	2470±753	2260±620	0.463
Vitamin C (mg)	133±42.3	89.5±44.1	0.0241 *
Vitamin D (mcg)	2.00±1.31	2.35±1.34	0.527

4.3.1.2 Resting cardiovascular variables and forearm blood flow

Resting ABP and HR values measured using the Finapres at the start of each visit are shown in Table 4.3. Two-way repeated measures ANOVA revealed no significant effect of visit day for any of these variables. There was a main effect of ethnicity on resting SBP, which was higher in WEs than SAs [$F(1,21)=5.09$, $p=0.0349$], and WEs also tended to have lower resting HR [$F(1,21)=4.01$, $p=0.0583$] and higher resting MABP [$F(1,21)=3.90$, $p=0.0615$] but there was no ethnic difference in resting DBP [$F(1,21)=2.14$, $p=0.158$]. There was no main effect of ethnicity or day on baseline forearm blood flow (FBF) as also shown in Table 4.3.

Table 4.3: Resting variables measured at the start of each visit for White European (WE, n=11) and South Asian (SA, n=12) groups. *Data are shown as mean± SD, with p values for main effects of ethnicity, visit day and ethnicity x visit day interaction (from 2-way repeated measures ANOVA: *p<0.05, #: p<0.1). HR: heart rate (bpm), MABP: mean arterial blood pressure (mmHg), SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (DBP), FBF: forearm blood flow, measured by venous occlusion plethysmography (ml.min^{-1}.100g tissue^{-1})*

				Main effects		
		Visit A	Visit B	Ethnicity	Day	Ethnicity x Day
HR (bpm)	WE	65.7±8.51	65.7±6.63	0.0583 #	0.791	0.792
	SA	71.3±7.62	72.1±9.07			
MABP (mmHg)	WE	84.8±6.82	85.3±8.81	0.0615 #	0.452	0.550
	SA	75.7±11.5	79.7±15.4			
SBP (mmHg)	WE	127±11.6	127±11.8	0.0349 *	0.520	0.485
	SA	112±16.1	117±21.8			
DBP (mmHg)	WE	63.6±6.85	64.3±7.91	0.158	0.456	0.660
	SA	57.9±9.71	60.9±12.5			
FBF (ml.min^{-1}.100g tissue^{-1})	WE	4.50±2.69	4.01±1.23	0.258	0.331	0.986
	SA	3.82±1.98	3.31±1.09			

4.3.2 Reactive Hyperaemia: VOP vs NIRS correlation

As indicated in Section 2.5.2 continuous NIRS monitoring does not provide absolute chromophore concentrations, hence NIRS Hb variables are presented as change from baseline at each time-point. As totalHb represents the combined volume of oxyHb and deoxyHb within the forearm, this was considered to be an index of FBF (see Section 4.1). Changes from baseline FBF and totalHb, measured by VOP and NIRS respectively, following release of 2 mins upper arm arterial occlusion, are shown in Figure 4.1. RH measured with the two techniques follows a very similar pattern, with an initial peak followed by steady decline to baseline within the first 30s (Figure 4.1). The mean time from end of arterial occlusion to the first VOP measure was 3.94±0.459s (time-adjusted in Figure 4.1); this represented the maximum FBF measure for all participants and was strongly correlated with peak ΔtotalHb (p=0.002, r=0.527, Figure 4.2A). There was also a strong correlation between FBF and ΔtotalHb at matched time-points from peak

to recovery measured by VOP and by NIRS respectively in both WEs ($p<0.0001$, $r=0.785$, Figure 4.2B) and SAs ($p<0.0001$, $r=0.813$, Figure 4.2C).

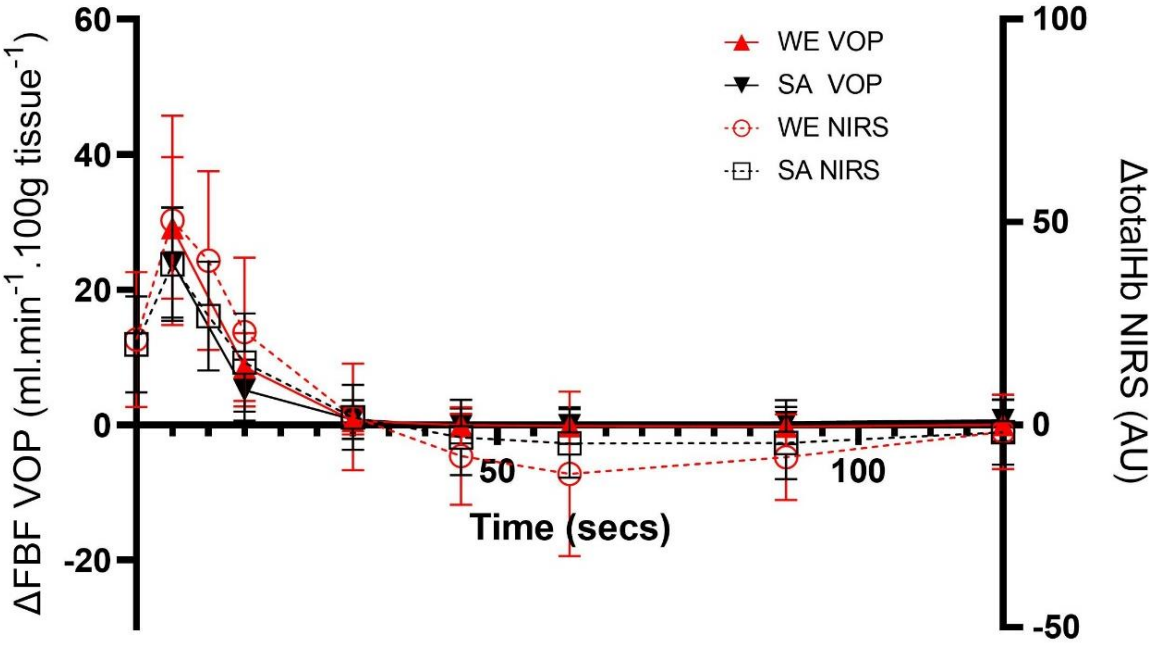

Figure 4.1: Reactive hyperaemia following release of upper arm arterial occlusion, measured by venous occlusion plethysmography and near-infrared spectroscopy. *Traces represent responses measured with NIRS (ΔtotalHb, dotted lines, right y axis) and VOP (forearm blood flow (FBF): solid lines, left y axis) in White Europeans (WEs, red) and South Asians (SAs, black). Data presented is mean ± SD.*

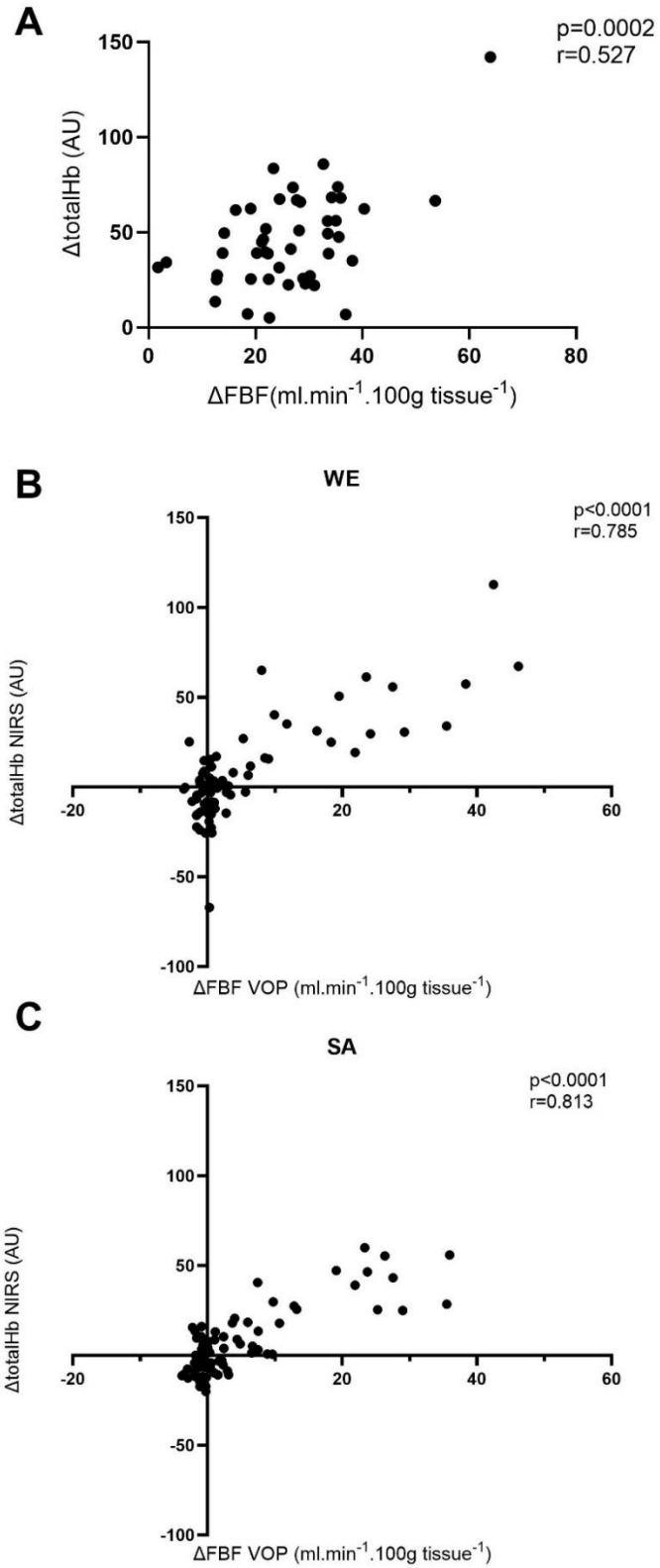

Figure 4.2: Correlation between changes in totalHb and forearm blood flow during reactive hyperaemia. *(A) at peak RH and throughout response in (B) White Europeans (WE, p<0.0001, r=0,785) and (C) South Asians (SA, p<0.0001, r=0.0813)) at matched time-points following release of 2 min upper arm arterial occlusion, as measured with NIRS (totalHb) and venous occlusion plethysmography respectively; values are shown as change from baseline.*

4.3.3 Reactive Hyperaemia: Ethnic Differences

Table 4.4 summarises peak changes observed in FBF and NIRS Hb variables during RH. Table 4.5 shows the time between end of arterial occlusion and peak or nadir for each NIRS variable. As no significant effect of visit day was found for any variable, subsequent ethnic comparisons and graphs displayed represent the mean across the 2 visit days for each participant.

Table 4.4: Changes in forearm blood flow (FBF), measured by venous occlusion plethysmography (VOP), and near-infrared spectroscopy measures at peak reactive hyperaemia in each visit for White European (WE, n=11) and South Asian (SA, n=12) groups. *Data are shown as mean±SD, with p values for main effects of ethnicity, visit day and ethnicity x visit day interaction (from 2-way repeated measures ANOVA: *p<0.05, #: p<0.1)*

				Main effects		
		Visit A	Visit B	Ethnicity	Day	Ethnicity x Day
Peak ΔFBF (ml.min^{-1}.100g tissue^{-1})	**WE**	30.2±13.8	27.8±13.4	0.202	0.311	0.842
	SA	25.9±8.27	22.3±8.94			
Peak ΔtotalHb (AU)	**WE**	54.2±34.8	50.5±29.1	0.188	0.938	0.599
	SA	39.2±11.8	42.0±20.2			
Peak ΔoxyHb (AU)	**WE**	67.3±42.0	66.2±34.0	0.0621 #	0.712	0.837
	SA	48.7±16.6	44.9±16.0			
Nadir ΔdeoxyHb (AU)	**WE**	-46.9±19.8	-40.9±15.1	0.0273 *	0.252	0.622
	SA	-32.4±10.5	-30.0±14.3			

Table 4.5: Time from end of arterial occlusion to peak change in near-infrared spectroscopy measures during reactive hyperaemia in each visit for White European (WE, n=11) and South Asian (SA, n=12) groups. *Data are shown as mean±SD (seconds), with p values for main effects of ethnicity, visit day and ethnicity x visit day interaction (from 2-way repeated measures ANOVA)*

				Main effects		
		Visit A	Visit B	Ethnicity	Day	Ethnicity x Day
Time to peak SO2 (s)	**WE**	13.7±3.10	13.2±3.38	0.584	0.756	0.355
	SA	9.98±2.60	10.5±2.91			
Time to peak ΔtotalHb (s)	**WE**	5.27±4.15	5.36±2.42	0.149	0.688	0.784
	SA	3.80±1.12	4.28±2.13			
Time to peak ΔoxyHb (s)	**WE**	10.1±2.91	11.3±2.49	0.237	0.477	0.360
	SA	9.78±1.78	9.63±2.93			
Time to nadir ΔdeoxyHb (s)	**WE**	10.5±3.11	11.1±2.70	0.240	0.738	0.767
	SA	11.9±2.35	11.9±3.47			

Figure 4.3 shows peak FBF measured by VOP and peak ΔtotalHb during RH, as mean across the 2 visits for each participant. Unpaired Student's t-tests showed no ethnic differences in peak FBF (WE: 29.0±10.4, SA: 24.1±7.36, p=0.202) or peak ΔtotalHb (WE: 52.3±25.7 AU, SA: 40.6±14.7 AU, p=0.188).

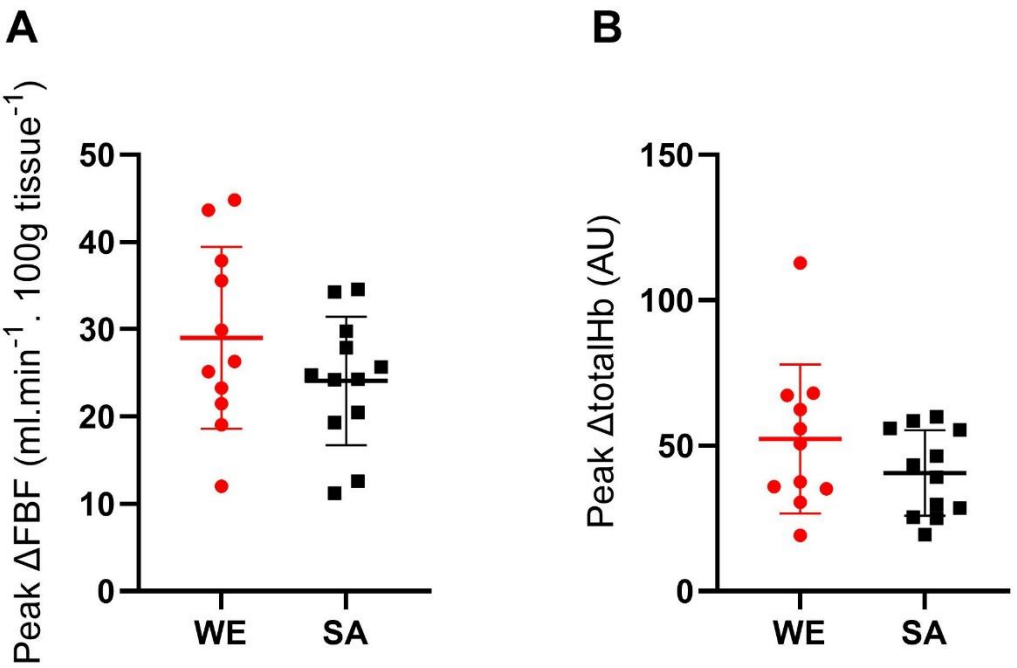

Figure 4.3: Change in (A) forearm blood flow (FBF) and (B) totalHb at peak reactive hyperaemia, measured by venous occlusion plethysmography (VOP) and near-infrared spectroscopy (NIRS) respectively, *in White Europeans (WE, n=11) and South Asians (SA, n=12). Data presented is mean±SD*

Table 4.6 shows measures calculated from SO_2 traces (as represented in Figure 2.4). Two-way repeated measures ANOVA showed that there was no significant effect of visit day on any SO_2 measure; there was a significant ethnicity x day interaction for baseline SO_2 [$F(1,20)=6.48$, $p=0.0193$], though post-hoc comparisons found no significant differences between groups. Figure 4.4 presents findings from the mean across the two visits for each participant, which were compared by unpaired Student's t-tests. Importantly, the rate of desaturation (Slope 1) was

greater for WEs (-0.123±0.0366 %/s) than SAs (-0.0904±0.0296 %/s, p=0.0283, Figure 4.4C), but there was no ethnic difference in the minimum SO_2 at the end of occlusion (WE: 45.4±5.09%, SA: 48.0±5.55%, p=0.261, see Figure 4.4B). Peak SO_2 was also significantly greater in WEs than SAs, when presented as absolute peak (WE:73.6±1.69%, SA: 69.7±3.42%, p=0.0029, Figure 4.4E) and change from baseline (SO_2 hypr; WE:13.4±2.81, SA: 10.2±2.25, p=0.0066, Figure 4.4F). Furthermore, the 10s reperfusion slope (Slope 2) was significantly greater in WEs (2.46±0.483%/s) than SAs (1.95±0.347%/s, p=0.0086, Figure 4.4D).

Table 4.6: Measures calculated from oxygen saturation (SO_2) traces in each visit for White European (WE, n=11) and South Asian (SA, n=12) groups. *Data are shown as mean±SD, with p values for main effects of ethnicity, visit day and ethnicity x visit day interaction (from 2-way repeated measures ANOVA; *:p<0.05, **:p<0.01). Slope 1: desaturation slope; SO_2 min: minimum SO_2; SO_2 max: maximum SO_2; Slope 2: 10s reperfusion slope; SO_2 hypr: hyperaemic reserve (SO_2 max- baseline)*

				Main effects		
		Visit A	Visit B	Ethnicity	Day	Ethnicity x Day
Baseline SO_2 (%)	**WE**	59.4±2.30	60.9±3.02	0.306	0.710	0.0193 *
	SA	59.2±3.67	58.0±4.99			
SO_2 min (%)	**WE**	45.6±5.62	45.2±6.61	0.319	0.616	0.856
	SA	48.2±6.59	47.3±6.01			
SO_2 max (%)	**WE**	72.1±2.05	73.9±2.30	0.0045 **	0.719	0.003 **
	SA	70.7±3.58	68.3±3.54			
SO_2 hypr (%)	**WE**	13.7±3.10	13.2±3.38	0.0066 **	0.980	0.440
	SA	9.98±2.60	10.5±2.91			
Slope 1 (%/s)	**WE**	-0.115±0.041	-0.131±0.049	0.0283 *	0.539	0.319
	SA	-0.092±0.035	-0.088±0.038			
Slope 2 (%/s)	**WE**	2.39±0.559	2.53±0.716	0.0086 **	0.654	0.182
	SA	2.09±0.500	1.82±0.357			

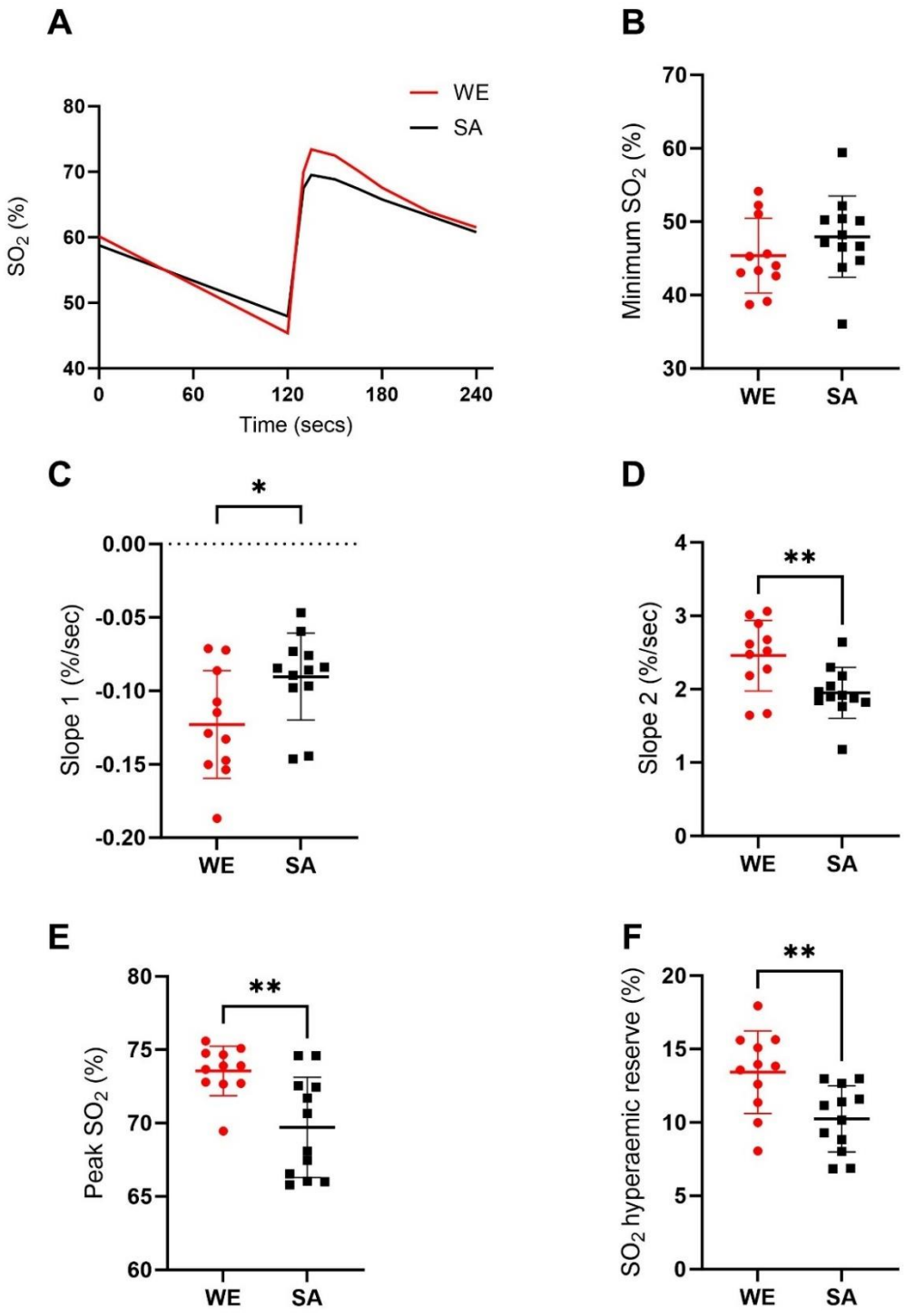

Figure 4.4: Ethnic differences in aspects of tissue oxygen saturation (SO_2, %) during occlusion and reactive hyperaemia. *Individual data represents mean for each participant across both visits. White Europeans (WE, n=11, red) and South Asians (SA, n=12, black) alongside group mean ± SD. (A) NIRS traces during and following 2 mins upper arm occlusion. Unpaired Student's t-tests revealed ethnic differences in (B) minimum SO_2 (SO_2 min), (C) slope 1 (desaturation slope), (D) slope 2 (10s reperfusion slope), (E) absolute peak SO_2 and (F) hyperaemic reserve *: p<0.05, **: p<0.01*

Figure 4.5 presents change in oxyHb and deoxyHb following release of arterial occlusion. The time courses of the change in oxyHb and deoxyHb were very similar in WEs and SAs (Figure 4.5 A&C). However the nadir ΔdeoxyHb during RH was significantly greater in WEs (-43.9±15.1 AU) than SAs (-31.2±9.77 AU, p=0.0273) and there was a trend for WEs to have higher peak ΔoxyHb than SAs, although any difference did not reach statistical significance (WE: 66.7±31.9 AU, SA: 46.8±14.1 AU, p=0.0621).

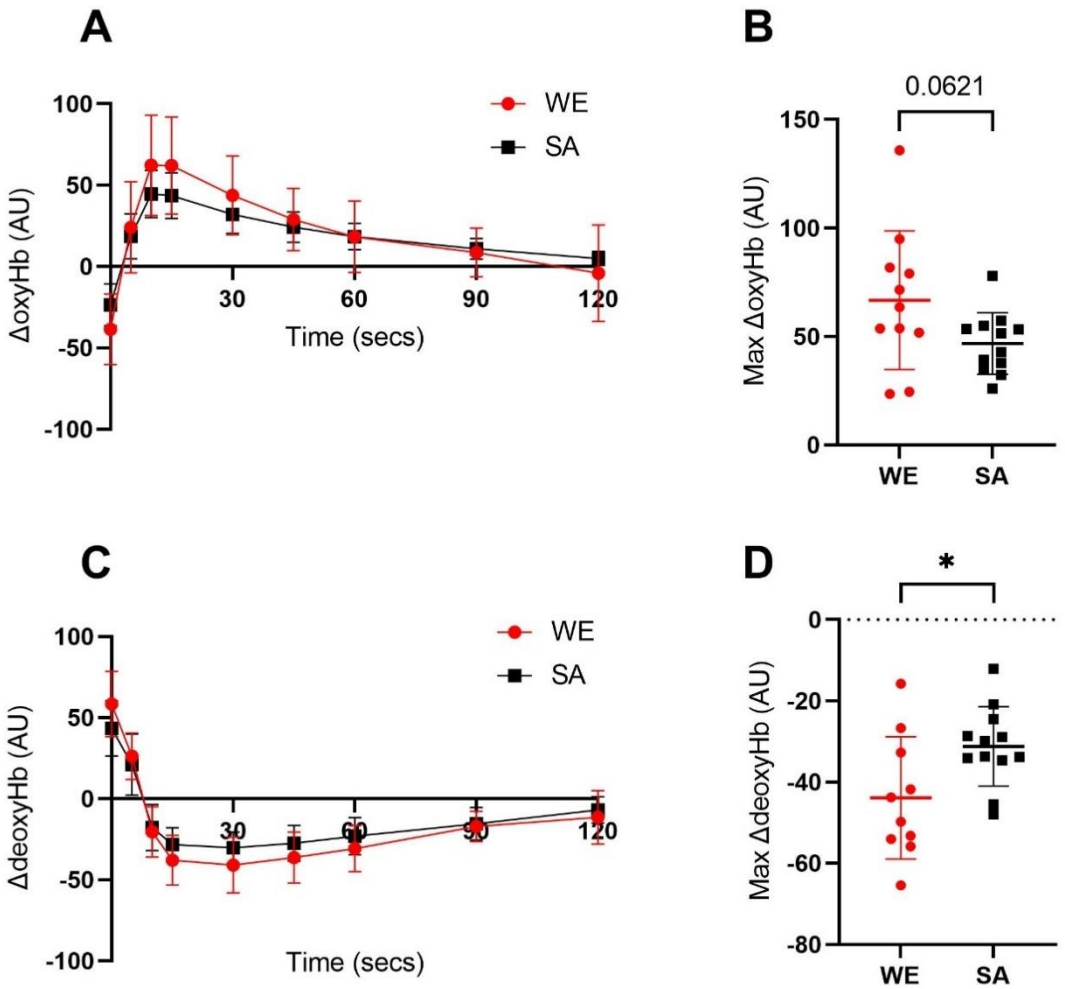

Figure 4.5: Ethnic differences in reactive hyperaemia traces and maximum change in oxyHb and deoxyHb. *Traces (A and C) represent mean±SD. Unpaired students' t-tests showed significant ethnic difference for change in (D) deoxyHb, though change in (B) oxyHb did not reach statistical significance. White European (WE, n=11, red) and South Asian (SA, n=12, black) *: p<0.05*

4.4 Discussion

The present study demonstrated a strong correlation between changes in FBF measured with VOP and changes in totalHb recorded by NIRS at corresponding time-points throughout RH in both WE and SA women. This is consistent with our hypothesis that continuous NIRS-derived totalHb can provide a useful index of forearm blood flow. Contrary to our hypothesis, there was no difference between SA and WE women in FBF or ΔtotalHb at peak RH. However, the rate of Hb desaturation (slope 1) during arterial occlusion and the peak SO_2 (SO_2 max and SO_2 hypr), as well as reperfusion rate (slope 2) during RH were all greater in WEs than SAs, as detected using NIRS. The nadir ΔdeoxyHb was also greater during reperfusion, and peak ΔoxyHb tended to be greater in WEs than SAs. Taken together, these findings suggest that NIRS may provide a useful tool for observing differences in tissue oxygenation at the microcirculatory level which cannot be discerned using traditional techniques such as VOP, and that microvascular responses following arterial occlusion are greater in WE than SAs.

Firstly, it is important to note that the present study comprised groups of young adult WE and SA women who did not differ in age or BMI, but there were differences in height and weight between the groups, WEs being taller and heavier than SAs as has previously been documented in adolescents (Nightingale et al., 2011).

There were also some key differences between WE and SA populations in the present study in terms of their general health and lifestyle. Of note, there was a higher prevalence of parental CV conditions amongst SAs than WEs, as could be expected due to the higher prevalence of hypertension and CVD amongst SAs (Whitty et al., 1999, Tillin et al., 2012, Rana et al., 2014). Furthermore, young WE women were more physically active than young SA women as reported previously in comparisons between WEs and SAs in older women (Babakus and Thompson,

2012) and children (Duncan et al., 2008, Duncan et al., 2012). There were also some ethnic differences in nutrient intake, specifically WEs had higher consumption of fibre and vitamin C. Low fibre intake amongst SA women has been previously demonstrated in older SA women (Sachan DS, 1999) and those with gestational diabetes (Croxford et al., 2021), but there seems to be no published evidence of differences in vitamin C intake between WEs and SAs. The difference in Vitamin C intake is likely to be explained in part by the fact that WEs more frequently consumed fruit. Taken together, our findings highlight the possibility that a diet relatively deficient in components known to promote health, and physical inactivity, amongst young SA women may contribute to their increased CVD risk and potentially to some of the findings in relation to RH.

4.4.1 NIRS-derived totalHb as an index of FBF

The primary aim of the present study was to compare change in FBF, measured with VOP, and change in totalHb, recorded by NIRS, at corresponding time-points throughout RH. In relation to RH, it should be noted that the earliest time at which FBF could be measured following release of arterial occlusion using VOP was actually within ~3-5s of releasing the venous occlusion and it is this value that is taken as peak RH in VOP studies (Engelke et al., 1996, Ormshaw et al., 2018, Harel et al., 2008). Importantly, by using continuous NIRS recording the peak ΔtotalHb was actually shown to be reached ~3-5s after release of arterial occlusion, and it was these peak values obtained with the two techniques that were well correlated. Moreover, when the two measurements were temporally aligned, the time-course of FBF and ΔtotalHb corresponded very closely throughout recovery (Figure 4.1). This was true in both WEs and SAs, and there was a strong correlation between change in NIRS-derived totalHb and FBF measured with VOP at matched time-points throughout RH recovery (Figure 4.2), as

hypothesised. Previous studies using NIRS to calculate FBF from the rate of increase in totalHb measured during venous occlusion have demonstrated that these values are closely correlated with instantaneous measurements of FBF obtained using VOP both at rest and at intervals following arterial occlusion (Harel et al., 2008) and handgrip exercise (De Blasi et al., 1994, Van Beekvelt et al., 2001, Homma et al., 1996a). Furthermore, there is evidence of close correlation between continuously monitored totalHb and FBF calculated using Doppler ultrasound of the brachial artery (Bopp et al., 2014, Soares et al., 2019b). The present findings therefore add to the evidence that NIRS-derived ΔtotalHb may, indeed, be a useful index of FBF.

Importantly, the present study differs from those that have previously compared FBF obtained by VOP with NIRS-derived totalHb as the present study used *continuous* recordings of totalHb as an index of FBF, rather than applying venous occlusion to calculate FBF from totalHb in absolute terms. The fact totalHb recorded by NIRS can provide an accurate measure of FBF provided the first heart-beat following venous occlusion is used, as inclusion of subsequent cardiac cycles can lead to underestimation of muscle blood flow, as is the case for FBF recorded by VOP (Cross and Sabapathy, 2017, Junejo et al., 2019) is important, because it helps to validate NIRS as a valuable technique. However, this methodology for using NIRS is again limited to intermittent recordings of FBF because each measurement is made following venous occlusion. On the basis of the present findings, it is reasonable to propose that continuous recordings of ΔtotalHb by NIRS, provides an important opportunity to accurately record the time-course of an index of FBF, even though totalHb must be expressed in relative rather than absolute terms (ΔtotalHb).

4.4.2 Ethnic differences in Reactive Hyperaemia

The present study found no significant differences between WE and SAs for peak change in FBF nor ΔtotalHb following release of arterial occlusion (Figure 4.3). This contrasts with previous evidence of impaired forearm vasodilator responses to arterial occlusion in young SA relative to WE men measured with VOP (Ormshaw et al., 2018), and of impaired cutaneous RH in SAs compared to WEs in mixed gender (Petrofsky et al., 2012) and young female (Ali et al., 2022) groups. To our knowledge, the present study is the first to compare RH in forearm muscle between young WE and SA women. The proportional differences between group means for both ΔFBF (19.6%) and ΔtotalHb (26.3%) at peak RH suggested that there may be a physiologically relevant difference between the two ethnic groups. However, the variability in peak changes in FBF and totalHb between individual WE and SA women suggests much larger group sizes would be required to discriminate this. Indeed, power calculations revealed that for unpaired Student's t-tests comparing peak ΔFBF and ΔtotalHb between WE and SAs, at least 70 women would be required per group to identify a significant difference between the groups (for power= 85%, significance level=0.05). This indicates that for RH at least, the blunting of endothelium-dependent responses on which RH is largely dependent (Rosenberry and Nelson, 2020) may not be as pronounced in young SA women relative to WE women, as it is in young SA men relative to WE men.

The use of NIRS also enabled monitoring of tissue oxygen saturation (SO_2) during and after release of arterial occlusion, as has been widely reported (Rogers et al., 2023, Rosenberry et al., 2018b, Kragelj et al., 2001). To our knowledge the present study is the first to use this technique to make ethnic comparisons for SO_2 measures (Table 4.6, Figure 4.4). Firstly, the present study showed that the rate of oxygen desaturation during arterial occlusion was greater in WEs

compared to SAs. Given the rate of change in SO_2 during arterial occlusion must reflect the rate at which O_2 is being taken up by local tissue cells, this indicates a lower rate of muscle oxygen consumption in SAs than WEs (Boushel et al., 2001, Rogers et al., 2023). This suggests that there may be differences in skeletal muscle composition or vascularity between WEs and SAs, though any such ethnic differences have not previously been explored. Since there is evidence of a strong positive correlation between resting oxygen uptake and the proportion of fast-twitch glycolytic fibres (Zurlo et al., 1994), it may be that WEs have a greater proportion of fast-twitch glycolytic fibres compared to SAs, thereby allowing a greater rate of muscle oxygen consumption. Furthermore, there may be a greater vascular density in WEs compared to SAs which may facilitate O_2 uptake, given that capillary density is increased by exercise training (Coyle et al., 1991) and WE women had higher level of physical activity SA women. The fact that the minimum SO_2 reached at the end of the occlusion period did not differ significantly between ethnic groups, reflects the variability between individual data within the relatively small group size. A power calculation showed that 60 women per group would be required to detect a significant difference in SO_2 between WEs and SAs using unpaired Student's t-test (for power= 85%, significance level= 0.05).

Nevertheless, a second main finding in relation to SO_2 was that the peak increase in SO_2 following occlusion release was significantly higher in WE than SA women, as was the hyperaemic reserve (from baseline SO_2 to peak SO_2). Since the SO_2 peak occurred at 10-15s, and was higher in WEs than SAs, whereas, as discussed above, the peak FBF and ΔtotalHb of RH occurred at ~3-5s and did not differ between WEs and SAs, this raises the possibility that different sections of the microcirculation may behave differently in WE and SAs. Whether this is the case may be deduced by considering the changes in oxyHb and deoxyHb. During arterial occlusion, oxyHb fell below baseline, whereas there was a concomitant increase in deoxyHb

above baseline levels, as expected on the basis of off-loading oxygen from haemoglobin to support resting tissue oxygen consumption (Rosenberry and Nelson, 2020). However, at ~3-5s following release of occlusion, when peak ΔtotalHb and FBF were reached, both oxyHb *and* deoxyHb were higher than their baseline levels (see Figure 4.5), oxyHb having risen rapidly immediately following occlusion, while deoxyHb was still falling towards its minimum value during recovery. This suggests that, as well as vasodilation of terminal arterioles in response to substances released as a consequence of local hypoxia, proximal arterioles and small arteries which make the major contribution to vascular resistance must have also reached their peak dilatation within 5 seconds. Efflux of deoxygenated blood from the venous vessels apparently had little counteracting influence on the peak increase in totalHb; indeed, dilatation of the small venules in response to the local dilator metabolites may even have contributed to the increase in totalHb (Marshall and Tandon, 1984). However, peak vasodilatation of arterial resistance vessels within the first 5 seconds can be explained because terminal arteriolar dilatation leads to rapid antidromic propagation to more proximal arterial vessels (Bagher and Segal, 2011, Segal, 2005). Since there was no significant difference in *peak* ΔtotalHb or FBF between ethnic groups, it is reasonable to deduce that the contribution of small arteries and proximal arterioles to peak RH is similar between young WE and SA women.

However, the fact that peak SO_2 occurred at ~10-15s after occlusion release, over a period when there is a continuing contribution of local metabolic dilator influences (Crecelius et al., 2013b), and was larger in WE than SA women, suggests that the terminal arterioles may dilate less in response to local metabolites in SA women and so are less effective in restoring tissue oxygenation than in young WE women. Consistent with this proposal, the rate of reperfusion during the first 10s (Slope 2) was also higher in WEs than SAs, suggesting microvascular reactivity was slower in young SA than WE women (Soares et al., 2019a, McLay et al., 2016a).

Interestingly, this proposal is consistent with reports that FMD of the brachial artery is greater in young WEs than in young SAs (Murphy et al., 2007, Junejo et al., 2020a, Roberts et al., 2023). Indeed, the fact that there is a strong correlation between FMD and the 10s reperfusion slope, has led to the proposal that the increased shear stress that arises from the increased FBF upon release of arterial occlusion contributes to, or provides the stimulus for, FMD of the brachial artery (Soares et al., 2019b, Soares et al., 2020, McLay et al., 2016a). This proposal is consolidated by findings in children that the reperfusion slope was correlated with shear stress, but not with FMD or with peak RH measured by laser Doppler (Kranen et al., 2023).

It is well established that FMD is mainly NO-dependent and is blunted in those with endothelial dysfunction (Green et al., 2011), and there is evidence to suggest that NO plays a role in vasodilation of terminal arterioles in response to local metabolic stimuli (Lamb et al., 2018). Furthermore, a role of NO has been postulated in the recovery phase of the RH, but not in peak flow (Tagawa et al., 1994, Bank et al., 2000). Thus, in the context of the present study, the finding that there was a difference between young WE and SA women in maximum SO_2 during RH but not in peak FBF or ΔtotalHb may indicate a lower NO bioavailability in SAs than WEs. Importantly, studies in BAs have implicated a reduction in NO bioavailability as a major contributory factor to their impaired vasodilator responses relative to WEs (Mata-Greenwood and Chen, 2008, Brothers et al., 2019). Further, impaired NO-dependent vasodilation in the forearm has previously been demonstrated in SA men relative to WE men and in the cutaneous circulation of SA women with hypertensive parents relative to WE women (Murphy et al., 2007, Ali et al., 2022). In future studies on young women it will be important to use NOS inhibitors to elucidate the role of NO in the slower reperfusion slope and lower peak SO_2 during RH in young SA women, for the results may inform strategies to improve vascular function in this group.

In relation to the other defining characteristics of the SA and WE women of the present study, it is well established that lifestyle choices such as physical activity have been linked to improved vascular function (Pasqualini et al., 2010, Boutcher and Boutcher, 2005, Sinoway et al., 1986). This raises the possibility that the enhanced dilator responses in young WE relative to SA women may be at least partly driven by the higher levels of physical activity in WE women (Lanier et al., 2016). Specifically, exercise training has been shown to improve FMD and vasodilatation of terminal arterioles (Early et al., 2017, Sindler et al., 2013), and as such may be particularly beneficial to SA women in whom dilator responses are impaired and physical activity levels are low. Furthermore, the greater consumption of vitamin C in WEs compared to SAs may also contribute to their differing vascular responses, given evidence that RH is augmented by supplementary vitamin C (Caruana and Marshall, 2015). As such, dietary interventions to augment vasodilator responses may be particularly advantageous in SAs.

4.4.3 Limitations

Despite attempts to mitigate the inherent limitations of NIRS and VOP techniques throughout the present study, there are some considerations which should be made when interpreting results. Firstly, NIRS signals can be influenced by adipose and skin tissue thickness (Homma et al., 1996b), which were not accounted for in the present study; the impact of this is unclear. There is some evidence to suggest higher adiposity levels in SA compared to WE children and adolescents (Nightingale et al., 2011, Ehtisham et al., 2005), although a recent systematic review found no differences in subcutaneous or visceral fat between WE and SA women aged 37-73 years (Iliodromiti et al., 2023). Future studies should measure skin and adipose tissue thickness using dual-energy X-ray absorptiometry or ultrasound to identify potential differences between young SA and WE women despite participants being within the healthy BMI range, as ethnic

differences in adipose thickness can affect the comparison of NIRS signal (Lu et al., 2019). Nevertheless, the strong correlation between time-matched RH measures from VOP and NIRS in both WE and SA populations suggests that NIRS does provides a similarly useful measure across WE and SA ethnic groups.

Furthermore, as the present study formed part of a wider experimental protocol in which all subsequent measurements were made with NIRS, RH was always assessed first with VOP and then NIRS, rather than the order being randomised as would have been optimal. Despite this, the protocol allowed at least 15 mins between RH measures, allowing baseline levels to be restored before subsequent cuff inflation for the second test of RH. Importantly, previous evidence shows no significant difference between RH measures repeated as soon as baseline is restored (Barton et al., 2011). Thus, it seems relatively unlikely the RH response measured by NIRS were affected by events that happened during RH monitored with VOP.

NIRS and VOP measures were conducted on the same forearm, with monitoring equipment positioned as similarly as possible in order to limit possible discrepancy between observations made using the two techniques. As the study was conducted across two experimental visits for each participant, photographs were taken of equipment set-up before removal during the first visit and best efforts were made to ensure that these were consistently positioned in the same location during the second visit. Nonetheless, it must be accepted that exact positioning could not be guaranteed. The present study tested inter-visit differences and as no significant differences were found, made comparisons between the WEs and SAs using the mean values for data across the 2 visit days to minimise any potential influence of variable probe positioning.

4.4.4 Conclusion

The present study demonstrates that continuously recorded changes in totalHb obtained by NIRS provides a potentially useful index of FBF during RH, with the additional benefit over VOP that the time-course of FBF responses can be made continuously and in real-time rather than at intervals, with calculations of FBF being made off-line. Further, given RH is a commonly used tool for assessing vascular function in clinical populations, NIRS appears a promising technique for enabling non-invasive continuous observations. In addition, NIRS provides useful information on changes in tissue oxygenation which could enable insight into oxygen consumption not only during RH but in other contexts such as exercise; these issues are explored in later studies presented in this thesis. By using NIRS the study showed that young WE women have a greater rate of resting oxygen consumption in forearm muscle than SA women; this is a novel finding. The present study also showed for the first time that peak change in SO_2 during RH and reperfusion rate are greater in WE than SA women, suggesting that there are differences in their microvascular responsiveness following a period of tissue ischaemia. It is clear that these differences warrant further investigation within a larger population in order to better understand the relevance of such differences for their CVD-risk and the mechanisms by which they are mediated.

Chapter 5:

Comparing the effect of an acute dose of cocoa flavanols on forearm vasodilator responses in young South Asian and White European women

5.1 Introduction

The systematic review presented in Chapter 3 highlighted that there is a lack of studies comparing effects of CFs within the microvasculature and argued that future studies in this area should be more specific in the age, gender and ethnicity of the populations they recruit. The present study set out to test the effect of a CF intervention within a group of young women of either WE or SA ethnicity, in order to determine whether the effects differ between ethnic groups, given previous evidence of impaired vasodilator responses in young SAs, shown in Chapter 4 and elsewhere (Ali et al., 2022, Hirst and Marshall, 2018, Ormshaw et al., 2018, Petrofsky et al., 2012).

Microvascular vasodilator responses provide an important insight into endothelial function and can provide an important predictor of future CVD (Anderson et al., 2011). The forearm is commonly used for assessing this due to its ease of accessibility. Furthermore, the skeletal muscle vasculature is regulated by a complex array of factors in order to ensure that the oxygen delivery meets the demands for tissue metabolism, as well as contributing to the regulation of systemic vascular resistance and the distribution of cardiac output (Segal, 2005, Segal and Kurjiaka, 1995, Thomas and Segal, 2004), therefore is a prime site for measuring microvascular vasodilator responses. From the comparison of techniques in Chapter 4, it seems that NIRS provides a promising tool for assessing forearm microvascular responses between ethnic groups in RH and further proposed that ΔtotalHb provides a reliable index of FBF, when directly compared with FBF measured by VOP (see Chapter 4). NIRS also holds advantages over VOP in that it provides not only continuous recordings of totalHb as an index of FBF, but also continuously monitors tissue oxygenation levels.

RH, EH and mental stress are commonly used stimuli to evoke forearm vasodilation, with different underlying mechanisms, as outlined in more detail in Chapter 1. Briefly, RH is the vasodilation occurring following the release of arterial occlusion and this is thought to be regulated by a combination of myogenic and metabolic factors, such as NO, prostaglandins and adenosine (Rosenberry and Nelson, 2020). On the other hand, during exercise, skeletal muscle blood flow increases proportionally to the increased metabolic demand of the tissue, this is mediated by a combination of local controls, such as metabolic, endothelial, and muscle pump mechanisms, with some additional central regulation (Joyner and Casey, 2015). Finally, vasodilation in forearm muscle in response to mental stress is evoked by a combination of changes in sympathetic nerve activity, the action of circulating adrenaline and release of endothelial vasodilators, due to the elevated ABP increasing shear stress, of which NO is thought to be the most important (Halliwill et al., 1997, Dietz et al., 1994). More recent evidence highlights the importance of nNOS which is located in endothelial, vascular smooth muscle cells and muscle fibres, in the forearm vasodilator response to stress, with nNOS inhibition shown to attenuate responses in healthy individuals (Khan et al., 2015). However, this was not the case in hypertensives, whose responses were already blunted compared to normotensives, suggesting a reduced contribution of NO in hypertensives (Khan et al., 2015).

Overall, such vasodilatory responses to RH, exercise and mental stress are typically impaired in individuals with endothelial dysfunction, which is a common predictor of CVD and future cardiovascular events (Vita and Keaney, 2002, Celermajer et al., 1994a). Specifically in young SAs, who are at higher risk of future CVD (Rana et al., 2014, Jain et al., 2017), there is evidence, particularly in men, of impaired forearm dilator responses during RH (Hirst and Marshall, 2018, Ormshaw et al., 2018) and mental stress (Ormshaw et al., 2018). This is believed to be, at least partially, mediated by reduced NO bioavailability in SAs (Murphy et al., 2007). As such,

nutritional interventions that can improve NO bioavailability, for example CFs, may present as an effective strategy to improve vasodilator responses in SAs and potentially reduce overall future risk of CVD.

CF supplementation has been shown to lead to significant reductions in resting blood pressure (Ried et al., 2017, Ried et al., 2010) and improvements in brachial FMD (Ebaditabar et al., 2020, Sun et al., 2019) in young and older adults, as well as other at-risk groups, such as type-2 diabetics, hypertensives and overweight individuals. There is also some evidence, although less established, that CF can improve vasodilator responses within the microvasculature (Woodward et al., 2018). The mechanism by which CFs improve endothelial function is largely thought to be modulated by NO bioavailability, since peak circulating NO and flavanol metabolites coincide with improvements in FMD (Heiss et al., 2003). Furthermore, increased dilator responses with CF supplementation were significantly reduced by NOS inhibition, suggesting a cause-effect relationship between NO availability and improvements in endothelial function after CF intake (Schroeter et al., 2006, Fisher et al., 2003a). The upstream signalling pathway leading to eNOS activation is thought to be initiated through (-)-epicatechin binding to GPERs on the endothelial cell membrane (Moreno-Ulloa et al., 2014, Moreno-Ulloa et al., 2015), and as such they may be expected to exert greater effects in women than men due to the greater presence of oestrogen. Furthermore, there is evidence of CFs reducing oxidative stress and inhibiting NO breakdown (Aprotosoaie et al., 2016b), for example by inhibition of NADPH oxidase, which produces O_2^- (Steffen et al., 2008, Steffen et al., 2007b). Taken together, CFs are thought to act by increasing NO bioavailability both by increasing production and inhibiting degradation.

Interestingly, evidence suggests that CF may exert a greater effect in individuals with elevated CVD risk. For example, a greater reduction in blood pressure was observed in hypertensives,

compared to normotensive individuals (Ried et al., 2017). Indeed, a key hallmark of CVD is reduced NO bioavailability (Yang et al., 2009), and there is evidence of impaired EDD, and a reduced contribution of NO to vasodilator responses in hypertensives (Khan et al., 2015), as well as in SA men (Murphy et al., 2007). As such, it is hypothesised that CF efficacy in facilitating vasodilator responses is likely to be mediated by increasing NO bioavailability in these populations (Ramirez-Sanchez et al., 2018). This may be of particular relevance in women, since CF actions are thought to be initiated through their binding to GPER (Moreno-Ulloa et al., 2015). Furthermore, CVD risk is associated with greater oxidative stress, and hence the presence of ROS (Rajendran et al., 2013), thus CFs' enhancement of antioxidant pathways (Aprotosoaie et al., 2016b) may be particularly relevant to at risk groups. On this basis, it could be expected that CFs would have a greater effect in SAs, who have elevated CVD risk compared to WEs, and in particular, SA women (Ahmed and El-Menyar, 2015).

It is apparent from the systematic review carried out in Chapter 3, that there is a lack of robust studies investigating the effect of CFs in the microvasculature. For example, findings are heterogenous due to the variety of vascular beds studied, as well as differences in the age range and gender of populations included, with the majority of studies focussing on male or mixed populations. Furthermore, many studies do not report the ethnicity of participants despite evidence of differences in responses between ethnic groups (Kim and Brothers, 2020). As such, there is a clear need for more studies in this area which focus on narrower age ranges, female populations and specific ethnic groups, particularly those at higher risk of CVD.

5.1.1 Aims and hypotheses

The aim of the present study was to compare vasodilator responses in the microvasculature following high- and low-flavanol cocoa, in order to determine the effect of an acute dose of CFs

in young WE and SA women. Following on from the previous chapter, responses to arterial occlusion, rhythmic handgrip and mental stress tasks were performed using NIRS in order to obtain simultaneous measures of tissue flow indices and tissue oxygen saturation in the forearm.

We hypothesise that:

(i) Peak changes in SO_2, oxyHb and totalHb during RH will be larger in WEs than SAs and will be higher following the high- compared to low-flavanol cocoa

(ii) Peak changes in SO_2, oxyHb and totalHb following rhythmic handgrip will be greater in WEs than SAs, and higher following the high- compared to low- flavanol cocoa

(iii) Peak changes in SO_2, oxyHb and totalHb during mental stress will be greater in WEs than SAs, and higher following the high- compared to low- flavanol cocoa

(iv) If there is an effect of cocoa on vasodilator responses, this will be greater in SAs compared to WEs

5.2 Methods

The study was conducted in accordance with approval by the University of Birmingham Ethnics Committee (ERN17_1755), and participants signed a consent form (Appendix 1) committing to the experiment and use of their data in line with the Participant Information Sheet (Appendix 2).

5.2.1 Participants

23 healthy women aged 18-26 years participated in this study; all were students in the UK and of either SA *(n=12)* or WE *(n=11)* ethnicity, with both parents of the same ethnic origin, and were the same women recruited in Chapter 4. Participants were recruited according to the inclusion criteria outlined in Section 2.1. Participants consented to the study and completed questionnaires

regarding their general health and lifestyle as well as a food frequency questionnaire as described in Chapter 2.

Subjects were asked to eliminate polyphenol-rich foods from their diet (such as certain fruits, vegetables, nuts, olive oil, tea and cocoa; full list in Appendix 4) for 24 hours prior to each laboratory visit. They were given a list of foods that could be consumed, such as meat, fish, dairy products, potatoes, bananas and lettuce. 24 hours was considered a practical duration for these restrictions so as to minimise burden on volunteers as most polyphenol metabolites are excreted within 24hrs (Borges et al., 2018). Participants were also asked to refrain from vigorous exercise, not to consume any alcohol or caffeine during this time and were required to fast for 12 hours prior to each laboratory visit.

All study days were arranged within the first 8 days of the subject's menstrual cycle, with the majority (n=40 out of 46 visits) conducted within the first 5 days, when oestrogen levels are lowest, thus minimising the cardiovascular effects of oestrogen and eliminating any cumulative contributions of oestrogen fluctuations (Hashimoto et al., 1995).

5.2.2 Study design

In this randomised, placebo-controlled, cross-over design, double-blind trial, each subject attended the temperature-controlled laboratory (20-22ºC) on two occasions and the same experimental protocol was conducted at both visits (Figure 5.1). Following the initial baseline monitoring and RH measured with VOP and NIRS (as detailed in Chapter 4), they received either the high-flavanol, or the low-flavanol cocoa drink. These were randomly allocated and coded so that neither the researcher nor the subject knew which intervention was being used. Randomisation was carried out by computerised random generator, and counter-balanced within each ethnicity so that equal numbers were allocated to each order of interventions.

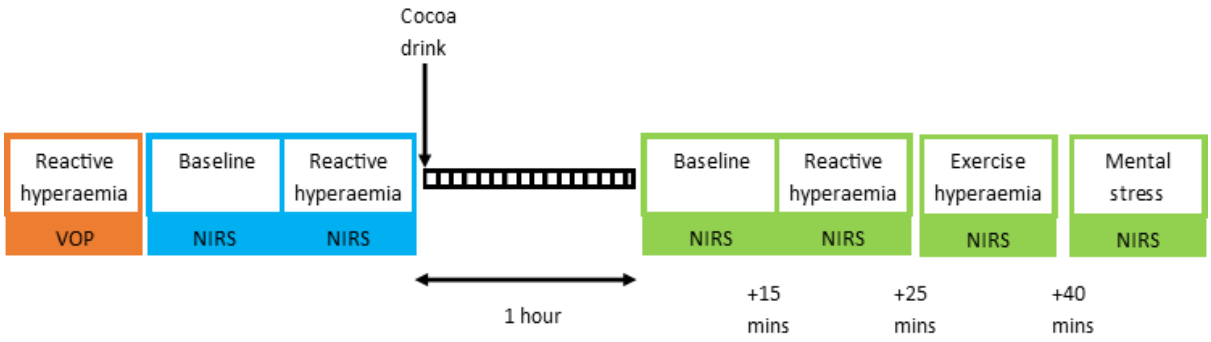

Figure 5.1: Experimental protocol. *Sequence and time-course of measures conducted during each experimental visit*

The subject was given the cocoa drink to consume within 10 min; it was freshly prepared as described below (Section 5.2.3). The next part of the protocol was started 1 hour after the drink was consumed so as to coincide with the peak concentration of flavanols in the plasma (Sansone et al., 2017). During this period the subject was asked to remain seated on the couch, if possible, to avoid the need to remove the NIRS probes; if they needed to go to the toilet, probes were removed and reattached using the photographs taken of their positioning.

At 50 mins after consuming the drink, the subject returned to the reclined position on the couch. All recordings were reinstated and a 5 min baseline period was observed 1 hr after finishing the cocoa. NIRS recordings were then made during arterial occlusion and RH, during rhythmic handgrip and EH, and during mental stress, to observe vasodilator responses in the forearm, with at least 10 mins rest between each. RH was evoked by inflation of the upper cuff to occlude the brachial artery for 2 mins. Exercise hyperaemia was the response to 2 min rhythmic handgrip contractions (1s contraction, 1s relaxation) at 60% MVC, followed by 3 min recovery. The mental stress task was an 8 min PASAT; briefly, this is a pre-recorded audio sequence of numbers 1-9 which progressively increases in speed and participants are tasked with adding each

number on the recording to the previous number and giving the answer aloud to be marked by the experimenter. Further details regarding all of these vasodilator stimuli are provided in Chapter 2.

5.2.3 Flavanol interventions

The Cocoa powders used were commercially available (Barry Callebaut, Zurich, Switzerland); these were stored in the freezer (–20°C). The low-flavanol powder was a fat-reduced alkalised cocoa powder (10/12 DDP Royal Dutch), and the high-flavanol was a non-alkalised fat-reduced powder (Natural Acticoa). Each 12g portion was stored in an individual zip-lock bag which was weighed before and after the emptying of contents and weights recorded. The powder was mixed with 300ml Buxton water at room temperature and blended for 30s to ensure the powder was fully dissolved before being transferred into a sealed cup with a straw; these were opaque so that the fluid was not visible to the subject, and beverages were identical in texture, taste and consistency to ensure effective double blinding. After all data analysis had been completed, experimenters were unblinded to the interventions. Buxton water was used as this is low in nitroso species, therefore minimises external influences on NO bioavailability (Lundberg and Govoni, 2004); if participants wished to drink water during the study, they were also given Buxton water. The high-flavanol drink contained 695mg total flavanols (150mg epicatechin), whereas there were ‘none-detected’ in the low-flavanol drink. The two drinks were matched as closely as possible in other nutrient content, including caffeine and theobromine (Table 5.1). The levels of flavanol monomers, procyanidin and methylxanthines within each cocoa powder have previously been tested by high-performance liquid chromatography and total polyphenol levels measured by Folin-Ciocalteu reagent calorimetric assay, as described in previous work (Miller et

al., 2008). The flavanol doses used in this study are safe and have been shown to improve vascular function in young, healthy adults (Baynham et al., 2021, Gratton et al., 2020).

Table 5.1: Composition of the high and low- flavanol cocoa powders per individual 12g dose

Compounds	Cocoa (12g)	
	High Flavanol	Low Flavanol
Total polyphenols (mg)	1246.8	260.0
Total flavanols (mg)	695.0	5.6
Procyanidins (dimers-decamers, mg)	459.6	ND
(-)-Epicatechin (mg)	150.0	< 6
(-) and (+)-Catechin (mg)	85.4	< 6
Theobromine (mg)	262.8	278.4
Caffeine (mg)	27.6	22.2
Fat (g)	1.7	1.3
Carbohydrates (g)	2.7	1.2
Protein (g)	2.7	2.7
Fiber (g)	1.8	4.0
Energy (kcal)	41.4	36.6

5.2.4 Vasodilator stimuli

5.2.4.1 Reactive hyperaemia

As described in Section 2.4.1, the cuff around the upper arm was inflated to 200mmHg to exceed SBP and hence occlude arterial inflow to the forearm for 2 mins. Upon release the subject was required to resist any temptation to move the arm for the subsequent 2 mins during which NIRS recordings were made.

5.2.4.2 Exercise hyperaemia

As indicated in Section 2.4.2, at the start of the protocol Maximum Voluntary Contraction (MVC) was measured by using a handgrip dynamometer. The subject performed rhythmic handgrip in time with a metronome (1s contraction, 1s relaxation) for 2 mins, aiming for each

contraction to reach 60% MVC. Following exercise, the subject retained this position of the dominant arm for a further 3 mins and NIRS monitoring was continued during recovery.

5.2.4.3 Mental stress

Briefly, for the PASAT the subject was played an 8 min pre-recorded audio sequence of numbers 1-9, which progressively increases in speed, and they must add sequential numbers, giving the answer verbally before the next number is played on the recording. Before beginning the task, the subject listened to a pre-recorded set of instructions and was given the opportunity to ask questions before undertaking a short practice test. Further details regarding the PASAT are outlined in Section 2.4.3.

5.2.3 Data acquisition and analysis

NIRS outputs were extracted from traces offline as described in Section 2.5.2 and stored in Excel for collation of responses from all participants. For all NIRS-derived Hb measures, values are expressed as change from baseline. On the basis that totalHb provides an index of FBF (see Chapter 4), forearm vascular conductance (FVC) index was calculated by dividing ΔtotalHb by MABP at the same time point; this was included only during exercise and mental stress during which changes in MABP were expected.

Since the response to mental stress within the control group (revealed after all experiments had been completed) showed heterogeneity in the direction of ΔFVC index, with a positive or negative change representing vasodilators and vasoconstrictors respectively, participants were also grouped by the direction of their mean change in FVC during mental stress (Ormshaw et al., 2018). There was an even split of individuals who showed overall dilator and constrictor effects, and this was similar across ethnic groups. Given that vasoconstriction during stress has been

associated with increased future CVD risk (Chida and Steptoe, 2010), additional exploratory analysis was conducted to investigate whether CFs would have differentiated effects on vasodilators versus vasoconstrictors.

5.2.4 Statistical analysis

All statistical analysis were performed, and figures were created, using Graphpad Prism Version 9.2.0. Alongside the comparison of habitual diet already made, the FFQ was also used to obtain information on specific flavonoid intake, for which ethnic comparisons were made using unpaired Student's t-tests. Resting NIRS and cardiovascular variables were compared before and after cocoa by two-way repeated measures ANOVA for main effects of ethnicity or time, with separate analysis conducted for the high- and low-flavanol interventions. Post-cocoa resting levels were then compared between the two interventions using two-way repeated measures ANOVA for main effects of ethnicity or cocoa. Two-way repeated measures ANOVA for main effects ethnicity and cocoa was also used to compare RH, exercise, and mean stress responses. Where appropriate, post-hoc comparisons were made by Šídák's multiple comparisons test to compare group means. Wilcoxon t-tests used to compare mean stress cardiovascular and NIRS variables with baseline (considered arbitrarily 0), and two-way repeated measures ANOVA was used within WE and SAs (or vasodilator and constrictors) separately for main effects of time and cocoa to assess changes across the 4 time-points. A sample size of 12 was selected based on power calculations (for power 85%, significance level=0.05) to detect an effect of CFs on dilator responses. The reported 'n' values reflect occasional missing data or outliers in peak values (classified as greater than two standard deviations from the mean of group); for example, one WE participant was excluded throughout, and one SA excluded during exercise, due to NIRS

equipment malfunction. A significance level of $p<0.05$ was used for all statistical analyses, and all values reported are mean±SD.

5.3 Results

5.3.1 Population characteristics

Population characteristics were compared as detailed in Chapter 4. WEs also had significantly greater maximum grip strength than SAs ($p=0.0014$). As well as the comparisons of habitual diet previously described (Table 4.2), WEs also tended to consume more flavanones ($p=0.0576$), flavanols ($p=0.0797$) and total flavonoids ($p=0.0675$) than SAs, but any difference did not reach statistical significance ($p> 0.05$) (Table 5.2).

Table 5.2: Habitual daily flavonoid intake estimated from Food Frequency Questionnaire in groups of White European (WE, n=11) and South Asian (SA, n=12) women. *Data shown as mean±SD, alongside p values from unpaired students' t-tests (#: p<0.1)*

	White European (WE, n=11)	South Asian (SA, n=12)	p value
Flavonols (mg)	28.9±15.9	21.7±8.51	0.187
Flavanones (mg)	27.1±13.9	15.5±13.6	0.0576 #
Flavones (mg)	2.68±1.89	4.89±5.85	0.245
Anthocyanins (mg)	15.9±8.00	11.8±7.17	0.215
Flavanols (mg)	501±443	244±187	0.0797 #
Total flavonoids (mg)	756±459	298±189	0.0675 #

5.3.2 Effect of Cocoa Flavanols at Rest

Two-way repeated measures ANOVA comparing resting NIRS variables before and one hour after cocoa interventions revealed no main effect of ethnicity ($p>0.174$) nor pre- versus post-cocoa for either the high- or low-flavanol interventions ($p>0.154$) (Figure 5.2). However, comparison of resting levels one hour after consumption of high- and low-flavanol cocoa drinks showed that SO_2 [$F(1,21=3.85, p=0.0630$] and oxyHb [$F(1,21)=4.14, p=0.0548$] tended to be higher following the high-flavanol cocoa compared to the low, but there were no effects of cocoa or ethnicity on other variables (Table 5.3).

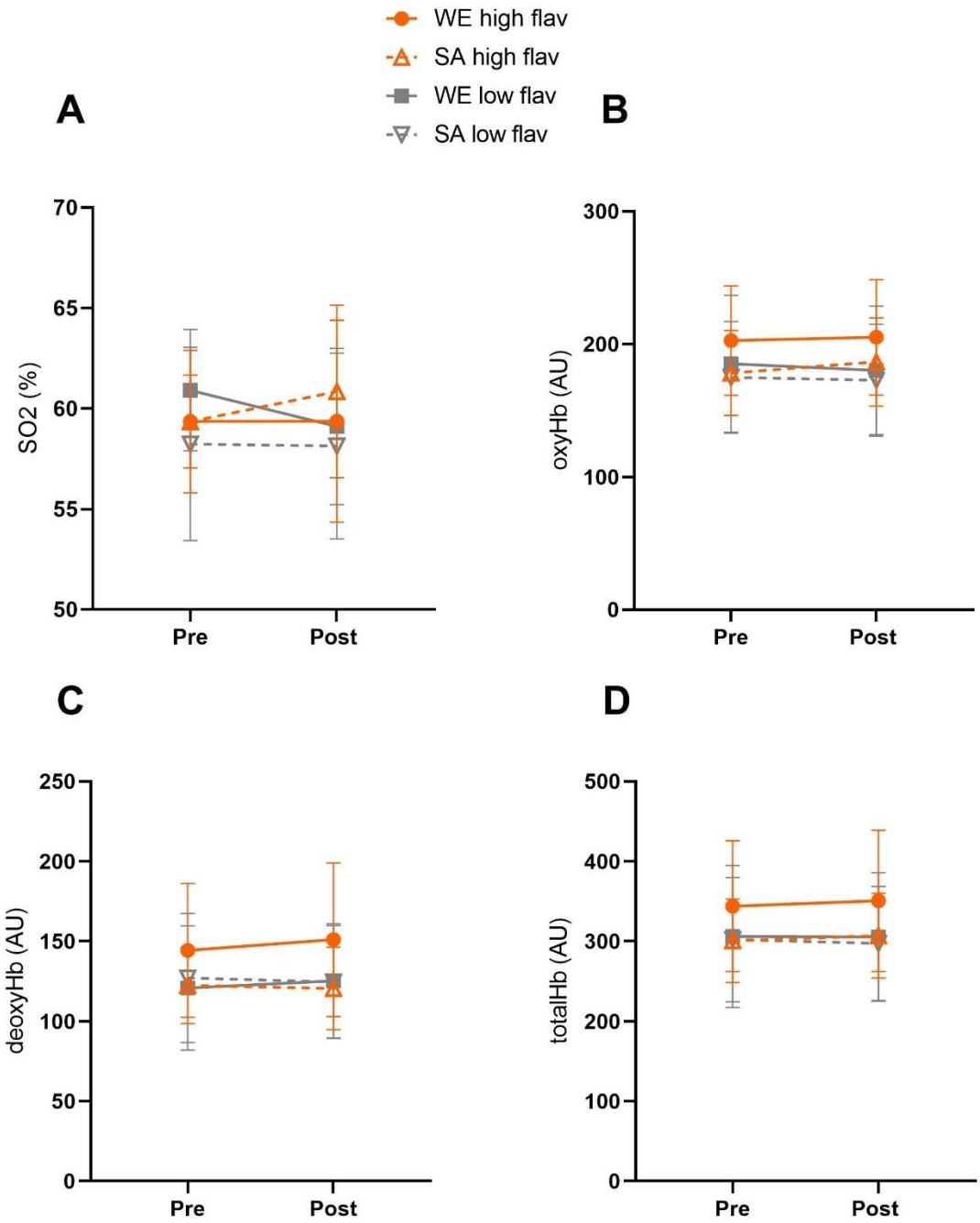

Figure 5.2: Baseline near-infrared spectroscopy (NIRS) variables before and one hour after high- (orange) and low-flavanol (grey) cocoa intervention. *(A) oxygen saturation (SO_2, %), (B) oxygenated (oxy-), (C) deoxygenated (deoxy-), (D) total haemoglobin (Hb) measured in arbitrary units (AU). WE: White European (n=11, solid lines), SA: South Asian (n=12, dotted lines)*

Table 5.3: Resting near-infrared spectroscopy measures one-hour following high- and low- flavanol cocoa interventions in groups of White European (WE, n=11) and South Asian (SA, n=12) women. *Data presented as mean±SD and p values for main effects of ethnicity, cocoa and ethnicity x cocoa interaction (from 2-way repeated measures ANOVA: #: p<0.1).*

				Main effects		
		High flavanol	**Low flavanol**	**Ethnicity**	**Cocoa**	**Ethnicity x Cocoa**
SO_2 (%)	**WE**	59.4±5.03	59.1±3.89	0.883	0.0630 #	0.120
	SA	60.9±4.29	58.1±4.62			
oxyHb (AU)	**WE**	205±43.5	180±48.4	0.387	0.0548 #	0.567
	SA	187±33.2	173±42.1			
deoxyHb (AU)	**WE**	151±48.2	130±33.3	0.186	0.300	0.131
	SA	121±25.8	125±35.2			
totalHb (AU)	**WE**	351±88.3	306±80.5	0.338	0.101	0.282
	SA	307±52.8	297±71.2			

Two-way repeated measures ANOVA comparing blood pressure and HR before and one hour after cocoa interventions showed no difference between baselines before and after the low-flavanol intervention (p>0.174), whereas HR was lower [F(1,21)=10.3, p=0.0042] and MABP [F(1,21)=6.53, p=0.0185], SBP [F(1,21)=6.58, p=0.0180] and DBP [F(1,21)=8.92, p=0.007] were higher one hour after the high-flavanol cocoa compared to before the intervention (Figure 5.3). In addition, two-way repeated measures ANOVA comparing resting values after the two interventions (for main effects ethnicity and cocoa) showed that WEs had a lower resting HR than SAs [F(1,21)=6.02, p=0.0230]. HR tended to be lower following the high- compared to low-flavanol drink [F(1,21)=3.66, p=0.0695] and DBP was higher following high- compared to low-flavanol drink [F(1,21)=5.11, p=0.0345, Table 5.4], but there was no main effect of ethnicity or cocoa on any other variables.

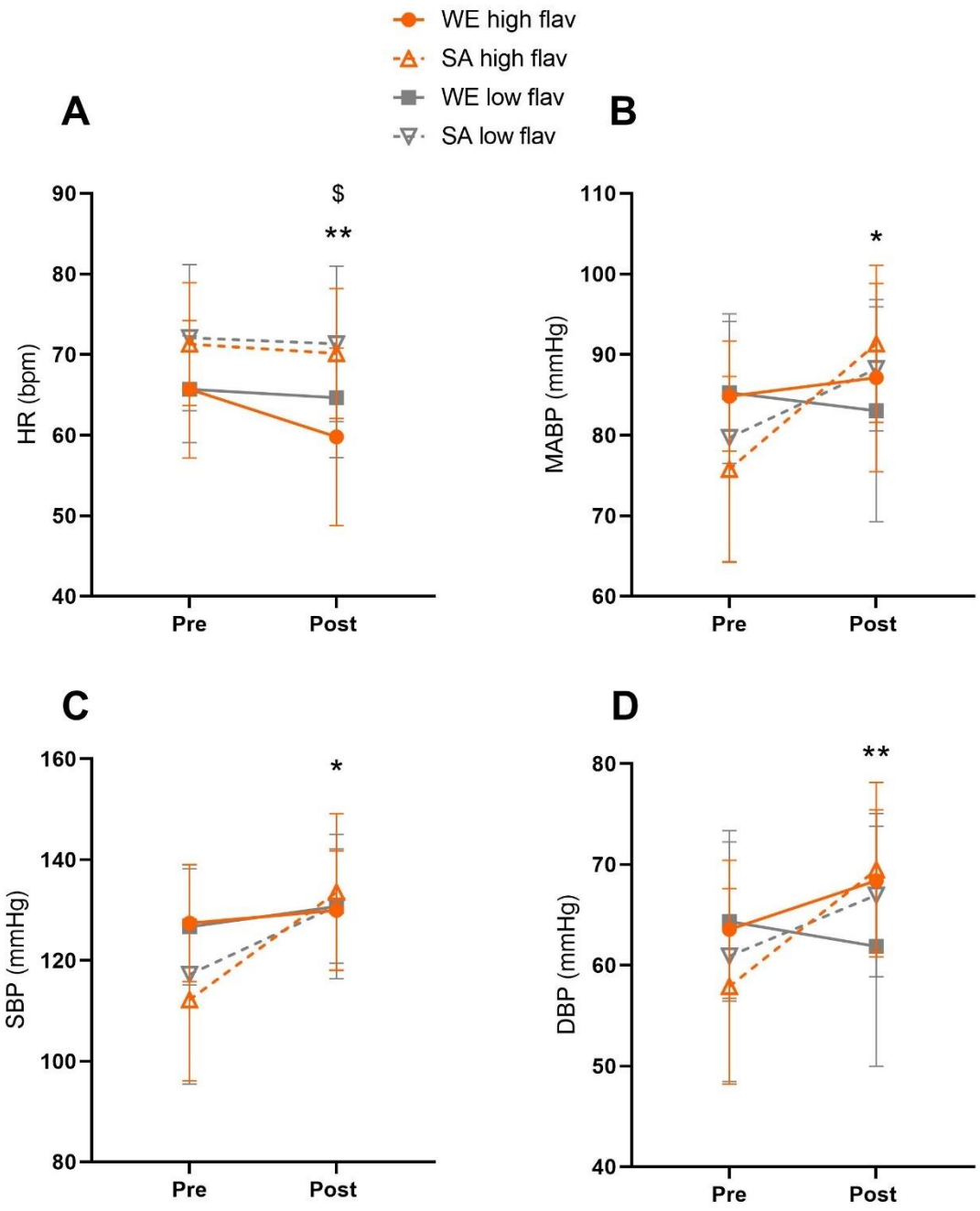

Figure 5.3: Baseline cardiovascular variables before and one hour after high- (orange) and low-flavanol (grey) cocoa intervention. *(A) Heart rate (HR, bpm), (B) mean arterial blood pressure (MABP, mmHg), (C) systolic blood pressure (SBP, mmHg), (D) diastolic blood pressure (DBP, mmHg). WE: White European (n=11, solid lines), SA: South Asian (n=12, dotted lines). (from 2-way repeated measures ANOVA for high- flavanol cocoa *: p<0.05, **: p<0.01 main effect pre versus post, $: p<0.05 main effect ethnicity)*

Table 5.4: Resting blood pressure and heart rate one hour following high- and low-flavanol cocoa interventions in groups of White European (WE, n=11) and South Asian (SA, n=12) women. *Data presented as mean±SD and p values for main effects of ethnicity, cocoa and ethnicity x cocoa interaction (from 2-way repeated measures ANOVA: *p<0.05, #: p<0.1). HR: heart rate (bpm), MABP: mean arterial blood pressure (mmHg), SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (DBP)*

				Main effects		
		High flavanol	**Low flavanol**	**Ethnicity**	**Cocoa**	**Ethnicity x Cocoa**
HR (bpm)	**WE**	59.8±11.0	64.7±7.45	0.0230 *	0.0695 #	0.260
	SA	70.1±8.06	71.3±9.66			
MABP (mmHg)	**WE**	87.1±11.7	83.0±13.8	0.229	0.163	0.841
	SA	91.3±9.74	88.2±7.70			
SBP (mmHg)	**WE**	130±11.8	130±14.0	0.600	0.646	0.396
	SA	134±15.6	131±11.3			
DBP (mmHg)	**WE**	68.4±7.04	62.2±12.0	0.387	0.0345 *	0.355
	SA	69.5±8.65	67.0±8.11			

5.3.3 Effect of Cocoa Flavanols on Reactive Hyperaemia

Figure 5.4 shows changes in NIRS variables during RH for combined groups of WEs and SAs; there were no differences in responses following high- and low-flavanol cocoa.

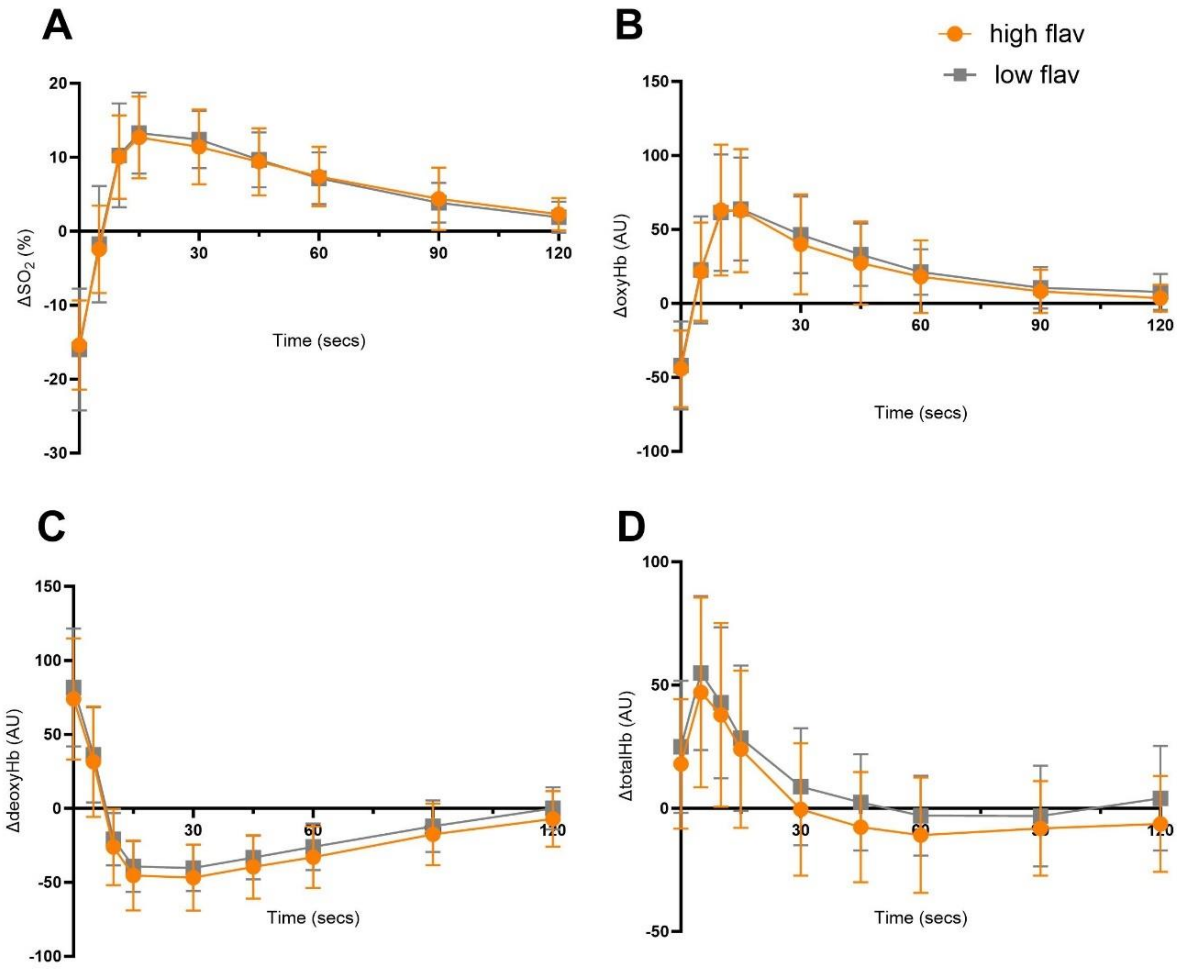

Figure 5.4: Reactive hyperaemia traces following high- (orange)and low- flavanol (grey) cocoa interventions*, for combined groups of White Europeans (WEs) and South Asians (SAs) showing (A) ΔSO₂ (%), (B) ΔoxyHb (AU), (C) ΔdeoxyHb (AU) and (D) totalHb (AU), measured with NIRS. Data shown is mean±SD (n=22)*

The changes shown in the individual ethnic groups are described in more detail below. Figure 5.5 shows changes in tissue oxygen saturation (SO_2) during and following release of 2 min upper arm arterial occlusion. Two-way repeated measures ANOVA showed a main effect of ethnicity on SO_2 peak [$F(1,20)=6.33$, $p=0.0205$] and hyperaemic reserve [$F(1,20)=7.13$, $p=0.0147$] and a significant ethnicity x cocoa interaction [$F(1,20)=6.37$, $p=0.0202$] for Slope 2, with post-hoc tests showing that WEs had a steeper reperfusion slope than SAs following the low-flavanol cocoa only ($p=0.0171$). There was no main effect of ethnicity nor cocoa on any other SO_2 outcomes ($p>0.149$).

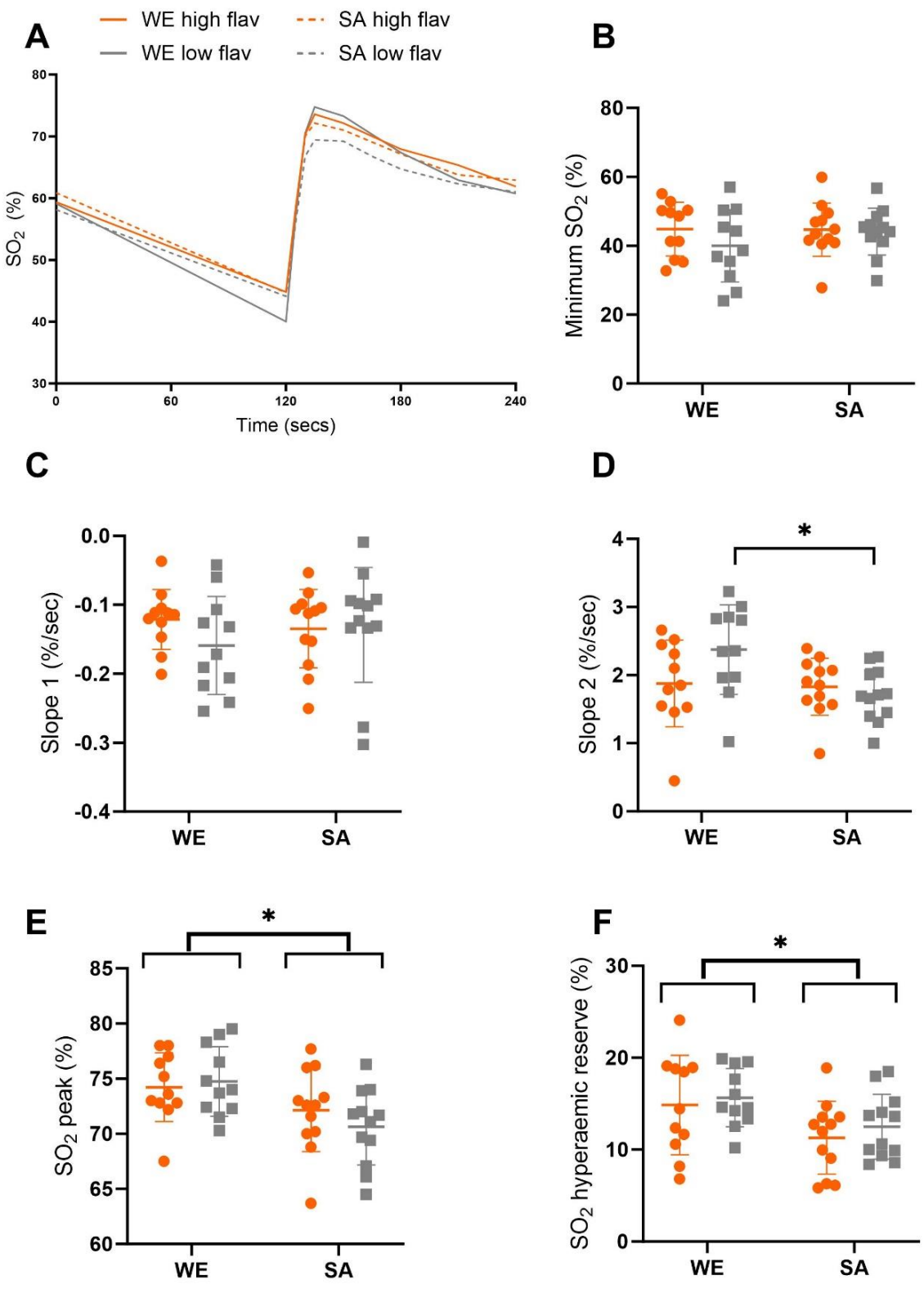

Figure 5.5: Ethnic differences in tissue oxygen saturation (SO2, %) during and after release of upper arm arterial occlusion (reactive hyperaemia) following high- (orange) and low-flavanol (grey) cocoa interventions. *(A) SO2 profile during and following release of occlusion, (B) minimum SO2, (C) desaturation slope, (D) reperfusion slope, (E) peak SO2 and (F) hyperaemic reserve. WE: White European (n=10), SA: South Asian (n=12) *: p<0.05*

The ΔoxyHb values are shown in Figure 5.6. Peak ΔoxyHb during RH was higher in WEs than SAs [$F(1,20)=7.76$, $p=0.0114$], and the maximum ΔdeoxyHb during RH tended to be larger in WEs [$F(1,20)=4.14$, $p=0.0554$]. Peak ΔtotalHb also tended to be higher in WEs than SAs, though this difference did not reach statistical significance [$F(1,20)= 3.99$, $p=0.0595$]. There was however no main effect of cocoa on any variable ($p>0.315$).

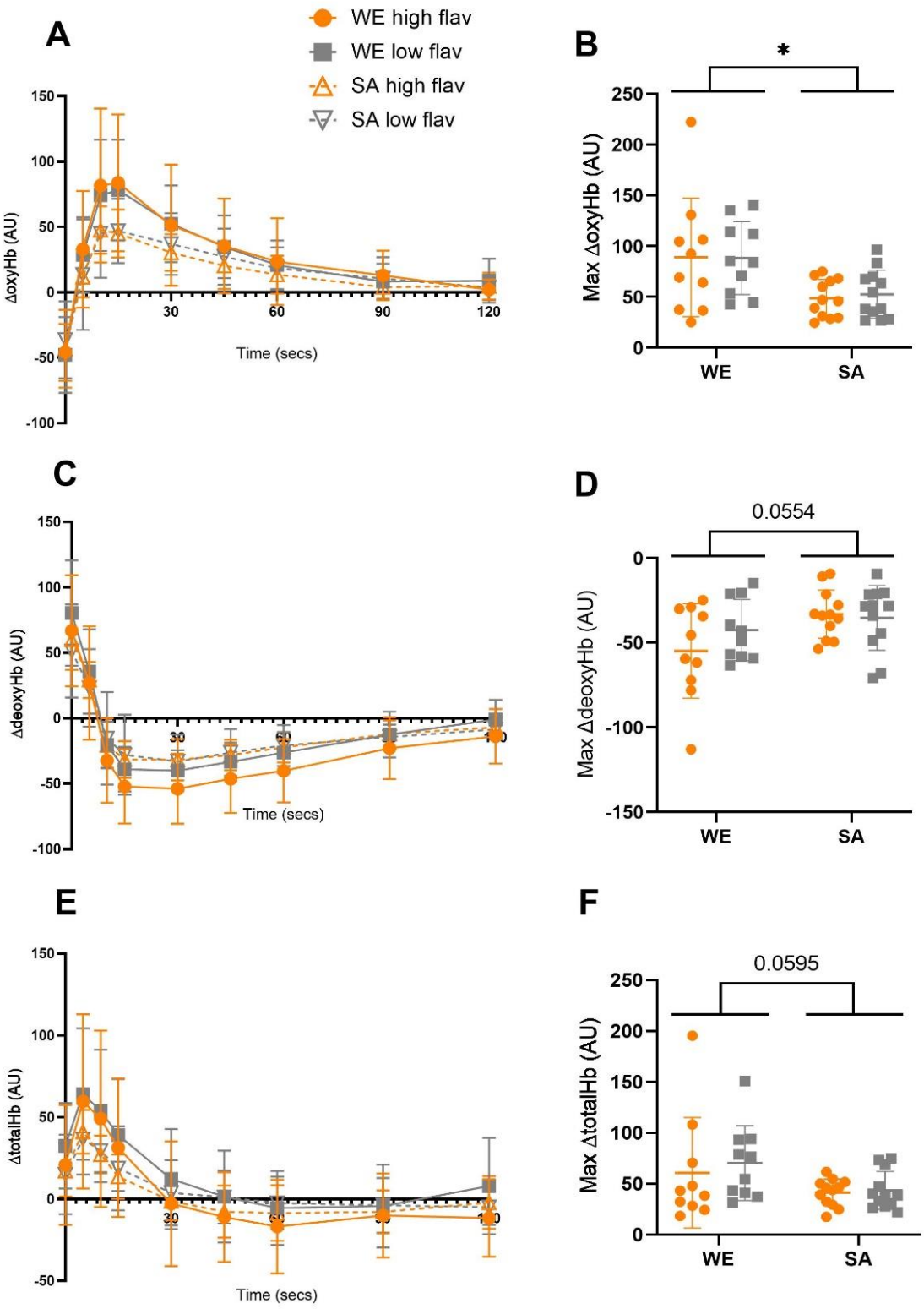

Figure 5.6: Ethnic differences in reactive hyperaemia traces and peak change in haemoglobin measures following high- (orange) and low-flavanol (grey) cocoa interventions*. (A)Δ oxyHb trace following release of occlusion, (B) peak ΔoxyHb, (C) ΔdeoxyHb trace, (D) maximum ΔdeoxyHb, (E) ΔtotalHb trace, and (F) peak ΔtotalHb. WE: White European (n=10, solid lines), SA: South Asian (n=12, dotted lines) *:p<0.05*

5.3.4 Effect of Cocoa Flavanols on Exercise Hyperaemia

Figure 5.7 shows changes in the systemic cardiovascular variables during the 2 min rhythmic handgrip activity and 3 min recovery period. There was no main effect of ethnicity ($p>0.470$) or cocoa ($p>0.152$) on the maximum increase from baseline during the activity on any variable.

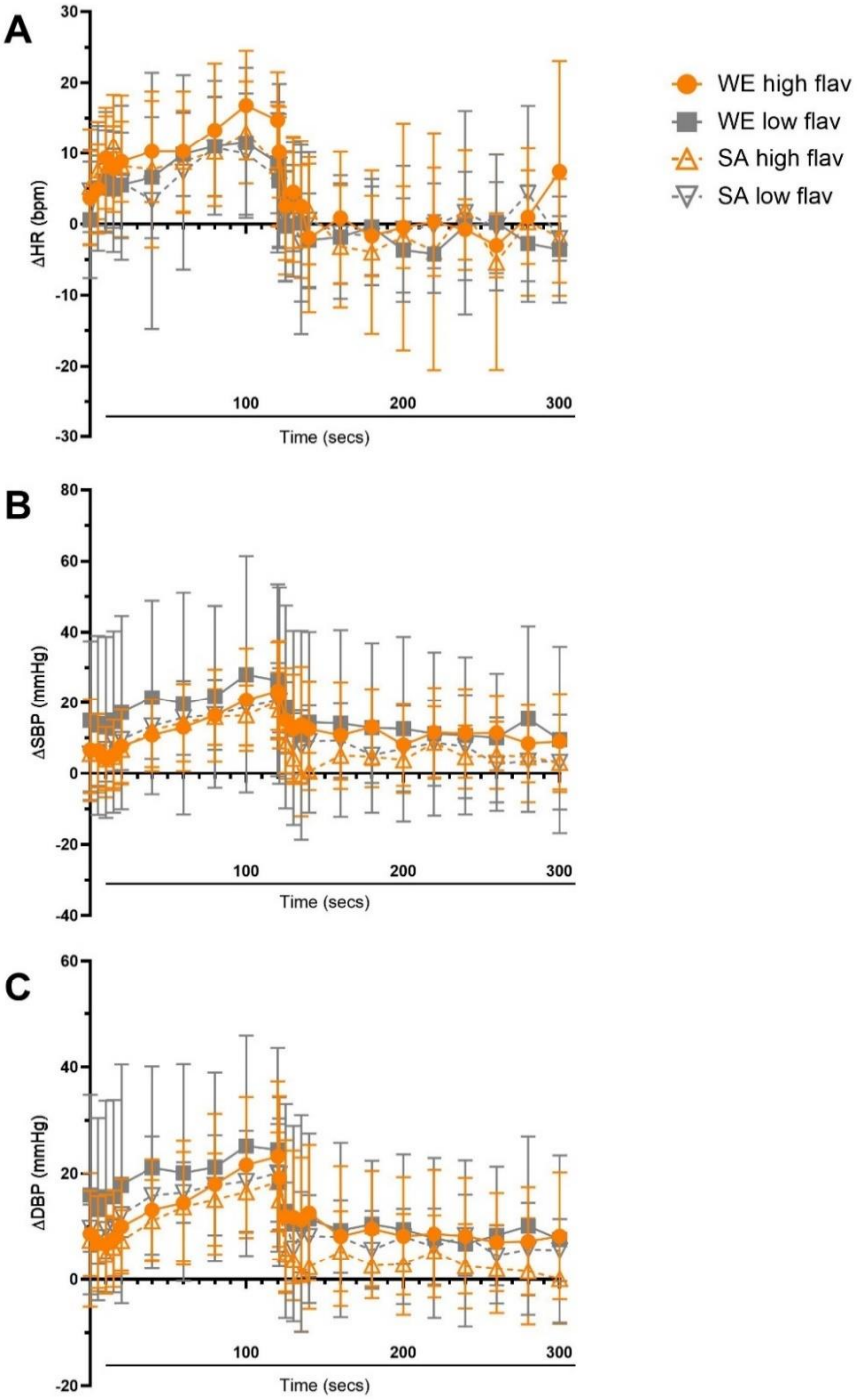

Figure 5.7: Changes in (A) heart rate, (B) systolic blood pressure and (C) diastolic blood pressure during 2 min rhythmic handgrip and exercise hyperaemia following high- (orange) and low- (grey) flavanol interventions*. Data shown as mean±SD. HR: heart rate, SBP: systolic blood pressure, DBP: diastolic blood pressure. WE: White European (n=10, solid lines), SA: South Asian (n=12, solid lines).*

Figure 5.8 shows changes in NIRS variables in combined groups of WEs and SAs during the 2 min rhythmic handgrip activity and 3 min recovery period. There was no significant effect of flavanol interventions on responses during or after exercise.

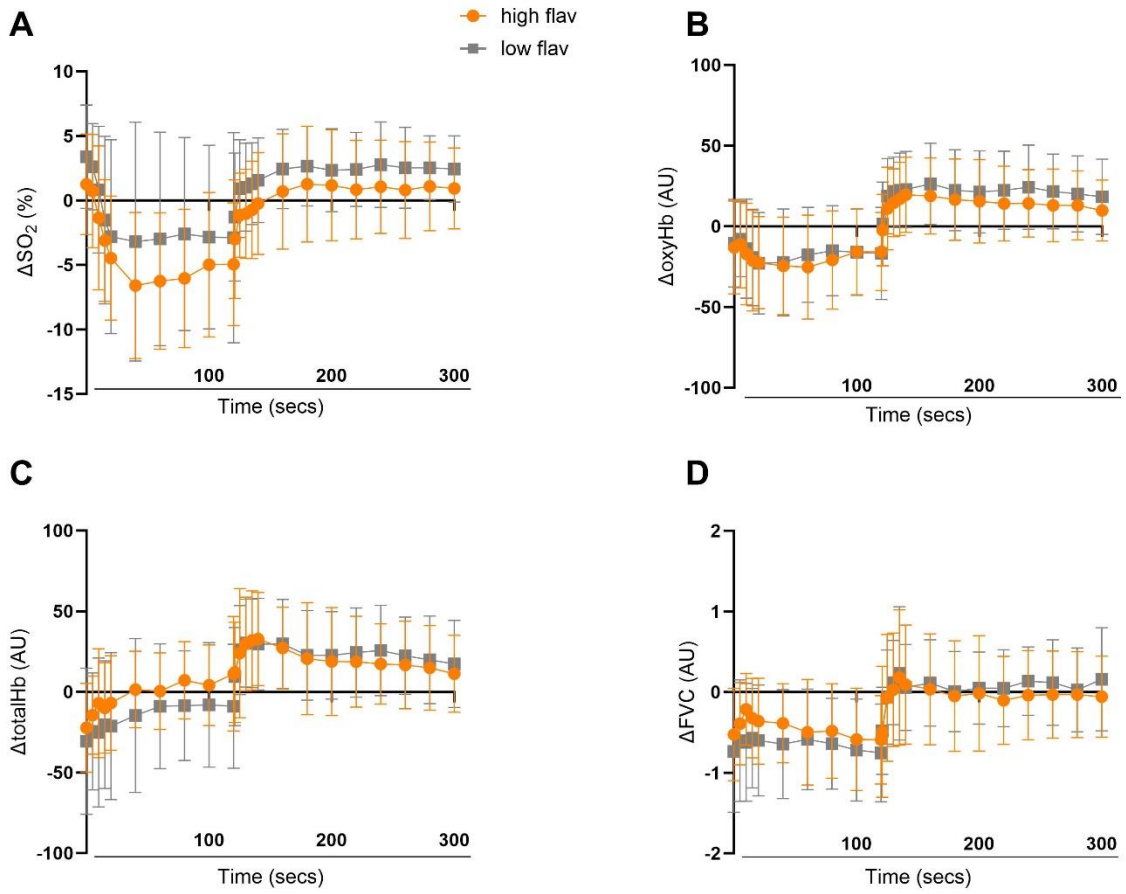

Figure 5.8: Changes in variables measured with near-infrared spectroscopy during 2 min rhythmic handgrip and exercise hyperaemia following high (orange) and low-flavanol (grey) cocoa interventions *for combined groups of White Europeans (WEs) and South Asians (SAs) showing (A) ΔSO₂ (%), (B) ΔoxyHb (AU), (C) ΔdeoxyHb (AU) and (D) totalHb (AU), measured with NIRS. Data shown is mean±SD (n=21)*

The changes in the individual ethnic groups are described in more detail below. Firstly, two-way repeated measures ANOVA showed that peak ΔoxyHb following exercise was greater amongst WEs than SAs [$F(1,19)=11.0$, $p=0.0036$], see Figure 5.9 A&B. The same was true for post-exercise SO_2 [$F(1,19)=5.21$, $p=0.0342$] see Figure 5.9 C&D. However, there was no main effect of cocoa on either peak ΔoxyHb or SO_2 ($p>0.373$, see Figure 5.9 B&D).

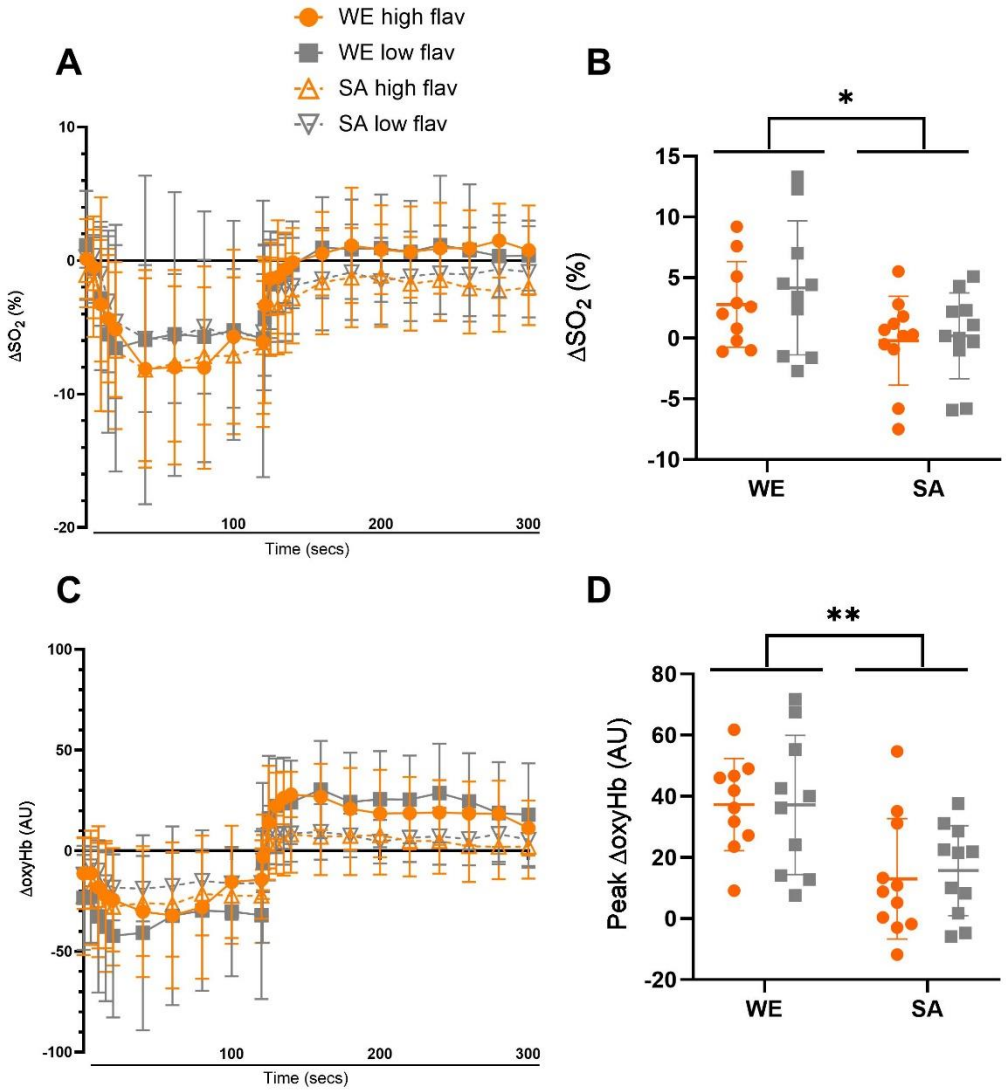

Figure 5.9: Ethnic differences in oxygen saturation (SO_2, %) and ΔoxyHb during 2 min rhythmic handgrip and exercise hyperaemia following high- (orange) and low-flavanol (grey) cocoa interventions. *(A) SO_2 trace, (B) peak SO_2, (C) ΔoxyHb trace, (D) peak ΔoxyHb. Data presented as mean± SD. WE: White European (n=10), SA: South Asian (n=11). *: p<0.05, **: p<0.01*

Further, two-way repeated measures ANOVA revealed a significant main effect of ethnicity on peak post-exercise ΔtotalHb [F(1,19)=7.86, p=0.0113] and ΔFVC [F(1,19)=5.57, p=0.0292] see Figure 5.10 A&B and C&D respectively, but there was no main effect of cocoa on either ethnicity (p>0.275).

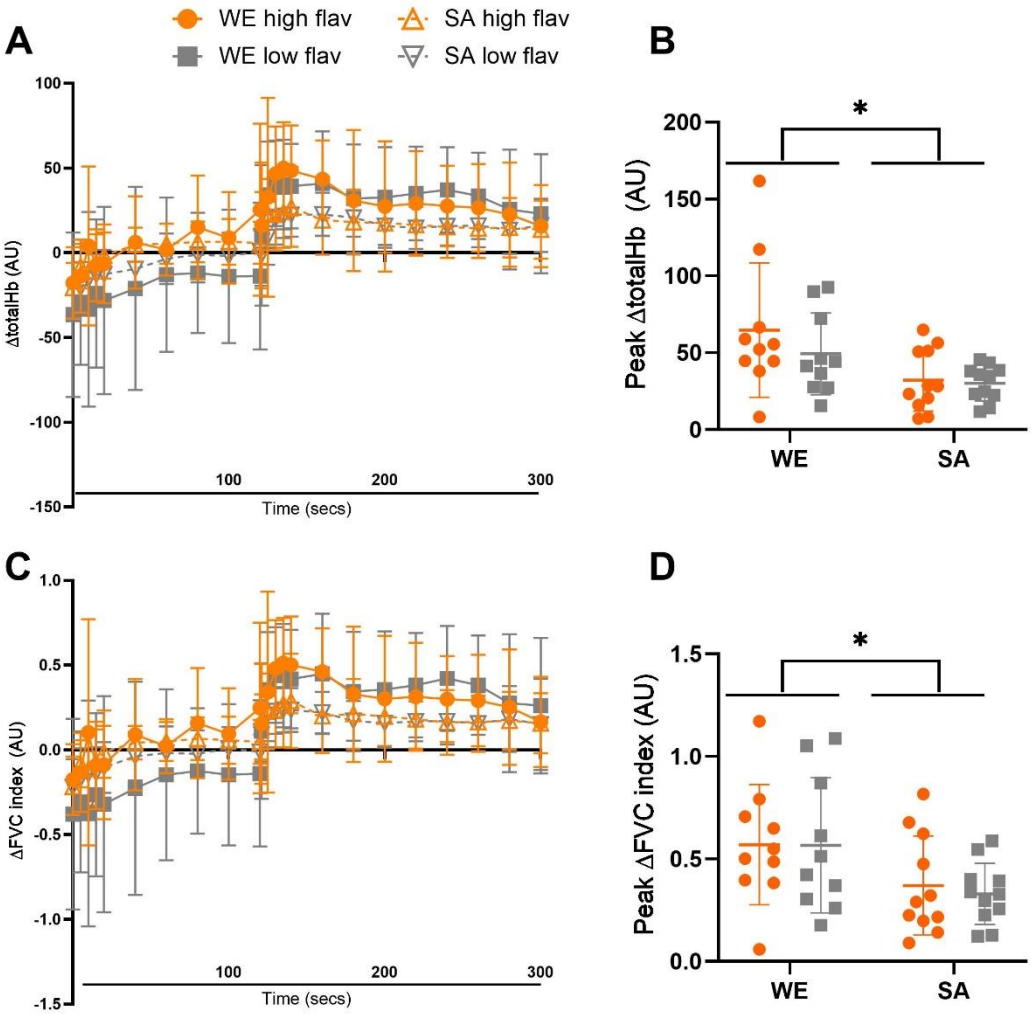

Figure 5.10: Ethnic differences in ΔtotalHb and ΔFVC index during 2 min rhythmic handgrip and exercise hyperaemia following high- (orange) and low-flavanol (grey) cocoa interventions *(A) ΔtotalHb trace, (B) peak hyperaemic ΔtotalHb, (C) ΔFVC index trace, (D) peak hyperaemic ΔFVC index. Data presented as mean± SD. WE: White European (n=10), SA: South Asian (n=11). *:p<0.05*

5.3.5 Effect of Cocoa Flavanols in Mental Stress

Wilcoxon t-tests comparing mean cardiovascular measures during stress to rest (0) showed significant elevations in all measures following high- and low-flavanol cocoa interventions and in both WEs and SAs. Table 5.5 shows PASAT scores and systemic cardiovascular variables measured during the stress task. Two-way repeated measures ANOVA showed there was no main effect of ethnicity ($p>0.129$) or cocoa ($p>0.183$) on any variable during the task, though there was a significant ethnicity x cocoa interaction for MABP [$F(1,21)=7.11$, $p=0.0145$], with post-hoc tests showing that MABP was lower following the high-, compared to low-flavanol cocoa only in SAs ($p=0.0257$).

Table 5.5: Cardiovascular variables measured during mental stress task and task performance following high- and low-flavanol cocoa interventions in groups of White European and South Asian women. *Data presented as mean±SD and p values for main effects of ethnicity, cocoa and ethnicity x cocoa interaction (from 2-way repeated measures ANOVA). HR: heart rate (bpm), MABP: mean arterial blood pressure (mmHg), SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (DBP)*

				Main effects		
		High flavanol	Low flavanol	Ethnicity	Cocoa	Ethnicity x Cocoa
PASAT Score (/228)	WE	132±6.14	124±12.2	0.843	0.582	0.590
	SA	132±10.0	132±11.5			
HR (bpm)	WE	70.0±14.7	71.7±13.7	0.129	0.276	0.792
	SA	79.2±13.8	80.3±13.1			
MABP (mmHg)	WE	100±10.1	96.6±10.3	0.599	0.287	0.015 *
	SA	92.0±7.95	101±12.6			
SBP (mmHg)	WE	145±12.4	139±17.9	0.905	0.183	0.826
	SA	144±20.2	139±16.1			
DBP (mmHg)	WE	77.3±10.1	72.7±6.63			
	SA	79.1±9.94	80.1±10.0	0.175	0.427	0.237

Figure 5.11 shows the time-course of changes in ABP, HR and NIRS variables in combined WE and SA groups during the 8 min mental stress task following high- and low-flavanol cocoa. Two-way repeated measures ANOVA showed that there was a significant effect of high flavanol cocoa on SO_2 [$F(1,84)=11.2$, $p=0.0012$, see Figure 5.11A] and ΔoxyHb [$F(1,84)=7.37$,

p=0.0081, see Figure 5.11B], both variables being greater during the stress task with high- compared to low-flavanol cocoa. There was no significant effect of cocoa or time on ΔtotalHb, ΔFVC index, ΔHR or ΔABP.

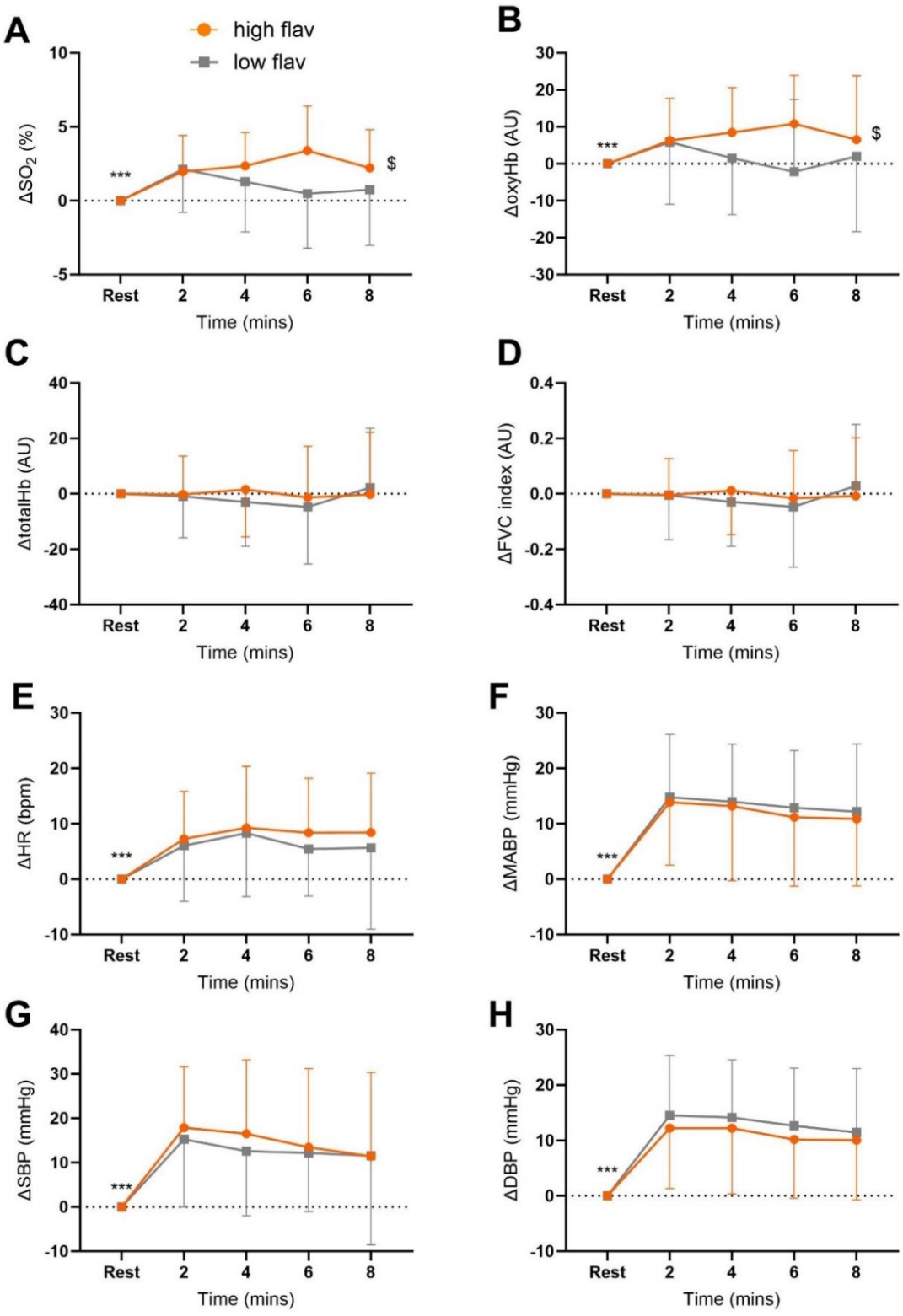

Figure 5.11: Changes in cardiovascular and near-infrared spectroscopy measures during mental stress task following high- (orange) and low-flavanol (grey) cocoa interventions *for combined groups of White Europeans (WEs) and South Asians (SAs) showing (A) ΔSO₂ (%), (B) ΔoxyHb (AU), (C) ΔdeoxyHb (AU) and (D) ΔtotalHb (AU), (E) ΔHR (bpm), (F) ΔMABP (mmHg), (G) ΔSBP (mmHg) and (H) ΔDBP.. Data shown is mean±SD (n=22). *: p<0.01 stress vs baseline, $: p<0.05 high- vs low- flavanol cocoa.*

Figure 5.12 shows the time-course of changes in systemic cardiovascular variables during the stress task within WE and SA groups (shown on the left and right respectively). Two-way repeated measures ANOVA showed that amongst SAs there was a main effect of time on ΔMABP [$F(3,30)=3.58$, $p=0.0253$] and ΔSBP [$F(3,30)=3.77$, $p=0.0209$], both values being greatest at the 2 min time-point and steadily decreasing through the rest of the stress response. There was no main effect of time on any measure in WEs ($p>0.209$) and no effect of cocoa on systemic cardiovascular variables in either WEs or SAs.

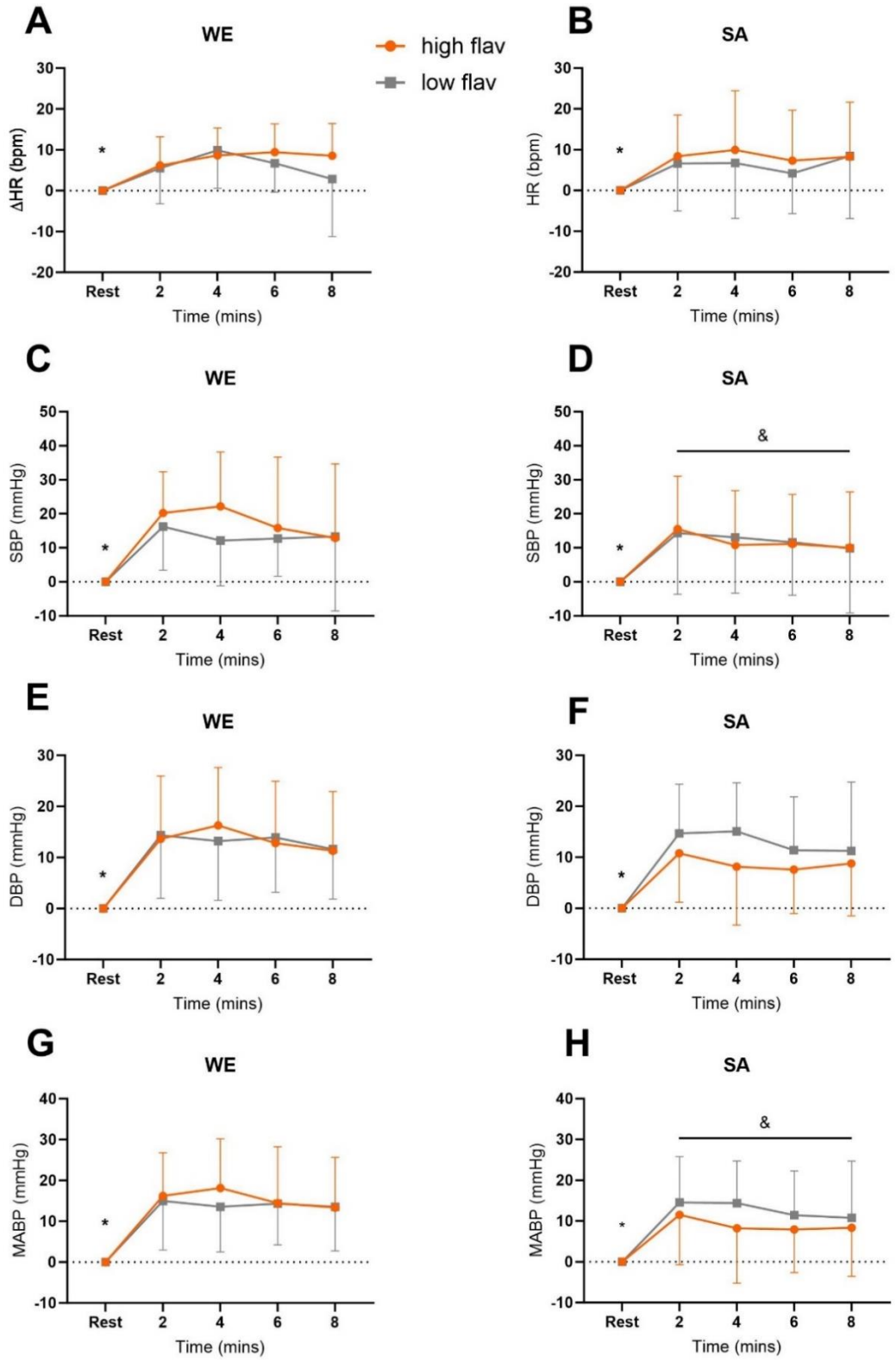

Figure 5.12: Time-course of cardiovascular responses during mental stress task following high (orange) or low (grey) flavanol cocoa in groups of White Europeans (WEs, n=10) and South Asians (SAs, n=12). *(A and B) ΔHR, (C and D) ΔSBP, (E and F) ΔDBP, (G and H) ΔMABP *: p<0.05 stress versus rest, &: main effect time during stress. HR: heart rate (bpm), SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (mmHg), MABP: mean arterial blood pressure (mmHg)*

In regard to NIRS variables, Wilcoxon t-tests comparing mean SO_2 during stress with rest (0) showed that SO_2 during stress was significantly elevated in both WEs (p=0.0371) and SAs (p=0.001) following the high-flavanol cocoa, but no significant increases were detected in the low-flavanol groups (WE: p=0.0840, SA: p=0.176, Figure 5.13). Mean ΔoxyHb during stress was significantly greater than baseline in SAs after high-flavanol only (p=0.0068), but this was not the case for WEs (p=0.432) after high flavanol. There was no difference from baseline during stress for the other NIRS variables in the low- or high-flavanol groups of WEs or SAs, (p>0.339), including for ΔtotalHb (p>0.375) or ΔFVC index (p>0.275), Figure 5.13 C-H.

Figure 5.13 shows the time-course of changes in forearm SO_2, oxyHb, totalHb and FVC index during mental stress, these were compared by two-way repeated measures ANOVA within WEs and SAs separately. Amongst WEs there was a main effect of time on ΔtotalHb [F(3,27)=3.46, p=0.0302] and on ΔFVC index [F(3,27)=3.47, p=0.0299, Figure 5.13 E&G]. In SAs, there was a significant cocoa x time interaction for ΔtotalHb [F(3,33)=3.11, p=0.0395] and ΔFVC index [F(3,33)=3.25, p=0.0341, Figure 5.13 F&H], post-hoc tests showing these were significantly higher at 8 min than 2 min in the low-flavanol group (ΔtotalHb: p=0.0187; ΔFVC index: p=0.0182). In terms of changes in SO_2, a significant cocoa x time interaction was found amongst WEs [F(3,27)=5.30, p=0.0053], Figure 5.13A, post-hoc tests showing that at 6 min time-point, ΔSO_2 was significantly higher in the high-, than low-flavanol group (p=0.0308). Amongst SAs, ΔSO_2 appeared to be greater throughout stress following the high-flavanol cocoa although this difference did not reach statistical significance [F(1,11)=3.44, p=0.0905, Figure 5.13B]. There was also a significant time x cocoa interaction for ΔoxyHb in WEs [F(3,27)=3.65, p=0.0249], post-hoc tests showing the difference between time-points 2 and 6 mins for the low flavanol (p=0.0211). There was no main effect of time or cocoa on ΔoxyHb in SAs (Fig 5.13D).

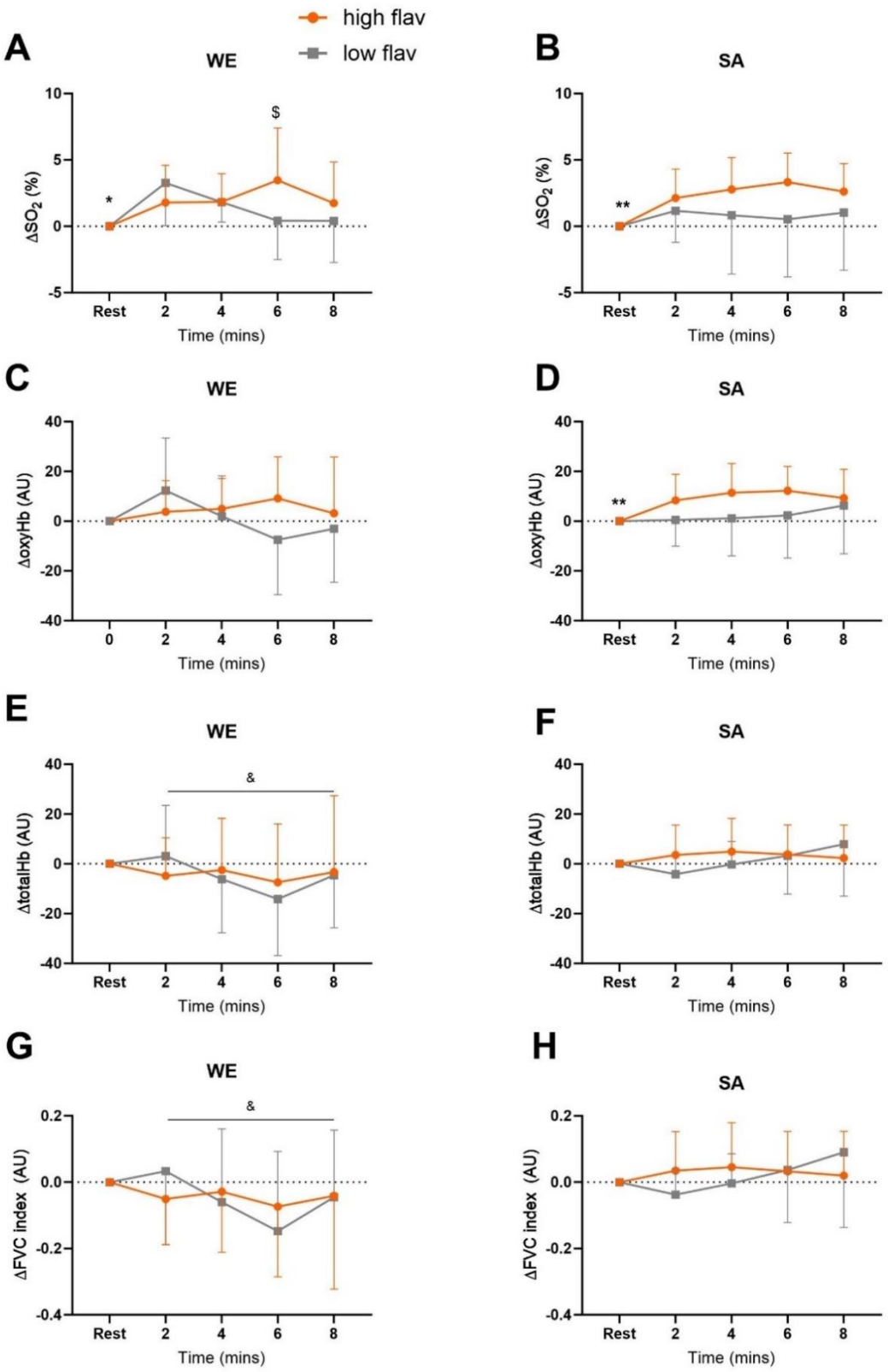

Figure 5.13: Time-course of changes in near-infrared spectroscopy measures in the forearm during mental stress task following high- (orange) or low-flavanol (grey) cocoa in groups of White Europeans (WEs, n=10) and South Asians (SAs, n=12) *(A and B) SO2 (%), (C and D) oxyHb, (E and F) totalHb, (G and H) FVC index *: p<0.05 stress versus rest, **: p<0.01 stress vs baseline, $: p<0.05 versus 2-min (low-flavanol), &: p<0.05 main effect time*

In addition to considering values recorded at each time point, two-way repeated measures ANOVA was also used to compare the mean of stress responses in each variable across the 4 time points. These comparisons revealed no main effect of ethnicity ($p>0.136$) or cocoa ($p>0.132$) on the change in SO_2, oxyHb, totalHb or FVC index. The mean stress responses within individuals with the low- and high-flavanol cocoa are shown in Figure 14.

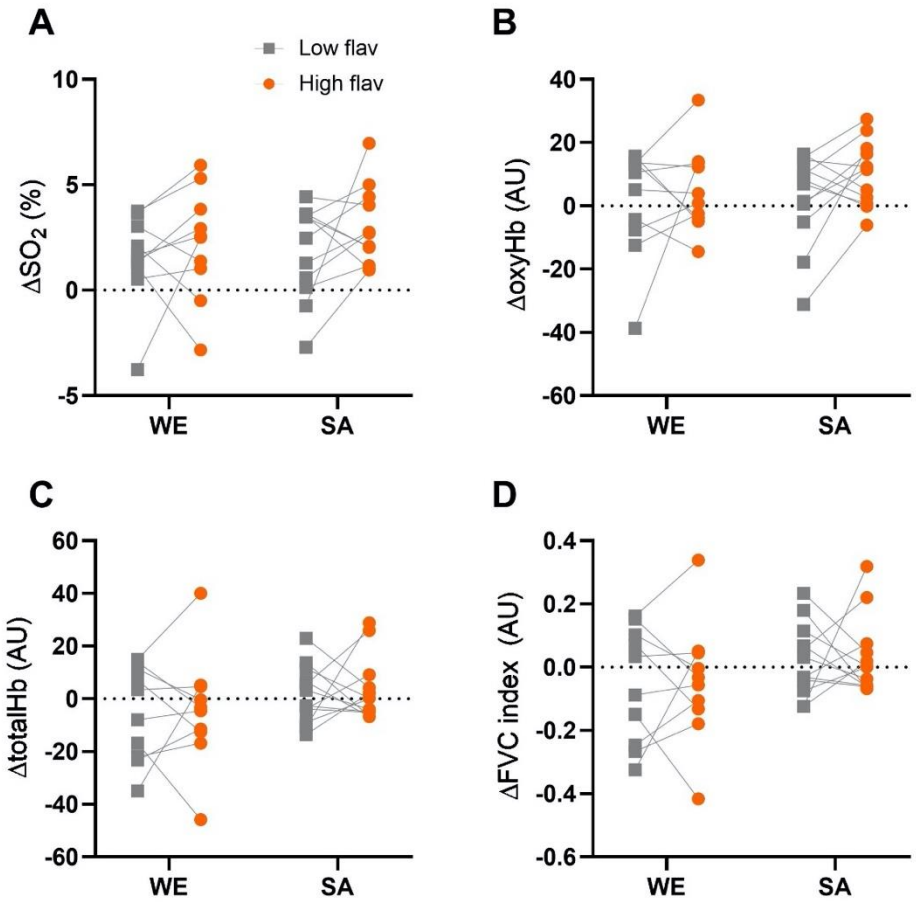

Figure 5.14: Mean change in near-infrared spectroscopy variables during mental stress task for individual participants following low- (grey) and high- (orange) flavanol cocoa *amongst White Europeans (WE, n=10) and South Asians (SA, n=12). (A) tissue oxygen saturation (SO_2, %), (B) ΔoxyHb, (C) ΔtotalHb, (D) ΔFVC index*

It is apparent from Figure 5.14 that amongst both WEs and SAs there was considerable variability in the responses shown by individuals. Considering changes in the low flavanol group, there was an even split of individuals showing increases and decreases in FVC during stress (Figure 5.14). Thus, based on the direction of mean ΔFVC index during stress after the low

flavanol cocoa drink, participants were split into vasodilators and vasoconstrictors (i.e. positive or negative change in FVC respectively, as described in Section 5.2.3). Additional analyses were then conducted to determine whether the effect of CFs differed between vasodilators and vasoconstrictors.

The proportion of vasodilators and vasoconstrictors within WE and SA groups was the same (50% each) and there were no clear differences between the patterns of their responses. Therefore, participants from both ethnic groups were combined into a single vasodilator (n=11) and vasoconstrictor group (n=11).

Wilcoxon t-tests comparing mean stress with rest (0) showed, as expected, that totalHb and FVC index were significantly greater and lower than rest (0) in the low flavanol group amongst vasodilators and vasoconstrictors, respectively. SO_2 was also elevated during stress amongst all except the low-flavanol constrictor group. Mean ΔoxyHb was not significantly different from rest (0) in either group (Figure 5.15).

Two-way repeated measures ANOVA also revealed a main effect of cocoa on SO_2 amongst vasodilators [$F(1,40)=7.68$, $p=0.0084$], SO_2 being higher following the high flavanol cocoa (Figure 5.15A). A similar effect tended to occur amongst vasoconstrictors, though the difference did not reach statistical significance [$F(1,40)=3.44$, $p=0.0712$, Figure 5.15B]. For ΔoxyHb there was no main effect of cocoa or time in either the vasodilator or vasoconstrictor group. There was a significant time x cocoa interaction [$F(3,30)=3.40$, $p=0.0303$] in the vasoconstrictor group, post-hoc tests showing that ΔoxyHb at the 6 min time-point was significantly greater following the high- than low-flavanol cocoa ($p<0.0001$, Figure 5.15D). Furthermore, amongst vasoconstrictors there was a main effect of cocoa on ΔtotalHb [$F(1,40)=8.58$, $p=0.005$,] which was elevated throughout stress in the high- compared to low-flavanol group (Figure 5.15F).

Indeed, some individuals who showed a negative change in totalHb with the low-flavanol cocoa showed a mean positive change following high-flavanol cocoa. For ΔFVC index there was no effect of cocoa, but a main effect of time on ΔFVC index [$F(3,30)=5.31$, $p=0.0047$] amongst vasoconstrictors was shown, ΔFVC index highest at the 2 min time-point and declining during the task (Figure 5.15H).

Finally, Figure 5.16 shows changes in systemic cardiovascular variables throughout stress in the vasodilator and constrictor groups. Mean stress values were significantly different from rest (0) for all variables and in both groups. Two-way repeated measures ANOVA showed no significant effect of cocoa on any measures in vasodilators, and only on HR amongst vasoconstrictors, HR being higher during stress with the high- than low-flavanol cocoa [$F(1,10)= 7.24$, $p=0.0226$, Figure 5.16B]. However, there was a main effect of time on MABP in vasodilators [$F(2,27)=3.85$, $p=0.0205$], MABP being higher at the 2min time-point and progressively decreasing during stress (Figure 5.16C); there was no main effect of time on any other variables in either group.

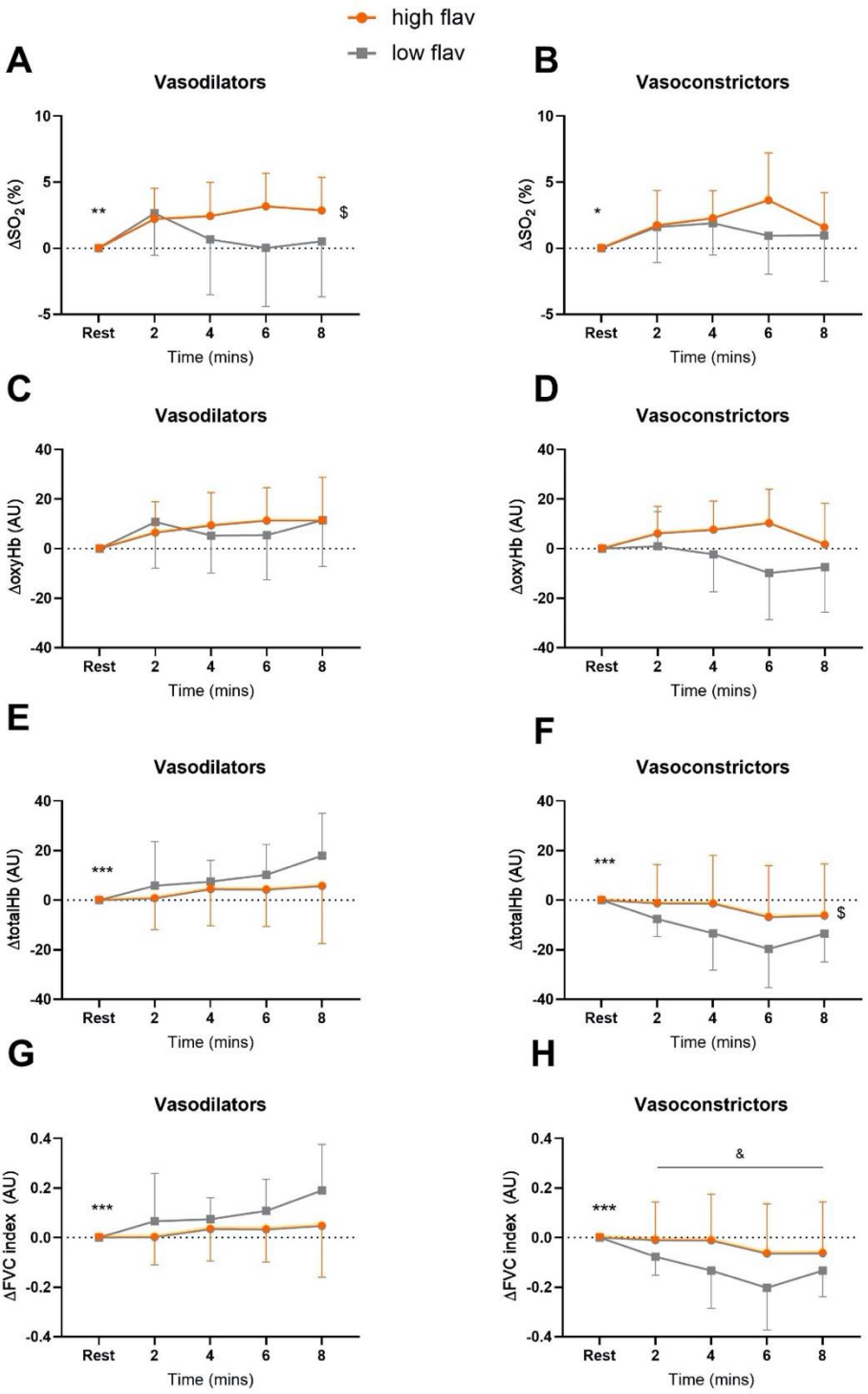

Figure 5.15: Time-course of changes in near-infrared spectroscopy measures in the forearm during mental stress task following high- (orange) and low-flavanol (grey) cocoa interventions in groups vasodilators (n=11) and vasoconstrictors (n=11). *(A and B) ΔSO₂ (%), (C and D) ΔoxyHb, (E and F) ΔtotalHb, (G and H) ΔFVC index. *: p<0.05 stress versus rest, **: p<0.01 stress vs rest, ***p<0.001 stress vs rest, $: p<0.05 main effect cocoa, &: p<0.01 main effect time*

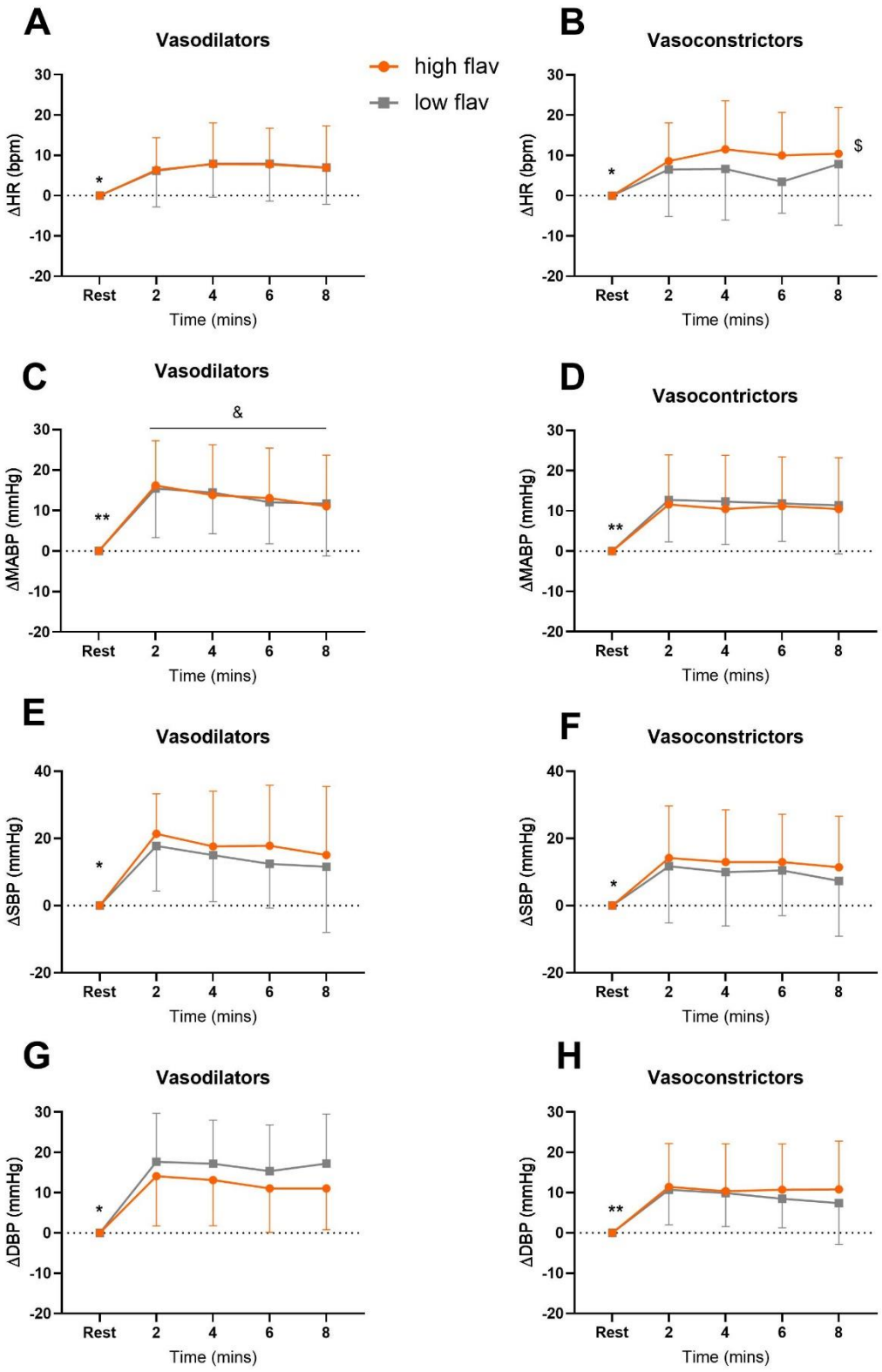

Figure 5.16: Time-course of changes in blood pressure and heart rate during mental stress task following high- (orange) and low-flavanol (grey) cocoa interventions in groups of vasodilators (n=11) and vasoconstrictors (n=11) *(A and B) ΔHR (bpm), (C and D) ΔMABP (mmHg), (E and F) ΔSBP (mmHg), (G and H) ΔDBP (mmHg) *: p<0.05 stress versus rest, **: p<0.01 stress versus rest; $: p<0.05 main effect cocoa, &: p<0.05 main effect time*

5.4 Discussion

The findings of the present study support the hypothesis that peak RH and EH responses would be greater in WEs than SAs. However, there was no significant effect of cocoa on either of these responses, contrary to the hypothesis. In relation to mental stress, there was no apparent effect of ethnicity or cocoa, which also challenged the hypothesis. However, when data were grouped according to whether individuals' showed forearm vasodilation or vasoconstriction during mental stress, the present study showed that after high flavanol cocoa, there was a greater increase in SO_2 during stress within the vasodilator group, and a smaller decrease in totalHb and FVC index in the vasoconstrictor group.

5.4.1 Ethnicity effects on vasodilator responses in the microvasculature

The findings presented in this chapter strengthen the view that EDD responses are blunted in young WE and SA women, despite no difference in resting values, as measured with NIRS. The peak SO_2 and ΔoxyHb during RH were greater, and the ΔdeoxyHb and ΔtotalHb also tended to be higher in WE than SA women. This aligns with previous evidence of blunted peak RH in the forearm of young SA men relative to WE men using VOP (Ormshaw et al., 2018), and consolidates the ethnic differences between SA and WE women described in Chapter 4. Furthermore, an impaired vasodilator response to exercise in young SA women has also been observed, with smaller peak SO_2, ΔoxyHb, ΔdeoxyHb and ΔtotalHb following 2 mins rhythmic handgrip contractions in young SA relative to WE women; this difference in EH is a novel finding. However, it must be acknowledged that maximum grip strength was greater in WEs than SAs. It has been proposed from studies comparing males and females that greater grip strength, and thus compressive force, results in greater hyperaemia following handgrip contractions (Aiku

and Marshall, 2019, Hunter et al., 2006). Therefore, greater absolute compressive force in the WE women may have contributed to the greater post-exercise hyperaemia in WE compared to SA women. Nonetheless, the change in SO_2 during exercise did not differ between ethnic groups, suggesting the same level of oxygen consumption was required by both groups irrespective of absolute compressive force, and suggesting that the differences in vasodilator responses are in fact, attributable to differences in other mediators of the dilator response, such as NO or prostaglandins.

By contrast , the present study found no difference in forearm vascular response to 8 min mental stress task between individuals of both ethnic groups, despite evidence of impaired vasodilator responses to sound stress in young SA men (Ormshaw et al., 2018). Importantly, Ormshaw et al demonstrated that, although both vasodilation and vasoconstriction occurred in response to mental stress in both WE and SA men, far more SA than WE men exhibited vasoconstrictor responses (Ormshaw et al., 2018). Thus, on the basis of the present results, it seems there may be sex-dependent differences in the direction of forearm vascular responses to acute mental stress between WEs and SAs, with far more WE women than WE men showing forearm vasoconstriction. Nevertheless, both WE and SA women showed elevations in ABP and HR during stress, as is characteristic of the alerting response (Hilton, 1982, Brod et al., 1959), with no difference in the magnitude of this response between the ethnicities.

As previously alluded to in Chapter 4, the ethnic differences in EDD responses may reflect genetic differences, which are confounded by lifestyle factors (Goyal and Sanghera, 2021, Jain et al., 2017), given low levels of physical activity and diets low in cardioprotective nutrients have been reported in SAs (Babakus and Thompson, 2012, LeCroy and Stevens, 2017). Importantly, the present study also found that WEs tended to consume more total flavonoids, and flavanols,

per day compared to SAs. Since diets rich in flavonoids, particularly flavanols, have been inversely linked to CVD risk (Parmenter et al., 2020, An et al., 2022), it is possible that lower flavonoid intake of SAs contributes to their impaired vascular function and smaller EDD responses during RH and EH. Whilst no studies have directly investigated the mechanisms contributing to ethnic differences in these vasodilator responses, it is likely that reduced NO bioavailability contributes at least in part, as has previously been established in BAs (Brothers et al., 2019). This is supported by the fact that NOS inhibition reduced basal vasodilation and responses evoked by ACh, to a greater extent in young WE versus SA men (Murphy et al., 2007). Seen against this background, the present finding that the proportion of SA women who showed vasodilator responses to mental stress was no smaller than amongst WE women is surprising, given the vasodilator response to mental stress was shown to be NO-dependent and that the NO contribution was impaired in hypertensives who, like SAs, are likely to display endothelial dysfunction (Halliwill et al., 1997, Khan et al., 2015). The issue of mechanisms underlying vasodilator responses is discussed further below.

5.4.2 Cocoa flavanol effects on vasodilator responses in the microvasculature

The present study identified no significant effect of CFs on forearm dilator responses as assessed by NIRS following arterial occlusion, rhythmic handgrip or during mental stress in young healthy women when WEs and SAs were grouped together or when they were separated by ethnicity. No effect of CFs was observed in any of the NIRS variables at rest. Although resting values recorded with NIRS are considered somewhat arbitrary (Barstow, 2019), the fact that there was no difference between values measured from the exact same site (without removal of probes) immediately before and one hour after ingestion of either low- or high-flavanol cocoa, it seems reasonable to deduce that in the present study, high flavanol cocoa had no effect on muscle

microvasculature at rest. Other studies comparing microvascular tone at rest have presented mixed results; some report an increase in resting FBF following an acute dose of CFs as measured by VOP (Baynham et al., 2021), and cutaneous blood flow of the forearm as measured by Laser Doppler (Neukam et al., 2007), whilst others reported no effect of CFs on the cutaneous or skeletal muscle microvasculature, measured by Laser Doppler or VOP respectively, at rest (Heiss et al., 2015, Hammer et al., 2015).

The effect of acute CFs on RH has been the focus of fewer studies compared to the well-defined facilitatory role in FMD, the dilator response evoked in the brachial artery in response to RH in the forearm (Ebaditabar et al., 2020, Sun et al., 2019), and evidence is mixed. Consistent with our findings, some studies demonstrated no effect of CFs on resting vessel diameter or peak RH within the cutaneous circulation in healthy subjects and type-2 diabetics (Bapir et al., 2022), or in peripheral artery disease patients (Hammer et al., 2015). By contrast, Heiss et al showed increased peak RH in the cutaneous and forearm circulations of healthy young, and elderly men, despite no effect of high flavanol cocoa at rest (Heiss et al., 2015); this difference may partly be due to gender differences in the populations studied. Furthermore, given that CFs are proposed to act predominantly via NO (Fisher et al., 2003a, Ramirez-Sanchez et al., 2010), but the RH response likely involves an interaction of NO with other factors such as prostaglandins (Engelke et al., 1996), it may be difficult to show an effect of CFs on the NO-component of the response. Importantly, referring back to Chapter 4 which showed Slope 2 was significantly steeper in WEs compared to SAs, the present findings suggest that this difference is ameliorated by high-flavanol cocoa, but not low-flavanol cocoa. This suggests that CFs may increase the rate of reperfusion only within the SAs, as could be expected based on their reduced NO bioavailability compared to WEs (Murphy et al., 2007).

Turning to exercise, the present study found no effect of CFs on the change in ABP or HR during exercise, nor the post-exercise forearm vasodilator response in WE and SA women considered together or separately. The effect of CFs on EH has been investigated previously using NIRS: in the leg muscle during steady state (~50% maximum effort) or time-trial cycling, no significant effect of chronic supplementation with CFs was shown on changes in oxyHb or SO_2 in trained young males (Shaw et al., 2020, Decroix et al., 2018b). Similarly, in CAD patients there was no difference in the hyperaemia evoked by wrist flexion-extension exercise following 6 weeks CF supplementation (Farouque et al., 2006). If it is assumed that CFs act by facilitating NO bioavailability (Ramirez-Sanchez et al., 2010), a lack of effect on EH may not be surprising, since a review of studies comparing peak EH before and after NOS inhibition reported mixed findings (Rådegran and Hellsten, 2000), suggesting that NO is not solely responsible for EH, and that when NOS is inhibited, other pathways, such as PGs and ATP/adenosine may be upregulated in order to compensate (Schrage et al., 2004). Indeed, more recent evidence suggests that EH is mediated by a combination of factors released from muscle fibres including K^+, adenosine, prostaglandins, and endothelial factors such as prostaglandins and EDHF, with NO playing at most a minor role (Joyner and Casey, 2015, Clifford and Hellsten, 2004). Thus, any effect CFs have on the contribution of NO to EH is likely to be outweighed by other components of the response, such that CFs may indeed have little effect even in SAs whose NO bioavailability is reduced.

The increases in HR and ABP observed during the mental stress task are attributable to effects of sympathetic activation and parasympathetic withdrawal on HR and a balance of sympathetic vasoconstriction and vasodilatation in limb muscle, as characterised by the alerting response (Brod et al., 1959, Hilton, 1982). The present study showed no significant effect of CFs on HR, SBP or DBP during stress in young WE women, though CFs were found to reduce MABP during

stress in young SAs only. Previous studies conducted in young WE males (Baynham et al., 2021) and females (Regecova et al., 2019), have reported no differences in absolute changes in ABP or HR during mental stress tasks following acute CFs. Furthermore, changes in blood pressure following acute CF supplementation are not reported in young, healthy populations (Vlachopoulos et al., 2005, Marsh et al., 2017, Sansone et al., 2017), though there is evidence of this effect in older adults (Heiss et al., 2015). This suggests that effects may be greater in at-risk populations, consistent with our observation of blood pressure-lowering effects in young SA but not WE women.

Furthermore, the present study found no significant effect of CFs on forearm vascular responses to mental stress using NIRS in young WE or SA women, when all WEs and SAs were considered together or in ethnic-dependent groups. This contrasts with previous evidence of a greater change in FBF following CFs during mental stress in young, healthy males, who also showed increased FBF at rest following CFs (Baynham et al., 2021). Nonetheless, the variability in the directional change in FVC during mental stress between individuals in the present study led us to also compare effects within groups of individuals who showed either forearm vasodilatation or vasoconstriction in response to mental stress (vasodilators and vasoconstrictors respectively, (Ormshaw et al., 2018)). Since there were the same proportions of vasodilators and vasoconstrictors amongst WEs and SAs, comparisons were made irrespective of ethnicity. The typical vasodilator response to mental stress is mediated by NO from eNOS attributable to increased shear stress (Pike et al., 2009, Martin et al., 1996, Halliwill et al., 1997) and nNOS-derived NO (Khan et al., 2015, Seddon et al., 2008) whereas forearm vasoconstriction during stress is characterised by an increase in muscle sympathetic nerve activity (Donadio et al., 2012, Carter and Ray, 2009, Edwards et al., 1998). It has been proposed that in those who show vasodilation during mental stress, the effect of increased muscle sympathetic nerve activity is

overcome by the dilator influences of endothelium-dependent NO (Ormshaw et al., 2018, Halliwill et al., 1997, Donadio et al., 2012). Indeed, Ormshaw et al proposed that this might explain why forearm vasoconstriction was more common in young SA men than WE men, given evidence that NO-dependent dilatation is blunted in SAs (Ormshaw et al., 2018). This is something which has not previously been considered in the context of CFs.

Interestingly, it was noted that CFs attenuated the decline in totalHb induced by mental stress only in the vasoconstrictor group, but no effect was shown on the vasodilator group. The apparent trend, but lack of significant effect of CFs on ΔFVC index in the vasoconstrictor group (Figure 5.15H) is likely due to the greater variability of ΔFVC data, which is calculated from ΔtotalHb and ΔMABP. Indeed, power calculations showed that 29 participants per group would be enough to detect a physiologically relevant difference (where power=85% and significance level p=0.05) between high and low flavanol interventions in ΔFVC data.

In contrast with what was observed in the vasoconstrictor group, CFs did not have a significant effect on ΔtotalHb or ΔFVC index in the vasodilator group. This may suggest that the NO contribution to their muscle vasodilator response to mental stress could not be increased further by the dose of CFs used in the present study. Nevertheless, CFs did augment the increase in SO_2 achieved during mental stress in the vasodilators. Considering the lack of apparent effect on the forearm vasodilation, we can postulate that this may be due to a reduction in forearm O_2 consumption. Importantly, as well as the role NO plays in vasodilation, it also limits endothelial cell and muscle fibre oxygen consumption via inhibition of mitochondrial cyctochrome-c oxidase (Shibata et al., 2005, Clementi et al., 1999). As such, if CFs increase NO bioavailability (Ramirez-Sanchez et al., 2010), the present findings in the vasodilator group, suggest that CFs induce greater inhibition of tissue oxygen consumption, so contributing to the increased SO_2.

It must be acknowledged that given that the present study was not designed to compare mental stress responses between vasodilators and vasoconstrictors, the interpretations outlined above should be taken with caution. However, it is clear that regardless of ethnicity, the effect of CFs was different in individuals who showed forearm vasodilator and vasoconstrictor responses, and this is something which warrants further investigation in order to understand differing contributions of CFs to individuals who demonstrate each type of response. This would involve specifically recruiting vasodilator and vasoconstrictors *a priori* and could also involve NOS inhibition in order to establish the contribution of NO to the stress response in each group.

Overall, acute administration of CFs had much less effect than was hypothesised: no effect on the EDD responses during RH or EH in SAs or WEs and a blunting effect on the mixed subgroup of WE and SA women who showed forearm vasoconstriction in response to mental stress, but not on those who showed the EDD. The relative lack of CF effects compared with what has been reported in studies of skeletal muscle performed on men (Baynham et al., 2021, Heiss et al., 2015) may partly be due to the timing of the present experiments with the female menstrual cycle. Thus, the present tests were done during the early follicular phase of the menstrual cycle, when oestrogen levels are lowest (Wenner and Stachenfeld, 2020). Since oestrogen receptor expression fluctuates with oestrogen levels during the menstrual cycle, the expression of oestrogen receptors is also lowest during this period (Gavin et al., 2009). As CFs are proposed to act via endothelial GPERs (Moreno-Ulloa et al., 2015), it is possible that there were insufficient receptors available for CFs to act upon in the low oestrogen phase of the cycle. In order to address this issue, it would be important to ascertain the potential relationship between circulating oestrogen levels and the effects of CFs in females, by studying the effects of CFs on vasodilator responses known to be mediated by, or dependent on, NO in different menstrual phases. This has not previously been explored in the context of FMD, nor microvascular

responses. Indeed, it has been argued that studies of vascular function in women should not control for menstrual phase, as testing during the low-oestrogen phase may lead to under or over estimation of the tested stimuli and loss of external validity to conditions in everyday life where oestrogen levels are higher the majority of the time (Stanhewicz and Wong, 2020). On this basis, it is possible that CFs may have a greater effect on the vascular responses tested if the experiments had been conducted at a different point in the menstrual cycle, when oestrogen levels are higher. Importantly, there is also evidence of membrane-bound oestrogen receptors in males (Cooke et al., 2017), which enables CF activity, though these do not show phasic variations as they do in women, hence CF responses are likely to be more consistent over time in men and may partly account for gender differences.

5.4.3 Limitations and future directions

As previously alluded to in Chapter 4, there are some limitations of the NIRS technique which may limit our interpretations of effects on vascular responses, for example differences in adipose thickness or skin pigmentation can impact on recordings of oxyHb and deoxyHb (Barstow, 2019). Nonetheless, since the effects of CFs here are considered within-subject, and NIRS measures are given relative to the baseline of each individual, this is more relevant to consider when drawing comparisons between ethnic groups than when interpreting the effect of CFs. In fact, since studies using VOP to measure FBF have shown effects of CFs on RH and stress responses within male groups (Baynham et al., 2021, Heiss et al., 2015), it would be useful to use VOP in young women to determine whether the general lack of CF effect reported in the present study is the result of methodological issues or is genuinely a gender disparity in the effects of CFs, perhaps reflecting time of the menstrual cycle as discussed above. Further, it should be noted that the sample size used in the present study was calculated based on the

magnitude of effects identified with VOP, but it is now clear that the variability of the NIRS data is much greater than measures obtained by VOP. Thus, a larger group size may be required to differentiate when using NIRS whether CFs are able to improve vasodilator responses in women. Nonetheless, the group size used sufficiently detected ethnic differences in RH and EH, implying that effects of CFs may be more subtle and require larger groups to be adequately powered. As mentioned above, it would also be useful to test at different points throughout the menstrual cycle, in order to improve the external validity of findings since the low-oestrogen phase represents such a short window of the response and effects of CFs may be masked during this period (Stanhewicz and Wong, 2020).

Another limitation of the present study is that although the concentrations of flavonoids within the CF interventions administered were known, the present study did not test their levels in the blood to confirm effective absorption. Previous studies have shown that circulating flavanol metabolites are significantly increased within 1 hr of consumption, and this is paralleled by increased plasma nitroso species for 1-3 hrs following consumption (Schroeter et al., 2006). Thus, the addition of blood sampling and chemiluminescence assays to future studies testing the effect of CFs on the microvasculature would provide additional insights into the mechanisms underlying any effects of CFs. In order to tighten the conditions of the present study, participants were asked to eliminate flavanol-rich foods from their diets for 24 hours prior to the study, thereby eliminating any potential background effects. This should allow for excretion of most flavanols before beginning the study, but there is evidence that metabolites may be present for up to 48 hours following intake (Borges et al., 2018, Rodriguez-Mateos et al., 2014). Thus, to ensure greater robustness in the washout period, the flavonoid-restricted window prior to testing should be increased to 48 hours.

In future studies, it would be of interest to explore the role of CFs in mental stress between individuals who show vasodilator and vasoconstrictor responses, since the present study highlighted that this might be very relevant for discerning effects of CFs across populations. Since pressor responses are associated with increased CVD risk (Chida and Steptoe, 2010), it would be important to ascertain whether the effect of CFs on the mental stress response within the vasoconstrictor group may be reproduced in a potentiating effect on vasodilator responses during RH and EH. If these individuals are considered to have impaired endothelial function, their reduced NO bioavailability and increased oxidative stress may provide a prime target for CFs in a wider population study.

5.4.4 Conclusion

The present study highlights differences in RH and EH in the forearm between young WE and SA women showing for the first time that peak dilator responses are smaller in SA women. This may be partly linked to differences in diet and lifestyle between ethnic groups, something that should be further explored. However, no effect of acute CFs was observed on either response in WE or SA women using NIRS. By contrast, no ethnic difference was found in the systemic haemodynamic changes or in the forearm vascular response to mental stress, nor was an effect of acute CFs on these responses in either group discerned. However, some differential effects of CFs during mental stress were apparent when individuals were grouped according to whether they showed forearm vasodilatation or vasoconstriction in response to mental stress. The present study showed, for the first time, that acute administration of CFs attenuated the vasoconstrictor response to mental stress within this subgroup.

On the basis of the present findings, it is apparent that the role of CFs within the microvasculature of the forearm may be more complex than the more widely reported effects in

conduit arteries. Furthermore, our findings in mental stress strongly suggest that additional considerations of individual responses should be made when assessing effects of CFs, since vasodilators and constrictors did not respond in the same way. Finally, the issue of whether the low-oestrogen phase is appropriate for assessing the vascular effect of CFs should be addressed through studies conducted during different phases of the menstrual cycle.

Chapter 6:

Comparing the diet and lifestyle of young South Asians and White Europeans

6.1 Introduction

It is well-documented that South Asian (SA) ethnicity is a major risk factor for the development of cardiovascular disease (CVD), with incidence of CVD-associated death estimated to be two- to three- times higher in SAs compared to the wider population (Gupta et al., 2006, Rana et al., 2014, Aambo and Klemsdal, 2017, Jain et al., 2017). Studies also indicate higher prevalence of CVD risk factors, such as hypertension, in SAs compared to White Europeans (WE) (Whitty et al., 1999, Williams et al., 1993). Furthermore, type-2 diabetes is estimated to occur in 22% of SAs, compared to just 7% of the native European population (Tillin et al., 2012) and symptoms of CVD appear to manifest at younger ages in SAs (Agyemang and Bhopal, 2002, Enas and Mehta, 1995).

Parental history of CVD is a commonly reported risk-factor for CV events in offspring (Lloyd-Jones et al., 2004), with CVD prevalence estimated to be two- to five- fold higher in those with positive family history compared to control (Thelle and Førde, 1979). There is also extensive evidence implicating the hereditary nature of CVD risk factors, such as hypertension (Ranasinghe et al., 2015, Stamler et al., 1979) and type-2 diabetes (Katulanda et al., 2015, Alcolado and Alcolado, 1991). Interestingly, offspring of hypertensive parents show impaired vasodilator responses to acetylcholine infusion, suggesting they are predisposed to endothelial dysfunction and progression of CVD (Taddei et al., 1996, Hirst and Marshall, 2018).

Taken together, the ethnic differences and evidence supporting the hereditary nature of CVD, hypertension and type-2 diabetes supports a potential role for genetics in determining CV-risk, particularly in SAs (Goyal and Sanghera, 2021). However, lifestyle factors such as smoking, physical activity (PA) and diet are also shown to contribute heavily towards the risk of CVD

(Lanier et al., 2016). There is most likely an interaction between genetics and lifestyle in determining CV health (Hartiala et al., 2021, Livingstone et al., 2021).

A significant link has been identified between physical inactivity and CVD risk and it is proposed that elimination of a sedentary lifestyle may reduce CVD risk by 15-39% (Lanier et al., 2016). Studies suggest that there is a dose-response relationship between PA levels and CVD-risk, with high leisure PA reducing CVD-risk by 20-30%, and moderate PA reducing risk by 10-20% compared to low activity levels (Li and Siegrist, 2012). Furthermore, sedentary time is thought to correlate with CVD incidence in a non-linear association (Pandey et al., 2016). Higher sitting time has been shown to increase risk of CVD, type-2 diabetes and hypertension, even when PA is adjusted for (Bailey et al., 2019, Henschel et al., 2020). It is therefore recommended that adults avoid sedentary behaviour by partaking in at least 150 minutes of moderate-intensity activity per week, and exercise should also be encouraged in children to promote a healthy lifestyle from a young age (Piercy et al., 2018).

Importantly, there is evidence of ethnic differences in physical activity; SA culture is commonly associated with low levels of PA, and studies suggest that at least 40% of SAs do not meet recommended activity levels (Babakus and Thompson, 2012, Daniel and Wilbur, 2011). In comparison to WE populations, physical inactivity has been shown to be approximately 20% higher amongst SAs (Williams et al., 2011) and SA total activity levels have been reported to be 31% lower (Afaq et al., 2019). Mean daily sitting time amongst SAs is also high, with studies indicating at least eight hours of sedentary time per day within SA males and females (Mahmood et al., 2020, Dey et al., 2021). Ethnic differences in activity levels are also apparent in children in the UK, with SAs found to have lower daily step counts than WEs and poorer fitness, which is at least partly attributable to reduced PA (Owen et al., 2009, Nightingale et al., 2016). These studies

suggest that from a young age SAs are not as exposed to PA, and that continues into their adult life, which is likely to also contribute to their increased risk of CVD. However, studies formally comparing PA between young healthy SA and WEs adults are limited, as most studies focus on older adults or mixed demographics.

Diet is also a key modifiable risk factor for prevention of CVD (Collaborators, 2016). Current recommendations relate to dietary patterns encompassing a balanced intake of multiple foods and nutrients (Casas et al., 2018, Lanier et al., 2016). For example, the Mediterranean diet, which is rich in vegetables, whole grains, and healthy fats such as olive oil but low in carbohydrates and processed foods, is widely considered protective against CV events (Sofi et al., 2010, Estruch et al., 2018). Furthermore, the DASH diet, characterised by high intake of fruit and vegetables, low-fat dairy, grains, nuts, fish and poultry, but low intake of saturated fat, sugar, and sodium, has also been linked to reduced CVD risk (Mertens et al., 2018, Jones et al., 2018). Commonalities of these healthy dietary patterns include high intake of fibre, antioxidants, vitamins and polyphenols, combined with reduced intake of salt, saturated fats and refined sugar (Mozaffarian, 2016). Specifically, CVD risk is strongly inversely associated with fruit and vegetable consumption; a 4% reduction in CV-mortality has been observed for each additional daily serving of fruit and vegetables (Miller et al., 2017, Wang et al., 2014a). Furthermore, consumption of fruits and vegetables rich in flavonoids can be particularly beneficial for CV health, including reducing risk of hypertension (Kong et al., 2023) and reducing CVD incidence and mortality (Micek et al., 2021, Parmenter et al., 2020). A comprehensive dose-response meta-analysis showed that consumption of 500 mg of daily flavonoids was associated with 27% lower CVD risk (Micek et al., 2021). In particular, dietary polyphenols have been shown to improve endothelial function and modulate oxidative stress, which are likely key protective mechanisms against CVD (Yamagata and Yamori, 2019). Conversely, high intake of saturated fats can also be

linked to increased prevalence of CVD, with a recent meta-analysis proposing a 17% decline in CV events when dietary saturated fat is reduced (Hooper et al., 2020).

Studies directly comparing dietary patterns between WE and SA populations are very limited, with no studies quantifying intake of flavonoids in SA populations. A review suggests that SA immigrants have lower consumption of protein and monosaturated fat, as well as micronutrients such as potassium, sodium, and vitamin A compared to Western populations, but with mixed findings for other nutrients (LeCroy and Stevens, 2017). The SA diet is also traditionally high in saturated fats, such as ghee and cooking oil, and migration to Western countries leads to further increases in fat and energy consumption due to the addition of other Western high-fat food items (Cainzos-Achirica et al., 2019, Simmons and Williams, 1997, Holmboe-Ottesen and Wandel, 2012). SAs also have some of the lowest consumption of fruit and vegetables worldwide, with studies suggesting that fewer than 4% of SAs consume the recommended 400g/day of fruit and vegetables (Jayawardena et al., 2020). As such, it is very likely that dietary choices may also contribute to increased CVD risk amongst SAs.

Identifying modifiable risk factors (such as diet and physical activity) within the young healthy population is particularly critical, as behavioural changes around PA and diet, are likely to help mitigate CVD risk and future health outcomes. To our knowledge, no studies have directly quantified and compared dietary patterns, PA and sedentary time between young healthy WE and SA. Furthermore, specific key aspects of diet, such as intake of flavonoids, now known to be important contributors to cardiovascular health, have never been quantified and compared across these two ethnic groups.

6.1.1 Aims and Hypotheses

The main goal of the present study was to directly compare dietary intake (macronutrients and targeted micronutrients, such as flavonoids), PA and sedentary time within young men and women of SA and WE ethnicity.

We hypothesise that:

(i) WEs will be more physically active than SAs, with higher total METmins/week

(ii) SAs will have higher mean daily sitting time than WEs

(iii) SAs will have lower intake of CV-protective nutrients (such as antioxidants and vitamins from fruit and vegetables) and higher intake of nutrients associated with increased CV-risk (such as salt and saturated fat)

6.2 Methods

6.2.1 Participants

We recruited a total of 80 men and women (n=40 WE and n=40 SA), aged 18-26 years, from February 2021 to September 2022. Participant ethnicity was self-reported (ONS, 2018) and all participants were UK residents at the time of study, with both parents of the same ethnic origin. Each participant signed a consent form (Appendix 1) committing to the study and the use of their data as outlined in the Participant Information Sheet (Appendix 3), in accordance with approval granted by the University of Birmingham Ethic Committee (ERN17_1755). Each volunteer was assigned a random four-digit ID number for use throughout the study to ensure anonymisation.

6.2.2 Data collection

There were several aspects to the survey that the participants were required to complete, which were all issued using SmartSurvey (SmartSurvey). The main mandatory component consisted of a general health and lifestyle questionnaire (Appendix 5), including questions relating to family heritage and health; the EPIC Food Frequency Questionnaire (FFQ); and International Physical Activity Questionnaire (IPAQ). The general questionnaire collected information on participants' ethnic origins, including their duration of residence in the UK, employment status, and their parental health, with a particular interest towards hypertension and diabetes, both of which are known to have a hereditary component (Stamler et al., 1979, Alcolado and Alcolado, 1991). The EPIC-FFQ is widely used to assess participants' average weekly consumption of various foods during the last year in order to estimate their nutrient intake (Mulligan et al., 2014). The IPAQ relates to participants' activity levels during a seven-day period and responses are used to quantify their physical activity at vigorous to light intensities, as well as overall (Craig et al., 2003).

Upon completion of this initial questionnaire participants were then sent a link for the three-day food diary, which they were asked to complete for two weekdays and one weekend day (Appendix 6). The food-diary was used alongside the FFQ to provide a more detailed insight into participants' daily diet and to provide an alternative means of calculating nutrient intake during a shorter time-period. Participants were asked to provide quantities and preparation methods, as well as daily water intake, estimated sitting time, and steps (where applicable, if participant uses a fitness tracker). Unfortunately, the uptake for this part of the survey was lower than for the initial questionnaire and some responses had to be excluded due to insufficient detail for accurate analysis.

6.2.3 Data analysis and interpretation

The FFQ data was interpreted using FETA software (Mulligan et al., 2014). Spreadsheets were prepared using the questionnaire data and processed using this software as explained in Section 2.6.1. Some details of the FFQ, including average weekly fruit and vegetable portions were analysed independently of the FETA outputs.

Food-diary responses were manually inputted to Dietplan6 software to obtain total nutrient consumption during the three-day period for each participant, as explained in Section 2.6.2. This was then used to calculate daily intake for each nutrient and flavonoid type of interest.

Results from the IPAQ were interpreted manually using Excel, using instructions provided to estimate energy expenditure(TrinityCollegeDublin), as explained in Section 2.6.3.

In relation to parental health, for participants who reported having a parent with diabetes, this was assumed to be type-2 unless stated otherwise, due to the overriding prevalence of type-2, which accounts for an estimated 85% of cases (Barceló and Rajpathak, 2001).

Macronutrients were compared as mean values as well as a proportion of total energy intake. To calculate this, weights of carbohydrates, protein and fat were converted to calorie content; 1g carbohydrates or protein is 4 kcal and 1g fat is 9kcal (Buchholz and Schoeller, 2004), so weights in grams were multiplied by the relevant factor to calculate kcal content, which was then divided by total calorie intake for each participant.

6.2.4 Statistical Analysis

All statistical analysis was performed and figures were created using Graphpad Prism Version 9.2.0. Anthropometric measures, IPAQ, FFQ and food diary results were analysed using two-way analysis of variance (ANOVA) for main effects of ethnicity and/or gender, followed by post-hoc

Tukey's pairwise comparisons where an ethnicity x gender interaction was identified. Smoking habits and parental health were compared by Fisher's exact test. Pearson's correlation coefficients were used to assess the relationship between FFQ and food diary data for each nutrient assessed. Outliers (classified as greater than two standard deviations from the mean of each group) were identified prior to group comparisons. One outlier was excluded from anthropometric measures and a total of ten were excluded from IPAQ calculations (at all intensities). For the FFQ and food-diary, outliers were calculated independently for each nutrient; if the same participant was an outlier for three or more nutrients, they were excluded across all analysis for that questionnaire, however for individuals who were only outliers in a single nutrient, only the relevant data points were excluded.

A significance level of $p<0.05$ was used for all statistical analyses, and all values reported are mean±SD.

6.3 Results

6.3.1 Population characteristics

Anthropometric measures are shown in Table 6.1, n=39 WE (20 men, 19 women) and n=40 SA (20 men, 20 women). Main effects analysis showed significant ethnic differences in age [$F(1,75)=5.46$, $p=0.0221$], height [$F(1,75)=11.3$, $p=0.0012$], and BMI [$F(1,75)=10.4$, $p=0.0019$], though no effect of ethnicity on weight [$F(1,75)=0.925$, $p=0.339$]. There was also a significant ethnicity x gender interaction for BMI [$F(1,75)=6.12$, $p=0.0156$]; post-hoc tests highlighted that SAs had a higher BMI than WEs only in women ($p=0.0008$).

Table 6.1: Anthropometric data presented for groups of White European (WE) and South Asian (SA) men (M) and women (F). *Data shown is mean ± SD alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: p<0.001)*

		White European (WE)	South Asian (SA)	Main Effects		
		n=39, M:20, W:19	n=40, M:20, W:20	*Ethnicity*	*Gender*	*Ethnicity x gender*
Age (yrs)	**Overall**	**23.2 ± 1.56**	**22.1± 2.20**	*0.0221 *	*0.289*	*0.626*
	F	23.5 ± 1.54	22.3 ± 2.31			
	M	22.9 ± 1.56	22.0 ± 2.13			
Height (cm)	**Overall**	**174 ± 10.2**	**169 ± 10.7**	*0.0012 **	*<0.0001 ****	*0.187*
	F	167 ± 6.10	160 ± 4.79			
	M	181 ± 8.48	178 ± 6.53			
Weight (kg)	**Overall**	**70.8 ± 13.4**	**72.3 ± 13.5**	*0.339*	*<0.0001 ****	*0.158*
	F	61.5 ± 9.40	65.6 ± 13.8			
	M	80.0 ± 10.1	78.9 ± 9.50			
BMI (kg/m²)	**Overall**	**23.2 ± 3.16**	**25.2 ± 3.63**	*0.0019 **	*0.0967*	*0.0156 *
	F	22.1 ± 3.33	25.5 ± 4.36			
	M	24.4 ± 2.56	24.9 ± 2.79			

There was no difference between the proportion of non-smokers in WE and SA populations (WE: 69.2%, SA: 75.0%, p=0.622). However, men were significantly more likely to be smokers compared to women (men: 43.6%, women: 12.5%, p=0.0026).

The prevalence of parental CV conditions was higher in SAs than in WEs. 62.5% of SAs reported at least one parent with CV condition (hypertension, type 2 diabetes, high cholesterol), compared to 20.0% of WEs (p=0.0002); there was no gender difference in parental CV health (p>0.999). Hypertension was the most commonly reported condition in both ethnic groups; the prevalence of parental hypertension was significantly higher in SA compared to WE populations (SA: 40.0%, WE: 15.0%, p=0.0230). There was no difference in the prevalence of parental hypertension between men and women (M: 22.5%, F: 32.5%, p=0.453).

All except two WE participants were born in the UK, and all were born in Europe. Contrarily, 50.0% of SA participants were born outside of Europe (10 men and 10 women). Within this

group, 25.0% have lived in the UK for over ten years, 20.0% for six to ten years, and 25.0% for five years or less (30.0% of respondents did not indicate when they moved to the UK).

75.0% of WE and 65.0% of SA participants were students, the remaining participants were in a mixture of full- and part-time employment in the workplace and working from home. The group sizes were too small to warrant further analysis on this basis.

6.3.2 Physical activity and sitting time

When categorised according to IPAQ guidelines, mean total weekly activity for WEs was in the 'high' active category, and the majority of WEs (75.0%) were in this category, with the remaining 25.0% categorised as 'moderate'. Comparatively, mean total activity for SA was classified as 'moderate', with 59.0% of SAs in this group. Only 33.3% of SAs were classified as 'high' active, and 7.70% were in the 'low' active group, all of whom were women. WEs had significantly higher total METmin/week than SAs [$F(1,65)=28.5$, $p<0.0001$], though there was no main effect of gender on total METmin/week [$F(1,65)=0.317$, $p=0.576$], nor was the ethnicity x gender interaction significant [$F(1,65)=0.00929$, $p=0.924$] (Figure 6.1, Table 6.2).

Simple main effects analysis showed that WEs have significantly higher METmin/week than SAs across all intensities, as shown in Figure 6.1 and Table 6.2 [Vigorous: $F(1,65)=13.8$, $p=0.0004$, moderate: $F(1,65)=7.16$, $p=0.0094$, walking: $F(1,65)=19.8$, $p<0.0001$]. At vigorous intensity there was also a main effect of gender, with men being more active than women [$F(1,65)=7.64$, $p=0.0074$].There was no significant effect of gender on METmin/week at moderate [$F(1,65)=0.986$, $p=0.324$] or walking [$F(1,65)=2.00$, $p=0.162$] intensities.

Table 6.2: Weekly physical activity at vigorous, moderate and walking intensities as well as total activity, as calculated from IPAQ questionnaire, for groups of White European and South Asian men (M) and women (F) *(METmins/week, mean±SD). Presented alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, **:p<0.01, ***:p<0.001,****: p<0.0001).*

		White European (WE)	South Asian (SA)	*Main Effects*		
METmin/week		n=35, M:17, F:18	n=35, M:18, F: 17	*Ethnicity*	*Gender*	*Ethnicity x gender*
Total	**Overall**	**4850 ± 2810**	**2050 ± 1110**	*<0.0001 **** *	*0.576*	*0.924*
	F	4730 ± 2990	1880 ± 1160			
	M	4970 ± 2700	2220 ± 1070			
Vigorous	**Overall**	**1880 ± 1700**	**732 ± 916**	*0.0004 *** *	*0.0074 ** *	*0.0803*
	F	1180 ± 1060	579 ± 647			
	M	2580 ± 1960	885 ± 1120			
Moderate	**Overall**	**1120 ± 1420**	**436 ± 454**	*0.0094 ** *	*0.324*	*0.113*
	F	1440 ± 1680	359 ± 496			
	M	783 ± 1030	514 ± 409			
Walking	**Overall**	**1870 ± 1210**	**883 ± 445**	*<0.0001 **** *	*0.162*	*0.378*
	F	2110 ± 1080	941 ± 548			
	M	1610 ± 1310	825 ± 319			

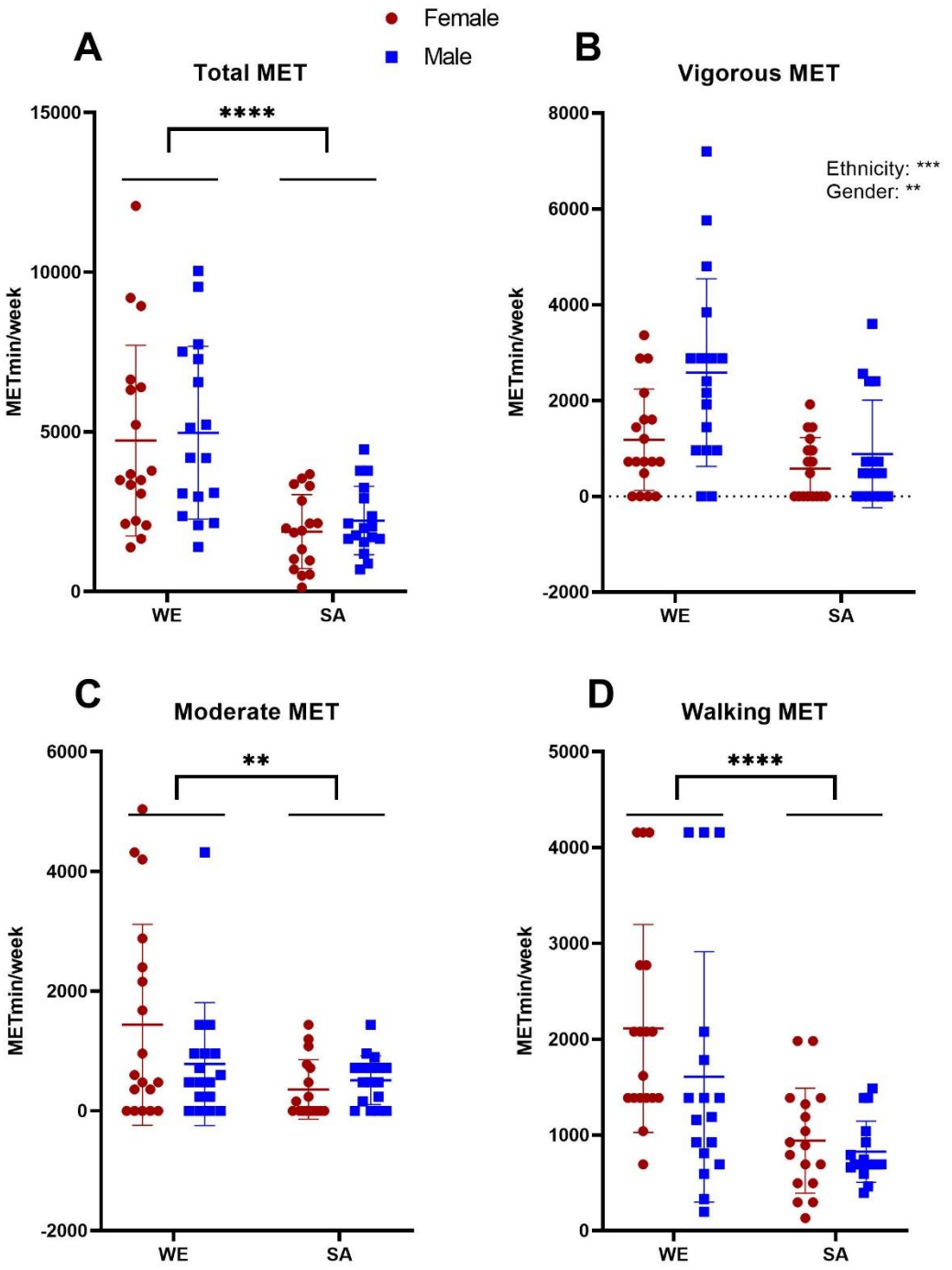

Figure 6.1: Distribution of physical activity within groups of White European (WE) and South Asian (SA) women (red) and men (blue), *as derived from the International Physical Activity Questionnaire for (A) total, (B) vigorous, (C) moderate and (D) walking activity. Main effects of gender and/or ethnicity are presented from 2-way ANOVA (**: p<0.01, ***: P<0.001, ****: p<0.0001).*

Figure 6.2 shows the distribution of sitting time for men and women of both ethnic groups, as assessed by the IPAQ. Daily sitting time was significantly higher in SAs (469±19.4mins) compared to WEs (387±21.5mins, F(1,65)=6.92, p=0.0107). Although women (447±24.2mins) tended to sit more than men (408±21.7mins) there was no significant effect of gender on sitting time [F(1,65)=1.70, p=0.197], nor was the ethnicity x gender interaction between these factors significant [F(1,65)=0.00450, p=0.947].

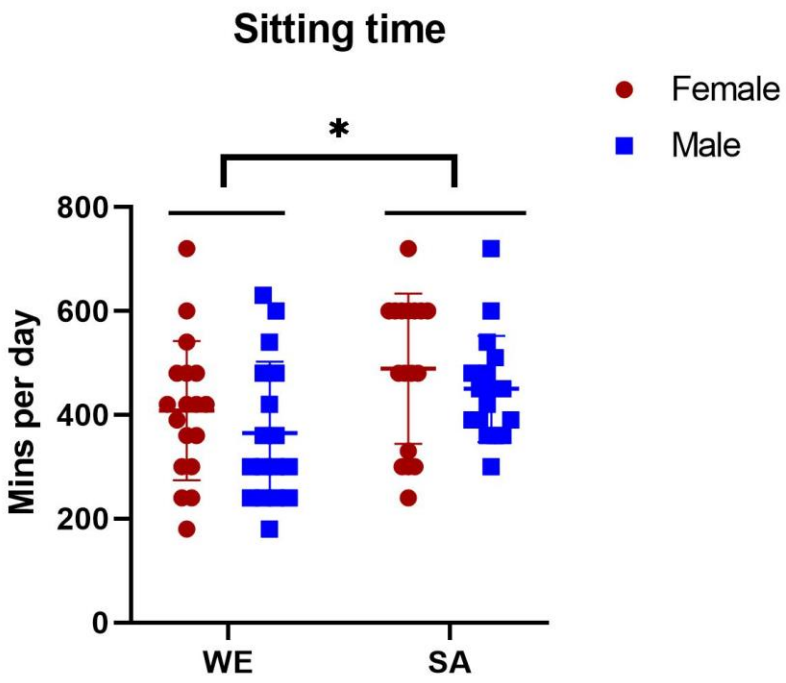

Figure 6.2: Distribution of sitting times within groups of White European (WE) and South Asian (SA) women (red) and men (blue), *as derived from the International Physical Activity Questionnaire. 2-way ANOVA revealed there is a significant main effect of ethnicity (p=0.0107).*

6.3.3 Dietary comparisons

6.3.3.1 Food Frequency Questionnaire: Weekly Portions

Table 6.3 and Figure 6.3 present the mean number of times per week WEs and SAs consumed vegetables, salad, fruit, fish and meat, ascertained by the FFQ. WEs ate significantly more vegetables [$F(1,76)=13.1$, $p=0.0005$] and salad [$F(1,76)=5.66$, $p=0.0198$] than SAs. There was no gender difference in consumption of either vegetables [$F(1,76)=0.222$, $p=0.639$] or salad [$F(1,76)=0.450$, $p=0.504$]. WEs also tended to eat more fruit than SAs, though the difference was not statistically significant [$F(1,76)=2.03$, $p=0.158$]. There was also no gender difference in fruit consumption [$F(1,76)=0.0292$, $p=0.865$], though there was a significant gender x ethnicity interaction [$F(1,76)=7.80$, $p=0.0066$]. Post-hoc analysis showed that the only significant difference was between WE and SA women ($p=0.0197$). There was no main effect of ethnicity [$F(1,76)=0.928$, $p=0.338$] or gender [$F(1,76)=0.928$, $p=0.338$] on fish consumption. There was no effect of ethnicity on meat consumption [$F(1,76)=0.00275$, $p=0.958$], but men consumed significantly more meat than women [$F(1,76)=13.1$, $p=0.0005$].

Main effects analysis revealed significant effects of both gender and ethnicity on alcohol consumption, though the ethnicity x gender interaction was not significant [$F(1,76)=1.76$, $p=0.189$]. Mean weekly alcohol consumption was significantly higher in WE (12.1±2.60 units) than SA (2.43±0.875 units) groups [$F(1,76)=54.8$, $p<0.0001$, Figure 6.3]. Men (8.98±1.32 units) also had significantly higher alcohol consumption than women (5.50±1.06 units, $F(1,76)=7.14$, $p=0.0092$).

Table 6.3: Mean weekly intake of vegetables, salad, fruit, fish and meat for groups of White European (WE) and South Asian (SA) men (M) and women (F) (*mean±SD times per week), alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: P<0.001).*

		White European (WE)	**South Asian (SA)**	***Main effects***		
		n=40, M:20, F:20	n=40, M:20, F:20	*Ethnicity*	*Gender*	*Ethnicity x gender*
Vegetables	**Overall**	**5.95 ± 1.20**	**4.80 ± 1.64**	*0.0005 ****	*0.639*	*0.0879*
	F	6.15 ± 1.09	4.45 ± 1.96			
	M	5.75 ± 1.29	5.15 ± 1.18			
Salad	**Overall**	**3.35 ± 2.08**	**2.38 ± 1.56**	*0.0198 *	*0.504*	*0.131*
	F	3.8 ± 2.22	2.2 ± 1.80			
	M	2.9 ± 1.89	2.55 ± 1.32			
Fruit	**Overall**	**5.03 ± 2.11**	**4.44 ± 1.96**	*0.158*	*0.865*	*0.0066 **
	F	5.6 ± 1.98	3.75 ± 2.05			
	M	4.45 ± 2.11	5.05 ± 1.67			
Fish	**Overall**	**1.68 ± 1.49**	**1.40 ± 1.01**	*0.338*	*0.338*	*0.433*
	F	1.7 ± 1.38	1.65 ± 0.988			
	M	1.65 ± 1.63	1.15 ± 0.988			
Meat	**Overall**	**4.25 ± 2.43**	**4.23 ± 2.21**	*0.958*	*0.0005 ****	*0.0704*
	F	2.95 ± 2.11	3.8 ± 1.70			
	M	5.55 ± 2.01	4.65 ± 2.60			

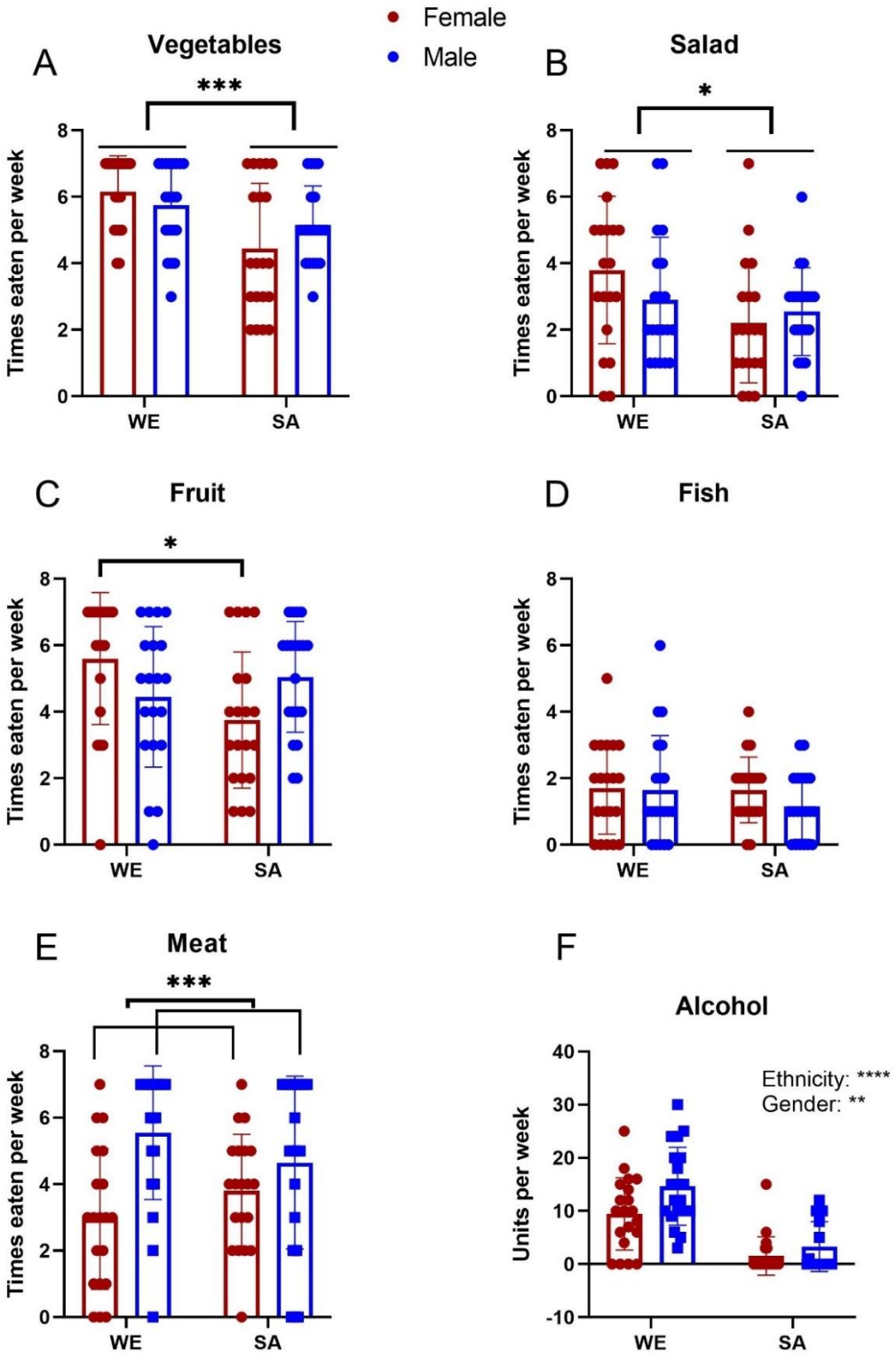

Figure 6.3 Mean weekly intake of (A) vegetables, (B) salad, (C) fruit, (D) fish, (E) meat and (F) alcohol for groups of White European (WE) and South Asian (SA) women (red) and men (blue*), as derived from Food Frequency Questionnaire. Main effects of gender and/or ethnicity are presented from 2-way ANOVA (*: p<0.05, ** p<0.01, ***: P<0.001).*

6.3.3.2 Habitual Nutritional Intake: Food Frequency Questionnaire

Table 6.4 and Figure 6.4 present nutrient intakes as estimated from the FFQ, representing habitual diet during the last year. Sodium was the only nutrient for which there was a main effect of ethnicity, with WEs consuming significantly more than SAs [$F(1,67)=7.086$, $p=0.0097$]. WEs also tended to consume more dietary vitamin D than SAs, though this did not reach statitical significance [$F(1,65)=3.58$, $p=0.063$]. Considering macronutrients as a proportion of total energy intake, SAs were found to obtain significantly more of their total energy from carbohydrates than WEs [WEs: 44.8±5.65%, SAs: 48.6±5.44; $F(1,67)=8.49$, $p=0.0049$].

There was a main effect of gender on intake of several nutrients; males consumed significantly more kcal [$F(1,67)=12.0$, $p=0.0009$], protein [$F(1,67)=12.0$, $p=0.0009$], carbohydrates [$F(1,67)=11.0$, $p=0.0015$], sugar [$F(1,66)=7.06$, $p=0.0099$], fat [$F(1,67)=6.26$, $p=0.0148$], saturated fat [$F(1,66)=9.86$, $p=0.0025$], sodium [$F(1,67)=8.30$, $p=0.0053$], cholesterol [$F(1,67)=7.13$, $p=0.0095$], folate [$F(1,67)=4.06$, $p=0.0481$] and vitamin D [$F(1,65)=4.36$, $p=0.0408$] than women. There was no significant ethnicity x gender interaction for any of the above nutrients, though there was for vitamin C [$F(1,65)=5.56$, $p=0.0214$], with post-hoc analysis identifying no significant differences between any groups, this being lowest in SA women.

Table 6.4: Habitual nutrient intake, estimated from the Food Frequency Questionnaire, for groups of White European (WE) and South Asian (SA) men (M) and women (F) *(mean±SD), alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: P<0.001).*

		White European (WE)	**South Asian (SA)**	***Main Effects***		
		n=35, M:17, F:18	n=36, M:18, F:18	*Ethnicity*	*Gender*	*Ethnicity x gender*
Energy (kcal)	**Overall**	**1770±522**	**1670±575**	*0.356*	*0.0009 *****	*0.438*
	F	1620±391	1410±557			
	M	1940±599	1920±480			
Protein (g)	**Overall**	**80.5±30.7**	**72.9±23.5**	*0.201*	*0.0009 *****	*0.900*
	F	70.7±23.8	62.1±19.0			
	M	90.9±34.3	83.8±22.9			
Carbohydrates (g)	**Overall**	**197±59.9**	**201±68.7**	*0.813*	*0.0015 ** *	*0.357*
	F	181±44.3	171±68.5			
	M	215±70.1	232±55.5			
Sugar (g)	**Overall**	**93.3±35.0**	**93.6±38.3**	*0.968*	*0.0099 ** *	*0.242*
	F	87.3±26.9	77.1±30.3			
	M	99.7±41.8	109±39.2			
Fat (g)	**Overall**	**71.5±24.6**	**67.4±29.2**	*0.496*	*0.0148 **	*0.399*
	F	66.5±19.0	57.0±28.5			
	M	76.8±29.1	77.8±26.7			
Saturated fat (g)	**Overall**	**27.2±10.3**	**24.9±12.1**	*0.328*	*0.0025*	*0.299*
	F	24.6±7.78	19.5±9.30			
	M	29.9±12.0	30.0±12.4			
Fibre (g)	**Overall**	**14.7±5.18**	**14.7±4.59**	*0.981*	*0.689*	*0.0932*
	F	15.4±5.36	13.5±4.88			
	M	13.9±5.03	15.9±4.05			
Sodium (mg)	**Overall**	**2580±955**	**2060±769**	*0.0097 ** *	*0.0053 ** *	*0.703*
	F	2340±538	1740±694			
	M	2830±1220	2380±720			
Vitamin C (mg)	**Overall**	**101±46.7**	**106±49.5**	*0.627*	*0.176*	*0.0214 **
	F	106±51.1	85.2±40.8			
	M	94.9±42.3	127±49.7			
Vitamin D (mcg)	**Overall**	**2.72±1.83**	**2.04±1.28**	*0.0630*	*0.0408 **	*0.601*
	F	2.44±1.65	1.54±0.803			
	M	3.02±2.02	2.52±1.48			
Folate (mcg)	**Overall**	**253±87.0**	**240±64.9**	*0.470*	*0.0481 **	*0.258*
	F	246±89.7	212±64.8			
	M	261±86.0	268±53.0			

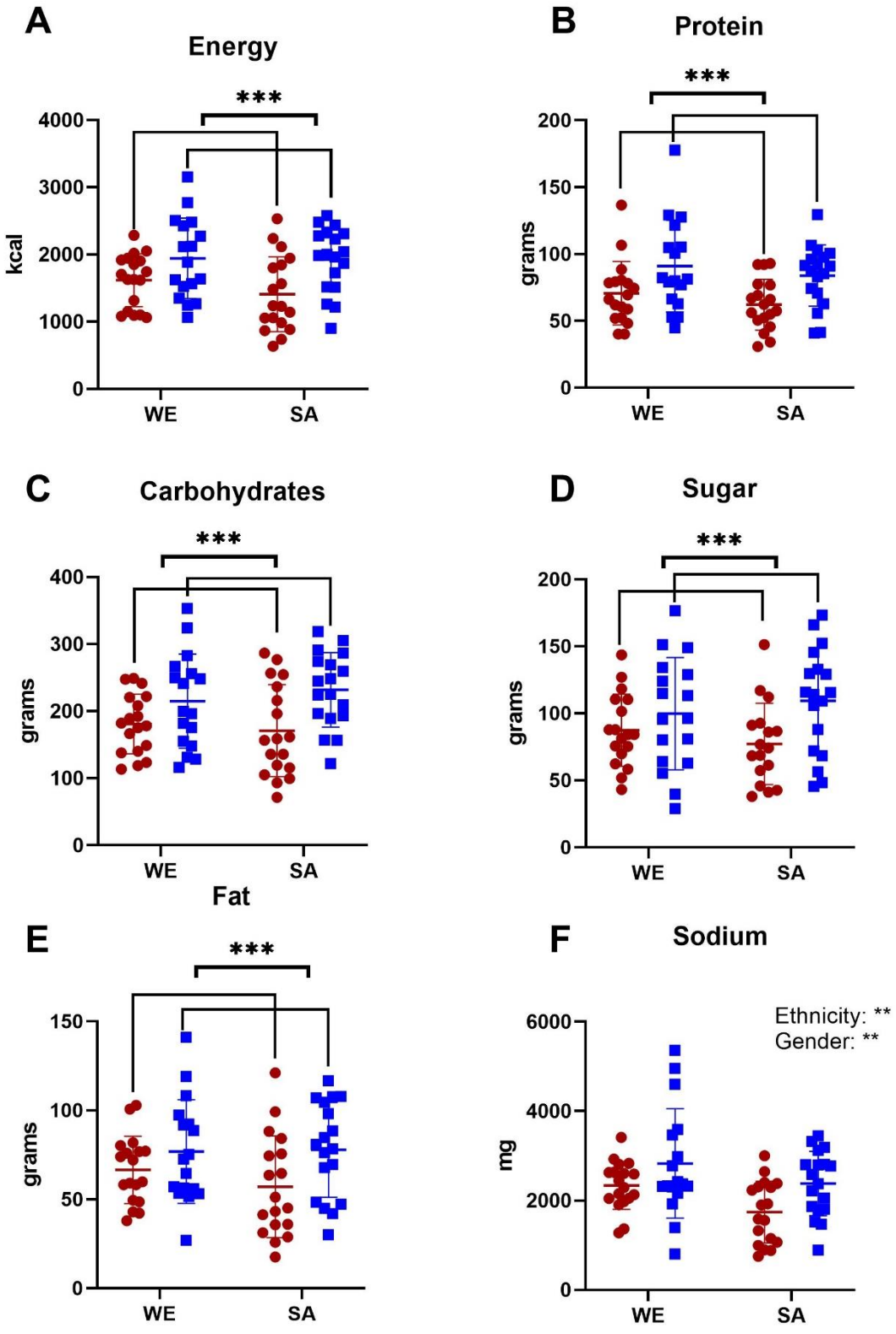

Figure 6.4: Habitual daily intake of various nutrients as estimated from Food Frequency Questionnaire for groups of White European (WE) and South Asian (SA) women (red) and men (blue). *Main effects of gender and/or ethnicity are presented from 2-way ANOVA (**:p<0.01, ***: P<0.001).*

6.3.3.3 Current Nutritional Intake: 3-day Food Diary

The three-day food diary provided an in-depth insight into diet at the time of completing the study (Table 6.5 and Figure 6.5). WEs were found to have higher daily intake of kcal [$F(1,46)=9.61$, $p=0.0033$], protein [$F(1,44)=7.26$, $p=0.0100$], carbohydrates [$F(1,46)=8.08$, $p=0.0066$], sugar [$F(1,45)=5.65$, $p=0.0218$], fat [$F(1,45)=8.11$, $p=0.0066$], saturated fat [$F(1,46)=12.7$, $p=0.0009$], fibre [$F(1,44)=6.00$, 0.0183], sodium [$F(1,46)=12.1$, $p=0.0011$], vitamin C [$F(1,45)=7.13$, $p=0.0105$] and folate [$F(1,44)=5.45$, $p=0.0242$] compared to SAs. Meanwhile, men consumed significantly more kcal [$F(1,46)=21.5$, $p<0.0001$], protein [$F(1,44)=21.4$, $p<0.0001$], carbohydrates [$F(1,46)=10.3$, $p=0.0025$], fat [$F(1,45)=19.5$, $p<0.0001$], saturated fat [$F(1,46)=11.4$, $p=0.0015$], fibre [$F(1,44)=6.70$, $p=0.0130$] and sodium [$F(1,46)=5.86$, $p=0.0195$] than women. The only nutrient for which there was a significant ethnicity x gender interaction was fat [$F(1,45)=5.54$, $p=0.0230$]; post-hoc analysis showed significant differences between WE and SA women ($p=0.0020$) and between SA men and women ($p=0.0003$).

Table 6.5: Estimated daily nutrient intake from three-day food diary for groups of White European (WE) and South Asian (SA) men (M) and women (F) *(mean±SD), alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: P<0.001, ****: p<0.0001).*

		White European (WE)	**South Asian (SA)**	***Main Effects***		
		n=29, M:12, F:17	n=21, M:10, F:11	*Ethnicity*	*Gender*	*Ethnicity x gender*
Energy (kcal)	**Overall**	**2270±757**	**1720±769**	*0.0033 ** *	*<0.0001 **** *	*0.169*
	F	2020±618	1190±391			
	M	2620±823	2310±651			
Protein (g)	**Overall**	**88.5±36.6**	**68.1±25.4**	*0.0100 ** *	*<0.0001 **** *	*0.819*
	F	71.9±18.6	52.3±16.1			
	M	111±43.3	87.3±21.3			
Carbohydrates (g)	**Overall**	**84.8±57.4**	**49.0±39.2**	*0.0066 ** *	*0.0025 ** *	*0.808*
	F	68.9±35.2	26.4±19.1			
	M	104±19.6	73.9±41.1			
Sugar (g)	**Overall**	**26.1±25.0**	**13.9±10.6**	*0.0218 * *	*0.0866*	*0.381*
	F	20.1±10.6	11.6±10.4			
	M	35.3±36.7	16.5±10.8			
Fat (g)	**Overall**	**95.3±29.8**	**73.3±34.2**	*0.0066 ** *	*<0.0001 **** *	*0.0230 * *
	F	88.5±20.0	48.5±15.3			
	M	104±38.6	101±27.8			
Saturated fat (g)	**Overall**	**34.9±13.0**	**24.0±10.1**	*0.0009 *** *	*0.0015 ** *	*0.279*
	F	31.9±9.76	17.4±6.77			
	M	39.1±16.1	31.3±7.78			
Fibre (g)	**Overall**	**19.4±6.75**	**14.9±7.06**	*0.0183 * *	*0.0130 * *	*0.784*
	F	17.1±3.84	12.9±7.11			
	M	22.6±8.53	17.4±6.56			
Sodium (mg)	**Overall**	**2700±1020**	**1810±888**	*0.0011 ** *	*0.0195 * *	*0.179*
	F	2490±997	1290±477			
	M	2980±1020	2370±902			
Vitamin C (mg)	**Overall**	**88.1±49.3**	**53.3±33.9**	*0.0105 * *	*0.519*	*0.897*
	F	92.4±40.6	56.5±35.9			
	M	82.4±60.4	49.8±33.1			
Vitamin D (mcg)	**Overall**	**2.35±1.74**	**2.40±1.79**	*0.870*	*0.729*	*0.723*
	F	2.50±1.83	2.40±1.51			
	M	2.12±1.65	2.40±2.17			
Folate (mcg)	**Overall**	**204±61.3**	**153±92.0**	*0.0242 * *	*0.289*	*0.969*
	F	195±62.6	141±93.1			
	M	218±59.6	166±93.9			

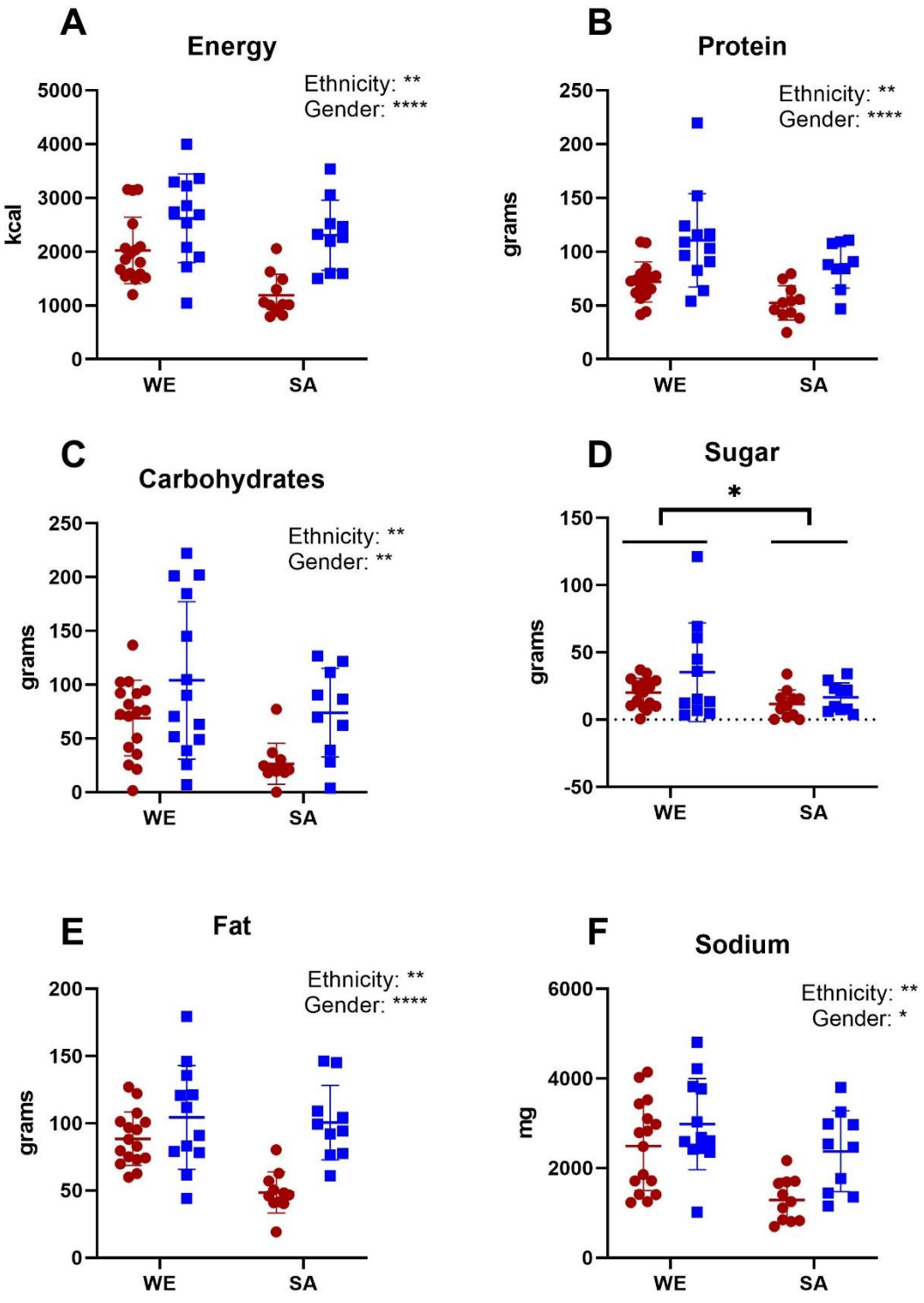

Figure 6.5: Daily intake of various nutrients as estimated from three-day food diary for groups of White European (WE) and South Asian (SA) women (red) and men (blue). *Main effects of gender and/or ethnicity are presented from 2-way ANOVA (*: p<0.05, ** p<0.01, ****: p<0.0001).*

6.3.3.4 Flavonoid intake

6.3.3.4.1 Habitual flavonoid intake: Food Frequency Questionnaire

In terms of flavonoid intake (Table 6.6 and Figure 6.6A), there was no main effect of ethnicity on consumption of any sub-group. Men consumed significantly more flavanones than women [F(1,64)=5.38, p=0.0236], and there was an ethnicity x gender interaction effect on flavanone consumption [F(1,64)=8.42, p=0.0051], with post-hoc analysis showing that SA men consumed significantly more than WE men (p=0.0220) and SA women (p=0.0026).

Table 6.6: Habitual flavonoid intake (by subclass and total) estimated from the Food Frequency Questionnaire for groups of White European (WE) and South Asian (SA) men (M) and women (F) *(mean±SD) , alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: P<0.001, ****: p<0.0001).*

		White European (WE)	**South Asian (SA)**		***Main Effects***	
		n=35, M:17, F:18	n=36, M:18, F:18	*Ethnicity*	*Gender*	*Ethnicity x gender*
Total flavonoids (mg)	**Overall**	**269±172**	**284±164**	*0.738*	*0.239*	*0.550*
	F	307±189	297±213			
	M	235±152	273±104			
Anthocyanins (mg)	**Overall**	**12.1±5.85**	**13.1±7.71**	*0.590*	*0.0976*	*0.152*
	F	11.9±6.13	10.5±7.05			
	M	12.3±5.71	15.5±7.69			
Flavanols (mg)	**Overall**	**219±170**	**228±156**	*0.790*	*0.117*	*0.652*
	F	259±175	251±205			
	M	177±159	206±88.9			
Flavanones (mg)	**Overall**	**13.5±13.0**	**17.2±12.9**	*0.204*	*0.0236 * *	*0.0051 ** *
	F	14.4±14.4	9.67±7.87			
	M	12.7±13.6	24.8±12.7			
Flavones (mg)	**Overall**	**3.17±2.59**	**2.48±2.03**	*0.213*	*0.959*	*0.133*
	F	2.75±2.31	2.90±2.35			
	M	3.58±2.85	2.02±1.53			
Flavonols (mg)	**Overall**	**18.4±7.06**	**19.3±7.18**	*0.638*	*0.591*	*0.562*
	F	18.5±8.63	18.3±7.93			
	M	18.4±5.35	20.2±6.49			

6.3.3.4.2 Current Flavonoid Intake: 3-Day Food Diary

Table 6.7 and Figure 6.6B show daily flavonoid intake estimated from the three-day food diary. WEs consumed more flavones [F(1,44)=4.83, p=0.0334] and flavonols [F(1,46)=16.5, p=0.0002] than SAs. Furthermore, WEs tended to have higher total flavonoid consumption than SAs [F(1,45)=3.60, p=0.0644], as well in the flavanol subgroup [F(1,46)=3.19, p=0.0807], though these were not statistically significant. The only significant gender difference was for anthocyanin intake, for which women consumed significantly more than men [F(1,44)=4.40, p=0.0417].

***Table 6.7: Estimated daily flavonoid intake (by subclass and total) estimated from 3-day food diary for groups of White European (WE) and South Asian (SA) men (M) and** women (F) (mean±SD), alongside p values for main effect of ethnicity, gender and ethnicity x gender interaction (from 2-way ANOVA, *: p<0.05, ** p<0.01, ***: P<0.001, ****: p<0.0001, #: p<0.1.*

		White European (WE)	**South Asian (SA)**	***Main Effects***		
		n=29, M:12, F:17	n=21, M:10, F:12	*Ethnicity*	*Gender*	*Ethnicity x gender*
Total flavonoids (mg)	**Overall**	**179±218**	**75.7±111**	*0.0644 #*	*0.319*	*0.939*
	F	204±261	99.2±147			
	M	146±148	49.9±46.4			
Anthocyanins (mg)	**Overall**	**11.3±11.7**	**8.15±9.21**	*0.321*	*0.0417 **	*0.866*
	F	13.7±13.7	11.1±11.2			
	M	7.78±6.87	4.18±2.76			
Flavanols (mg)	**Overall**	**161±244**	**50.8±109**	*0.0807 #*	*0.213*	*0.682*
	F	201±292	74.1±145			
	M	104±148	25.2±40.1			
Flavanones (mg)	**Overall**	**5.34±6.46**	**3.29±6.64**	*0.378*	*0.474*	*0.101*
	F	6.06±6.94	1.08±3.41			
	M	4.22±5.78	5.74±8.56			
Flavones (mg)	**Overall**	**0.585±0.381**	**0.294±0.480**	*0.0334 **	*0.808*	*0.599*
	F	0.625±0.372	0.276±0.222			
	M	0.525±0.406	0.312±0.660			
Flavonols (mg)	**Overall**	**24.0±16.4**	**7.82±8.17**	*0.0002 ***	*0.204*	*0.848*
	F	25.8±20.2	10.6±9.78			
	M	21.5±8.65	4.78±4.72			

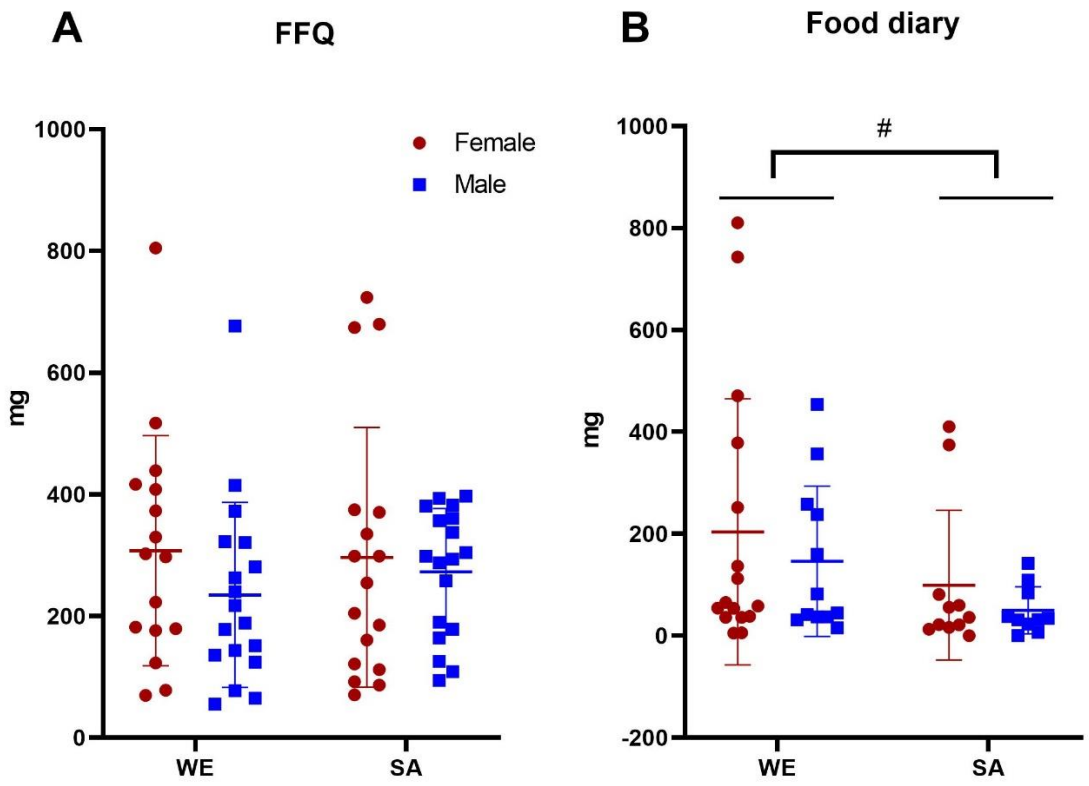

Figure 6.6: Total daily flavonoid intake, as estimated from (A) food frequency questionnaire (FFQ) and (B) three-day food diary for groups of White European (WE) and South Asian (SA) women (red) and men (blue). *Ethnic difference presented from 2-way ANOVA (p=0.0644) for food diary*

6.3.4 Correlation between FFQ and 3-Day Food Diary

Pearson's correlation coefficients were computed to assess the relationship between nutrient intakes estimated from the FFQ and food-diary. There was a significant positive correlation between FFQ and food-diary responses across the entire population [n=548, r=0.629 (CI: 0.575 to 0.677), p<0.0001], as well as within WE [n=312, r=0.682 (CI: 0.618 to 0.737), p<0.0001] and SA groups [n=236, r=0.550, (CI: 0.454 to 0.633), p<0.0001].

6.4 Discussion

The findings of the present study support the hypothesis that lifestyle factors in young adulthood might contribute to the increased prevalence of CVD within the SA population. Parental hypertension and CVD were significantly more common amongst SAs than WEs. Young SAs were also less physically active and had higher sitting times. Whilst differences in the habitual diets of SAs and WEs (as assessed by FFQ) were limited, the food-diary data highlighted ethnic differences in consumption of various nutrients. SAs were found to have lower consumption both of cardioprotective nutrients (such as fibre, folate, vitamin C and flavonoids) but also potentially harmful nutrients (such as alcohol, fat, sugar and sodium). This suggests that their increased risk of CVD might be associated with lower consumption of protective nutrients and foods, rather than increased intake of unhealthy nutrients and foods.

There were small statistically significant differences in height and BMI, but not weight, between the ethnic groups; this appears to be largely driven by differences within women, though both are very similar to the average BMI for 18-24 year olds in England of 24.7kg/m^2 for females and 24.5kg/m^2 for males (Statistica). The higher prevalence of parental hypertension and CVD amongst the SAs is in line with numerous previous studies demonstrating the commonality of such conditions within the SA population (Whitty et al., 1999, Tillin et al., 2012, Williams et al., 1993). The similarity in proportion of students within each ethnic group also suggests that ethnic differences in lifestyle choices are not attributable to student versus employed lifestyles. Taken together, these observations suggest that the group sampled is representative of typical young WE and SAs in the UK.

6.4.1 Physical Activity and Sitting Time

The observed ethnic differences in PA are supported by previous findings from self-reporting questionnaires that SAs are less physically active than WEs in the UK (Hayes et al., 2002, Williams et al., 2011). Lower MET scores for SAs across all intensities are also demonstrated by Afaq et al using more objective accelerometer-based data rather than IPAQ to assess activity levels (Afaq et al., 2019). The mean reported sitting time for SAs in this study was 469±19.4 mins per day, which corresponds with other reported values measured by self-reported questionnaires (e.g; 416 mins per day (Dey et al., 2021)) and accelerometers (482-587mins per day (Mahmood et al., 2020)). Similarly, the observed sitting time for WEs was 387mins per day, which corresponds closely to published data in students in Europe (397 mins per day, (Matusiak-Wieczorek et al., 2020)).

Both WEs and SAs are comfortably over the American Heart Association Guidelines of 600METmin/week to confer some protection against CVD (Tian and Meng, 2019). Despite this, there is also evidence of a dose-response relationship with further benefits to CV-health obtained with higher levels of PA and it has been proposed that each additional MET-hour of activity per week corresponds to a 1% reduction in CVD-risk (Kraus et al., 2019, Chomistek et al., 2013, Lear et al., 2017). In the context of the present study, that would translate into a 46% reduction in CVD-risk in WEs versus SAs. In terms of sitting time, various studies have observed differences in CVD incidence, mortality and endothelial function linked to sedentary times ranging from four to ten hours per day (Daniele et al., 2022, Ekelund et al., 2019, Zhao et al., 2020). Indeed, some meta-analyses associate increasing sedentary time with rising CVD-risk, particularly above six to seven hours sedentary time per day (Pandey et al., 2016, Patterson et al., 2018).

Importantly, the WE group in the current study is very close to this threshold (approx. 6.5 h sitting/day), whilst SAs exceed it (approx. 7.8 h sitting/day), suggesting that sedentary behaviour is most likely contributing to increased CVD risk in both populations but to a greater extent in SAs.

Overall, our findings align with existing evidence in adult populations (Williams et al., 2011, Afaq et al., 2019), as well as in children (Duncan et al., 2008, Duncan et al., 2012) that SAs are less physically active than WEs, suggesting that a lifetime of inactivity is likely to exaggerate their risk of CVD (Chomistek et al., 2013, Li and Siegrist, 2012, Henschel et al., 2020). Importantly, this strongly suggests that young SAs may benefit from becoming more physically active in order to improve their future CV health. However, SA culture does not traditionally encourage exercise from childhood and it is proposed that a lack of understanding amongst SAs on the health benefits of PA contributes to their low activity levels (Jepson et al., 2012). Furthermore, there are documented barriers to activity encountered by SAs, which include worries relating to personal safety, physical modesty, lack of confidence, family commitments, and lack of time due to long working hours (Cross-Bardell et al., 2015).

6.4.2 Habitual and Current Dietary Intake

Firstly, WEs were found to consume more vegetables and salad, and tended to consume more fruit, than SAs. This is supported by evidence from Khaw et al, who showed in an older population that SAs had lower plasma vitamin C concentrations than WEs, indicating lower intake of fruit and vegetables (Khaw et al., 2008). Importantly they also inferred from plasma vitamin C levels that fewer SAs than WEs met the recommended five daily portions of fruit and vegetables (>50umol/l vitamin C). This aligns with comparisons between SAs and New Zealanders across a range of age-groups, with a consistently lower proportion of SAs meeting

intake guidelines (Parackal et al., 2015). Global comparisons of diet have also demonstrated low fruit and vegetable intake in South Asian countries, with many not meeting recommended intakes (Jayawardena et al., 2020, Miller et al., 2017). Indeed, it is widely evident that Western populations also fall short of the recommended fruit and vegetable guidelines (Miller et al., 2017, Kalmpourtzidou et al., 2020, Parackal et al., 2015). In the present study if each 'time per week' reported in the FFQ is considered one portion, then both WEs (2.05±0.574) and SAs (1.68±0.685) consumed far less than the recommended 5 daily portions. However, this assumption may lead to underestimation of intake as each reported 'time per week' is likely to represent more than one portion, particularly for salad items. As such, it is difficult to draw comparisons between the findings of the present study and recommended intakes expressed as portions or weights due to the actual amounts of each food being unknown.

Generally, habitual nutrient intake as assessed by the FFQ, shows no ethnic differences in diet, except for WEs consuming more sodium and SAs obtaining a higher proportion of their total energy from carbohydrates. Higher carbohydrates intake amongst SAs has also been observed by another FFQ-based study, though in an older population (Pomerleau et al., 1998a). This study also showed lower intakes of fat and protein in Indians than Canadians, though these findings was not evident in the present study (Pomerleau et al., 1998a, Pomerleau et al., 1998b). Currently, evidence directly comparing habitual diet between WEs and SAs using FFQs is very limited and cannot be feasibly paralleled with this study due to variations in age range and location of populations sampled (Ghai et al., 2012). The use of FFQs to compare dietary intake between ethnic groups is also limited by the fact that FFQs, such as that used in the present study, are typically designed for Western diet and therefore may not accurately capture the diversity of the SA diet (Cade et al., 2002, Teufel, 1997). As such, the food diary was also used as an alternative tool for assessing dietary intake within this population.

Current dietary intake, as assessed by the food diary, highlighted more robust ethnic differences in consumption of energy, protein, carbohydrates, sugar, fat, saturated fat, fibre, sodium, vitamin C and folate, with WEs having higher consumption of all of these nutrients compared to SAs. Previous literature from food diaries ranging from four- to seven- days duration supports ethnic differences in nutrient intake (Sachan DS, 1999, Anderson et al., 2005), and several 24hr dietary recall studies also highlight differences in nutrient consumption between WE and SA adults (Parackal et al., 2015) and children (Stone et al., 2007). In support out our findings, Sachan et al showed that WEs consumed more calories, fat, saturated fat and sugar than SAs, though they noted no difference in carbohydrate, protein or vitamin C between ethnic groups (Sachan DS, 1999).

Importantly, some of the nutrients for which ethnic differences were observed have previously been suggested to be implicated with CVD risk (Mozaffarian, 2016). Firstly, lower sodium intake in SAs versus WEs, as observed in both the FFQ and food diary, is also supported by existing evidence (Anderson et al., 2005). The difference between WE and SA sodium intake in the present study is approximately 1g per day, a level thought to confer up to a 6% increase in CVD risk with increasing sodium consumption (Wang et al., 2020). Indeed, according to the present study only SA women consume less than the guideline maximum sodium intake of 2000mg per day (Wang et al., 2020), suggesting that in WEs and SA men sodium intake may elevate their CVD risk. Nonetheless, all groups are below the mean reported global sodium intake of 3.95g per day (Powles et al., 2013), though self-reported studies using dietary recall are thought to underestimate sodium intake, hence actual levels may be higher (O'Donnell et al., 2020).

Fibre is another dietary component implicated with CVD risk, and in the present study intake levels from both the FFQ and food diary are similar to those previously reported in Americans

[~15g (Soliman, 2019)], but both WE and SA men and women fall short of their recommended daily intakes of 38g for men and 25g for women (Soliman, 2019). This suggests that both WEs and SAs would benefit from increasing their fibre intake; every group consumes ~10g per day, or more, less than their recommended intake, an increment which has been shown to confer a 9% reduction in CV-mortality (Kim and Je, 2016). In particular, SA males consume an average of 17.4g per day which is ~20g below the recommended male intake of 38g, hence they would gain the greatest benefit from increasing fibre intake.

Evidence for other nutrients is mixed, with no clear recommendations on cardioprotective intake levels. For example, there is evidence to suggest a dose-response relationship between vitamin C intake and CVD-risk, with greater vitamin C intake thought to reduce CVD risk (Xu et al., 2022), though other studies suggest no significant association (Ashor et al., 2019). Furthermore, reducing saturated fat intake has been shown to prevent CV events in some cases (Hooper et al., 2020), but with no clear cardioprotective benefit according to others (Astrup et al., 2020, Valk et al., 2022). Evidence for carbohydrates is also variable, partly due to the heterogeneity of effects associated with different dietary sources such as total sugar, added sugar and starch (Huang et al., 2023, Dehghan et al., 2017, Ho et al., 2020)

Overall, the focus of the present study on a younger population within a narrower age range enables more reliable ethnic comparisons to be drawn without confounding effects of changing diet with age, such as is the case in many other studies. This is particularly relevant for SAs as generational differences and amount of time spent in Western countries has been shown to influence their dietary practices (Parackal et al., 2015). This may explain why the present study found more specific ethnic differences in carbohydrate, protein, fibre, vitamin C and folate which have not previously been documented. The present study showed that SAs consume less

quantities of nutrients that are shown to be beneficial towards CV health (for example fibre, vitamin C, folate) but they also consume less of nutrients that may be detrimental (such as saturated fat, sugar, alcohol and sodium).There is evidence to suggest that improving intake of cardioprotective nutrients is more beneficial towards CVD risk than reducing intake of harmful foods (GBD, 2019, Miller et al., 2022), hence the potential contribution of diet to elevated CVD-risk in SAs is likely due to their low consumption of protective nutrients.

6.4.3 Flavonoid intake

A novel aspect of the current study was to draw ethnic comparisons between the intake of flavonoids, which have been shown to protect against CVD, by reducing blood pressure and improving endothelial function (Wang et al., 2014b, Grassi et al., 2013). In both ethnic groups, total flavonoid intake assessed by both the FFQ and food diary is lower than the previously estimated global consumption of 400 mg per day (Parmenter et al., 2020), though mean global intake values are thought to vary between 150-600mg/day (Kong et al., 2023).

Total flavonoid intake estimated from the food diary in the present study was ~179mg/day and ~76mg/day for WEs and SAs respectively; the ~100mg difference between these is thought to confer a 7% reduction in CVD risk (Micek et al., 2021) and 4% reduction in CV-mortality with increasing flavonoid intake (Grosso et al., 2017). There is mounting evidence from epidemiological studies of a dose-response relationship between flavonoid intake and CVD, which is proposed to plateau around 250-500 mg (McCullough et al., 2012, Bondonno et al., 2019). Both WE and SA consumption is below this level, suggesting that both groups would benefit from increasing their daily flavonoid intake. It has also been proposed that flavonoid-associated CVD benefits may be greater in populations with higher CVD-risk (Bondonno et al.,

2019, Mink et al., 2007), therefore increasing flavonoid intake may be particularly beneficial for SAs.

6.4.4 Gender differences: Physical Activity and Diet

Although the current study did not aim to draw comparisons between men and women, the populations sampled also allowed for gender comparisons to be made. A greater proportion of males were smokers within both WE and SA populations, aligning with previous evidence (Wang et al., 2008). There was no difference in physical activity between men and women other than at vigorous intensity, this is in contrast with other studies which include wider and older range groups, suggesting that men are more physically active than women (Dagmar et al., 2011, Rosenfeld, 2017). Men consumed more alcohol than women, as has been previously established (Erol and Karpyak, 2015). Males also consumed more weekly portions of meat, as is widely documented (Rosenfeld and Tomiyama, 2021, Ritzel and Mann, 2021). Across both habitual diet and food diary, men had higher intake than women for many nutrients including carbohydrates, fat, sugar, protein and sodium; this is supported by existing literature, with physiological differences contributing to gender differences in energy requirement (Bennett et al., 2018). Indeed, our findings of consistent gender differences in intake of many nutrients across both the FFQ and food-diary highlights the relevance of both methods for assessing diet, particularly as these differences have been previously described (Bennett et al., 2018).

6.4.5 Comparison between methods of assessing dietary intake

The significant correlation between FFQ and food diary nutrient intake also supports the credibility of both methods and the mean Pearson's r coefficient of 0.629 for all nutrients was above the reported value of 0.4 which confers validity of techniques used (Al-Shaar et al., 2021).

However, it must be noted that the FFQ- food diary correlation coefficient was higher amongst WEs than SAs, and indeed the ethnic differences were predominantly observed in the food diary rather than FFQ responses. This poses the question in relation to the suitability of the FFQ for assessing SA diet. Furthermore, whilst the FFQ represents habitual diet across a year-long period, the food diary only represents diet in a very limited time point which may not be representative of their year-long dietary habits and hence a perfect correlation cannot be expected (LeCroy and Stevens, 2017).

Both FFQ and food diaries provide unique outlooks on dietary analysis, hence their use in combination within this study allows for a broad understanding of ethnic differences in diet, despite limitations of each. Firstly, as FFQs aim to assess diet across long periods of time, they require retrospective recall of dietary habits, for which interpretations may differ between respondents (Al-Shaar et al., 2021). Furthermore, the FFQ is limited by a closed list of food items; though the list is extensive, and an opportunity is given for participants to note additional foods they consume regularly, this way of assessing diet may not accurately capture items regularly consumed as part of an SA diet, as previously indicated (Cade et al., 2002, Teufel, 1997). Comparatively, there is no restriction on foods incorporated in the food-diary and participants are asked to input exactly what they have consumed, therefore it may be more valid for use across different cultures (LeCroy and Stevens, 2017). Food diaries are however limited by the level of detail in responses provided, and require respondents to accurately measure out quantities and portions used in order to gain a suitable insight into diet, therefore to gain the most valid responses participants should be provided with measuring equipment (Al-Shaar et al., 2021).

The estimation of flavonoid intake by both dietary assessment tools is limited by the reliability of the food composition database upon which it is based, leading to large variability in intake estimates due to assumptions being made where the identical food option was not included (Probst et al., 2018). This is particularly relevant for the FFQ, in which questions incorporate groups of food items and estimates of average weight, hence within some categories there will be a wide range of flavonoid content. For example, the EPIC-Norfolk FFQ used in the present study simply asks about 'wine' intake, despite considerable differences between the flavonoid composition of red and white wine (Kuhnle, 2018). Furthermore, the food composition database used for analysis of both assessment tools is not specific to the dietary habits of SAs, which is likely to exacerbate the challenges of accurately assessing their flavonoid intake (Probst et al., 2018). This highlights the need for FFQ and flavonoid databases better tailored to the SA diet, as well as a flavonoid-specific FFQ, to enable more accurate quantification of flavonoid intake in the present study.

6.4.6 Limitations

Firstly, all dietary measures are self-reported, which may result in mis-estimation of portions and nutrient intake (Aaby and Siddique, 2021). Although the food dairy asked participants to provide specific quantities, scales were not provided and therefore measures were reliant on participants providing their own equipment and the accuracy of their measurements cannot be guaranteed (Shim et al., 2014). Providing measurement equipment, or use of interviews and photographic monitoring may have enabled more accurate dietary assessment. Despite this, the present study did capture the previously documented gender differences, implying that techniques used sufficiently highlighted variability between populations even if the accuracy of values could be improved.

Furthermore, PA measures were also self-reported. Studies have highlighted that use of accelerometers to monitor activity levels can lead to more accurate representation of physical activity, as self-reported measures such as IPAQ tended to over-estimate active minutes and under-estimate sitting time (Colley et al., 2018, Dey et al., 2021). It has even been suggested that differences in physical activity between WEs and SAs may be less pronounced when measured objectively using accelerometers compared to self-reported (Yates et al., 2015). As such, the use of more objective measures such as accelerometers would benefit future studies.

Additionally, the timeframe during which the study was conducted spans the COVID-19 pandemic. This was shown to have altered diet, both negatively and positively, in a variety of populations (Bennett et al., 2021). There is also evidence of changes in physical activity levels in both adults and children during the pandemic (Mattioli et al., 2020, Rossi et al., 2021). The impact of the pandemic on diet and physical activity patterns means that responses may be inconsistent depending on when they were conducted and makes it difficult to answer questions relating to year-long diet which was likely to be changing in and out of lockdown. This may impact interpretation of absolute values, however, given that all participants were UK residents and subject to the same restrictions, any effects of the pandemic would be expected to affect both groups to the same extent, hence ethnic differences would likely remain consistent.

6.4.7 Conclusions

Overall, the findings of the present study suggest that the lifestyle choices in young healthy SAs, particularly in regard to reduced PA, increased sitting time and reduced intake of healthy foods and nutrients (such as fruits and vegetables, fibre, folate, flavonoids) may contribute to the elevated CVD-risk in this population. Key target areas for both WEs, and to a greater extent SAs, to reduce CVD risk include reducing daily sitting time by 1-2hours and consuming 2-3

additional portions of fruit and vegetables per day, particularly those high in fibre (such as bananas, avocados and leafy greens) and flavonoids (such as berries, apples and onions).

SA culture is strongly based around family, therefore it can be difficult to implement alterations to individual diet when large groups are eating together, and to promote physical activity when priorities are caring for others and spending time together. However, generational-differences in SA activity and dietary patterns suggest that young populations who have been brought up in Western countries are more impressionable with Western lifestyle choices and may therefore be more open to implementing healthy lifestyle changes (Bhatnagar et al., 2015, Smith et al., 2012).

Despite existing barriers to physical activity amongst SA, simple measures such as educating the population on the health benefits of an active lifestyle may help to increase participation. In addition, implementing more women-only facilities, as well as encouraging group activities and practical options such as walking would help to broaden accessibility and participation (Jepson et al., 2012, Cross-Bardell et al., 2015).

Chapter 7:

General Discussion

The focus of this project was on investigating the effect of CFs on microvascular vasodilator responses. The study conducted in young, healthy women showed that an acute CF intervention did not significantly improve forearm RH or EH, but that forearm vascular responses to mental stress could be modified by CFs, with this effect dependent upon whether individuals showed vasodilator or vasoconstrictor responses to mental stress without CFs (Chapter 5). This adds to the body of literature looking at the impact of CFs on the microvasculature, as reviewed in Chapter 3, and highlights that the beneficial effects previously shown in men might not translate to women. It also suggests that other factors must also be considered when evaluating the modulation of microvascular responses by CFs.

The project also aimed to compare vasodilator responses between young WE and SA women, and showed for the first time that the magnitude of both RH and EH was attenuated in young SA, compared to WE women (Chapters 4&5), whilst forearm vascular responses to mental stress did not differ between ethnic groups. It was proposed that the ethnic differences in RH and EH may be attributed, at least partly, to differences in diet and lifestyle between WEs and SAs. In agreement with this, a larger questionnaire-based study of young men and women presented in Chapter 6 highlighted that SAs were less physically active than WEs, and that SAs typically consumed less of foods and nutrients considered to be cardioprotective, including flavonoids. However, an acute CF intervention did not seem to be enough to improve microvascular responses in women (even SA women with impaired endothelial function), raising some important questions regarding individuals who may benefit from such interventions. These are discussed below.

7.1 The use of NIRS for assessing microvascular function

Before considering the observations made in the project regarding ethnic differences and the effects of CFs, it is important to discuss some of the methodological issues. Chapter 3 highlighted the heterogeneity of techniques and microvascular beds used in published studies assessing the effects of CF interventions. There are advantages and disadvantages to each technique, and they each provide slightly different insights into the function of the microvasculature. Thus, the experimental study presented in Chapter 4 compared RH measured with the gold standard VOP, and NIRS, in the forearm, hypothesising that NIRS-derived ΔtotalHb provides a useful index of FBF, which can be measured continuously, in contrast to the >5s intervals necessitated by the VOP technique. The strong correlation between NIRS-derived ΔtotalHb and FBF measured by VOP at the same time-points supported this hypothesis. Taken together with existing evidence of correlations between totalHb measured at intervals with NIRS (by assessing rate of change in totalHb following venous occlusion) and FBF measured with VOP (Harel et al., 2008), the present results reveal a new potential of NIRS as a tool for continuously monitoring blood flow. Correlations have also been reported between continuously recorded totalHb and brachial artery blood flow monitored with Doppler ultrasound (Bopp et al., 2014).

To consolidate the proposal that NIRS-derived ΔtotalHb provides a useful index of FBF these findings should be extrapolated beyond RH as a stimulus, by comparing ΔtotalHb and FBF, for example during EH or mental stress. The continuous nature of NIRS measures is of particular benefit in conditions such as exercise, since it allows changes to be observed *during* contractions, rather than being limited to after cessation of exercise in order to obtain measures, as is the case with VOP (Joyner et al., 2001, Junejo et al., 2019). Moreover, as well as an index of blood flow,

NIRS can also measure tissue SO_2, changes in which can be used to determine tissue oxygen consumption (Boushel et al., 2001, Rogers et al., 2023, Barstow, 2019). This provided the basis for some novel comparisons made between microvascular vasodilator responses in WEs and SAs, as discussed below.

The prognostic value of microvascular behaviour is important, with studies linking impaired microvascular vasodilator responses with future CVD and CAD risk (Anderson et al., 2011). SAs have an elevated CVD risk, in particular SA women being at risk of hypertension, type-2 diabetes, and early CAD (Ahmed and El-Menyar, 2015, Mishra and Monica, 2019). Furthermore, CAD in women is associated with microvascular endothelial dysfunction, rather than obstruction of the epicardial coronary arteries which occurs in men (Reis et al., 2001). Thus, focussing this project on microvascular function in SA women in comparison with WE women seemed pertinent.

7.2 Ethnic Differences in Vasodilator Responses

Chapters 4 and 5 demonstrated by using NIRS that RH was impaired in young SA, relative to WE women, consistent with existing evidence of endothelial dysfunction in young SA men (Ormshaw et al., 2018, Murphy et al., 2007, Hirst and Marshall, 2018). Using the same technique, EH was shown for the first time to be similarly impaired in SA compared to WE women. However, the forearm vascular responses to mental stress did not differ between WE and SA women (Chapter 5).

The present study also showed that the rate of desaturation during the arterial occlusion required to evoke RH, was greater in young WE than SA women, indicating a greater rate of skeletal muscle oxygen consumption in the WE women (Chapters 4&5). Similar disparities have previously been reported in young versus older adults, the rate of oxygen consumption being

greater in the young adults and followed by a greater peak RH (Rosenberry et al., 2018b). However, in that study the magnitude of peak RH was dependent on the level of desaturation reached during arterial occlusion and when the magnitude of desaturation was matched in young and older adults, by using a longer occlusion duration in the older adults, the difference in peak RH was abolished (Rosenberry et al., 2018b). The present study found no difference in the level of desaturation reached during arterial occlusion between WEs and SAs. This suggests that differences observed in peak RH, and the slower reperfusion rate in SA women, were the result of blunted microvascular reactivity in SAs, and strengthens the proposal of impaired EDD responses in this group.

This is also important in relation to EH; the impaired vasodilator response in young SA relative to WE women is something which has not previously been demonstrated. WEs did have a higher MVC than SAs, and a relationship has previously been proposed between grip strength and the magnitude of EH following handgrip contraction, due to increased compressive force (Aiku and Marshall, 2019, Hunter et al., 2006). However, the level of desaturation achieved during exercise did not differ between WE and SA women. Since the release of prostaglandins, which are responsible for ~50% of EH evoked by rhythmic handgrip contractions at the 60% MVC used, is O_2-dependent (Junejo et al., 2020b), this suggests that the release of prostaglandins and their contribution to the vasodilator stimulus is probably very similar. Together with the findings for RH, this supports blunted microvascular reactivity in SAs, compared to WEs, which may contribute to their future CVD risk.

7.3 The Role of Diet and Lifestyle in Vasodilator Responses

Together with the findings of Chapter 6, which demonstrated that young SA men and women were less physically active and had diets lower in nutrients beneficial for cardiovascular health

than WEs, it seems reasonable to deduce that their impaired microvascular function may be partly attributable to dietary and lifestyle factors, which exacerbate their underlying genetic predisposition (Goyal and Sanghera, 2021). This is important since vasodilator responses are shown to be improved with exercise training (Pasqualini et al., 2010, Boutcher and Boutcher, 2005, Sindler et al., 2013), and with Vitamin C supplementation (Eskurza et al., 2004), suggesting that lifestyle adaptations may help to mitigate the risk of future CVD, particularly in SAs.

Interactions between genetic and dietary factors have also been established through 'nutrigenomics', which explores the effect of diet in modulating gene expression and how this contributes to disease development (Hesketh, 2013). By characterising common genotypes and polymorphisms within individuals, such as those involved in plasma lipid regulation, and understanding how nutrients may modulate genetic expression, for example by epigenetic modifications, there is the potential for developing personalised nutritional approaches tailored to individuals with increased risk (Lombardi et al., 2020, Peña-Romero et al., 2018). Given the prevalence of genetic polymorphisms such as in lipoprotein lipase and apoE genes amongst SAs (Jain et al., 2017, Goyal and Sanghera, 2021), exploring how these may be modulated by diet would be of interest in this population, in order to target interventions that could reduce CVD risk.

Although it is clear that the implementation of lifestyle changes may help to mitigate the risk of future CVD in SAs, major barriers to increasing physical activity and changing dietary habits must be acknowledged. As well as the barriers encountered by the wider population, such as cost, lack of time, and motivation, SAs also report difficulties implementing dietary changes due to the culture of communal eating which makes it difficult for individuals to alter their diet (Cross-

Bardell et al., 2015) and not feeling comfortable using leisure facilities (Cross-Bardell et al., 2015, Jepson et al., 2012). These issues are likely to be especially prevalentt in SA women. The importance of community amongst SAs is clear, and a key motivator for participating in physical activity amongst this group is the social element (Jepson et al., 2012, Cross-Bardell et al., 2015). Thus, providing specialised facilities for group activities and establishing role models from the SA community may help to promote participation amongst SAs. It has also been noted that SAs were less motivated by the health benefits of physical activity than other external motivators (Jepson et al., 2012), potentially due to a lack of understanding. Therefore, initiatives to increase awareness about the importance of a healthy diet and lifestyle for overall health, and CVD risk in particular, may be beneficial and could help to encourage communities to support each other in implementing beneficial lifestyle changes.

7.4 Effect of Cocoa Flavanols on Reactive and Exercise Hyperaemia

Of particular relevance to the present project, is that CFs are considered to be a dietary intervention which may contribute to improved vascular function and improved cardiovascular health. Accordingly, the systematic review of the existing literature (presented in Chapter 3) concluded that there is evidence of a benefit of CFs on vasodilator responses within the microvasculature. Despite this, our findings in Chapter 5, indicate that CFs do not improve the responses of resistance vessels or more terminal vessels of the microcirculation during RH or EH in the forearm of young SA or WE women. In fact, the heterogeneity of findings across studies included in the systematic review, together with our own findings, raises the possibility that the lack of consistency may reflect differences in the populations studied. Notably, previous studies showing beneficial effects of acute CFs in the forearm microvasculature were mainly conducted in men (Baynham et al., 2021, Heiss et al., 2015), with a few in mixed groups of men and

women (Kim and Brothers, 2020, Santos et al., 2023). Further, there is evidence of differences between men and women, in the magnitude of their vasodilator responses particularly in relation to exercise (Aiku and Marshall, 2019) and mental stress (Carter and Ray, 2009, Yang et al., 2013). This strongly suggests that the efficacy of such interventions in men may not translate to women, and this is supported by the present finding that acute administration of CFs to young women at a dose used in previous studies on men and mixed gender groups, had no significant effect on RH or EH.

7.4.1 The Role of Oestrogen in Vascular Responses

Importantly, it should be noted that vascular responses in women differ depending on the point in the menstrual cycle at which they are tested (Hashimoto et al., 1995), with FMD and forearm vasodilator responses to infusion of endothelium-dependent dilators being larger in the late follicular phase, when oestrogen levels are highest and smaller during menses when oestrogen levels are at their lowest (Adkisson et al., 2010). The present study tested the effect of CFs during the low oestrogen phase, in order to minimise the effect of fluctuations in oestrogen levels on vascular responses, since the first day of menstruation is the only day which can be used to accurately determine the stage of menstrual cycle. By contrast, it has been suggested that the menstrual cycle should not be accounted for in studies of vascular function in women in order to maintain the external validity of studies and mimic the randomness with which women experience environmental stimuli in everyday life (Stanhewicz and Wong, 2020). With hindsight, as far as the present studies are concerned, it should be acknowledged that the early follicular phase may not have been the optimal time for assessing the effect of CFs on vascular function, but that ignoring phase of the menstrual cycle would not have been appropriate either. Indeed, it seems that oestrogen levels may be of particular relevance when comparing the effect

of CF intervention, given that the proposed mechanism by which CFs act is via GPERs which activate the NOS pathway (Moreno-Ulloa et al., 2015). Further, the density of oestrogen receptors on the vascular endothelium fluctuates with circulating hormone levels, being lowest during the low-oestrogen phase and this is also reduced in post-menopausal women (Gavin et al., 2009). Thus, in the future, it would be of particular interest to compare the effect of CFs in young, healthy women at different points of the menstrual cycle, and to compare these effects with those induced in men.

In this context, it is known that young and older men also have circulating levels of oestrogen and endothelial oestrogen receptors (Cooke et al., 2017). Comparison with the effects of CFs on vascular responses in post-menopausal women would also be useful, since oestrogen depletion has been associated with endothelial dysfunction in these women (Celermajer et al., 1994b, Lieberman et al., 1994, Gilligan et al., 1994a). Indeed, CFs have been shown to enhance FMD in post-menopausal women (Marsh et al., 2017).

It seems a reasonable hypothesise that the enhanced GPER expression when oestrogen levels are higher provides a greater potential for CFs to enhance vasodilator responses due to the greater availability of receptors to which (-)-epicatechin can bind. If this is the case, it may explain why CFs had little effect in the present study in experiments done when oestrogen levels were low. Alternatively, it may be that the effects of CFs on GPERs are diminished during the high oestrogen phase of the menstrual cycle due to competition from circulating oestrogen and already larger influences on eNOS activation, and that this would mask any potential improvement with CFs. If this were the case, then it would raise the question as to whether CFs have any significant effects on EDD in premenopausal women irrespective of the oestrogen levels.

7.4.2 Future Mechanistic Studies on Cocoa Flavanols

Clearly, the issue raised above also warrants future studies testing the density of GPER receptors in men and women, in order to ascertain the point of the menstrual cycle at which these are most closely aligned. This would involve harvesting endothelial cells from forearm vasculature at different points of the menstrual cycle and using immunofluorescent staining to quantify the density of receptors, as previously described (Gavin et al., 2009). Cells could also be stained for eNOS and phosphorylated eNOS in order to test the relationship between GPER expression, activated NOS and NO production, and to determine whether this varies between men and women. Obtaining these measures alongside CF interventions at different points of the menstrual cycle would enable a more causal mechanistic relationship to be established between levels of GPER expression and the modulation of endothelial function by CFs.

Furthermore, influences of CFs on other vasodilator pathways and factors have not been explored and should be addressed in future studies. The interplay of these pathways means that enhanced NO bioavailability may also augment synthesis or actions of prostaglandins and EDHF (Engelke et al., 1996, Boushel et al., 2002). By taking blood samples at peak RH and testing the levels of the NO metabolites, nitrite and nitrate, as well as prostaglandin metabolites and K^+ it would be possible to determine whether CF intervention altered these. It would also be possible to determine how these factors contribute to peak RH, while the addition of NOS inhibitors could be used to confirm whether any differences in prostaglandins and K^+ efflux are NO-dependent, or whether CFs directly influence these vasodilator factors.

7.5 The Role of Cocoa Flavanols in Mental Stress

In regards to the effect of CFs on forearm microvascular responses to mental stress, a novel aspect was uncovered which has not previously been explored in relation to the role of CFs. Though the study aimed to determine the effect of CFs within WE and SA women, it was apparent that within both groups there were individuals showing forearm vasodilator responses, a characteristic feature of the alerting response, which is NO-dependent (Halliwill et al., 1997), and others showing vasoconstriction, attributed to increased MSNA (Carteret al., 2005; Halliwill et al., 1997), the proportion of each being similar in both ethnic groups. The proportion of WE women showing vasoconstrictor responses was greater than that previously reported in men (Ormshaw et al., 2018), though this may also relate to the point of the menstrual cycle in which they were tested, since enhanced noradrenergic vasoconstrictor responses have previously been demonstrated in the low oestrogen phase (Srinivasa and Marshall, 2011).

Thus, Chapter 5 showed that CFs modulated forearm vascular responses to mental stress in the group of vasoconstrictors, but not vasodilators, attenuating the level of vasoconstriction, possibly by helping to reveal the NO-dependent dilatation of the alerting response. Impaired vasodilation in response to mental stress has previously been shown in hypertensives, BAs and SAs, with this being attributed to reduced NO bioavailability (Khan et al., 2015, Ormshaw et al., 2018, Cardillo et al., 1998a, Cardillo et al., 1998b). If CFs did attenuate the forearm vasoconstriction in these WE and SA women by facilitating the release and action of NO then it may be that in this subgroup, CFs were able to act on the GPERs even in the low oestrogen phase of the cycle. This raises the question of whether individuals who show forearm vasoconstriction in response to mental stress also show smaller dilator responses to other stimuli such as RH and EH, and

whether these responses may also be enhanced by CFs. A larger scale study would be required to test this possibility.

Importantly, increased pressor responses have been shown to correlate with forearm vasoconstriction during stress (Ormshaw et al., 2018). Moreover, exaggerated and maintained pressor responses to environmental stressors are associated in longitudinal studies with increased CVD risk (Chida and Steptoe, 2010, Steptoe, 1986). Repeated exposure to stress in everyday life has also been shown to accelerate progression of hypertension (Malan and Malan, 2017, Cinciripini, 1986, Steptoe and Kivimäki, 2012), and is considered particularly relevant in at-risk groups (Steptoe and Kivimäki, 2013, Steptoe, 1986). Thus, the potential of dietary interventions including CFs to minimise the impact of stressors on CVD progression is important to explore. Indeed, an individual's baseline response to mental stress is likely to be as important to take into account than their gender or ethnicity. Thus, future studies should first determine an individual's baseline responses to mental stress, in order to stratify groups according to forearm vasodilation and vasoconstriction and then to formally the effects of CFs between these groups.

7.6 Practical Implications of Cocoa Flavanol Interventions

CF absorption and metabolism and the potential benefits in vascular function varies greatly between individuals (Rodriguez-Mateos et al., 2015). Thus, ascertaining subpopulations for whom interventions would be most beneficial is of interest. It seems likely that the effect of supplementary CFs on microvascular function may be linked to an individual's habitual diet, for it was shown that the effect of CFs on hippocampal memory was greatest in those with the lowest habitual flavonoid intake (Brickman et al., 2023). Hence, this may also be true of effects on vascular function and studies should include a baseline assessment of flavanol intake so that this can be considered when testing the effect of an intervention. Dietary questionnaires may be

useful for gaining some insight to this, but more reliable measures could be obtained by screening plasma and urine for common metabolites (Fong et al., 2021, Ottaviani et al., 2019). These results could then be correlated with the effect of CF interventions of vasodilator responses, in order to deduce whether these factors are related, and thus to determine individuals who may particularly benefit from CF interventions.

Previous studies have proposed in relation to ABP that the greatest ABP-reducing effect of CFs was shown in those with higher resting ABP (Ried et al., 2017, Ried et al., 2012). This, together with the idea that CFs act via increasing NO bioavailability (Heiss et al., 2003), suggests that beneficial effects may be maximised in individuals with endothelial dysfunction and reduced NO bioavailability, providing receptors are available for CF-mediated effects (see above). Incidentally, such populations may include the same individuals who have low habitual flavonoid intake as suggested above, for diets low in fruit and vegetables, which are key sources of flavonoids, are associated with increased CVD risk and endothelial dysfunction (Miller et al., 2017). In order to determine whether this is the case, studies should recruit groups of healthy individuals, and those with endothelial dysfunction, measured by impaired vasodilator responses, and obtain measures of their habitual flavanol intake, as suggested above, before testing effects of CF interventions.

When considering CFs as a potentially useful dietary intervention, it should be noted that the doses used in acute studies are highly variable, but many, including the experimental study presented in Chapter 5, use doses well above the range of 400-600mg/day that is reported to confer cardiometabolic protection (Crowe-White et al., 2022). Crucially, the translation of CFs into everyday dietary consumption is important, since in practice, the recommended dose is likely to be reached from a combination of different foods consumed throughout the day rather

than from a single dietary component. Examples of foods with the highest CF content per serving include green tea, kidney beans, apples and blackberries; but, at least two portions of any of these would still be required to obtain 500mg CFs (Ottaviani et al., 2018). Further, commercially available chocolate is not the best source of CFs because they are lost during the manufacturing process meaning that the bioavailability is very low (Ludovici et al., 2017). Interestingly, the recommended intake level is substantially higher than those reported as daily consumption levels in the majority of populations studied. Accordingly, Chapter 6 reports daily flavanol intake of only ~50-200mg in the present questionnaire-based study of young healthy adults, while the mean global flavonoid intake is thought to vary between 150-600mg/day, although the contribution of flavanols to this value is also likely to vary (Kong et al., 2023). This implies that many populations would benefit from increasing their flavanol intake. Importantly, fruit and vegetables, which are good sources of flavanols, are also high in other cardioprotective nutrients such as vitamin C and fibre.

It should also be acknowledged that additional considerations are required when testing the effect of CF interventions. Firstly, bioavailability varies depending on the source of CFs and potentially by interactions with other dietary components (Rimbach et al., 2009). Rapid absorption by the gut wall means that maximum serum concentrations peak within the first couple of hours after consumption, although active metabolic products may persist for up to 48 hours beyond consumption (Rodriguez-Mateos et al., 2014), thus prolonging the potential vascular effects. As such, chronic intervention studies may be more relevant for determining the effect of CF supplementation over a period of days or weeks than testing the effect of an acute intervention.

7.7 Concluding remarks

Considering the findings from elements of this project together, it appears that the role of CFs in modulating microvascular responses in women may be more complex than the previously reported role in men and may be dependent on the availability of GPERs on the endothelium. Furthermore, it is clear that characteristics beyond gender and ethnicity should be considered when testing the effects of CF supplementation on vascular responses. For example, the effect of CFs may be greater in individuals with a low habitual flavanol intake. The present study also highlighted differences in the effect of CFs in mental stress, with improvements in vascular responses apparent only in individuals who showed forearm vasoconstriction; this is something which should be considered in future studies testing the effects of CFs on vascular responses.

Appendices

Appendix 1: Consent Forms

School of Sport, Exercise and Rehabilitation Sciences

Institute of Clinical Sciences, College of Medical and Dental Sciences

Participant Consent Form

Study Title: The effects of intake of cocoa flavanols on cardiovascular responses to vasodilator stimuli .

Investigators: Professor Janice Marshall, Dr Catarina Rendeiro and Sophie Richardson

Participant Name & ID: _____

Participant Address: _____

Telephone Number: _____ Date of Birth: _____

1. I have read the study information sheet and have discussed the experiment with one of the above named investigators, who have explained the procedures to my satisfaction. ☐
2. I understand that I am volunteering to participate in the experiment by my choice and that I may stop and withdraw from the experiment at any time. ☐
3. I confirm that I have not been treated for any cardiovascular, metabolic, neurological or respiratory conditions in the past. ☐
4. I confirm that I do not have any food allergies to the best of my knowledge. ☐
5. I understand that the data collected during the study may be looked at by responsible individuals from the University of Birmingham where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data and understand that any information will be kept strictly confidential. ☐
6. I understand that my digital data will be stored for a minimum of 10 years in accordance with University of Birmingham policies on password protected systems accessible only to research personnel associated with this study. I agree to this. ☐
7. I understand that my questionnaire data will be stored for a minimum of 10 years in accordance with University of Birmingham policies in a locked cabinet only accessible to the research personnel associated with this study. I agree to this. ☐
8. I would like to receive a summary of the study findings YES / NO (circle your response)
9. I agree to the arrangements described in the Information Sheet as they relate to my participation. I agree to participate in this study. ☐

Name of Participant (PRINT)	Date	Signature
Name of Researcher (PRINT)	Date	

School of Sport, Exercise and Rehabilitation Sciences

Institute of Clinical Sciences, College of Medical and Dental Sciences

Participant Consent Form

Study Title: Comparing the diet and lifestyle of young South Asian and White Europeans

Investigators: Professor Janice Marshall, Dr Catarina Rendeiro and Sophie Richardson

Participant ID (date of birth): _____

	Initial each box
10. I have read the study information sheet and discussed any questions I have with one of the above-named investigators, who have answered to my satisfaction.	☐
11. I understand that I am volunteering to participate by my choice and that I may stop and withdraw at any time.	☐
12. I understand that the data collected during the study may be looked at by responsible individuals from the University of Birmingham where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data and understand that any information will be kept strictly confidential.	☐
13. I understand that my digital data will be stored for a minimum of 10 years in accordance with University of Birmingham policies on password protected systems accessible only to research personnel associated with this study. I agree to this.	☐
14. I would like to receive a summary of the study findings YES / NO (circle your response)	
15. I agree to the arrangements described in the Information Sheet as they relate to my participation. I agree to participate in this study.	☐

_____	_____	_____
Name of Participant (PRINT)	Date	Signature
_____	_____	_____
Name of Researcher (PRINT)	Date	

Appendix 2: Participant Information Sheet- Experimental Study

UNIVERSITY OF BIRMINGHAM

Participant Information Sheet

The effects of intake of cocoa flavanols on cardiovascular responses to vasodilator stimuli

Investigator: Sophie Richardson

Supervisors: Professor Janice Marshall **and** Dr Catarina Rendeiro

An invitation to take part:

Thank you for taking the time to read this leaflet. We would like to invite you to take part in this study at the University of Birmingham. Before you decide if you want to participate or not, it is important that you understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with friends or relatives, if you wish. Please ask us if there is anything that is not clear or if you would like more information.

1. **What is the purpose of the study?**

The aim of this project is to examine whether flavonoids, which are small molecules naturally present in cocoa, as well as fruits and vegetables such as berries, tea, citrus fruits, apple (typically responsible for giving these foods their strong colours), can be used to protect the human vascular system. Impaired vascular function, as determined by measuring vasodilator responses to various everyday stimuli, can be linked to elevated blood pressure (hypertension) and prevalence of cardiovascular disease. Findings from this study could have implications for effective prevention of cardiovascular events in groups with increased cardiovascular risk, such as South Asians and in particular, South Asian women.

Am I suitable to participate in this study?

You are suitable to participate in this study if you are:

- Female (White or Black African or South Asian ethnicity (with ethnic roots in India, Pakistan, Bangladesh, Sri Lanka, or Nepal)
- Healthy
- Aged 18 to 26 years old
- Do not smoke
- Do not consume more than 21 units of alcohol per week
- Have no history of cardiovascular, respiratory, metabolic, liver, inflammatory diseases
- Do not suffer from blood-clotting disorders
- Do not have allergies or intolerances to any foods
- Are not on a weight reducing dietary regime
- Are not taking any dietary supplements, including fatty acids and vitamins
- Are not taking any long-term medication or been on antibiotics for the last 3 months
- You do not have an infection at present (e.g. cold)

- Experience a regular menstrual cycle (controlled by contraceptive pill or otherwise)

2. Do I have to take part?

No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason for up to two weeks after your last laboratory visit. You should feel under no pressure to participate and if at any time you are asked questions that you are not comfortable with answering (e.g., those asked in the Lifestyle Questionnaire) you are free to not disclose this information. However, please note that not answering some questions may mean you cannot participate. Please also bear in mind that all information collected will be kept strictly confidential.

3. What will happen to me if I agree to take part?

If you agree to take part, you will be invited for an initial screening and familiarisation visit. An investigator will explain the nature of the procedures to you in detail. This process will take place in the laboratory so that you can see all the measurements and techniques that will be carried out. You are encouraged to ask questions prior to and throughout the study protocol if there is anything you do not understand or feel comfortable with. You will then be asked to sign a consent form. You will also be asked to complete a general health and lifestyle questionnaire, and Cohen's Perceived Stress Score (PSS) during this time and have your height and weight measured. This visit will last approximately 30 minutes.
If all the above is fine then your participation in the study can start and you will be booked in for your 2 visit days, which will last approximately 4 hours, 1hr of which you will be free to complete your own work whilst resting in the laboratory. We will arrange these to be within days 1-8 of your menstrual cycle, to minimise the influence of oestrogen, as levels will be lowest during this period. Hence your visits should be separated by about 4 weeks. In one of your visits you will be given a cocoa drink, which is rich in flavonoids and on the other visit a cocoa drink that does *not* contain flavonoids.

4. What will I be asked to do?

<u>Before the experimental Visits</u>

The day before the study day, you will be asked to **refrain from eating certain foods**, such as fruits, vegetables, coffee, tea, chocolate (a detailed list of what you can and cannot eat will be given to you). You will also be asked to **refrain from drinking alcohol and caffeinated drinks** the day before the study. You will be asked to **refrain from vigorous exercise** for 24 hours prior to testing. You will be asked to **fast for 12 hours before your morning visit** to the laboratory. This means that you must not eat or drink anything other than water between 8 pm on that evening and your arrival at the laboratory at 8 am the following morning (or 9 pm to 9 am etc).

<u>During Experimental Visits</u>

We will ask you to attend the laboratory on **2 occasions** in a fasted state. During each visit, at your arrival you will be weighed and asked to lay down and rest for approx. 20 min, during this time you will be asked to complete a 24 h dietary recall questionnaire in which you will have to describe everything that you ate or drank the day before. Measures of blood pressure, and forearm blood flow response to reactive hyperaemia (see below) will then be taken immediately, after which you be given a cold cocoa drink (high or low flavonoid), which you will be asked to drink within 10 minutes. You will then be asked to relax for approximately 1hr, during which time you will be free to complete your own work etc. After this we will repeat the reactive hyperaemia, as well as measuring exercise hyperaemia and response to mental stress (these tests are described below). After all the measurements are finished, you will be given coffee/ tea and a snack.

Details on the experimental techniques:

We will be using near infrared spectroscopy (NIRS) to measure blood flow and the level of oxygenation supplied by blood flow in the muscles of the forearm and in the cerebral cortex of the brain. NIRS is a completely non-invasive technique that is used worldwide for these purposes, both clinically and for research purposes. It is completely safe. It simply involves passing a near infra red light, which is at the red end of the spectrum, and has a wave length longer than the eye can normally see – it is used for example with a special camera to film animals during the night for wildlife videos. When a NIRS beam is directed by a small probe into tissue, it penetrates only a few millimetres and is reflected back to the probe by haemoglobin, the red pigment in blood. Haemoglobin that is carrying oxygen, or that has given up its oxygen to the tissue cells reflects light at different frequencies. Thus, NIRS gives a measure of both tissue oxygenation and blood flow.

NIRS probes will be placed on your forearm and on your forehead and each will be held in place by a band that is wrapped around your arm, or head respectively.

Reactive hyperaemia: This test will measure the size of the vasodilatation that can occur in the blood vessels that supply the muscles of your forearm. This test will be performed while you are lying down. A blood pressure cuff will be placed on your upper arm (just above the elbow) and will be inflated for 5 mins and then be rapidly deflated. During the cuff inflation you may feel a tingling sensation ('pins and needles') or numbness from the pressure of the inflated cuff on your arm. This will stop once the cuff is deflated. Reactive hyperaemia is the increase in muscle blood flow that occurs following deflation of the cuff

Mental stress: You will be asked to complete an 8 minute task adding single digit numbers that you will hear on a recorded CD and to say the answer out loud. We will measure tissue oxygenation and blood flow in your forearm and brain.

Exercise hyperaemia: At the beginning of the session, we will determine your maximum grip strength with your dominant hand by asking you to squeeze the handgrip dynamometer as hard as you can. Then we will ask you to perform rhythmic forearm contractions at 60% of your maximum for 3 min while we record tissue oxygenation and blood flow in your forearm and brain.

Blood pressure: We will measure your arterial blood pressure at the beginning of the session with a standard sphygmomanometer**.** We will then record blood pressure continuously throughout all the tests by means of a small cuff that is wrapped around your finger.

Electrocardiogram (ECG): We will measure your heart rate and electrical activity by putting small spot electrodes on your chest as is done routinely in medical practice.

5. What are the possible disadvantages and risks of taking part?

There are no known risks associated with taking cocoa drinks provided within this study: they are produced by a food company for human consumption. These drinks are however low in sugar and fat and have a bitter taste which may be unpleasant to some people.

Investigators will observe you carefully throughout the study and you are encouraged to notify an investigator immediately if you have any worrisome symptoms in addition to those symptoms described above.

6. What are the possible benefits of taking part?

At your request, you will find out about your blood pressure, resting heart rate and vascular function. If you are studying for a degree that requires you to commit time to research studies then you will collect "research

hours" for your participation in this study providing you complete both sessions as requested and complete the questionnaires.
Importantly, you will also become familiar with the purposes of research that is currently being performed at the university and the methodology that is used.

7. Will my taking part in this study be kept confidential?

Yes, your participation in this study will be kept confidential.

8. What will happen to the results of the research study?

The results of this study are expected to be published anonymously in a scientific journal and within a PhD thesis; however, names of participants will never be published. Any information that we obtain from this study about you, including your name, will be confidential. This information will be stored according to University regulations, and only accessible to researchers of the study. All data will be handled in accordance with DPA 2018 At the start of the study, you will be given a unique ID number, all your data will be stored and analysed using that unique ID number.

9. Can I change my mind?

If, at any point before or during the study, you wish to withdraw, you may do so. You do not need to give any reason for this, participation is voluntary.

10. What will happen if I wish to withdraw from the study?

As indicated above, you are free to withdraw from the study at any time, including following data collection, without giving a reason. If the data collected until the time of withdrawal could be used, you will specifically be asked to give your consent to having the data included in any analysis. Additionally, you can withdraw your data from the study for up to two weeks following completion of the data collection, by notifying us via email or telephone. If you withdraw, the data collected to date cannot be erased but it will not be used in any data analysis or publications unless you give permission. Finally, if you took part in this study for research hours, you will receive the amount of hours you completed.

11. Can I obtain feedback from the study?

Yes, if you wish to know the results of the study a summary of the results can be provided once the study has concluded. On the Consent Form, there is a space to indicate if you would like to receive a study summary.

12. Do I have to sign anything?

Yes, if you agree to participate we will ask you to sign a Consent Form. This is to show that you have understood what is involved and that you have read this Information Sheet. You can still withdraw at any time without having to give us an explanation.

13. Who is organising and funding the research?

The research is organised by Dr Catarina Rendeiro and Professor Janice Marshall.

14. Do you have any further questions or concerns?

If you have any further questions about the study please feel free to contact:

Sophie Richardson: [redacted]
Professor Janice Marshall: [redacted]
Dr Catarina Rendeiro: [redacted]

***Detailed ingredient composition of the cocoa powders can be provided if you wish.**

Appendix 3: Participant Information Sheet- Questionnaire Study

UNIVERSITY OF BIRMINGHAM

Participant Information Sheet

Comparing the diet and lifestyle of young South Asians and White Europeans

Thank you for taking the time to read this leaflet. We would like to invite you to take part in this study at the University of Birmingham. Before you decide if you want to participate or not, it is important that you understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with friends or relatives, if you wish. Please ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?
These questionnaires form part of a wider study investigating whether flavonoids, which are small molecules naturally present in cocoa, as well as fruits and vegetables such as berries, tea, citrus fruits, apple (typically responsible for giving these foods their strong colours), can be used to protect the human vascular system. Impaired vascular function, as determined by measuring vasodilator responses to various everyday stimuli, can be linked to elevated blood pressure (hypertension) and prevalence of cardiovascular disease. Findings from this study could have implications for effective prevention of cardiovascular events in groups with increased cardiovascular risk, such as South Asians.
Specifically, these questionnaires aim to compare the diets and lifestyles of young South Asian and White Europeans. This is important as diet and lifestyle play an important role in cardiovascular health, therefore ethic differences in these may contribute to any potential differences identified in vascular function between ethnic groups.

Am I suitable to participate in this study?
You are suitable to participate in this study if you are:

- White European or South Asian ethnicity (with ethnic roots in India, Pakistan, Bangladesh, Sri Lanka, or Nepal)
- Aged 18 to 26 years old

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason for up to two weeks after your last survey day. You should feel under no pressure to participate and if at any time you are asked questions that you are not comfortable with answering (e.g. those asked in the Lifestyle Questionnaire) you are free to not disclose this information. However, please note that not answering some questions may mean you cannot participate. Please also bear in mind that all information collected will be kept strictly confidential.

What will happen to me if I agree to take part?
If you agree to take part, you will be sent a link for the online surveys (conducted via Smart Survey), please complete these as honestly and accurately as possible.

What will I be asked to do?
The surveys you will be asked to complete are detailed below.

General health and lifestyle questionnaire; contains questions concerning your general health, ethnicity and family history
Food frequency questionnaire; this reflects your diet across the last year, we appreciate that this can be highly variable but please try to complete as accurately as possible
International Physical Activity Questionnaire (IPAQ); short survey in which you are asked to estimate your average weekly activity at varying intensities, please base your responses on an average week
3-day food diary; you will be sent a link for a survey asking to recall everything you have eaten during the last 3 days, this can be accessed at any time to log food intake so please complete in as much detail as possible

What are the possible disadvantages and risks of taking part?
There is the possibility that participants may consider some questions to be of a sensitive nature (e.g., food and exercise habits, ethnicity), yet questionnaires completed are no more demanding than questions and activities you might experience in everyday life. If you require any additional support with some of the issues linked to food intake and/or ethnicity, contact details are provided at the bottom of this information sheet.

What are the possible benefits of taking part?
This provides an opportunity to become familiar with the research that is currently being conducted at the University of Birmingham and the methodology that is used. A copy of your dietary analysis can also be made available to you if you wish so that you can better understand your intake of various nutrients.

Will my taking part in this study be kept confidential?
Yes, your participation in this study will be kept confidential.

What will happen to the results of the research study?
The results of this study are expected to be published anonymously in a scientific journal and within a PhD thesis; however, names of participants will never be published. Any information that we obtain from this study about you, including your name, will be confidential. This information will be stored according to University regulations, and only accessible to researchers of the study. All data will be handled in accordance with DPA 2018. At the start of the study, you will be given a unique ID number, all your data will be stored and analysed using that unique ID number.

Can I change my mind?
If, at any point before or during the study, you wish to withdraw, you may do so. You do not need to give any reason for this, participation is voluntary.

What will happen if I wish to withdraw from the study?
As indicated above, you are free to withdraw from the study at any time, including following data collection, without giving a reason. If the data collected until the time of withdrawal could be used, you will specifically be asked to give your consent to having the data included in any analysis. Additionally, you can withdraw your data from the study for up to two weeks following completion of the data collection, by notifying us via email. If you withdraw, the data collected to date cannot be erased but it will not be used in any data analysis or publications unless you give permission.

Can I obtain feedback from the study?
Yes, if you wish to know the results of the study a summary of the results can be provided once the study has concluded. On the Consent Form, there is a space to indicate if you would like to receive a study summary.

Do I have to sign anything?
Yes, if you agree to participate we will ask you to complete a Consent Form. This is to show that you

have understood what is involved and that you have read this Information Sheet. You can still withdraw at any time without having to give us an explanation.

Who is organising and funding the research?
The research is organised by Dr Catarina Rendeiro and Professor Janice Marshall.

Do you have any further questions or concerns?
If you have any further questions about the study please feel free to contact:

Sophie Richardson: [redacted]

Appendix 4: List of foods to avoid before experimental study

Flavonoid rich foods to avoid for 24 hours prior to each study day

Please **EXCLUDE** these foods from your diet for **24 hours** prior to the study days:

- Any kind of fruit juice (orange, apple, grapefruit, berry etc.)

- Most Fruits, in particular: orange, grapefruit, berries, apple, pear, grapes, peach, apricot, nectarine, plum, any kind of exotic fruit.
- Most Vegetables, in particular: onion, leek, peppers, tomato, broccoli, aubergine, beetroot, green beans, carrots.
- Any pre-prepared food containing the above.
- Any soya containing-products
- Black , green tea, any kind of herbal tea or fruit tea
- Any kind of wine, beer, cider or any alcoholic beverages
- All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade etc
- Olive oil
- All nuts
- Jams and preserves
- Pesto Sauce
- Dark or milk chocolate
- Cocoa/ Chocolate beverages
- Coffee (caffeinated or decaffeinated)

An indication of foods that you **CAN** eat during the pre-trial period is given below:

- Meat, poultry and fish
- Dairy products (milk, cheese, yogurt; not fruit containing yogurts)
- Bananas
- Croissants/ Pastries (not containing vegetables, fruits, fruit preserves or chocolate)
- Cabbage
- Cucumber
- Lettuce
- Potatoes, Rice and Pasta
- Sweetcorn
- Eggs and Butter

REMEMBER:

Please fast for 12 h before the study visit (approximately from 8 pm the day before). During this period you should only consume water.

Please do not exercise for 24 h before the study visit.

Please do not consume alcohol for 24 h before the study visit.

Appendix 5: General Health and Lifestyle Questionnaire

The University of Birmingham

School of Sport, Exercise and Rehabilitation Sciences

General Health, Lifestyle and Ethnicity Screening Questionnaire

Name:		Title:
Address:		
Gender : Male / Female / Other	Ethnicity: White / South Asian / Other	Date of Birth:
Weight (kg):	Height (m):	BMI:
E-mail:	Mobile: (indicate what is the best time to call)	Occupation:

Please answer the following questions to the best of your knowledge. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed study is appropriate for you. Your answers will be kept strictly confidential.

1.	When did you last see your doctor? (Put X where appropriate) In the: Last week..... Last month..... Last six months..... Year..... …More than a year.....		
2.	Have you ever been diagnosed with inflammatory diseases such as rheumatoid arthritis?	YES	NO

3.	Have you been diagnosed as suffering from heart disease, stroke or any other disease of the circulation?	YES	NO
4.	Have you ever been diagnosed with an illness that may affect the ability of your blood to clot?	YES	NO
5.	Have you been diagnosed as suffering from any respiratory disease or condition?	YES	NO
6.	Do you suffer from asthma?	YES	NO
7.	Do you suffer from thyroid disorder?	YES	NO
8.	Has your doctor ever said you have diabetes?	YES	NO
9.	Has your doctor ever said you have high blood pressure?	YES	NO
10.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
11.	Have you ever had viral hepatitis?	YES	NO
12.	Do you suffer from any other illness (e.g. mental health; liver disease)? If yes, please give details:_____ _____	YES	NO
13.	Are you currently taking any medication? If yes, specify what is and for what reasons:_____ _____	YES	NO
14.	Do you regularly take any painkillers, for example aspirin? If yes, please give details:_____ _____	YES	NO
15.	Have you had a cold or feverish illness in the last month?	YES	NO
16.	Have you been on antibiotics in the last 3 months?	YES	NO
17.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
18.	Do you smoke?	YES	NO
19.	Do you drink alcohol? If yes, approx. how many units per week do you drink? _____	YES	NO

20.	Do you have any known food allergies? Or any other types of allergies (e.g. pollen) If yes, please give details:_____	YES	NO	Do NOT know
21.	Do you take currently any form of dietary supplement e.g. fish oils, evening primrose, vitamins or minerals? If yes, please give details:_____ _____	YES	NO	
22.	When was the last time you have taken dietary supplements and for how long?_____			
23.	Are you currently on a weight reducing or other diets? If yes, please give details:_____ _____	YES	NO	
24.	How many portions of fruits and vegetables do you consume in average per day? (your best guess)			
25.	Do you drink coffee? How many cups of coffee do you consume in average per day?	YES	NO	
26.	Do you drink tea? How many cups of tea do you consume in average per day?	YES	NO	
27.	Do you exercise regularly or take part in team sports? If yes, which form of exercise, how often (times per week), and at what intensity (light, moderate, vigorous)? _____ _____	YES	NO	
28.	Have you been involved in a human study in the last 3 months? If yes, please give details:_____ _____	YES	NO	

Ethnicity

A) Were you born in the UK? **Yes/No**
B)
C) If not, please provide details of your country of birth and how long you have lived in the UK:

_____

_____

D) What ethnicity would you consider yourself? (Choose from options below and please sub-specify e.g. White- British/White-Scottish/South Asian-Pakistani, etc)

White ___; if selected specify

___ White/British/Welsh/English/Scottish/Northern Irish;

___ White/Irish

___ Any other White background

South Asian ___; if selected specify

___ Afghaniskani;

___ Bangladeshi

___ Nepalese

___ Indian

___ Pakistani

___Sri Lankan

___ Any other South Asian background

E) What ethnicity do your parents consider themselves? (Use the specifications provided in the previous question). Please state their place of birth.

Father: _____

Mother: _____

F) Are you aware of any long-term conditions either of your parents suffer from? (e.g. high blood pressure) Do they take any regular medication?

Father: _____

Mother: _____

How did you hear about the study?

_____

Would you like to be sent information about future studies? (If yes provide an email for future contact)

_____

Thank you for completing this questionnaire

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signature: .....

Date: .....

Appendix 6: Food Diary

3-day dietary record diary

- Record EVERYTHING you eat and drink on the tables provided
- Eat as you normally would – if you alter your diet, the results will be invalid
- Begin each day on a new page – if you fill up a page before the day is over, indicate that the day continues on a new page
- You must record what you eat on the three days of the week before your visit, including TWO WEEKDAYS and ONE SATURDAY OR SUNDAY
- The information obtained from completing this record will only be as accurate as the information you provide. Therefore, you must try to be as specific as possible in describing what you eat

Column 1: Time

- Record the time of day whenever you eat or drink

Column 2: Food

- Describe each food as specifically as possible. For example:
- Include all beverages consumed, such as water, alcohol, coffee, tea, etc.
- Do not say "fish," specify type (e.g., salmon, cod, tuna, etc.)
- Specify if you ate white or dark meat in poultry
- Specify the fat level of milk (e.g., skim, whole, soy, etc.)
- State the type of bread (e.g., white, whole wheat, cereals, etc.)
- Do not forget to include all condiments (e.g., butter on bread, oil in salads, salt added to vegetables)
- Fresh, frozen or canned fruit and vegetables
- Include all the components of mixed dishes (e.g., do not just say sandwich, but specify the kind of meat and bread used, as well as any condiments like mayonnaise or mustard)

Column 3: Amount

- Try to be as accurate as possible in describing amounts. Although technically this would mean weighing and measuring all the foods you eat, this level of detail is not mandatory. However, you MUST provide some indication of the quantity:
- Express weights in ounces or grams
- Express volumes in milliliters
- With some foods, you can obviously just provide a number (e.g., 1 slice of bread, 1 apple, 2 eggs, etc.)
- The following information may be helpful in estimating amounts:

- 60 g of meat = approximately the size of a deck of cards
- 1 cup = 250 mL
- 1 tablespoon = 15 mL
- 1 teaspoon = 5 mL

Column 4: Other details

- When appropriate, use this column to provide other information about the food that may be useful. For example:
- Who is the manufacturer and/or what is the brand name?
- Are the vegetables steamed or fried?
- Are the eggs scrambled with milk or poached?
- You may not need to fill out this column for every food

PLEASE USE A NEW PAGE FOR EACH DAY

Date: ________________________________
(year/month/day)

Subject Initials: ______
Randomization Number: ______

Time	Food/Drink	Amount	Other

Appendix 7: Food Diary Analysis Assumptions

Category	Type	Assumption made unless otherwise stated:
Beverages	Milk	Semi-skimmed, average
	Tea	Infused, containing semi-skimmed milk
	Coffee	Infused, containing semi-skimmed milk
	Beer	Lager
Carbohydrates	Rice	White, boiled
	Chips	Oven cooked
	Wraps	White
	Bagels	White
Meat	Sausages	Pork
Dairy	Cheese	Cheddar
	Butter	Unsalted
	Eggs	Chicken (if cooked, boiled)
Fruit and vegetables	Apple	Eaten with skin
	Spinach	Baby
	Kiwi	Golden
	Tomatoes	Cherry
	Grapes	Red
	Lettuce	Assumed iceberg
Other	Cooking oil	Vegetable
	Pesto	Green
	Jam	Raspberry
	Weetabix	Fortified

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