Vascular Effects of Isometric handgrip training (IHG): Influence of ethnicity and ageing

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A thesis presented to the Institute of Cardiovascular Science of

the University of Birmingham for the degree of Doctor of Philosophy

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March 2021

ABSTRACT

It is established that isometric handgrip (IHG) training reduces resting arterial blood pressure (ABP) in hypertensives and normotensives. However, the mechanisms are unclear. The aim of this project was to test whether IHG training with one arm improves endothelium-dependent vasodilation (EDD) including that mediated by nitric oxide (NO) and prostacyclin (PGI_2) in young White European (WE) men, and in young South Asian (SA) and older WE men who have recognised endothelial dysfunction.

IHG training was performed with the dominant hand, (four 3-minute contractions at 30% of their maximum voluntary contraction (MVC) at 5 min intervals) 4 days/week for 4 weeks. MVC was increased in the trained arm, but not the non-trained arm of all groups; resting ABP was reduced in older men only. However, IHG training increased muscle performance in the non-trained *and* trained arm of all groups during rhythmic handgrip at 60% MVC for 3 min. Further, exercise hyperaemia evoked by these contractions, and reactive hyperaemia were increased in both the trained arm *and* non-trained arm of young and older WE, but not in the non-trained arm of young SA men. In addition, IHG training augmented the increased venous efflux of NO metabolites from the non-trained arm during reactive hyperaemia in older men, but decreased it in young WE and SA men, whilst augmenting the increase in PGI_2 efflux in young SA men only. Finally, during a single bout of IHG training, limb blood flow increased progressively with repeated IHGs in contralateral arm and ipsilateral leg of WEs, and to a lesser extend in forearm of SAs. These responses were attenuated by cyclooxygenase (COX) inhibition in WEs but enhanced in SAs implicating vasodilator and vasoconstrictor COX products respectively.

These new findings indicate that just 4 weeks of IHG training improves muscle performance and endothelial dilator function in young healthy WE men and particularly in older WE men, but not in young SAs. The acute effects of IHG training on blood flow in the resting limbs are consistent with shear stress providing the stimulus for improved endothelium-dependent dilatation in young and older WEs but suggest the release of vasoconstrictor COX products may limit the beneficial effects in young SAs. The mechanisms require further investigation. However, in older WE men, IHG training may provide a useful, non-pharmacological tool for improving EDD and

reducing ABP and cardiovascular risk, but more intense or longer IHG training may be required even in young SA men to achieve such effects.

Keywords: Isometric handgrip training, hypertension, arterial blood pressure, endothelium dependent vasodilation, South Asian ethnicity, ageing.

ACKNOWLEDGMENTS

I would like to thank several people who contributed to this thesis. First, I would like to thank my parents who have supported me by all means throughout my life. I would also like to thank my supervisor Professor Janice Marshall for her guidance, support, and endless patience, especially in critical time that things were not going according to plan. I am also indebted to the Alexander S. Onassis Foundation for providing the meritorious scholarship which allowed this academic journey. Many thanks also belong to the academic and technical staff of the College of Medical and Dental Sciences. Finally, I would like to express my gratitude to all these who have contributed to the completion of the thesis and are not included here. They know who they are!

PUBLICATIONS

Papers under preparation:

- Tsitoglou KI, Marshall, JM Martin U. Comparison of the effects of Isometric handgrip (IHG) training on endothelial dilator responses in young White European (WE) and South Asian (SA) men: changes in the contribution of cyclooxygenase (COX) products.

- Tsitoglou KI, Marshall, JM Martin U. The effects of isometric handgrip (IHG) training of one forearm on reactive and exercise hyperaemia in the untrained contralateral arm: Differences between young White European (WE) and South Asian (SA) men.

- Tsitoglou KI, Marshall, JM Martin U. The effects of isometric handgrip (IHG) training of one forearm on reactive and exercise hyperaemia in the untrained contralateral arm: Differences between healthy young and old people.

Abstracts:

- Tsitoglou KI, Marshall, JM Martin U (2016). Comparison of the effects of Isometric handgrip (IHG) training on endothelial dilator responses in young White European (WE) and South Asian (SA) men: changes in the contribution of cyclooxygenase (COX) products. Presented to British Hypertension Society as a poster presentation at Annual Meeting, Dublin September 2016. Journal of Human Hypertension; (2016) 30, 633–656. **IF: 2.88**

- Tsitoglou KI, Marshall, JM Martin U (2016). The effects of isometric handgrip (IHG) training of one forearm on reactive and exercise hyperaemia in the untrained contralateral arm: Differences between young White European (WE) and South Asian (SA) men. Submitted as Oral Communication to Physiology 2016; Annual meeting of The Physiological Society, Dublin; July 2016.

- Tsitoglou K.I., Martin U, & Marshall JMM. The effects of isometric handgrip (IHG) training of one forearm on reactive and exercise hyperaemia in the untrained contralateral arm: Differences between healthy young and old people. Presented to British and Irish (BHIS) Hypertension Society as an oral presentation at Annual Meeting, Glasgow September 2017. Journal of Human Hypertension; (2017) 30, 633–656. **IF: 2.88**

- Tsitoglou K.I., Martin U, & Marshall JMM. Effects of isometric handgrip training (IHG) training on endothelium-dependent vasodilation in older (O) men: contribution of cyclooxygenase (COX) products. Presented to British and Irish (BHIS) Hypertension Society as a poster presentation at the Annual Meeting, Glasgow September 2017. Journal of Human Hypertension; (2017) 30, 633–656. **IF: 2.88**

- Tsitoglou K.I., Martin U, & Marshall JMM (2017) Mechanisms by which Isometric Handgrip training may improve endothelial dilator function in young healthy men. Presented to the American College of Sports Medicine annual meeting in Denver, Colorado, US. University of Birmingham, Medical School Overseas Travel Fund Recipient. Medicine & Science in Sports & Exercise 49:903 · May 2017.

- Tsitoglou K.I., Martin U, & Marshall JMM (2017) Investigation of mechanisms underlying the blunted endothelium-dependent vasodilation in young South Asian (SA) men relative to White European (WE) men. Presented to British Microcirculation Society and UK cell adhesion society joint meeting as poster presentation. BMS-UKCAS Travel Award recipient for poster presentation.

- Tsitoglou KI, Marshall, JM Martin U (2016), Comparison in young White European (WE) and South Asian (SA) men of endothelium-dependent cutaneous vasodilator responses: contribution of cyclooxygenase (COX) products. FASEB J 30, 952.8. Elizabeth-Dawson Award recipient for poster presentation.

- Tsitoglou KI, Marshall, JM Martin U (2016). Effects in young men of isometric handgrip training (IHG) on endothelium-dependent vasodilator responses in the contralateral arm: changes in the contribution of cyclooxygenase (COX). FASEB J 30, 1240.18. Elizabeth-Dawson Award recipient for poster presentation.

- Tsitoglou KI, Marshall, JM Martin U (2016). Effects of isometric handgrip (IHG) training of one forearm on reactive and exercise hyperaemia in the ipsilateral and contralateral arm of White European Young Men. FASEB J 30, 1240.19. Elizabeth-Dawson Award recipient for poster presentation.

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I. ABBREVIATIONS

AA – Arachidonic Acid

ABPM – Ambulatory blood pressure measurement

ACE – Angiotensin converting enzymes

ACC – American College of Cardiology

ACSM – American College of Sports Medicine

AE – Aerobic exercise

AHA – American Heart Association

ANOVA – Analysis of variance

ANS – Autonomic nervous system

AOBPM – Automated office blood pressure measurement

AOBPM – automated office blood pressure measurement

ABP –Arterial Blood pressure

BMI – Body mass index

BPM – Beats per minute

BPV – Blood pressure variability

CAD – Coronary artery disease

CBF – Calf Blood Flow

CHEP – Canadian Hypertension Education Program

CVD – Cardiovascular disease

CVC – Cutaneous Vascular conductance

CVR – Cutaneous Vascular Resistance

DASH – Dietary approaches to stop hypertension

DBP – Diastolic blood pressure

EMG – Electromyography

EDD – Endothelium Dependent Dilatation

FBF– Forearm Blood flow

FMD- Flow mediated dilatation

FVC – Forearm Vascular Conductance

FVR – Forearm Vascular Resistance

HBPM – home blood pressure measurement

HR – Heart rate

HRV – Heart rate variability

HTN – Hypertension

Hz – Hertz

IHG – Isometric handgrip

ILE – Isometric leg exercise

KG – Kilogram

MAP – Mean arterial pressure

mmHg – Millimetres of mercury

LDF – Laser Doppler Flow

MSNA – Muscle sympathetic nerve activity

MVC – Maximum voluntary contraction

OBPM – Office blood pressure measurement

PNS – Parasympathetic nervous system

PP – Pulse pressure

PU – Perfusion Units

Q – Cardiac output

RCF- Red Cell Flux

RCT – Randomized controlled trial

RE – Resistance exercise

RHG- Rhythmic Handgrip

ROS – Reactive oxygen species

RRI – R-R interval

SBP – Systolic blood pressure

SD – Standard deviation

SE-Standard Error

SNS – Sympathetic nervous system

VOP – Venous Occlusion Plethysmography

TPR – Total peripheral resistance

VO2 Max- Maximal oxygen consumption

WHR – Waist-to-hip ratio

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1. General Introduction

1.1 General Overview

The present study investigated the effects of the standard IHG training protocol on resting levels of ABP and endothelium-dependent vasodilator responses in healthy young normotensive WE and SA men and in older normotensive WE men, these groups being chosen because EDD is known to be blunted in SAs and in older individuals (Brandes et al., 2005, Herrera et al., 2010, Vanhoutte et al., 2017, Murphy et al., 2007, Ormshaw et al., 2018). In order to provide an appropriate background, the sections below deal firstly, with the factors responsible for EDD, and endothelial dysfunction, with particular focus on endothelial dysfunction in aging, HTN and SA ethnicity. The second part deals with the evidence that has led to the use IHG training as a therapy for HTN and reviews the studies performed to date on the mechanisms that may be involved.

The advances of medical science and practice in the past 50 years have shown a huge improvement in reducing the risk for cardiovascular mortality. Hypertension (HTN) is diagnosed when arterial blood pressure (ABP) is ≥140/90 (systolic/diastolic) mmHg and more recently 130/80 mmHg and is considered to require medical attention (Whelton and Carey, 2017) because this represents an established risk for the development of cardiovascular disease (CVD). HTN is one of the leading risk factors for CVD and mortality globally and is the leading factor responsible for 9.4 million deaths (Lim et al., 2012). In the UK, HTN currently affects 1 in 4 adults and worldwide is expected to rise to approximately to 1.56 billion by 2025 (Kearney et al., 2005). Ethnicity is an independent factor for HTN and South Asians (SAs) in particular with ethnic origins in the Indian sub-continent (King-Shier et al., 2019) are established as an ethnic group with a high prevalence of raised blood pressure (Gupta, 2004b). Further, in South Asians, CVD risk factors present earlier than in White Europeans, particularly when residing in western countries (Wild et al., 2007, Rana et al., 2014). In addition, cardiovascular risk rises during the aging process as a consequence of increasing systolic pressure and pulse wave velocity (PWV) - a marker for arterial stiffness, above 50 years of age (Lim and Townsend, 2009) accompanied by increases in diastolic blood pressure and peripheral vascular resistance (PVR), which is the primary indicator of essential HTN (Nilsson et al., 2014, Nowak et al., 2018).

HTN is categorized into essential or primary hypertension and secondary hypertension. Essential or primary hypertension is generally regarded a disease of adulthood with a prevalence of 30% (Gillespie and Hurvitz, 2013) and is considered the most frequent type of hypertension (95%). It is confirmed when no obvious cause can be established (Lenfant et al., 2003). Secondary hypertension is an elevated level of resting ABP that can be attributed to conditions affecting other physiological systems (i.e. kidneys or endocrine system)(Onusko, 2003). Once diagnosed, HTN is generally managed by anti-hypertensive medication, but there is increasing recognition that changes to life style including diet and exercise can be used to avoid development of HTN, to reverse early signs of HTN and to achieve more effective control of HTN when used in conjunction with medication (Brook et al., 2013a, Franco et al., 2004).

Of the various exercise regimes that might be used, isometric handgrip (IHG) training has emerged as a protocol that lowers ABP in normotensives as well as non-medicated and medicated hypertensives (Kiveloff and Huber, 1971, Buck and Donner, 1985, Wiley et al., 1992, Saito et al., 1986, Ray and Carrasco, 2000). Indeed, on the basis of substantial evidence from experimental studies and clinical trials, IHG training following a standardised protocol has been introduced as part of the ACC/AHA and Canadian hypertension education program (CHEP) guidelines, directing individuals to perform four 2-minute contractions separated by 1-minute of rest at 30-40% of MVC for 3 sessions per week for 8-10 weeks (Whelton and Carey, 2017, Leung et al., 2017).

Although IHG training is effective in reducing ABP, the mechanisms by which it might work have remained elusive. There is no evidence that IHG training reduces resting levels of sympathetic nerve activity to vasculature (Ichinose et al., 2006, Ray and Carrasco, 2000), a main contributor to resting ABP in normotension and to raised ABP in HTN (Mancia, 2014). There is evidence that endothelium-dependent dilatation (EDD) is increased by IHG training in the vasculature of the trained arm (McGowan et al., 2006B, McGowan et al., 2006A, Badrov et al., 2016), and improved EDD is a recognised way of reducing ABP in HTN (Badrov et al., 2013A, McGowan et al., 2006B, McGowan et al., 2006A). However, whether IHG training affects EDD in the non-trained limbs or systemically has received very little attention.

1.2 Endothelial function

The endothelium consists of a thin single layer of endothelial cells mainly found on the interior surface of blood vessels and lymphatic vessels and is essential for the normal functioning of the cardiovascular system. The endothelial cells present numerous functions of which the most prevalent include: a) operating as a selectively permeable barrier regulating blood-tissue exchange, b) controlling vascular tone, c) delivering anticlotting and pro-clotting factors, and d) triggering angiogenesis (new blood vessel formation) contributing to the maintenance of intravascular homeostasis (Freestone et al., 2010). Therefore, endothelial cells are actually an "organ" (~1kg in weight) (Augustin et al., 1994) which can be measured for its function and dysfunction by assaying the secreted products or by determining their effects on their vasculature.

Vascular tone is regulated by the degree of smooth muscle contraction in the tunica media. The endothelium lining the arteries and the arterioles is one of the major factors regulating smooth muscle contraction. Endothelial cells synthesize and release vasodilators including nitric oxide (NO) prostaglandins, (PGs), endothelium-dependent hyperpolarising factors (EDHFs) and vasoconstrictors including endothelin (ET).

1.1.1 Nitric Oxide (NO)

NO is synthesized by the enzyme NO synthase (eNOS) which cleaves NO from the amino acid L-arginine, see Figure 1. NO diffuses from the luminal surface of the endothelium and towards the smooth muscle cells of the vascular wall where is causes relaxation, but it also diffuses into the blood stream where it can be taken up by red blood cells or metabolised to nitrite/nitrate compounds (Gladwin et al., 2004).

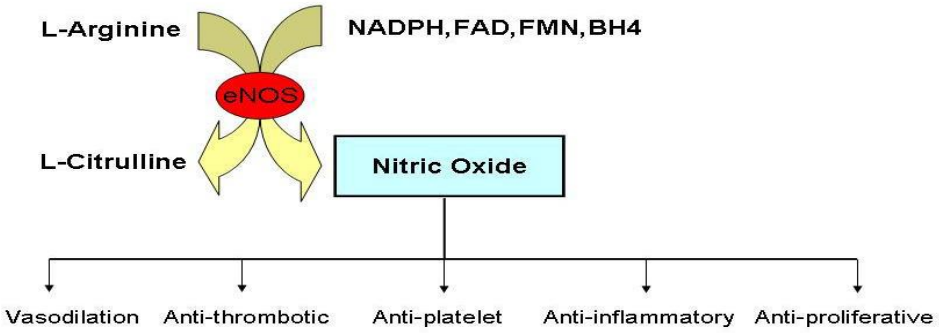


Figure 1.1- **Synthesis of NO and its actions. eNOS activity is accelerated by L-arginine and from co-factors released by caveolae (NADPH, FAD, FMN, BH4) leading to the subsequent conversion of L-arginine to L-citrulline and Nitric Oxide. L-citrulline recycling is tightly connected with L-arginine regeneration and further eNOS activation(Flam et al., 2007). NO is then involved to vasodilation and applies anti-thrombotic, anti-platelet, anti-inflammatory and anti-proliferative properties to the vasculature.**

NO signals numerous pathways generally through the activation of guanylate cyclase to produce cGMP, by decreasing Ca intracellularly and decreasing the sensitivity of actin-myosin to Ca (Lincoln et al., 2001). Thus, NO can hyperpolarize, or repolarize vascular smooth muscle cells by activating, in either a cyclic-GMP-dependent or cyclic-GMP-independent manner, potassium channels such as ATP-sensitive potassium channels (K-ATP), large conductance calcium-activated potassium channels (BKCa), inwardly rectifying potassium channels (K_{IR}) and/or voltage activated potassium channels (KV), so inducing relaxation. NO also affects other ionic channels of smooth muscle, including chloride and cationic channels and affects the membrane potential (Feletou, 2006, Feletou and Vanhoutte, 2006b). The synthesis of NO can be inhibited by L-arginine analogues such as L-arginine methyl ester (L-NAME) and L-arginine derivatives NG-nitro-L-arginine (L-NOARG). Their use provides a way of testing the functional role of NO (Pfeiffer et al., 1996).

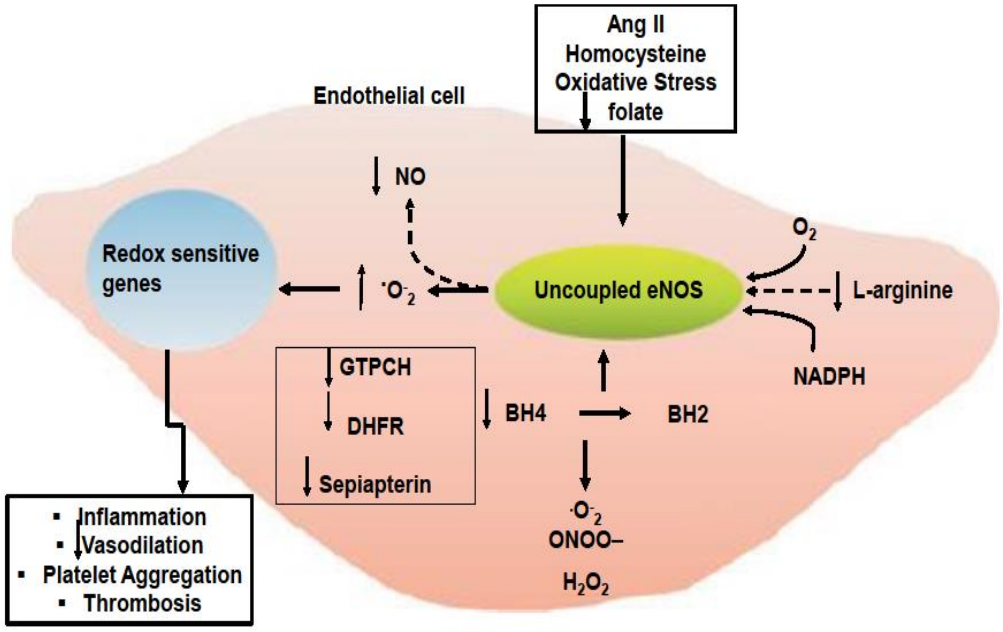


Figure 1.2 **Uncoupling of endothelial nitric oxide synthase**, because of reduced bioavailablity of tetrahydrobiopterin and ⁄ or decreased l-arginine, shifts the nitroso-redox balance favouring production of O_2 rather than nitric oxide, resulting in increased endothelial reactive oxygen species formation and activation of redox-sensitive genes that contribute to endothelial dysfunction. GTPCH, GTP-cyclohydrolase; DHFR, dihydrofolate reductase (Montezano and Touyz, 2012).

Functional roles of NO: It has generally been shown by using NOS inhibitors that in healthy blood vessels, tonic release of NO results in a continuous state of vasodilation providing shear stress is present (Vallance and Chan, 2001). *In vivo*, the effects of NOS inhibition have shown that NO exerts a tonic vasodilator influence on peripheral vasculature and therefore on resting levels of ABP, on vascular resistance in the major beds including limb muscles (Joyner and Dietz, 1997). In addition, the effects of NOS inhibitors indicate that NO contributes to greater or lesser extents to endothelium-dependent dilatation induced by agonists and to flow mediated dilation (FMD), reactive hyperaemia and exercise hyperaemia (Pyke et al., 2004, Pyke and Tschakovsky, 2007, Pyke and Tschakovsky, 2005, Walker et al., 2007).

To elaborate, NOS inhibition attenuates vasodilator responses in human forearm induced by graded doses of ACh and other known endothelium-dependent dilators (Taddei et al., 1997b, Taddei et al., 1998b, Taddei et al., 2001a, Crecelius et al., 2010) indicating these responses are partly NO-dependent.

FMD is generally evoked by occluding blood flow in the brachial artery by inflating a sphygmomanometer cuff around the forearm for 5 minutes. Release of the cuff leads to an increase in blood flow (reactive hyperaemia) in the forearm as the resistance vessels respond to the fall in intravascular pressure and ischaemia, and the increase in forearm blood flow (FBF) increases the shear stress in the brachial artery. The change in brachial artery diameter as a percentage increase from control is measured with a Doppler ultrasound probe located on the centre of the artery proximal to the site of occlusion (Raitakari and Celermajer, 2000). The change in vessel diameter induced by shear-stress is almost entirely blocked by prior NOS inhibition (Joannides et al., 1995) indicating FMD is largely attributable to NO.

Reactive hyperaemia can be assessed as just described following release of arterial occlusion, by using a Doppler ultrasonic probe to measure blood velocity in the centre of the blood stream in the brachial artery and brachial artery diameter: FBF is calculated as the product of lumen cross-sectional area and Doppler velocity (Pyke and Tschakovsky, 2007). More commonly, reactive hyperaemia is measured by using the technique of venous occlusion plethysmography (VOP) when FBF is computed from the rate of change in circumference of the forearm immediately following occlusion of the venous drainage. This technique is described in detail in Chapter 2. Judging from effects of NOS inhibition, NO makes a minor contribution to peak reactive hyperaemia in human forearm (Engelke et al., 1996, Tagawa et al., 1994, Nugent et al., 1999).

Exercise hyperaemia is the increase in blood flow that occurs during and following a period of muscle contraction. The effects of NOS inhibition indicate that NO makes a relatively minor contribution during or after short periods of muscle contraction once the effects on resting blood flow are taken into account (Wilson & Kapoor, 1993; Endo et al 1994; Joyner & Dietz 1997). However, there is evidence NO makes a small but significant contribution at higher intensities and during longer periods of contraction (Gilligan et al., 1994, Schrage et al., 2004, Katz et al., 1996, Dyke et al., 1995, Duffy et al., 1999b, Duffy et al., 1999a).

By contrast, in studies in which cutaneous blood flow in the forearm was recorded by using the laser Doppler technique, the influence of NOS inhibitors indicated that NO makes little contribution to ACh-evoked dilatation or reactive hyperaemia in cutaneous

circulation (Holowatz et al., 2005, Lorenzo and Minson, 2007, Zhao et al., 2004, Wong et al., 2003, Kellogg et al., 2003).

1.2.1 Prostaglandins (PGs):

PGs are metabolites derived from arachidonic acid (AA) and generated by cyclooxygenase (COX) (Moncada and Vane, 1978, Furchgott and Zawadzki, 1980, Moncada et al., 1991). Prostacyclin (PGI_2) is the major PG synthesised in endothelial cells (Moncada and Vane, 1978). In endothelium, the AA normally resides in the phospholipid bilayer of membranes. The AA is discharged into the cytosol either catalysed by phospholipase A_2 (PLA_2) or diacylglycerol (DAG) lipase breaking down the phospholipid. The AA is transformed into prostaglandin H_2 (PGH_2) by cyclooxygenase (COX). Thereafter, PGI_2 synthase catalyses PGH_2 into PGI_2.

In a similar manner to NO, PGI_2 release is closely related with $[Ca^{2+}]i$. AA is liberated from the cell membrane by phospholipase A_2 (PLA_2) which is activated by an increase in $[Ca^{2+}]i$. caused either by a variety of endothelial cell agonists or by shear stress (Koller et al., 1993, Bhagyalakshmi and Frangos, 1989, White and Frangos, 2007, Feletou et al., 2011).

PGI_2 stimulates IP receptor (I2 receptor is a receptor belonging to the prostaglandin (PG) group of receptors) activity on vascular smooth muscle wall and in most arteries under physiological conditions causes relaxation by increasing cyclic AMP (cAMP) levels, *see* Figure 1.3 (Morgado et al., 2012). Like cGMP, cAMP decreases $[Ca^{2+}]i$ in vascular smooth muscle cells and decreases the sensitivity of actin-myosin to Ca (Lincoln et al., 2001). Additionally, depending on the artery and/or the species, PGI_2 can also via cAMP induce hyperpolarisation, resulting in one or more types of potassium channels opening. Therefore, there is an association between PGI_2-induced relaxation and ATP-sensitive potassium channels (K-ATP), large conductance calcium-activated potassium channels (BK_{Ca}), inwardly rectifying potassium channels (KIR) and/or voltage activated potassium channels (KV) (Feletou, 2006, Feletou and Vanhoutte, 2009).

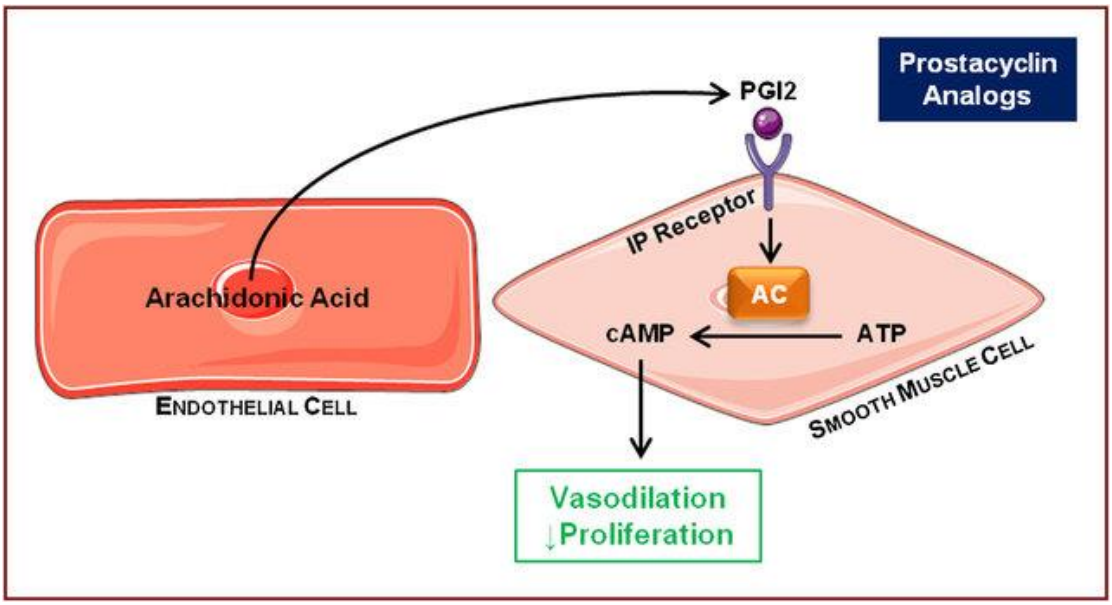


Figure 1.3 **Prostacyclin (PGI2) signalling pathway**. PGI2 is produced from arachidonic acid metabolites in endothelial cells and acts on smooth muscle cells through prostacyclin receptor (IP) mediation, which leads to an increase in cyclic adenosine monophosphate (cAMP) concentration, resulting in vasodilation and reduced proliferation. Therefore, one of the pulmonary arterial hypertension-specific therapies is the use of prostacyclin analogues, as they can mimic prostacyclin signalling in smooth muscle cells. AC: adenylyl cyclase; ATP: adenosine triphosphate (Ribeiro, 2016).

Functional Roles of PGs: COX inhibitors (ibuprofen, aspirin, celecoxib, rofecoxib etc.) in humans have been used in many studies on the forearm to investigate the contribution of PGs to endothelium-dependent dilatation. Generally such studies, involving VOP, demonstrated that COX inhibition has no effect on FBF indicating PGs do not exert a significant tonic influence on vasculature (Carlsson and Wennmalm, 1983, Carlsson et al., 1987, Kilbom and Wennmalm, 1976). However, after COX inhibition, in some studies the peak of reactive hyperaemia was attenuated and the total response of excess flow was blunted (Carlsson et al., 1987, Carlsson and Wennmalm, 1983), while in other studies there was an influence mainly on restoration of flow after the peak (Engelke et al., 1996, Crecelius et al., 2013). Thus, there is some controversy, but it seems PGs do make a significant contribution to reactive hyperaemia.

The contribution of PGs is further extended to exercise hyperaemia. The first report of PGs contribution to exercise hyperaemia was by Kilbom and Wennmalm (1976) who by using VOP to measure FBF in men and women, showed that post-contraction hyperaemia following rhythmic or isometric forearm contractions at moderate to heavy

loads was reduced by 30-50% after COX inhibition. The first attempt to determine the contribution of PGs *during* exercise was made by Wilson and Kapoor (1993). Since VOP cannot be applied reliably when muscles are contracted, FBF was measured during 4-5s breaks in 5 min periods of graded rhythmic contractions. In young men and women, COX inhibition reduced increases in FBF induced during contractions at light and medium workload by ~20% and abolished the 2- to 3-fold increase in prostaglandin E2 (PGE_2) and prostaglandin I2 (PGI_2) efflux (Wilson and Kapoor, 1993). On the contrary, Shoemaker et al. (1996), who used Doppler ultrasound recordings of brachial artery diameter and blood velocity to assess FBF in young men, showed that COX inhibition had no effect on hyperaemia evoked during rhythmic forearm contractions at 10% MVC. Therefore, they concluded PGs are not involved essentially in hyperaemia during exercise. This was similarly concluded by Mortensen et al. (2007), who recorded blood flow by thermodilution in young men performing knee extensor exercise at 20% maximum. In some contrast, Schrage et al. (2004), who used Doppler ultrasound in a group of men and women, discovered that infusion of COX inhibitor when hyperaemia induced by rhythmic forearm contractions at 10% MVC was already established, caused a short-lasting, 12% attenuation in FBF. They suggested PGs do contribute to exercise hyperaemia, but when their influence is removed, other dilator(s) compensate (Schrage et al., 2004).

The simplest explanation for these discrepancies is that PGs are released and do contribute to exercise hyperaemia evoked by medium to strenuous exercise but have little or no role in light exercise. Certainly, microdialysis samples demonstrated PGE_2 concentration in the interstitium was unchanged during light knee extensor exercise, but increased during moderate workloads (Boushel et al., 2002). Further, graded cycling exercise in young men was accompanied by graded increases in interstitial PGE_2 and PGI_2 (Karamouzis et al., 2001).

An alternative explanation (Shoemaker et al., 1996) is that PGs contribute to muscle vasodilation during recovery from exercise rather during exercise per se, and that VOP reveals this contribution even when used during breaks between rhythmic contractions (Wilson and Kapoor, 1993) because this technique basically measures “recovery flow”. However, this seems unlikely to be the full explanation for Junejo et al. (2020) demonstrated that at the peak of post-contraction hyperaemia, immediately following

isometric or rhythmic contractions at moderate intensity (60% MVC) there was substantial release of PGI_2 and PGE_2 from forearm of young and older men, which must have been formed during the contractions. COX inhibition greatly attenuated PG release and reduced post contraction hyperaemia for both types of contraction by at least 20% in both young and older men. Furthermore, in both groups, the release of PGI_2 and PGE_2 and post contraction hyperaemia were similarly attenuated by breathing 40% O_2, whereas combined 40% O_2 and COX inhibition had no greater effect. Therefore, it was suggested that the release of PGs during moderate intensity (60% MVC) isometric and rhythmic contractions is O_2 -dependent in both young and older men. Recent studies on WE and Black African (BA) men and women showed that COX inhibition also reduced peak post-exercise hyperaemia by 30% following rhythmic forearm contractions at 60% MVC in WE and BA men and WE women, but not in BA women in whom exercise hyperaemia was smaller (Aiku and Marshall, 2019).

Putting these observations on light and moderate exercise intensities together, it seems probable that PGs do contribute to hyperaemia between contractions in rhythmic exercise, as well as during post-contraction hyperaemia following contractions, providing the PG levels reached during the period of contraction are sufficiently raised (Aiku and Marshall, 2019).

COX inhibitors have also been used to investigate the contribution of PGs to endothelium dependent dilatation evoked in the skin by ACh or during reactive hyperaemia. However, the results were equivocal: COX inhibition attenuated ACh-evoked dilatation and reactive hyperaemia in the skin in some studies (Noon et al., 1998, Holowatz et al., 2005, Medow et al., 2008, Kellogg et al., 2005), but not others (Berghoff et al., 2002, Dalle-Ave et al., 2004) and even *augmented* reactive hyperaemia in one study by Medow et al. (2007).

Interactions between NO and PGs: As indicated above, the vasodilator effects of NO and PGs are mediated via elevations in cGMP and cAMP respectively (Vanhoutte and Mombouli, 1996). Interestingly, cGMP has been demonstrated to have an inhibitory effect on the degradation of cAMP (Maurice and Haslam, 1990), which may explain synergistic contributions of NO and PGs to vasodilation (De Wit, 1993). Such synergism is of particular physiological interest because it improves the sensitivity of the vasodilator mechanisms, but it also demonstrates that even a small reduction in

cGMP by inhibition of NOS could have a large effect on the remaining dilatation, whether that was originally mediated by cGMP, cAMP or both. Further, concurrent inhibition of NO and prostanoid formation could reduce vascular smooth muscle cell levels of cAMP and cGMP to such an extent that inhibiting the synthesis of further vasodilator mechanisms coupled to these cyclic nucleotides may not have a further effect (Hellsten et al., 2012).

On the other hand, COX inhibition can also enhance NO release. Bolz and Pohl (1997) showed that by attenuating agonist-induced [Ca^{2+}] increases in endothelial cells, cAMP can modulate the production of PGI_2. Further, it can also modulate the production of NO. As a result, in conditions of low levels of PGI_2, e.g. following COX inhibition, an increased NO production occurs. Since this does not underlie a feedback inhibition by cGMP, NO might compensate for the lack of PGI_2 in the control of vascular tone under these circumstances. Therefore, after COX inhibition, an endothelial NO production occurs. This indicates that NO might compensate for the lack of PGI_2 when COX is inhibited (Bolz and Pohl, 1997).

In view of these interactions, it is clear that NOS inhibition may not simply reveal the contributions of NO to a given vasodilator response, while COX inhibition may not simply reveal the contribution of PGs. The potential contribution of NO and PGs is further complicated by EDHFs.

1.2.2 EDHFs

Given that both NO and PGI_2 can activate K^+ channels and induce hyperpolarisation, they can be considered as an endothelium-derived hyperpolarizing substances. However, endothelium-dependent hyperpolarization can also occur in the presence of inhibition of both COX and NOS and it is these mechanisms that are generally considered to represent the effects of EDH or of EDHFs (Feletou, 2006, Feletou and Vanhoutte, 2006b, Garland et al., 2017). The EDHF-mediated responses in most arteries involve activation of small and intermediate K_{Ca} channels (SK_{Ca} and/or IK_{Ca}) in vascular smooth muscle (Marrelli et al., 2003, Garland, 1996, Corriu et al., 1996, Ding, 2002, Gluais et al., 2005a), but not of big K_{Ca} (BK_{Ca}) (Zygmunt et al., 1997, Chataigneau et al., 1998). It is the rise in $[Ca^{2+}]_i$ after endothelial receptor stimulation by an agonist or shear stress that activates the K_{Ca} channels on endothelial cells to

hyperpolarize the endothelial cells. Endothelial cells protrude through holes in the internal elastic lamina in arterioles to contact with vascular smooth muscle cells. Gap junctions are located at these sites where endothelial cells meet vascular smooth muscle cells. Inositol trisphosphate (IP_3) has been thought to be a signal that passes through these gap junctions to endothelial cells to mediate vasodilation. However, Garland et al. (2017) demonstrated that it was Ca^{2+}, rather than IP_3, that entered vascular smooth muscle cells through voltage-gated Ca^{2+} channels, subsequently passing back through gap junctions into endothelial cells, and initiating vasodilation mediated by endothelial cells. The magnitude of these Ca^{2+} signals in endothelial cells depends on IP_3 receptors (*see Figure 1.4*). This evidence resolves a long-standing controversy over how vascular smooth muscle cells communicate with endothelial cells to trigger feedback vasodilation to balance vasoconstriction (Garland et al., 2017, Feletou et al., 2011).

There are two mechanisms suggested to explain how activation of endothelial SK_{Ca} and/or IK_{Ca} results in hyperpolarization of smooth muscle cells: (1) hyperpolarization of the endothelial cells directly hyperpolarises vascular smooth muscle by means of gap junctions; and (2) accumulation in the intercellular cleft of K^+ ions released from endothelial cells through K_{Ca}, leads to hyperpolarization of smooth muscle by increasing K^+ conductance through inwardly rectifying K^+ channels (K_{IR}) and/or stimulating Na^+/K^+-ATPase (Feletou and Vanhoutte, 2006b).

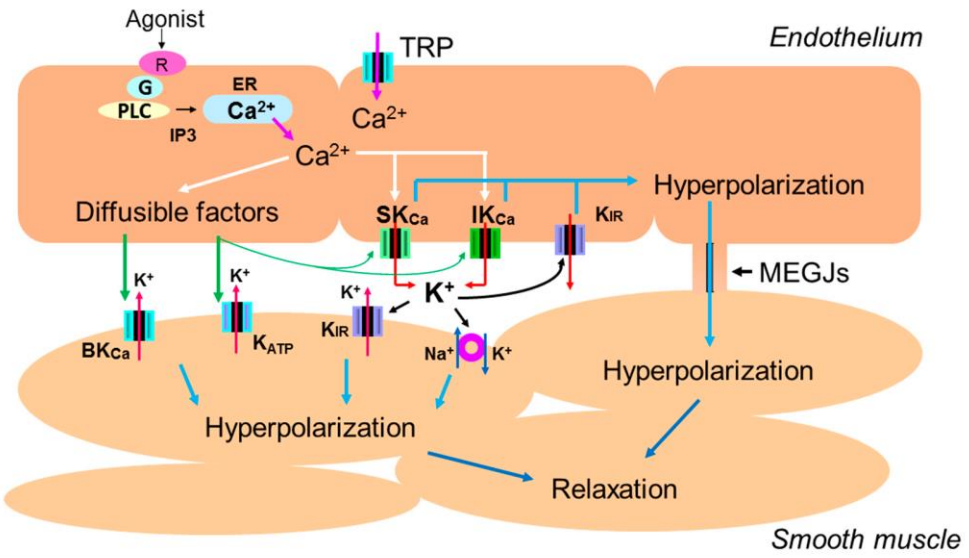


Figure 1.4 **Endothelium-dependent hyperpolarization of vascular smooth muscle cells.** Endothelial stimulation with agonists or by shear stress augments the intracellular release from the endoplasmic reticulum (ER) and Ca^{2+} influx through endothelial nonselective cation channels of the transient receptor potential (TRP) family. The rise in the endothelial Ca^{2+} concentration subsequently activates small (SK_{Ca}) and intermediate conductance (IK_{Ca}) Ca^{2+}-activated K^+ channels, generating endothelium-dependent hyperpolarization (EDH). The EDH then spreads to adjacent smooth muscle cells via myoendothelial gap junctions (MEGJs), leading to vasorelaxation in a number of vascular beds. In some vascular beds, diffusible factors hyperpolarize vascular smooth muscle cells via the opening of potassium channels and/or activation of Na^+/K^+-ATPase. Diffusible factors also act on endothelial potassium channels to generate or amplify EDH in certain vascular beds in specific conditions (Goto and Kitazono, 2019).

In arteries that demonstrate EDHF-mediated responses as judged by the effects of K_{Ca} channel antagonists, the endothelium-dependent hyperpolarization of vascular smooth muscle and hyperpolarization of endothelial cells follow the same time course (Beny, 1990, Emerson and Segal, 2000). Further a close relationship lies between the expression of myo-endothelial gap junctions and the incidence of EDHF-mediated responses (Sandow et al., 2002, Dora et al., 2003, Sandow et al., 2004), such that the number of myo-endothelial gap junctions (MEGJs) increases as the size of the artery decreases (Sandow and Hill, 2000), a trend that mimics the greater contribution of EDHF-mediated relaxation to endothelium-dependent relaxation in smaller arterioles (Hwa et al., 1994, Shimokawa et al., 1996). Indeed, EDH is implicated in propagated dilatation which is triggered at the level of terminal arterioles and capillaries and propagates proximally (Segal, 2015).

Functional role of EDHFs: By using selective antagonists, the activation of K_{IR} channels was shown to contribute substantially to total and peak reactive hyperemia in the forearm, while Na^+/ K^+-ATPase was found to contribute to total, but not peak reactive hyperaemia. Together, these signaling pathways contribute to most of the total RH (~90%) response (Crecelius et al., 2013).

With regards to exercise hyperemia, propagated EDH and dilatation has been shown to be of key importance in exercise hyperemia in studies on microcirculation (Segal, 2015). Also, in studies on forearm, K_{IR} channels activation was shown to contribute to the hyperemia at the onset and during steady state muscle contractions. In the presence of inhibition of K_{IR} channels and Na^+/ K^+-ATPase, combined COX and NOS inhibition caused a small attenuation of hyperaemia at the onset and a further modest attenuation of steady state exercise hyperaemia. Since Na^+/ K^+-ATPase inhibition had no greater effect than inhibition of K_{IR} channels, it was proposed that PGs and NO make a moderate contribution to the initial increase (10-15s) and steady state increase in muscle blood flow that is independent of K_{IR} channels (Crecelius et al., 2014, Crecelius et al., 2013). The primary stimuli for the opening of K_{IR} channels during muscle contractions require further investigation, however, K^+ and circulating ATP are considered as potential candidates (Crecelius et al., 2013, Crecelius et al., 2014).

Apart from the production of NO, PGI_2 and K^+ ions, the endothelial cells can generate other relaxing factors which induce vasodilation via hyperpolarization: these include epoxyeicosatrienoic acids (EETs) and Hydrogen peroxide (H_2O_2).

1.2.2.1 EETs

EETs derived from cytochrome P450 2C have been shown to induce endothelium-dependent relaxations of various blood vessels (Quilley and McGiff, 2000, Fleming, 2004, Widmann et al., 1998, Oltman et al., 1998). EETs act mainly by increasing the open-state probability of BK_{Ca} through a G protein-signalling cascade (Li and Campbell, 1997), although the presence of a specific cell membrane receptor(s) for EETs in vascular smooth muscle has not been established (Feletou and Vanhoutte, 2006b). EETs can also stimulate smooth muscle vanilloid transient receptor potential channel (TRPV4), which enhance the frequency of calcium sparks and as a result of spontaneous transient outward currents, induce hyperpolarization and relaxation of smooth muscle cells (Earley et al., 2005).

Evidence for the involvement of EETs in vasodilation comes from the fact that in several arteries, inhibitors of cytochrome P450 monooxygenases inhibit endothelium-dependent vasodilator responses that are resistant to the existence of inhibitors of NO synthases and cyclooxygenases (Fleming, 2004). Similarly, endothelium-dependent hyperpolarisations and relaxations can be blocked by antisense oligonucleotides aimed towards cytochrome P450-2C8-9 (Fisslthaler et al., 1999, Bolz et al., 2000). Contrariwise, these responses are raised by agents that augment the endothelial expression of cytochrome P450 (Fisslthaler et al., 1999, Popp et al., 1996). Bradykinin, pulsatile stretch, and shear stress have all been shown to release EETs from the endothelium (Fisslthaler et al., 1999, Popp et al., 1996, Campbell et al., 1996, Gauthier et al., 2005, Huang et al., 2005).

In humans, the effect of a cytochrome P450 inhibitor, suggested that EETs play a role in the control of the radial artery diameter *in vivo* (Bellien et al., 2006) and in exercise hyperaemia in the lower limb (Hillig et al., 2003). However, in the forearm of healthy volunteers and patients with various pathologies, the cytochrome P450 blocker sulfaphenazole did not affect basal tone, or decrease bradykinin-induced vasodilation alone or in the presence of inhibitors of cyclooxygenase and NO synthase suggesting that EETs may not be involved in basal vascular tone or in these non-NO-non-PGI_2 – mediated responses in the upper limb (Passauer et al., 2003, Passauer et al., 2005, Fichtlscherer et al., 2004).

1.2.2.2 H_2O_2

Another potential EDHF candidate is H_2O_2. Reactive oxygen species (O_2^-) are produced in considerable amounts by endothelial and smooth muscle cells. Superoxide either spontaneously, or enzymatically through dismutation by superoxide dismutase (SOD) is dismuted to H_2O_2, which, depending on the tissue and experimental conditions can induce dilator or constrictor responses and hyperpolarise or depolarise smooth muscle (Ellis and Triggle, 2003). The role of H_2O_2 as an EDHF derives from the observation that, in some blood vessels, agonist-and flow-induced dilatation that are non-NO-non-PGI_2-mediated phenomenon are moderately or wholly prevented by catalase and followed by endothelial generation of H_2O_2 (Shimokawa and Matoba, 2004).

To summarise, the current view is that endothelium-dependent relaxation, independent of generation of NO and PGI_2, mediated by EDH plays a major role in vascular

regulation (Moncada and Vane, 1978, Feletou, 2006, Garland et al., 2017, Crecelius et al., 2014, Segal, 2015). It can function as a backup system in larger arterial vessels when NO and PG availability is reduced but plays a major role physiologically at the microcirculatory level. Notably, dilatation that is triggered by K^+ and involves activation of K_{IR} and Na-K ATPase has been implicated in reactive hyperaemia, exercise hyperaemia and in hypoxic dilatation in the forearm of healthy humans (Crecelius et al., 2013, Crecelius et al., 2014, Racine et al., 2018) while EETs and H_2O_2 also induce vasodilation via these mechanisms (Feletou and Vanhoutte, 2009).

1.3 Endothelial dysfunction

Endothelial dysfunction occurs when the layer of endothelial cells starts to malfunction. In animal models of CVD and in human subjects who have CVD or are at risk of CVD, endothelium-dependent dilator responses are blunted or may even present as endothelium-dependent vasoconstriction. Oxidative stress can be considered as a common attribute in endothelial dysfunction (Griendling and FitzGerald, 2003a, Griendling and FitzGerald, 2003b). Notably, several reactive oxygen species (ROS) are important to vascular physiology and pathophysiology: NO, O_2^-, the hydroxyl radical ($^{\cdot}OH$), H_2O_2 and peroxynitrite ($ONOO^-$), which are generated under both normal and stress conditions such as inflammation or injury. Superoxide (O_2^-) is produced by diverse enzymes (i.e., nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cytochrome P450 monooxygenases, enzymes of the mitochondrial respiratory chain) in vascular smooth muscle and endothelial cells and are also generated by NOS and COX. Either automatically or enzymatically (through dismutation by SOD), O_2^- is converted to the uncharged H_2O_2. H_2O_2, in the presence of the enzyme catalase or glutathione peroxidase is dismuted into water and oxygen (Griendling and FitzGerald, 2003a, Griendling and FitzGerald, 2003b). However, in the presence of transition metals (copper, iron) or O_2^-, H_2O_2 produces highly reactive hydroxyl radicals (OH^-) via the Fenton or Haber-Weiss reaction, which can be extracted by mannitol or dimethylthiourea (Griendling and FitzGerald, 2003a, Griendling and FitzGerald, 2003b). When NO and O_2^- are generated in close proximity, they combine to form peroxynitrite ($ONOO^-$). a dynamic oxidant which is capable of oxidising sulfhydryl groups, nitrating and hydroxylating aromatic groups, including tyrosine, tryptophan and guanine (Zou et al., 2004). As a consequence of ROS generation, the

three major endothelium-dependent vasodilator pathways are limited, i.e. NO, prostacyclin, and EDHF. These effects are covered in brief below.

Effects on NOS pathway: Firstly, as indicated above NOS can contribute to ROS generation and this occurs when eNOS is uncoupled, a phenomenon that is associated with endothelial dysfunction in animal models of CVD including deoxycorticosterone acetate-salt hypertension(Landmesser et al., 2003), angiotensin II-induced hypertension (Mollnau et al., 2002), myocardial ischemia/reperfusion injury (Moens et al., 2008a) and aging (Yang et al., 2009). As indicated in Section 1.2.1, BH_4 is a major co-factor for eNOS isoforms. It is responsible for normal eNOS coupling, when two monomers of eNOS form a stable dimer. BH_4 binding to eNOS shifts the NOS haem iron to a high-spin state, augmenting arginine binding, the substrate for NO generation and converting to BH_3, which stabilizes NOS dimer formation (Moens and Kass, 2006, Ketonen and Mervaala, 2008, Moens et al., 2008b). When BH_4 levels are low, NOS dimer formation is decreased and its catalytic activity becomes functionally 'uncoupled' such that enzymatic molecular oxygen reduction by eNOS does not couple to L-arginine, leading in the generation of harmful O_2^- instead of NO (Figure 1.2). The availability of BH_4 is determined by the enzyme GTP-cyclohydrolase I (GTPCH I) which generates BH_4 *de nova* from GTP, or via a salvage pathway from, sepiapterin via recycling of BH_2 to BH_4, involving dihydrofolate reductase (Moens and Kass, 2006). Several factors affect BH_4 bioavailability and thus NOS uncoupling, including oxidative stress, which not only reduces expression of GTPCH but depletes NADPH, which is required for *de nova* synthesis and is involved in re-cycling, by oxidising BH_4 to inactive BH_2 (Moens and Kass, 2006).

Secondly, the eNOS uncoupling that is caused by reduced BH_4 availability increases generation of O_2^- which combines with NO that is still being generated by uncoupled eNOS to form peroxynitrite $ONOO^-$ which oxidises BH_4 to BH_2 causing further eNOS uncoupling. Furthermore, $ONOO^-$ attenuates guanylyl cyclase so attenuating cGMP production by NO and disables PGI_2 synthase by tyrosine nitration (see section 1.3.2); $ONOO^-$ additionally augments oxidative stress by inhibiting SODs (Zou et al., 2004, Munzel et al., 2005).

Thirdly, NO synthesis can be impaired by decreased availability of the substrate L-arginine for eNOS. It is considered unlikely that plasma L-arginine *per se* falls below

the concentrations required for eNOS activity, but a decreased intracellular L-arginine caused by arginase may lead to eNOS uncoupling. The endothelial expression of arginase effectively out-competes the availability of arginine for eNOS (Bachetti et al., 2004) so downregulating eNOS activity (Caldwell et al., 2018) and uncoupling it. Additionally, endogenous asymmetric dimethylarginine (ADMA) levels, can competitively inhibit eNOS in animals, and in human forearm vasculature (Vallance et al., 1992). Oxidative stress is associated with elevated levels of plasma ADMA, while exogenous administration of ADMA has been shown to contribute to development of endothelial dysfunction and CVD (Achan et al., 2003, Sydow et al., 2003, Kietadisorn et al., 2012).

Finally, in normal physiological conditions, the endothelial generation of endothelin-1 (ET-1) and/or its action on vascular smooth muscle is firmly kept under control by counter-regulatory systems involving NO. This is important because vasoconstriction and pressor responses initiated by ET-1 are different from those generated by most other vasoconstrictors in that they are progressively developing and long lasting even after washing out the peptide (DE Nucci, 1988, Yanagisawa et al., 1988). However, generation of NO normally inhibits the generation of ET-1 from Big-ET by ET converting enzyme (Boulanger and Luscher, 1990, Vanhoutte, 2000). Moreover, the effective and sustained vasoconstriction induced by ET-1 is efficiently attenuated by both exogenous and endothelium-derived NO, in a cyclic GMP-dependent manner (Miller et al., 1989, Lillestll et al., 1998). Ultimately, ET-1 released by endothelial cells on human coronary arteries for example, stimulates in an autocoid manner, endothelial ET_B receptors, which are linked to NO generation (Schini et al., 1991, Halcox et al., 2007). Therefore, under normal conditions, any overgeneration of ET-1 is counterbalanced by the increased release of NO, which downregulates the production of ET-1 and diminishes its vasoconstrictor effects via ET_A receptors on vascular smooth muscle (Vanhoutte, 2009, Vanhoutte, 2000, De Mey and Vanhoutte, 2014). Thus, when the availability of NO is limited by ROS in conditions of endothelial dysfunction, this is associated with an increase in the production and vasoconstrictor action of ET-1 (Taddei et al., 2001b).

1.3.1 COX pathway

Most endothelium-dependent contractions are averted by non-selective inhibitors of COXs (Katusic et al., 1988, Miller and Vanhoutte, 1985, Luscher and Vanhoutte, 1986) demonstrating the crucial role of these enzymes in the phenomenon. Bioassay studies presented that the vasoconstrictor prostanoids involved are generated by endothelial rather than vascular smooth muscle COX (Yang et al., 2003). Two isoforms of the enzyme have been identified in blood vessels (Feletou et al., 2011), COX-1 and COX-2. Molecular biology experiments and studies using preferential and selective inhibitors of the two isoforms of the enzyme, suggest that up-regulation of COX-1 is the major source of endothelium derived contraction factors (EDCFs) in mouse arteries (Zhou et al., 2013, Tang et al., 2005) and aorta of spontaneously hypertensive rats (SHR-laboratory rat which is an animal model of essential (or primary) hypertension, used to study cardiovascular disease) and diabetic rats (Ge et al., 1995, Traupe et al., 2002, Ospina et al., 2003, Wang et al., 2003, Yang et al., 2003, Gluais et al., 2006, Virdis et al., 2013). Shortly after the discovery of endothelium-dependent relaxation, it appeared that under certain conditions and in certain blood vessels, the endothelial cells produced contractions and not relaxations (Vanhoutte, 1982). Bioassay studies revealed that the endothelium can secrete very labile and more stable polypeptide-like contracting factors (Rubanyi and Vanhoutte, 1985), which in analogy with the contracting factors, were termed endothelium-derived contracting factor(s) (EDCFs). EDCFs are released by physical and chemical stimuli (i.e., hypoxia, pressure, and stretch) and autacoids, local and circulating hormones. The mechanism of EDCFs to hypoxia involves withdrawal of nitric oxide. The COX pathway can generate thromboxane A_2, prostaglandin H_2, and superoxide anions (Luscher et al., 1992). The enhanced generation of vasoconstrictor prostanoids by the endothelium diffuse to contract the underlying vascular smooth muscle (Feletou and Vanhoutte, 2009, Vanhoutte et al., 2009). However, in human essential hypertension, COX-2 is overexpressed and appears to be the major isoform responsible for endothelial dysfunction (Virdis et al., 2013).

As shown below in figure 1.5, COX transforms arachidonic acid into endoperoxide, PGH_2, which is released during endothelium-dependent contractions. Endoperoxides *per se* can activate vascular smooth muscle and thus are considered as plausible endothelium-derived contracting factor(s) EDCF candidates (Ito et al., 1991, Asano et

al., 1994, Vanhoutte et al., 2005). Endoperoxide diffusing from the endothelium may be processed in the vascular smooth muscle into PGI_2, which then stimulates IP receptors, the receptors which are known to be the receptors for the classical vasoconstrictor product of the COX pathway – thromboxane (TXA_2), or PGH_2 may directly stimulate IP receptors (Zhou et al., 2013). However, the majority of endoperoxide is converted in the endothelial cells into PGI_2, TXA_2, PGD_2, PGE_2 and/or PGF_{2a} by their selective synthases (Bos et al., 2004). Moreover, as indicated above, the expression of the PGI_2 synthase gene is the most abundant in the endothelial cells (Gluais et al., 2005b, Tang and Vanhoutte, 2008). In oxidative stress, PGI_2 synthase is inhibited by $ONOO^-$ (section 1.3-Endothelial dysfunction), which can change the balance such that PGH_2 is converted to TXA_2 rather than PGI_2 (Smyth, 2010). However, it has also been shown that during endothelium-dependent contractions to ACh, the release of PGI_2 outweighs that of other PGs (Gluais et al., 2005b) and it has been shown that in such conditions, PGI_2 does not induce relaxation, but causes contraction (Rapoport and Williams, 1996, Gluais et al., 2005b) because the IP receptor becomes insensitive and PGI_2 stimulates TP receptors (Vanhoutte, 2009). Thus, it is reasonable to conclude that PGH_2, PGI_2 and/or TXA_2 are the main mediators of these EDCF-mediated responses (Vanhoutte et al., 2005, Vanhoutte and Tang, 2008, Bos et al., 2004). For example, PGI_2 seems important in rat aorta (Ge et al., 1995, Blanco-Rivero et al., 2005, Gluais et al., 2005b) in rat and mouse aortae, and TXA_2 in canine basilar artery and in SHR aorta (Katusic et al., 1988, Auch-Schwelk and Vanhoutte, 1992, Gluais et al., 2006, Gluais et al., 2007). Thus, the precise nature of the COX-dependent EDCFs apparently varies among species and vascular beds depending on the relative expression of respective prostaglandin synthases, the extent of oxidative stress and the vasoactive mediators involved (Vanhoutte et al., 2017).

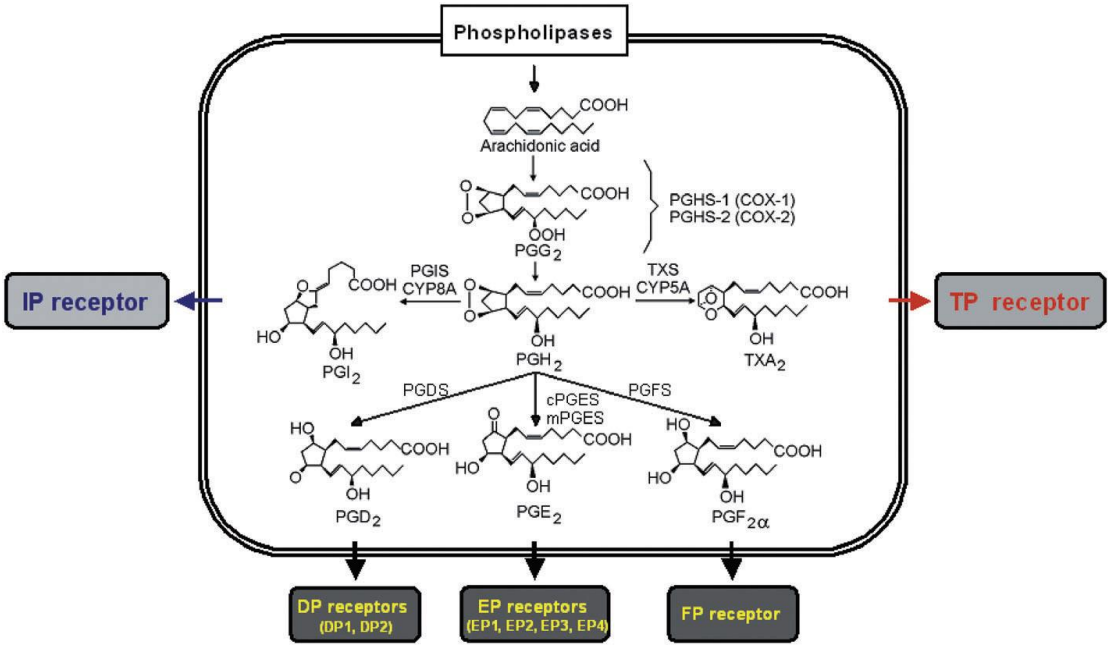


Figure 1.5 **Cyclooxygenases and arachidonic acid metabolism**. Prostacyclin and thromboxane synthases belong to the cytochrome P-450 superfamily (in the human, CYP8A1 and CYP5 respectively). The preferential receptors for the five primary prostaglandins and their subtypes are indicated: IP, DPs, EPs, FP and TP for prostacyclin, prostaglandin D_2, prostaglandin E_2, prostaglandin F_{2a} and thromboxane A_2 respectively. PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; TXA_2, thromboxane A_2; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PGF_{2a}, prostaglandin F_{2a}; COX, cyclooxygenase; PGHS, prostaglandin H synthase; PGIS, prostacyclin synthase; TXS, thromboxane synthase; PGDS, prostaglandin D synthases; cPGES, cytosolic prostaglandin E_2 synthase; mPGES, membrane prostaglandin E_2 synthase; PGFS, prostaglandin F_2 synthase (Feletou et al., 2011).

Additionally, in studies on humans, the COX inhibitors aspirin and indomethacin were shown to potentiate the vasodilator response to acetylcholine in the forearm of patients with hypertension but not in normotensive subjects (Taddei et al., 1997a, Taddei et al., 1997c, Taddei et al., 1995, Monobe et al., 2001). This suggests that EDCF-mediated responses also are part of endothelial dysfunction in human hypertension. Further, in patients with CAD and atherosclerosis, aspirin or the TP receptor inhibitor terutroban were shown to improve endothelium-dependent FMD and reactive hyperaemia in forearm circulation, or calf vasodilator responses to ACh, suggesting that endothelium-derived prostanoids acting via TP receptors contribute to the endothelial dysfunction associated with CAD and/or atherosclerosis (Belhassen et al., 2003, Husain et al., 1998). Consistent with this evidence, selective antagonists of TP receptors also improved FMD in the forearm of patients with atherosclerosis (Lesault et al., 2011). By

contrast, TP receptor antagonists had no effect on reactive hyperaemia in whole forearm or forearm skin of young healthy men (Pasche, 2013).

1.3.1.1 EDHF

The contribution of K_{Ca} channel to EDHF-mediated responses is also reduced by the chronic action of superoxide anion (Kusama et al., 2005), and oxidative stress reduces EDH signalling via myoendothelial and smooth muscle gap junctions by interacting with connexins 37, 40 and 43 (Griffith et al., 2005). In a similar manner, $ONOO^-$ diminishes the EDHF component of flow mediated vasodilation in mice coronary arteries (Liu et al., 2006). Further effects of ROS include the promotion of vascular smooth muscle cell contraction by assisting with the activation of releasing mechanisms and enhancing the sensibility of the contractile proteins to calcium ions (Jin et al., 1991, Suzuki and Ford, 1992). Additionally, superoxide anion, rather than PGs generated by hydroperoxidase activity of cyclooxygenase, is an endothelium-derived contracting factor in canine cerebral arteries (Katusic and Vanhoutte, 1989).

To summarize, endothelial dysfunction and oxidative stress are associated with a gradual imbalance between vasodilator and vasoconstrictor signals with a shift towards vasoconstrictors. It remains unclear how this balance varies in blood vessels of different sizes in diseased states. There is evidence (Vanhoutte et al., 2017) from studies on isolated vessels on changes in endothelial function in large arteries, but the effects of endothelial dysfunction on the behaviour of smaller arteries and microcirculation still remains largely uninvestigated. This is an important deficit because it is these vessels that are functionally important in determining changes in tissue vascular resistance. The following sections provide a brief review of what is already known about how endothelial dysfunction that occurs during aging, in hypertension and in those of SA ethnicity, affects regulation of vascular resistance.

1.4 Aging and Endothelial function

Advancing age is the most potent independent correlate of endothelial dependent-dilatation (Franklin et al., 1997), with age-related attenuation in endothelial function in both larger arteries and the microcirculation leading to a host of haemodynamic alterations (Donato et al., 2018). These include augmented large and resistance arterial tone and increases in large artery stiffness (Lakatta and Levy, 2003, Vaitkevicius et al., 1993). There is also a progressive decrease in brachial FMD with aging (Celermajer et

al., 1994) and a decrease in endothelium-dependent vasodilator responses to agonists (Seals et al., 2011). For example, there is attenuation in the contribution of NO to endothelium-dependent dilatation as judged from the attenuated effects of NOS inhibition on ACh-induced forearm vasodilation with aging, decreased plasma levels of NO metabolites and the finding that infusion of the substrate for NOS, L-arginine can improve ACh-induced dilatation (Toprakci et al., 2000, White et al., 1997, Taddei et al., 2001a, Taddei et al., 1997a). Increased levels of ET were also shown in response to head-up tilt in older people (White et al., 1997), and the levels of ET-1 in endothelial cells increased with age to an extent that paralleled attenuation of FMD (Donato et al., 2009), while ET-1 receptor inhibition indicated enhanced ET-1 induced vasoconstrictor tone in healthy older subjects (Thijssen et al., 2007). These findings are consistent with up regulation of ET-1 production and action when NO availability is reduced as discussed above.

Further, as might be expected from Section 1.3, the production of reactive oxygen species (ROS) is increased in the arterial wall of older subjects as evidenced by increased nitro tyrosine in endothelial cells, indicating increased generation of $ONOO^-$ in endothelial cells, while the activity of the pro-oxidant enzyme, NADPH oxidase, but not xanthine oxidase, increased (Donato et al., 2007). More recently, reductions in essential antioxidant enzymes, such as superoxide dismutases (SODs), catalase and glutathione (GSH), were reported to contribute to age-related arterial oxidative stress in humans (Donato et al., 2018). Consistent with eNOS uncoupling associated with oxidative stress and attenuated levels of the co-factor BH_4 (1.3.1), it was shown that administration of BH_4 improved FMD in sedentary older men (Eskurza et al., 2005).

Additionally, it was demonstrated that *ex vivo*, NOS inhibition attenuated shear stress-induced increases in O_2^- generation in the arterioles of old rats providing direct evidence that O_2^- was generated by eNOS uncoupling (Sindler et al., 2009). Further, aged mice had increased levels of nitrotyrosine co-localised with e-NOS in arteriolar endothelium, and an increased ratio of monomer to dimer NOS, while sepiapterin, the pre-cursor for BH_4 enhanced ACh induced arterial dilatation (Yang et al., 2009).

It has also been demonstrated that exercise hyperaemia during relatively weak contractions at 10% MVC is attenuated in older subjects, reflecting attenuated contributions of NO and PGs (Schrage et al., 2007). Acute improvements in the

hyperaemia were induced by administration of the anti-oxidant ascorbic acid during forearm exercise (Kirby et al., 2009). Thus, it was suggested that ascorbic acid acts to improve NO bioavailability due to direct scavenging of free radicals (e.g. O_2^-) (Nishikimi, 1975) or through stabilizing BH_4 (Heller et al., 2001). An alternative possibility is that age-associated oxidative stress increases ET-mediated vasoconstriction, and ascorbic acid acutely reverses this adverse effect of ET on local vasodilator function, subsequently resulting in greater hyperaemic responses during exercise in older adults (Bohm and Pernow, 2007, Van Guilder et al., 2007).

A further possibility is that vasoconstrictor PGs generated by the COX pathway during exercise limit exercise hyperaemia in older subjects, given the effects of aging on the COX pathway discussed in section 1.3.2. In line with this idea, after the age of 60 years, COX inhibition augmented ACh-induced forearm dilatation indicating production of COX products with EDCF effects (Taddei et al., 1997c). There appears to have been no study yet on the effects of TP receptor antagonism of ACh-induced dilatation, or exercise hyperaemia in older subjects.

1.5 Endothelial Dysfunction in Human Hypertension

It is recognised that reactive oxygen species are increased in essential, hypertension (HTN) (Feletou and Vanhoutte, 2006a). There is augmented generation of ROS, reduced antioxidant mobilization, and an attenuated capability to scavenge oxygen-derived free radicals all contributing towards increased oxidative stress (Touyz, 2004). The renin-angiotensin system contributes to the production of ROS in HTN through activation of NADPH oxidase (Lassegue and Griendling, 2004).

An early study in the field showed that in patients with essential HTN, forearm vasodilation to bradykinin and ACh demonstrated similar responses to NOS. After NOS blockade, they were of comparable amplitude in normotensive and hypertensives (Panza, 1994, Panza et al., 1995). These results indicated that the impaired endothelium dependent dilatation in HTN is not specific to one particular agonist or its receptor. Subsequently it was shown in subjects ranging from 20-80 years of age, that the attenuating effect of NOS inhibition on ACh-induced forearm vasodilation was impaired 20-30 years earlier in patients with HTN than in normotensives (Taddei et al., 1997c) suggesting that in this respect at least, HTN facilitates early aging.

Accordingly, the ability of COX inhibition to augment ACh-induced dilatation also occurred 20-30 years earlier in HTN as did the ability of L-arginine and Vitamin C to augment ACh-induced dilatation in forearm and coronary arteries (Solzbach et al., 1997, Taddei et al., 1998a, Taddei et al., 1997a, Taddei et al., 2001b). The finding that selective COX-2 inhibitors depressed endothelium-dependent dilatation in patients with essential hypertension (Bulut et al., 2003), suggests that it is the COX-1 isoform that is responsible for the production of vasoconstrictor prostanoids. Further, in the patients with coronary artery disease mentioned above (section 1.3.2), at least some of whom were hypertensive, the impaired ACh-induced forearm vasodilation was recovered by S 18886, a powerful and specific antagonist of the TP receptor (Belhassen et al., 2003, Simonet and TJ., 1998), substantiating the idea that at least in this population, COX products act on TP receptors to limit endothelium-dependent dilatation.

Consistent with the evidence (section 1.3.1-Endothelin-1), that the production and vasoconstrictor action of ET is enhanced in conditions of endothelial dysfunction, plasma levels of ET are not increased in most HTN patients (Schiffrin, 2005), but ETA and mixed ETA-ETB antagonists do reduce blood pressure in mild to moderately hypertensive patients (Krum et al., 1998, Nakov et al., 2002). These findings indicate an increased functional role for ET-1, consistent with reduced NO availability. Further, in patients with essential HTN, but not in normotensive controls, the combined blockade of ETA and ETB receptors, or selective blockade of the ETA receptor enhanced FBF and increased ACh-induced vasodilation suggesting that ET limits endothelium-dependent dilatation in HTN (Cardillo et al., 2002, Cardillo et al., 1999, Ghiadoni et al., 2000).

It has also been shown that ouabain, an inhibitor of Na^+-K^+-ATPase, attenuated ACh-induced forearm vasodilation in hypertensives but not in normotensives(Taddei et al., 1999). However, in the presence of Vitamin C which restored ACh-induced response to equal that of the normotensives, the attenuating effect of ouabain was lost. These findings indicated that an EDHF contributes to endothelium dependent dilatation in HTN and partly compensates for the decreased importance of NO (Taddei et al., 1999): when the availability of NO was restored the contribution of EDHF was reduced to that of a normotensive. Reduced availability of NO in essential HTN has been attributed to insufficient levels of BH_4 (Higashi et al., 2002), impaired $_L$-arginine transport (Schlaich

et al., 2004b), and to enhanced serum levels of ADMA (Perticone et al., 2005, Takiuchi et al., 2004, Vallance et al., 1992).

The finding that the COX inhibitor, indomethacin and the anti-oxidant, vitamin C had similar augmenting effects on ACh responses in HTN patients when administered individually and had no greater effect when co-infused led to the proposal that oxygen free radicals are mainly produced by a COX-dependent mechanism in essential HTN (Taddei et al. (1998a). Further understanding of the involvement of prostanoids (TXA_2 and PGH_2, see section 1.2.2) in HTN will require further investigation with PGH_2/THA_2 (TP) receptor agonists, or TXA_2 synthase inhibitors when they become available for human use (Tang and Vanhoutte, 2010).

Overall, these data demonstrate that, even though, essential hypertensive patients present increased oxidative stress and impaired NO availability, affecting both basal and agonist-stimulated release, alterations in COX activity and its products are likely to be implicated only in agonist-stimulated release (Versari et al., 2009). Interestingly, it was demonstrated that whereas NOS inhibition had little effect on ACh, or Bradykinin-induced forearm vasodilation in HTN patients in contrast to the marked attenuating effect in normotensives, P450 monoxoygenase inhibition with sulphenazole attenuated both dilator responses in HTN patients, but not in normotensives (Taddei et al (2006). These results indicate that defects in the NOS and COX pathway in HTN are replaced in part by an increased role for the EDHF, EET.

1.6 South Asian Ethnicity and Endothelial Function

Several factors contribute to the higher prevalence of CVD in SA population groups. An overview is presented below focussing on issues of relevance to this thesis. The health survey for England in 2004 demonstrated a high prevalence of CVD in SA populations, especially in Pakistani men (9.1%) when compared to the general population (7.9%) ischaemic heart disease and stroke combined (NHS Health and Social Care Information Centre, 2004).

The correlation of lower socio-economic class and poorer health outcome is well established (Kaplan, 1996, Kaplan et al., 1996). Accordingly, within the UK, cardiovascular disease (CVD) trends observed in migrant SA populations demonstrated that there was increased risk of CVD in South Asians in lower socioeconomic classes

linked with fewer years of education (Tillin et al., 2008). Indeed, the UK census data in 1991, presented increased all-cause mortality and mortality due to ischaemic heart disease in SAs relative to WEs in comparable socioeconomic classes (Maxwell, 1997). The Whitehall II study also demonstrated higher rates for coronary artery disease in SAs, even though all the applicable investigations and secondary prevention measures were utilized similarly by SAs and others (Britton et al., 2004). Importantly, post-myocardial infarction SA populations demonstrated similar improvements in outcome and decline in mortality when compared to WE populations (Liew et al., 2006).

However, a higher prevalence of coronary artery (CAD) disease was also shown amongst SA compared to WEs in a prospective population study, which indicated that higher prevalence of risk factors such as insulin resistance, diabetes, metabolic syndrome and socio-economic status did *not* explain the excess risk demonstrated by the SA group (Nair and Prabhakaran, 2012). Indeed, there is much contrary evidence indicating that the higher cardiovascular risk in people with South Asian descent cannot be completely explained by conventional risk factors such as tobacco use, diet and glucose intolerance; rather, an interplay between genes and the environment may be the reason for increased development of CAD in SAs (Gupta, 2006).

The genetic background for the higher CAD risk is evidenced by SAs tendency to higher lipoprotein (a) levels compared to other ethnic groups (Hoogeveen et al., 2001). Thus, hyperinsulinemia with consequent insulin resistance and hyper-triglyceridemia has been linked with SA ethnicity. Further, SA men had higher concentrations of small HDL particles and smaller overall HDL particle size which have cardioprotective benefits, consistent with a higher CAD risk as shown in the Framingham Offspring Study (Bhalodkar et al., 2004, Enas et al., 1996). In addition, studies on SA men demonstrate increased small, dense LDL particles and defective cholesterol transport (Kulkarni et al., 1999, Superko, 2001). There is also association between increased total body fat and waist circumference in SA and high-sensitivity C-reactive protein, a strong marker of cardiovascular risk and inflammation, indicating a latent proinflammatory state contributes to excess CAD risk (Chandalia et al., 2003).

Obesity, defined as BMI>30kg/m^2, is also highly prevalent in migrant SA populations (McKeigue et al., 1991). However, SAs present with relatively higher body fat and a lower lean body mass, indicating that BMI of <25 kg/m^2 may not necessarily represent

'normal' body composition (Hughes et al., 1990). In fact, waist to hip ratio is comparable in WEs and SAs, mainly due to small waist size in SAs (Misra and Vikram, 2004). The increased abdominal fat, combined with the increased intra-abdominal fat and body fat patterning may be significant determinants of dyslipidemia and insulin resistance (Banerji et al., 1999, Raji et al., 2001). Indeed, the thrifty gene hypothesis suggests that genetically susceptible individuals, when exposed to a lifestyle with a high-energy diet and attenuated energy expenditure which is common with urbanization induces insulin resistance and attendant complications, including CAD. It has therefore been suggested that this profile is a potential explanation for the CAD excess in SAs (McKeigue et al., 1989).

According to this hypothesis, the protective genetic traits that are beneficial in times of famine in SAs, are harmful in settings of excess energy consumption. This was demonstrated in urban centers in India where SA had higher CAD rates when compared to rural settings (McKeigue et al., 1989). The urban environment is also linked with increased abdominal obesity, type 2 diabetes and insulin resistance in SAs relative to other populations (McKeigue et al., 1991). Further, Type II diabetes has a prevalence of 2% in rural areas, but up to 16% in those residing in North America or the United Kingdom (Banerji et al., 1999). Moreover, the hyperinsulinemia that follows insulin resistance is implicated in the development of premature CAD (Gupta, 2006).

The fact that SAs with established CAD were found to have higher prevalence of metabolic syndrome, despite having a lower BMI (Gupta, 2004a) suggests that SAs present higher CAD risk at lower BMI than do European populations. Thus, the World Health Organization recommended different BMI cutoff points for overweight (23 kg/m^2) and obesity (25 kg/ m^2) in people of SA origin (WHO, 2004). Subsequently, the cutoff for abnormal waist circumference has also been lowered, to 90 cm in Asian men compared to WE men (≥ 94 cm) (WHO, 2004, International Diabetes Federation, 2006).

Against this background, the fact that the SA population in the UK has a higher prevalence of hypertension is particularly relevant to this PhD project (Cappuccio et al., 1997, Primatesta et al., 2000). Notably, prevalence rates for hypertension were higher in SA men than WE men (Cruickshank et al., 1991, Miller et al., 1988, Williams et al., 1993, Cappuccio FP, 1998). Additionally, alterations in lifestyles and

environmental factors were proposed to play a major role in the development of hypertension in SAs (Gupta et al., 1996).

Further, endothelial vasodilator function was depressed even in apparently healthy SAs aged 20-65 years compared with matched WEs, and this was accompanied by increased insulin resistance and dyslipidaemia in the SAs (Raji et al., 2004). Endothelial dysfunction is an essential part of the syndrome of insulin resistance, and presents before the onset of hyperglycemia (Balletshofer et al., 2000). Moreover, insulin has a vasodilator action mediated by NO generation (Scherrer et al., 1994, Steinberg et al., 1994). Hence, it is possible that the endothelial dysfunction observed in SAs partly results from impaired insulin-mediated eNOS activation.

In this context, Murphy et al. (2007) demonstrated that young healthy SA men aged 20-40 years showed an attenuated branchial FMD response and increased plasma insulin relative to matched WE men, and a reduced ability of NOS inhibition to increase vascular tone, consistent with reduced NO availability in young SAs. Although FMD is not such a good prognostic indicator for CVD as reactive hyperaemia (Anderson et al., 2011), peak reactive hyperaemia was also shown to be attenuated in healthy young normotensive SA men aged only 18-24 years compared to matched WE men (Ormshaw et al., 2018). Since NO, PGs, adenosine, and EDHF are known to contribute to peak reactive hyperaemia while NO maintains reactive hyperaemia (Carlsson et al., 1987, Tagawa et al., 1994, Engelke et al., 1996, Crecelius et al., 2013) see section 1.2.1) these blunted responses in young SAs may indicate blunted contributions of NO, adenosine and/or PGs (Ormshaw et al., 2018). Further, forearm vasodilator responses to environmental stressors were also depressed in young SA men relative to WE men, SAs generally showing forearm vasoconstriction on repetition of the stressor (Ormshaw et al., 2018). Since the mechanism of forearm vasodilation in mental stress is dependent on shear stress-induced release of NO, the β_2-adrenoreceptor effect of adrenaline, which is also NO-dependent and the action of ACh released by shear stress acting on the endothelium (Dietz et al., 1994, Halliwill et al., 1997, Seddon et al., 2008), these results are all consistent with early endothelial dysfunction in young SA men, that precedes overt cardiovascular disease. Indeed the fact that NO-mediated component of stress-induced forearm vasodilation was impaired in young normotensive subjects with hypertensive parents, in young hypertensives and in black African Americans (Cardillo

et al., 1998, Schlaich et al., 2004a, Khan et al., 2015), suggest that young SAs are part of this group with early endothelial dysfunction (Ormshaw et al., 2018).

There is also evidence that cutaneous endothelium-dependent dilatation is depressed in young SAs, for ACh-induced cutaneous dilatation was blunted in healthy young SA men compared to WEs (Hirst and Marshall, 2018). Interestingly, a study on young normotensive offspring of people with essential HTN, who have greatly increased risk of developing HTN relative to offspring of normotensives presented impaired forearm vasodilator responses to ACh compared with matched offspring of normotensive subjects (Taddei et al., 1996), demonstrating endothelial dysfunction occurs earlier in these subjects and before the development of hypertension. In contrast to frank hypertensive patients, the forearm dilator responses of those with hypertensive parents were not enhanced by COX inhibition, but by the eNOS substrate L-arginine suggesting a defect in the NOS pathway, rather than in production of COX-derived EDCFs, (Taddei et al., 1996, Versari et al., 2009). However, (Hirst and Marshall, 2018) showed that COX inhibition attenuated ACh-induced cutaneous dilatation and reactive hyperaemia in those with normotensive parents but not in those with hypertensive parents, suggesting that endothelial dysfunction develops differently in cutaneous circulation and is associated with an earlier depression of COX-generated vasodilator products rather than attenuated NO availability (Hirst and Marshall, 2018).

Since the heritable predisposition of ABP in both WEs and SAs is 40-60% (Shih and O'Connor, 2008, Wang et al., 2008, Tozawa et al., 2001, Ranasinghe et al., 2015), these results raise the possibility that the earlier evidence of endothelial dysfunction in SA men is associated with their increased risk of hypertension.

Taken together, the findings presented above demonstrate that SA ethnicity is certainly linked with higher CVD risk. Conventional risk factors such as diet and smoking make a contribution to development of CVD in SAs. However, emerging risk factors such as obesity, insulin resistance, diabetes, hypertension and endothelial dysfunction are also linked to development of CVD disease in SAs. Further, SA ethnicity seems to be a cofactor for development for HTN as well as CVD, and CVD seems to develop earlier in those of SA ethnicity. Early onset of endothelial dysfunction has been suggested as potential explanation for early development of CVD in SAs (Murphy et al., 2007). However, the contribution of NO, PGs and EDHF to endothelial dysfunction in young

SA remains to be established, as does the question of whether lifestyle intervention can reduce risk of CVD in SAs.

1.7 Exercise as a Lifestyle Intervention

It is widely accepted that physical activity is associated with a reduction in CVD mortality and a reduction in all-cause mortality (of ~35% and 33% respectively) in comparison to individuals who engage in a sedentary lifestyle (Nocon et al., 2008). For individuals with HTN seeking to decrease their BP and normotensives seeking to maintain their ABP, the American College of Cardiology (ACC)/American Heart Association (AHA) recommendations include performing 90-150 minutes per week of aerobic exercise (AE) at 65-75% of heart rate reserve and/or dynamic resistance exercise (RE) for 90-150 minutes per week, comprising 6 exercises of 3 sets per exercise for 10 repetitions, at an intensity of 50-80% heart rate reserve, or one-repetition at 100% maximum. More recently, isometric handgrip (IHG) training has been introduced as part of the guidelines, directing individuals to perform four 2-minute contractions separated by 1-minute of rest at 30-40% of MVC for 3 sessions per week (Whelton and Carey, 2017, Leung et al., 2017).

A review of the literature on the effects of different types of exercise training on cardiovascular risk, focussing on isometric training is provided in the sections below.

1.8 Isometric Exercise

Isometric exercise is characterized by a sustained muscle contraction without a change in the length of the working muscle (Fleck, 2004). In fact, humans, are unable to perform pure state contractions, thus isometric contractions are classified as contractions involving minimal change in muscle length (Mitchell and Wildenthal, 1974).

The first study to examine the relationship between arterial blood pressure (ABP) and isometric exercise training was performed by Kiveloff and Huber (1971). A population of 8 individuals with HTN performed 5-8 weeks of maximal whole-body isometric training for durations of 6 seconds at maximum voluntary contraction (MVC), 3 times daily: a decrease of 16-24 mmHg in systolic blood pressure (SBP) and 2-14mmHg in diastolic blood pressure (DBP) was achieved. This decrease was comparable to that and achieved in individuals receiving pharmacotherapy who noted a decrease of 4-28

mmHg and 2-14mmHg in SBP and DBP, respectively. Among HTN individuals who were on medication and prescribed similar maximal isometric training, baseline BP was not affected suggesting the effects were not additive (Kiveloff and Huber (1971). However, working at maximum MVC is potentially dangerous due to the associated acute increase in BP during contraction.

The hypotensive effect of isometric exercise in the workplace was noted by Buck and Donner (1985). Individuals with an occupation entailing greater isometric activity tended to have lower ABP than individuals whose occupation required lower isometric activity. These results are difficult to interpret because occupational isometric activity was rated as a categorical variable, and so the data collection made the length of exposure to isometric activity difficult to quantify and there was no indication of individual differences in exposure to isometric activity. Nevertheless, this report became the basis for many subsequent interventional studies.

Several modalities of isometric exercise have been studied including both IHG and bilateral isometric leg exercise (ILE) as ways of decreasing ABP. IHG has become a popular training modality as it has been shown to decrease BP in a short period of time with minimal time commitment (Carlson et al., 2014).

The first interventional IHG training study to examine effects on ABP was performed by Wiley et al. (1992). A population of normotensive healthy volunteers aged (19-56 years) were recruited. Two protocols were tested: the first consisted of 4 sets of 2-minute unilateral contractions performed at 30% MVC separated by 3 minutes of rest performed 3 days per week for 5 weeks, while the second consisted of 4 sets of 45-second unilateral contractions performed at 50% MVC 5 days per week for 5 weeks. In both cases, participants had their ABP measured at 5 weeks following completion of the IHG intervention, followed by a 5-week detraining period. The first group showed a decrease of 13/15 (SP/DP) mmHg, while the second group showed a fall of 9.5- and 8.9-mm Hg respectively. After 5 weeks of detraining, ABP values returned to baseline in both protocols (Wiley et al., 1992). Consistent with this study, but using a duration twice as long (10 weeks) it was demonstrated in normotensive individuals that IHG training comprising five 3-minute contractions at 30% MVC, three times a week reduced resting ABP by ~10/5 mmHg (Garg et al., 2014).

Turning to ILE, Somani et al. (2018) recruited 46 healthy young normotensive individuals divided into two groups, and had them perform either 4 sets of 2-minute IHG contractions at 30 % MVC or ILE contractions at 32% MVC, both performed by using a leg extension dynamometer. The interventions lasted 10 weeks and the protocol was performed 3 times per week. It was found that ILE and IHG elicited similar decreases in ABP. These findings on ILE training were supported by another study (Wiles et al., 2017) in which 28 normotensive young males performed a crossover design study for 4 weeks at-home consisting of 4 sets of 2-minute bouts of isometric wall squat exercise 3 times per week and a 4-week washout period. The results showed that the training part of the protocol was successful in significantly reducing SBP, DBP and mean ABP, as well as cardiac output and heart rate. This ILE protocol which rivals IHG for its convenience was judged to be relatively safe and in accordance with ACSM guidelines in terms of the evoked rise in ABP (Wiles et al., 2018, Wiles et al., 2017).

Summarising these effects, two systematic reviews and meta-analyses Carlson et al. (2014) and Inder et al. (2016), have shown that isometric training produces clinically meaningful reductions in ABP. Further, another meta-analysis which included both handgrip and double-leg extension exercises indicated that less than 1 hour of isometric exercise per week at 20-30% MVC, is sufficient to achieve a 10mmHg fall in SBP (Owen et al., 2010), making it attractive for individuals with a busy life style.

1.9 Summary of IHG training programmes

The section below covers what is known about the effects IHG training including the "prescription of IHG training programs" and the factors that affect the response of individuals to IHG training. A summary of the methods and results of these training studies is provided in Table 1.

On the basis that IHG training studies using programmable handgrip devices had generally been shown to reduce clinic ABP within 8 weeks, (Millar et al., 2013, Peters et al., 2006, Taylor et al., 2003, Wiley et al., 1992, McGowan et al., 2006B) investigated whether use of a simple inexpensive spring-loaded handgrip device could produce hypotensive effects. The study was performed on men and women, average age 66 years who used such devices at 30% MVC for 2 minutes, performed 4 times with each hand alternately, 3 times per week for 8 weeks, 2 days in the laboratory and once at home. A

control group did no IHG training and simply had their ABP measured twice per week in the laboratory. This initial protocol reduced SP from 122 to 112 mm Hg and DP from 70 to 67 mm Hg, while the controls showed no change in ABP. These findings indicated that IHG training could be used to reduce ABP without the use of expensive equipment and potentially in the home.

Subsequently, a similar protocol using IHG training was shown to reduce ABP in normotensives (McGowan et al., 2007, Badrov et al., 2016, Somani et al., 2018), non-medicated hypertensives (Taylor et al., 2003, Carlson et al., 2016) and in medicated hypertensives (Badrov et al., 2013A, Millar et al., 2013). The decreases of ABP derived from these studies indicated SBP was reduced by ~ 5 mmHg and DBP by ~ 2 mmHg. The greatest reductions in ABP were shown in hypertensives, specifically in those with the highest ABP (Millar and McCartney, 2007). Thus the CHEP (2017) and the ACC/ AHA Guidelines (AHA) endorsed this training programme as treatment for HTN (Leung et al., 2017, Arnett et al., 2019). Such an endorsement for IHG training would make a beneficial addition to future NICE guidelines in the UK for the management and treatment of hypertension in adults. Unfortunately it is not part of the guidelines for 2019 (NICE, 2019).

1.9.1 Factors determining the effects of Isometric Training

Regarding, the frequency and length of training regime required to induce efficient ABP reductions, it is generally used 3 times per week for 8 to 10 weeks duration (Badrov et al., 2013A, Leung et al., 2017). However, several studies have reported that ABP decreases linearly during the training period (e.g. McGowan et al. (2007), although they have generally reported ABP only at the midpoint and endpoint. The length of the hypotensive effects of IHG training also remains largely unexplored: one study revealed the hypotensive effects endured for 7-10 days after termination of training (Millar and McCartney, 2007). Therefore, further investigation is required to explore the minimum training period to induce the hypotensive effects and to establish their duration when training ceases.

Badrov et al. (2013B) observed that training frequency affects the speed at which individuals achieve a hypotensive response to IHG training. Thus, in 35 young, normotensive women, a subgroup who trained at 30% MVC, 3 times per week, showed

a significant decrease in BP at week 8 of 6mmHg, but no greater change in ABP occurred in the subgroup who trained 5 times per week at 4 or 8 weeks. These findings suggest a maximum hypotensive response is reached regardless of training dose, and the time to maximum effect is dependent on training frequency. These findings cannot be extrapolated to the general population due to the absence of men in the study population.

On the other hand, (Carlson et al., 2016) found that the intensity of IHG also plays a role. A mixed group of 40 men and women with HTN (35-65 years) were randomly assigned to IHG training at either 5% or 30% MVC. Both groups performed 4, 2-minute unilateral IHG exercises separated by 3-minutes of rest 3 days per week: at 8 weeks of IHG training, both SBP and mean ABP were significantly decreased in the 30% MVC group, but not in the 5% MVC group.

1.9.2 Individual differences in responses to IHG training

In addition to exercise intensity, frequency and duration of training, several other factors which have been demonstrated to predict the hypotensive effect of IHG training. They include baseline BP, pharmacological intervention, response to stressors and age, and possibly, sex (Lawrence et al., 2015).

Firstly, resting ABP at the beginning of IHG training is a strong predictor of how effective IHG training will be in lowering ABP. In a multilevel analysis performed by Millar and McCartney (2007), individuals with medicated HTN partaking in IHG training (8-10 weeks, 3x/week) showed an average decrease of 6/3 mmHg, but participants who entered the study with higher BP experienced the greatest reductions in BP. It was also found that reductions in SBP were lost in only 7-10 days following cessation of IHG training.

Another factor is whether or not the participants are taking HTN medications. To date, no study has directly examined the effect of pharmacological intervention *per se* or specific drug classes on the ABP response to IHG training. However, it seems that the changes in ABP seen in individuals with similar baseline ABP are much greater among those who are *not* medicated compared to those who are. For example, Stiller-Moldovan et al. (2012) reported a smaller change in ABP of ~3 mmHg following 8 weeks of IHG training in HTN patients than McGowan et al. (2006B) whose

participants took fewer HTN drugs, and had higher SBP at baseline (134±4 mmHg vs 114±13 mmHg): showed reductions of MAP of ~ 4 mmHg, despite IHG training being of comparable duration and frequency. Similar findings were made by analysis of results obtained in 3 different studies, by using hierarchical linear modelling: it was shown that participants with higher initial SP showed greater rates of ABP decline during IHG training (Millar and McCartney (2007). It was therefore deduced that individuals with higher blood pressure stood to achieve greater benefits from this.

There is also evidence that other interventional stimuli may also be able to identify responders and non-responders to IHG training. Thus, Millar et al. (2009a) tested whether a cold pressor test or serial subtraction task could predict the responsiveness to IHG training. They found that among a group of 17 normotensive older individuals who had completed an IHG training study 6 months previously, the magnitude of IHG training-induced decrease in ABP was directly correlated with the pressor response to a serial subtraction task, but not the cold pressor test. Similar findings were made by Badrov et al. (2013B), who tested SBP reactivity to serial subtraction, cold pressor test and IHG (a single sustained 30% MVC for 2 minutes on the non-dominant arm) before and after 10 weeks IHG training. The fall in resting ABP was correlated with the pressor response to the stress test and IHG, but not to the cold pressor test. Somani et al. (2018), also found that individuals who showed the largest ABP response to a 2-minute isometric exercise test (either ILE or IHG) showed the greatest reduction in resting ABP following IHG training.

To date, only one study has examined whether IHG training is effective in reducing ABP in healthy, older, non-medicated men and women (aged 50-80 years). The study discussed above using the inexpensive handgrip device (Millar et al. (2008) showed that IHG training was effective in reducing resting ABP, but within group analysis indicated the hypotensive effect was greater in the oldest quartile and greater in women than men.

In some contrast, Badrov et al. (2016) found that 8 weeks of unilateral IHG reduced ABP equally in young normotensive male and female participants (aged 21-23 year). Similarly, Somani et al. (2018), found there were no differences in the magnitude in decreases of SBP and DBP between young women and men performing a 10-week IHG or ILE training. This contrast between these two sets of findings and those of Millar et

al, 2008 raises the question of whether aging particularly enhances the hypotensive effect of IHG training in women.

Table 1.3: **Review of IHG training studies effects on resting ABP**

Author Year	Population	Training Protocol	Duration	Significant Findings
Wiley et al. (1992)	Normotensive 20-35 years N=10	4, 45-sec bilateral IHG, 50% MVC, 5x/wk	5 weeks	↓SBP10 mmHg, ↓DBP 9 mmHg
	Unmedicated pre-hypertensive 29-52 years N=18	4, 2-min unilateral IHG,30% MVC, 3X/wk	8 weeks	↓SBP13 mmHg, ↓DBP15 mmHg
Ray and Carrasco (2000)	Normotensive 19-35 years N=16	4, 3-min unilateral IHG, 30% MVC, 4X/wk	5 weeks	↓DBP 5mmHg, No change in MSNA
Taylor et al. (2003)	75% medicated hypertensive, 25% unmedicated hypertensive 69±6 years N=17	4, 2-min bilateral IHG, 30% MVC, 3X/wk	10 weeks	↓SBP19 mmHg ↓ LF:HF HRV
Peters et al. (2006)	Unmedicated pre-hypertensive and hypertensive 52±5 years N=10	4, 45-sec bilateral IHG, 50% MVC, 3X/wk	6 weeks	↓SBP13mmHg, ↓DBP 2 mmHg
McGowan et al. (2006A)	Medicated hypertensive 62±4 years N=9	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↑ brachial artery FMD, trained limb ↓SBP 15 mmHg
	Medicated hypertensive 66±6 years N=7	4, 2-min bilateral IHG, 30% MVC, 3X/wk		↑ brachial artery FMD, both arms ↓SBP10 mmHg

McGowan et al. (2006B)	Medicated hypertensive 67±6 years N=7	4, 2-min unilateral IHG, 30% MVC 3X/wk	8 weeks	↑brachial artery endothelium independent FMD, trained limb
		4, 2-min unilateral IHG, 30% MVC	Acute	↓ brachial artery FMD
Millar and McCartney (2007)	Medicated hypertensives 38-77 years N=43	4, 2-min unilateral or bilateral IHG, 30% MVC, 3X/wk	8 weeks	↓SBP5.7mmHg ↓ DBP 3 mmHg
McGowan et al. (2007)	Normotensive 28±14 years N=20	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↓ SBP 5 mmHg ↔brachial artery blood flow, ↔brachial artery diameter
Millar et al. (2008)	Normotensive 66±1 years N=56	4, 2-min bilateral 30-40% MVC performed on a spring handgrip trainer, 3X/wk	8 weeks	↓SBP 10 mmHg ↓DBP 3 mmHg
Devereux et al. (2010)	Normotensive males 21±2 N=13	4, 2-min bilateral ILE, 3X/wk, 95% HRpeak	4 weeks	↓ SBP 5 mmHg ↓ DBP 3 mmHg ↓ MAP 3 mmHg
Mortimer and McKune (2011)	Normotensive females 48±2 years N=18	4, 45-sec bilateral IHG, 30% MVC	5 days	↔BP
Araujo et al. (2011)	Normotensive 64±9 years N=41	4, 2-min bilateral IHG, 30% MVC	Acute	↑ SBP 16 mmHg ↑ DBP 7 mmHg ↑ HR 3 BPM SBP, DBP, HR return to baseline within 3 mins
Stiller-Moldovan et al. (2012)	Medicated hypertensive 60±9 years N=20	4, 2-min bilateral IHG, 30% MVC, 3X/wk	8 weeks	↔Resting or ambulatory BP
(Stiller-Moldovan, 2012)	Medicated hypertensive 70±5 years N=20	4, 2-min bilateral IHG, 30% MVC, 3X/wk	8 weeks	↔BP No PEH pre or post intervention
		4, 2-min bilateral IHG, 30% MVC	Acute	No PEH
Badrov et al. (2013A)	Normotensive female 23±4 years N=12	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↓SBP by 6 mmHg ↑Resistance vessel endothelial function by 42%
	Normotensive female 27±6 years N=11	4, 2-min unilateral IHG, 30% MVC, 5X/wk	8 weeks	↓SBP by 6 mmHg ↑Resistance vessel endothelial function by 57%

Badrov et al. (2013B)	Hypertensive 83% medicated, 17% unmedicated 65±6 years N=24	4, 2-min bilateral IHG, 30% MVC, 3X/wk	10 weeks	↓ SBP 8 mmHg ↓ DBP 5 mmHg ↓ PP 4 mmHg
Millar et al. (2013)	Medicated hypertensive 65±6 years N=23	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↓ SBP 5 mmHg ↓ MAP 3 mmHg ↑ Non-linear HRV ↔Linear HRV
Garg et al. (2014)	Normotensive 30±6 years N=30	5, 3-min bilateral IHG, 30% MVC, 3X/wk	10 weeks	↓ SBP 10 mmHg ↓ DBP 5 mmHg
Gill et al. (2015)	Normotensive 23±3 years N=40	4, 2-min bilateral ILE, 30% EMGpeak 3X/wk	3 weeks	↓SBP 7 mmHg women ↓DBP 2 mmHg men
Moon et al. (2015)	CAD patients 63±9 years N=30	3-min unilateral IHG, 30-40% MVC	Acute	↑ Central SBP ↑ Central DBP ↑ PWV
Hess et al. (2016)	Normotensive 39±11 years N=22	4, 2-min unilateral IHG, 5% MVC, 3X/wk	6 weeks	↓ SBP 4 mmHg ↔DBP
		4, 2-min unilateral IHG, 10% MVC, 3X/wk		↓ SBP 5 mmHg ↔DBP

Badrov et al. (2016)	Normotensive Male 21±2 years Female 23±4 years N=20	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↓ SBP 8 mmHg ↓DBP 2 mmHg ↑ FMD equally in males and females
Goessler et al. (2016)	Male CAD patients 68±7 years N=30	4, 2-min bilateral IHG, 30% MVC	Acute	↔DBP, SBP, ABP
Carlson et al. (2016)	Hypertensive 65% medicated, 45% unmedicated 52±8 years N=20	4, 2-min unilateral IHG, 30% MVC, 3X/wk	8 weeks	↓ SBP 7 mmHg ↓ DBP 5 mmHg
	Hypertensive 65% medicated, 45% unmedicated 54±8 years N=20	4, 2-min unilateral IHG, 5% MVC, 3X/wk	8 weeks	↔SBP, DBP
Baross et al. (2017)	Normotensive 20±2 years N=48	3, 10-second bilateral IHG, 20% MVC+ 30-min walking 6.5 km/hr, 4X/wk	6 weeks	↓SBP 10 mmHg
		3, 10-second bilateral IHG, 20% MVC, 4X/wk	6 weeks	↓SBP 5 mmHg
		30-min walking 6.5 km/hr	6 weeks	↓SBP 4 mmHg

Somani et al. (2018)	Normotensive 24±6 years N=46	4, 2-min bilateral IHG, 30% MVC, 3X/wk	10 weeks	↓SBP 4 mmHg
		4, 2-min bilateral ILE, 20% MVC, 3X/wk		↓SBP 7 mmHg
Wiles et al. (2017)	Normotensive males 30±7 years N=28	4, 2-min 95% HRmax bilateral isometric wall squat, 3X/wk	4 weeks	↓SBP 4 mmHg ↓DBP 3 mmHg
Goessler et al. (2018)	Normotensive adults 33±1 years N=60	4, 2-min bilateral IHG, 30% MVC, 7X/wk at home	8 weeks	↓ Daytime SBP 3 mmHg ↓ Nighttime SBP 3 mmHg ↓ Office SBP 4 mmHg ↓ Office DBP 4 mmHg
		150 min moderate aerobic exercise total /wk at home		↔Daytime or nighttime BP ↓ Office SBP 6 mmHg ↔Office DBP
Wiles et al. (2018)	Normotensive males 28±7 years N=20	4, 2-min 95% HRmax isometric wall squat, 2X/wk	2 weeks	BP stayed within ACSM safe range during isometric wall squat

N=number of participants, IHG=isometric handgrip training, SBP=systolic blood pressure, DBP=diastolic blood pressure, MAP=mean arterial pressure, MVC=maximum voluntary contraction, HR=heart rate, MSNA=muscle sympathetic nerve activity, PP=pulse pressure, PWV=pulse wave velocity, BP=blood pressure, LF=low frequency, HF=high frequency, HRV=heart rate variability, , FMD=flow mediated dilation, ILE=isometric leg extension, CAD=coronary artery disease, PEH=post-exercise hypotension, EMG=electromyography, week=wk, ACSM=American College of Sports Medicine

1.10 Mechanisms of IHG training induced Blood Pressure Reduction

To date, researchers have only hypothesized on the mechanisms responsible for the decrease in ABP associated with IHG training. Common theories include changes in autonomic function, enhanced vascular endothelial function, inhibition of ROS activity and the effects of increased shear stress. These possibilities are reviewed below:

1.10.1 Autonomic Function

The results of studies that have investigated changes in autonomic activity following IHG training are equivocal. It has been argued that most of the variance may be explained by differences in pharmacological treatment of the subjects since different

classes of antihypertensive medications affect the neural pathways involved in the baroreceptor reflex, and may, or may not affect the mechanisms by which IHG training acts in a non-medicated individual (Stiller-Moldovan et al., 2012).

For example, traditional measures of autonomic function such as heart rate variability (HRV) and ABP variability have not consistently shown improvement with IHG training. In a study on older hypertensives (SBP>140mmHg and/or DBP >85mmHg, 75% of whom were medicated), IHG training with 4 sets of 2-min unilateral contractions at 30% MVC 3 days/week for 10 weeks, decreased SBP by 19 mmHg and DBP by 7 mmHg. These effects on ABP were accompanied by changes in the High and Low frequency components of HRV and ABP variability which suggested increased vagal control of the heart and decreased sympathetic control of heart and blood vessels Taylor et al. (2003). In contrast, Stiller-Moldovan et al. (2012) conducted a similar study on older individuals with well-controlled HTN and found no change in HRV despite significant reduction in resting ABP. Similar findings were reported by Millar et al. (2013). Thus, it could be that in individuals with well-controlled HTN, IHG training may not have the potential to improve their autonomic function as much, but it would also seem that changes in HRV do not fully explain the hypotensive effects of IHG training. A study performed by Badrov et al. (2013A) supported this idea in that IHG training for 8 weeks in normotensive women, reduced resting ABP but did not change any index of autonomic activity as measured by HRV.

As far as sympathetic activity to vasculature is concerned, healthy normotensive male subjects who performed 2 minutes of IHG contractions at 10% MVC showed a substantial 30% increase in muscle sympathetic nerve activity (MSNA) as recorded from the tibial nerve (Saito et al., 1986). This was confirmed by (Ray and Carrasco, 2000) in healthy normotensive subjects who performed IHG training comprising four 3-minute contractions at 30% MVC, four times per week for 5 weeks, but they also showed that although resting ABP was significantly decreased, there was no change in baseline MSNA. Similar findings were reported by (Saito et al., 1986). Thus, it seems unlikely the hypotensive effect of IHG training is due to a decrease in sympathetic activity to muscle vasculature, one of the major contributors to peripheral vascular resistance. However, given the evidence that HTN is driven by sympathetic over-

activation (Mancia, 2014), it would be helpful if MSNA were to be recorded in a study of IHG training in hypertensives.

1.10.2 Endothelial-dependent dilator function

The idea that the ABP reduction elicited by IHG training is caused by increased endothelium-dependent vasodilation occurring locally, or systemically has been investigated in several studies. In brief, in 16 medicated subjects with HTN who performed 4, 2-minute unilateral or bilateral IHG contractions at 30% MVC 3 times per week for 8 weeks, FMD in the brachial artery was increased in both forearms during the bilateral protocol, but only in the trained arm with unilateral IHG training (McGowan et al., 2006A). Further, the same group (McGowan et al., 2006B) showed that the same unilateral training protocol in medicated hypertensives improved FMD in the trained arm, but had no effect on peak reactive hyperaemia or peak response to the endothelium-independent dilator sodium nitroprusside, as assessed by forearm blood flow (FBF) calculated from the product of brachial artery diameter and blood velocity measured by ultrasound. They therefore concluded that the change in FMD in medicated hypertensives could be attributed to augmented endothelium dependent dilatation of the brachial artery of the trained arm, but with no change in endothelium-dependent or -independent dilatation of the resistance vasculature.

By contrast, McGowan et al. (2007), observed no change in FMD in the trained arm of young normotensive men and women following 8 weeks of IHG training with the standard protocol. On the other hand, Badrov et al. (2013A) found that in young normotensive women who performed IHG training 3 or 5 times per week for 8 weeks, the decrease in resting ABP was accompanied by an increase in peak forearm reactive hyperaemia by 42% and 57%, respectively as assessed by ultrasound. Neither study investigated responses in the non-trained arm. These findings indicate that at least in young normotensives, IHG training 3 times and 5 times per week for an 8 week of IHG training does improve endothelial function in the resistance vessels of the trained arm, but they give no indication of whether similar changes might occur in the contralateral arm, or systemically.

1.10.3 Reactive oxygen species

Peters et al. (2006) hypothesized that the repeated ischemia reperfusion episodes caused by IHG training protocols increases antioxidant activity, decreasing ROS, and changing the balance of vasodilator and vasoconstrictor substances acting on the blood vessels, so reducing ABP. They tested this idea in a group of hypertensives who performed 4 sets of 45-second unilateral IHG at 50% MVC 3 times/week for 6 weeks, which induced a reduction in SP and DP of 13 and 2 mmHg respectively. There was a blunting of the increase in plasma levels of ROS induced by a 12 min graded exercise test from +132% to +36% (-266%); resting ROS levels did not change but there was an increase in resting levels of oxidized glutathione (+61%), indicating increased antioxidant protection. Since ROS can limit the availability of endothelial NO, as indicated above, these results would be consistent with IHG training increasing the availability of NO and supporting endothelial-dependent dilation. Unfortunately, no vascular responses were tested in this study.

1.10.4 Shear Stress

There is substantial evidence *in vitro* that sustained and repeated increases in shear stress increase the expression and activity of NOS and COX in endothelial cell layers (Lu and Kassab, 2011, Laughlin et al., 2008). Consistent with this evidence it was shown *in vivo*, that dogs who exercised regularly on a treadmill for 10 days demonstrated increases in eNOS mRNA and protein expression and increased release of nitrite a stable metabolite of NO from coronary endothelium in response to ACh (Sessa et al., 1994).

Turning to human studies of relevance to the present thesis, it was shown that 3 months of predominantly lower limb exercise involving a graded walking programme augmented forearm vasodilator responses to ACh in older men who were previously sedentary (DeSouza et al., 2000). This study suggested that the improvements in endothelial dilator function may occur at sites remote from the muscles undertaking training. Further, Green et al. (2002) showed that graded cycling exercise in young healthy men produced graded increases in mean blood flow and peak systolic blood flow in the forearm that were attenuated by NOS inhibition. They proposed this reflected local synthesis and release of NO in the forearm stimulated by increased shear

stress arising from either increased heart rate or pulsatile flow. A subsequent study revealed that changes in HR induced by pacing in the absence of exercise, did not increase mean FBF or systolic flow and NOS inhibition had no additional effect compared to control. These results indicated that during exercise, changes in pulsatile pressure or flow including shear stress act as an important stimulus for NO release in non-exercising limb (Green et al. (2004).

Further studies by the same group showed by analysing recordings of blood velocity made with Doppler ultrasound, that brachial artery blood flow and shear rate were augmented with progressive levels of rhythmic handgrip exercise in healthy individuals. In fact, there was an intensity-dependent increase in antegrade shear, with negligible levels of retrograde rate, during rhythmic handgrip exercise (Green et al., 2005). Further, similar to these observations regarding redundancy and blockade effects during exercise-hyperaemia (Joyner and Casey, 2015), the increase in blood flow and the shear rate patterns observed in the brachial artery during incremental handgrip exercise were modestly attenuated in the presence of a NO synthase blocker (Green et al., 2005).

Subsequent studies investigated the effects of exercise training on endothelium-dependent dilator function, recognising that exercise training improves dilator function and induces structural changes in the vasculature, but that the contribution of NO to endothelium-dependent dilatation seemed to increase with short term training, but return to normal with longer training (see Tinken et al. (2008). They therefore hypothesised that during short-term exercise training the increased shear stress improves endothelium-dependent dilator function by releasing NO and upregulating NOS, the dilatation then normalizing shear stress. However, longer exercise leads to structural enlargement of the vasculature so normalising shear stress and restoring the stimulus for NO production to baseline. To test this hypothesis, Tinken et al. (2008) investigated both brachial and popliteal artery function and structure across 8 weeks of exercise training in healthy volunteers, by using FMD as an index of NO-dependent conduit artery dilator function and maximal dilator capacity of the vasculature served by the brachial artery following ischaemic exercise as an index of vascular remodelling. The results supported the hypothesis that exercise training resulted in an initial

improvement in FMD at 2 and 4 weeks, which returns towards baseline by 8 weeks, while dilator capacity increased progressively over the 8 weeks.

To investigate the importance of shear stress in improving FMD acutely, Tinken et al. (2009), investigated FMD in both arms before and after 30-min bilateral rhythmic handgrip exercise, cycle exercise, and bilateral forearm heating, stimuli which had similar effects on anterograde flow in the brachial artery but different effects on retrograde flow. In one arm, shear rate was prevented from increasing by inflating a sphygmomanometer cuff to 60mmHg. In the non-cuffed arm, FMD was similarly augmented by 30 minutes of each stimulus, whereas in the cuffed arm there was no change in FMD after any of the stimuli (Tinken et al., 2009). The results indicated that the increase in anterograde flow and shear stress, does indeed increase endothelial NO activity acutely given FMD is virtually abolished by NOS inhibition (Green et al., 2004).

The role of shear stress during exercise training was investigated by Tinken et al. (2010) who tested the effects of 8 weeks of bilateral rhythmic handgrip training while shear stress was prevented from increasing in one arm by inflating a cuff around the upper arm to 60mmHg. They found that FMD increased in the brachial artery of the control arm at 2, 4 and 6 weeks of training, although not at 8 weeks, but there were no changes in the cuffed arm. Meanwhile maximum dilator capacity following ischaemic exercise increased progressively in the non-cuffed arm only (Tinken et al., 2010). These results indicated that endothelium dependent dilatation in the brachial artery is augmented by the increased shear stress associated with rhythmic handgrip training. However, no tests were made of whether endothelium-dependent dilatator function to submaximal dilator stimuli were increased in the resistance vessels. Further the design of these studies meant that any effects of increased shear stress in augmenting endothelium-dependent dilatation in non-trained limbs were not tested. In addition, no attention has been paid to whether PGs might contribute to the beneficial effects of exercise training even though shear stress is known to increase COX activity and PG release (Bhagyalakshmi and Frangos, 1989, Berthiaume and Frangos, 1992, Koller et al., 1993).

Nothing similar has been done to investigate the role of shear stress during IHG training. However, there is evidence to suggest that blood flow may increase in non-exercising limbs during isometric contraction of one limb. If this happens during the

protocol that is generally used in IHG training, then such findings could provide evidence in support of shear stress being involved in the beneficial effects of such training. This evidence is reviewed below.

1.11 Vascular responses in resting limbs during isometric contractions

The metaboreflex plays a pivotal role in the control of the cardiovascular system during exercise. The term was coined to describe the response evoked by muscle afferent nerves stimulated by metabolites accumulating in the muscle interstitium and it is sometimes referred to as the 'the muscle chemoreflex' (Mitchell, 1990, Kaufman et al., 1983). In humans, the metaboreflex was first demonstrated by Krogh and Lindhard (1917) who demonstrated a rise in heart rate and ABP in the absence of signals from the brain, during muscle activity. These key findings were investigated further by Alam and Smirk in the 1930s who found that the rise in ABP and heart rate (Alam and Smirk, 1937, 1938) evoked by isometric contraction remained elevated during post-exercise circulatory occlusion (PECO) of the exercising arm, returning to pre-exercise levels when occlusion was released. These findings indicated a metabolic stimulus that was maintained during occlusion and was making a major contribution to the increase in ABP by sending afferent signals to the brain. Similarly, it was shown that circulatory occlusion potentiated the increase in mean systemic arterial pressure evoked by static contraction (Staunton et al., 1964), consistent with increasing the accumulation of metabolites in the muscular interstitium, causing greater stimulation of the sensory receptors in the active limb, and potentiating the metaboreflex.

Animal models have helped to identify the sensory afferents which mediated the response as group III and IV muscle sensory nerves (Kaufman 2012). The factors implicated in stimulating the metaboreceptors in the exercising limb include K^+, adenosine, PGI_2, lactate H^+, (McCloskey and Mitchell, 1972, Kaufman et al., 1983). The metaboreflex has been shown to elevate ABP via an increase in sympathetic nerve activity to the heart and to the major vascular beds including skeletal muscle: it is generally considered that the metaboreflex helps to maintain adequate perfusion to exercising skeletal muscle by reducing blood flow to inactive muscle groups (Murphy et al., 2011). Indeed, early studies, involving acute IHG exercise of one arm indicated

that reflex vasoconstriction occurred in the contralateral forearm, when that forearm was truly at rest (Lind et al., 1964).

1.11.1 Responses reported in resting limbs.

Despite the evidence just reviewed, vascular responses in the resting limbs during static contractions have proven to be equivocal, in that increases in blood flow and vasodilation have been reported in non-exercising limbs during single limb exercise (Eklund, 1974, Sanders et al., 1989, Matsukawa, 2013). For example, healthy male subjects who performed three 2-minute IHG dominant arm contractions at 33% MVC, showed a 2.5 fold increase in FBF in the non-dominant arm during the first minute of contraction (Eklund, 1974). Vascular resistance in the non-exercising arm significantly decreased during the first minute of the contraction, indicating vasodilation, but then increased steadily towards baseline values, consistent with reflex vasoconstriction. Lind et al. (1964) made similar observations but found that the increase in blood flow was dependent on the strength of the contraction. They suggested the increased blood flow responses was partly attributable to increased perfusion pressure (ABP) when another group of muscles contracts at higher force, but also noted that increases in blood flow most often occurred when EMG activity was evident in the "resting limb". Cotzias and Marshall (1993) substantiated this idea, demonstrating that graded vasoconstriction occurred in the non-exercising forearm during graded IHG of the contralateral arm, when there was no EMG activity in the resting limb, whereas vasodilation occurred when their EMG activity could be detected.

However, Sanders et al. (1989) reported that initial vasodilation occurred in the non-contracting forearm in the *absence* of EMG activity in the first minute of IHG contraction of the contralateral arm, as indicated by a fall in forearm vascular resistance and an increase in forearm blood flow. It has been proposed that vasodilation occurring during IHG in limbs that are not intended to be contracting, reflects exercise hyperaemia due to inadvertent contractions of that limb, or is a response to circulating adrenaline, or to nerve-released or locally released ACh or NO (Reed et al., 2000, Martin et al., 1996, Wilson et al., 2016). Therefore, another aim for this PhD project that a session of IHG training will be tested for any vasodilatory and EMG activity changes on the ipsilateral leg and investigate the changes in prostaglandin production

after COX inhibition. These issues are discussed in more detail in the Introduction to Chapter 6.

1.12 Statement of the problem

As demonstrated above very little attention has been paid to the changes that may occur in the non-trained arm during unilateral IHG training and yet such remote effects are very relevant given the substantial evidence that IHG training reduces resting ABP and given the fragmentary evidence that IHG training facilitates endothelium dependent dilation in the trained arm. Hence the aims for this PhD project were:

1) following IHG training of 4-5 weeks at 30% MVC changes in muscle and cutaneous vasodilation will occur in both the unilateral and contralateral arm and
2) whether any differences between young WEs and SAs and between young and older WEs will be demonstrated after IHG training of 4-5 weeks and
3) also, to investigate effects of IHG training of 4-5 weeks on plasma samples of prostacyclin, nitrite/nitrate for nitric oxide, potassium and isoprostane on the contralateral arm to explore mechanisms of IHG training adaptations.

Therefore, the primary aim of the present study was to investigate the effect of IHG training of the dominant arm for 4-5 weeks on responses evoked acutely in the dominant arm during exercise hyperaemia and reactive hyperaemia, known endothelium-dependent dilator responses, by using VOP to record FBF. As the published findings indicate shear stress-mediated effects of IHG training could occur in non-trained limbs, a secondary, but important, aim was to investigate the effects of unilateral IHG training, on blood flow and vascular resistance responses evoked by exercise hyperaemia, reactive hyperaemia in the contralateral arm. In addition, the laser doppler technique was used to investigate the effects of IHG training on the cutaneous circulation of the contralateral arm by reactive hyperaemia and the established endothelium-dependent dilator ACh, delivered by iontophoresis.

To investigate these hypotheses young, WE and SA men as well as healthy sedentary older WE men were recruited to the study on the basis that endothelium dependent dilatation is blunted in both SAs and older subjects. It was hypothesised that endothelium-dependent dilator responses in the trained and non-trained arm would be

enhanced by IHG training and would be particularly improved in young SAs and older WEs.

Since COX products have been implicated in endothelium dependent dilatation and since COX products are known to induce vasoconstriction rather than vasodilation in conditions of endothelial dysfunction, the effects of COX inhibition were tested in the cutaneous circulation of the non-trained limb before and after IHG training. It was hypothesised that any contribution of vasoconstrictor COX products would be attenuated or changed to show involvement of vasodilator COX products particularly, in young SA and older WE men.

In addition, potential changes in the role of other factors implicated in endothelium-dependent responses were tested by assaying K^+, PGI_2, NO metabolites and 8-isoprostane, a well-established marker of oxidative stress (Milne et al., 2005, Montuschi, 2007) released into the venous efflux of the non-trained arm at peak reactive hyperaemia, before and after IHG training. It was hypothesised that shear rate was likely to be maximal immediately arterial occlusion was released at peak reactive hyperaemia. Further, that an increased release of all four substances would occur at peak reactive hyperaemia, but the release of K^+, PGI_2, NO metabolites would be enhanced following IHG training, particularly in young SA and older WE men, whereas release of isoprostane, as a marker of endothelial oxidative stress, might be decreased.

In addition, limb blood flow and vascular resistance, together with EMG activity and cutaneous perfusion were recorded in the contralateral forearm and ipsilateral calf of young WEs and SAs during a single bout of acute IHG training before and after COX inhibition. It was hypothesised that increased shear stress would cause vasodilation and increased blood flow in the contralateral forearm and ipsilateral limb in the absence of EMG activity, that the responses would be larger in WEs than SAs and attenuated more by COX inhibition in WEs.

2. General Materials and Methods

2.1 Subjects

All experiments were performed on recreationally active young White European (WE) and South Asian men (with family roots in India, Pakistan, Nepal, Sri Lanka) and on recreationally active older WE males recruited from the under- and postgraduate student body of the University for young participants, and from the 1000 Elders Group (a data base held by the Institute of Immunity and Infection, University of Birmingham,) and employees of the University of Birmingham. They were all healthy - free from any cardiovascular, respiratory and/or metabolic disease (diabetes etc.); young WEs and SAs were 18-25 years old and the older WEs were 55-70 years old.

2.2 Questionnaires

The subjects had to be non-obese, non-smoking individuals with limited alcohol consumption of < 21 units of alcohol per week (Table 2.1) according to guidelines from the international diabetes federation (International Diabetes Federation, 2006). This information together with other personal information was collected by using questionnaires completed in their own time and in the familiarisation visit.

Ethnicity was self-declared according to the grouping provided by the Office for National Statistics (on-line); all were UK residents with both parents of White European ethnicity or South Asian ethnicity, ethnicity being defined as common ancestry, language, society, culture and/or nation. Subjects were generally classified as 'recreationally active', based on an activity status questionnaire. For most studies, this included time spent in different activities (Appendix 1). For the last two studies (see Chapter 4 and 5), the International Physical Activity and Recreation questionnaire (I-PARQ) (see Appendix 2) was used to provide a deeper understanding of the physical activity status of each individual as well as suitability for participation to the study (Warburton, 2011). Those who were designated as 'trained' or 'sedentary' (≥ 150 minutes moderate-to-vigorous physical activity (MVPA)/week = physically active for adults and ≥ 150 minutes moderate physical activity (MPA)/week = physically active for older adults) were excluded (Cristi-Montero, 2017). Each subject also answered questions on date of birth, medical history namely, smoking status, history of disease, family history of disease including hypertension and other CVD. They also answered questions on alcohol consumption, dietary patterns and participation in other studies see (Appendix 1).

In addition, at the first meeting, recordings were made of height (cm.), weight (kg.), body mass index (BMI), waist to hip ratio (WHR), maximum grip strength in both the dominant and non-dominant arms by using hand dynamometer (Lafayette-Model 78010) mounted on an iron base and, resting ABP after 10 mins of rest on supine position by using an arm blood pressure monitor (a standard arm blood pressure monitor [M4 (HEM-722C1-E, OMRON Matsusaka Co, Ltd, Japan; EU representative: OMRON Healthcare Europe B.V., Wegalaan 57, NL-2132 JD Hoofddorp). In addition, the familiarization visit was used to record maximum voluntary contraction (MVC) for handgrip. The participant was asked to sit in the experimental position and grip the handgrip dynamometer (Lafayette 70718, Loughborough, U.K.) with the dominant hand as powerfully as possible and to hold the contraction for 5s. The force measured at the end of the 5s was recorded as MVC. This was done 3 times separated by 30s to confirm reproducibility and avoid snap contraction readings. The average of the 3 contractions for each arm was taken as 100% MVC for that arm. This procedure was repeated with the non-dominant hand. Women were not included in any of the studies to avoid the effect changes in hormonal levels associated with the menstrual cycle and/or contraceptive pill might have had on cardiovascular responses. All studies were approved by the University of Birmingham's Science, Technology, Engineering, and Mathematics Ethical Review Committee (application number ERN_14-1395) and complied with the declaration of Helsinki. Two different set ups were used for 2 different types of experiment. The first experimental set up was forearm blood flow (FBF) by venous occlusion plethysmography; The other was used to measure forearm cutaneous red cell flux (RCF) with a laser Doppler probe.

Inclusion criteria	Exclusion criteria
❖ **Young Caucasian (18-25 years) and older Caucasian adults (55-70 years)** ❖ **Young South Asian (18-25 years)** ❖ **Absence of any cardiovascular, respiratory and/or metabolic disease (diabetes etc.)** ❖ **Recreationally active, non-obese, non-smoking** individuals with a **limited alcohol consumption to 21 units of alcohol per week.**	❖ Other Asian background (Chinese, etc.) ❖ Identified with cardiovascular risk markers, (BMI, WHR) ❖ Under medication ❖ Trained or sedentary individuals ❖ Women

Table 2.1: **Inclusion and Exclusion criteria**.

2.3 Anthropometry

Subjects were assessed wearing light clothing and without shoes. Height (cm) was measured with a wall-mounted stadiometer (STAT 7X, Ellard Instrumentation Ltd., Monroe, WA, USA). Body mass was measured (kg) using a medical scale (SECA, Williams Medical Supplies (WMS). BMI (kg/m^2) was calculated by dividing body mass by height squared. To calculate waist to hip ratio (WHR), waist circumference (cm) was measured around the narrowest point between the hips and ribs. Hip circumference (cm) was measured at the greatest gluteal protuberance (WHO, 2008). Each measurement was repeated twice by the same researcher to decrease intra-observer variability and the average of the two measurements were taken.

2.4 Preparation for studies

For each study, the subject was required to attend an initial familiarization visit before the experiments at which the questionnaires mentioned above were completed. (Appendix 3). Subjects who fitted the eligibility criteria were then shown the equipment and its use to facilitate habituation to the experimental surroundings. All experimental procedures were explained to the subjects before they agreed to participate and gave

informed consent (see Appendix 2). Subjects were required to abstain from alcohol and strenuous exercise for 24h before experiments proper (see below), and from heavy meals and caffeinated drinks for 12h. Where appropriate, experiments were performed in a randomised single-blind manner.

2.5 Experimental Conditions

Each experiment was performed at room temperature (19-24^{o} C) while the subject was seated comfortably on a couch with backrest at ~65^{o} to the horizontal. Room temperature varied by no more than ~1-2^{o} during the course of each experiment. Both arms were supported at approximately the level of the heart. Generally, the legs were stretched out horizontally. The exception was the studies presented in Chapter 6 in which one leg was slightly raised for the placement of the recording equipment and in one part of the study in each of Chapters 3 and 4 in which the subject was seated on a chair with the feet resting on the floor and the backrest at ~85^{o} to the horizontal. To minimize any distractions, the room was organised so that the subject was unable to see the recordings, and noise and the visual distractions were kept at an absolute minimum. Participants could watch a movie, a documentary or listen to relaxing music if they wished, to avoid falling asleep.

2.6 Arterial Blood Pressure and Heart Rate

When the subject had been resting for ~15min, resting Arterial blood pressure (ABP) was measured three times at intervals of 2 min from the subject's dominant arm by using a standard arm blood pressure monitor [M4 (HEM-722C1-E, OMRON Matsusaka Co, Ltd, Japan; EU representative: OMRON Healthcare Europe B.V., Wegalaan 57, NL-2132 JD Hoofddorp).

Thereafter, ABP was continuously recorded at heart level, by means of an automatically calibrating Finapres monitor (Ohmeda 2300, Englewood, USA), which consists of a small finger-cuff that was fastened around the middle phalanx of the middle finger of the non-exercising hand. This monitor uses the principles of infrared photo-plethysmography in combination with an inflatable bladder. The photo-sensor detects the scatter and absorption of the infrared light while the bladder inflates and deflates in response to changes in volume and pressure, allowing a continuous measurement of ABP. Before the fourth study (Chapter 6), one of the Finapres machines malfunctioned

and a portable model called Portapres (Finapress Medical Systems B.V., Portapress ®) was used instead that functions on the same principles.

Heart Rate (HR) was monitored from the Finapres, computed on-line from the intervals between systolic pressure peaks, using ADInstruments Labchart software (version 8). Mean arterial pressure (MABP) was also derived on-line from the ABP recording by Lab Chart software and was displayed on the computer.

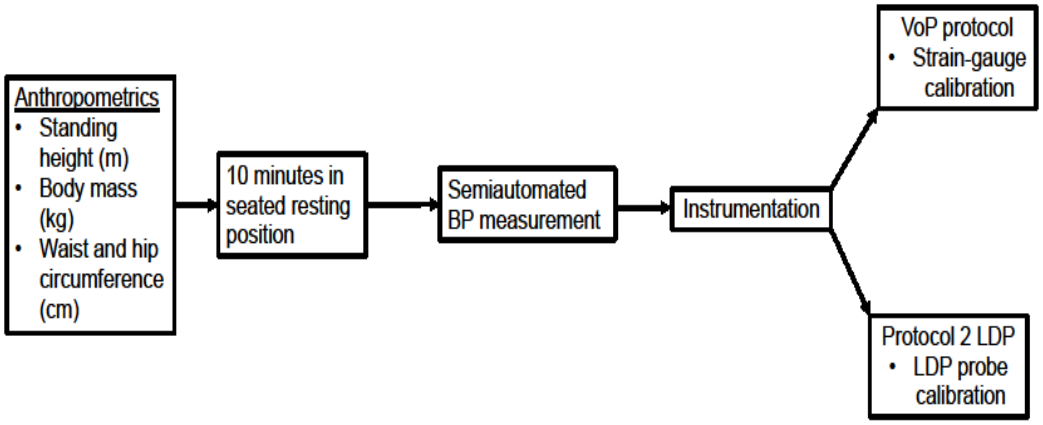


Figure 2.1: **Overview of laboratory testing protocol.**

BP=blood pressure, VOP = Venous Occlusion Plethysmography, LDP= Laser Doppler Perfusion

2.7 Experimental Recordings

2.7.1 Limb blood flow

Strain gauge venous occlusion plethysmography (VOP) was used to record limb blood flow. Depending on the protocol, the equipment was placed on the forearm for forearm blood flow recordings (FBF), or on the calf, to record calf blood flow (CBF). VOP is a non-invasive technique, which was established over 100 years ago. The modern version of VOP involves wrapping an appropriate-sized Indium-Gallium silastic strain-gauge

around the widest circumference of the limb and fastening it securely in place (Joyner et al., 2001). The principles underlying VOP are described in brief below.

2.7.2 Principles of venous occlusion plethysmography

Venous occlusion plethysmography (VOP) was initially used to record blood flow in the spleen by Schafer and Moore (Schafer and Moore, 1896). Later, the technique was adapted to record limb blood flow by Hewlett and Von Zwaluwenburg (Hewlett, 1909). The principle underlying the technique is that occlusion of the venous drainage of a circulation for example, an arm or a leg by inflating a cuff on the upper arm or thigh respectively to a pressure that is higher than venous pressure, but below diastolic pressure is that blood can continue to flow into the limb but cannot leave it (Wilkinson and Webb, 2001, Joyner et al., 2001). Consequently, the venous vessels fill with blood with each heart beat due to continuing arterial inflow, thus increasing limb volume: the rate of change in limb volume therefore reflects the arterial inflow (Joyner et al., 2001, Wilkinson and Webb, 2001). Clearly, the rate of change of limb volume depends on the difference between arterial and venous pressures; venous pressure increases as the venous vessels fill with blood, and this in turn slows the rate of arterial inflow. Therefore, the initial rate of change in limb volume reflects the arterial blood flow at the time the veins were occluded.

Originally, VOP involved using a water-filled container in a form of a rigid box. The recording limb was sealed within the container and blood flow was measured by inflating the venous occlusion cuff and measuring the displacement of water surrounding the limb (Joyner et al., 2001, Whitney, 1953). This technique had two major limitations. Firstly, the subject had to remain still during the whole experimental time as any movement interfered with the displacement of water around the limb (Whitney, 1953). Furthermore, maintaining a watertight seal around the limb was extremely difficult; any leak seriously limited the accuracy of the technique (Whitney, 1953, Joyner et al., 2001).

In view of these problems, Whitney devised a way of addressing them by recording changes in limb circumference rather than limb volume (Whitney, 1953). He reasoned that if the limb were an ideal cylinder, the percentage change in an area of any given transverse section of the limb will always be twice the percentage change in

circumference [Δ(total) Velocity/Velocity=2 Δ(total) Conductance/Conductance, or Δ(total) Velocity=2 Δ(total) Conductance]. Assuming the length of the cylinder (limb) remains unaltered, the volume change following venous occlusion would be accommodated within the cross-sectional area. Thus, Whitney measured changes in limb volume by using a thin rubber tubing filled with mercury and attached to a strain-gauge and through which a small amount of current was applied and was fastened around the widest part of the limb. Following occlusion of the venous outflow, the circumference of the limb increased, lengthening the tubing and causing an increase in the electrical resistance which can be recorded as a change in voltage. Therefore, Whitney demonstrated that Ohm's Law could be used to calculate circumference (length) if one measured the change in voltage produced when a constant current is delivered through a conductor (Whitney, 1953). He compared the two methods (the comparison of the gauge method with water plethysmographic method) with Ohm's Law (voltage = current x resistance) to provide the basis for the use of one such device (i.e., mercury filled rubber strain-gauge), which could record change in limb circumference. Subsequently, he used it to measure the change in volume and he demonstrated that both methods showed close similarities. The strain gauge method eliminated the disadvantages of water plethysmographic method mentioned above. The "initial" gradient (i.e. the rate of change) of this recording was employed to calculate resting blood flow (Greenfield et al., 1963, Whitney, 1953) and was usually estimated over at least 3-4 heart beats (Tschakovsky et al., 1995). In order to calibrate the device, an adjusting screw on the strain-gauge was turned by a known amount so increasing or decreasing stretch on the mercury-filled tubing and decreasing or increasing respectively, its length by a known amount so that the change in voltage was recorded (Joyner et al., 2001, Whitney, 1953).

Blood flow was typically expressed relative to dl of tissue volume per unit time; but can be expressed as ml/min if the volume of the limb is calculated by using the volume displacement method (Wilkinson and Webb, 2001).

In the 1970s, Hokanson noted that if the length of a strain-gauge is exactly the same as the circumference of the limb, the linearly proportional relationship between resistance and volume can be expressed as: Δ(total) Resistance (R)/Resistance (R)= Δ(total) Velocity (V)/Velocity (V) or Δ(total) Resistance (R) = Δ(total) Velocity (V) (Hokanson

et al., 1975). By utilizing this observation, a system was developed that allowed one arm of the Wheat-bridge to be altered by 1% to provide a simpler electrical calibration of the mounted strain-gauge (Hokanson et al., 1975). The commercially available Hokanson's plethysmograph equipment allows a step deflection of the plethysmograph to be applied and provides a 2-point calibration (c) signal; the difference between the two values not only signifies a 1% change in volume, but also a similar change in the length of the mounted strain-gauge (i.e. limb circumference) (Tschakovsky et al., 1995). This removed the need to separately account for the initial limb circumference and simplified the algorithm to:

This can simply be expressed as:

$$\text{Limb Blood Flow (ml/dl/min)} = \frac{2\ x\ b\ x\ 60\ (\frac{s}{\text{min}})}{c}$$

Where; b = ''initial'' gradient of the plethysmograph trace

c = value of calibration deflection (voltage difference between 0 and 1% deflection)

s= seconds

It might appear that the initial gradient of the plethysmograph trace rises linearly following venous occlusion; and this has sometimes been suggested in the literature (Greenfield et al., 1963, Wilkinson and Webb, 2001).

However, experiments conducted by Tschakovsky et al. (1995) using the Hokanson rapid cuff inflation system, provided evidence that following inflation of the venous cuff to 50 mmHg there is a decrease in the rate of rise of plethysmograph trace (i.e. arterial inflow) over subsequent beats as recorded simultaneously with Doppler ultrasound, particularly when blood flow is raised. This demonstrated that FBF measured by strain gauge is reliable and valid only when it is calculated on the first beat following cuff inflation: inclusion of subsequent beats where the appearance of mechanical factors is considered to reduce the validity of the FBF estimation (Tschakovsky et al., 1995). The recommendations of Tschakovsky et al were further investigated by Junejo et al. (2019). They explored the influence on the measured values of FBF of an apparently negligible delay (~1-2s) introduced by inflation of the venous occlusion cuff manually vs. by automatic rapid cuff inflation as done in some studies.

This study was also relevant because Wythe et al. (2015) used the rapid cuff inflator system with a delay of 4 s before calculating FBF that take as long as 1-2 s to inflate the venous occlusion cuff which leads to underestimation of FBF as shown from (Tschakovsky et al., 1995) and further validated by Junejo et al. (2019).

The VOP method was preferred in the present study rather than other methods such as doppler ultrasound because ultrasound demonstrates random error during repeated measurements (errors on probe operator, improper alignment of the ultrasound beam with the artery and the doppler processing and frequency estimation) (Tschakovsky et al., 1995). It is rather difficult to obtain an accurate measurement in vessel diameter smaller than 3±4 mm. The vessel diameter that is commonly used is 5±10 mm and even then, the accuracy of the doppler can reach at best 20-30%. In addition, it requires high level of training to use this equipment (Wilkinson and Webb, 2001). Further, VOP is one of the most powerful techniques to measure changes in blood flow for over 120 years (Joyner et al., 2001). Thus, venous occlusion plethysmography method was selected and used in the present study as described below:

2.7.3 Application of VoP in the present studies

For each recording of blood flow, a small cuff (Omron, paediatric cuff, SS 12-18 cm, 5-7 inch) on the wrist or ankle was first inflated to >200mmHg to occlude blood flow to the hand or foot, so allowing the measurement to be dominated by skeletal muscle of the limb. A manually operated sphygmomanometer (Accosson, mercurial sphygmomanometer, A.C. Cossor & son (surgical) LTD, Accoson Works, 5/6 Parkway, Harlow business park, Harlow, Essex, CM19 5QP, UK) was used to inflate this cuff. A larger cuff which was wrapped around the upper arm or thigh (Hokanson, SC 10D, Bellevue WA, 98005, USA) was then inflated to ~50 mmHg, restricting the venous drainage but allowing arterial flow. This was done, with the rapid cuff inflator system (E20, Rapid Cuff-inflator, D.E. Hokanson Inc., USA). The smaller cuff was always inflated ~5-10 s before the larger cuff.

An electrically calibrating strain-gauge plethysmograph device (EC6 Plethysmograph, D.E. Hokanson Inc., USA) was used to measure the rate the change of limb circumference (Hokanson et al., 1975). As shown in Figure 2.2, the device was calibrated by generating a step deflection of 1% in the voltage-output of the trace. As

indicated above, this method of 2-point calibration (0 and 1%) simulates a 1% change in the length of the mounted strain gauge (i.e., limb circumference), and a similar change in the limb volume (Tschakovsky et al., 1995). This was done 3 times and the average calibration value along with the initial gradient of the slope of plethysmograph trace was then used to calculate blood flow using the algorithm from above:

$$\dot{:}\text{Limb Blood Flow (ml/dl/min)} = \frac{2\ x\ b\ x\ 60\ (\frac{s}{\text{min}})}{c}$$

When the plethysmograph recording trace moved off scale during forearm exercise (See Figure 2.3), due to the change in shape of the forearm muscles. Thus, the device was re-balanced to move the plethysmograph trace back to the middle of the chart. This does not affect the calibration of the strain-gauge but allows a VOP recording of FBF to be made immediately when handgrip contractions ceased and at intervals afterwards.

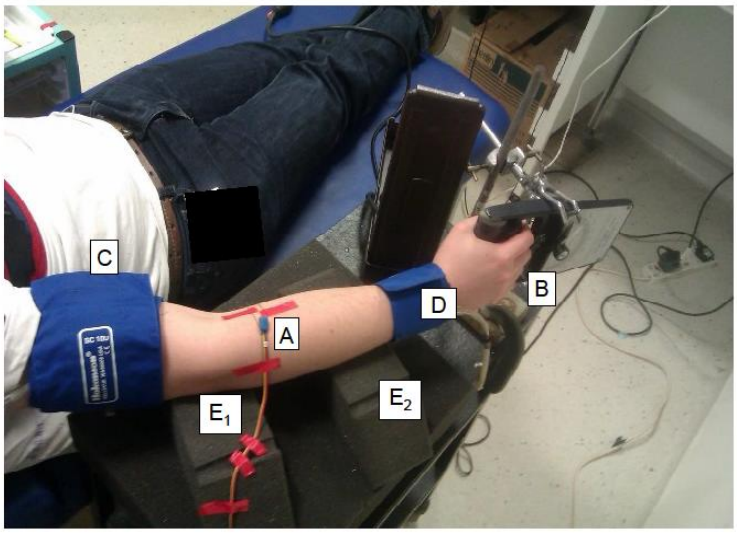


Figure 2.2: **Arrangement used for venous occlusion plethysmography and handgrip contractions**. Strain-gauge (A) was placed around the widest part of the forearm and secured with paper tape. Handgrip dynamometer (B) was secured with a clamp and elbow of each subject allowed to rest on two foam blocks (E_1, E_2). Handgrip dynamometer (B) was connected with a visual display -unit that was used to perform rhythmic and isometric handgrip contractions at required MVC intensities. This setup aided recording of blood flow at the level of the heart and avoided interference of the strain-gauge with the surface of the couch. Hokanson sphygmomanometer cuff on upper arm (C), which was attached to rapid inflation system (Plethysmograph- EC6; Rapid cuff inflation system-E20; Cuff inflator air source-AG101). Paediatric cuff (D) wrapped around the wrist, which was inflated manually to restrict blood flow to the hand.

2.7.4 Digital and forearm red cell flux (RCF)

A Laser Doppler Perfusion monitor, (Moor Instruments Ltd (Laser-Doppler Perfusion and Temperature Monitor, DRT4, Millwey Axminster, Devon, UK) and probe were used to record cutaneous RCF. The probe of 10mm circumference (DPITV2; Moor Instruments) is mounted at the end of a cable containing a bundle of fibres. For example, at 2 cm spacing, the light penetration into tissue is estimated to be approximately 0.5–1 mm deep (Rajan et al., 2009). In most experiments, the laser-doppler probe (DPITV2; Moor Instruments) was used to monitor red cell flux (RCF) after being placed into an iontophoresis chamber which was attached to the anterior surface of the forearm by using double-sided adhesive discs as described below.

2.7.5 RCF recordings during iontophoresis of ACh

The method of iontophoresis was applied as described previously (Morris and Shore, 1996, Hendry and Marshall, 2004). A Perspex ring-shaped iontophoresis electrode chamber (30 mm total diameter and 7 mm height with an 8 mm diameter inner 'drug' chamber; Moor Instruments, Axminster, Devon, U.K.) was attached to the skin by means of a double-sided adhesive ring. A sticky pad containing electrogel, which acted as an indifferent electrode was placed next to the Perspex chamber to complete the circuit. The drug chamber was then filled with approximately 0.5-0.8 ml dilutant of ACh (A6625-Sigma Aldritch Acetylcholine chloride ≥99%). The laser Doppler probe was then inserted into the chamber to allow continuous measurements of cutaneous RCF. The indifferent electrode and the Perspex chamber were connected to a battery-charged iontophoresis controller (MIC 1; Moor Instruments) that provided the current for iontophoresis. Both the Doppler probe and iontophoresis controller were connected to a Dell computer (Dell Inc, USA) via a Powerlab data acquisition hardware (Power-Lab, AD Instruments Inc., Colorado Springs, CO USA), so that RCF and the iontophoresis current could be recorded. The polarity of the iontophoresis electrodes was set according to the drug applied. Thus, ACh is positively charged, and the electrode connected to the plastic adhesive disk was negatively charged while the sticky patch connected to the indifferent electrode was positively charged to create a circuit. The recordings were made while the subject sat in a comfortable armchair with arms supported at heart level on a table with the dominant arm rested on a foam block. An area on the anterior skin of the non-trained non-dominant arm was identified with the

laser-doppler probe (DPITV2; Moor Instruments) when RCF response signal was close to zero. This avoided interference from superficial veins. If hairs were present, they were removed by a disposable razor (2-blade disposable razor, Sainsbury's) to avoid interference with the laser signal. Then the skin area was cleansed with an alcohol swab. ABP and HR were measured and baseline RCF was recorded for approximately 5 min. The reactive hyperaemia protocol was then applied (see below) and another equilibration period followed, and the iontophoresis period followed (see below). At the end of the experimental session, ABP and HR were measured again, and RCF was recorded for a further 2 min or until the ACh effects started to withdraw.

For reactive hyperaemia protocol see section 2.4.7. Approximately 5 min later, ACh was delivered into the skin by 8 separate pulses of 20 s each, at intervals of 60s; the first 7 pulses were 100 mA and the final pulse was 200 mA, using the iontophoresis protocol used previously (Hendry and Marshall, 2004, Morris and Shore, 1996, Brandes et al., 2005). The changes in RCF evoked by train of pulses of ACh were recorded.

The subject then consumed an orange flavoured drink containing 600 mg soluble aspirin to inhibit COX (see Chapter 2 section 2.5.1) and ~25 min later, the protocol was repeated with the iontophoresis well attached to a different site on the non-dominant, non-trained arm. This was necessary because the dilatation induced by ACh lasted for at least 30 min.

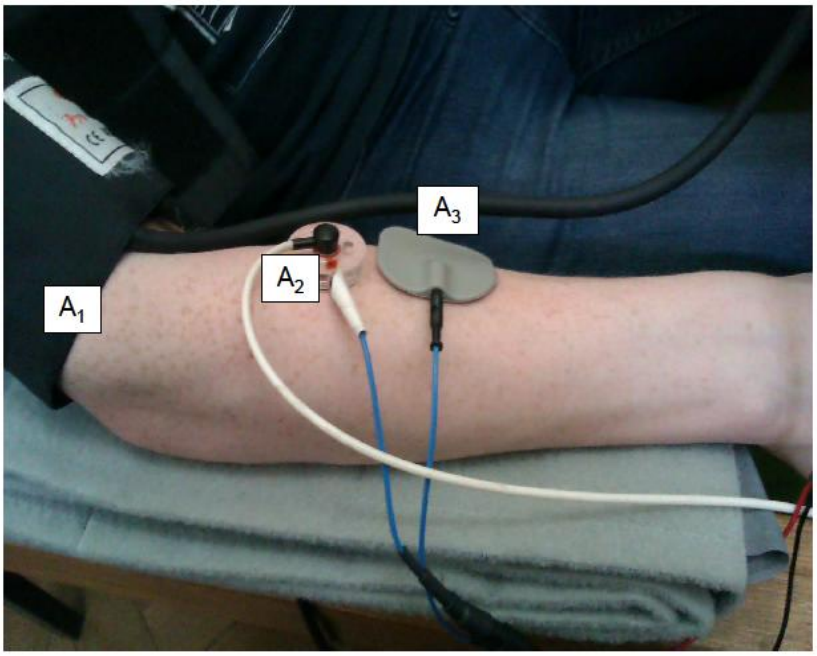


Figure 2.3: **Arrangement used for red cell flux (RCF) ACh iontophoresis and reactive hyperaemia.** Upper arm cuff (A_1) connected to mercury sphygmomanometer used for reactive hyperaemia. Laser-doppler probe (A_2) mounted into a drug chamber filled with ~0.5 ml ACh on the non-dominant arm. A sticky pad (A_3) which acted as an indifferent electrode was placed next to the chamber.

2.7.6 Reactive Hyperaemia

Forearm blood flow (FBF) was measured after arterial occlusion, also known as reactive hyperaemia in both experimental protocols. During the VoP protocol, the upper arm cuff of the dominant arm was inflated to >200mmHg for 3 min by the rapid cuff inflation system. When released the larger cuff (Hokanson, SC 10D, Bellevue WA, 98005, USA) was inflated to ~50 mmHg, restricting the venous drainage but allowing arterial flow. The wrist cuff was inflated to 200mmHg ~5-10 sec before releasing the upper arm cuff. Then, immediately the upper arm cuff was deflated, it was re-inflated with the rapid cuff inflation system to 50mmHg so that FBF could be recorded. During the cutaneous RCF recording protocol, an upper arm cuff was manually inflated to 200 mmHg for three minutes and deflated to measure cutaneous blood flow changes after occlusion recorded by the laser Doppler probe (DPITV2; Moor Instruments) as described on section 2.4.6.

2.7.7 Rhythmic Handgrip Contractions

Rhythmic Handgrip contractions were performed with the handgrip dynamometer (Lafayette 70718, Loughborough, U.K.) at 60% MVC intensity (1:1 duty cycle). Termination criteria comprised of inability to maintain the force for 5s and/or a decrease by 5% from MVC despite vigorous verbal encouragement. A web-based metronome was used to aid subjects with rhythmic contractions.

The dynamometer was calibrated before the familiarisation visit and subsequently before each experiment so that 1.0 volt on the display-unit represented subject's 100% MVC; allowing each subject to visualize the percentage of force produced and maintain the level requested.

Figure 2.1 demonstrates that the elbow of the subject's exercise arm rested on a polystyrene block and as the dynamometer was fastened to the bench, this allowed the subject to release their grip when asked to do so and completely relax the arm, thus facilitating blood flow measurements. Two different protocols were carried out: Protocol 1 involved VOP recordings and Protocol 2 involved LDP recordings. Both were completed in random order before and after IHG training. The details of the protocols are described in Chapter 3, but an overview of the study design is presented below (Figure 2.3)

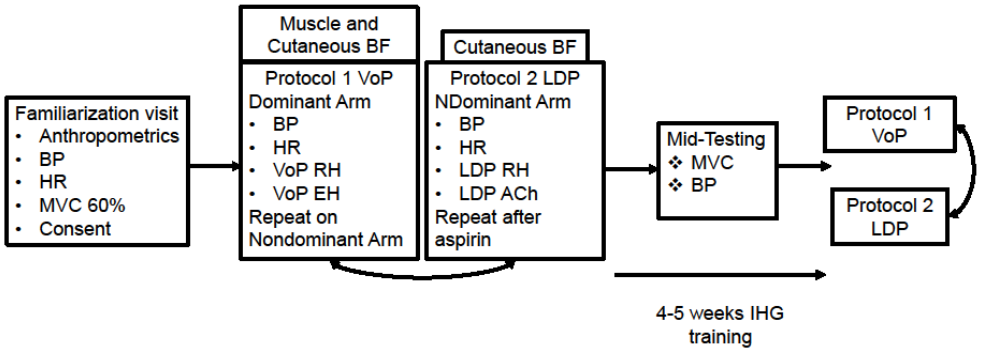


Figure 2.4: **Overview of study design.**

BP=blood pressure, HR= Heart Rate, MVC=Maximum Voluntary Contraction, VoP=Venous Occlusion Plethysmography, LDP=Laser Doppler Perfusion, RH= Reactive Hyperaemia, EH= Exercise Hyperaemia, ACh=acetylcholine, IHG=isometric handgrip training; subjects trained with their dominant arm for 4 times for per week for 4 to 5 weeks.

2.7.8 Isometric Handgrip training

The subject was then shown by the researcher how to perform static (isometric) exercise contractions at 30% MVC with the dominant arm by using a handgrip dynamometer (Lafayette 70718, Loughborough, U.K.) with a voltage-output and an interfaced visual display-unit (Figure 2.3). This demonstration was used for the subject to understand the handgrip exercise training program which was commencing after the first two experimental sessions. The isometric handgrip training program (see below) required each subject to perform handgrip training at 30% MVC for 30s, 4 times at 3-min intervals for 4 days per week for 4-5 weeks with a commercially available portable handgrip dynamometer (CAMRY, Model: EH101, ISO 9001 certified by SGS). The subjects were provided also with a handgrip exercise training record to monitor their training sessions (appendix I). MVC was measured in both arms after 2 weeks of training and maximum MVC was readjusted at 60% in case of increased 100% MVC. During the MVC measurements, three MVCs were requested from each subject and the average score was used to estimate changes in their MVC. After the training period of 4-5 weeks, their maximum voluntary contraction (MVC) was measured again to record any training adaptations in both arms. MVC readjustment was repeated after 4 weeks of IHG training before the final two experimental sessions (Figure 2.4).

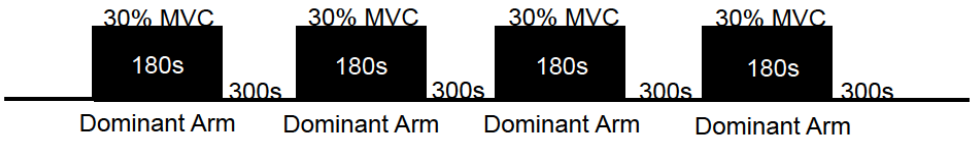


Figure 2.5: **IHG training protocol schematic**

MVC=maximum voluntary contraction was tested before and after IHG training protocol; s: seconds for each isometric handgrip test (180s) and the resting interval between each set (300s).

2.7.9 Aspirin

In most studies, aspirin was given to inhibit COX activity. For this purpose, 600mg of Aspirin (A; Aspirin dispersible tablets, Sainsbury's, UK) dissolved in orange squash (Sainbury's double strength orange squash with no added sugar, 1.5L) was given at an appropriate stage in the experiment as described by Win and Marshall, 2005. The dose of aspirin has previously demonstrated to provide near-maximal COX-inhibition from 0.5 to 1.5 hrs (Heavey et al., 1985), so inhibiting prostaglandin (PG) synthesis. An overview of the laboratory procedures followed during the experimental protocols is presented below (Figure 2.5).

2.8 Data Acquisition

Data were collected to a desktop computer (Dell Inc, USA) using a Powerlab data acquisition hardware (Power-Lab, AD Instruments Inc., Colorado Springs, CO USA) and Lab-Chart data acquisition software (version 7.3.3, AD Instruments, USA) at the sampling frequency of 400Hz. The data was transferred then to Microsoft Excel (2010).

Heart rate (HR) was computed from the pulsatile pressure recording via lab chart software from the interval between successive systolic blood pressure peaks; MABP was calculated online Labchart software.

FBF and CBF were calculated off-line from the plethysmograph outputs as discussed above (section 2.4.3). Forearm vascular conductance (FVC was calculated as (FBF/MABP). Calf vascular resistance (CVR) was calculated as (MABP/CBF) in the Microsoft Excel spread sheets to which all data were exported. The Flow and ABP data used for calculating FVC and CVR were generally extracted from the same cardiac cycle. If this was not possible due to automatic re-calibration of the Finapres monitor, MABP (and HR) were taken from the adjacent cardiac cycle. Their values were expressed in either conductance units (CU) or resistance units (RU), respectively.

2.9 Blood Sampling

Blood was taken from a sample of subjects who participated in the present study. The details are described on Chapter 5.

2.9.1 Blood Sample Collection

An intra-venous cannula (22 – 24 G, BD Venflon, BD) shown in Figure 5.2, was inserted into the branchial vein of the subject's non training forearm via a needle (BD

Venflon Pro™ Peripheral IV Catheters 20g Pink, 32mm). The cannula was connected to a multiple-sample luer-adapter (BD Vacutainer Multiple-Sample Luer-Adapter, BD) via a plastic tube connector (10 cm BD Connecta, BD).

On each occasion, a sample of 10-15 ml was taken. Blood was collected into 10 ml vacutainers (EDTA, BD Vacutainer, BD) for prostaglandins (PG) analysis. In order to prevent ex-vivo formation of PGs, 1 μl/ml of 10 μM Indomethacin (Sigma-Aldrich, UK) stock was added to the vacutainers immediately after the collection; indomethacin stock was prepared using ethanol. An extra 4 ml of blood were collected into 5 ml vacutainers (Heparin BD Vacutainer, BD) for NO (nitrate/nitrite) analysis at rest and after 3 min. of arterial occlusion during the experimental time. The intra-venous cannula with its connector tube was periodically flushed with 3 ml bolus of 0.9% sterile-saline (BD PosiFlush SP Syringe, BD) to stop blood clotting. At each time point of blood sample collection, 0.8 ml of blood was initially drawn out and discarded in order to get rid of the dead-space volume from the connector tube. All blood samples were kept on ice during the period of experimentation and the addition of indomethacin. In total, ~40 ml of blood was collected during each experiment.

2.9.2 Blood storage and analysis

All vacutainers were then centrifuged (Mistral 3000i, MSE Ltd) at 2500 rpm at 4°C for 20 min; After supernatant plasma collection, samples were labelled and transferred into 1ml Eppendorf tubes (Sigma-Aldrich, UK). 0.005% of butylated hydroxytoluene (BHT) (10μl of 5mg/ml solution in ethanol per 1ml sample) (Sigma-Aldrich, UK) was added in plasma samples intended for isoprostane (8-iso Prostaglandin $F_{2\alpha}$) assay to avoid oxidation of 8-isoprostane; BHT stock was prepared using ethanol. All plasma samples were then stored in a - 80°C freezer for later analysis of PGI_2, $PGF_{2\alpha}$ metabolites, NO (NO_3NO_2) metabolites, and K^+ levels.

2.10 ELISA techniques and preparation

The plasma samples were stored to be analysed with commercially available ELISA kits (Cayman chemicals, US) for PGI_2, isoprostane and nitric oxide. The procedures involved in the analysis of the ELISAS are detailed further in Chapter 5.

2.11 Statistical Analysis

All results are presented as Mean ± SEM. Appropriate Analysis of Variance (ANOVA) was used to detect age, time, treatment, condition, and their interaction effects. Once a significant main effect was identified, the data were further analysed using an appropriate post-hoc test (Tukey's HSD) to detect the exact point of difference. Where appropriate other comparisons were made by using paired or unpaired Student's t-test, and coefficient of determination. Statistical significance was assumed when $P<0.05$.

3. Effects of IHG Training in White Europeans and South Asians

- Ipsilateral and Contralateral Forearm blood flow and muscle performance
- Contralateral Forearm Cutaneous perfusion (contribution of COX products)

3.1 INTRODUCTION

As discussed in the General Introduction (see section 1.6 South Asian Ethnicity and Endothelial Function), the prevalence of cardiovascular disease (CVD) in South Asian (SA) populations including those with ethnic roots in India, Pakistan, Bangladesh, Sri Lanka and Nepal, is higher than in White European (WE) populations, especially when living in western countries (Wild et al., 2007, Rana et al., 2014). Thus, SA ethnicity is considered to confer a high risk for cardiovascular disease (CVD) (Gupta et al., 1996, Gupta et al., 2004, Gupta, 2004b). Moreover, hypertension is an emerging public health problem in India (Gupta et al., 2004). Controlling the increasing prevalence of CVD would require limiting the risk factors related to hypertension, or early treatment of hypertension because hypertension is a common risk factor for further CVD such as heart failure, arrhythmia and stroke.

Isometric handgrip (IHG) training has been shown to reduce resting arterial pressure in patients with hypertension and even in normotensives (Badrov et al., 2013A, Millar P.J., 2009, Ray and Carrasco, 2000, Williams et al., 2007, Brook et al., 2013b, Millar et al., 2014), see Section 1.9, (Summary of IHG training programmes- General Introduction). The most widely utilized IHG training protocols have consisted of four 2 min handgrips at 30-50% MVC, separated by 1-4 min and repeated 3-4 times per week. (Millar et al., 2014). These protocols have generally been followed for 4, or 8 weeks and have resulted in a ~10% reduction in both systolic and diastolic blood pressure in normotensives, pre-hypertensives and hypertensives (Kelley and Kelley, 2010).

The IHG training-induced reduction in ABP must be attributed to one or both of the factors that contribute to ABP: cardiac output and total peripheral resistance. No significant changes in cardiac output have been reported (Millar et al., 2014). However, IHG training has been shown to improve FMD in the brachial artery, which is taken to be an index of endothelial function that is NO-dependent (Lawrence et al., 2015, Green et al., 2017). This might suggest that IHG training reduces TPR, by improvement in endothelium-dependent dilation. However, an enhanced FMD has only been reported in the trained arm (Millar et al., 2014, McGowan et al., 2006A, McGowan et al., 2006B, McGowan et al., 2007), not in the non-trained arm of non-medicated or medicated hypertensives (Millar et al., 2014).

Further, FMD provides information only about the behaviour of a large distributing artery.

There have so far been no studies on the effects of IHG training on resistance vessels and microcirculatory vessels: those that contribute to the regulation of vascular resistance and tissue blood flow. Further, relatively little has been done on young healthy subjects with no overt CVD. Thus, the primary aim of this first study was to test in young subjects, the effect of IHG training of the dominant arm on reactive hyperaemia and exercise hyperaemia, dilator responses evoked in whole forearm vasculature following release of arterial occlusion (reactive hyperaemia) and forearm exercise (exercise hyperaemia). Endothelium-dependent dilator influences contribute to both of these responses (Section 1.12.2). Since an effect on TPR would imply an effect on resistance vessels beyond the trained arm, we also tested the effects of IHG training on the non-trained, as well as the trained arm. In fact, our underlying hypothesis was that IHG of the dominant arm would increase the shear stress in the contralateral arm, and legs as a consequence of acute increases in ABP associated with each episode of IHG at 30% MVC (Cui et al., 2007). This aspect of the hypothesis was investigated in the study described in Chapter 6. For the purposes of the present study, the working hypothesis was that IHG training augments reactive and exercise hyperaemia in the trained *and* non-trained arm. This was tested by using VOP to record whole forearm blood flow (FBF).

A second aim of the present study was to test in young subjects, the effect of IHG on vasodilator responses evoked in microcirculation of the skin by iontophoresis of ACh and during reactive hyperaemia. ACh-induced dilatation and reactive hyperaemia have generally been attributed to NO, PGs and/or EDHF (endothelium-derived hyperpolarizing factor; (Kellogg et al., 2005). Whether dilator PGs actually contribute to ACh-evoked dilatation in forearm cutaneous circulation is controversial (Khan et al., 1997, Lorenzo and Minson, 2007, Morris and Shore, 1996). However, it seems generally agreed that NO does *not* actively contribute to ACh-induced dilatation, or reactive hyperaemia in human skin (Wong et al., 2003, Holowatz et al., 2005, Medow et al., 2007). Thus, for the present study, we hypothesised that IHG training of the dominant-arm would increase the dilator contribution of PGs to ACh-induced dilatation and reactive hyperaemia in the non-

trained arm. To test this hypothesis, we aimed to examine the effect of aspirin given orally at a dose sufficient to cause cyclooxygenase (COX)-blockade was tested on these responses in the non-trained arm before and after IHG training; cutaneous perfusion was recorded as red cell flux (RCF) by laser doppler fluximetry (LDF).

Since, as discussed in the General Introduction, SAs are at a greater risk of CVD than WEs, we hypothesised that reactive and exercise hyperaemia in the whole forearm and ACh-evoked dilatation and reactive hyperaemia in cutaneous microcirculation would be smaller in SAs than WEs and would be augmented less by IHG training in SAs. We also hypothesised that vasodilator PGs would make smaller contributions to cutaneous vasodilator responses in SAs than WEs before IHG training, but that their contribution would increase in both ethnicities after IHG training.

3.1.1 HYPOTHESES AND AIMS

To summarize, the hypotheses of the present study were to:

1. We hypothesised that IHG training of the dominant-arm would increase the dilator contribution of PGs to ACh-induced dilatation and reactive hyperaemia in the non-trained arm.
2. we hypothesised that reactive and exercise hyperaemia in the whole forearm and ACh-evoked dilatation and reactive hyperaemia in cutaneous microcirculation would be smaller in SAs than WEs and would be augmented less by IHG training in SAs.
3. We also hypothesised that vasodilator PGs would make smaller contributions to cutaneous vasodilator responses in SAs than WEs before IHG training, but that their contribution would increase in both ethnicities after IHG training.

and the aims to address these hypotheses were to:

1. To test in young subjects, the effect of IHG training of the dominant arm on reactive hyperaemia and exercise hyperaemia, evoked in the trained and non-trained arm, dilator responses evoked in whole forearm vasculature following release of arterial occlusion and rhythmic forearm exercise.

2. To test in young subjects, the effect of IHG training of the dominant arm on vasodilator responses evoked in microcirculation of the skin of the contralateral, non-trained arm, by iontophoresis of ACh and during reactive hyperaemia.

3. To test whether reactive and exercise hyperaemia in the whole forearm and ACh-evoked dilatation and reactive hyperaemia in cutaneous microcirculation would be smaller in SAs than WEs and would be augmented less by IHG training, since SAs are at greater risk of CVD than WEs.

4. To test whether vasodilator PGs make a larger contribution to these cutaneous responses after IHG training than before and whether the augmentation is greater in WEs than SAs.

3.2 METHODS

Twenty subjects (10 WEs; 10 SAs: 18-25 years old) participated in this study. Data were collected and recordings were made as described in Chapter 2; the variables recorded are described in brief below.

3.2.1 GENERAL PROTOCOL

The experimental protocol as shown in Figure 3.1, involved each subject coming to the laboratory on six separate days. The first session was a familiarization visit in which each participant signed an informed consent form and anthropometric measurements were taken. The latter are shown in Table 3.1. After the familiarization visit, each subject returned for two experimental sessions (see below) followed by a period of 4-5 weeks during which the subject performed IHG training. Midway through this period, the subject returned to the laboratory for an interim visit. At the end of the period, the subject returned for two experimental sessions that were repeats of those performed before IHG training. Depending on subject availability, the final two sessions were sometimes continued into the fifth week to ensure, the subject continued the IHG training until the date of the final session.

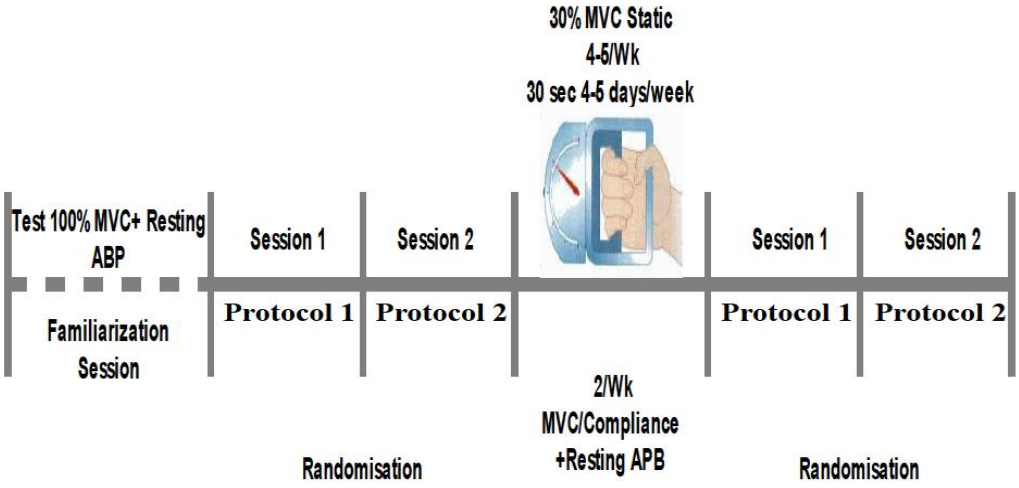


Figure 3.1: **Schematic diagram of the General Protocol:** the order of Protocols 1 and 2 before and after IHG training was randomized.

As indicated in Chapter 2 section 2.2, at the familiarization visit, the subject was informed about the experimental methodology and equipment and 100% MVC was measured in their dominant and non-dominant arm. The subject was then shown how to perform rhythmic exercise with the dominant arm with dynamometer at

60%MVC. After the first two experimental sessions, he was then shown how to use the portable dynamometer to perform handgrip with the dominant arm at 30% MVC for 30s, 4 times at 3-min intervals. When he was proficient, he was asked to perform these static handgrips at 30% MVC for 30 min per day for 4 days each week for 4-5 weeks. An exercise training sheet was provided to each subject to help remind the subjects of the IHG sessions for each week and the intensity, frequency and duration of the IHG exercise.

During the interim visit after ~2 weeks of IHG training, the subject returned to the laboratory so that resting ABP could be measured and MVC in the dominant arm could be checked. If there were an improvement in the maximum force of MVC, an appropriate adjustment was made to the 30% MVC that was used for IHG training for the final 2 weeks.

Protocols 1 and 2 were carried out before and after IHG training in random order. Coming from the student population of the university of Birmingham, all the experimental sessions were running during the evening (after 16.00pm) for both ethnic groups and particularly due to school commitments (attending lectures, assignments etc.) and for the South Asian group, which was going through the Ramadan, a religious period for them which they had to abstain from any food or drink until sunset (summertime).

Protocol 1: Reactive and Exercise hyperaemia in forearm. At this session (Figure 3.2), when recording equipment had been put in place, the subject rested for 12 min and ABP was measured three times at intervals of 2 min from the subject's dominant arm by using a standard semi-automatic sphygmomanometer. Thereafter, ABP was recorded continuously by means of a finger cuff and Finapres monitor from the non-dominant hand; HR was derived from the pulsatile ABP signal. FBF was recorded from the dominant arm (ipsilateral arm) by VOP (see Chapter 2 section 2.4.3.). For each measurement of FBF, the wrist cuff was inflated to 200mmHg, and the upper arm cuff was rapidly inflated to 50 mmHg and held at this pressure for 10-15 s so that the slope of the increase in forearm circumference could be measured (see Section 3.3.2). The cuffs were then deflated.

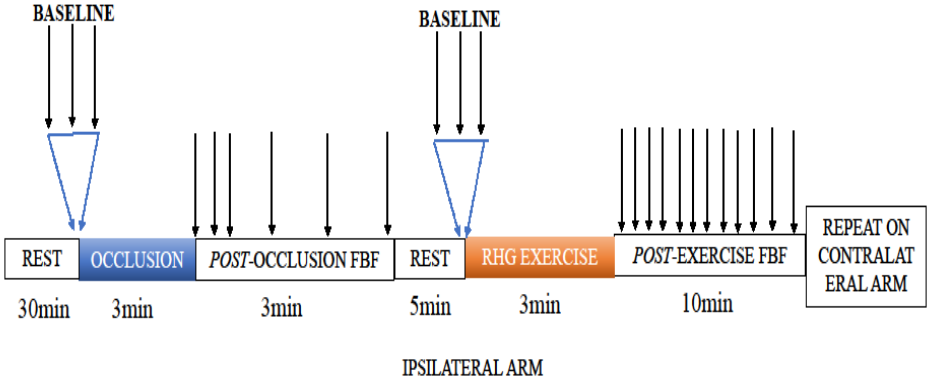


Figure 3.2: **Schematic diagram of Protocol 1.** Black arrows indicate FBF recordings made during rest, after 3 min of arterial occlusion and after 3 min of rhythmic handgrip at 60%MVC. This protocol was performed in both arms.

For reactive hyperaemia, FBF was measured 3 times under baseline conditions to give resting FBF Figure 3.2). The upper arm cuff was then inflated to 200mmHg for 3 min and released to induce reactive hyperaemia: FBF was recorded immediately the cuff was deflated at 0 min and then at 0.5, 1, 2, 3, 4 min. After a rest period of ~5 min, each subject was then requested to perform rhythmic handgrip exercise at 60% MVC for 3 min contracting for 1s at 1s intervals by using an online metronome. This was a very difficult task for all subjects particularly before IHG training. Thus, verbal encouragement was used to ensure they continued to contract at 1s intervals even when the force achieved was not 60%MVC; the output of the dynamometer was continuously recorded for later analysis (see below). Immediately after handgrip exercise, FBF was measured at 0, 30s, and then at 1 min intervals from 1-10 min.

The protocol was then repeated to allow reactive and exercise hyperaemia to be recorded in the non-dominant arm. This entailed re-arranging the VOP equipment and the ABP monitoring equipment on the opposite arms.

Protocol 2: Reactive hyperaemia and ACh-induced dilatation in cutaneous circulation

As shown in Figure 3.3 when the equipment had been arranged, the subject rested for 12 min, and ABP was measured three times from a standard sphygmomanometer as described above. Thereafter, ABP was recorded continuously from a finger cuff on the dominant arm and RCF was recorded continuously from the anterior surface of the non-dominant arm by using LDF, the probe being inserted into a Perspex iontophoresis well (see section 2.3.5). For reactive hyperaemia, the sphygmomanometer cuff around the upper part of the non-dominant arm was inflated to 200mmHg for 3 min and released to induce reactive hyperaemia whilst recording RCF. Approximately 5 min later, ACh was delivered into the skin by iontophoresis (8 separate pulses of 20 s each, at intervals of 60s; the first 7 pulses were 100 µamp and the final pulse was 200 µamp see (Hendry and Marshall, 2004, Morris and Shore, 1996). The changes in RCF evoked by ACh were recorded (see Section 3.6).

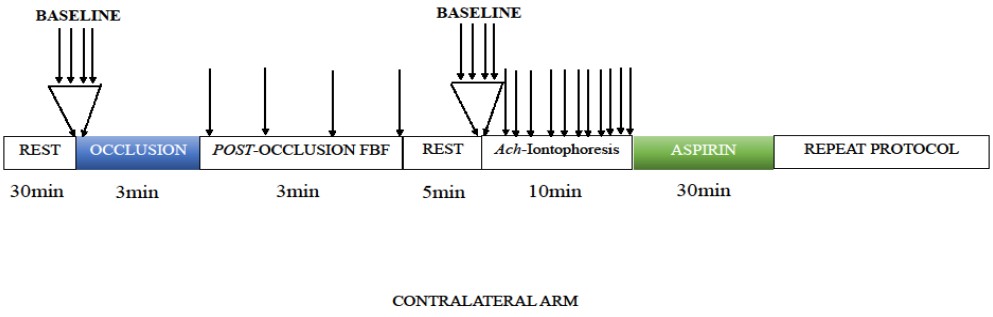


Figure 3.3: **Schematic diagram of Protocol 2.** RCF was recorded before and after release of arterial occlusion (reactive hyperaemia) and during iontophoresis of ACh. Black arrows indicate RCF recordings during rest, after 3min of arterial occlusion and during *ACh* iontophoresis. This was repeated 30 min after aspirin administration. This protocol was performed in the non-trained arm.

The subject then consumed an orange flavoured drink containing 600 mg soluble aspirin to inhibit COX (see Chapter 2 section 2.3.8) and ~25 min later (when aspirin reached its maximum concentration), the protocol was repeated with the LDF

probe and iontophoresis well attached to a different site on the non-dominant, arm. This was necessary because the dilatation induced by ACh lasted for at least 30 min.

3.2.2 MEASUREMENTS of FBF and RCF

VOP measurements of FBF during reactive and exercise hyperaemia were performed as described in Chapter 2 (see section 2.5) using the Hokanson plethysmograph. Figure 3.4 (A) shows the gradient of plethysmograph trace used for calculation of baseline FBF. Figure 3.4 (B) shows an example of the slope used for calculation of FBF immediately after 3 min of rhythmic handgrip exercise. The gradient was calculated from the "*initial*" rising slope of the plethysmograph deflection following venous occlusion by using the best-fitting line option on Lab-Chart. The gradient was drawn over the initial complete pulsatile beat after cuff inflation as recommended (Tschakovsky et al., 1995, Junejo et al., 2019): as can be seen, the slope would have been lower and FBF underestimated if calculated from subsequent beats.

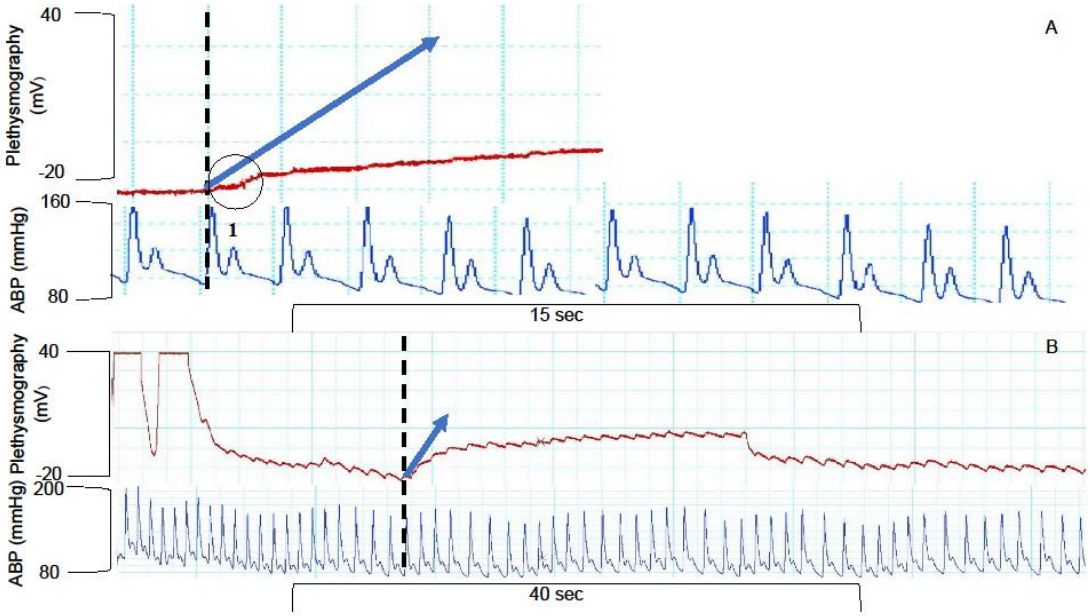


Figure 3.4: **Recordings of Plethysmograph trace and pulsatile ABP before and following automatic rapid inflation of venous occluding cuff under baseline conditions (A) and following handgrip rhythmic contractions (B).** Dashed lines represent time of inflation to 50 mmHg while blue line and black circle (A) represents the gradient used for FBF calculation. Pulsatile ABP aids visualization of pulsatile increments on plethysmograph trace.

RCF was reported as perfusion units (PU), a 1volt calibration signal representing 100 PU, Figure 3.5 shows an example of the change in RCF recorded

during the reactive hyperaemia that followed 3 min of arterial occlusion at 200 mmHg. RCF values were extracted at peak hyperaemia (midway through the pulsatile recording) and at 10, 30, and 60s following the peak. Figure 3.6 shows an example of RCF recorded during the ACh iontophoresis protocol. RCF was measured as the maximum average value during the interval between successive pulses and following the final pulse. These values were expressed as change from baseline RCF.

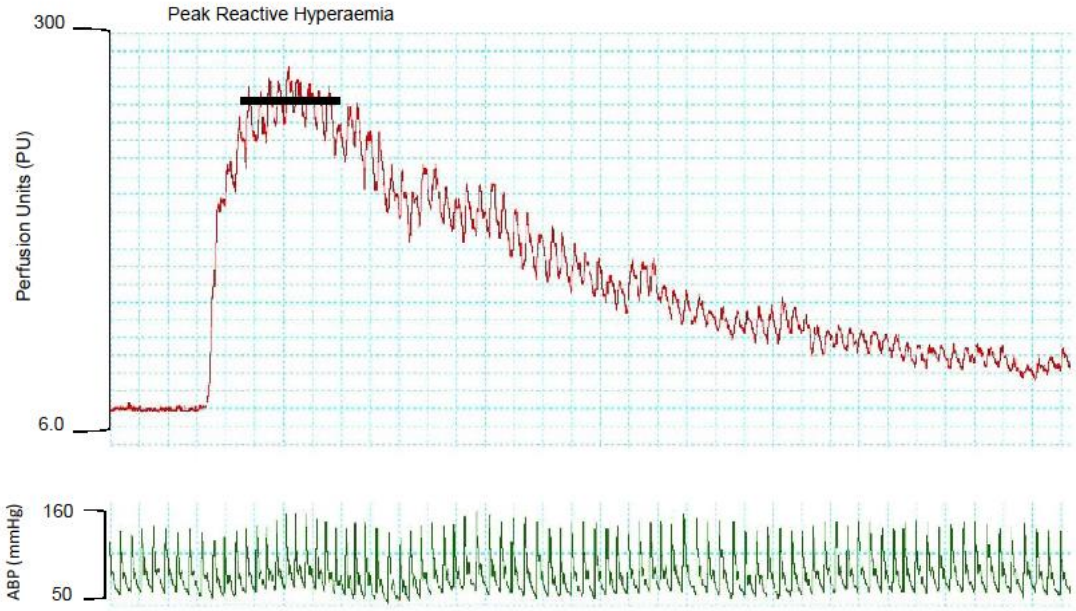


Figure 3.5: **Recording of cutaneous RCF with corresponding ABP recording before and after release of arterial occlusion cuff.** RCF was measured at Peak and at intervals thereafter. The horizontal black line indicates the peak response after cuff release.

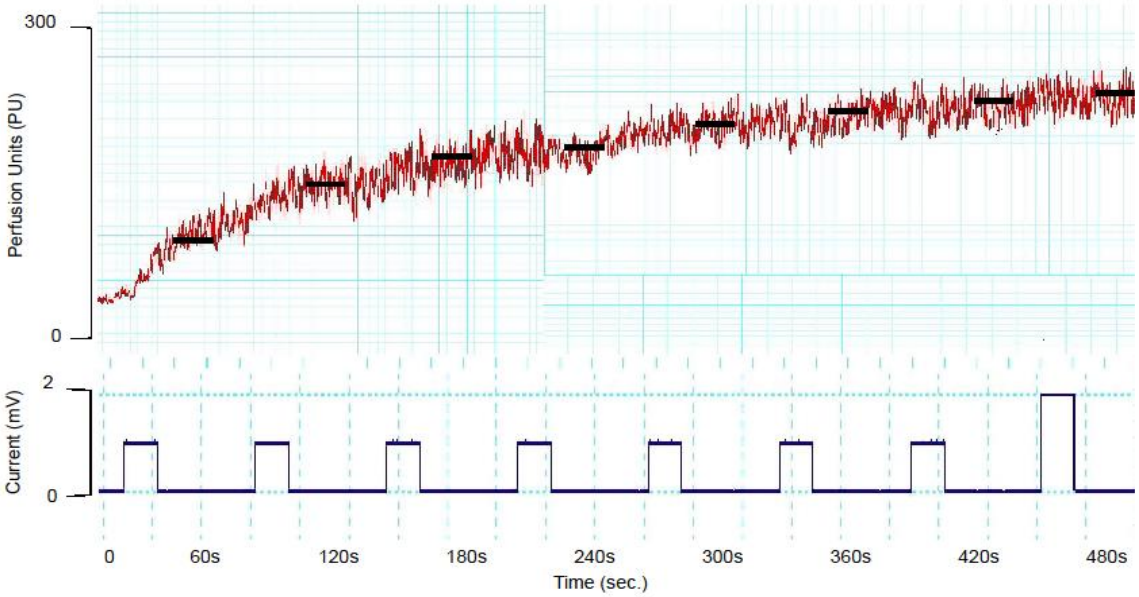


Figure 3.6: **RCF recording during ACh iontophoresis:** Purple boxes show the pulses induced by the indifferent electrode which augmented RCF response from baseline: seven pulses at 100 mA for 20s followed by a pulse of 200 mA. The time bar indicates the time period used to record the RCF response. The black vertical lines indicate peak RCF response where the RCF trace response was taken after each pulse.

3.2.3 DATA ANALYSIS

All data were expressed as means ± SEM. Forearm vascular conductance (FVC) was calculated as FBF/ABP recorded over the same time period; FVC was expressed in conductance units (CU). Baseline levels of ABP, HR, FBF, FVC, RCF before and after IHG training were compared within WE and SA groups by using Student's paired t-tests. A Shapiro-Wilk's test ($p>.0001$) (Razali, 2011, Shapiro, 1965) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that baseline levels of ABP, HR, FBF, FVC, RCF before and after IHG training were approximately normally distributed for both ethnic groups, with a skewness for before training BS ABP: 0.612 (SE=0.687), BS HR: -0.524 (SE=0.687), BS FBF (non-dominant arm): 0.623 (SE= 0.378), BS FVC (non-dominant arm): 0.404 (SE=0.378),BS RCF (no aspirin): 0.652 (SE=0.378), BS RCF (aspirin): 0.324 (SE=0.374), BS FBF (dominant arm): 0.730 (SE=0.378), BS FVC (dominant arm): 0.413 (SE=0.378) and a kurtosis for before training BS ABP: – 0.406 (SE=1.334), BS HR: -0.830 (SE=1.334), BS FBF (non-dominant arm): -0.500 (SE=0.741), BS FVC (non-dominant arm): -0.522 (SE=0.741), BS RCF (no aspirin): 0.448

(SE=0.741), BS RCF (aspirin): -0.787 (SE=0.733), BS FBF (dominant arm): 0.332 (SE=0.741), BS FVC (dominant arm): -0.391 (SE=0.741) for WEs and a skewness for BS ABP: 1.09 (SE= 0.687), BS HR: -0.158 (SE=0.687), BS FBF (non-dominant arm): 0.117 (SE= 0.374), BS FVC (non-dominant arm): -0.318 (SE=0.374), BS RCF (no aspirin): 0.453 (SE=0.374), BS RCF (aspirin): 0.568 (SE=0.374), BS FBF (dominant arm): 0.658 (SE=0.374), BS FVC (dominant arm): 0.324 (SE=0.374), and a kurtosis for BS ABP: 2.172 (SE=1334), BS HR: -0.279 (SE= 1.334), BS FBF (non-dominant arm): -0.457 (SE=0.733), BS FVC (non-dominant arm): -0.933 (SE=0.733), BS RCF (no aspirin): 0.160 (SE=0.733), BS RCF (aspirin): 1.123 (SE= 0.733), BS FBF (dominant arm): 0.495 (SE=0.735), BS FVC (dominant arm): -0.787 (SE= 0.733) for SAs. Similarly, after IHG training baseline levels of ABP, HR, FBF, FVC, RCF were approximately normally distributed for both ethnic groups, with a skewness for BS ABP: 0.239 (SE= 0.687), BS HR: -0.410 (SE=0.687), BS FBF (non-dominant arm): 0.546 (SE= 0.378), BS FVC (non-dominant arm): 0.066 (SE=0.378), BS RCF (no aspirin): 0.227 (SE=0.378), BS RCF (aspirin): 0.589 (SE= 0.378), BS FBF (dominant arm): 0.210 (SE= 0.378), BS FVC (dominant arm): 0.227 (SE= 0.378) and a kurtosis for after IHG training BS ABP: -0.863 (SE=1.334), BS HR: 0.708 (SE=1.334), BS FBF (non-dominant arm): -0.514 (SE= 0.741), BS FVC (non-dominant arm): -0.378 (SE= 0.741), BS RCF (no aspirin): -1.350 (SE= 0.741), BS RCF (aspirin): 1.215 (SE=0.741), BS FBF (dominant arm): - 0.734 (SE=0.741), BS FVC (dominant arm): - 0.739 (SE=0.741) for WEs and a skewness for BS ABP: 0.501 (SE= 0.687), BS HR: -0.350 (SE=0.687), BS FBF (non-dominant arm): 0.813 (SE=0.374), BS FVC (non-dominant arm): 0.806 (SE=0.374), BS RCF (no aspirin): 0.665 (SE=0.374), BS RCF (aspirin): 0.325 (SE=0.374), BS FBF (dominant arm): 0.201 (SE=0.374), BS FVC (dominant arm): 0.324 (SE=0.374), and a kurtosis for BS ABP: -0.925 (SE= 1.334), BS HR: -1.855 (SE= 1.334), BS FBF (non-dominant arm): -0.703 (SE=0.733), BS FVC (non-dominant arm): -0.677 (SE=0.733), BS RCF (no aspirin): 0.426 (SE=0.730), BS RCF (aspirin): -1.025 (SE=0.733), BS FBF (dominant arm): -0.745 (SE=0.733), BS FVC (dominant arm): -0.787 (SE=0.733) for SAs (Cramer, 2004, Doane, 2011). All comparisons between WEs and SAs were made by unpaired t-tests. The proportion of subjects with hypertensive parents were compared between the two ethnicities by using Fisher's Exact Test. Two-way repeated measures ANOVA for detection of time, treatment, time*treatment effects

were used to compare changes in FBF, FVC, RCF evoked by experimental interventions before IHG training and after IHG training. The time points for these comparisons were 0, 10, 30 and 60 s. for RH comparisons and 0 s, 10s, 30s, 60s, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min. for EH comparisons. Comparisons of these indices between WEs and SAs were made by using Two-way repeated measures ANOVA for detection of time, treatment, time *treatment effects. The group interaction was compared with time*treatment variable. The term "treatment" was used to compare the values of each ethnic group for aspirin and IHG training effect as demonstrated on FBF, FVC, RCF values. In addition, peak values of FBF and FVC during reactive and exercise hyperaemia before and after IHG training were compared within WEs and SAs by Student's paired t-test.

Measurements of MVC in kilonewtons (kN) were compared within groups by using Student's paired t-test. Work done during the 3 min periods of rhythmic contractions was calculated by using the integral function on Lab Chart, as Tension Time Index (TTI) kN.s and is referred to as "actual" TTI. As indicated in Results, individual subjects generally did not manage to perform 60% MVC at 1 cycle/ 2 s for the whole 3 min period: their ability to achieve 60% MVC waned during the 3rd min. Their "expected" TTI was calculated as the product of 60% MVC during the first contraction of the 3 min rhythmic contractions x 90s to give the TTI that would have been achieved had they not fatigued.

The percentage changes in peak FBF, peak FVC, expected and actual TTI before vs after IHG training were calculated as the value recorded after IHG training minus value before training divided by value before training and expressed as percentage. Percentage improvements in actual/expected TTI were calculated by dividing the actual TTI value with the corresponding expected TTI value and multiplied by 100. The two ethnic groups were compared by un-paired t-tests for all above variables on this paragraph. In all cases, statistical significance was set at $P<0.05$.

3.3 RESULTS

There were no significant differences between the anthropometric characteristics of WEs and SAs (Table 3.1). Cardiovascular baselines are presented in Table 3.2 for protocols 1 and 2. Systolic and diastolic blood pressure and baseline HR and MABP did not change significantly after 4 weeks of training. However, baseline FBF and FVC were increased in both the trained and non-trained arm in WEs, while only FVC in the trained arm was increased in SAs. Further, baseline RCF in the non-trained arm was decreased after IHG training in WEs, but there was no change in SAs.

3.3.1 Effects of IHG training on MVC

IHG training after 2 weeks increased MVC in the trained arm of WEs (28±2.0 vs 31.0±2.15 kN; P<0.05) and SAs (27.08±2.44 vs 29.55±2.58; P<0.05) but not in the contralateral, non-trained arm of WEs (25.51±0.89 vs 26.59 ±0.86 kN) or in SAs (25.60±2.52 vs 26.29±2.48 kN). After 4-5 weeks of IHG training, MVC increased in the trained arm of WEs (28±2.0 vs 32.86±2.22 kN; P<0.05) and SAs (27.08±2.44 vs 31.98 ± 3.15 kN; P<0.05), but not in the contralateral, non-trained arm of WEs (25.51±0.89 vs 26.59 ±0.86 kN) or SAs (25.60±2.52 vs 26.29±2.48 kN) see Figure 3.7.

Protocol 1

3.3.2 Forearm vascular responses during reactive hyperaemia

In WEs, IHG training of the dominant arm for 4 weeks increased peak reactive hyperaemia FBF and FVC (Figure 3.8 and 3.9, LHS) while increased FBF and FVC *during* the whole period of reactive hyperaemia in the trained (dominant) arm. In the trained arm, peak FBF was 41.73±3.45 before, and 50.8±3.84 $ml.100ml^{-1}.min^{-1}$ (P< 0.0001) after IHG training, while peak FVC was 0.49±0.04 and 0.66±0.07 CU (P< 0.0001) respectively. Similarly, peak FBF increased from 41.37±2.74 to 52.94±2.98 $ml.100ml^{-1}.min^{-1}$ (P< 0.0001) in the non-trained arm, while peak FVC increased from 0.52±0.03 to 0.63±0.05 CU (P< 0.0001).

By contrast, in SAs, IHG training peak reactive hyperaemia FBF was also increased from 42.77±4.64 to 52.83 ± 3.06 ml.100ml^{-1}.min^{-1} (P< 0.0001) whereas peak FVC was not changed (0.59±0.07 to 0.71±0.06 CU, P< 0.0001) while during whole response FBF increased but not FVC in the trained arm and. In the non-trained arm, there were no significant changes in FBF or FVC *at peak or during* reactive hyperaemia (FBF: from 45.22±4.53 to 48.89± 3.83 ml.100ml^{-1}.min^{-1}; FVC was 0.62±0.08 before, and 0.64±0.07 CU after IHG training, P< 0.0001) (Figures 3.8 and 3.9, RHS). The analysis for "*at peak or during*" time periods used two-way repeated measures ANOVA during taking all *time points* (during) and the *peak* which was the initial time point (peak BF after occlusion). The report from the statistical package *JMP* (JMP®, Version 15.1. SAS Institute Inc., Cary, NC, 1989–2021) allowed for both "*at peak or during*" time periods after two-way repeated measures ANOVA.

In WEs, the percentage increase in peak FBF caused by IHG training was 24.9±9.5% in the trained and 32.9±12.4% (P< 0.0001) in the non-trained arm, with no significant difference between the two arms. Further, in SAs, IHG training improved peak FBF in the trained and non-trained arms by 30.7±9.9% and 14.3±10.8% (P<0.0001) respectively, with no differences between the two arms (Figure 3.10). The FVC percentage changes after IHG training were also not significantly different between the non-dominant arm (40.63±17.74%) (P< 0.0001) and the dominant arm (30.94±15.01%) of WEs and between the dominant (38.33±16.56%) (F >0.001, P> 0.0001) or non-dominant arm (23.84±22.57%) (P< 0.0001) of SAs. Further, no differences were found in FBF and FVC percentage changes between WEs and SAs in either the dominant or the non-dominant arm (Figure 3.10).

3.3.3 Muscle performance during rhythmic contractions

Since subjects were asked to perform rhythmic contractions at 60% MVC for 3 min in Protocol 1, before and after IHG training, this task was adjusted according to the individual's MVC before and after IHG training (see 3.3.1 above). Work performed during rhythmic contraction of the dominant and non-dominant arms at 60% MVC before and after IHG training is shown as Actual TTI in Table 3.3 and Figure 3.11. The stippled columns in Figure 3.11 show the Expected TTI that would have been achieved had the subjects been able to contract at 60% MVC for the full

3 min. Expected TTI increased significantly in the dominant arm of both WEs (15.39±9.27 vs 17.75±1.20 kN.s), and SAs (14.57±1.32 vs 17.27±1.70 kN.s), reflecting in part (apart from vascular factors), the increase in MVC in that arm. However, subjects also fatigued less during the 3 min after IHG training than before. As can be seen from Figure 3.11, actual TTI increased significantly (P>0.001) after IHG training in the trained arm of WEs (8.88±1.27 vs 17.08±1.78 kN.s) and in SAs (7.34±1.07 vs 13.36±1.93 kN.s). There was no difference between actual TTI of the trained arm of WEs and SAs before (8.88±1.27 vs 7.34±1.07 kN.s) or after IHG training 17.08±1.78 vs 13.36±1.93 kN.s).

Surprisingly, expected TTI also increased slightly in the non-dominant (non-trained) arm of WEs (13.67±4.98 vs 14.36±4.66 kN.s; P<0.05) with a similar trend in the SAs (13.24±1.24 vs 14.3±1.26 kN.s, p=0.06). Further, actual TTI of the non-dominant arm increased significantly in both the WEs (7.21±1.28 vs 11.06±1.16 kN.s) and SAs (5.6±9.03 vs 8.94 ±1.30 kN.s); see Figure 3.12, even though it was the dominant arm that underwent the training. Similarly, to the trained arm, the increase in muscle performance after IHG training was almost identical in the WEs and the SAs (Figure 3.12).

When calculated from the actual TTI before IHG training, there were improvements of 107.63±19.32% and 93.7±27.74%, in performance of the dominant (trained) arm in WEs and SAs respectively (Figure 3a). There were also improvements of 77.65±23.26%, and 73.14±24.53% in the performance of the non-dominant (non-trained) arm in WEs and SAs respectively (see Table 3.3 and Figure 3.12 a). Further, the difference between the expected and actual TTI expressed as percentage of actual/expected presented in Table 3.3 and Figure 3.12 c, indicate that actual TTI was much closer to the expected TTI after, than before IHG training in the trained arm and non-trained arm of both WEs and SAs. In the trained arm, the actual/expected TTI expressed as a percentage, improved from 56.0±.5.9 % to 95.8±7.2% and from 49.3±4.7 % to 76.1±8.0 % in WEs and SAs, respectively) indicating an improvement in fatigue resistance after IHG training. Similarly, in the non-dominant arm, the actual/expected TTI percentage change was improved from 51.9±8.1% to 76.6±6.4% and from 41.1±4.6% to 61.5±6.8% in WEs and SAs, respectively), suggesting an improvement in fatigue resistance in the *non-trained*

arm. No differences were found before or after IHG training between the two ethnic groups.

3.3.4 Systemic and vascular responses evoked by rhythmic contractions

ABP and HR in the WEs and SAs, increased significantly from baseline values during rhythmic handgrip contraction of both the dominant and non-dominant arm and returned to resting values after cessation of handgrip contractions (Figures 3.13 and 3.14). The increases in ABP and HR evoked by rhythmic contractions of the trained and non-trained arms were not significantly changed before vs after IHG training in either WEs or SAs (Figures 3.13 and 3.14).

In WEs, IHG training increased FBF and FVC throughout post-exercise hyperaemia in both the trained and the non-trained arm Figures 3.15 and 3.16 (LHS), the peak FBF in the trained arm increasing from 70.96±6.48 to 110.17±6.64 $ml.100ml^{-1}.min^{-1}$, and peak FVC increasing from 0.67 ± 0.06 to 1.03 ± 0.11 CU. Further, in the *non-trained arm*, FBF and FVC were increased during post-exercise hyperaemia, peak FBF increasing from 75.50 ± 6.47 to 102.64± 8.51 $ml.100ml^{-1}.min^{-1}$, and peak FVC increasing from 0.72± 0.05 to 0.89±0.06 CU.

Similarly, in the SAs, IHG training increased FBF and FVC during post-contraction hyperaemia in both the trained and non-trained arms Figures 3.15 and 3.16 (RHS) in the trained arm. Peak FBF increased from 74.19±7.26 to 109.64±5.43 $ml.100ml^{-1}.min^{-1}$, and peak FVC increased from 0.71±0.07 to 0.99±0.06 CU in the trained arm. However, in the non-trained arm of SAs, peak FBF did not increase significantly (86.86±9.02 before, 93.92±8.99 $ml.100ml^{-1}.min^{-1}$ after) and neither did peak FVC (0.86 ± 0.10 vs 0.87±0.13 CU, Figure 3.15, 3.16).

Any differences between the increases in FBF/FVC shown in the two arms did not reach statistical significance in WEs (FBF: 66.34±18.49% vs 40.71±12.15% and FVC: 47.27±22.57% vs 17.47±10.83%) as shown in Figure 3.17. There were no significant differences between ethnicities for the changes in FBF and FVC. IHG training affected the % increase in FBF and FVC evoked during exercise hyperaemia more in the trained arm than non-trained arm of SAs (FBF: 56.2±13.2% vs 16.0±12.0% and FVC: 47.3±17.9% vs 10.6±21.2% respectively).

3.3.5 Cutaneous vascular responses

Baseline cutaneous RCF was lower in the non-dominant (non-trained) arm of WEs after IHG training than before (Table 3.2). Before IHG training, COX inhibition with aspirin caused a significant decrease in baseline RCF (Table 3.4), whereas after IHG training, COX blockade had no significant effect (Table 3.4). By contrast, in SAs, IHG training had no effect on baseline RCF, and COX blockade had no effect on baseline RCF before IHG training. However, COX blockade had a significant effect in RCF baseline in SAs after IHG training (Table 3.4). Due to the effects of IHG training and COX inhibition on baselines in WEs, all responses evoked in cutaneous circulation are expressed as change from baseline RCF.

Reactive hyperaemia. Before training, release of arterial occlusion evoked substantial reactive hyperaemia in cutaneous circulation of the non-dominant (non-trained) arm of both WEs and SAs, the peak response being significantly higher in WEs than in SAs (WEs: 70.11± 6.17 vs SAs: 56.08±10.07, $P<0.05$).

Before IHG training, in WEs, COX inhibition had no effect on the changes evoked in RCF during reactive hyperaemia. (Figure 3.18): Peak ΔRCF was 70.11±6.17 before and 58.81±6.57 PU ($P=0.3584$) after COX inhibition. Similarly, in SAs, before IHG training, COX inhibition had no effect on reactive hyperaemia (Figure 3.18): Peak RCF was 56.08±10.07 ΔRCF before, and 64.93±6.48 PU after COX inhibition (Figure 3.18) ($P=0.9482$). The number of subjects demonstrating the highest peak ΔRCF value during reactive hyperaemia was 5/10 for each ethnic group.

After IHG training in WEs, ΔRCF during reactive hyperaemia was greater than before IHG training: peak reactive hyperaemia was 76.7± 4.72 after vs 70.11±6.17 PU before IHG, $P<0.0001$; Figure 3.19). Similarly, in SAs, ΔRCF *during* reactive hyperaemia was greater after IHG training than before, but this was not associated with an increase in peak reactive hyperaemia: ΔRCF at 10s was greater after training: 29.10±4.94 vs 19.15±5.94 before IHG training ($P<0.0001$; Figure 3.19). The number of subjects demonstrating the highest peak ΔRCF value during reactive hyperaemia was 8/10 for WEs and 6/10 for SAs.

After IHG training, COX blockade again had no significant effect on reactive hyperaemia in either WEs or SAs (Figure 3.18): peak reactive hyperaemia was 76.7±5.6 vs 85.0±4.2 PU before vs after COX blockade in WEs (P=0.96) and in SAs, peak RCF was 58.8± 6.2 and 64.5±9.8 PU before vs after COX inhibition (P=0.5471).

ACh-evoked dilatation. In WEs before IHG training, iontophoresis of ACh evoked a graded increase in cutaneous RCF in the non-trained arm and COX blockade had no effect on this response (Figure 3.20-LHS). IHG training itself had no effect on the ACh-induced cutaneous dilatation in WEs (Figures 3.20 and 3.21). However, after IHG training, COX blockade enhanced the ACh-evoked ΔRCF in the non-trained arm of WEs (Figure 3.21-LHS).

In SAs, before IHG training, ACh-evoked similar cutaneous dilatation to that seen in WEs (Figure 3.21-RHS) and COX blockade had no significant effect on the ACh-evoked response. After IHG training, the ACh-response was not changed as in WEs (Figures 3.21), but any trend for COX blockade to increase the ACh response did not show any difference at all: (Figure 3.21-RHS; P=0.976).

Anthropometric Characteristics	White Europeans (n=10)	South Asians (n=10)	P value
Age	23.9±0.7 yrs	22.6±1.0 yrs	P=0.15
BMI (kg/m^2)	22.35±0.87	23.5±1.08kg/m^2	P=0.28
Waist:hip ratio (cm)	0.95±0.04 cm	0.89±0.02 cm	P=0.19
Physical activity level	01 Low 10 Average 9 High= 20	05 Low 10 Average 0 High= 15	P=0.71
Parental Hypertension	06/10	08/10	P=0.63

Table 3.1 **Anthropometric Characteristics in White European (LHS) and South Asian (RHS) men.** Values are shown as mean ± SEM. **Physical activity level**: calculated from number of participants achieving one of the 3 levels of PA 0-2 Low (x1), 3-4 Average (x2), ≤5 High (x3) which was multiplied by the number of the people who achieved that level; **Parental cardiovascular disease**: subjects who reported that their parents and grandparents diagnosed with cardiovascular disease and presented related symptoms (M.I., angina, etc.) and risk factors (high blood pressure, diabetes etc.).

	WE (n=10)	WE (n=10)	SA (n=10)	SA (n=10)
	Before IHG Training	After IHG Training	Before IHG Training	After IHG Training
Systolic pressure (mmHg)	124.5±1.82	125±1.8	116.6±2.6	115.9±6.7
Diastolic pressure (mmHg)	69.8±1.67	72±1.6	65.1±2.7	74±2.3
MABP (mmHg)	88.03±2.6	89.4± 2.3	82.2±2.5	83.3±2.5
FBF (non-trained arm) $ml.min^{-1}.100ml^{-1}$	4.95 ±0.6	6.53±0.68*	5.3±0.6	6.8±1.1
FBF (trained arm) $ml.min^{-1}.100ml^{-1}$	5.1±0.5	7.84±0.8*	6.1±0.4	7.1±0.7
FVC (non-trained arm) Conductance units (CU)	0.06±0.01	0.08±0.01*	0.08±0.01	0.09±0.01
FVC (trained arm) Conductance units (CU)	0.06±0.01	0.10±0.01*	0.07±0.01	0.10±0.01*
RCF (PU) (non-trained arm)	22.8±0.1	15.4±1.9***	12.5±0.1	13.5±0.1

Table 3.2 **Cardiovascular baselines in White European (LHS) and South Asian (RHS) men for Protocol 1 and 2 before and after IHG training.** Values are shown as mean ± SEM. ***, *: before vs after IHG training between WEs or SAs; P<0.001, P<0.05 respectively. Aspirin effect: ǂǂ:P<0.001; Baseline Aspirin Vs after IHG aspirin: ǂP<0.01.

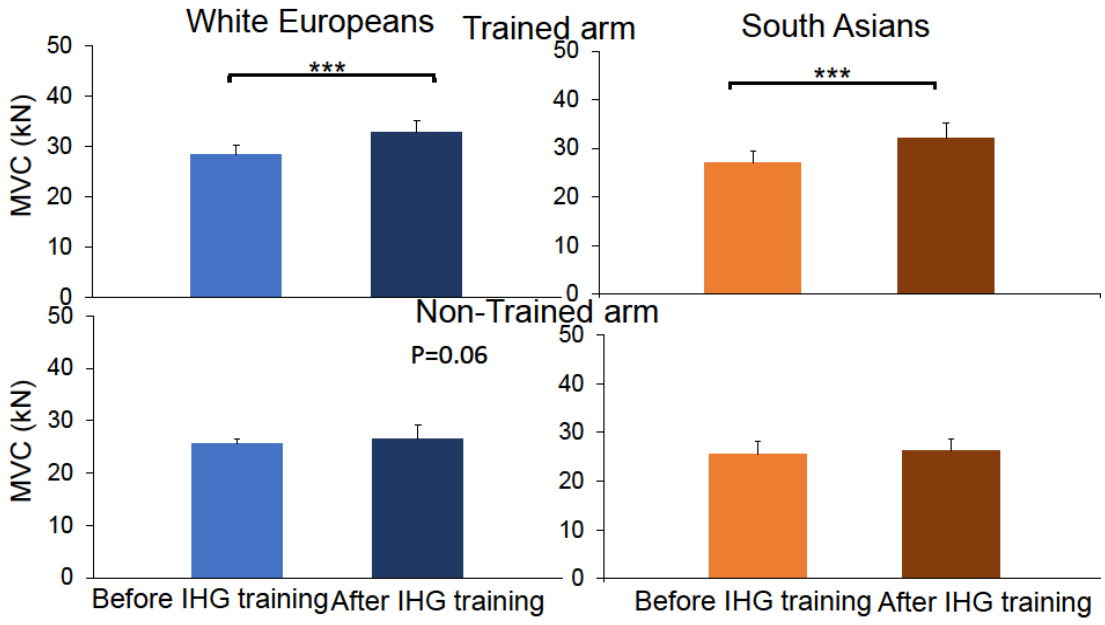


Figure 3.7: **Effect of IHG training on MVC in dominant, trained arm (above) and non-dominant, non-trained arm (below) before and after training in White European (LHS) and South Asian men (RHS).** Values shown as mean ±SEM. *, **, ***: before vs after training P<0.05, P<0.001 respectively.

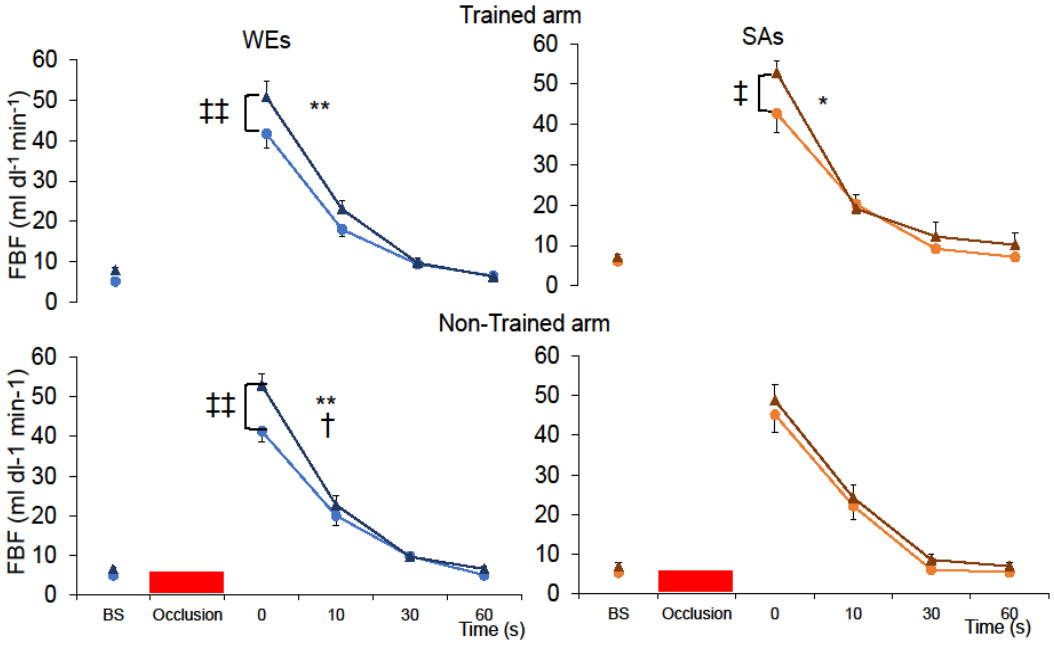


Figure 3.8: **FBF recorded at rest and following arterial occlusion for 3 min in trained and non-trained arm of** WEs (LHS): BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), (before (light blue) and after (dark blue) IHG training and in SAs (RHS): BS: before (circular orange dot)and after IHG training (triangular dark orange dot), before (light orange) and after (dark orange) IHG training. *,**,***: before vs after training: $P<0.05$, $P<0.01$,$P<0.001$, †, ††: time*treatment IHG training, $P<0.01$ $P<0.001$, ‡, ‡‡: peak responses before vs after training $P<0.05$, $P<0.01$ respectively.

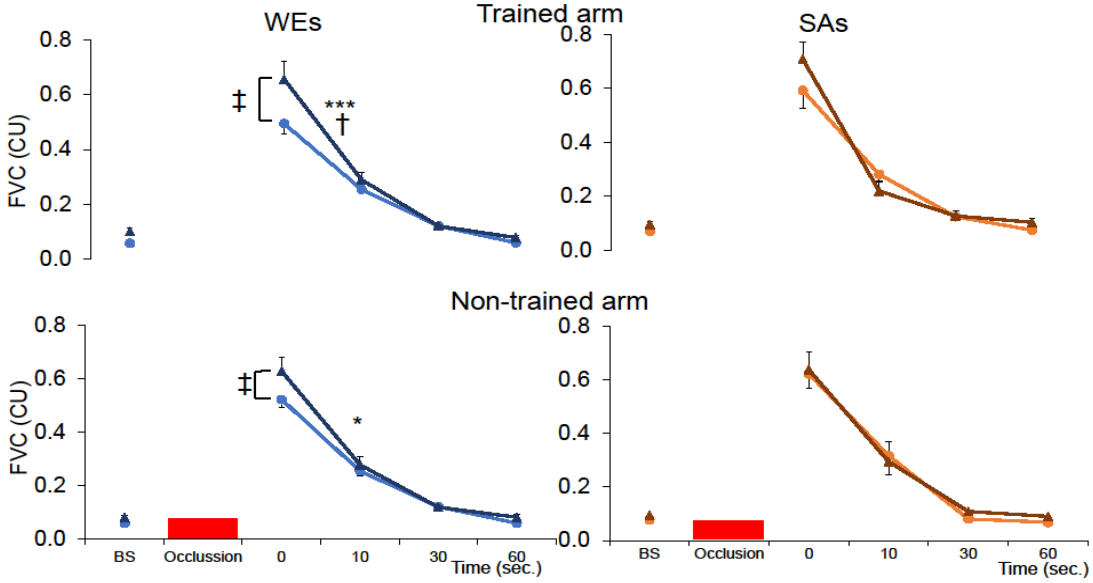


Figure 3.9: **FVC recorded at rest and following arterial occlusion for 3 min in the trained arm and in the non-trained arm** in White Europeans (LHS): BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), before (light blue) and after (dark blue) IHG training and in South Asians (RHS): BS: before (circular orange dot)and after IHG training (triangular dark orange dot), before (light orange) and after IHG (dark orange) training. *,**,***: before vs after training: $P<0.05$, $P<0.01$,$P<0.001$, †, ††: time*treatment IHG training, $P<0.01$ $P<0.001$, ‡, ‡‡: peak responses before vs after training $P<0.05$, $P<0.01$ respectively.

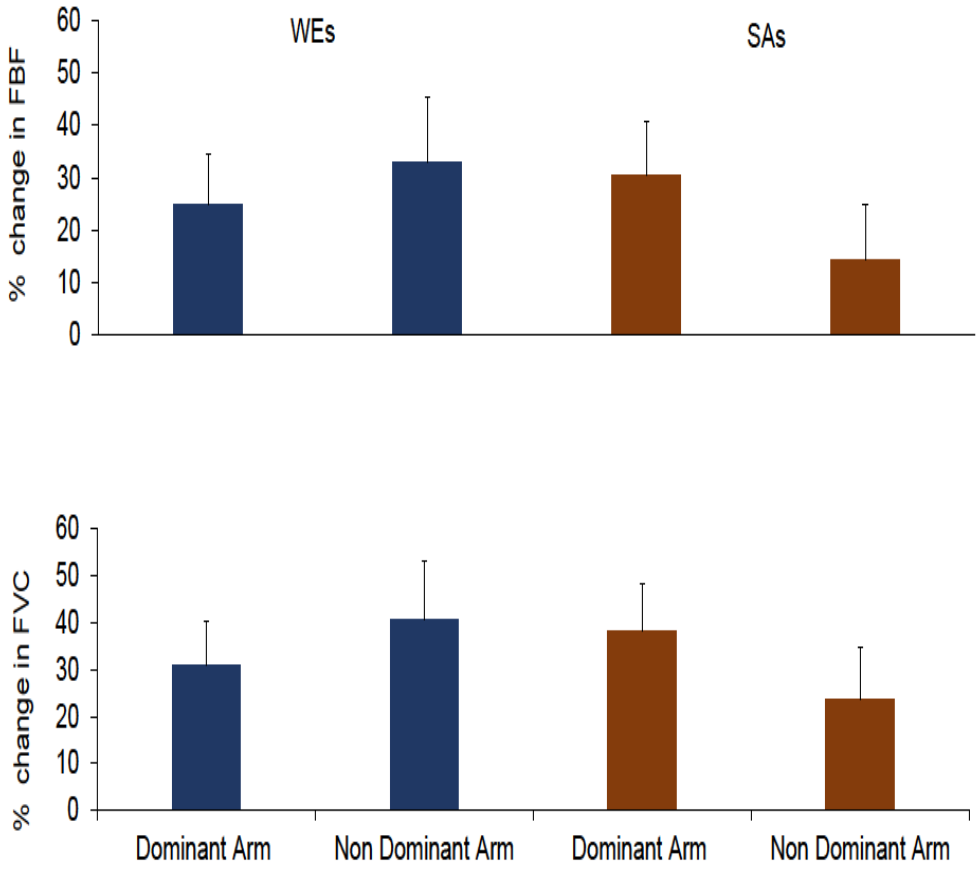


Figure 3.10: **Percentage change caused by IHG training in peak FBF (upper panel) and FVC (lower panel) during reactive hyperaemia in trained (dominant) and non-trained (non-dominant) arm of White Europeans (LHS) and South Asians (RHS)**. Values are shown as mean ± SEM.

	Actual TTI (KN)		(%) change in Actual TTI	Expected TTI (KN)		Actual/Expected as percentage	
	Before IHG training	After		Before IHG training	After	Before IHG training	After IHG training
			Dominant Arm				
WEs	8.9±1.3	17.1±1.8***	107.6±19.3***	15.4±0.1	17.8±1.2***	55.9±5.9	95.8±7.2***
SAs	7.3±1.1	13.4+1.9**	93.7±27.7***	14.6±1.3	17.3±1.7**	49.3±4.7	76.1±7.9**
			Non Dominant Arm				
WEs	7.2±1.3	11.1±1.1**	77.7±23.7***	13.7±0.5	14.6±0.7*	51.9±8.1	76.6±6.4*
SAs	5.6±0.9	8.9±1.3**	73.1±24.5***	13.2±1.3	14.3±1.3	41.1±4.6	61.7±6.8**

Table 3.3 **Effects of IHG training on muscle performance in dominant, trained arm and non-dominant, non-trained arm of WEs (LHS) and SAs (RHS).** Values are shown as mean ± SEM. ***, **, *: P<0.001, P<0.01, P<0.05, Before vs after IHG training.

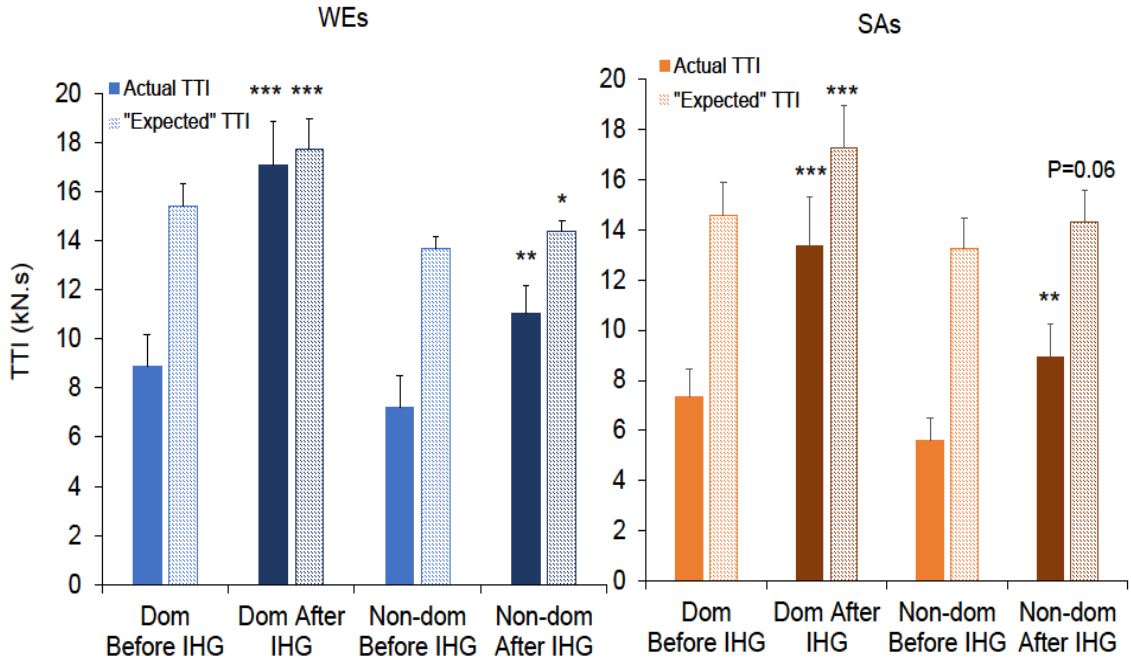


Figure 3.11: **Effect of IHG training on Expected Time-Tension index (TTI, above) and Actual TTI (below) in dominant arm that underwent IHG training and non-dominant, non-trained of White European (LHS) and South Asian men (RHS).** Values shown as mean ±SEM. *, **, ***: before vs after IHG training $P<0.05$, $P<0.01$; $P<0.001$ respectively.

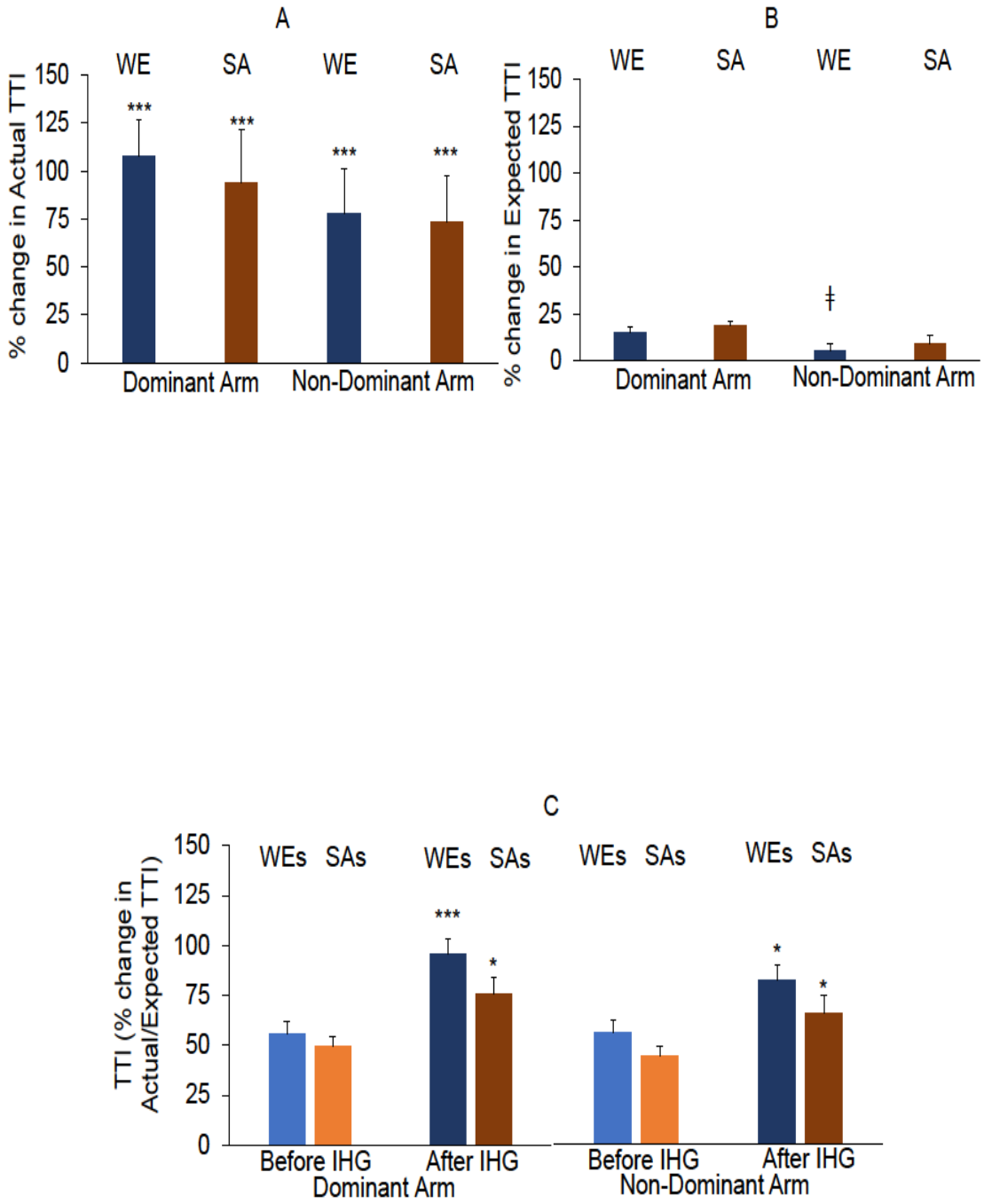


Figure 3.12: **Actual TTI and Expected TTI (A&B) in dominant and non-dominant arm after IHG training in White European (WE) and South Asian (SA) men shown as percentage changes; Actual and Expected/Expected % (C) before and after training (below).** Values shown as mean ±SEM. *, **,***: before vs after training, ǂ, ♯ : dominant vs non-dominant arm changes after IHG training, $P<0.05$, $P<0.01$ $P<0.001$ respectively.

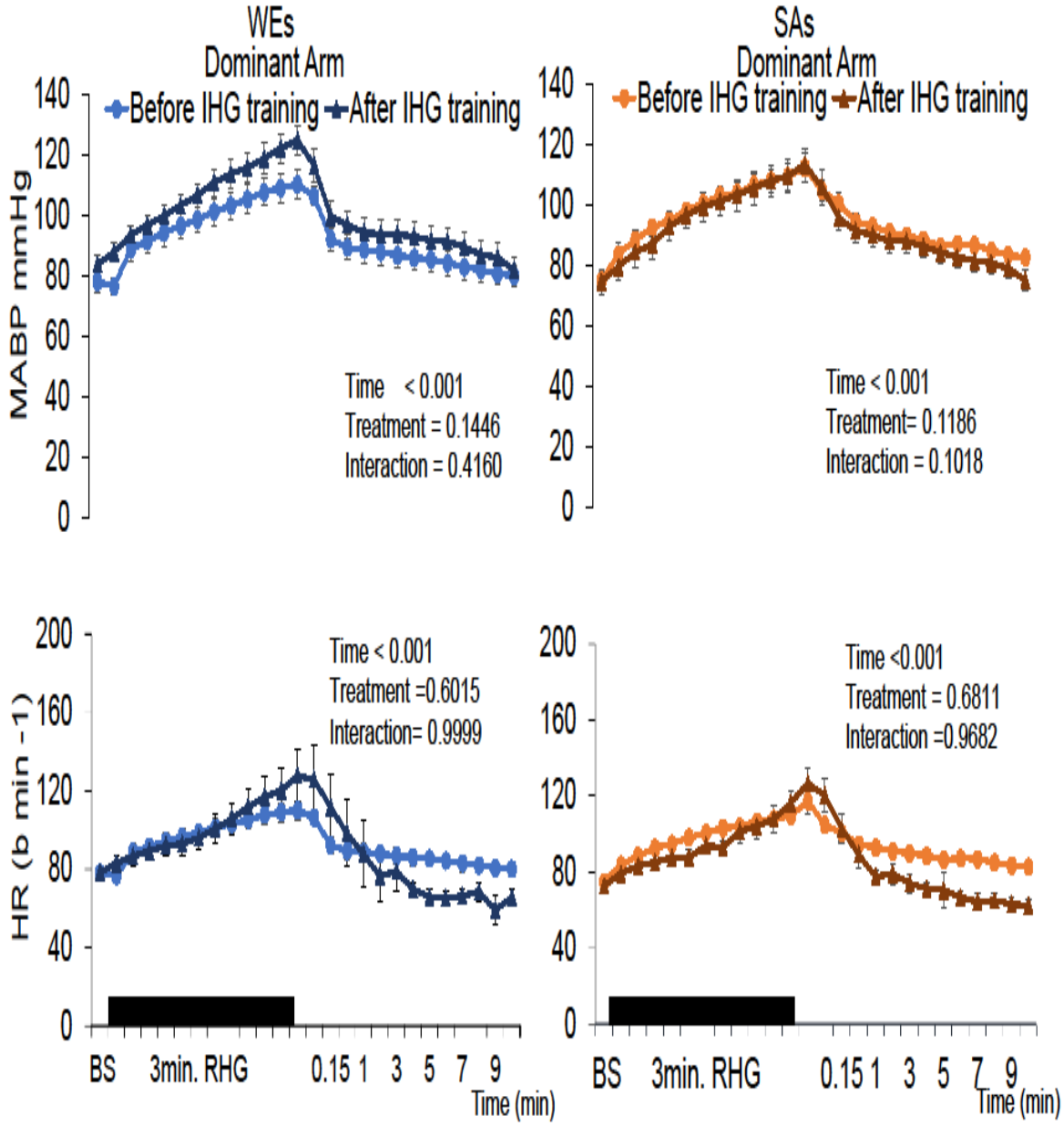


Figure 3.13: **MABP responses at rest, during rhythmic handgrip exercise of the dominant arm contracting at 60% MVC over 120 sec period, recovery that underwent IHG training (above) and HR (below) in White Europeans (LHS) and South Asians (RHS).** *, ***: before vs after training $P<0.05$, $P<0.001$ respectively.

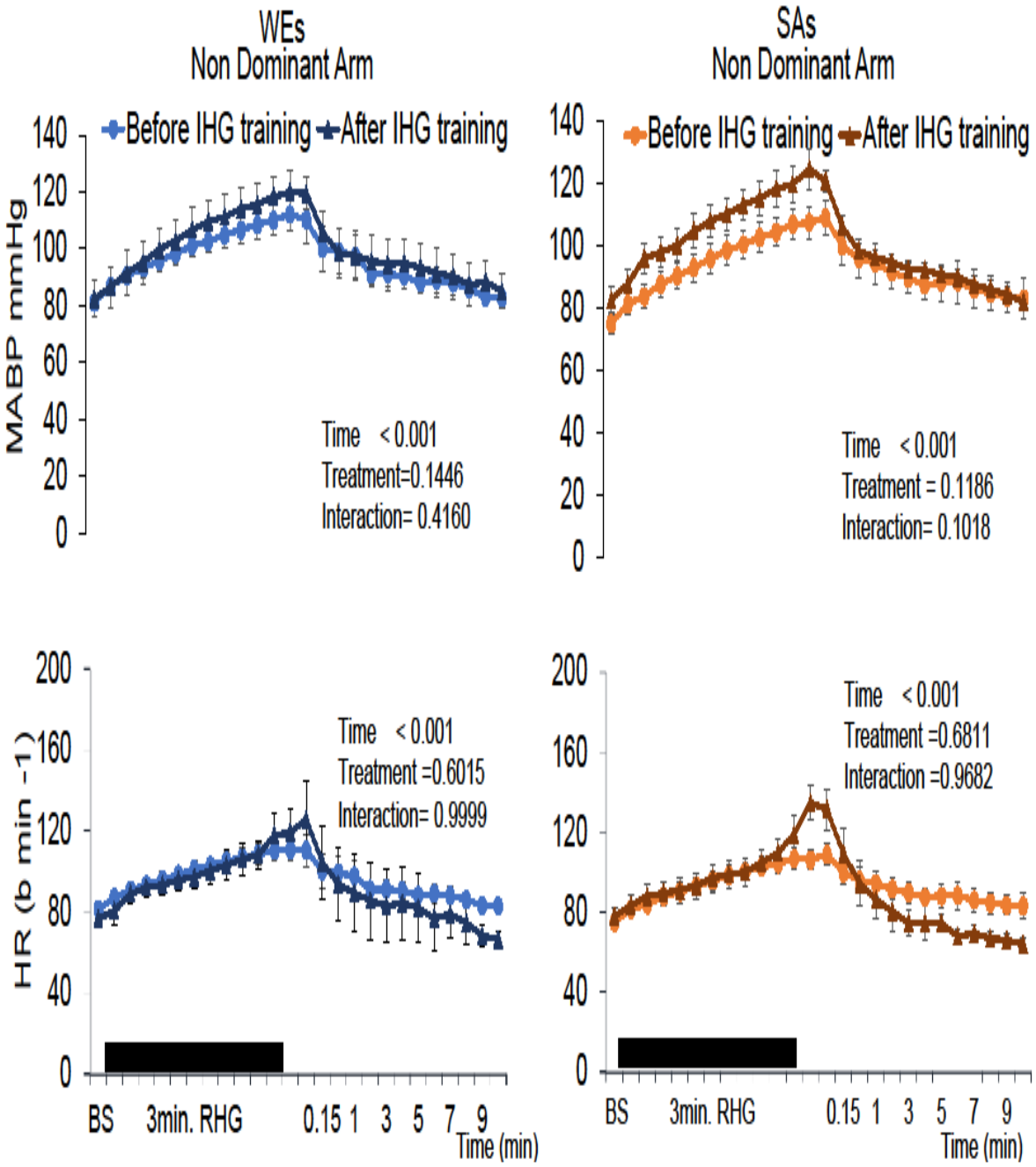


Figure 3.14: **MABP responses at rest, during rhythmic handgrip exercise of the non-dominant arm contracting at 60% MVC over 120 sec period, recovery that underwent IHG training (above) and HR (below) in White Europeans (LHS) and South Asians (RHS).** *, ***: before vs after training $P<0.05$, $P<0.001$ respectively.

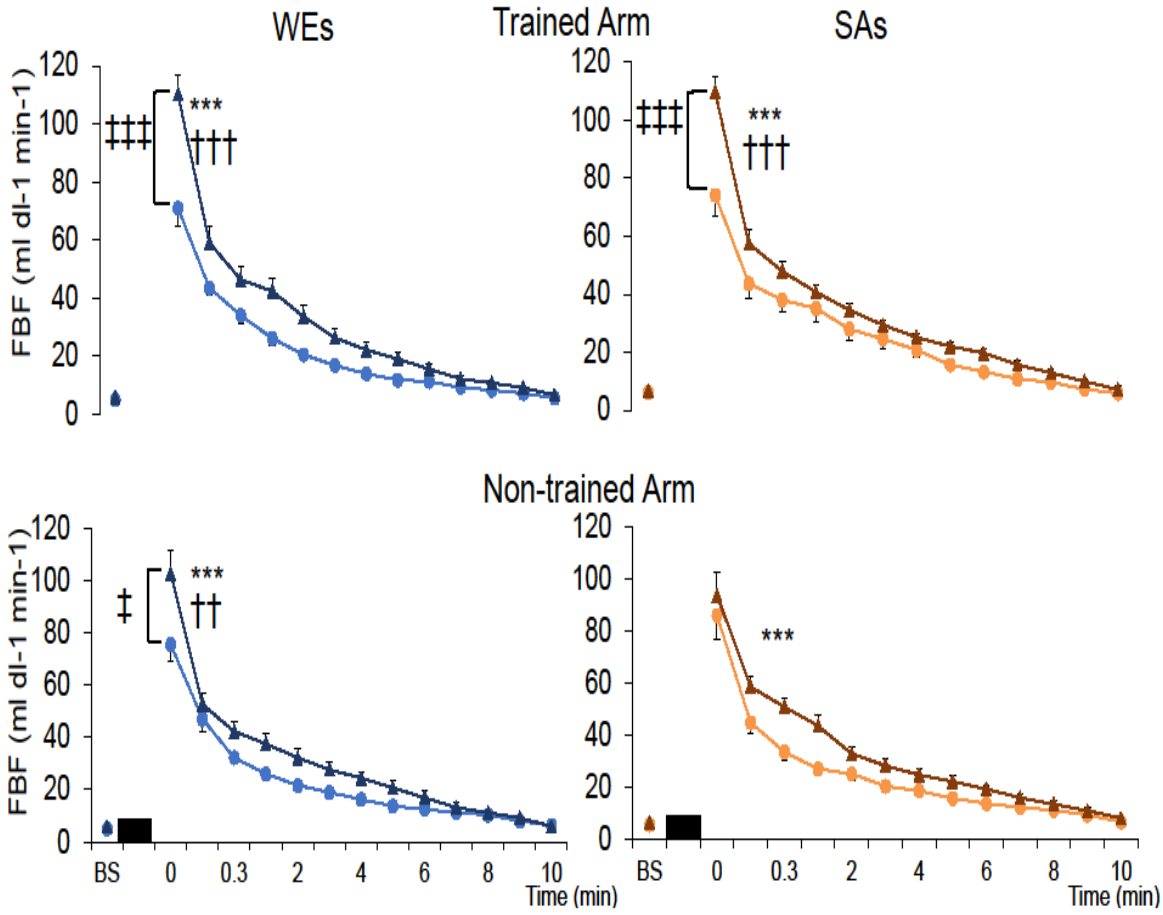


Figure 3.15**: Effects of rhythmic contraction at 60%MVC for 3 min on FBF in White Europeans (LHS) and South Asians (RHS) before and after IHG training in the trained arm (above) and non-trained arm (below).** White Europeans: BS: before IHG training (circular light blue dot), after IHG training (triangular dark blue dot), before (light blue line) and after (dark blue line) IHG training, South Asians: BS: before IHG training (circular orange dot), after IHG training (triangular dark orange dot), before (light orange) and after (dark orange). ***: before vs after training: $P<0.0001$, †, ††, †††: time*treatment IHG training, $P<0.05$, $P<0.001$, $P<0.0001$; ‡, ‡‡, ‡‡‡: peak responses before vs after training $P<0.05$, $P<0.01$, $P<0.001$ respectively.

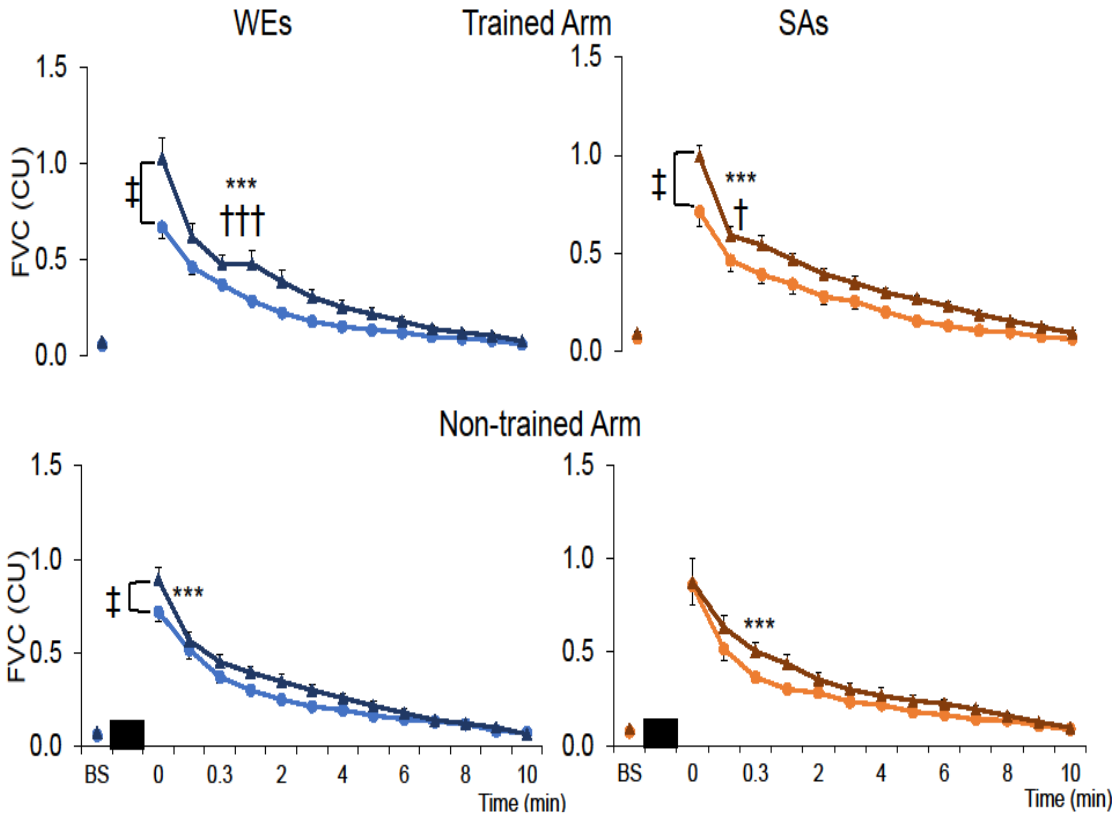


Figure 3.16: FVC recorded at rest and after rhythmic contraction at 60%MVC for 3 min in White Europeans (LHS) and South Asians (RHS) before and after IHG training in the trained arm (above) and non-trained arm (below). White Europeans: BS: before IHG training (circular light blue dot), after IHG training (triangular dark blue dot), before (light blue line) and after (dark blue line) IHG training, South Asians: BS: before IHG training (circular orange dot), after IHG training (triangular dark orange dot), before (light orange) and after (dark orange). ***: before vs after training: $P<0.0001$, †, ††, †††: time*treatment IHG training, $P<0.05$, $P<0.001$, $P<0.0001$, ‡, ‡‡, ‡‡‡: peak responses before vs after training $P<0.05$, $P<0.01$, $P<0.001$ respectively.

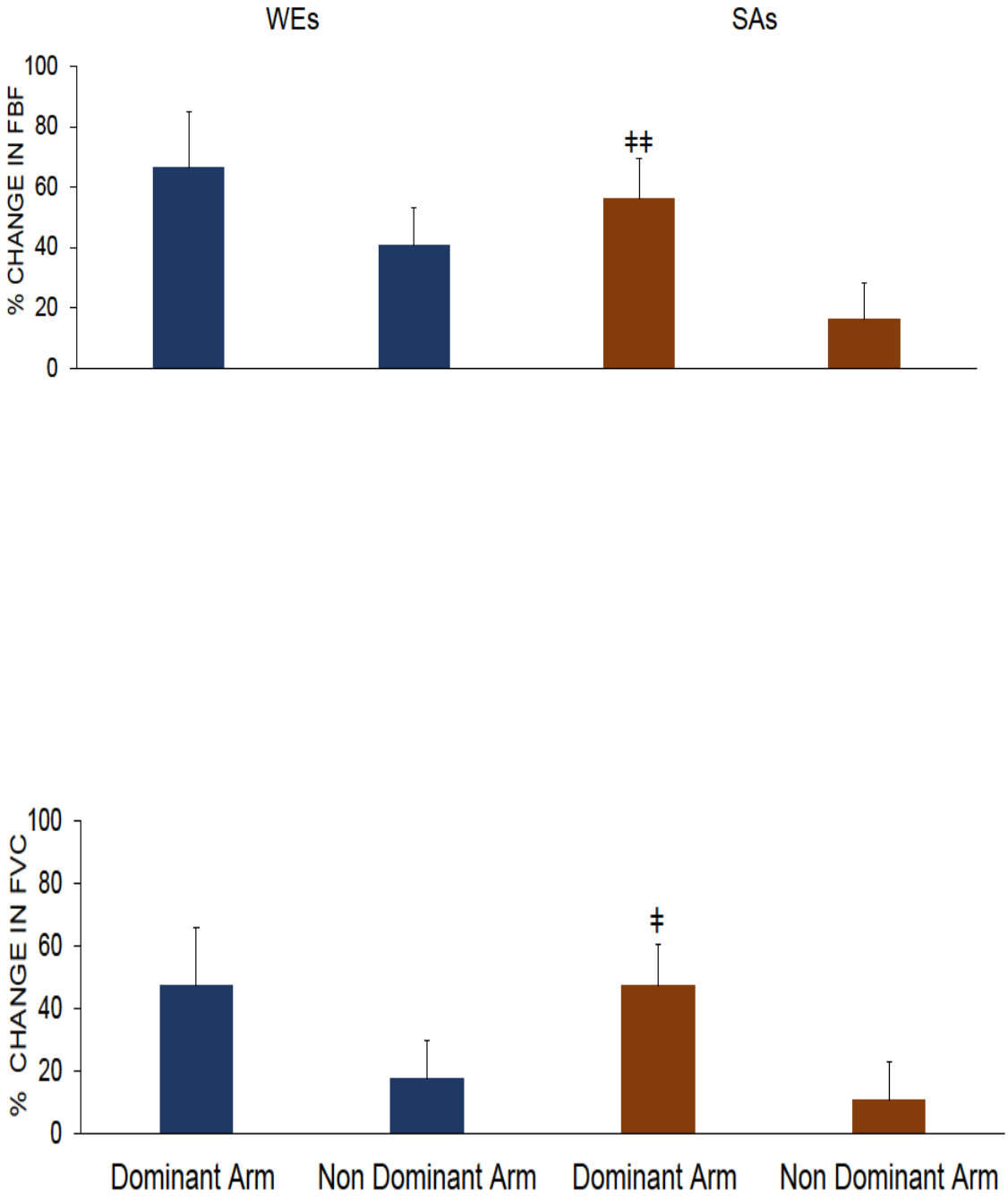


Figure 3.17: **Percentage increase in peak FBF (upper panel) and FVC (lower panel) following rhythmic contraction at 60%MVC for 3 min caused by IHG training** in WEs (LHS) and SAs (RHS) ǂ, ♯ : dominant vs non-dominant arm changes after IHG training, P<0.05, *P*<0.01, *P*<0.001 respectively.

	WE (n=10)	WE (n=10)	SA (n=10)	SA (n=10)
	Before IHG Training	After IHG Training	Before IHG Training	After IHG Training
Baseline RCF (PU) (non-trained arm)	22.8±0.1	15.4±1.9***	12.5±0.1	13.5±0.1
After Aspirin RCF (PU) (non-trained arm)	16.3±0.1ǂǂ	14.6±1.8	13.8±0.1	15.4±0.1ǂǂ

Table 3.4 Effect of Aspirin on baseline cutaneous RCF in the non-trained arm of White European (LHS) and South Asian (RHS) men before and after IHG training. Values are shown as mean ± SEM. Values are shown as mean ± SEM. ***, *: before vs after IHG training within WEs or SAs; P<0.001, P<0.05 respectively. Aspirin effect: ǂǂ:P<0.001; Baseline Aspirin Vs after IHG aspirin: ǂP<0.01.

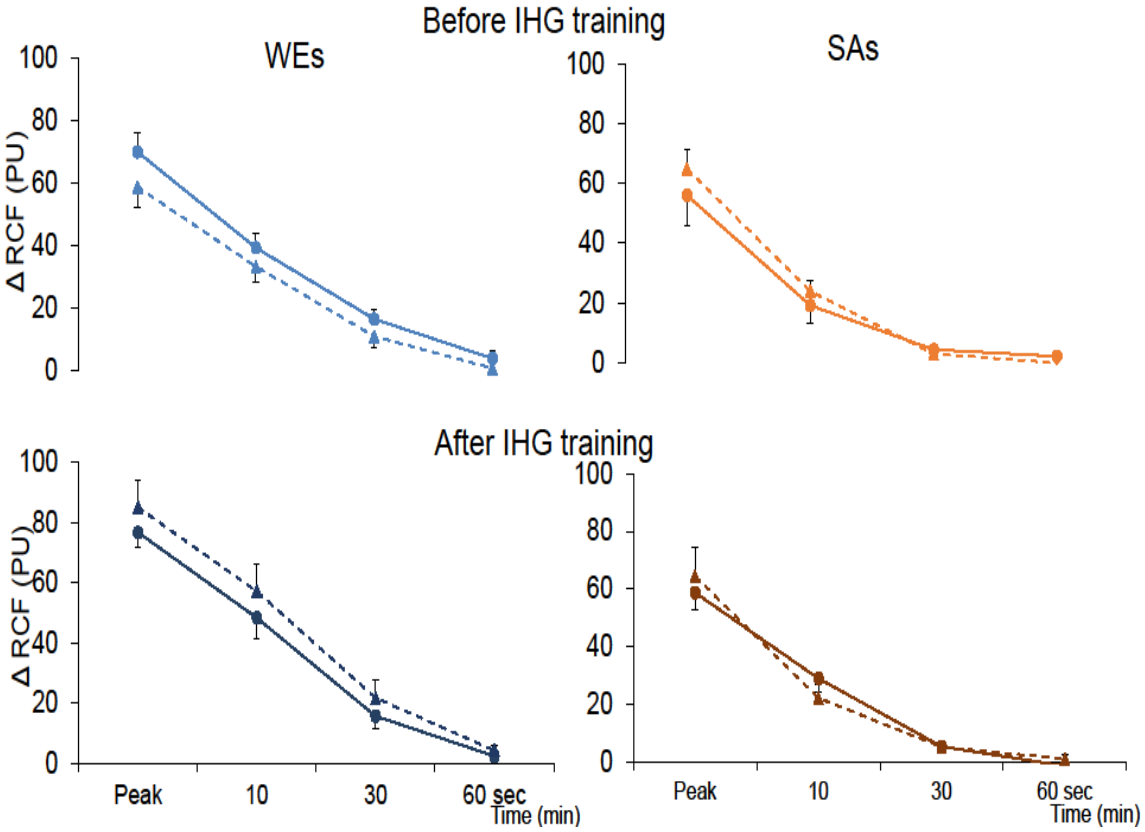


Figure 3.18: **Effect of aspirin on change (Δ) in RCF recorded after arterial occlusion for 3 min in cutaneous circulation of non-trained arm of White Europeans (LHS) and South Asians (RHS) before (above) and after (below) IHG training.** In each case values recorded after COX inhibition are connected by dashed lines. Values shown as mean±SEM.

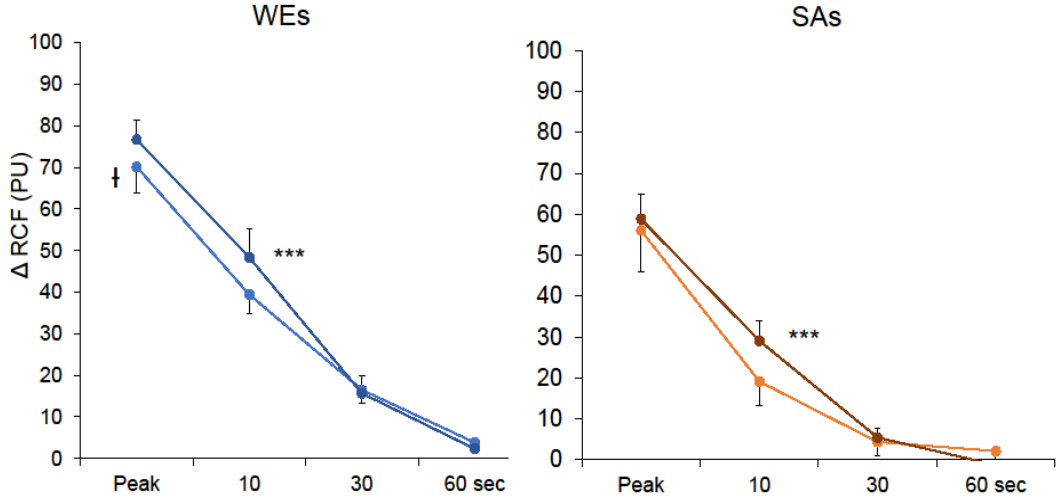


Figure 3.19: **Δ(change) RCF recorded after arterial occlusion for 3 min in White Europeans (LHS) and South Asians (RHS) before and after IHG training in the cutaneous circulation of the non-trained arm.** White Europeans: before (light blue) and after (dark blue) IHG training; South Asians: before (light orange) and after (dark orange) IHG training. Values shown as mean ± SEM. ***: before vs after training: $P<0.0001$ ǂ: WE VS SA, $P<0.05$.

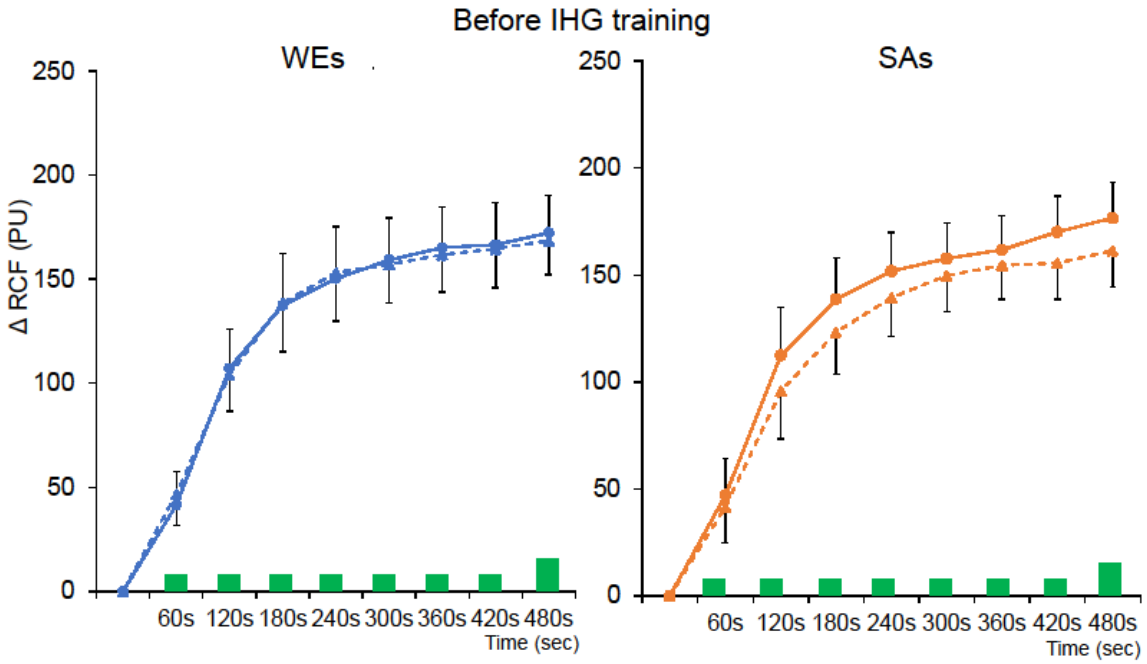


Figure 3.20: **Change (Δ) in RCF recorded during ACh iontophoresis in White Europeans (LHS) and South Asians (RHS) in the cutaneous circulation of the non-trained arm before and after COX inhibition (dashed lines).** Values shown as mean±SEM.

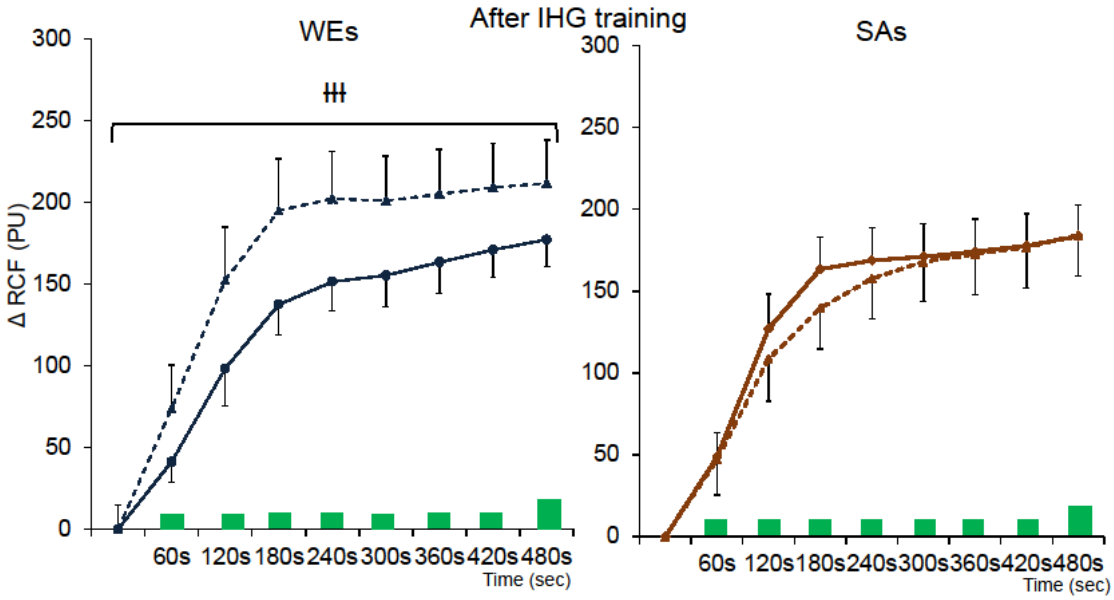


Figure 3.21**: Δ RCF recorded during ACh iontophoresis in White Europeans (LHS) and South Asians (RHS) in the cutaneous circulation of the non-trained arm after IHG training. White Europeans: baseline (dark blue) and COX (dark blue-dashed line); South Asians: baseline (dark orange) and (dark orange-dashed line).** Values shown as mean±SEM., ǂǂ: P<0.0001 after COX inhibition.

3.4 DISCUSSION

The present study showed that in both young WE and SA men, IHG training of one arm for 4 weeks improved peak muscle power in the trained arm, but not in the non-trained arm and had no effect on resting SP, DP or mean ABP. In addition, the present study made several novel findings. Firstly, IHG training was accompanied by an increase in resting FVC vascular conductance in the trained, and non-trained arm of WEs, but only in FVC in SAs (table 3.2). Secondly, following IHG training, there was an increase in muscle performance during rhythmic contractions at 60% MVC in the trained and non-trained arm of both WEs and SAs (Figure 3.7). Furthermore, there was a concomitant increase in the post-contraction hyperaemia in both the peak and the whole response evoked by rhythmic contractions at 60% MVC in the trained arm of both WEs and SAs but increased reactive hyperaemia in the non-trained arm of WEs only (Figures 3.15, 3.16, 3.8, 3.9). Thirdly, in both WEs and SAs, IHG training increased reactive hyperaemia in the trained forearm, but augmented reactive hyperaemia in the non-trained arm of WEs only (Figures 3.8, 3.9). Fourthly, in the cutaneous circulation of the non-trained forearm, peak changes in RCF evoked during reactive hyperaemia before IHG training, were higher in WEs compared to SAs, but COX inhibition with aspirin had no effect in either ethnicity (Figures 3.18, 3.19). Further, IHG training augmented reactive hyperaemia in cutaneous circulation of WEs but not in SAs, although COX inhibition still had *no* effect on reactive hyperaemia in cutaneous circulation in either WEs or SAs (Figures 3.18). Fifthly, cutaneous vasodilator responses evoked by ACh were similar in WEs and SAs before IHG training and were not affected by COX blockade in either ethnicity. However, IHG training had no effect on ACh-evoked dilation in WEs or SAs, but COX blockade augmented the ACh responses after IHG training in WEs, but not SAs (Figures 3.20, 3.21). To summarize the findings of this study, a list is presented below:

- Both young WE and SA men, IHG training of one arm for 4 weeks improved peak muscle power in the trained arm, but not in the non-trained arm and
- Had no effect on resting SP, DP or mean ABP

- IHG training was accompanied by an increase in resting FVC vascular conductance in the trained, and non-trained arm of WEs, but only in FVC in SAs
- After IHG training, there was an increase in muscle performance during rhythmic contractions at 60% MVC in the trained and non-trained arm of both WEs and SAs
- There was a concomitant increase in the post-contraction hyperaemia in both the peak and the whole response evoked by rhythmic contractions at 60% MVC in the trained arm of both WEs and SAs but increased reactive hyperaemia in the non-trained arm of WEs only
- In both WEs and SAs, IHG training increased reactive hyperaemia in the trained forearm, but augmented reactive hyperaemia in the non-trained arm of WEs only
- In the cutaneous circulation of the non-trained forearm, peak changes in RCF evoked during reactive hyperaemia before IHG training, were higher in WEs compared to SAs, but COX inhibition with aspirin had no effect in either ethnicity
- IHG training augmented reactive hyperaemia in cutaneous circulation of WEs but not in SAs, although COX inhibition still had no effect on reactive hyperaemia in cutaneous circulation in either WEs or SAs
- Cutaneous vasodilator responses evoked by ACh were similar in WEs and SAs before IHG training and were not affected by COX blockade in either ethnicity
- IHG training had no effect on ACh-evoked dilation in WEs or SAs, but COX blockade augmented the ACh responses after IHG training in WEs, but not SAs.

Subject compliance and characteristics: To study the effects of IHG training specific compliance measures were taken to ensure that the training programme was completed as intended. After the screening session and the initial two visits the participants were provided with a portable hand dynamometer to train for 4-5 weeks and an exercise training sheet as described in the methods section. To ensure compliance, each subject visited the laboratory after 2 weeks of training so that tests

could be made of whether there were changes in their MVC. Each subject was expected to have increased their MVC during that time: In fact, 9/10 of WEs and 10/10 SAs had improved. In those subjects, the MVC was readjusted to 30% of the new MVC for the next 2 weeks of training. At the end of the 4-week training period, maximum MVC in the trained arm was increased in *all* subjects indicating their compliance to the IHG training program.

There were no differences between WEs and SAs groups regarding their characteristics such as ABP, BMI, and WHR. Interestingly, more of the SAs had parents with evidence of CVD, which was generally essential hypertension, but included also CVD incidents (M.I.) (Table 3.1; Fisher's exact test did not show any significant differences between the CVD history of the parents of the two ethnic groups) compared to WEs subjects (WEs parental CVD :6/10 vs SAs parental CVD: 8/10).

Effects on resting ABP: The lack of effect of IHG training on resting SP, DP and MABP in the young normotensive subjects of the present study must first be compared with the reports in the literature that IHG training reduces ABP. Firstly, it would not be surprising if IHG training reduced ABP more easily in people who were already hypertensive (Lawrence et al., 2015). The fact that IHG training reduced resting ABP in some previous studies on normotensive people may simply be explained by the fact that the 4 weeks IHG training used in the present study was a relatively short period compared to the 8 weeks used in many previous studies on normotensive people, for the effect on ABP seems to be dose-dependent (Badrov et al., 2013A, McGowan et al., 2007, Millar et al., 2014, Wiles et al., 2010, Wiley et al., 1992, Howden et al., 2002). However, a fall in ABP of ~4mmHg was reported at 4 weeks of IHG training in several previous studies on groups of normotensive men and women aged 19-35 years (McGowan et al., 2007, Wiley et al., 1992, Ray and Carrasco, 2000). It was suggested this reflected predominantly, a decrease in total peripheral resistance (TPR), since exercise training has no effect on cardiac output (McGowan et al., 2007). It may be that the disparity between these studies and the present study reflects the fact that the normotensive subjects of those studies were older (27.5±14.2) than those of the present study (WEs: 23.1±0.7; SAs: 22.6 ±1.0 years). Certainly, the present finding that IHG training for 4 weeks increased

baseline forearm conductance (FVC) in the trained and non-trained arm of both WEs though not SAs, suggests the contribution muscle vascular resistance makes to TPR in young men can decrease within this time consistent with previous suggestions that ABP falls due to a decrease in TPR rather than CO (Millar et al., 2014). Interestingly, Ray and Carrasco (2000) demonstrated that the reductions in diastolic pressure (DP) and MABP reductions induced by IHG training for 5 weeks in their study on normotensive subjects was not attributable to a decrease in resting muscle sympathetic nerve activity (MSNA). Further the increase in MSNA evoked by isometric handgrip at 30%MVC was also not changed by IHG training in that study. Thus, they suggested that changes in MSNA are unlikely to be important during IHG training and that other vascular adaptations might be involved. The present results are not inconsistent with this view and suggest that endothelium-dependent dilator dilatation may be involved (see below).

Muscle Performance in trained arm. The present finding that IHG training of the dominant arm enhanced the magnitude of MVC in both WEs and SAs has been reported previously when utilizing isometric exercise programs on the arm in humans of unspecified ethnicity. Such improvements in muscle performance are mainly attributable to changes in the physiological properties of spinal motoneurons, interneurons and associated reflex pathways and descending pathways (Gabriel et al., 2006) and/or the morphological properties of these neurons rather than to muscle hypertrophy or psychological factors (Yue and Cole, 1992). Non-trained people generally cannot achieve their true maximum contraction, but resistance training allows this to happen (Dowling et al., 1994, Knight and Kamen, 2001, Gandevia, 2001). Indeed, it has been reported before that repeated handgrip testing at 2-week intervals is a training stimulus wherein subjects 'learn' to maximally activate muscles (Gabriel et al., 2001). The situation in non-trained individuals, is known as 'incomplete motor unit activation' and explains the potential limitations to motor unit recruitment or firing rate (Gabriel et al., 2006). Indeed, the twitch activation technique demonstrated that muscle is not fully activated during maximum performance in untrained subjects, but was improved with training with an associated higher discharge rate of the motor unit (Knight and Kamen, 2001). Moreover, (Roland, 1985), suggested that voluntary maximal muscle contraction may require programming of the primary motor cortex, and other regions of the

frontal cortex in order to achieve high effort levels, reporting evidence for low-level activation of the supplementary motor area even during submaximal isometric contractions (Roland et al., 1980). Thus, it is possible that the motor cortex is involved in causing improved motor unit activation during the early stages of IHG training, by improving motoneuron excitability (Gabriel et al., 2006).

A consequence of the increase in MVC of the trained arm was that the "expected" TTI, requested of the subjects who were asked to rhythmically contract at 60%MVC for 3 min, was also increased, assuming they could maintain contractions of 60% MVC for the full 3 min. However, before IHG training, both ethnic groups achieved a lower Time-Tension index (TTI) during rhythmic handgrip contractions of the dominant arm at 60% MVC for 3 min than the 'Expected' TTI, i.e., they "fatigued" during the 3 min, whereas after IHG training, they were much closer to reaching the "expected TTI" even though the "expected TTI" was increased. This suggests that IHG training not only improved muscle strength in the trained arm as measured by MVC, but reduced fatigue and/or improved actual performance in both ethic (WEs and SAs) groups. These changes could be explained by improved firing rate of the motoneurons and also by their synchronization rather than by muscle adaptations to fatigue (Gabriel et al., 2006). Alternatively, or in addition, it is possible that the improved performance in the trained arm is explained by improved vasodilation and muscle blood flow during contractions (see below).

Exercise hyperaemia and reactive hyperaemia in trained arm. Regarding the haemodynamic response of the *trained arm* after IHG training, both the post-exercise increases in FBF (post-exercise hyperaemia) evoked by sub-maximal (60% MVC), rhythmic contractions in that same arm *and* the concomitant increase in FVC, which reflects the magnitude of the post-exercise vasodilation were greater after, than before IHG training. This is consistent with previous evidence that IHG training and other kinds of exercise training improve exercise hyperaemia in the trained limb (Tinken et al., 2010, Green et al., 2004, McGowan et al., 2006A, McGowan et al., 2007, Badrov et al., 2013A). Notably, Sinoway et al. (1987) showed that in 6 young subjects, 4 weeks of rhythmic forearm training with the non-dominant arm at 70% MVC at 30 contractions/min for 30 min four times/week induced a 40% improvement in the exercise hyperaemia evoked by maximal rhythmic exercise of

that arm and a corresponding reduction in forearm vascular resistance. As far as we are aware, the finding that *isometric* (IHG) training of one arm augments exercise hyperaemia evoked by *rhythmic* exercise of that arm is novel. However, there is evidence that young rock climbers who regularly undertake isometric contractions, showed larger forearm vasodilator responses to isometric and rhythmic contractions than sedentary healthy young individuals; their reactive hyperaemia response was also larger (Ferguson and Brown, 1997). Interestingly, in the present study, the extent of the improvement following rhythmic contractions was similar in WEs and SAs, so there is no reason to suggest that the SAs who have greater future risk of CVD (Rana et al., 2014) are less able to respond to the training effects on muscle vasodilation.

The increase in peak FVC in the trained arm during exercise hyperaemia could be explained by the rise of the dilator responses through individual arteriolar resistance vessels in muscle (O'Leary, 1991, Lautt, 1989, Brown, 2002). Alternatively or in addition by an increase in the density of the vascular bed, i.e. by training-induced angiogenesis or arteriogenesis (Brown and Hudlicka, 2003, Hellsten et al., 2008). Thus, 4 weeks of IHG training is certainly long enough for angiogenesis related adaptations to occur in the trained forearm. Augmentation in shear stress, which is a primary factor to angiogenetic adaptation to exercise induces upregulation of vascular endothelial growth factor (VEGF) and other pro- and angiogenic factors (Hoier et al., 2012). Moreover, even 2 weeks of passive leg exercise enhanced levels of these factors and their by-products in leg muscles and by 4 weeks there was an increase in capillary density. However, there were no tests in that study of whether the increase in capillary density was associated with an increased arteriolar density or an increase in muscle vascular conductance during exercise or reactive hyperaemia.

Considering the reactive hyperemia that followed 3 min arterial occlusion, IHG training augmented reactive hyperaemia in the trained arm of WEs and SAs but augmented peak FVC in WEs only. Previous studies showed that arterial occlusion for 3 min produces near maximal peak reactive hyperaemia: extending the occlusion duration to 5, 10 or 20 min had little effect on the peak but decreased the rate at which the dilatation waned and extended recovery time (Carlsson et al., 1987,

Tagawa et al., 1994). Indeed, as reactive hyperaemia is largely mediated by endothelium-dependent dilator substances (see Chapter 1, section 1.10.2), the finding that the peak of the response was augmented in WEs, but not SAs could be explained if endothelium-dependent dilatation following 3 min occlusion had greater potential to be improved in the WEs over the 4 week training period and that any effect on the SAs was less pronounced, consistent with their greater risk of CVD. On the other hand, if IHG training for 4 weeks induced a substantial increase in vascular density, it might have been expected that increase in FVC would have been augmented in the trained arm of the SAs, as well as in the WEs. Thus, it could be that IHG training induced greater angiogenesis in WEs than SAs.

It may be noted that in previous studies involving the same IHG training protocol as in the present study, Badrov et al. (2013A) showed in normotensive, young women, that peak reactive hyperaemia evoked in the trained arm following 5 min arterial occlusion was not changed at 4 weeks when they trained 3 times/week, but was augmented when they trained 5 times/week for 4 weeks and was augmented further at 8 weeks. On the other hand, McGowan et al. (2007) showed in a mixed group of young normotensive men and women, that neither 4, nor 8 weeks IHG training affected FMD evoked in the brachial artery by 5 min arterial occlusion of the trained arm. Thus, the present results are consistent with those of Badrov et al. (2013A) and indicate that 4 weeks IHG training does improve endothelium-dependent dilatation at the level of vessels that contribute to vascular resistance (conductance), at least in WEs.

These proposals should be tested more rigorously in future studies by recording an estimate of maximal vascular conductance in the trained forearm by assessing peak dilatation induced by an agent such as papaverine that produces maximal vasodilation, which would help distinguish between an increase in the size of the vascular bed and increased dilatation of existing blood vessels. Whether or not the IHG training protocol was sufficient to induce angiogenesis in the present study, there is evidence from previous studies that exercise training augments exercise hyperaemia in the trained muscle by facilitating endothelium-dependent, NO and PG-dependent dilatation (e.g., (Green et al., 2004, Spier et al., 2007). This is consistent with other aspects of the present findings, as discussed in Chapter 5.

Irrespective of the mechanisms that improve vasodilation during exercise and reactive hyperaemia following IHG training, and whether or not angiogenesis occurred, the improved vasodilation in the trained arm after IHG training may have contributed causally to the improved muscle performance over the 3 min of rhythmic contractions at 60%MVC, by improving oxygen (O_2) delivery. Previous studies suggested that exercise training can improve leg muscle O_2 extraction (Mourtzakis et al., 2004), with increased effectiveness in previously sedentary individuals (Saltin et al., 1968). During exercise, O_2 extraction, which is the amount of O_2 transferred per unit time between capillaries and mitochondria, is a product of the diffusive capacity of the muscle for O_2 and the difference between oxygen tension in the capillary and the mitochondria (Wagner, 2012). Animal studies have shown that exercise training not only increases capillary density but increase the mitochondrial reticular network providing a larger oxygen sink, and leading to enhanced O_2 diffusion and O_2 tension in the contracting myocyte (Poole and Mathieu-Costello, 1996); which might in turn be expected to allow increased performance and reduce fatigue. In future studies the effects of IHG training on the relationship between tissue oxygenation and forearm blood flow during muscle contraction could be investigated by using NIRS (near-infrared spectroscopy) (Celie et al., 2012, Gayda, 2014).

The fact that the present results demonstrated an improvement in performance in the *trained* arm raises the possibility of improved skeletal muscle oxidative capacity and reduced accumulation of factors that contribute to the sensation of fatigue (Allen et al., 2008). Metabolic by-products such as H^+, lactate, and inorganic phosphate are considered major contributors to peripheral fatigue. It would be interesting in future studies to test whether IHG training reduces the accumulation of substances like H^+, lactate, and inorganic phosphate.

Muscle performance in the non-trained arm. A major, novel finding of the present study is that although IHG training had no detectable effect on MVC in the non-trained arm of either ethnicity, there was substantial improvement in muscle performance in the non-trained arm of both WEs and SAs. An improvement in MVC (power) might have been expected in line with of 'cross-transfer' or "cross-education": a phenomenon that is over a century old, and for which the mechanism/s

remains unclear. The cross-transfer phenomenon is that training of one limb leads to improvement in maximal force of the contralateral limb (Gabriel et al., 2006). For example, it was reported that, 8 weeks of isometric handgrip training increased strength of the contralateral elbow flexors by as much as 25% (Moritani and deVries, 1979). Moreover, the cross-transfer effect has been reported to be effective in improving strength in both the upper limbs and lower limbs (Hellebrandt et al., 1951, Shields et al., 1999, Yuza et al., 2000). However, more recent meta-analyses of studies on cross transfer focusing on well-controlled studies involving unilateral resistance (isometric) training such as that used in the present study indicated that the effect on contralateral muscle strength is rather small. Thus, in 13 studies which involved isometric training of at least 50% MVC for a minimum of at least 2 weeks, the improvement in contralateral muscle strength was only ~7.8% which was ~35% of the strength gain in the trained limb (Munn et al., 2004). This estimate was updated to an improvement of ~8%, amounting to ≤ 50% of the improvement in the trained limb when 16 well-controlled studies were included (Carroll et al., 2006). Further, this estimate compared reasonably well with that made in a large randomized controlled trial on training of elbow flexors which achieved a 7% improvement in strength of the non-trained arm compared with ~25% of the improvement on the trained side (Munn et al., 2005).

Judging from the estimates just described, we might have expected at most, an improvement of ~1.8 kN in WEs and SAs on MVC of 25.5 kN in WEs and 25.6 kN in SAs respectively, which would have been very difficult to detect with subject groups of only 10. But, in fact, the estimated "Expected" performance over the full 3 min of contractions at 60%MVC was significantly improved from 13.67 to 14.36 kN.s in the non-trained arm of WEs, whereas no significant improvement were seen in SAs. Given "Expected" performance was calculated as the product of 60% MVC and 90s - the time over which subjects were asked to perform contractions, it seems likely that that there was a small cross-transfer effect of 4 weeks IHG training in the WEs at least, that might be detected if a larger group of subjects were used. Although "Expected" performance was not improved in SAs, IHG training at 30%MVC for 4 weeks *did* cause substantial improvement in *actual* muscle performance in the non-trained arm during acute bouts of rhythmic handgrip exercise at 60% MVC for 3 min from 7.21 to 11.06 kN.s and from 5.86 to 8.94 kN.s of WEs and SAs respectively,

amounting to ~77.7% and ~73.1% increases respectively. As far as we are aware, this is a novel finding for very few studies have been performed on the effects of unilateral training on *performance* in the contralateral limb (Carroll et al., 2006, Gabriel et al., 2006).

As indicated above, the mechanisms involved in cross-transfer remain unclear. They are considered to include 'spill-over' of neural drive of the strength training of one arm to the neural control system of the contralateral arm, or neuromuscular adaptations from the trained unilateral arm that induces changes in its control neural system which can be accessed for the contralateral arm (Carroll et al., 2006). The evidence suggests that peripheral muscular adaptations are not likely to play a major part (Carroll et al, 2006). It seems likely that these same factors might contribute to the increased muscle performance seen in the present study, but it also seems likely that factors associated with the behavior of the vasculature are also involved (see below).

Irrespective of the mechanisms, the increase in the actual performance of the *non-trained* arm caused by IHG training was that both WEs and SAs were much closer to reaching their "expected" TTI, at 60%MVC for 3 min. *Before* IHG training, WEs and SAs achieved 56 and 49% of Expected TTI in the dominant arm respectively and only 52 and 41% in the non-dominant arm, whereas after IHG training these percentages increased to 96 and 70% in the trained arm of WEs and SAs respectively and as much as 71 and 62% respectively in the non-trained arm. Thus, the non-trained arms of both WEs and SAs showed substantial improvements in muscle performance characterized by reduced fatigue.

Exercise hyperaemia and reactive hyperaemia in non-trained arm. There were substantial improvements in the peak exercise hyperaemia evoked by acute rhythmic forearm contractions at 60%MVC for 3 min in the WEs but not in SAs, amounting to improvement of ~40% in WEs. Similarly, the increase in peak FVC was improved in WEs, but not SAs, indicating the improvement in maximal vasodilation to this exercise stimulus was smaller in SAs. In addition, there was substantial augmentation in reactive hyperaemia and FVC in the non-trained arm of WEs, but not SAs.

Interestingly, it was previously shown that *rhythmic* handgrip training at 30%MVC until exhaustion increased muscle endurance and exercise-induced blood flow in the contralateral non-trained, forearm (Yuza et al., 2000, Yasuda and Miyamura, 1983). In that study, rhythmic training at 30% MVC to exhaustion 5 times/week for 4 weeks augmented muscle endurance and maximal exercise hyperaemia in the trained arm by 125%, and +30% respectively, and also increased endurance and peak exercise hyperaemia in the non-trained arm by 40%, and 19% respectively (Yuza et al., 2000). Further, in an earlier study, the same research group showed that before training, blood flow increased in the "resting" arm during and after contraction of the contralateral arm and this response was accentuated following a 6-week rhythmic training (Yasuda and Miyamura, 1983). As far as they could discern, the increase in blood flow in the non-exercising arm was not secondary to inadvertent muscle contraction or EMG activity. They therefore speculated that the dilatation in the non-trained limb reflected the action of humoral factors from the contracting arm, or neurogenic dilatation (Yasuda and Miyamura, 1983) as reported by others (Eklund et al., 1974). This in turn led them to propose that the improvements in muscle performance and vasodilation that occurred in the non-trained limb following a period of rhythmic hand grip training (Yuza et al., 2000) were secondary to, or at least, related to, the improvements in muscle blood flow associated with the muscle contractions (Yasuda and Miyamura, 1983, Yuza et al., 2000).

The present results may be consistent with these findings and proposals, but they are novel in showing that *isometric* handgrip (IHG) training at much more modest intensity (30% MVC for 30s, 4 times at 3 min intervals only, rather than rhythmic contractions at 30% MVC to exhaustion) increased muscle performance, improved fatigue resistance, and both exercise and reactive hyperaemia in the contralateral non-trained limb of WEs, but not in SAs. It may be that either the *isometric* handgrip (IHG) training was not potent enough to induce similar changes in SAs and/or the early cardiovascular risk factors reported in this group (Rana et al., 2014) were inhibiting the *isometric* handgrip (IHG) training adaptation.

In previous studies involving a similar IHG training protocol to the present study, as indicated above, peak reactive hyperaemia evoked in the *trained* arm

following 5 min arterial occlusion was increased at 4 weeks (Badrov et al., 2013A) in normotensive young women, but they did not test reactive hyperaemia in the *non-trained* arm. Furthermore, McGowan et al, (McGowan et al., 2007) showed in a mixed group of young normotensive men and women, that 4, or 8 weeks IHG training did not affect FMD evoked in the brachial artery of the non-trained arm by 5 min arterial occlusion. They therefore concluded that IHG training for 4-8 weeks does *not* improve *systemic* endothelium-dependent dilatation in normotensive individuals. Considering the present results, it now seems reasonable to argue that FMD of the brachial artery, which provides a measure of endothelium-dependent dilatation in a distributing artery, does not reveal important effects on endothelium-dependent dilatation of downstream arteriolar vessels. Indeed, given that both exercise-induced and reactive hyperaemia are endothelium-dependent dilator responses (Clifford and Hellsten, 2004, Joyner and Wilkins, 2007) and that when recorded by VOP they reflect dilatation of the resistance arterioles that regulate blood flow in the whole forearm, the present results indicate that IHG training does improve endothelium-dependent dilatation in arteriolar resistance vessels in the non-trained arm and therefore, in vasculature that is remote from the training stimulus, in young WE men, but that any effects over this 4 week IHG training period are smaller or negligible in young SA men.

As discussed in the General Introduction (1.12.4), it is a reasonable hypothesis that the increase in ABP evoked by IHG at 30% MVC and repeated regularly during the IHG training protocol increases shear stress and that this acts as the stimulus for improved endothelial function in the trained and non-trained arm. Certainly, chronic increases in shear stress have been shown to up-regulate endothelial dilator function, and it has been suggested that nitric oxide (NO) and prostaglandins (PGs) are involved (Tinken et al., 2010, Tinken et al., 2008, Green et al., 2004, Green et al., 2017). Both NO and PGs are further explored in chapter 5 for their involvement during reactive hyperaemia and the effects of IHG training. Thus, the findings of the present Chapter are consistent with that hypothesis, but it seems the remote effects on endothelium-dependent dilatation occur much more readily in young WE men than in young SA men who have higher risk of CVD. Protocol 2, which was performed on cutaneous microcirculation was a first attempt to test this hypothesis more directly and to investigate the mediators that may be involved (see below).

Effects on ABP and HR responses evoked by rhythmic forearm contractions. The present finding that IHG training of one arm did not change ABP evoked by rhythmic contraction of the trained and non-trained arm in either WEs, or SAs apparently contrasts with results of previous studies. Thus, exercise training of one limb *attenuated* the exercise pressor response and increase in MSNA evoked by rhythmic contractions (Sinoway et al., 1996, Fisher and White, 1999). They concluded this attenuation could be attributed to a reduction in central command and to a reduction in the reflex increase in MSNA induced by muscle afferent stimulation (Sinoway et al., 1996, Fisher and White, 1999). However, the apparent disparity may be explained by differences in study design, for both these studies used *rhythmic* training of the forearm or leg for 4, or 6 weeks respectively, rather than isometric training. In both studies, fatigue resistance increased in the trained limb, but there was no change in MVC in the trained limb, in contrast to the increase in MVC induced in the trained limb by IHG training in the present study. Thus, the magnitude of the isometric contraction tested at the end of the training period was the same as it was at the beginning (Fisher and White, 1999, Sinoway et al., 1996). By contrast, the fact that IHG training in the present study increased MVC in the trained arm and increased muscle performance during rhythmic contractions at 60%MVC in both arms, indicates that the stimulus to central command and to muscle afferents was greater after IHG training. On the other hand, the fact that exercise hyperaemia was also increased in both the trained and non-trained arm, may well have offset any neurally- mediated increase in the evoked pressor responses. This could be explored in future studies by comparing the pressor response evoked by rhythmic contractions evoked at the same absolute workload, before and after IHG training.

Reactive hyperaemia and ACh-induced dilatation in cutaneous circulation of the non-trained arm before IHG training. In the present study, peak reactive hyperaemia evoked in cutaneous circulation of the non-trained forearm before IHG training, was greater in WEs throughout reactive hyperaemia than SAs, but ACh-induced dilatation was not different between the two groups. This contrasts with the recent study from our laboratory on young men, which showed that ACh-evoked dilatation evoked in the skin was blunted in SAs relative to WEs, whereas reactive hyperaemia was not (Hirst and Marshall, 2018). However, when those findings were considered according to whether the parents of the young men were hypertensive

(OH) or normotensive (ON), it was clear that in both ethnicities, both dilator responses were blunted in OHs relative to ONs (Hirst and Marshall, 2018). Thus, it may be the fact that there were uneven numbers of OH and ON in the ethnic groups of the present study (5/10 and 2/10 OH in WEs and SAs respectively), explains the disparity between the two sets of results.

In the present study, before IHG training, baseline RCF was lower after COX inhibition than before in WEs, but not SAs. The most obvious interpretation of this finding is that COX dilator products contributed to resting vasodilator tone in WEs but not SAs. This interpretation must be treated with some caution as the site used for RCF recordings after COX administration was different than before, because the dilator responses evoked by ACh in forearm cutaneous circulation are so long-lasting (see Methods section 2.4.5) and the laser probe was therefore moved before the next part of the protocol. Thus, we cannot be absolutely sure whether by chance, sites with lower baseline RCF were chosen after COX inhibition. Nevertheless, after IHG training, baseline RCF was lower in WEs than before training and under this condition, there was no effect of COX inhibition, and again, there was no effect on the RCF values in SAs. Unless this finding also reflects the chance effect of site selection for RCF recordings, the obvious interpretation of this result is that IHG training removed a dominant dilator effect of COX products on vasodilator tone in WEs. As far as we are aware, these observations are novel. This issue was explored in Chapter 5.

Seen against this background, the finding that COX inhibition had no significant effect on reactive hyperaemia in WEs or SAs *before* IHG training, suggests that additional generation of COX dilator and constrictor products made no contribution to this response in either ethnicity. In previous studies on mixed group of men and women, the effects of COX inhibition on reactive hyperaemia in cutaneous circulation were variable: Binggeli et al. (2003) reported it was attenuated, whereas Medow et al. (2007) reported reactive hyperaemia was *augmented* after COX inhibition. In the recent study by Hirst and Marshall (2018), COX inhibition attenuated reactive hyperaemia in young ON men, but not in OH men and, in OH men who were SA there was a trend for reactive hyperaemia to be augmented by COX inhibition. This prompted Hirst and Marshall (2018) to suggest the outcome of

previous studies may have been affected by the numbers of OH and ON amongst the subject groups because this characteristic was not commented upon. Similarly, the outcome of the present study may have been affected by the different numbers of OH in the two ethnic groups.

Similarly, before IHG training, ACh-induced dilator responses were not altered by COX inhibition with aspirin in either WEs or SAs. The most obvious interpretation of this finding is that any contribution newly generated COX products made to the ACh response was also negligible. The outcomes of previous studies on the effect of COX inhibition on ACh-induced dilatation in the skin were again, highly variable. For example, COX inhibition attenuated ACh-evoked dilatation in men (Noon et al., 1998) and in mixed male/female groups (Holowatz et al., 2005, Kellogg et al., 2005, Medow et al., 2008) but had no effect in mixed groups of men and women (Berghoff et al., 2002, Dalle-Ave et al., 2004). The recent study from our laboratory, showed that COX-inhibition attenuated ACh-induced dilatation in young ON men, but had no effect in young OH men and this outcome was found in both WEs and SAs (Hirst and Marshall, 2018). They therefore suggested, as for reactive hyperaemia, that the variability in effects of COX inhibition on ACh responses in published studies could be explained, at least in part, by lack of consideration of familial history of hypertension. Thus, the fact that the numbers of ON and OH were different in the WE and SA groups of the present study, may explain why no attenuating effect of COX inhibition was identified in either ethnicity.

Effects of IHG training on reactive hyperaemia and ACh-induced dilatation in cutaneous circulation. Reactive hyperaemia in the skin is predominantly mediated by shear stress and myogenic dilatation (Carlsson et al., 1987, Meijer et al., 2008). Thus, the simplest explanation for the finding that IHG training increased reactive hyperaemia, in the cutaneous circulation of the non-trained arm of WEs but not SAs is that the shear stress stimulus proposed to augment endothelium-dependent dilatation in the muscle vasculature of the non-trained arm was great enough to have a significant effect in the cutaneous circulation of WEs, but not SAs. The fact that COX inhibition did not affect reactive hyperaemia in WEs or SAs after IHG training suggests that the improved reactive hyperaemia in the WEs may have reflected

increased NO bioavailability in WEs rather than increased PG availability and that such an effect did not occur in SAs.

By contrast, ACh-evoked dilatation is an agonist-induced response mediated by receptor coupling. After IHG training, COX inhibition augmented ACh-evoked responses in the WEs, but not in SAs. These findings clearly contrast with the hypothesis that IHG training would enhance the contribution of dilator PGs to both reactive hyperaemia and ACh-induced dilatation. The most obvious interpretations of these results might be that IHG training revealed or augmented a contribution of vasoconstrictor products to ACh-induced dilator responses in WEs. However, it seems very unlikely that IHG training would enhance vasoconstrictor influences of COX products in WEs against a background of generally augmenting vasodilator responses in these subjects, as discussed above. An alternative explanation is that the effects of COX inhibition are dependent on the interactions between the COX and NO pathway and that IHG training alters the balance of these interactions in WEs. There is substantial evidence of reciprocal interactions between the COX and NOS pathways (Salvemini et al., 2013). Notably, Medow et al. (2007) provided evidence in human cutaneous circulation that COX products normally inhibit the contribution of NO to reactive hyperaemia, such that when COX is inhibited, the contribution of NO is revealed. Similarly, most studies indicate that NOS inhibition has little or no effect on ACh-induced cutaneous dilatation (Khan, 1987, Noon et al., 1998, Holowatz et al., 2005), but COX inhibition revealed a contribution of NO (Holowatz et al., 2005).

If these are the interpretations of the effects of IHG training on ACh-induced responses in WEs, it would suggest that the inhibitory effects of COX inhibition on NO availability are smaller in SAs, or the availability of NO is lower in SAs. Moreover, if it is proposed that IHG training increased the bioavailability of NO and augmented the contribution of NO to ACh-induced dilatation in WEs, this could explain why COX inhibition enhanced ACh-induced dilatation in WEs after IHG training, but not before. These proposals are explored in Chapter 5, which concerns the effects of IHG training on the efflux of NO and PGs.

In conclusion, IHG training for 4-5 weeks in young healthy WE and SA men had no effect on SP, DP, MABP in either group. However, muscle performance

during rhythmic contractions, exercise and reactive hyperaemia were all improved in the trained arm, after IHG training in both ethnicities. Further, the study showed for the first time that TTI during rhythmic contractions was also improved by IHG training in both groups. The mechanisms involved require further elucidation, but may include cortical, sub-cortical, spinal and possibly, muscular, mechanisms. Alongside these improvements in muscle performance, IHG training augmented reactive and exercise hyperaemia in the non-trained arm of young WE men but had minimal effects on these responses in young SA men. Regarding the effects of IHG training on cutaneous circulation, reactive hyperemia was augmented by IHG training in WEs but not in SAs. Responses evoked by ACh in cutaneous circulation were not different between WEs and SAs before or after IHG training and training had no effect on the ACh response in either ethnicity. However, whereas COX inhibition had no effect on reactive hyperaemia before or after IHG training in either WEs or SAs, COX inhibition augmented the ACh-induced response in WEs after IHG training, but not in SAs.

Taken together, these results indicate that 4-5 weeks IHG training has less pronounced effects on endothelium-dependent dilator responses in the non-trained arm of young SA men than young WE men, consistent with the fact that SAs have greater risk of CVD than WEs (Murphy et al., 2007). The augmentation of reactive and exercise hyperaemia in the whole forearm and in reactive hyperaemia of forearm cutaneous circulation in WEs are consistent with the proposal that IHG training increases endothelium-dependent responses by increasing shear stress in the non-trained arm throughout the training period.

4. **Effects of IHG training in older men compared with young men**

- Ipsilateral and Contralateral Forearm blood flow and muscle performance
- Contralateral Forearm Cutaneous perfusion (contribution of COX products)

4.1 INTRODUCTION

As discussed in the General Introduction (section 1.4-Aging and Endothelial Function), aging is considered the most important risk factor which affects cardiovascular disease (CVD) prevalence (Kovacic et al., 2011b, Kovacic et al., 2011a, Costantino et al., 2016). Increased peripheral vascular resistance (PVR) is presented as early evidence of vascular aging and is usually demonstrated as increased brachial and central systolic blood pressure as well as increased pulse wave velocity PWV (Lim and Townsend, 2009). PWV serves as a marker for arterial stiffness in people above 50 years of age (Lim and Townsend, 2009, Vlachopoulos et al., 2010). Increases in peripheral vascular resistance (PVR) and diastolic blood pressure (DBP) are also considered to be the cardinal manifestation of essential hypertension (Nilsson et al., 2014). Not surprisingly, the prevalence of essential hypertension increases with age, to two out of four adults aged 50 or older (Nowak et al., 2018). Indeed, in older American people (>65 years old) hypertension prevalence is increased by ~70% as a risk factor developed during their lifetime-among other lifestyle habits leading to metabolic disorders (bad dietary habits, lack of exercise, sleep deprivation): the presence of hypertension has been implicated in development of other CVDs (Nilsson et al., 2014). Similarly, this was also shown in older Chinese people (>75 years old) with 52% of them developing CVD with hypertension as a predominant factor (Wang, 2021). Apart from hypertension, aging itself is also associated with CVD and contributes towards age-related morbidities (Nowak et al., 2018). Therefore, if we seek to reduce the prevalence of CVD it is important to delay or reverse these age-related changes.

As discussed in the General Introduction, IHG training for 4-5 weeks was shown to be effective in reducing ABP in normotensive men and women of young to middle age, as well as in patients with hypertension, whether they are medicated (McGowan et al., 2006A, McGowan et al., 2006B) or not (Badrov et al., 2013A). The study described in Chapter 3 showed that in *young*, normotensive WE men, IHG training for 4 weeks had no effect on resting ABP. However, it increased endothelium-dependent dilatator responses in both the trained and in the non-trained arm, suggesting that it has widespread beneficial effects on endothelial function. By contrast, the IHG training protocol was much less effective in young SA men, who are known to show early signs of endothelial dysfunction (Murphy et al., 2007, Hirst and Marshall, 2018). However,

in both WEs and SAs, IHG training induced an increase in muscle performance in the non-trained, as well as the trained arm. Thus, the primary aim of the study described in this Chapter was to establish whether IHG training has comparable effects in older normotensive WE men who we expected to not only have raised ABP, but endothelial dysfunction relative to young WE men.

It is generally recognised that the changes in arterial wall structure and functioning that accompany aging are associated with endothelial dysfunction (Herrera et al., 2010), which has been attributed to an imbalance between endothelial vasodilator/constrictor substances. For example, in coronary arteries *in vivo* (Egashira et al., 1993), and whole forearm circulation, endothelium-dependent dilator responses to agonists such as ACh, decrease with age and the attenuating effects of NOS inhibition and COX inhibition may both be blunted (Singh et al., 2002, Lyons et al., 1997, Taddei et al., 1995) or COX inhibition may accentuate ACh-induced forearm dilatation (Taddei et al., 1997c). It has also been shown that endothelial dilator function is blunted more by aging in men than in women (Celermajer et al., 1994). Whether, early attenuation of such endothelial dysfunction can prevent or delay clinical CVD still needs to be fully evaluated, but the results of dietary intervention, are very positive (Ordovas, 2006b, Ordovas, 2006a).

There is evidence that in cutaneous circulation, vasodilator prostanoids contribute to the ACh-induced dilatation and reactive hyperaemia (Khan et al., 1997, Noon et al., 1998) while, other evidence suggests that vasoconstrictor COX products are involved (Medow et al., 2007). The extent to which NO and EDHF contribute is not clear and there seems to be interaction between these factors (Holowatz et al., 2005, Medow et al., 2007). The results of Chapter 3 suggest that in young WE men, IHG training of one arm changes the contribution of COX products to endothelium-dependent responses in the cutaneous circulation of the contralateral forearm. Thus, a second aim of the studies described in this Chapter was to assess the effects of IHG training on cutaneous vasodilator responses in older WE men and to establish whether the role of COX products it changed.

4.1.1 HYPOTHESES AND AIMS

To summarize, the hypotheses of the present study were to:

1. We hypothesised that IHG training of the dominant arm would increase muscle peak performance in both young and older WEs.
2. We hypothesised that reactive and exercise hyperaemia in the whole forearm and ACh-evoked dilatation and reactive hyperaemia in cutaneous microcirculation would be smaller in older than young WEs and would be augmented less by IHG training in older WEs.
3. We also hypothesised that vasodilator PGs would make smaller contributions to cutaneous vasodilator responses in older than young WEs before IHG training, but that their contribution would increase in both groups after IHG training.

And the aims to address these hypotheses are:

1. To test whether in older WE men, IHG training of the dominant arm increases muscle performance of the trained *and* non-trained arm, as in young WE men.
2. To test whether in older WE men, the effect of IHG training of the dominant arm on reactive hyperaemia and exercise hyperaemia in the trained and non-trained arm are smaller than in young WE men.
3. To test whether in older men, the effects of IHG training of the dominant arm on vasodilator responses evoked in cutaneous microcirculation of the contralateral, non-trained arm by iontophoresis of ACh and during reactive hyperaemia, are smaller than in young men.
4. To test whether in older men, vasodilator PGs make a larger contribution to these cutaneous responses after IHG training than before.

4.2 METHODS

This study was performed on 10 older men (55-70 yrs) recruited according to the inclusion and exclusion criteria and the physical activity questionnaire (IPAQ) described in Chapter 2 (section 2.1). HR, MABP, FBF and cutaneous RCF were measured as described in Chapter 2 (section 2.3).

4.2.1 GENERAL PROTOCOL

The experimental protocol was the same as that used on young men and described in Chapter 3. In brief, each subject came to the laboratory on six separate days, for a familiarization visit for two experimental sessions before IHG training, an interim visit midway through the 4–5-week IHG training period and for the final two experimental sessions that were repeats of those performed before IHG training. To summarise, in Protocol 1, reactive hyperaemia following 3 min arterial occlusion and exercise hyperaemia following 3 min rhythmic contraction at a "nominal" 60% MVC were recorded in the dominant and non-dominant forearm by using VOP. In Protocol 2, reactive hyperaemia following 3 min arterial occlusion and dilatation evoked by iontophoresis of ACh were recorded in the cutaneous circulation of the non-dominant arm by using Laser Doppler fluximetry before and after COX inhibition with aspirin (600 mg p.o). IHG training was carried out with the dominant arm at 30% MVC for 30s, 4 times at 3-min intervals, 4 days each week for 4-5 weeks. After 2 weeks of IHG training, MVC in the dominant arm was checked and if there were improvement in the absolute force at MVC, an appropriate adjustment was made to the 30% MVC that was used for handgrip training. During each Protocol, ABP and HR were recorded continuously and during rhythmic handgrip contractions in Protocol 1, the output of the dynamometer was also recorded.

4.2.2 MEASUREMENTS of FBF and RCF

Measurements of forearm blood flow before and at intervals during reactive and post-contraction hyperaemia were performed as described in Chapter 3 (section 3.3.2).

4.2.3 DATA ANALYSIS

All data were expressed as means ± s.e.m. Analyses were performed on the older men presented in this Chapter as described for the young men in Chapter 3 (section 3.3.3),

but comparisons were also made between data collected in the older WE men and those collected in the young WE men of Chapter 3. To summarise, baseline levels of ABP, HR, FBF, FVC, RCF before and after IHG training were compared within the older group, by using paired t-tests; comparisons between young and older were compared by unpaired t-tests. The proportions of older and younger men who had hypertensive parents were compared by Fisher's Exact test. Two-way repeated measures ANOVA for detection of time, treatment, time*treatment effects were used to compare changes in FBF, FVC, RCF evoked by experimental interventions before IHG training. Comparable analyses were performed after IHG training. Comparisons on these indices between young and older men were made by using two-way repeated measures ANOVA for detection of time, treatment, time *treatment effects. Measurements of dynamometer force (MVC) before and after IHG training were compared within group by using Student's paired t-test. As in Chapter 3, work done during the 3 min periods of rhythmic contractions was calculated by using the integral function on Lab Chart, as Tension Time Index (TTI) in kilonewtons (KN) termed "actual TTI". As in the study of Chapter 3, older men did not manage to perform 60% MVC at 1 cycle/ 2 s for the whole 3 min period. Thus, "expected" TTI was calculated as a product of 60% MVC during the first contraction of the 3 min rhythmic contractions x 90s before and after IHG training.

Percentage changes in peak FBF, peak FVC, expected and actual TTI before vs after IHG training and percentage improvements in actual/expected TTI were calculated as described in Chapter 3 as a subtraction of the value recorded after IHG training from that recorded before training, divided by the value before training and expressed as percentage. Further, compacted means for iontophoresis were calculated as subtraction of the RCF values with the baseline RCF value. The two age groups were compared by un-paired t-test. Statistical significance was $P<0.05$.

4.3 RESULTS

Anthropometric characteristics of the 10 older men, compared with the 10 young men of Chapter 3 are shown in Table 4.1. Apart from age, there were no differences between the groups. Cardiovascular baselines before and after IHG training are presented in Table 4.2. Baseline ABP values were not different between older and young men before IHG training. However, systolic, diastolic blood pressure and MABP were reduced

after 4 weeks of IHG training in the older men only, such that SP was then lower in older than young men. Baseline FBF, and FVC in dominant and non-dominant arm, did not differ between older and young men. However, whereas in the young men, FVC and FBF were decreased in both arms and RCF was decreased in the contralateral arm by IHG training, this did not happen in older men (Table 4.2).

4.3.1 Effects of IHG training on MVC

As expected, MVC after 2 weeks of IHG training increased in the trained arm (25.46±1.55 vs 27.1±1.67 kN) but not in the non-trained arm (24.48±1.43 vs 25.25 ± 1.47). MVC after 4 weeks of IHG training, increased in the trained arm of older men (25.46±1.55 vs 28.45±1.64 kN; Figure 4.1). Interestingly, there was also a significant increase in MVC in the non-trained arm of older men (24.48±1.43 vs 25.31±1.41 kN; $P<0.05$).

Protocol 1

Forearm vasculature responses during reactive hyperaemia

In older men, IHG training of the dominant arm for 4 weeks increased reactive hyperaemia in the trained arm (Figure 4.2): peak FBF was 23.95±2.01 before, and 45.65±6.60 ml.100ml^{-1}.min^{-1} after IHG training, while FVC, was 0.28±0.02 before, and 0.58±0.14 CU respectively (Figures 4.2 and 4.3, top). Further, in older men, IHG training increased hyperaemia in the non-trained arm: peak FBF was 20.37±1.45 before, and 28.61±1.78 ml.100ml^{-1}.min^{-1} after IHG training, while FVC, was 0.23±0.03 before, and 0.34±0.04 CU after IHG training (Figures 4.2 and 4.3 below).

Comparing older with young men, peak FBF and peak FVC were blunted in older men relative to young men both before and after IHG training: peak FBF in young men was 41.73±3.45 before, and 50.8±3.84 ml.100ml^{-1}.min^{-1} after IHG training, while peak FVC was 0.49±0.04 and 0.6*±0.07 CU respectively. Further, in young men, IHG training did not affect the increase in FVC in the non-trained arm, whereas it did in the older men (see Figures 4.2 and 4.3 RHS).

IHG training increased peak FBF during reactive hyperaemia by 52.44±10.34% and by 44.92±10.12% in the trained and non-trained arm of older men, whereas while in young men, peak FBF was increased by 24.97±9.45% and by 32.95±12.36% respectively

(Figure 4.4). The effect of IHG training on the FBF response of the dominant arm was greater in the older, than young men by ~39% (figure 4.4).

4.3.2 Muscle performance during rhythmic contractions

As indicated in Methods (section 4.2.1) since MVC in the trained arm was increased by IHG training, the absolute force at which older men were asked to perform rhythmic contractions at 60% MVC for 3 min after IHG training was adjusted according to individual MVC before and after IHG training, as for young men in Chapter 3. Work performed during rhythmic contractions at 60%MVC of the dominant and non-dominant arms for 3 min before and after IHG training is shown as actual TTI in Table 4.3 and Figure 4.5. In older men, actual TTI increased significantly after IHG training in the dominant (trained) arm (6.05±0.64 vs 13.48±0.78 kN). The actual TTI values in the dominant arm were lower than those of young men both before (8.88±1.27 kN) IHG training and after training (17.08±1.78 kN).

Further, actual TTI also increased in the *non-dominant* arm of older men (5.03±0.65 vs 13.47±0.74 kN) as it did in young men (Figure 4.5), even though it was the dominant arm that underwent the training. Actual TTI in the non-dominant, non-trained arm was not different in the older and young men before IHG training (young men: 7.21±1.28 kN) but was significantly greater in older men after IHG training (13.47±0.74 kN).

As noted in Chapter 3, performing 60% MVC contractions at 1s intervals was a very strenuous task, and thus, older men fatigued during the 3 min. Figure 4.5 shows in the stippled columns, the "expected" TTI of the older men had they been able to attain the task requested over the full 3 min before and after IHG training. In the older men, "expected" TTI was substantially increased in the dominant (trained) arm (from 8.67±0.55 to 14.99±0.82 kN) and also in the non-dominant arm (from 6.71±0.47 to 13.44±0.69 KN, see Figure 4.5 RHS).

As can be seen in Figure 4.6, percentage increase in actual TTI in the dominant (trained) arm of older men was lower than in young men +53.28±1.93% and +92.34±4.11% (Figure 4.6-A, above LHS) respectively. For the non-trained arm, the percentage improvement was not different between the older and young men: 33.21±0.84% vs 43.11±1.31%). Interestingly, actual TTI improvement was similar in the trained and non-trained arms of older men (Figure 4.6-A LHS above).

The percentage increase in "expected" TTI was greater in the dominant than in the non-dominant arms in both groups (Y: 15.31±3.04% vs 5.35±2.51%; O: 11.44±1.74 vs 0.23±2.37%) (Figure 4.6-B RHS above). Further, the percentage increase in actual/expected TTI was significantly greater after IHG training (from 44.15±2.53 to 57.95±2.52 %) in the trained arm of older men as in young men, but the effect was smaller in the older men (57.95±2.52 vs 95.83±7.16%). Interestingly, IHG training also enhanced the actual/expected TTI percentage in the non-trained arm in the older people (49.97±2.49 vs 36.92±3.63%) as in the young men. Again, the effect was blunted compared to the young men (49.97±2.49 vs 76.58±6.43%) (Figure 4.6-C below).

4.3.3 Systemic and vascular responses evoked by rhythmic contractions

ABP and HR in the older men, increased significantly from baseline values during rhythmic handgrip contractions at 60% MVC of both the dominant and non-dominant arm and returned to resting values after cessation of handgrip contractions, before and after IHG training of the dominant arm (Figures 4.7 and 4.8). In contrast to the young men, there was a treatment*time interaction for the increase in ABP evoked by contractions of the trained arm, indicating the evoked increase in ABP was smaller after IHG training (Figure 4.7). Further, in contrast to the young men, the increase in HR evoked by rhythmic contractions of the non-trained arm was smaller in older men after IHG training (Figure 4.8).

Exercise hyperaemia

In the older men, IHG training increased post-contraction hyperaemia in both the trained and non-trained arms whether considered as FBF or FVC (Figures 4.9 and 4.10 respectively). In the trained arm, peak FBF increased from 36.63 ± 2.6 to 65.1 ± 6.83 $ml.100ml^{-1}.min^{-1}$, while peak FVC increased from 0.41± 0.03 to 0.65 ±0.08 CU. Further, in the non-trained arm, peak FBF increased from 28.8 ± 1.71 to 43.1±2.55 $ml.100ml^{-1}.min^{-1}$ while peak FVC in the untrained arm increased from 0.30±0.03 to 0.44±0.05 CU. When considered as percentage change (Figure 4.11), the increase in post-contraction FBF induced by IHG training was similar in the dominant and trained arm of older and young men (87.9±27.8 vs 66.3±18.5%) respectively, and in the non-dominant arm (69.9±21.6 vs 40.7±12.2%) respectively. Similarly, this was demonstrated in FVC percentage induced by IHG training was similar in the dominant

and trained arm of older and young men (47.27±22.57 vs 66.61±20.11%) (P> 0.43, effect sizes: young: -2.58, old -3.42) respectively, however in the non-dominant non-trained arm the increase was higher in the older compared to the young (17.47±10.83 vs 49.50 ± 12.24%) even though not significantly different P> 0.07, effect sizes: Y-2.95 O -3.48) (Figure 4.11).

4.3.4 Cutaneous vascular responses

Cutaneous RCF was not changed by IHG training in older men, in contrast to the decrease in RCF that occurred in the young men (Table 4.4).

In older men, IHG training had no effect on baseline RCF in the non-trained arm and COX blockade had no effect on RCF before IHG training (see Tables 4.2, 4.4). This contrasts with young men in whom COX inhibition reduced RCF before IHG, but not after (Table 4.4). Due to these effects of IHG training and COX inhibition on baselines, all responses evoked in cutaneous circulation are expressed as change from baseline RCF.

Reactive hyperaemia. After release of arterial occlusion, there was an increase in RCF in older men, but the change in RCF was much smaller than in young men (peak ΔRCF: 39.0 ± 4.0 vs 70.11±2.53 PU) see Figures 4.12 and 4.13.

Before IHG training, reactive hyperaemia in cutaneous circulation was not significantly changed by COX inhibition in older men: peak RCF was 39.0 ± 4.0 PU before, and 46.66 ± 4.34 PU after aspirin (Figure 4.12). After IHG training however, peak RCF was increased from 45.17±5.55 to 51.69±3.75 PU (P <0.05). It may be noted that in older men, there was an apparent trend for COX inhibition to enhance reactive hyperaemia before IHG training (P> 0.40, effect size: 0.61), but after IHG training this reached statistical significance (P< 0.001, effect size: 1.40). However, when peak RCF was compared before and after IHG training there was no significant increase: 39.0 ± 4.0 before vs 45.17±5.55 PU after COX inhibition. Older men demonstrated attenuated reactive hyperaemia relative to young men both before and after IHG training (Figure 4.12).

ACh-evoked dilatation. Before IHG training, ACh-evoked dilatation was smaller in older men than young men (Figure 4.14; P<0.0001) COX blockade had no effect on the ACh-evoked response in older men (Figure 4.14-RHS) as in young men.

After IHG training, in older men as in young men, the ACh-response was not changed (Figure 4.14 vs 4.15). However, as in young men, COX blockade enhanced the ACh response in older men (Figure 4.15; $P<0.05$). When expressed as compacted means, the effect of IHG training was smaller on ACh-evoked dilatation in older than young men (O: 83.51±17.48 vs Y: 136.90±16.66 PU) and was smaller after COX inhibition in older men (104.7±24.57 PU vs 181.31±26.5 PU) (Figure 4.16).

Anthropometric Characteristics	Young (n=10)	Older (n=10)	P value
Age	23.9±0.7	62±1.5	P<0.0001
BMI (kg/m^2)	22.35±0.87	25.38±0.9	P=0.35
Waist:hip ratio (cm)	0.95±0.04	0.92±0.10	P=0.46
Physical activity level	01 Low 10 Average 09 High =20	01 Low 14 Average 06 High =21	P=0.88
Parental Hypertension	04/10	03/10	P=0.63

Table 4.1 **Anthropometric Characteristics in Young (LHS) and Older (RHS) men.** Values are shown as mean ± SEM. **Physical activity level**: calculated from number of participants achieving one of the 3 levels of PA 0-2 Low (x1), 3-4 Average (x2), ≤5 High (x3) which was multiplied by the number of the people who achieved that level; **Parental cardiovascular disease**: subjects who reported that their parents and grandparents diagnosed with cardiovascular disease and presented related symptoms (M.I., angina, etc.) and risk factors (high blood pressure, diabetes etc.).

	Young (n=10)		Older (n=10)	
	Before IHG Training	After IHG Training	Before IHG Training	After IHG Training
Systolic pressure (mmHg)	124.5±1.82	125±1.8	124.4±1.8	114.4±1.8**§
Diastolic pressure (mmHg)	69.8±1.67	72±1.6	78.0±1.8	69.3±1.6**
MABP (mmHg)	88.03±2.6	89.4± 2.3	93.5±1.7	84.3±1.6**
FBF (non-trained arm) $ml.min^{-1}.100ml^{-1}$	4.95 ±0.6	6.53±0.68*	4.73± 0.46	4.12± 0.36§
FBF (trained arm) $ml.min^{-1}.100ml^{-1}$	5.1±0.5	7.84±0.8*	5.7 ± 0.7	4.9± 0.6§
FVC (non-trained arm) Conductance units (CU)	0.06±0.01	0.08±0.01*	0.05±0.01	0.05±0.01§
FVC (trained arm) Conductance units (CU)	0.06±0.01	0.10±0.01*	0.07±0.01	0.06±0.01
RCF (PU) (non-trained arm)	22.8±0.1	15.4±1.9***	15.9±2.08	16.41±1.2

Table 4.2 **Cardiovascular baselines in Young (LHS) and Older (RHS) men for Protocol 1 and 2 before and after IHG training.** Values are shown as mean ± SEM. ***, *: before vs after IHG training between Young (Y) or Older (O) respectively. §: Y vs O: 1,2 symbols indicate P<0.01 P<0.001 respectively.

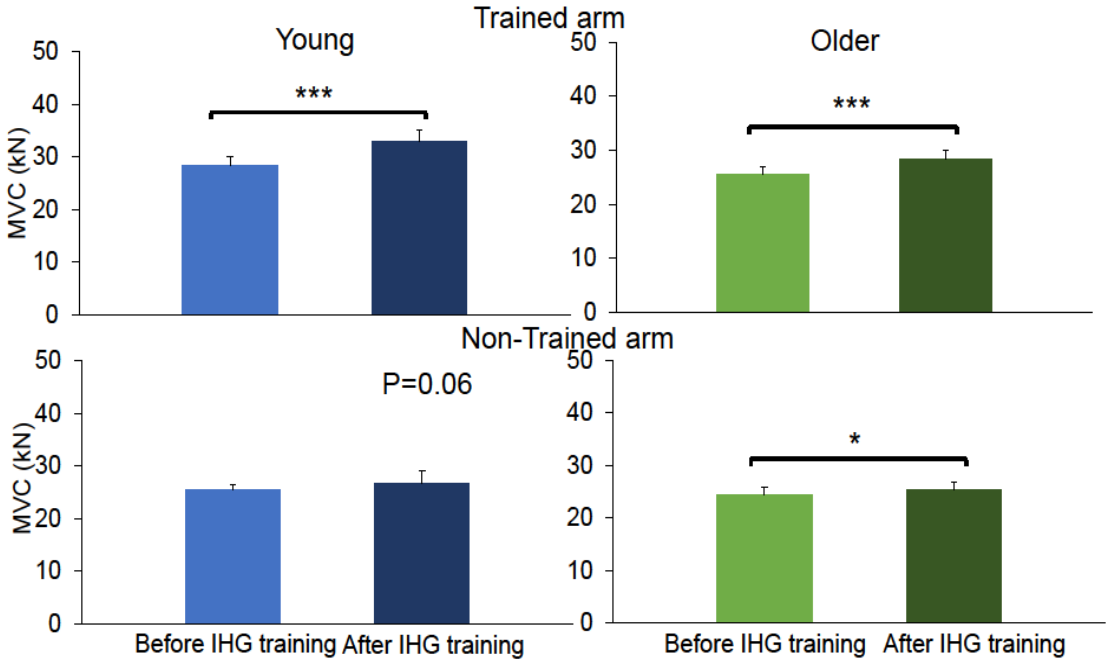


Figure 4.1: **Effect of IHG training on MVC in dominant, trained arm (above) and non-dominant, non-trained arm (below) before and after training in Young (LHS) and Older (RHS).** Values shown as mean ±SEM. *, **, ***: before vs after training P<0.05, P<0.001, P<0.0001 respectively.

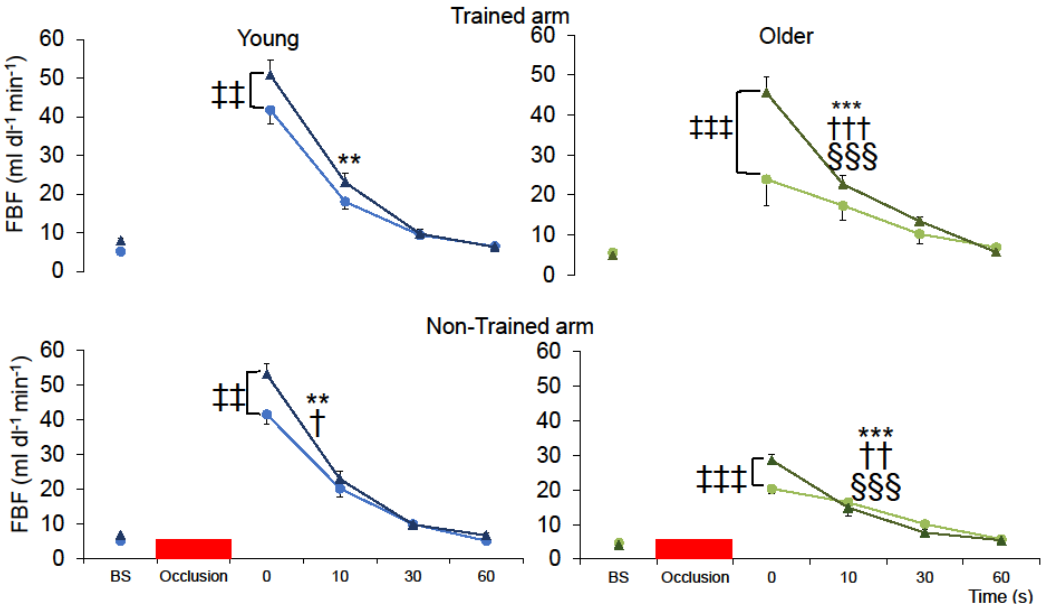


Figure 4.2. **FBF at rest and following arterial occlusion for 3 min in trained (above) and non-trained (below) arm before and after IHG training in young (LHS) and older (RHS) men**. Young: BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), before (light blue), after (dark blue) IHG training; Older: BS: before (circular light green dot) and after IHG training (triangular dark green dot), before (light green), after (dark green) IHG training. Values shown as mean ±SEM. *,**,***: before vs after training, †, ††, †††: time*treatment IHG training, ‡, ‡‡: peak responses before vs after training, § § §; Y vs O: 1,2,3,4 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

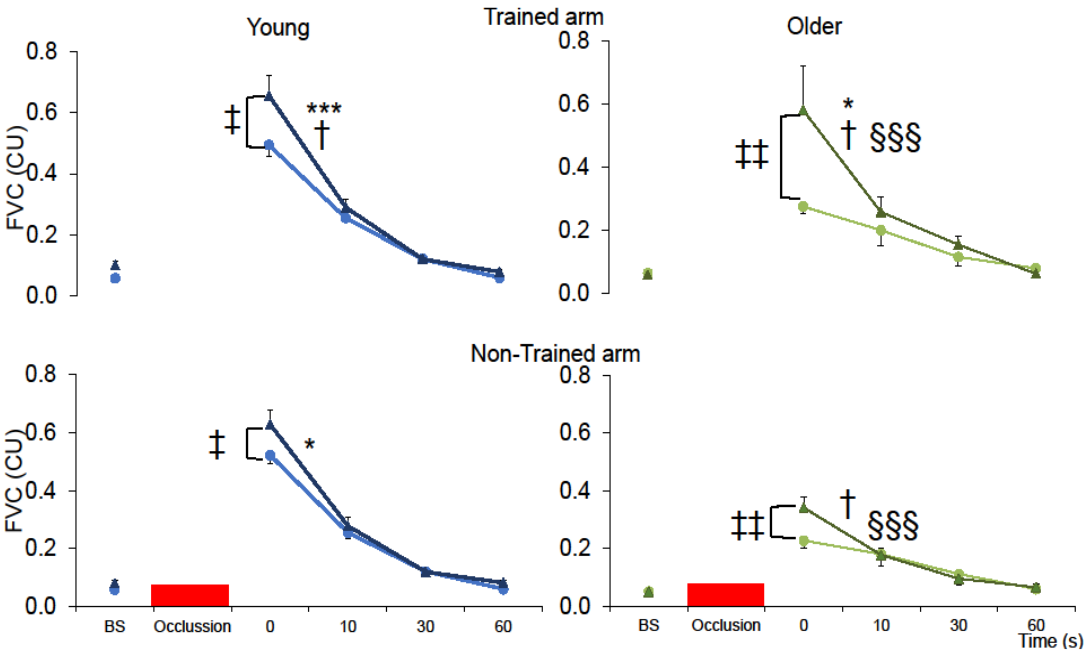


Figure 4.3. **FVC at rest and following arterial occlusion for 3 min in trained (above) and non-trained (below) arm before and after IHG training in young (LHS) and older (RHS) men**. Young: BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), before (light blue), after (dark blue) IHG training; Older: BS: before (circular light green dot) and after IHG training (triangular dark green dot), before (light green), after (dark green) IHG training. Values shown as mean ±SEM. *,**,***: before vs after training, †, ††, †††: time*treatment IHG training, ‡, ‡‡: peak responses before vs after training, § § §; Y vs O: 1,2,3,4 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

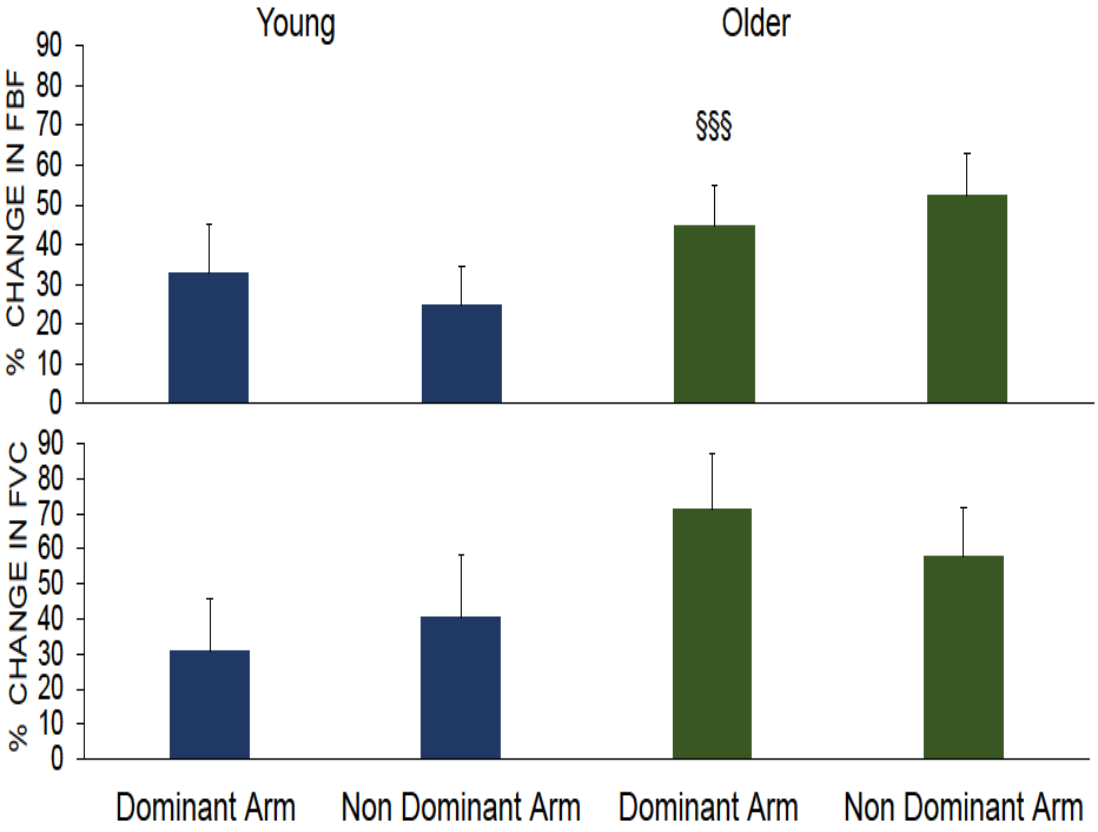


Figure 4.4: **Percentage change caused by IHG training in peak FBF (upper panel) and FVC (lower panel) during reactive hyperaemia in trained (dominant) and non-trained (non-dominant) arm of Young (LHS) and Older (RHS)**. Values are shown as mean ± SEM. §§§: Y vs O after IHG training: symbol indicates P<0.001 respectively. P<0.001.

	Actual TTI (KN)		(%) change in Actual TTI	Expected TTI (KN)		Actual/Expected as percentage	
	Before IHG training	After IHG training		Before IHG training	After IHG training	Before IHG training	After IHG training
			Dominant Arm				
Young	8.9±1.3	17.1±1.8***	92.3±4.1***	15.4±0.1	17.8±1.2***	55.9±5.9	95.8±7.2***
Older	6.05±0.6	13.48±0.8**	43.1±1.3***	8.67±0.5	14.99±0.8**	44.2±2.5	57.9±1.4***
			Non Dominant Arm				
Young	7.2±1.3	11.1±1.1**	53.3±1.9***	13.7±0.5	14.6±0.7*	51.9±8.1	76.6±6.4*
Older	5.0±0.7	13.8±0.8**	33.2±0.8***	6.7±0.5	13.4±0.7***	36.9±3.6	49.9±2.5**

Table 4.3 **Effects of IHG training on muscle performance in dominant, trained arm and non-dominant, non-trained arm of young (LHS) and older (RHS).** Values are shown as mean ± SEM. ***, **, * Before vs after IHG training: 1,2,3 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

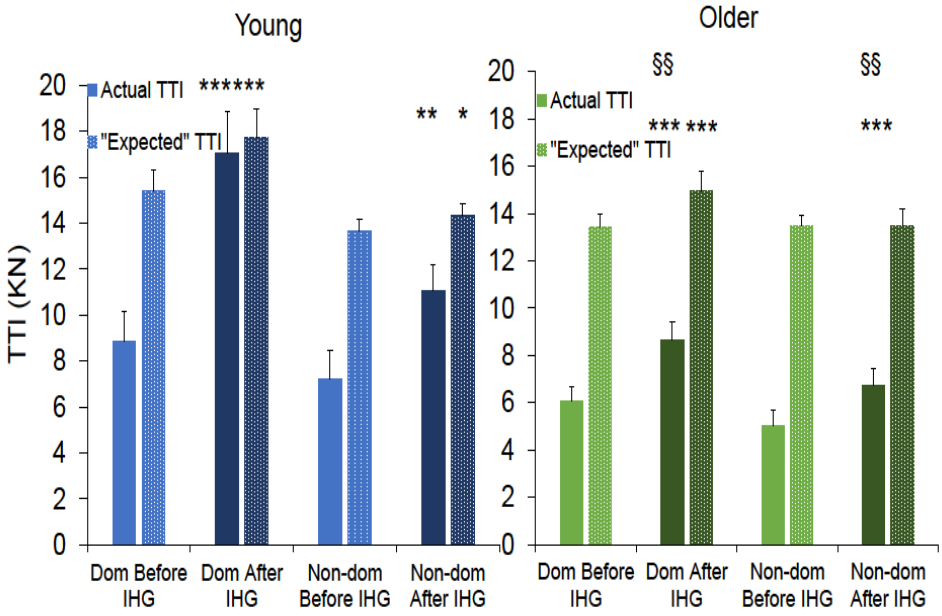


Figure 4.5: **Effect of IHG training on Expected Time-Tension index (TTI, above) and Actual TTI (below) in dominant arm that underwent IHG training and non-dominant, non-trained of Young (LHS) and Older men (RHS).** Values shown as mean ±SEM. *, **, ***: before vs after IHG training; §§: Y vs O after IHG training: 1,2 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

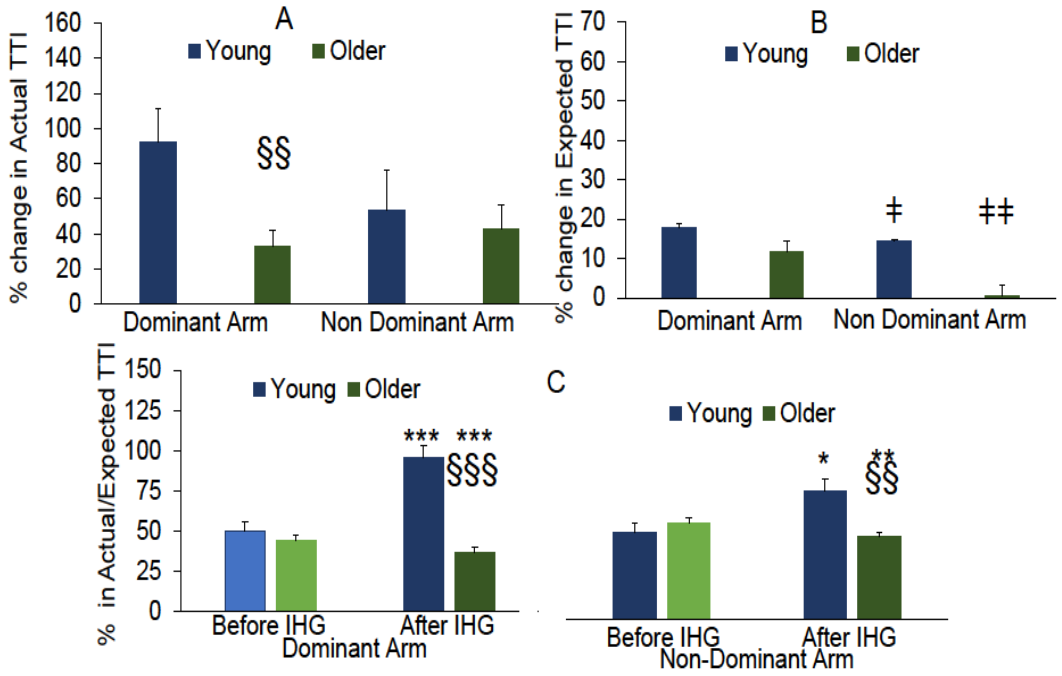


Figure 4.6: **Actual TTI (left top) and Expected TTI (right top) in dominant and non-dominant arm after IHG training in Young (Y) and Older (O) men shown as percentage changes; Actual and Expected/Expected % before and after training (below).** Values shown as mean ±SEM. *, **,***,****: before vs after training:; ǂ, ǂǂ: Dominant vs Non Dominant arm §, §§, §§§: Y vs O: 1,2,3 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

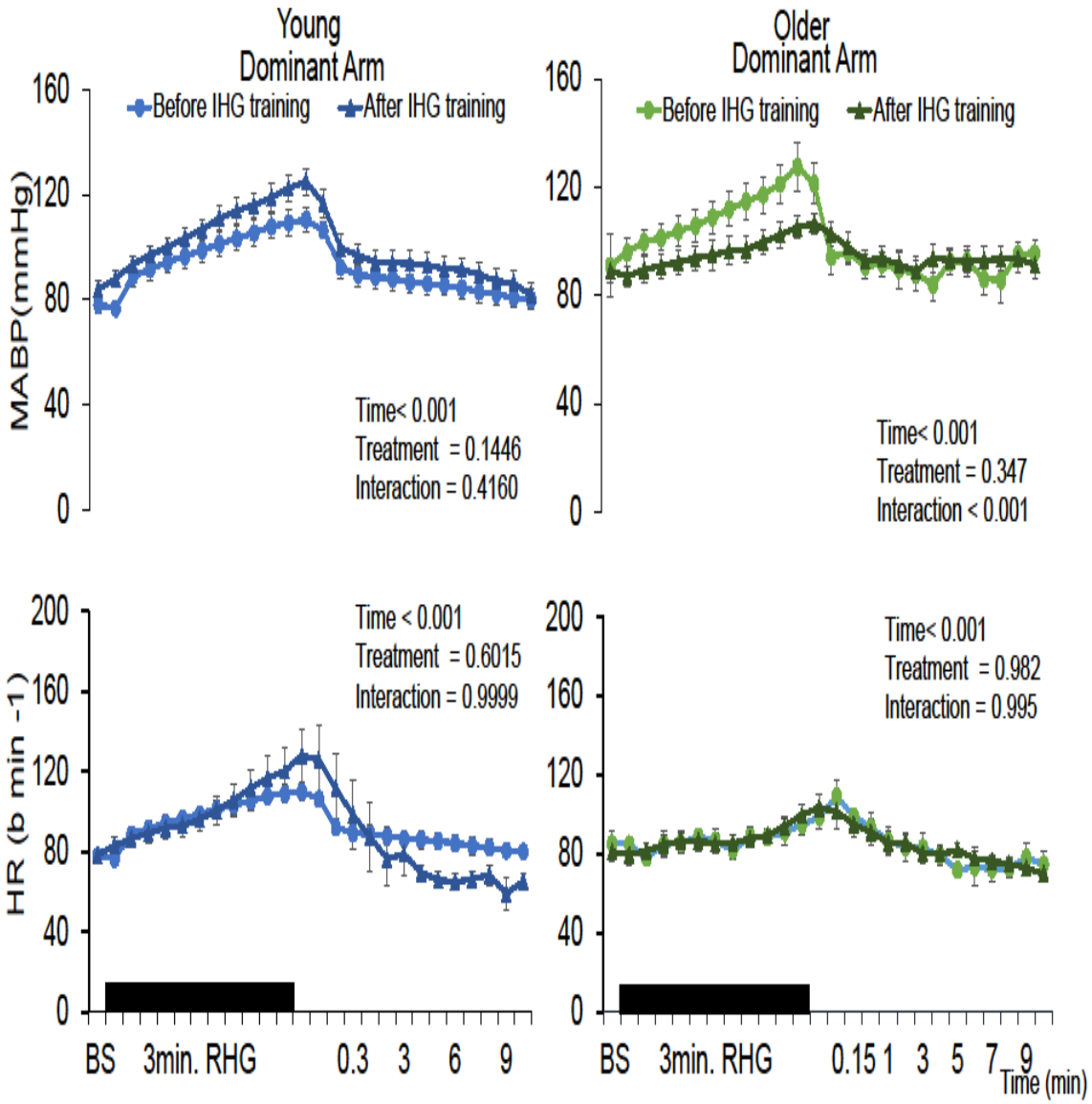


Figure 4.7: **MABP and HR responses at rest, during rhythmic handgrip exercise of the dominant arm contracting at 60% MVC over 120 sec period, recovery that underwent IHG training (above) and HR (below) in Young (LHS) and Older men (RHS).** Values shown as mean ±SEM.

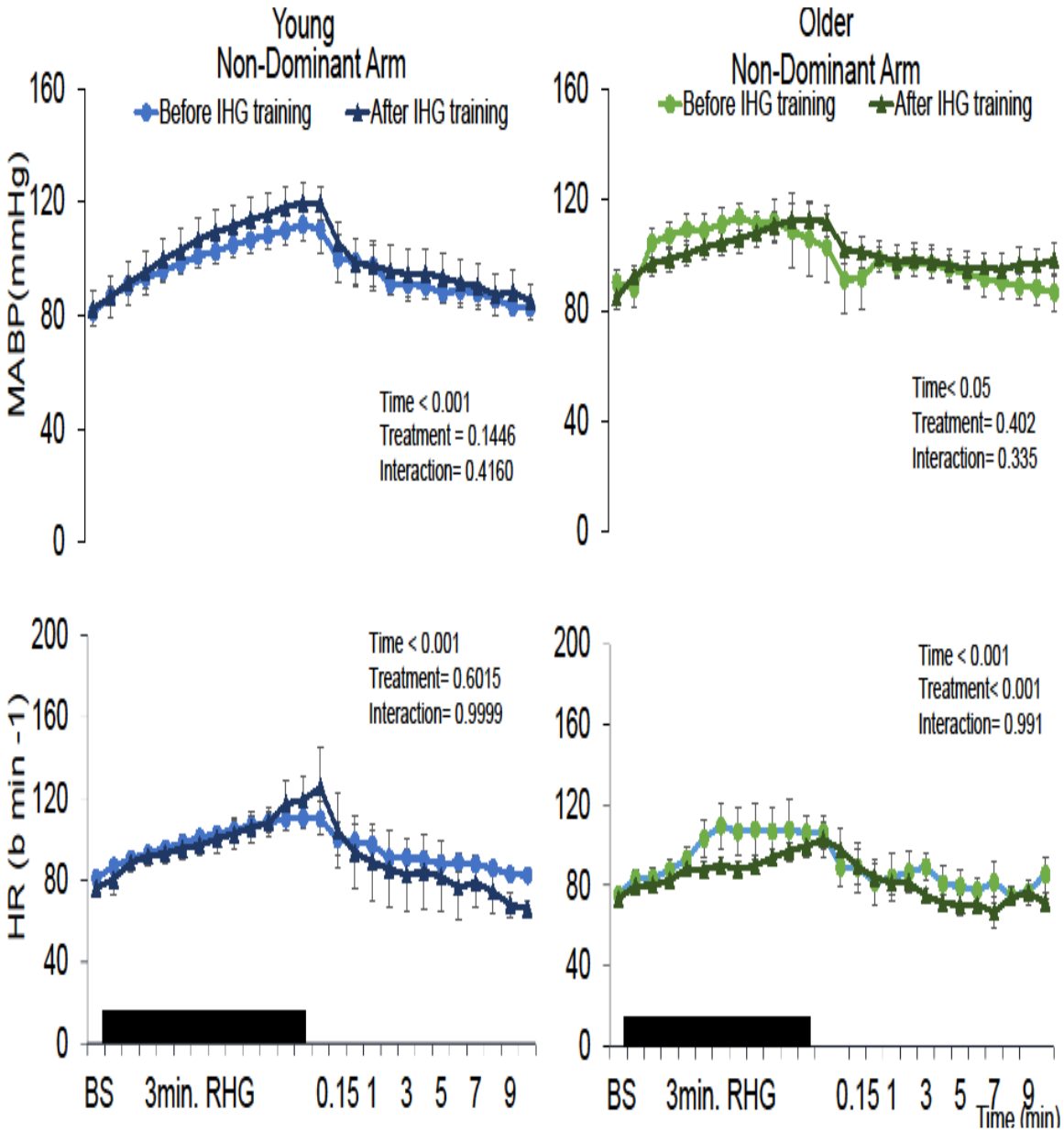


Figure 4.8: **MABP and HR responses at rest, during rhythmic handgrip exercise of the non-dominant arm contracting at 60% MVC over 120 sec period, recovery that underwent IHG training (above) and HR (below) in Young (LHS) and Older men (RHS).** Values shown as mean ±SEM.

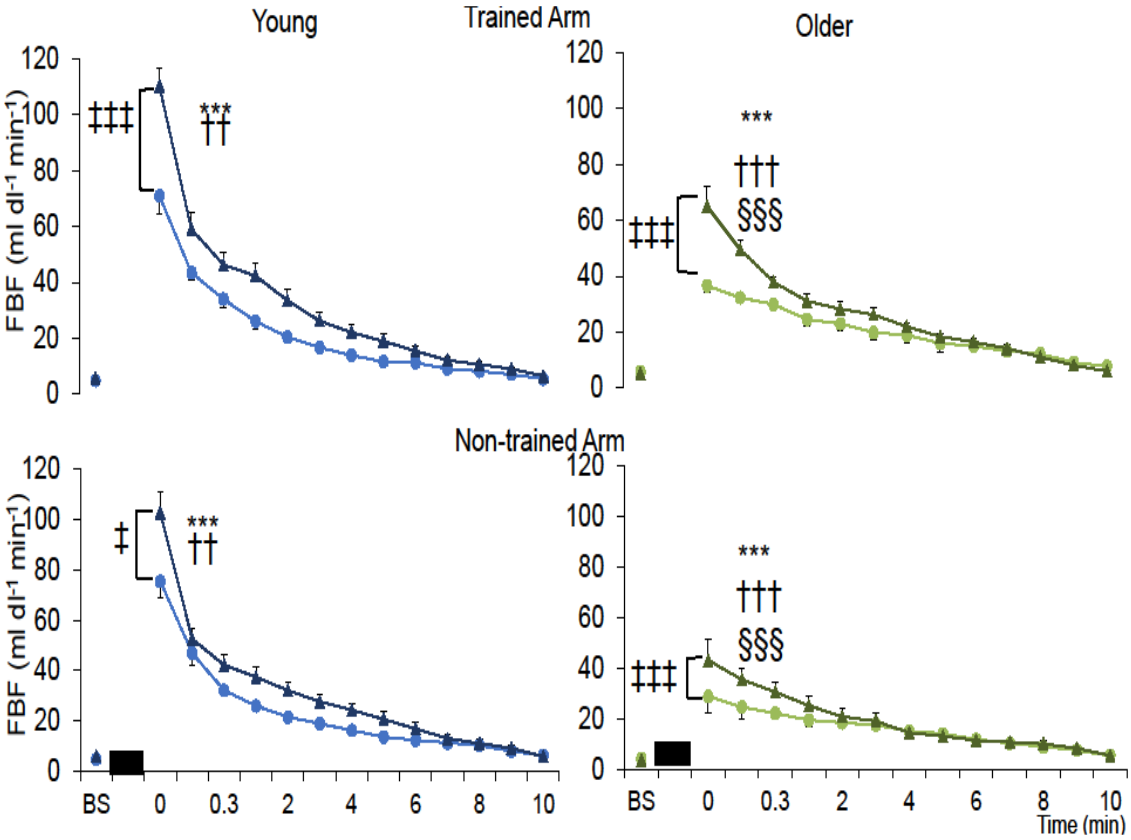


Figure 4.9: **FBF at rest and after rhythmic contraction at 60%MVC for 3 min of the trained arm (above) and non-trained arm (below) in Young (LHS) and Older men (RHS) before and after IHG training.** Young: BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), before (light blue line) and after (dark blue line) IHG training, Older: BS: before (circular light green dot) and after IHG training (triangular dark green dot), before (light green) and after (dark green) IHG training. Values shown as mean ±SEM. *,**,***: before vs after training, †, ††, †††: time*treatment IHG training, ‡, ‡‡: peak responses before vs after training, § § §; Y vs O: 1,2,3,4 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

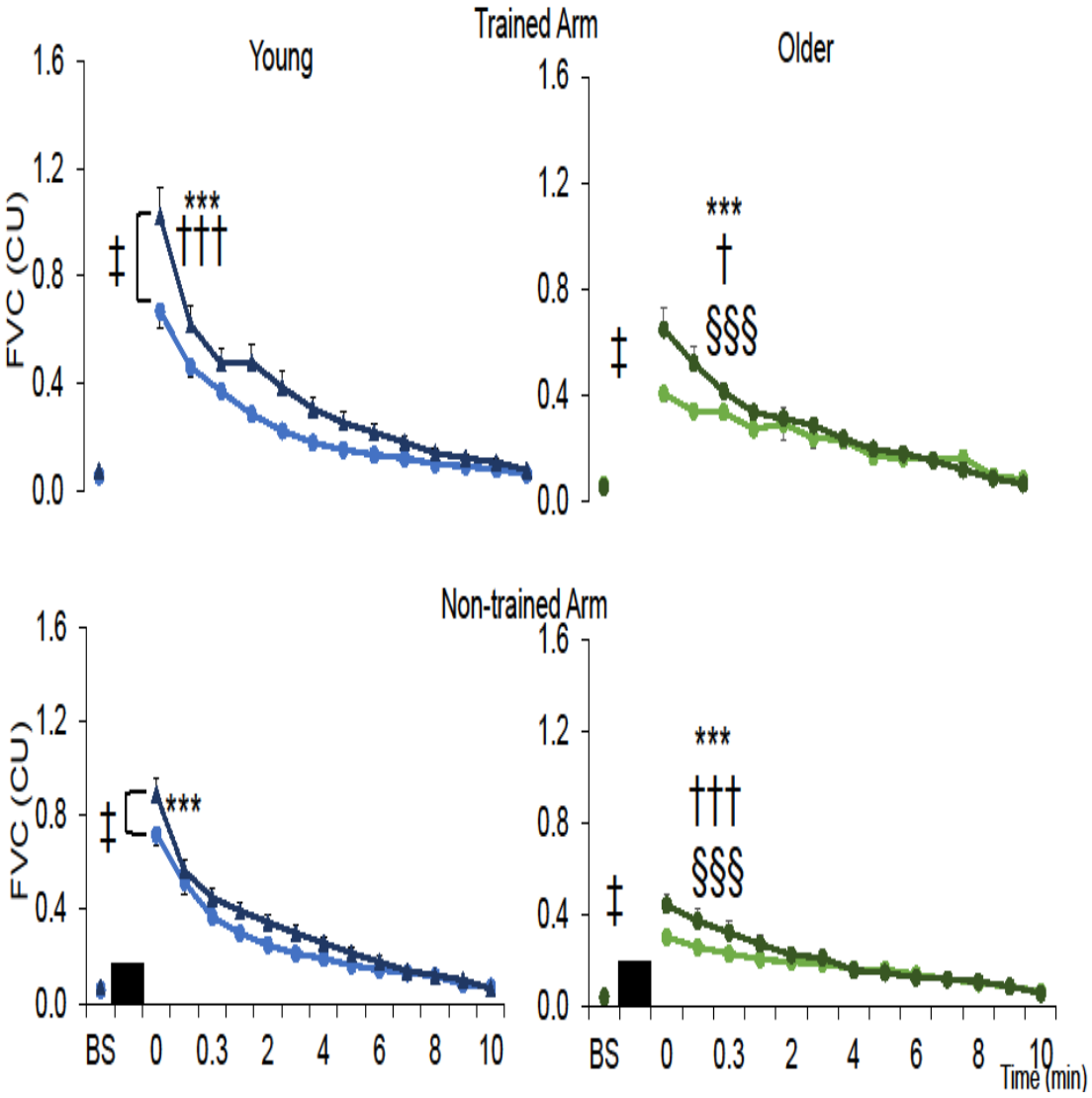


Figure 4.10: **FVC at rest and after rhythmic contraction at 60%MVC for 3 min in the trained arm (above) and non-trained arm (below) of Young (LHS) and Older men (RHS) before and after IHG training.** Young: BS: before (circular light blue dot) and after IHG training (triangular dark blue dot), before (light blue line) and after (dark blue line) IHG training, Older: BS: before (circular light green dot) and after IHG training (triangular dark green dot), before (light green) and after (dark green) IHG training. *,**,***: before vs after training, †, ††, †††: time*treatment IHG training, ‡, ‡‡: peak responses before vs after training § § §; Y vs O: 1,2,3,4 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

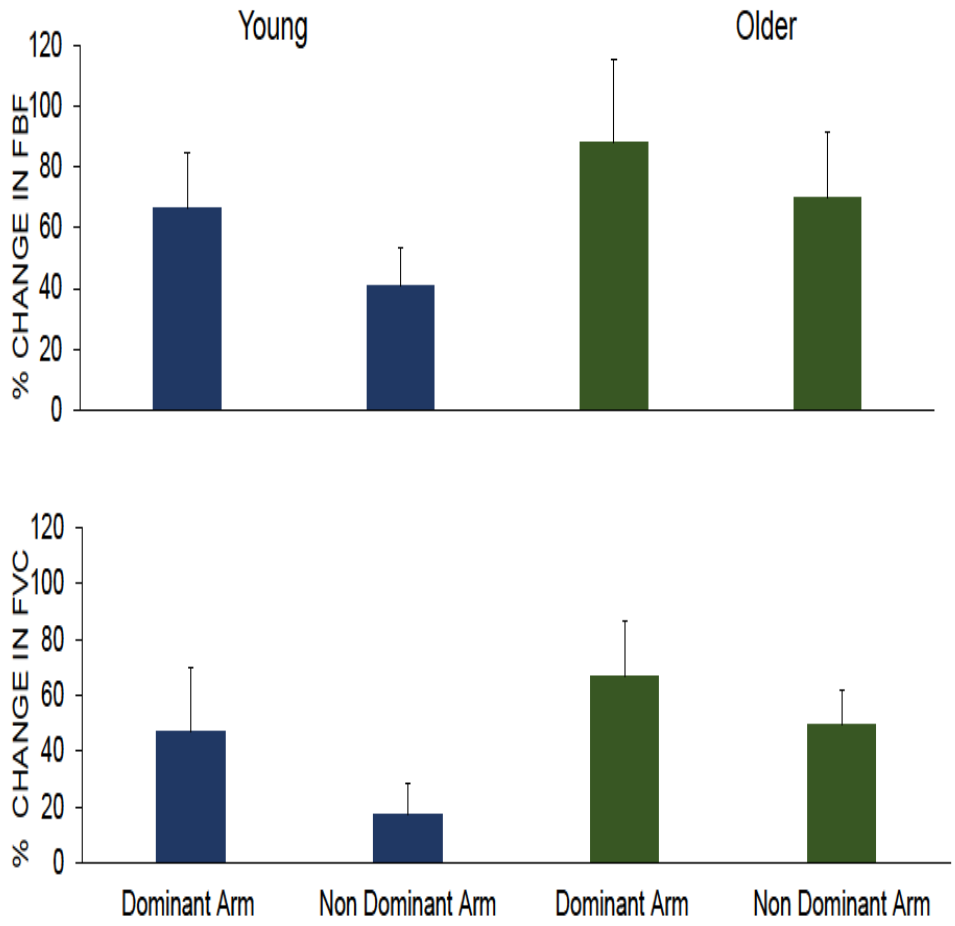


Figure 4.11: **Percentage change in peak FBF recorded after rhythmic contraction at 60%MVC for 3 min after IHG training in Young (LHS) and Older men (RHS) in trained (Dominant) arm and non-trained (non-dominant arm).** Values are shown as mean ±SEM.

	Young (n=10)	Young (n=10)	Older (n=10)	Older (n=10)
	Before IHG training	After IHG training	Before IHG training	After IHG training
Baseline RCF (PU) (non-trained arm)	22.8±0.1	15.4±1.9***	15.9±2.08	16.41±1.2
After Aspirin RCF (PU) (non-trained arm)	16.3±0.1ǂǂ	14.6±1.8**	16.2±1.4	18.2±2.6

Table 4.4 **Effect of Aspirin on baseline cutaneous RCF in the non-trained arm of Young (LHS) and Older (RHS) men before and after IHG training.** Values are shown as mean ± SEM. Values are shown as mean ± SEM. ***, *: before vs after IHG training within Y or O. Aspirin effect: ǂǂ; Baseline Aspirin Vs after IHG aspirin: ǂ: 1,2,3 symbols indicate P<0.05, P<0.01 P<0.001 respectively.

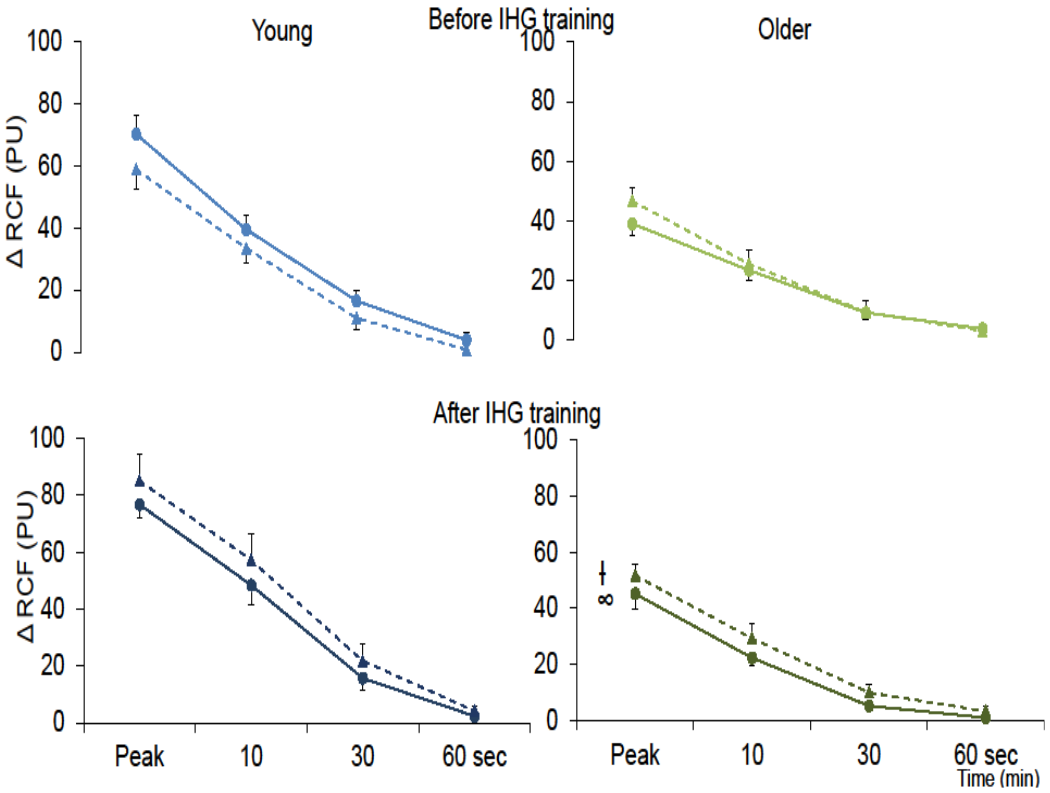


Figure 4.12: **Effect of aspirin on change (Δ) in RCF recorded after arterial occlusion for 3 min in cutaneous circulation of non-trained arm of young (LHS) and older (RHS) men before (above) and after (below) IHG training.** Values shown as mean±SEM. COX: dashed lines with triangular dots, ǂ: effect after COX, ∞: differences between Young Vs Older after COX: 1,2 symbols indicate $P<0.05$, $P<0.0001$ respectively.

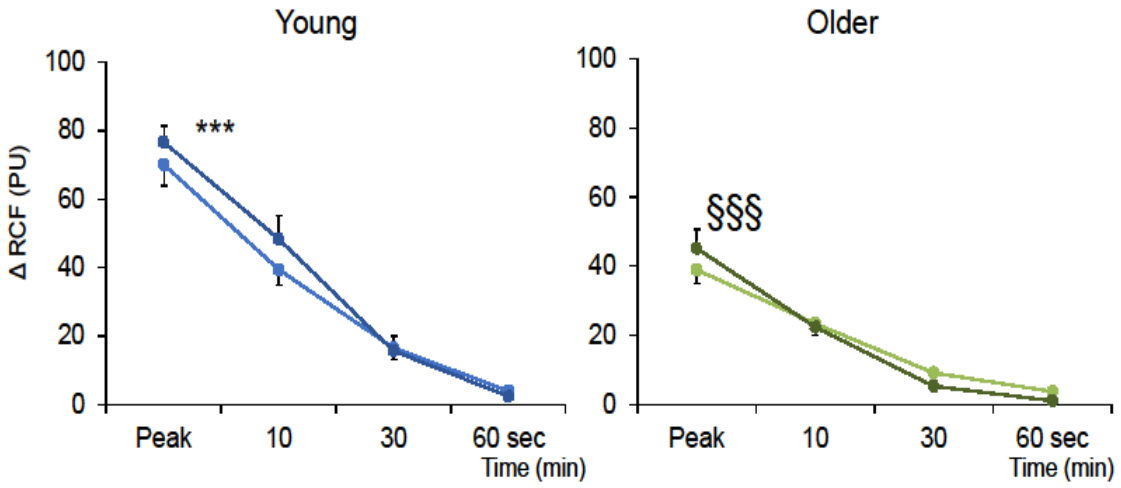


Figure 4.13: **Δ (change) RCF recorded after arterial occlusion for 3 min in Young (LHS) and Older (RHS) men before and after IHG training in the cutaneous circulation of the non-trained arm.** Young: before (light blue) and after (dark blue) IHG training; Older: before (light orange) and after (dark orange) IHG training. Values shown as mean±SEM. ***: before vs after training. §§§ Young Vs Older before & after IHG training: 1,2 symbols indicate *P*<0.0001 respectively.

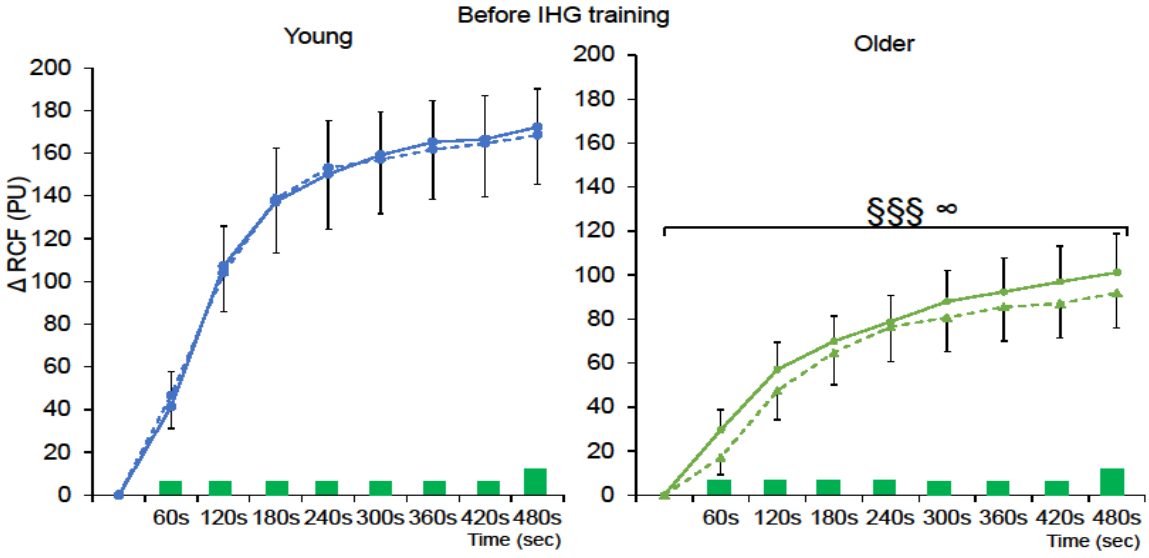


Figure 4.14: **Effect of COX inhibition on change (Δ) in RCF evoked by ACh iontophoresis in Young (LHS) and Older men (RHS) in the cutaneous circulation of the non-trained arm. Young: baseline (dark blue) and COX (dark blue-dashed line); Older: baseline (light green) and (dark green-dashed line).** Values shown as mean±SEM. §§§: Young vs Older before COX inhibition, ∞: older vs young after COX inhibition: 1,2 symbols indicate *P*<0.001 respectively.

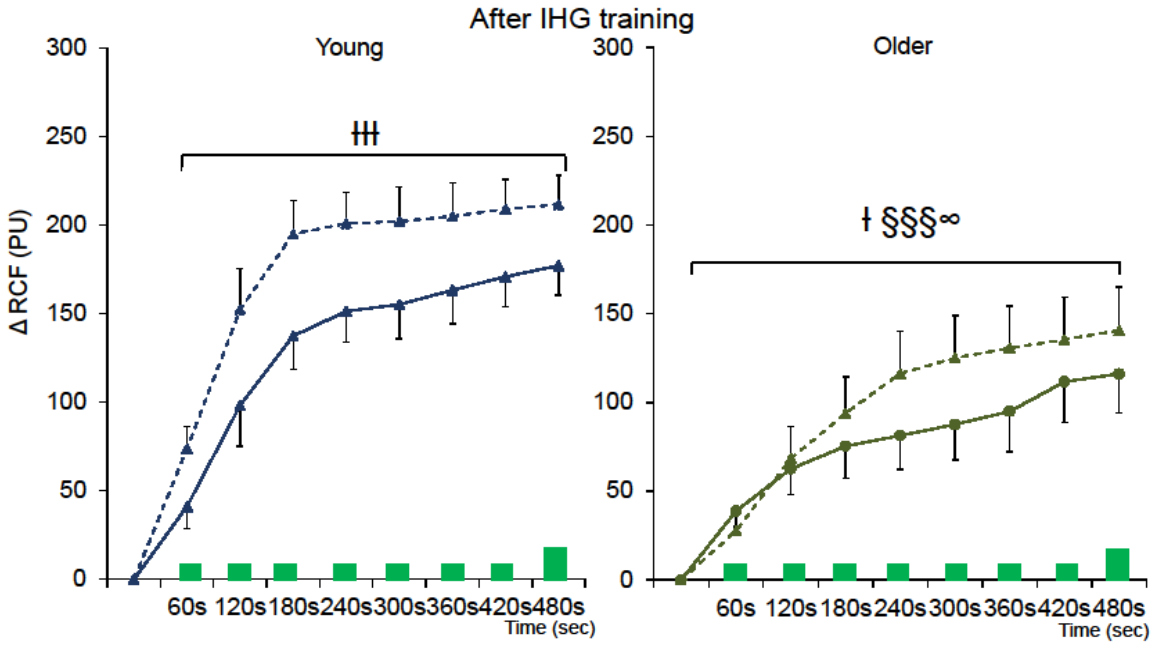


Figure 4.15: **Effect of COX inhibition on change (Δ) in RCF evoked by ACh iontophoresis in Young (LHS) and Older men (RHS) in the cutaneous circulation of the non-trained arm after IHG training. Young: IHG (dark blue) and IHG/COX (dark blue-dashed line); Older: IHG (light green) and IHG/COX (dark green-dashed line).** Values shown as mean±SEM. Ɨ: COX inhibition, §§§: older vs young before COX inhibition, ∞: older vs young after COX inhibition: 1,2,3 symbols indicate *P*<0.05, *P*<0.001, P<0.0001 respectively.

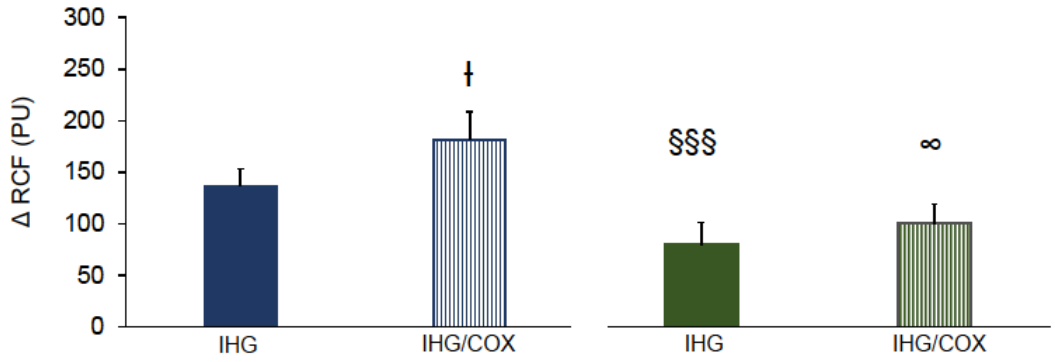


Figure 4.16: **Compacted means in RCF evoked by ACh iontophoresis in Young (LHS) and Older men (RHS) in the cutaneous circulation of the non-trained arm after IHG training. Young: IHG (dark blue) and IHG/COX (dark blue-dashed line); Older: after IHG (dark green) and after IHG/COX (dark green-dashed line).** Values shown as mean±SEM. Ɨ, Ⱨ: COX inhibition, §§§:, older vs young before COX inhibition, ∞: older vs young after COX inhibition: 1,2,3 symbols indicate P<0.05, P<0.001 , P<0.0001 respectively.

4.4 DISCUSSION

The present study showed that in older men, IHG training of the dominant arm for 4-5 weeks improved peak muscle power in both the trained arm and in the non-trained arm, but improved muscle performance during rhythmic contractions at 60% MVC in both the trained and non-trained arms, and reduced resting SP, DP and mean ABP. This was associated with no change in resting vascular tone in the trained or non-trained arm, but an increase in post-contraction hyperaemia evoked by rhythmic contractions at 60% MVC in both the trained and non-trained arms. Further, IHG training improved reactive hyperaemia in both arms. In addition, IHG training had no effect on reactive hyperaemia or ACh-induced dilatation in the cutaneous circulation of the non-trained forearm of older men, but after IHG training, COX inhibition augmented peak reactive hyperaemia and augmented ACh-evoked dilatation. In general, the control responses before IHG training were blunted in older, relative to the young men of Chapter 3 and thus IHG training improved muscle performance and forearm vasodilator responses in older men towards levels recorded in young men before training. To summarize the findings of this study, a list is presented below:

- In older men, IHG training of the dominant arm for 4-5 weeks improved peak muscle power in both the trained arm and in the non-trained arm, but improved muscle performance during rhythmic contractions at 60% MVC in both the trained and non-trained arms, and
- Reduced resting SP, DP and mean ABP.
- IHG training improved reactive hyperaemia in both arms.
- IHG training had no effect on reactive hyperaemia or ACh-induced dilatation in the cutaneous circulation of the non-trained forearm of older men.
- After IHG training, COX inhibition augmented peak reactive hyperaemia and augmented ACh-evoked dilatation.
- The control responses before IHG training were blunted in older, relative to the young men.

Subject characteristics and compliance: Apart from age, the subjects BMI, WHR and parental CVD history were similar. When using the PAR Q to determine whether the subject's physical activity level was suitable for participating in the study, the physical activity score was similar to the young men of Chapter 3.

Effects on resting ABP: The older men of the present study showed a fall in ABP of ~9mmHg after 4 weeks of IHG training. Specifically, MABP was reduced from 93.5± 1.7 to 84.3±1.6 mmHg, systolic BP was reduced from 124.4±1.8 to 114.9±1.8 and diastolic BP, from 78.0±1.8 to 69.3±1.6 mmHg, a fall of ~10 mmHg. A fall of ~4mmHg at 5 or 8 weeks of IHG training was reported in several previous studies on groups of normotensive men and women aged 19-35 years, mean age 27±2.38 respectively (McGowan et al., 2007, Wiley et al., 1992, Ray and Carrasco, 2000). As far as we are aware, this is the first study to show a fall in ABP after just 4-5 weeks of IHG training in normotensive, older men. The fact that IHG training had no significant effect on baseline FBF, or FVC in the trained or non-trained arm of older men, even though, it increased these variables in the young WE men, suggests that reduction in vascular resistance in muscle circulation is unlikely to have contributed to the older men. It may be there was a reduction in resistance in other vascular beds and/or in cardiac output.

Effects of IHG training on muscle performance in the trained and non-trained arms. After IHG training, MVC was increased from 25.31±1.41 to 28.45±1.64 kN, in the trained arm of older men, a 11.04% MVC increase which was similar to that shown by the young men, (14.57%). As discussed in Chapter 3, it is well established that resistance training can improve MVC in the trained limb: such improvements have been attributed to changes in the neural pathways to the muscles rather than to changes in the muscles themselves (Gabriel et al., 2001, Gabriel et al., 2006). Given resistance training allows the individual to achieve a force of contraction that is closer to true maximum (Roland 1985; Knight & Kamen, 2001), the present findings suggest neural remodelling is possible in older men, as in young men. As in the young men, the increase in MVC was accompanied by an increase in "Expected TTI" and importantly, by an increase in actual TTI in the trained arm over 3 min rhythmic contractions at 60% MVC. In other words, there was an increase in muscle performance and a reduction in fatigue in the trained arm of older, as well as young men.

A further, novel finding of the present study on older men, is that IHG training also increased MVC in the *non-trained* arm ($P<0.05$); IHG training also tended be increase MVC in the *non-trained* arm of young men ($P=0.06$). Thus, it seems the 'cross-transfer' effect on strength discussed in Chapter 3 (Gabriel et al., 2006), occurred in older men, and in contrast to the young men, the effect was large enough that it was detectable in a group of only 10 older men during 4 weeks of IHG training of one arm.

Not surprisingly, the increase in MVC was accompanied by improvement in Expected TTI for the non-trained arm during 3 min rhythmic contractions at 60% MVC. But, importantly, the actual TTI was improved in the non-dominant arm of older men after IHG training (6.71±0.47 vs 5.03±0.69, $P<0.001$) such that it was closer to the "expected" TTI (13.47±0.69 vs 13.48±0.74). This further demonstrated in the percentage of the expected TTI which improved from ~37% to ~50% of the expected whereas in the young men, it improved from ~50% to ~76%. Thus, muscle performance during rhythmic contractions was substantially improved in the non-trained arm of older men and fatigue was reduced. Indeed, in both the trained and non-trained arms of older men, muscle performance after IHG training more or less equalled that recorded in absolute terms in the young men before IHG training. As indicated in Chapter 3, in previous studies, improvement in muscle performance of the non-trained arm was induced in young men by training that involved handgrip rhythmic contractions to exhaustion (Yasuda and Miyamura, 1983, Yuza et al., 2000). The present study shows that an improvement in performance can occur in older men as well as young men, even when modest 30% MVC contractions are used for training. The effect of IHG training was smaller in older than young men in both arms. However, this improvement would be expected to suffice in older men to enhance physical functionality to cope with daily activities. This will be interesting to be tested in future studies.

Regarding the mechanisms underlying the increases in performance in the non-trained arm of older men, the contributing factors are likely to be similar to those discussed for the young WE men (Chapter 3); thus, the changes may be attributed both to the improved firing rate and synchronization of the motor neurons (Gabriel et al., 2006) and/or the improved vasodilation and muscle blood flow during contractions, (see below).

Effects of IHG training on exercise hyperaemia and reactive hyperaemia in trained and non-trained arms.

Firstly, it should be noted that before IHG training, in both the dominant and non-dominant arm, post-contraction hyperaemia was smaller in older, than young WE men. The fact that older men achieved a lower actual TTI in the dominant trained arm before IHG training than young WE men when they attempted 60% MVC for 3 min could partly explain the blunted post-contraction exercise hyperaemia in the older men, because they did less work. However, this would not explain the blunted exercise hyperaemia in the non-trained arm of older men before IHG training because they achieved similar TTI to the young men. Further, reactive hyperaemia was also blunted in both arms of older men relative to young men. Taken together, these findings are consistent with previous evidence that both exercise hyperaemia and reactive hyperaemia are blunted in older subjects relative to younger subjects, at least when comparisons are made in relatively sedentary individuals. For example, previous studies demonstrated reduced leg blood flow and vascular conductance during moderate intensity leg exercise in older sedentary individuals compared to young (Koch et al., 2003, Poole et al., 2003), while others demonstrated blunted reactive hyperaemia in older men and women relative to younger subjects (Wray and Ives, 2012).

Blunted exercise hyperaemia in older sedentary individuals has been attributed to age-related increased sympathetic vasoconstriction and the actions of circulating catecholamines on alpha$_1$ adrenoreceptors (Koch et al., 2003) and/or to mitochondrial limitation of O_2 consumption in the exercising muscle of older people (Poole et al., 2003). However, depressed endothelial dilator function has also been considered. Thus, it was reported that the contribution of NO and PGs to exercise hyperaemia is depressed in older subjects relative to young subjects (Schrage et al., 2007) and was improved by antioxidant administration in older subjects (Kirby et al, 2009). Moreover, others found that the attenuating effect of COX inhibition on reactive hyperaemia was accentuated in older relative to young subjects and suggested this is because older subjects are less able to compensate for loss of the actions of dilator PGs by increased influence of other dilator substances (Taylor et al., 2014). It seems most likely that in the present study, depressed endothelial function in the older men made

a major contribution to their blunted exercise and reactive hyperaemia, but this should be investigated in future studies by assaying endothelium-dependent factors in plasma (see Chapter 5), or by using COX or NOS inhibitors.

Regarding the effects of IHG training, as discussed in Chapter 3 for young men, the present finding that IHG training for 4 weeks augmented post-exercise hyperaemia in the trained arm following rhythmic contractions for 3 min and the associated increase in FVC of older men is consistent with previous evidence that IHG training and other kinds of exercise training improves exercise hyperaemia in the trained limb (Tinken et al., 2010, Green et al., 2004, McGowan et al., 2006A, McGowan et al., 2007, Badrov et al., 2013A). The extent of the increase was similar in the young and older men (56 % vs 43%).

The present finding that IHG training of one arm augmented reactive and exercise hyperaemia and the underlying forearm vasodilation in the contralateral, non-trained arm of older men is novel, as it is for young men. However, it should be noted that lower limb exercise training involving graded walking exercise over 6 weeks was reported to improve blunted-endothelial dilatation in the upper limbs of older men (Tanaka et al., 2000, DeSouza et al., 2000). Such results are consistent with the general hypothesis of the present studies that IHG training of one arm improves dilator responses in the contralateral arm as part of a systemic improvement in endothelial dilator function. Practically, the present results suggest that in older, relatively sedentary men with depressed endothelium-dependent dilator responses, IHG training of one arm greatly improves these responses at least in the contralateral arm, without the need to undertake strenuous physical activities. In future studies it would be important to investigate whether dilator responses are also improved in the legs, by IHG training.

Effects of IHG training on ABP and HR responses evoked by rhythmic forearm contractions

The present finding that IHG training reduced the increase in ABP evoked by rhythmic exercise of both the trained and non-trained arm of older men is novel. A reduction in the exercise pressor response could be explained by attenuated increases in sympathetic nerve activity, cardiac output and/ or reduced central command (Murphy et al., 2011)

and would also be blunted by the augmentation of exercise hyperaemia during the period of rhythmic contractions (Van Beekvelt et al., 2001). Since the exercise-induced increase in HR was attenuated during rhythmic contractions of the non-trained arm of older men, this suggests there was an attenuated central command (Murphy et al., 2011) following IHG training and that this was more pronounced in older, than young men. The involvement of these various factors should be investigated in future studies. Irrespective of the mechanisms, the finding that the pressor response to exercise was reduced by IHG training in older subjects would be advantageous given the increased cardiovascular risk associated with pressor responses in older people (Greaney et al., 2015, Sidhu et al., 2015, Hess et al., 2009).

Effects of IHG training on reactive hyperaemia and ACh-induced dilatation in cutaneous circulation of the non-trained arm. In the present study, both reactive hyperaemia and ACh-induced dilatation evoked in cutaneous circulation of the non-trained forearm before IHG training, were blunted in older men relative to young men. Blunted cutaneous reactive hyperaemic responses have been reported previously in older subjects (Tew et al., 2010) as has blunted ACh-mediated vasodilation (Holowatz et al., 2005).

Since the interactions between vasodilator and vasoconstrictor COX substances were reported to change with aging (Holowatz et al., 2005) the effects of COX inhibition on these two responses were compared between older and young men in the present study. In fact, COX inhibition had no effect on reactive hyperaemia, or ACh-induced dilatation in cutaneous circulation of older men before training suggesting that balance of vasoconstrictor and vasodilator COX products have no net effect, or that removal of their contribution is compensated by increased release of other dilators such as NO (Markwald et al., 2011). This contrasts with the results of Holowatz et al. (2005) who showed that COX inhibition raised baseline cutaneous perfusion in older, but not young subjects, and although vasodilator responses to ACh were attenuated by COX inhibition in older and young subjects, the attenuating effect was smaller in older subjects. Thus, they suggested that vasoconstrictor COX products contribute to baseline vascular tone in older subjects, and the ability of ACh to release vasodilator COX products is blunted in older subjects. In addition, they showed that the attenuating effect of NOS inhibition on ACh-induced dilatation was attenuated in older relative to young subjects (Holowatz

et al., 2005). These findings cannot be directly compared with those of the present study because they used a mixed group of men and women and there may be differences between the effects of aging on men and women that affected the results. It would be interesting in future studies to more extensively test the effects of NOS and COX inhibition in whole forearm and cutaneous circulation of forearm of older men.

Even though IHG training augmented reactive hyperaemia in cutaneous circulation of *young* men, it had had no effect on the magnitude of reactive hyperaemia or ACh-induced dilatation in cutaneous circulation of older men: both responses were still blunted compared to those in the young men. However, after IHG training COX inhibition augmented peak reactive hyperaemia and ACh-induced dilatation in older men as seen for the ACh response in young men. As discussed in Chapter 3, this suggests removal of the influence of a COX vasoconstrictor product or removal of an inhibitory influence on NOS activity which augmented vasodilator activity (Shirasaki et al., 1986).

The effect of IHG training on the cutaneous vascular responses of older people has not been tested before. However, several studies have tested the effect of a more strenuous exercise training. For example Black et al. (2008) demonstrated that in older people lower limb exercise training improves endothelium-dependent effects of NOS inhibition in ACh-induced dilation in cutaneous circulation. Further, the NO contribution to ACh induced dilatation was increased as judged from the effects of micro dialysis of L-NAME (Black et al., 2008). This finding would be consistent with one of the proposals made above: that IHG training enhances the expression or synthesis of NO, such that a greater NO-contribution to endothelium-dependent dilator responses is revealed following COX inhibition in older men, as proposed for young men in Chapter 3. It would be interesting to test this possibility more directly, by investigating the effects of NOS and COX inhibition singly and combined on ACh responses and reactive hyperaemia in older subjects. But these proposals were explored in a different way in the study of Chapter 5, which concerns the effects of IHG training on the efflux of NO, PG, and other potential mediators during reactive hyperaemia in older and young men.

To summarize, the present study on IHG training in older healthy, but sedentary men showed that this training regime for just 4 weeks can improve the muscular and

hemodynamic responses not only on the trained, but also the non-trained forearm. The effects of IHG training on the non-trained arm are entirely novel and the fact that there was reduction in muscle fatigue in both arms indicates this training modality could be particularly beneficial for the elderly. The blunted endothelium-dependent dilator responses seen in cutaneous circulation of older men compared to young men before IHG training were expected. However, the finding that the improvements induced by IHG training managed to upregulate these responses so that they became more similar to those of untrained young men raises the possibility that lengthening the period of IHG training might have achieved even bigger effects. Interestingly, the adaptations that occurred in whole forearm (mainly skeletal muscle) seem to be greater than those that occurred in the cutaneous circulation. The mechanisms by which IHG training affects cutaneous and whole limb responses were investigated further in Chapter 6.

5. Effects of IHG Training on prostacyclin, nitric oxide, isoprostane and potassium release from the non-trained arm in young WE and SA men and older WE men

- Contralateral Forearm blood flow release of PGI_2, NO_3/NO_2, 8-iso PGI_{2a} and K^+ during muscle performance

5.1 INTRODUCTION

As discussed in the General Introduction (section 1.9 Summary of Isometric Handgrip training), isometric handgrip (IHG) training of one arm for 4-5 weeks has been shown to decrease ABP in hypertensive patients and in normotensives. The results of Chapters 3 and 4 showed that IHG training for 4-5 weeks had no effect on ABP in *young* normotensive WE, or SA men, but reduced ABP in older normotensive WE men. However, the key novel findings of the studies presented in these chapters were that IHG training augmented reactive and exercise hyperaemia in the *non-trained* forearm of young WE men, with smaller effects in the young SAs. Since reactive and exercise hyperaemia are endothelium-dependent vasodilator responses these results suggest remote beneficial effects on endothelial function in young men which is smaller in SAs who are at higher risk of CVD. Further, IHG training greatly augmented blunted reactive and exercise hyperaemia in the non-trained arm of *older* WE men which is important because as discussed in Chapter 4, aging is associated with endothelial dysfunction.

Taken together, these new findings raise the question of whether IHG training increases the release, or action of endothelium-dependent dilator substances from the non-trained arm and whether any changes are different between young WE and SA men and older WE men. It was suggested in Chapters 3 and 4 that the stimulus for improved endothelial function may be increased shear stress. Certainly, shear stress was shown to be important in improving endothelium-dependent dilatation during rhythmic handgrip training (Green et al., 2004, Tinken et al., 2009). Shear stress is known to up regulate endothelial release of NO, PGs and EDHFs (Campbell and Fleming, 2010). Further, NO, PGs and EETs, which are recognised EDHFs, together with K^+ and hyperpolarisation have all been implicated in exercise hyperaemia (Hillig et al., 2003, Crecelius et al., 2014) and reactive hyperaemia (Tagawa et al., 1994, Carlsson et al., 1987, Crecelius et al., 2013). Moreover, endothelial dysfunction in young SAs has been associated with impaired NO-induced dilatation (Murphy et al., 2007) while endothelial dysfunction with aging has been associated with reduced NO availability and attenuated involvement of PGs (Schrage et al., 2007). On the other hand, it is widely accepted that EDHFs may compensate for reduced availability of NO and PGs (Vanhoutte et al., 2009) in endothelial dysfunction. In addition, endothelial dysfunction is associated with oxidative stress (Feletou et al., 2010) and isoprostanes are sensitive markers of

oxidative stress (Montuschi, 2007). But, isoprostanes may also act as EDHFs (Stojiljkovic et al., 2002, Janssen, 2002).

Therefore, in view of this background, the primary aim of the present study was to investigate the effect of IHG training on plasma levels of NO. Prostacyclin (PGI_2), nitrate/nitrate (NO_3NO_2), isoprostane (8-iso PGI_{2a}) metabolites plus K^+ were assayed in young WE and SA men as well as in older healthy WE men and the changes in their corresponding efflux that occurs from the non-trained forearm during reactive hyperaemia before and after IHG training. Reactive hyperaemia was chosen so that we could directly assess any changes in the release of these factors during a vasodilator response we had monitored in Chapters 3 and 4. Reactive hyperaemia was chosen rather than exercise hyperaemia because exercise hyperaemia is partly mediated by substances released from the contracting muscle fibres, whereas reactive hyperaemia is more likely to be mediated by substances released by the endothelium as a consequence of hypoxia and shear stress.

5.1.1 HYPOTHESES

The hypotheses were as follows:

1. Before IHG training, young WE men, will show greater efflux of PGI_2, NO (NO_3NO_2) metabolites, K^+ and lower efflux of isoprostane (8-iso $PGF_{2\alpha}$) from the *non-trained arm* during reactive hyperaemia than young SA men.
2. IHG training of the dominant arm will increase the efflux of PGI_2, NO metabolites (NO_3NO_2), K^+ and lower efflux of isoprostane (8-iso $PGF_{2\alpha}$) during reactive hyperaemia in the non-trained arm of both young WE and young SA men, but the changes will be greater in WE men.
3. Before IHG training, isoprostane (8-iso $PGF_{2\alpha}$) metabolites during reactive hyperaemia will be greater in older men compared to young men. However, older men will show lower levels of efflux of PGI_2, NO (NO_3NO_2) metabolites and K^+ during reactive hyperaemia than young men.

4. IHG training of the dominant arm will increase the efflux of PGI_2 metabolites, NO metabolites (NO_3NO_2) and K^+ but decrease the efflux of isoprostane (8-iso $PGF_{2\alpha}$) during reactive hyperemia in older, as well as young men.

And the aims to address these hypotheses were:

1. To test whether in young WE men there is a greater efflux of PGI_2, NO (NO_3NO_2) metabolites, K^+ and lower efflux of isoprostane (8-iso $PGF_{2\alpha}$) from the *non-trained arm* during reactive hyperaemia than in young SA men.
2. To test whether IHG training of the dominant arm will increase the efflux of PGI_2, NO metabolites (NO_3NO_2), K^+ and lower efflux of isoprostane (8-iso $PGF_{2\alpha}$) during reactive hyperaemia in the non-trained arm of both young WE and young SA men, but the changes will be greater in WE men.
3. To test whether isoprostane (8-iso PGF2α) metabolites during reactive hyperaemia will be greater in older men compared to young men. However, older men will show lower levels of efflux of PGI2, NO (NO_3NO_2) metabolites and K^+ during reactive hyperaemia than young men.
4. To test whether IHG training of the dominant arm would increase the efflux of PGI_2 metabolites, NO metabolites (NO_3NO_2) and K^+ but decrease the efflux of isoprostane (8-iso PGF2α) during reactive hyperemia in older, as well as young men.

5.2 METHODS

This study was performed on 12 young men (6 WEs and 6 SAs; 18-25 yrs) and 6 older WE men (55-70yrs) who agreed to give blood samples selected randomly from the studies described in Chapters 3 and 4.

5.2.1 GENERAL PROTOCOL

The protocol was as described on Chapter 3 and 4 except that 2 venous samples were taken during one of the red cell flux (RCF) or venous occlusion plethysmography (VOP) experiments before and after arterial occlusion at the start of experimental sessions before and after IHG training for 4-5 weeks (sections 2.7.2 and 2.7.3). This was performed separately from blood flow experiments. A graphical representation of the protocol is shown on Figure 5.1. A brief description follows: an intra-venous cannula (22 – 24 G, BD Venflon, BD) as described on Chapter 2 (section 2.9.1), was inserted into the branchial vein of the subject's non training forearm via a needle (BD Venflon Pro™ Peripheral IV Catheters 20g Pink, 32mm). The cannula was connected to a multiple-sample Luer-adapter (BD Vacutainer Multiple-Sample Luer-Adapter, BD) via a plastic tube connector (10 cm BD Connecta, BD). On each occasion, a sample of 10-15 ml was taken at rest and immediately after release of a 3 min of period of arterial occlusion (i.e., peak of reactive hyperaemia). The composition of the solution into which the blood was collected in order to prevent further metabolism is described in chapter 2 (section 2.9.2) as are the centrifugation and storage procedures.

5.2.2 ENZYME LINKED IMMUNO-SORBENT ASSAYS (ELISAs)

5.2.2.1 PROSTACYCLIN

Analysis of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2 was carried out by using a commercially available ELISA kit (Item-515211, Cayman Chemical Company). In brief, this assay is based on the competition between 6-keto-PGF1α and 6-keto-PGF1α tracer (6-keto-PGF1α acetylcholinesterase conjugate) for a restricted number of 6-keto-PGF1α-specific rabbit antiserum binding sites (Figure 5.2). The 6-keto-PGF1α antiserum binds to the mouse monoclonal anti-rabbit immunoglobulin-G (IgG) previously attached to the plate wells. The concentration of bound PGF1α is inversely proportional to the concentrations of bound tracer. Unbound reagents were washed away; Ellman's reagent was added to develop the plate. The yellow substance produced

as a result of the enzymatic reaction was measured by Spectrophotometry: 120 min after development of plate in the dark, it was read at 420 nm using a plate reader (VeraMax micro-plate reader, Molecular Devices LLC). Each sample was analysed in triplicate and a mean absorbance was calculated: values were converted into concentrations of 6-keto-PGF1α by using the standard curve generated by a logit transformation [logit (B/B_0) = In[$B/B_0/(1-B/B_0)$]. Then, the concentration of each sample was determined by the calculation of the equation obtained from the standard curve plot.

5.2.2.2 ISOPROSTANE

Analysis of 8-*iso* $PGF_{2\alpha}$, the stable metabolite of isoprostane (8-isoprostane), was carried out by using a commercially available ELISA kit (Item-516351, Cayman Chemical Company). In brief, this assay is based on the competition between 8-*iso* Prostaglandin $F_{2\alpha}$ and 8-*iso* Prostaglandin $F_{2\alpha}$ tracer (8-*iso* Prostaglandin $F_{2\alpha}$ acetylcholinesterase conjugate) for a restricted number of 8-*iso* Prostaglandin $F_{2\alpha}$ specific rabbit antiserum binding sites (Figure 5.3. The 8-*iso* Prostaglandin $F_{2\alpha}$ antiserum binds to the mouse monoclonal anti-rabbit immunoglobulin-G (IgG) previously attached to the plate wells. The concentration of bound 8-*iso* PGF2α is inversely proportional to the concentrations of bound tracer. Unbound reagents were washed away; Ellman's reagent was added to develop the plate. The yellow substance produced as a result of the enzymatic reaction was measured by Spectrophotometry: 120 min after development of plate in the dark, it was read at 420 nm using a plate reader (VeraMax micro-plate reader, Molecular Devices LLC). Each sample was analysed in triplicate and a mean absorbance was calculated: values were converted into concentrations of 8-*iso*-PGF_{2a} using the standard curve by a logit transformation [logit (B/B_0) = In[$B/B_0/(1-B/B_0)$]. Then, the concentration of each sample was determined by the calculation of the equation obtained from the standard curve plot.

5.2.2.3 NITRATE/NITRITE ASSAY

Colorimetric Assay of Nitrite/Nitrate, as an index of plasma NO concentration, was carried out by using a commercially available kit (Item-780001, Cayman Chemical Company). This assay based on a two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step involves the addition of the Griess reagent which converts nitrite into a deep purple azo compound. This involves

the conversion of nitrate to nitrite and the addition of Griess reagent. The photometric measurement of the absorbance due to this azo chromosphere accurately determines nitrite concentration (Green et al., 1982). Plasma samples were ultra-filtered using 30kDa filters (Amicon Company), which were rinsed with UltraPure water (Millipore Company). Plasma was then centrifuged at 10000 x *g*, 20 min rpm at 4°C (Eppendorf centrifuge 5417R) to reduce absorbance caused by the presence of haemoglobin and improve Griess Reagent colour formation. 180 min after development of the plate in the dark, it was read at 540nm using a plate reader (VeraMax micro-plate reader, Molecular Devices LLC). Each sample was analysed in triplicate and a mean absorbance was calculated: values were converted into concentrations of Nitrate/Nitrite using the standard curve. Standard curve was generated through a two-step process. Firstly, the blank wells were averaged and then subtracted from the absorbance values of all the other wells. Secondly, the absorbance at 540-550 nm was plotted as a function of nitrate concentration. The nitrate standard curve was then used to determine the total nitrate/nitrite (NO_3/NO_2) concentration (Figure 5.4).

5.2.2.4 POTASSIUM IN PLASMA (K^+)

Potassium (K^+) concentrations in plasma samples were analysed by the Clinical Biochemistry Department at Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust by using a Roche Modular automated analyser. This analyser comprises of indirect ion selective electrode (ISE) module, a c702 high through-put clinical chemistry module (spectrophotometry) and two e602 immunoassay modules. Plasma samples were defrosted, labelled and transferred into tubes received from the hospital. Each tube contained 500 to 1000μL of plasma. The corresponding barcode labelling was attached on each tube to identify each sample and labelling was added to an Excel spread for the hospital staff to add the results of the measurement. The plasma was transferred to the hospital on the same day for the analysis.

5.2.2.5 ANALYSIS OF EFFLUX

The effluxes of PGI_2, NO (NO_3NO_2), 8-iso $PGF_{2\alpha}$ and K^+ were calculated for each efflux by multiplying the concentration of each substance measured in plasma at baseline and at the peak of reactive hyperaemia by the corresponding FBF value

measured with VOP at these time points before and after IHG training in the experiments described in Chapter 3 and 4.

STATISTICAL ANALYSIS

The data collected for each variable (6-keto-PGF1α, 8-iso PGF2α, NO_3/NO_2, K^+) for each condition were analysed within subject groups (young WEs and SAs and older WEs) by using One-way ANOVA to detect differences between baseline values and those collected at the peak of reactive hyperaemia. When ANOVA indicated statistical significance at $P<0.05$, post hoc paired t-tests with Bonferroni correction was used to compare values within groups at different time points before and after IHG training. One-way ANOVA was also used to compare groups before and after IHG training followed by un-paired t-tests with Bonferroni correction to identify times at which groups differed. Statistical significance was set at $P<0.05$.

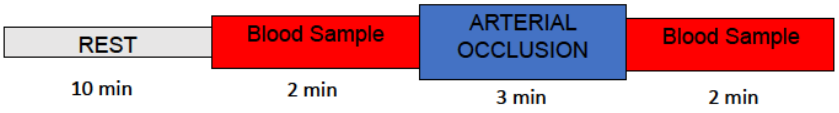


Figure 5.1: **Schematic diagram of protocol.** Blood sample was taken from non-dominant, non-trained arm before and immediately after release of arterial occlusion for 3 min before and after 4-5 weeks of IHG training.

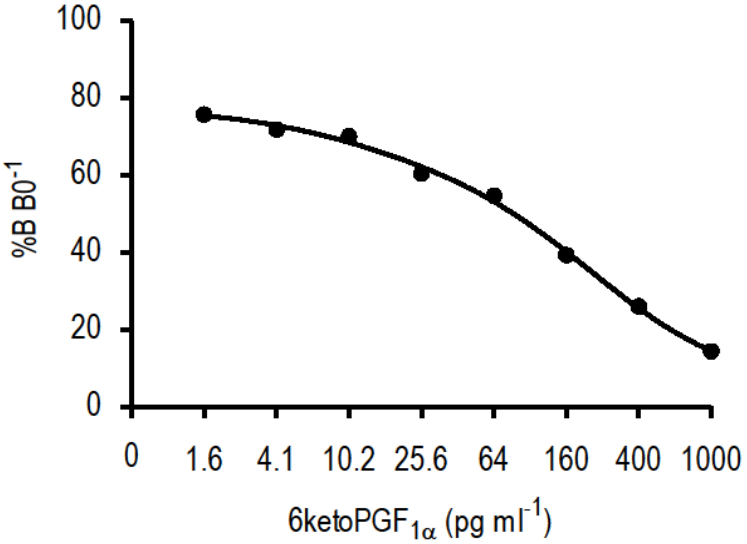


Figure 5.2: **Standard curve** calculated from the standards prepared for 6-ketoPGF$_{1\alpha}$ ELISA. (Cayman Company 515211).

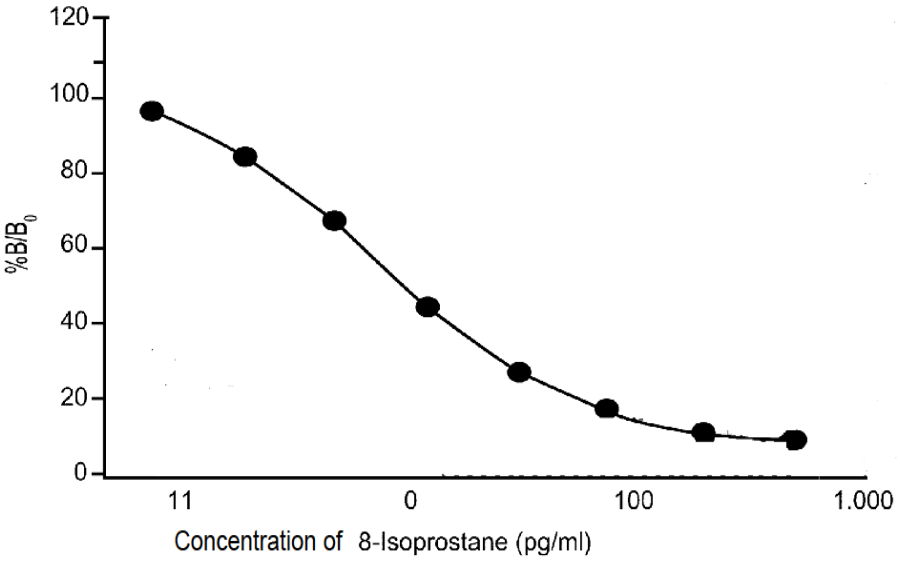


Figure 5.3: **Standard curve** calculated from the standards prepared for 8-*isoprostane* ELISA. (Cayman-Chemical-Item No. 516351)

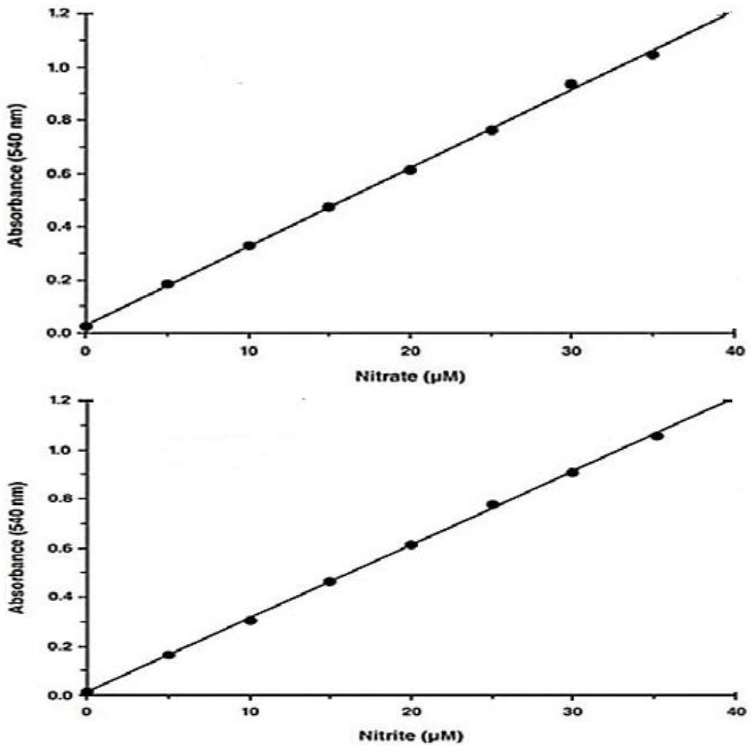


Figure 5.4: **Standard curve** calculated from the standards prepared for nitrate ELISA (Cayman-Chemical-Item No. 780001).

5.3 RESULTS

PGI_2 measured as 6-keto-PGF_{1a}: The concentrations of PGI_2 measured in the venous efflux of the *non-trained arm* in young WEs, SAs (Table 5.1) and older WEs (Table 5.2) showed no changes during reactive hyperaemia before vs after IHG training (Table 5.1). However, when the venous PGI_2 efflux, calculated as the product of concentration and FBF (see Methods 5.2) at baseline and at the peak of reactive hyperaemia before and after IHG training was compared within groups, ANOVA showed there was a significant effect of reactive hyperaemia before and after IHG training ($P<0.0001$). Moreover, post-hoc analysis showed that within young WEs and within older WEs (Table 5.4; Fig. 5.5), there was a significant increase in PGI_2 efflux during reactive hyperaemia both before and after IHG training, but after IHG training only, in young SAs (Table 5.3; Fig. 5.5). Within each group the PGI_2 efflux at the peak of reactive hyperaemia did not differ significantly before vs after IHG training. Comparison between young WEs and SAs by ANOVA showed that there were significant differences before IHG training ($P<0.01$). Further, a comparison between young WE and older men by ANOVA showed significant changes before IHG training ($P<0.001$). Similarly, after IHG training comparisons between young WEs and SAs and between young and older WE men also shown significant differences ($P<0.001$). But, post hoc analysis showed no significant difference between either the young WEs and SAs or the young vs older WEs (Fig. 5.5).

NO_3NO_2 (Nitrate/Nitrite): NO_3NO_2 concentration did not change significantly during reactive hyperaemia in both young WE, and SA men before (young WEs effect size: 1.40, young SAs effect size: 1.29) and after IHG training (effect size: 1.51, young SAs effect size: 1.29) (Table 5.1). By contrast, in older WE men, NO_3NO_2 concentration was not changed during RH before IHG training (older effect size: 1.47), but baseline and reactive hyperaemia values were significantly different from young WEs after IHG training (older effect size: 1.19) (Table 5.2).

Considering NO_3NO_2 efflux, one-way ANOVA within groups showed significant effects of reactive hyperaemia on NO_3NO_2 efflux before and after IHG training in all three groups ($P<0.001$; Figure 5.6). In young WEs and young SAs post-hoc analysis showed NO_3NO_2 increased from baseline during reactive hyperaemia before, but not after IHG training (Table 5.3; Figure 5.6).

Analysis of one subject's from the older WE men indicated an efflux of 7583.83 μM.dl^{-1}.min^{-1} during reactive hyperaemia before IHG training. This value was rejected as it was more than 3 SDs away from the mean and considered as outliers. This left only five subjects for analysis before IHG training. NO_3NO_2 efflux during reactive hyperaemia did not reach statistical significance before IHG training in the older group (Table 5.4; Figure 5.6). After IHG training, in the older group there was a trend for an increased reactive hyperaemic response as post-hoc analysis demonstrated (Table 5.4; Figure 5.6). A power calculation was used to determine the sample size required to achieve statistical significance for the NO (NO_3NO_2) efflux response evoked during reactive hyperaemia for older WEs before IHG training. Based on pilot data indicated that a subgroup of size of six was sufficient to detect differences during reactive hyperaemia at approximately 20% in older WE men with 80% power at *P* less than 0.05 (https://clincalc.com/stats/samplesize).

Potassium (K^+) Efflux: K^+ concentrations measured in the venous efflux did not show any changes during reactive hyperaemia before or after IHG training either in young WE and SAs or in older WE men (Table 5.1).

Within young WEs, ANOVA showed a significant effect on K^+ efflux before and after IHG training (P<0.001) and *post-hoc* analysis showed K^+ efflux increased during reactive hyperaemia both before and after IHG training, but with no change in the efflux before vs after IHG training (Table 5.3; Figure 5.7). Similarly, in young SAs, there were significant increases in K^+ efflux during reactive hyperaemia before and after IHG training (P<0.001), but no change in efflux before vs after IHG training. The post-hoc analysis showed that there were no differences between the two ethnic groups for K^+ efflux during reactive hyperaemia (Table 5.3; Figure 5.7).

In older WE men there were also significant increases in K^+ efflux during reactive hyperaemia before and after IHG training (P<0.0001), but post-hoc analysis showed no differences before vs after IHG training. The increases in K^+ efflux was not different when compared to young WEs (Table 5.4; Figure 5.7).

8-isoprostane $PGF_{2\alpha}$: The concentrations of 8-*iso* $PGF_{2\alpha}$ in venous plasma were not changed before vs after occlusion before or after IHG training in young WEs or SAs (Table 5.1; Figure 5.8), or in older WEs (Table 5.2).

By contrast, within group ANOVA showed there was a significant effect of reactive hyperaemia on the efflux of 8-*iso* $PGF_{2\alpha}$ before and after IHG training in both young WEs and SAs ($P<0.0001$ in each case). Moreover, post-hoc analysis showed that within young WEs there was a significant increase in 8-*iso* $PGF_{2\alpha}$ efflux during reactive hyperaemia both before and after IHG training. However, in young SAs 8-*iso* $PGF_{2\alpha}$ efflux increased significantly during reactive hyperaemia before, but not after IHG training (Table 5.3; Figure 5.8). Comparisons between young WEs and SAs by ANOVA showed a significant change ($P<0.001$). But no changes were identified after post-hoc analysis in either condition.

In older WEs 8-*iso* $PGF_{2\alpha}$ efflux within group ANOVA showed there was a significant effect of reactive hyperaemia before and after IHG training ($P<0.001$). Post-hoc analysis demonstrated that 8-*iso* $PGF_{2\alpha}$ efflux increased during reactive hyperaemia before, but not after IHG training. Interestingly, the baseline of 8-*iso* $PGF_{2\alpha}$ efflux was significantly higher after, vs before IHG training (Table 5.4; Figure 5.8).

Plasma	Before IHG training				After IHG training			
	Baseline		After Occlusion		Baseline		After Occlusion	
	WEs	SAs	WEs	SAs	WEs	SAs	WEs	SAs
PGI_2 (pg/ml^{-1})	27.6±8.0	22.4±3.8	20.7±4.8	18.1±4.3	33.7±7.1	26.8±8.3	29.4±5.9	32.5±9.6
NO_3NO_2 (μM/ml)	45.3±5.1	39.8±7.1	6.4±4.3	5.4±0.9	47.0±4.6	7.3±5.9	8.0±3.4	6.2±5.6
K^+ (mmol/L)	8.6±0.7	9.4±1.3	8.5±0.2	8.4±1.2	9.6±1.6	7.8±0.3	9.7±1.0	8.8±0.6
8-iso $PGF_{2\alpha}$ (pg/ml^{-1})	13.8±3.2	14.2±3.8	11.4±1.9	12.9±2.5	15.5±4.8	10.3±1.6	18.2±5.3	16.3±5.6

Table 5.1**: Concentrations of PGI_2, 8-iso $PGF_{2\alpha}$ in pg/ml^{-1}, K^+ in mmol/L and NO_3NO_2 in μM measured in venous plasma samples recorded before and immediately after arterial occlusion (3 min) before and after IHG training (4-5 weeks) between young White Europeans vs South Asians.** Values are shown as mean ± SEM. There were no significant differences within or between groups at baseline or after release of occlusion before or after

Plasma	Before IHG training				After IHG training			
	Baseline		After Occlusion		Baseline		After Occlusion	
	Young	Older	Young	Older	Young	Older	Young	Older
PGI_2 (pg/ml^{-1})	27.6±8.0	22.6±3.5	20.7±4.8	28.7±4.1	33.7±7.1	27.9±4.2	29.4±5.9	24.9±3.1
NO_3NO_2 (µM/ml)	45.3±5.1	76.4±12.8	6.4±4.3	76.2±11.9	47.0±4.6	145.8±68.7§	8.03±3.4	176.5±94.7§
K^+ (mmol/L)	8.6±0.7	9.7±0.7	8.5±0.2	8.7±0.6	9.6±1.4	8.6±0.1	9.7±0.9	10.9±1.8
8-iso $PGF_{2\alpha}$ (pg/ml^{-1})	13.8±3.2	22.2±5.0	11.4±1.9	24.5±5.1	15.5±4.8	29.6±6.3	18.2±5.3	33.9±10.5

Table 5.2: **Concentrations of PGI_2, 8-iso $PGF_{2\alpha}$ in pg/ml^{-1}, K^+ in mmol/L and NO_3NO_2 in µM measured in venous plasma samples recorded before and immediately after arterial occlusion (3 min) before and after IHG training (4-5 weeks) between young Vs older.** Values are shown as mean ± SEM. §: young vs older ($P<0.05$; ANOVA with post-hoc Bonferroni).

Plasma	Before IHG training				After IHG training			
	Baseline		After Occlusion		Baseline		After Occlusion	
	WEs	SAs	WEs	SAs	WEs	SAs	WEs	SAs
PGI_2 $pg.dl^{-1}.min^{-1}$	3.8±1.1	2.9±0.4	16.1±3.8‡	15.1±4.4‡	5.6±1.4	4.1±1.4	23.9±4.9‡	22.6±5.3‡
NO_3NO_2 µM. $dl^{-1}.min^{-1}$	6.9±0.5	5.4±0.9	38.9±7.2‡	24.9±3.0‡	0.9±0.7†	1.0±0.8†	6.4±2.9†	4.8±3.7†
K^+ $mmol.dl^{-1}.min^{-1}$	1.2±0.3	1.4±0.3	6.9±0.9‡	6.7±0.8‡	1.5±0.1	1.2±0.1	7.8±0.9‡	6.9±1.1‡
8-iso $PGF_{2\alpha}$ $pg.dl^{-1}.min^{-1}$	2.0±0.5	1.8±0.4	9.2±1.7‡	11.4±2.8‡	2.2±0.3	1.6±0.2	13.3±1.8‡	13.2±4.3

Table 5.3: **Effects of release of arterial occlusion on venous efflux of PGI_2, 8-iso $PGF_{2\alpha}$, K^+ in $mmol.dl^{-1}.min^{-1}$, and NO_3NO_2 before and after IHG training in young WEs and SAs men (n=6 in each group).** Values are shown as mean ± SEM. ‡: baseline vs after occlusion; †: before vs after IHG training. ($P<0.05$; ANOVA with post-hoc Bonferroni).

Plasma	Before IHG training				After IHG training			
	Baseline		After Occlusion		Baseline		After Occlusion	
	Young	Older	Young	Older	Young	Older	Young	Older
PGI_2 $pg.dl^{-1}.min^{-1}$	3.8±1.1	3.5±0.5	16.1±3.8‡	15.5±2.5‡	5.7±1.4	5.1±1.3	23.9±4.9‡	17.3±2.9‡
NO_3NO_2 $\mu M.dl^{-1}.min^{-1}$	6.9±0.5	14.2±5.8	38.9±7.2‡	46.4±13.0‡	0.9±0.7†	28.9±91†	6.4±2.9†‡	120.5±39.1†
K^+ $mmol.dl^{-1}.min^{-1}$	1.2±0.3	1.5±0.3	6.9±0.9‡	4.9±0.9‡	1.5±0.1	1.5±0.2	7.8±0.9‡	7.1±1.0‡
8-iso $PGF_{2\alpha}$ $pg.dl^{-1}.dl.min^{-1}$	2.0±0.5	3.4±0.7	9.2±1.7‡	13.4±2.9‡	2.2±0.3	5.2±1.3†	13.3±1.8‡	23.3±6.9

Table 5.4**: Effects of release of arterial occlusion on venous efflux of PGI_2, 8-iso $PGF_{2\alpha}$, K^+ in $mmol.dl^{-1}.min^{-1}$,and NO_3NO_2 before and after IHG training in young and older(n=6) men.** Values are shown as mean ± SEM. ‡: baseline vs after occlusion; †: before vs after IHG training (P<0.001; post-hoc adjustment: P<0.0125).

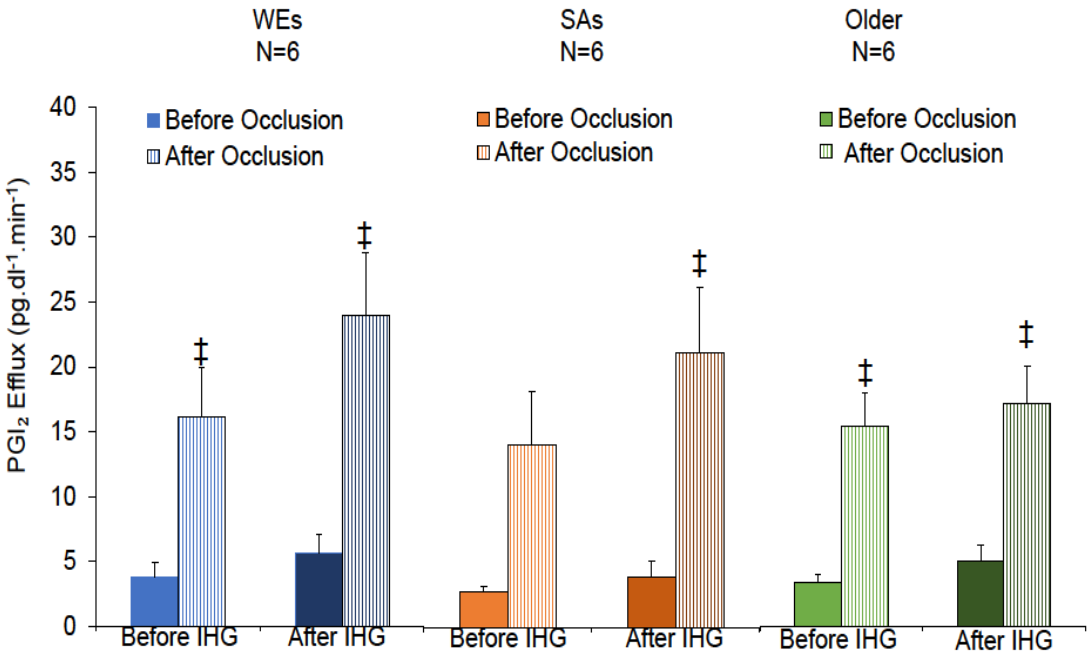


Figure 5.5 **Venous efflux of PGI_2 during reactive hyperaemia before and after IHG training in young WEs, SAs and older WEs.** ‡: baseline vs occlusion within group before or after IHG training, P<0.05 ANOVA with post hoc Bonferroni.

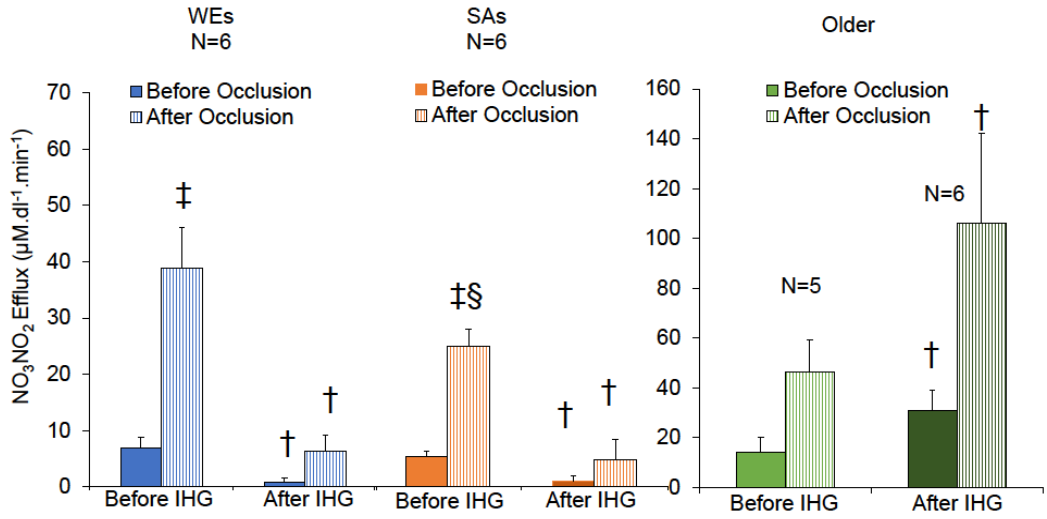


Figure 5.6 **Venous efflux of NO_3NO_2 during reactive hyperaemia before and after IHG training in young WEs, SAs and older WEs.** ‡: baseline vs occlusion; †: before vs after IHG training, §: WEs vs SAs, or young WE vs older WE, P<0.05 ANOVA with post-hoc Bonferroni.

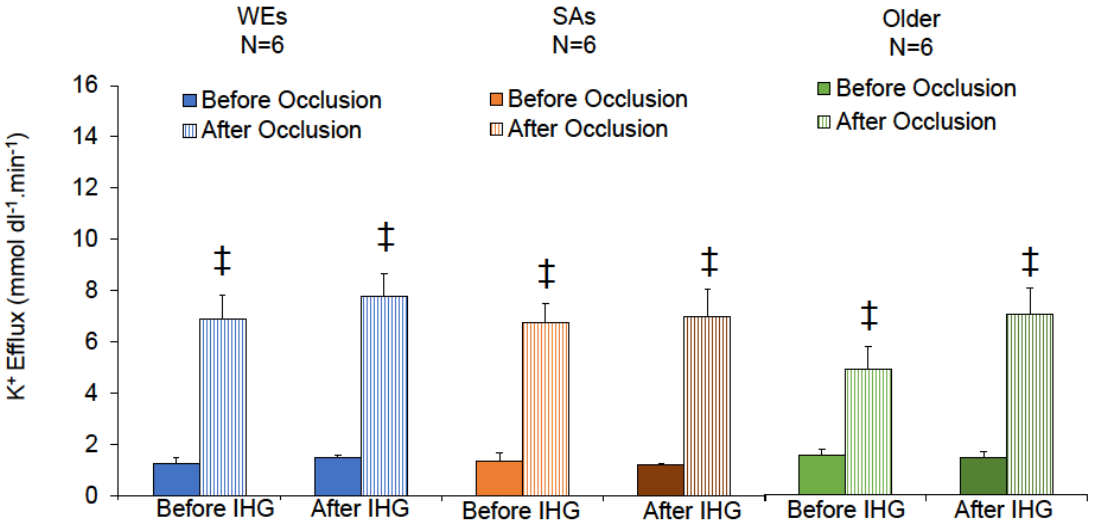


Figure 5.7 **Venous efflux of K^+ during reactive hyperaemia before and after IHG training.** ‡: baseline vs occlusion within group before or after IHG training, P<0.05 ANOVA with post hoc Bonferroni.

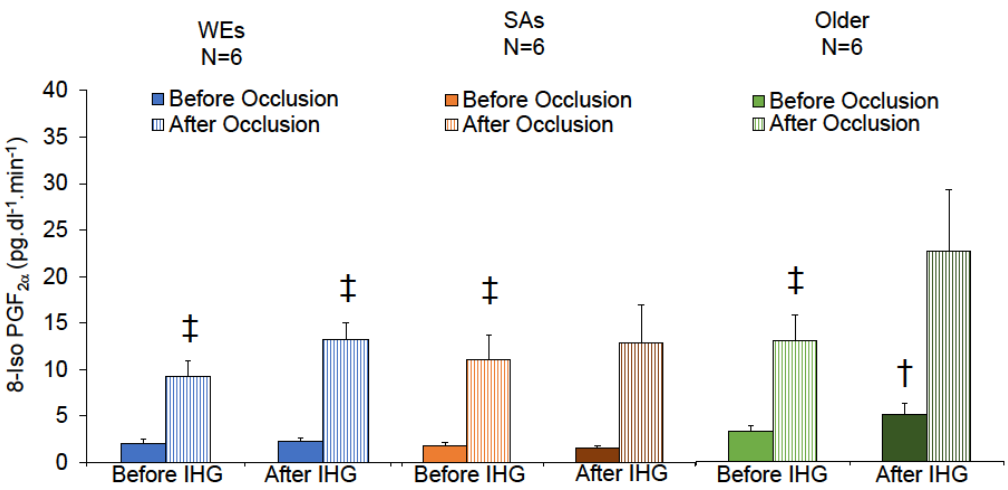


Figure 5.8 **Venous efflux of 8-iso $PGF_{2\alpha}$ during reactive hyperaemia before and after IHG training in young WEs, SAs and older WEs.** ‡: baseline vs occlusion; †: before vs after IHG training, §: WEs vs SAs, or young WE vs older WE, $P<0.05$ ANOVA with post-hoc Bonferroni.

5.4 DISCUSSION

The present study investigated the effects of IHG training on the release of several potential mediators of reactive hyperaemia in the non-trained arm, which might be associated with the improvements in reactive hyperaemia that occurred following IHG training in the young WE men and older WE men. In the young WE men, the effluxes of PGI_2 metabolite, K^+ and 8-iso PGI_{2a} were increased in the non-trained forearm in reactive hyperaemia both before and after IHG training with no significant difference between the values measured before and after IHG training. By contrast, the efflux of NO_3NO_2 metabolite was increased in reactive hyperaemia before, but not after IHG training and baseline NO_3NO_2 metabolite efflux was also decreased after IHG training. Similar changes in the efflux of all 4 substances occurred in young SA as in the WE men *before* IHG training although PGI_2 metabolite showed only a trend for an increase in reactive hyperaemia. After IHG training, there were again similar changes in SAs to those seen in young WE men, but, 8-iso PGI_{2a} efflux showed only a trend to increase in reactive hyperaemia. In older WE men the effluxes of PGI_2, K^+ and 8-iso PGI_{2a} metabolites showed an increase in reactive hyperaemia before and after IHG training. However, efflux of NO_3NO_2 metabolites showed only a trend for an increase in reactive hyperaemia before IHG training, but after IHG training, baseline efflux of NO_3NO_2 metabolites was higher than before IHG training and reached a higher value in reactive hyperaemia. These results are discussed in more detail below in relation to the changes observed in reactive hyperaemia in the non-trained arm discussed in Chapters 3 and 4. To reiterate: IHG training of the dominant arm augmented the peak increase in FBF and FVC recorded in the forearm during reactive hyperaemia in the non-trained arm of young *and* older WE men but had no significant effect on these changes in young SA men. To summarize the findings of this study, a list is presented below:

- In the young WE men, the effluxes of PGI_2 metabolite, K^+ and 8-iso PGI2a were increased in the non-trained forearm in reactive hyperaemia both before and after IHG training.
- No significant difference between the values measured before and after IHG training.
- The efflux of NO_3NO_2 metabolite was increased in reactive hyperaemia before, but not after IHG training and baseline NO_3NO_2 metabolite efflux was also decreased after IHG training.

- Similar changes in the efflux of all 4 substances occurred in young SA as in the WE men before IHG training although PGI_2 metabolite showed only a trend for an increase in reactive hyperaemia.
- After IHG training, there were again similar changes in SAs to those seen in young WE men, but 8-iso PGI2a efflux showed only a trend to increase in reactive hyperaemia.
- In older WE men the effluxes of PGI_2, K^+ and 8-iso PGI2a metabolites showed an increase in reactive hyperaemia before and after IHG training.
- Efflux of NO_3NO_2 metabolites showed only a trend for an increase in reactive hyperaemia before IHG training, but after IHG training, baseline efflux of NO_3NO_2 metabolites was higher than before IHG training and reached a higher value in reactive hyperaemia.

Contribution of PGI_2, NO_3NO_2, K^+ and 8-iso PGI_{2a} to reactive hyperaemia before IHG training

In previous studies, the contribution of various factors to reactive hyperaemia has mainly been deduced from the effects of selective antagonists. For example, in a mixed group of men and women of non-specified ethnicity, reactive hyperaemia in forearm and release of a PG-like substance, which could not be identified with certainty at the time, were both attenuated by ibuprofen a COX inhibitor (Kilbom and Wennmalm, 1976). Thus, they concluded dilator PGs contribute to reactive hyperaemia. Subsequently, it was shown that both ibuprofen and the adenosine receptor antagonist theophylline attenuated reactive hyperaemia and an associated efflux of adenosine in the forearm of a mixed group of men and women aged 19-41 years (Carlsson et al., 1987). As the combined effect of ibuprofen and theophylline on reactive hyperaemia was no greater than their independent effects, they proposed that the contributions of PGs and adenosine to reactive hyperaemia are interdependent in some way (Carlsson et al., 1987). By contrast, Taylor et al. (2014) reported that COX inhibition with indomethacin *potentiated* reactive hyperaemia in a mixed group of young men and women, suggesting that either COX inhibition reveals dilator effects of other mediators such as NO or adenosine, or that *vasoconstrictor* COX products limit reactive hyperaemia.

On the other hand, it was concluded that NO contributes to the recovery phase of reactive hyperaemia rather than the peak, judging from the effects of NOS synthase

inhibition in young men who were presumably Japanese since the study was performed in Japan (Tagawa et al., 1994). A similar proposal was made by Engelke et al. (1996) who showed that COX, or NOS inhibition attenuated the peak or recovery phases of reactive hyperaemia respectively in a mixed group of young men and women. Opening of K_{ATP} channels and efflux of K^+ was also implicated in the recovery phase by the actions of K_{ATP} channel blockade in men and women (mean age 42 years; (Bank et al., 2000). More recently, it was concluded that hyperpolarisation due to opening of K_{IR} channels and stimulation of Na^+/K^+-ATPase, is responsible for ~90% of reactive hyperaemia in young men and women, judging from the effects of antagonists of these mechanisms alone or in combination, on the peak and recovery of reactive hyperaemia (Crecelius et al., 2013). Neither NO nor K^+ efflux was assayed in these studies.

Very few studies have been performed on reactive hyperaemia in older subjects, but the study of Taylor et al. (2014), mentioned above, showed that COX inhibition attenuated reactive hyperaemia in older men and women, in contrast to the augmentation seen on the young subjects, leading them to suggest that dilator PGs make a larger contribution in older, than young subjects. They speculated this may be because NO normally inhibits PG generation by COX (Bauersachs et al., 1996) and NO availability is reduced in older subjects (Taddei et al., 1997a), such that COX inhibition reveals a larger contribution of dilator PGs to reactive hyperaemia in older adults (Taylor et al., 2014).

As far as we are aware, the present study is the first one in which the efflux of several of the substances implicated in reactive hyperaemia has been assayed in samples taken from groups of *male* subjects of restricted age range and of known ethnicity. We can therefore propose that PGI_2, NO and K^+ contribute in some way to reactive hyperaemia in the forearm of young WE and SA men since the efflux of all three substances was significantly increased above baseline in both groups immediately following release of arterial occlusion. Indeed our findings provide direct evidence for the findings that reactive hyperaemia was attenuated by COX inhibition (Engelke et al., 1996, Carlsson et al., 1987, Kilbom and Wennmalm, 1976, Addor et al., 2008), that the recovery phase was attenuated by NOS inhibition (Engelke et al., 1996, Bank et al., 2000, Tagawa et al., 1994, Dakak et al., 1998, Nugent et al., 1999) and by K_{ATP}, while the peak and recovery were attenuated by inhibition of K_{IR} and Na/K ATPase (Bank et al., 2000,

Banitt et al., 1996, Engelke et al., 1996, Tagawa et al., 1994, Crecelius et al., 2013); the latter group of antagonists would inhibit the efflux of K^+ or its ability to cause dilation.

The fact that the efflux of NO_3NO_2 was smaller during reactive hyperaemia in young SA than WE men is consistent with the evidence that NO availability was decreased in young SA men: their forearm dilator responses to ACh were smaller and the effect of NOS inhibition on baseline blood flow was blunted relative to young WE men (Murphy et al., 2007). In fact, the present evidence adds to the view that endothelial dysfunction is already present in apparently healthy young SA men (Murphy et al., 2007).

In addition, although reactive hyperaemia was depressed in older, WE men relative to young WE men, it is reasonable to propose that PGI_2 and K^+ contribute to reactive hyperaemia in older WE men, whereas NO probably does not or contributes less, given the efflux of PGI_2 and K^+ was increased during reactive hyperaemia, but NO_3NO_2 was not significantly raised above baseline level. This agrees with previous evidence discussed above that COX inhibition attenuated reactive hyperaemia in older people (Taylor et al., 2014). In fact, the present results add *direct* evidence to the indirect evidence that NO availability is lower in older than young men [e.g.(Taddei et al., 1995, DeSouza et al., 2000)]. In both studies forearm dilator responses induced by ACh were depressed in older men relative to young men and in the study of Taddei et al. (1995), l-arginine augmented the dilator responses in older, but not young men, indicating NOS is substrate limited in older men.

It could be also argued that the isoprostane 8-iso PGI_{2a} may contribute to reactive hyperaemia given its efflux was increased following release of arterial occlusion in all three groups. For, although isoprostane 8-iso PGI_{2a} is a recognised marker of oxidative stress and has been shown to have both constrictor and dilator effects (Janssen, 2002), infusion of several different vasodilators including bradykinin, the NO donor sodium nitroprusside or the Ca^{2+} entry blocker diltiazem all increased forearm blood flow in groups of normotensive and hypertensive men and women (mean age 45 and 31 years respectively) and there was an associated efflux of 8-iso PGI_{2a} (Fong et al., 2010). It was therefore proposed that 8-iso PGI_{2a} was released by increased shear stress acting on endothelial cells secondary to the increased blood flow, consistent with evidence that increased shear stress activates NADPH oxidase which is an important source of reactive oxygen species (De Keulenaer et al., 1998). This proposal was, in turn, consistent with evidence that superoxide generates 8-iso PGI_{2a} non-enzymatically, or

via COX, from arachidonic acid and can function as an EDHF (Janssen, 2002). In future studies, it would be interesting to test whether 8-iso PGI_{2a} does contribute to reactive hyperaemia in young WEs, SAs and/or older WEs by using an antagonist of its generation.

Contribution of PGI_2, NO_3NO_2, K^+ and 8-iso PGI_{2a} to reactive hyperaemia after IHG training.

After IHG training, the increases in efflux of all the assayed metabolites during reactive hyperaemia were similar to those that occurred before IHG training, except for NO_3NO_2 efflux, which was *lower* at baseline and showed smaller increases during reactive hyperaemia than before IHG training in both young WE and SA men, but was *increased* at baseline and showed a larger increase during reactive hyperaemia in older WE men. Given IHG training augmented reactive hyperaemia in young WE men, these findings indicate that NO is *not* the dominant factor contributing to this augmentation in young WE men, although increased NO release might be responsible for the augmented reactive hyperaemia in the older, WE (Chapter 3 and 4). In fact, these findings raise the question of *how* IHG training may have decreased NO efflux at baseline and during reactive hyperaemia in young WE, and SA men, and had the opposite effect in older WE men. Additionally, it clearly indicates that NO_3NO_2 contributes minimally to the FBF and FVC responses after IHG training in young WE and SA men.

Previous evidence derived mainly from animal studies suggested that NO availability increased initially during exercise training in the trained limb but then the vessels increase in diameter such that the shear stress stimulus for NO release is removed and NO availability is not augmented after longer term training (Green et al., 2004). More recently, it was shown that flow mediated dilatation of the brachial artery which is considered to be NO-dependent initially increased during an 8-week rhythmic handgrip training programme, but returned to control level by 8 weeks, whereas reactive hyperaemia progressively increased (Tinken et al., 2010). Further, no such changes occurred in the contralateral arm, which underwent the same training programme, but with external compression to prevent the increase in shear stress occurring in the brachial artery during each bout of rhythmic exercise (Tinken et al., 2010). These results were consistent with those of the animal studies but allowed the firm proposal that changes in shear stress associated with reciprocal changes in vascular re-modelling

are responsible for parallel changes in NO-dependent dilatation during exercise training, while vascular re-modelling and an increased size of the vascular bed explained the increased reactive hyperaemia (Tinken et al., 2010).

In view of these results, it could be that the reduction in NO release at baseline in the non-trained arm of young WEs reflected *lower* shear stress in that arm secondary to vascular remodelling. Certainly, baseline FVC and FBF were increased in non-trained arm of young WEs by IHG training, although the increase in mean FBF and FVC in young SA men did not reach statistical significance in SAs (see Chapter 3). Further, as argued in Chapter 3 it may be that in line with the augmented MVC and muscle performance in the *non*-trained arm of young WEs and SAs (Chapter 3), there was a parallel increase in size of vascular bed in the non-trained arm, that was particularly pronounced in young WE that contributed to both the increase in exercise and reactive hyperaemia. If so, the shear stress stimulus induced by sudden release of arterial occlusion in the non-trained arm could have led to a greater increase in FVC during reactive hyperaemia due to the increased size of the vascular bed, but with smaller increase in shear stress and therefore, smaller shear-stress induced NO release. If so, it could be that similar mechanisms were responsible for the depressed NO efflux during reactive hyperaemia in the non-trained arm of young SA men following IHG training, but the effects on vascular remodelling and therefore on baseline FVC and reactive and exercise hyperaemia were less marked.

On the other hand, the finding that baseline NO efflux and the increase in NO efflux during reactive hyperaemia in the non-trained arm were augmented in older WEs is consistent with evidence discussed by Green et al. (2004) that exercise training does increase NO-dependent dilatation in groups that have endothelial dysfunction. It should also be noted that lower body walking for up to 5-6 days/week for 3 months at 60-75% of maximum HR improved endothelium-dependent ACh-induced dilatation in the forearm of elderly sedentary men (DeSouza et al., 2000), which again indicated a remote effect on endothelial function which might have been mediated by shear stress. Further, shear-stress induced release of NO was increased in arterioles taken from the soleus muscle of sedentary aged rats that underwent aerobic exercise training for 10-12 weeks (Sindler et al., 2009). However, the present results are novel in demonstrating that training involving 4 isometric contractions of one arm at only 30% MVC 4

days/week for 4-5 weeks induced substantial improvements in endothelium-dependent dilatation and NO release in the non-trained arm.

The mechanism/s that might underlie the augmented RH seen in the non-trained arm of young WE men after IHG training, are not clear from the present results. Although there was no significant increase in efflux of PGI_2, K^+ or 8-iso PGI_{2a}, during RH after IHG training relative to before, there were trends for the mean effluxes to be greater (see Figures 5.7-5.8-5.9). This raises the possibility that the total of their effect was greater after IHG training than before. Alternatively, if the size of the vascular bed in the non-trained arm was indeed increased by re-modelling caused by IHG training as suggested above, it may be that a similar magnitude of dilatation in individual arterioles caused a larger increase in FVC and FBF during reactive hyperaemia. Another possibility is that the efflux of another substance that was not assayed, such as adenosine, or one of the EDHFs was increased after IHG training. In this context, it should be noted that in the study of Sindler et al. (2009) on rats, exercise training for 12 weeks, there was augmented endothelial release of O_2- from muscle arterioles, in part from uncoupled NOS, and the H_2O_2 generated from O_2- by superoxide dismutase contributed to the augmented shear-stress induced arteriolar dilatation. Thus, it could be that H_2O_2 contributed to the augmented reactive hyperaemia in young WE men. This could be tested in future studies by using inhibitors of superoxide dismutase (SOD).

Other potential mechanisms include products of the cytochrome P450 (CYP) pathway such as the Epoxyeicosatrienoic acids (EETs; (Campbell et al., 2006) which are recognized as EDHFs, which induce hyperpolarization and vasodilation (Campbell and Fleming, 2010). The production of EETs occurs in endothelial cells and is increased by shear stress, bradykinin, acetylcholine, cyclic stretch stimuli, all of which induce endothelium-dependent hyperpolarization and relaxation (Campbell and Fleming, 2010). Further FMD and reactive hyperaemia in cutaneous circulation was attenuated by sulfaphenazole which inhibits -CYP (Fischer et al., 2007). In addition, fluconazole which is also a -CYP inhibitor inhibited reactive hyperaemia in cutaneous circulation (Roustit and Cracowski, 2013). If the actions of EETs contributed to the augmented reactive hyperaemia in WE men after IHG it might have been expected that there would have been increased K^+ efflux during reactive hyperaemia following IHG training, but this did not occur in any of the groups. However, it could be that the same release of

EDHF made a larger contribution to reactive hyperaemia given the evidence that the role of EDHF is increased when NO availability is reduced (Vanhoutte, 2009). Thus, in future studies, it would be interesting to test the effects of sulfaphenazole and fluconazole on reactive hyperaemia before and after IHG training in all 3 groups.

A question then remains as to why IHG training did not augment reactive hyperaemia in young SA men. As for IHG training-induced adaptations in muscle performance and exercise hyperaemia, it seems that either the stimulus that triggered the augmented RH in the non-trained arm of young WE men during IHG training was not present in young SAs, or they did not respond to it at this intensity, frequency and duration of IHG training. One way of addressing this issue is to follow blood flow in the tissues of the contralateral arm during IHG in both young WE, and SA men, particularly as shear stress was shown to be a mediator for vascular adaptation that contributes to the improvement of vascular function during rhythmic contractions in previous studies (Tinken et al., 2010). The important question is whether the changes in blood flow in the contralateral arm during IHG training were similar in young WEs and SAs. This was done in the experiments described in Chapter 6.

Overall, the present results show for the first time that the vasodilator mediators PGI_2, K^+, NO_3NO_2, 8-iso PGI_{2a} that have been implicated in reactive hyperaemia are actually released during reactive hyperaemia in young WEs, SAs and older WEs. They also demonstrated that isoprostane 8-iso PGI_{2a} is released during reactive hyperaemia in all 3 groups and the possibility is raised that it may contribute to dilation. In addition, the present results show for the first time that IHG training for 4 weeks leads to a reduction in the efflux of NO at baseline and during reactive hyperaemia in the *non*-trained arm of both young WE and SA men, but to an augmented release at baseline and in reactive hyperaemia in older WE men. It is proposed that this augmented NO release contributes to the enhanced reactive hyperaemia in the older men. The present results provided inconclusive explanations for the fact that reactive hyperaemia in the non-trained arm was augmented by IHG training in young WE, but not in SA men. The possibility is raised that the disparity may be explained by SAs showing smaller blood flow changes and lower shear stress during IHG training bouts than WEs. This was tested in the following chapter.

6. **Responses evoked by isometric handgrip in contralateral arm and calf**

- Contralateral Forearm and Ipsilateral Leg blood flow during isometric contractions
- Contralateral Forearm and Ipsilateral Leg Cutaneous perfusion (contribution of COX products) during isometric contractions

6.1 Introduction

As discussed in the General Introduction (section 1.9), IHG training for 4-5 weeks reduced ABP in normotensive men and women of young to middle age, and in patients with hypertension, (McGowan et al., 2006B, McGowan et al., 2006A, Badrov et al., 2013A). The study described in Chapter 3 showed that in *young*, normotensive WE and SA men, IHG training for 4 weeks had no effect on resting ABP, but endothelium-dependent dilatator responses – reactive hyperaemia and exercise hyperaemia – increased in both the trained and in the non-trained arm of WEs, but only in the trained arm of SAs. These results suggested that IHG training has widespread beneficial effects on endothelial function in young WEs, but not in young SA men, who are known to show early signs of endothelial dysfunction (Murphy et al., 2007, Hirst and Marshall, 2018). However, in both studies, IHG training induced increased muscle performance during rhythmic forearm contractions in both the trained and non-trained arms in both WEs and SAs, which might also have been expected to increase endothelium-dependent dilatation in both groups. As explained in General Introduction, one of the hypotheses of the present study is that IHG training would increase endothelial dilator function remotely in the systemic circulation by providing a shear stress stimulus to endothelial function. Therefore, the main aim of the study presented in this chapter was to determine the changes in blood flow that occur in the contralateral arm during a single bout of IHG training and to establish whether they are different between young WE and SA men, such that the shear stress stimuli might be different. Since IHG training was effective in improving endothelium-dependent dilatation in the contralateral arm, a secondary aim was to test whether IHG training might increase blood flow in the ipsilateral leg.

There is evidence that isometric contraction of forearm *does* increase blood flow in the contralateral arm, although this is controversial, as are the mechanisms underlying any increase in flow. In brief, Lind et al. (1964) argued that during isometric handgrip, FBF increased in the contralateral arm only when there was concurrent electromyographic (EMG) activity in that arm, and that the magnitude of the increase in flow was directly related to the size of the handgrip contraction (Lind et al., 1964). They suggested that the increased FBF was exercise hyperaemia due to inadvertent muscle contraction. Subsequently, (Cotzias and Marshall, 1993), explored different intensity isometric contractions at 25%, 50%, 70% MVC whilst recording ABP, FBF and EMG activity in

the contralateral arm, and demonstrated that 2 minutes into contraction EMG activity was correlated with a fall in vascular resistance at 70%, but not at 25% MVC. Subjects demonstrated greater falls in forearm vascular resistance (FVR) when they did not attempt to control their EMG activity. However, when subjects avoided movement in the "resting" arm and there was minimum EMG activity, FVR increased instead at 25 and 75% MVC (Cotzias and Marshall, 1993). Taken together, these results suggested that at higher contraction intensities, vasodilation in the contralateral arm secondary to inadvertent contraction can overcome normal reflex vasoconstriction of the exercise pressor response (Murphy et al., 2011).

By contrast, Eklund et al. (1974) reported that vasodilation in the non-exercising arm peaked in the first minute of isometric contraction at 33% MVC whereas EMG activity peaked in the second minute of exercise. Nevertheless, FBF was still substantially raised at the end of the 2nd minute. These results were consistent with those of another study (Sanders et al., 1989) which also demonstrated that the highest EMG activity response was achieved at the second minute of isometric contraction at 30% MVC, while the vasodilation was at its greatest during the first minute. Similarly, in the lower limb, Eklund (1974) reported an initial 50% increase in calf blood flow (CBF) during the first minute of isometric forearm contraction at 30%MVC, but there was no significant change in calf vascular resistance (CVR) and in the second minute CBF decreased together with an increase in CVR. Further, Fisher and White (2003) reported initial vasodilation at 15 seconds in the contralateral leg during isometric handgrip at 30% MVC in the absence of EMG activity, followed by an increase in calf vascular resistance.

Turning to the mechanisms underlying the vascular responses in the "resting" limb, it has been established that muscle sympathetic nerve activity (MSNA) shows no change in the initial 30 s of isometric handgrip at 30% MVC, but then progressively increased concomitant with an increase in vascular resistance indicating the progressive increase in resistance is sympathetically-mediated vasoconstriction (Seals, 1989, Vissing et al., 1991).

The mechanisms underlying the *increase* in blood flow and vasodilation have been attributed to several different factors apart from exercise hyperaemia. Initially, Eklund and Kaijser (1976) reported the fall in vascular resistance in the contralateral limb

during isometric handgrip was diminished by ~50% by the beta adrenoreceptor blocker, propanol (Eklund and Kaijser, 1976). They deduced this reflected the action of nerve-released noradrenaline on beta adrenoreceptors because it was too soon for circulating adrenaline levels to increase. On the contrary, Sanders et al (1989) found that propanol did not affect the decrease in vascular resistance in resting forearm or leg, whereas Reed et al. (2000) reported that propanol attenuated the vasodilation in the resting limb when sympathetic activation had been prevented by stellate ganglion blockade implicating circulating adrenaline.

It has also been suggested that the initial vasodilation in resting forearm during contralateral isometric contraction is cholinergic since it was virtually abolished by local infusion of atropine (Sanders et al., 1989). Further, atropine attenuated the initial rapid vasodilation in the resting arm during one-legged voluntary exercise (Ishii et al., 2013), while others reported that rhythmic handgrip during ischaemia evoked vasodilation in the contralateral arm that persisted after autonomic ganglion blockade, but was attenuated by atropine or NO synthase inhibition (Dietz et al., 1997, Reed et al., 2000). Although sympathetic cholinergic fibres are present in animals such as the dog and the cat (Bolme and Fuxe, 1970), there is no histological or pharmacological evidence to support their presence in humans (Joyner and Halliwill, 2000). Thus, it was suggested that vasodilation in the resting limb during muscle contraction may be caused by mechanical stimulation of the endothelium and release of ACh from endothelial cells, which then triggers release of NO and vasodilation (Reed et al., 2000, Martin et al., 1996). It has recently been confirmed that increased blood flow, which is generally associated with increased shear stress does indeed activate the endothelium to release ACh, trigger NO synthesis and cause vasodilation (Wilson et al., 2016).

These findings are interesting, especially because endothelial cells release PGs in response to increased blood flow and shear stress (Bhagyalakshmi and Frangos, 1989, Berthiaume and Frangos, 1992). PGs have also been implicated in exercise hyperaemia during light as well as more strenuous forearm contraction (Schrage et al., 2004, Win and Marshall, 2005, Junejo, 2014) and so might be released in the "resting" limb if there is unintended contraction in that arm. Moreover, as presented in Chapter 5, reactive hyperaemia, which is at least partly caused by an increase in shear stress (Koller and Bagi, 2002, Tagawa et al., 1994), caused a significant increase in venous efflux of PGI_2

in young WE men, but not in young SAs. Moreover, following IHG training of one arm for 4 weeks, PGI_2 efflux during reactive hyperaemia in the contralateral arm was maintained in young WE men, but augmented in young SA men.

6.1.1 HYPOTHESES AND AIMS

To summarize, the hypotheses of the present study were:

1. We hypothesized that there was an increase in the blood flow in the contralateral arm and the cutaneous perfusion during each 3 min period of isometric contraction.
2. We hypothesized that there was an increase in the blood flow and the cutaneous perfusion in the ipsilateral leg during each 3 min period of isometric contraction.

And the aims to address these hypotheses were:

1. To test whether blood flow does increase in the contralateral arm of young WE and SA men during each 3 min period of isometric forearm contraction at 30% MVC, when these are repeated 4 times as during the IHG training protocol used in the present study.
2. To test whether blood flow increases in the ipsilateral leg during each 3 min period of isometric contraction.
3. To test whether any increases in blood flow in the contralateral arm and increases in cutaneous perfusion are larger in WEs than SAs and are partly dependent on dilator PGs in WEs and attenuated by COX inhibition with aspirin in WEs, but not in SAs.
4. To test whether any increases of blood flow in the ipsilateral leg and increases in cutaneous perfusion are partly dependent on dilator PGs and attenuated by COX inhibition with aspirin.

6.2 METHODS

The study was performed on 30 young men (20 WEs and 10 SAs; 18-25 years old): 10 WEs and 10 SAs took part in experiments on the contralateral arm to that which undertook isometric handgrip; another 10 WEs were recruited for experiments on the ipsilateral leg.

6.2.1 GENERAL PROTOCOL

All experiments were performed according to general principles described fully in Chapter 2. All were familiarized with the protocol and techniques, provided with the participant information sheet, and completed the health and lifestyle questionnaire. The baseline characteristics of the subjects can be found in Table 6.1. At this initial visit, each subject's 30% MVC was determined as were resting ABP and HR (see Chapter 2 sections 2.6 and 2.7.7, 2.7.8).

6.2.1.1 Experimental Recordings

For all the experimental sessions, the experimental set-up described in section 2.7 was used for FBF recordings from the non-dominant forearm (Protocol A) and ipsilateral calf (Protocol B) through venous occlusion plethysmography (VOP) (figure 6.1; 6.2; and 6.3). During the 1st isometric contraction of the dominant arm (see Protocol: section 2.7.7), ABP and HR were continuously recorded via a finger cuff (Finapres, Ohmeda 2300) wrapped around the middle finger of the non-dominant arm (Figure 6.1). During subsequent contractions of the dominant arm (in Protocol A), it was not possible to continuously record ABP from the non-dominant arm since VOP recordings from that arm interrupted the ABP recordings (see protocol below- schematic 6.1). In addition, cutaneous red cell flux (RCF) was recorded via a laser Doppler probe (MoorVMS-LFF2; see section 2.7.6) positioned on the anterior surface of the non-dominant forearm (FRCF), or lateral aspect of the ipsilateral calf (CRCF). The calf was raised to the level of the arms to try to ensure recordings were made at heart level (Figure 6.2).

Throughout these experiments, electromyography (EMG) activity was recorded either from the non-dominant arm or ipsilateral calf to provide an index of electrical and contractile activity in muscle. For this purpose, surface electrodes were positioned over

the major flexor muscle of the digits in the forearm – the flexor pollicis longus – and over the body of the gastrocnemius muscle in the calf. The appropriate area of the forearm or calf was cleaned using an alcoholic pad, hair in this area was trimmed and then a pair of surface EMG electrodes was attached to the skin with double sided adhesive discs over the longitudinal axis of the muscle (Figures 6.1, 6.2 and 6.3). The distance between the pairs of electrodes was constant for all experiments. The output of the EMG electrodes was connected to a dual Bio amp with a connection to the Power Lab.

6.2.1.2 Isometric Handgrip Exercise

A handgrip dynamometer (LAFAYETTE Hand Dynamometer Model 1018) was used by the first two subjects. However, for the remaining subjects, an electronic hand dynamometer (CAMRY EH101) was used instead as subjects found it difficult to grip the other dynamometer for 3 min due to foam padding around the handle. For the experimental protocol, the subject was instructed to perform 30% MVC with the dynamometer using their dominant hand, five times for 3 minutes, with 5-min intervals. The subjects were instructed verbally by the investigator when to start each contraction, and when to stop; a screen on the dynamometer informed the investigator and subject if they were achieving their 30% MVC. If the subjects deviated, they were urged to correct the error.

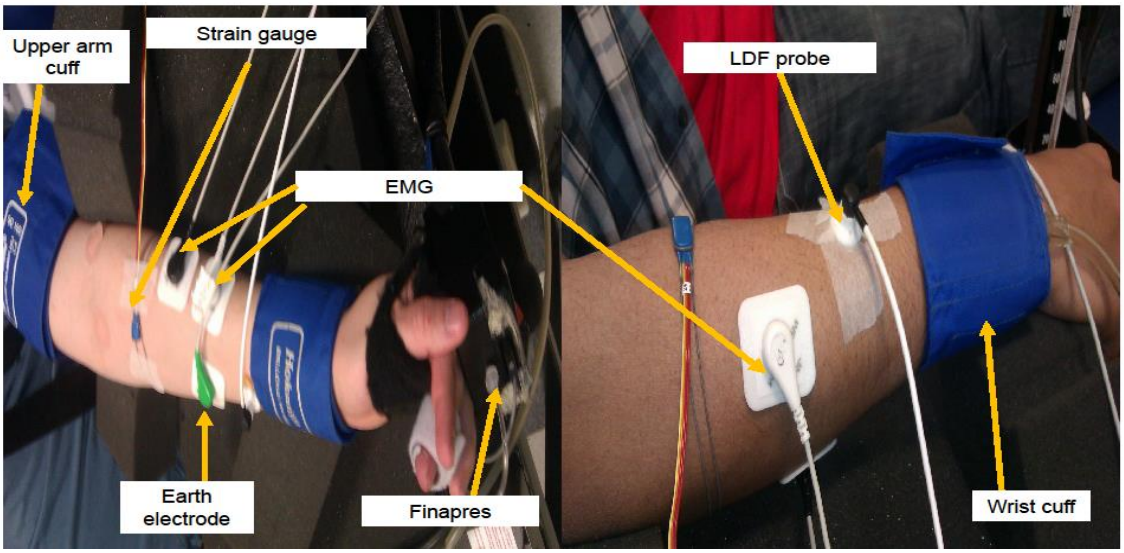


Figure 6.1 Arrangement of recording devices on contralateral non-dominant arm: inner arm view (LHS) and outer arm view (RHS). Non-dominant arm is equipped with strain gauge (for VOP), EMG electrode (for EMG recordings; LHS) and LDF probe (for RCF recording; RHS). Upper arm and wrist cuff used for VOP are shown on LHS and RHS.

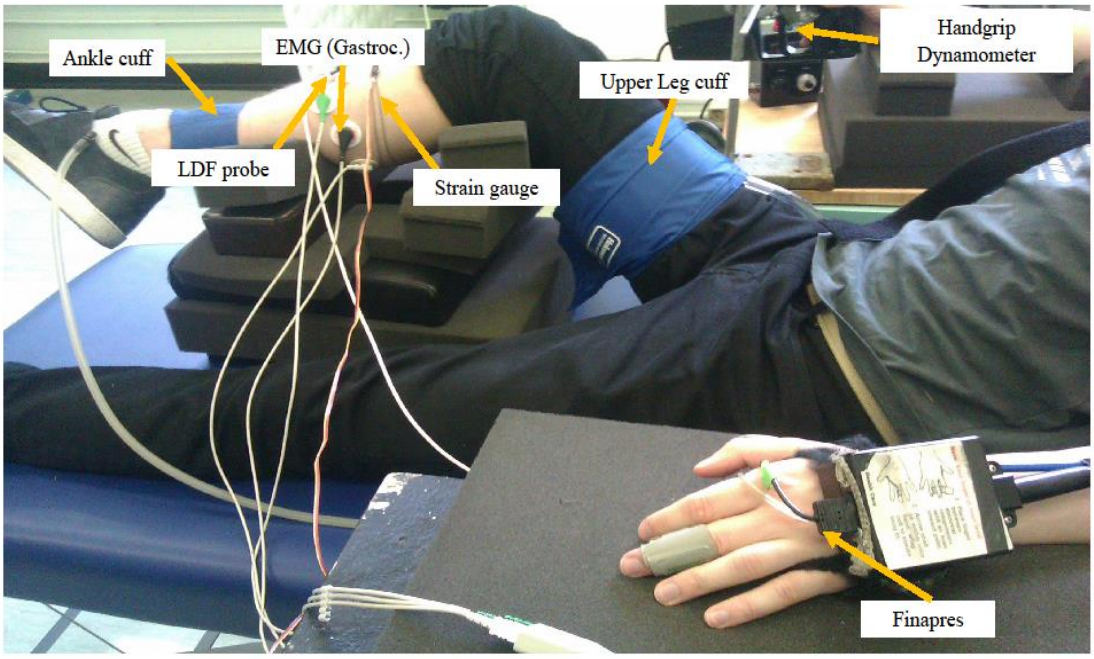


Figure 6.2 Complete experimental set up. The subject is in the 45-degree upright position throughout the whole experimental protocol. The ipsilateral leg is equipped for VOP, EMG and LDF recordings. It is raised to the level of the arms to maintain the recordings at the heart level.

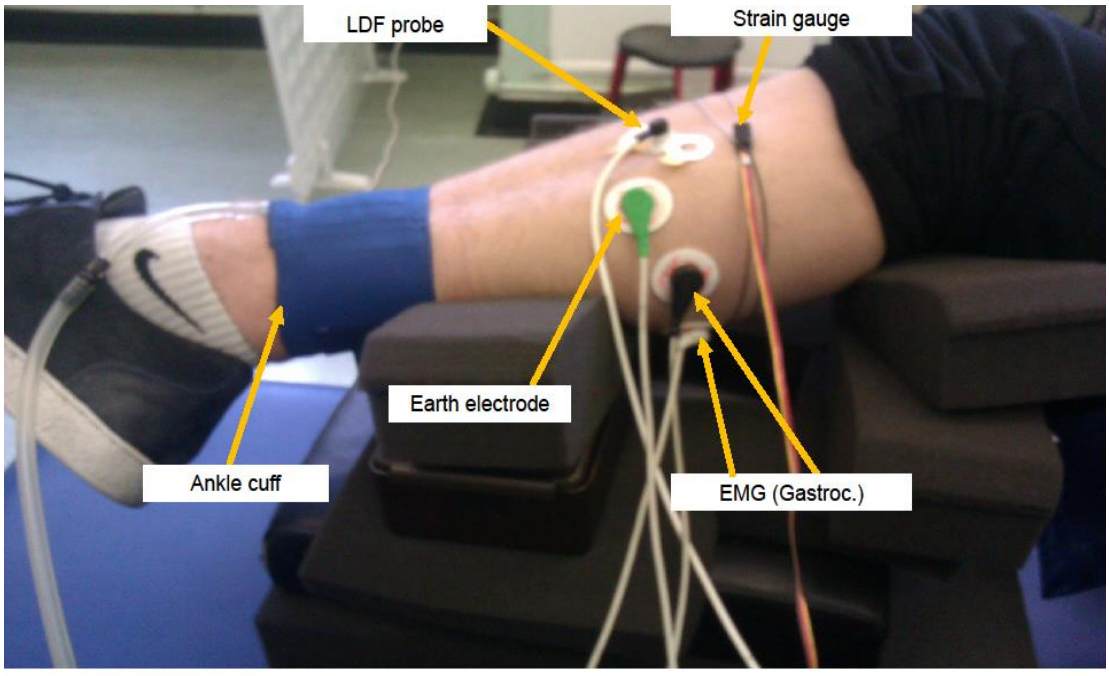


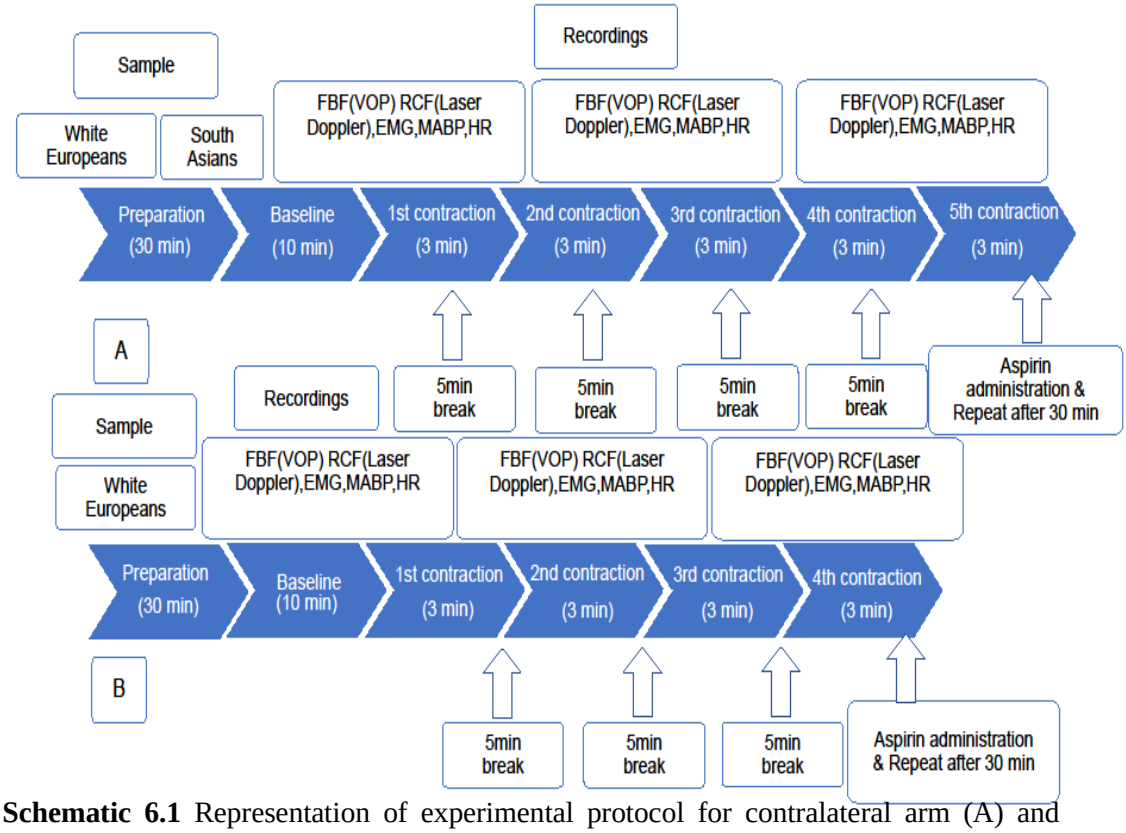
Figure 6.3 Ispilateral leg set up during 1st - 4th contractions. Calf is equipped with strain gauge (for VOP), EMG electrode (for EMG recordings) and LDF probe (for RCF recording; RHS).

6.3 Experimental Protocols

All subjects who took part in this study were involved in two different experiments. One was performed 30 min after a placebo drink and the other after a drink containing aspirin (600 mg; section 2.7.9); the order of the experiments was single-blind randomised for each subject. Before the protocol began, the subject was allowed 30 min to acclimatise to the environment and to allow COX inhibition to develop (Schematic 6.1). Before the protocol began, baseline readings of all recordings were taken (see section 3.3.2).

In Protocol A the dominant arm was contracted isometrically 5 times at 30% MVC for 3 min each at 5 min intervals and recordings were made from the non-dominant arm, (Figure 6.2). During the first contraction, ABP was continuously measured by using the Finapres on the non-dominant arm (see Figure 6.2). From the onset of exercise, MABP recordings were extracted for analysis every 15 s throughout the 3 min contraction period. The Finapres was removed for the 2nd-5th contractions inclusive, and FBF in the non-dominant arm was recorded by VOP at 15s intervals through the 3 min contraction. Cutaneous RCF was also recorded continuously from the forearm (FRCF) and EMG activitiy was recorded from flexor pollicis longus. Throughout the contractions, any visible movement in the contralateral arm, were noted.

In Protocol B ABP was recorded continuously from the non-dominant arm throughout 4 isometric contractions of the dominant forearm at 30% MVC for 3 min at 5 min intervals, while recordings were made from the calf ipsilateral to the dominant arm (Figure 6.3). CBF was recorded by VOP at 15 s intervals during each period of contraction, calf cutaneous RCF was recorded continuously from the calf (CRCF) and EMG activity was recorded from Gastrocnemius; any movements of the calf or leg were noted.



Schematic 6.1 Representation of experimental protocol for contralateral arm (A) and ipsilateral leg (B) respectively.

6.3.1 Data Analysis

All data were extracted and analysed by using LabChart v8.1.2 software essentially as described in section 3.3.3. During the first contraction in Protocol A, ABP recordings taken at baseline and at 15 s intervals during contraction 1, were used to calculate forearm vascular conductance (FVC) for the other 4 contractions, by dividing the relevant FBF measurement by the corresponding recording of MABP. A similar procedure was followed for calculation of forearm cutaneous vascular conductance (FCVC). This was justified on the basis that Cotzias and Marshall (1993) provided evidence, that the increase in ABP that occurs during a given force of handgrip contraction, was consistent upon repetition. For Protocol B, calf vascular conductance (CVC) and calf cutaneous vascular conductance (CCVC) were calculated by dividing each recording of CBF or calf RCF by ABP recorded at the same time.

Quantitative EMG analysis was also performed across each of the five contractions of the dominant arm for contralateral arm and each of the four contractions of the dominant arm for the ipsilateral leg, by calculating the area under the raw EMG

waveforms, using the integral function available on LabChart. This function summed the negative and positive values detected by the EMG, to give a single value every 15 s, for each contraction. Similarly, cutaneous RCF data were also extracted, as the average value every 15 s throughout the 3-min contractions. All data are expressed as mean ± standard error of the means (SEM).

In order to determine the overall increase in FBF, FVC, FRCF, FCVC, CBF, CVC CRCF and CCVC during the placebo and aspirin experiments in each ethnic group, compacted mean data were generated by taking an average of the recordings made at each 15 s across the 3-min periods of contraction for each subject; these values were then grouped for each ethnicity and each condition.

Statistical Analysis

Comparisons between baseline values in placebo and aspirin conditions were made by paired t-tests; those between ethinic groups were made by un-paired t-tests. Paired t-tests were also used to compare the compacted mean changes in FBF, FVC, CBF and CVC in WEs and SAs before and after aspirin during each isometric contraction expressed as change from the baseline values. Mixed between and within subjects repeated measures ANOVAs were used to compare absolute values in the two ethnic groups across the 3-min periods of contraction and to compare control responses and responses after aspirin. Post-hoc tests were used to calculate differences between time points for each contraction. Statistical significance was set at $p<0.05$.

6.4 RESULTS

Baseline Characteristics

There were no differences in the anthropometric characteristics between groups for Protocols A or B (See Tables 6.1 and 6.2) except that SAs were younger than WEs (22.8±0.9 vs 20.6±0.8 yrs).

There were also no differences for cardiovascular baselines between WEs and SAs except that FBF and FVC were lower in SAs than WEs. Further, FBF and FVC were reduced after aspirin in WEs (FBF:12.0±1.9 vs 3.8±0.7 ml.100ml^{-1}.min^{-1} and FVC: 0.16±0.03 vs 0.05±0.01 CU respectively), whereas only FBF values were significantly lower after aspirin in SAs (FBF: 6.7±0.9 vs 4.4±0.9 ml.100ml^{-1}.min^{-1} and FVC: 0.09±0.01 vs 0.06±0.01 CU; Table 6.4).

Comparisons between baselines for experiments in which recordings were made from the non-dominant arm and ipsilateral leg in WEs (Table 6.5) showed no differences between the two WEs groups except that baseline CBF and CVC were lower than baseline FBF and FVC respectively.

Mean arterial blood pressure

The effects of the 1st 3-min period of isometric contraction on MABP in Protocol A were similar in WEs and SAs (figure 6.4) and were not affected by aspirin. In protocol B, ABP changes were similar during each of the 4 contractions under placebo conditions and were not affected by aspirin (figure 6.11).

Forearm Vascular responses

In Protocol A, as shown in Figure 6.5, FBF increased significantly during each contraction in WEs and the increases in FBF in the 4th and 5th contraction were greater than occurred in the 2nd contraction. Similarly, FVC, increased during each contraction, the increase during the 4th and 5th contractions being greater than during Contraction 2. After aspirin, not only were baseline FBF and FVC decreased as shown on table 6.4, but also FBF and FVC reached significantly lower values after aspirin than before in contractions 2-5 (Figure 6.5). Mean ΔFBF and ΔFVC responses were also significantly reduced after aspirin during the 2nd, 4th, and 5th contractions, but not during the 3rd contraction (Figure 6.9).

In SAs, FBF and FVC also increased during each contraction and values of FBF attained during the 3rd, 4th and 5th contractions were greater than during contraction 2, (figure 6.6) as in WE. Aspirin decreased baseline FBF, but not FVC in SAs as shown in table 6.4, and in contrast to WEs, FBF and FVC reached significantly higher values during contractions 2 – 5 after aspirin than before (Figure 6.6). Similarly, mean ΔFBF and FVC during contraction 5 were greater than in contraction 2 in SAs. Moreover, ΔFVC responses were significantly increased after aspirin during the 2nd, 4th, 5th but not the 3rd contraction in SAs (Figure 6.9).

Comparisons between ethnic groups revealed significant differences between WEs and SAs. After placebo, FBF was significantly greater in WEs than SAs only during the 4th contraction, but FVC was greater in WEs than SAs during all contractions from the 2nd to the 5th (Figure 6.7). After aspirin, FBF was significantly higher in SAs than WEs through all contractions, while FVC was greater in SAs than WEs from the 2nd to 4th contractions, but not the 5th (Figure 6.8).

Forearm cutaneous vascular responses

Forearm Cutaneous RCF (FRCF) in the contralateral arm was unchanged during placebo in WE men. Similarly, there was little obvious change in FRCF after aspirin (table 6.4). However, there was a tendency for FRCF to be lower during isometric contractions after aspirin, this reaching significance during the 2nd and 5th contraction (Figure 6.5). There were no obvious changes in forearm cutaneous vascular conductance (FCVC) during the 2^{nd}-5^{th} contractions, however there was a trend for FCVC to be lower during contraction after aspirin in the 2nd and 5th after aspirin administration [particularly six FCVC values demonstrating statistical significance ($P>0.001$) during the 2nd contraction after aspirin administration and five FCVC values during the 5th contraction after aspirin administration ($P>0.001$), supporting the decrease of the FCVC during both the 2nd and 5th contractions] (Figure 6.5).

By contrast in SAs there was a tendency for baseline FRCF to be higher after aspirin than after placebo (Table 6.4) although this did not achieve statistical significance and a tendency for FRCF to be at higher levels after aspirin than before during each contraction, this reaching significance only during the 5th contraction (Figure 6.6). Similarly, baseline FCVC increased after aspirin in SAs, and FCVC tended to fall

during each contraction the difference between FCVC values before and after aspirin reaching statistical significance during the 3rd, 4th, and 5th contractions (Figure 6.6).

When expressed as change from baseline, ΔFRCF and ΔFCVC were smaller during isometric contractions in WEs after aspirin suggesting attenuated cutaneous vasodilation. By contrast, in SAs it seemed that the smaller ΔFRCF and ΔFCVC seen during isometric contractions after placebo relative to those in WEs were reversed to cutaneous vasoconstriction from higher baseline values, after aspirin (Figure 6.9), although any differences between placebo and aspirin did not reach statistical significance in SAs.

Between group comparisons showed that after placebo, FRCF was higher during all contractions in WEs than SAs, while FCVC was higher in WEs than SAs in contractions 2-4 (Figure 6.7). After aspirin, FRCF and FCVC were higher in SAs than WEs during contraction 2, but not during the rest of the contractions (Figure 6.8). Mean ΔFRCF and ΔFCVC responses were significantly different between WEs and SAs after placebo, with SAs showing smaller responses in both variables. After aspirin, ΔFRCF and ΔFCVC were not significantly different, between SAs and WEs (Figure 6.9).

Forearm EMG activity

EMG activity recordings from the flexor pollicus longus of the forearm remained close to resting levels (< 0.04 mv) during each contraction (30% MVC) after placebo, tending to show saw tooth increases/decreases in the 2nd and 3rd min of each contraction particularly in WEs (Figure 6.10). After aspirin in both WEs and SAs, these small changes in EMG were similar to those seen after placebo with no difference between conditions (Figure 6.10). This suggests minimum muscle activity in the non-dominant arm. There were no apparent contractions in the non-dominant arm of any subject.

Calf Vascular responses.

In Protocol B WE subjects, CBF and CVC baselines were not changed after aspirin relative to placebo administration (Table 6.6). CBF increased during contractions 1-4 and CBF values were significantly lower after aspirin (Figure 6.12), but analysis at different time points indicates this effect mainly reflected differences between the CBF values in the first 1 min and sometimes in the third min. of the 3 min contraction. Any

effects of isometric contraction on CVC were small after placebo, but in contractions 1 and 4, CVC was lower after aspirin than placebo in the first min in both contractions 1 and 4, and in the 3rd min in contraction 4 (Figure 6.12). Mean ΔCBF responses were significantly reduced after aspirin in the 4th, but not during the other contractions (figure 6.13). Similarly, ΔCVC responses were not significantly changed after aspirin during the 1st and 2nd contractions, but they were attenuated during the 3rd and 4th contractions (Figure 6.13).

Comparisons between the forearm and calf demonstrated that ΔCBF and ΔCVC were significantly smaller than ΔFBF and ΔFVC respectively after placebo and after aspirin as presented in figure 6.14.

Calf cutaneous vascular responses

Calf red cell flux (CRCF) showed a trend to increase during each contraction after placebo and after aspirin (Figure 6.12). These CRCF values were significantly different during the 1st and the 3rd contraction, CRCF being lower after aspirin. On the other hand, calf cutaneous vascular conductance (CCVC) showed a trend to increase during the 1st contraction after placebo and did not return to the original baseline value; from this higher baseline, CCVC showed a trend to decrease during contractions 2, 3 and 4. However, after aspirin, baseline CCVC was not raised after the 1st contraction and there were no further changes in CCVC during contractions 2-4. There was a significant difference between conditions for absolute values of CCVC only during the 3rd contraction, CCVC being lower after aspirin (Figure 6.12).

ΔCRCF and ΔCCVC responses are shown in figure 6.13. Neither ΔCRCF nor ΔCCVC showed any significant effect of aspirin vs placebo (figure 6.13).

Comparisons between forearm and calf cutaneous responses, showed smaller responses in ΔCRCF, ΔCCVC, than ΔFRCF and ΔFCVC during all contraction after placebo and after aspirin as shown in figures 6.15.

Calf EMG activity

EMG activity recordings from gastrocnemius muscle showed minimal changes during each contraction of the ipsilateral forearm both after placebo and after aspirin, any activity occurring in the 3rd min (figure 6.10). There were no apparent movements of the ipsilateral leg of any subject.

Anthropometric Characteristics	WE (n=10) (Contralateral arm)	SA (n=10) (Contralateral arm)	P value
Age	22.8±0.9	20.6±0.8	P<0.05
BMI (kg/m^2)	23.2±1.0	23.5±1.1	P=0.71
Waist:hip ratio (cm)	0.9±0.2	0.9±0.2	P=0.29
Physical activity level	03 Low 06 Average 01 High =18	05 Low 05 Average 00 High =15	P=0.58
Parental Hypertension	05/10	06/10	P=0.65

Table 6.1 **Anthropometric Characteristics in young WEs (LHS) and SAs (RHS).** Values are shown as mean ± SEM.

Anthropometric Characteristics	WE (n=10) (Protocol A)	WE (n=10) (Protocol B)	P value
Age	22.8±0.9	21.2±0.9	P=0.17
BMI (kg/m^2)	23.2±1.0	21.9±0.7	P=0.24
Waist:hip ratio (cm)	0.9±0.2	0.9±0.1	P=0.75
Physical activity level	03 Low 06 Average 01 High =18	01 Low 14 Average 07 High= 21	P=0.58
Parental Hypertension	05/10	04/10	P=0.66

Table 6.2 **Anthropometric Characteristics of young WEs recruited for Protocol A (LHS) and B (RHS) for recordings from contralateral arm and ipsilateral leg.** Values are shown as mean ± SEM.

	WE (n=10)	SA (n=10)
Systolic pressure (mmHg)	117.5±3.6	119.8±1.6
Diastolic pressure (mmHg)	69.1±2.7	73.1±3.7
MABP (mmHg)	71.4±2.9	71.1±2.0

Table 6.3 **Cardiovascular baselines in WEs (LHS) and SAs (RHS) for Protocol 1.** Values are shown as mean ± SEM.WEs vs SAs: ǂ: P<0.05.

	WE (n=10)	SA (n=10)
FBF ml.min^{-1}.100ml^{-1} before Aspirin	12.0±1.9	6.7± 0.9ǂ
FBF (ml.min^{-1}.100ml^{-1}) after Aspirin	3.8±0.7ǂǂ	4.4 ± 0.7ǂǂ
FVC Conductance units (CU) before Aspirin	0.16±0.03	0.09±0.01ǂ
FVC Conductance units (CU) after Aspirin	0.05±0.01ǂǂ	0.06±0.01
FRCF (PU) before Aspirin	28.2±5.3	20.9±2.8
FRCF (PU) after Aspirin	28.0±6.8	38.2±8.9
FCVC (CU) before Aspirin	0.37±0.27	0.27±0.19
FCVC (CU) after Aspirin	0.37±0.32	0.62±0.38

Table 6.4 **Cardiovascular baselines in WEs (LHS) and SAs (RHS) for Protocol 1.** Values are shown as mean ± SEM. Aspirin effect: ǂǂ:P<0.001 WEs vs SAs: ǂ: P<0.05.

	WE (n=10)	WE (n=10)
	Protocol A	Protocol B
Systolic pressure (mmHg)	117.5±3.6	115.2±2.1
Diastolic pressure (mmHg)	69.1±2.7	66.8±2.2
MABP (mmHg)	71.4±2.9	70.6±1.5
FBF or CBF (ml.min^{-1}.100ml^{-1})	12.0±1.9	6.0±1.2ǂ
FVC or CVC (CU)	0.16±0.03	0.07±0.01ǂ
FRCF or CRCF (PU)	28.2±5.3	27.7±4.9
FCVC or CCVC (CU)	0.37±0.27	0.33±0.03

Table 6.5 **Cardiovascular baselines in the contralateral arm (CA) (LHS) and the ipsilateral leg (IL) (RHS) for Protocol 2.** Values are shown as mean ± SEM. CA vs IL: ǂ: P<0.05.

	WE (n=10)	WE (n=10)
	Protocol A	**Protocol B**
FBF or CBF (ml.min^{-1}.100ml^{-1}) CBF before Aspirin	12.0±1.9	6.0±1.2ǂ
FBF or CBF (ml.min^{-1}.100ml^{-1}) after Aspirin	3.8±0.7ǂǂ	6.9±1.1
FVC or CVC before Aspirin	0.16±0.03	0.07±0.01ǂ
FVC or CVC (CU) after Aspirin	0.05±0.01ǂǂ	0.09±0.01
FRCF or CRCF (PU) before Aspirin	28.2±5.3	27.7±4.9
FRCF or CRCF (PU) after Aspirin	28.0±6.8	20.2±2.8
FCVC or CCVC (CU) before Aspirin	0.37±0.27	0.33±0.03
FCVC or CCVC (CU) after Aspirin	0.37±0.32	0.24±0.01ǂ

Table 6.6 **Effect of aspirin on cardiovascular baselines in Protocols A and B.** Values are shown as mean ± SEM. Protocol A: Contralateral arm (CA), Protocol B: Ipsilateral leg (IL), Vasculature responses: FBF, CBF, FVC, CVC, Cutaneous responses: FRCF, CRCF, FCVC, CCVC, Before vs after Aspirin: ǂǂ:P<0.001; contralateral arm vs ipsilateral leg: ǂ: P<0.05.

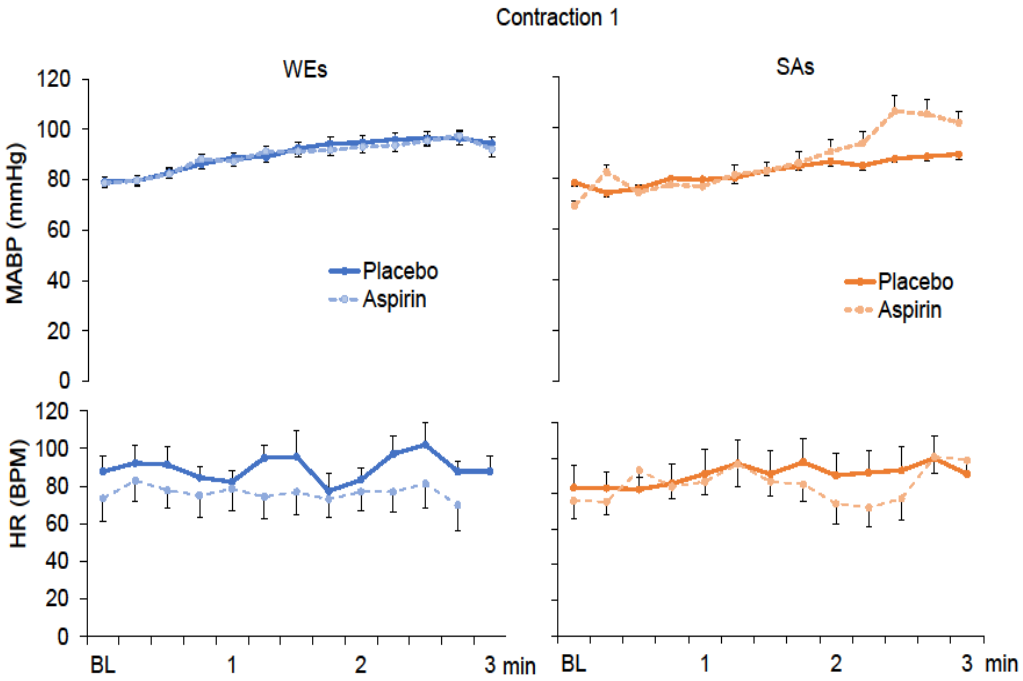


Figure 6.4 **Responses evoked in mean ABP (MABP) and HR by isometric contraction of dominant arm in WEs and SAs before and after aspirin.** Data are shown as mean and SEM.

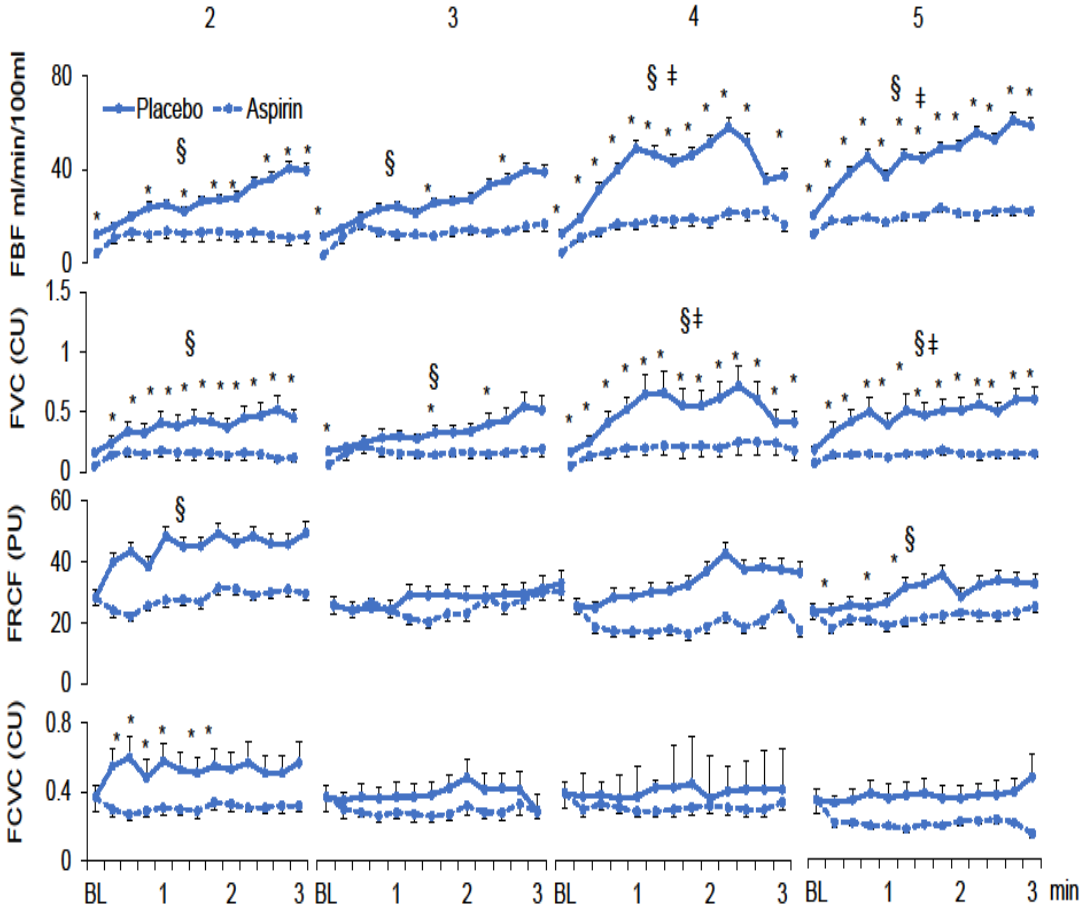


Figure 6.5 **Responses evoked in forearm vasculature by 4 successive isometric contractions in WEs**. Abbreviations for variables as shown in text. Data are shown after placebo and after COX inhibition with aspirin. §: placebo vs aspirin; *: placebo vs aspirin at time point; ǂ: vs 2nd contraction; $P<0.05$ in each case.

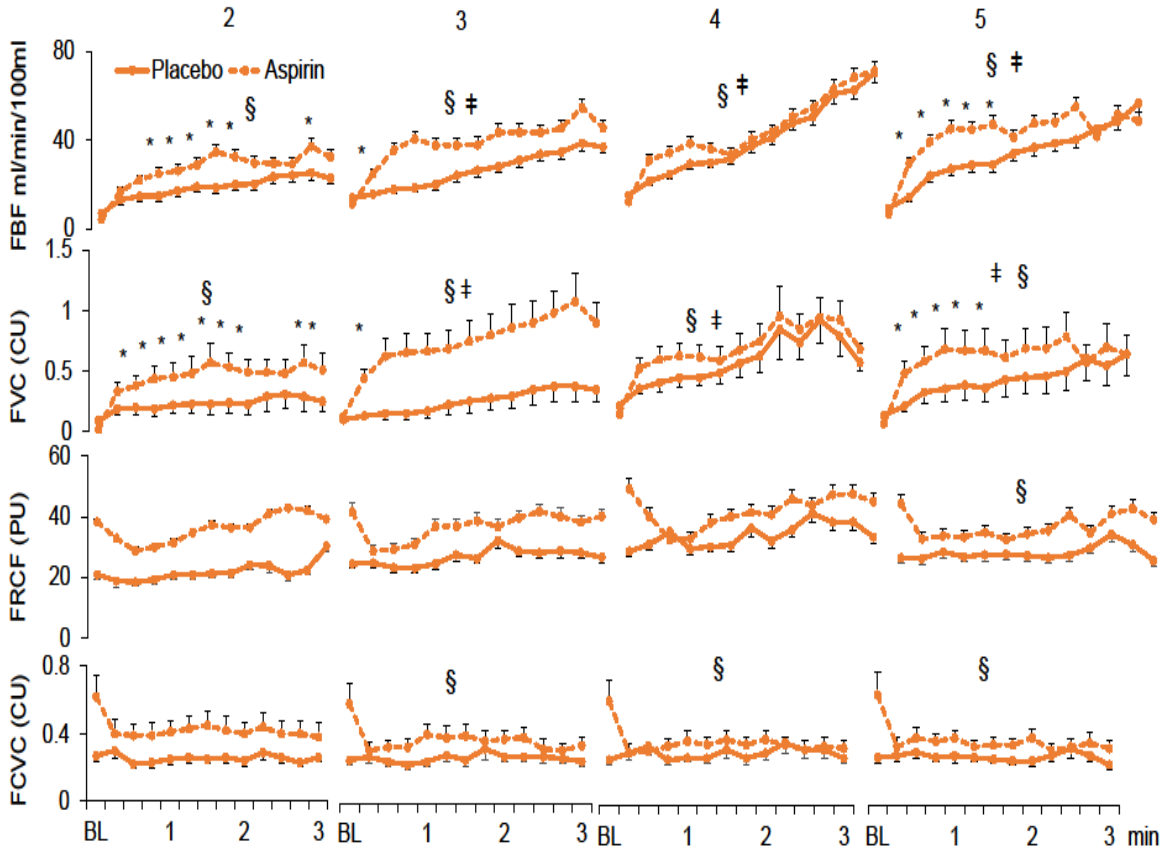


Figure 6.6 **Responses evoked in forearm vasculature by 4 successive isometric contractions in SAs. Abbreviations for variables are shown in text.** Data are shown after placebo and after COX inhibition with aspirin. §: placebo vs aspirin; *: placebo vs aspirin at time point; ǂ: vs 2nd contraction, P<0.05 in each case.

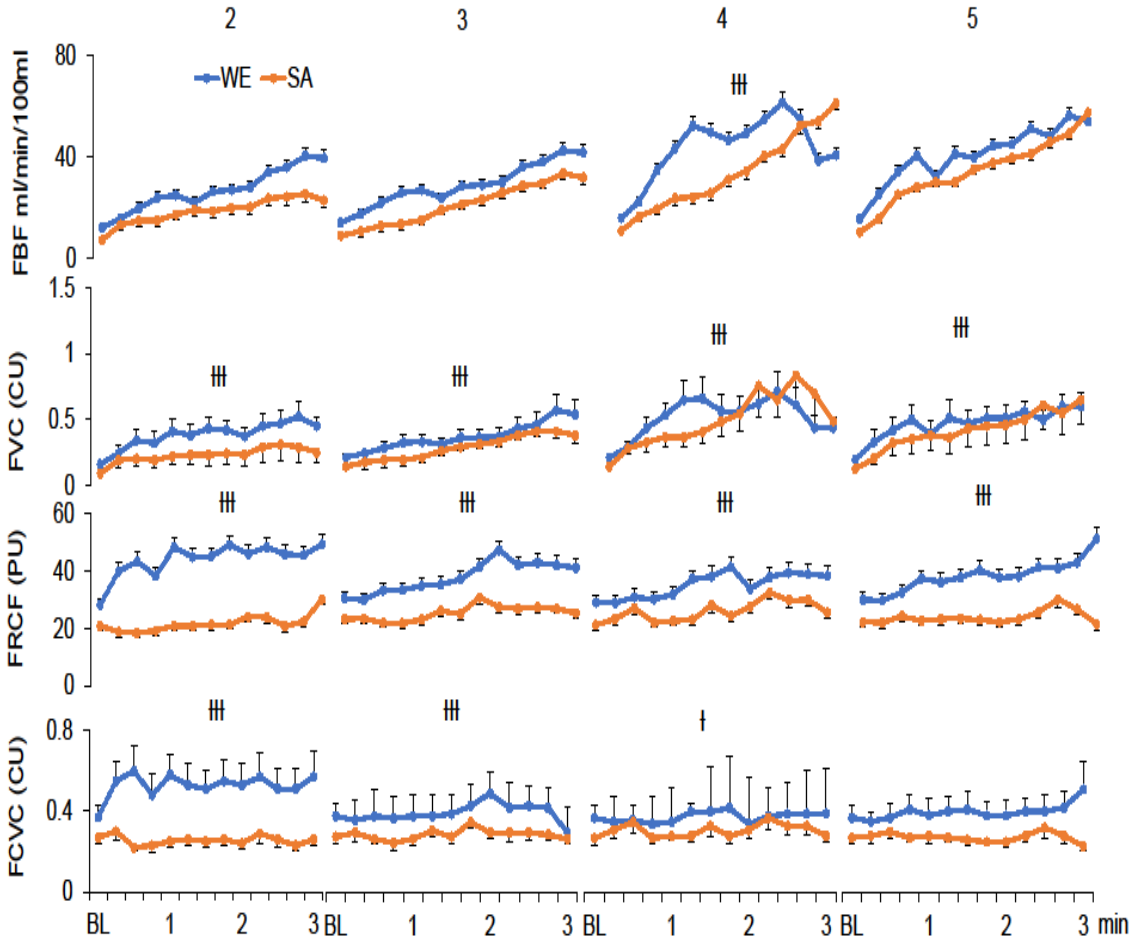


Figure 6.7 **Comparisons between WEs and SAs of responses evoked in forearm vasculature by 4 successive isometric contractions after placebo**. Data are shown as mean and SEM. Ŧ: $p<0.05$, ŦŦ: $p<0.001$ change between WEs and SAs.

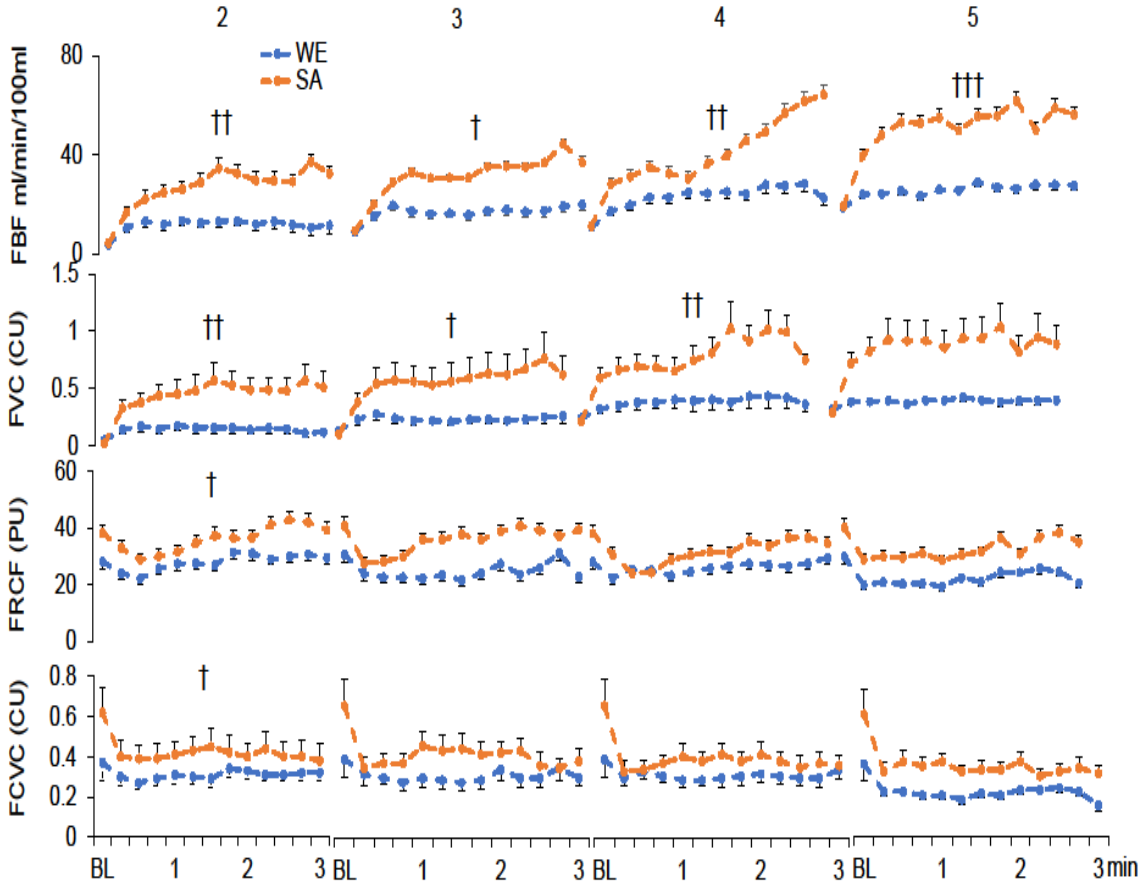


Figure 6.8 **Comparisons between WEs and SAs for responses evoked in forearm vasculature by 4 successive isometric contractions after aspirin**. Data are shown as mean and SEM. †: p<0.05 ††: p<0.01, †††: p<0.001 change after aspirin.

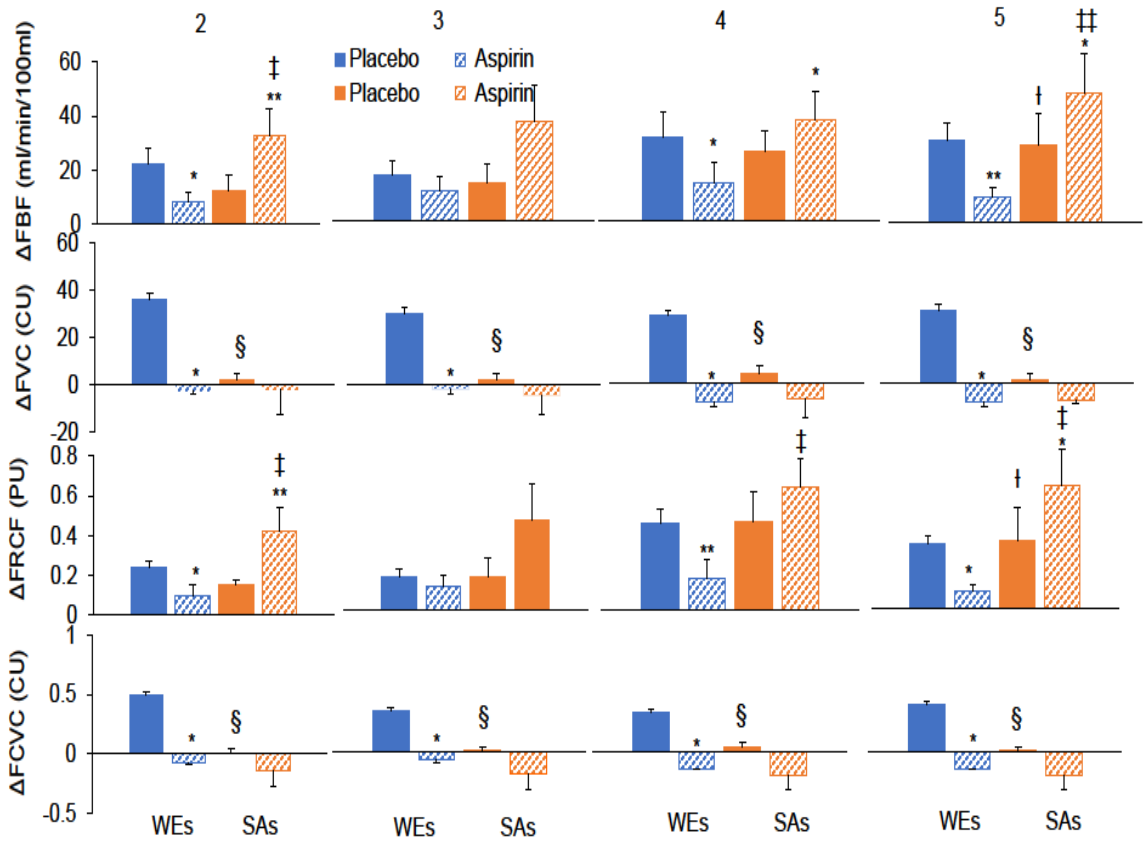


Figure 6.9 **Forearm vascular responses evoked in WEs placebo (blue bars), aspirin (blue stripped bars) and SAs placebo (orange bars), aspirin (orange stripped bars) before and after aspirin by successive forearm contractions expressed as Δ (change) from baseline**. Vascular responses: ΔFBF: forearm blood flow, ΔFVC: forearm vascular conductance, Cutaneous responses: ΔRCF: red cell flux, ΔFCVC: forearm cutaneous vascular conductance, PU: perfusion units, CU: conductance units. Data are shown as mean and SEM. *: p<0.05, **:p<0.01 aspirin vs placebo; §: p<0.001, WEs vs SAs, ‡:p<0.05 ,‡‡:p<0.001 WEs Vs SAs after aspirin, Ɨ: p<0.05 2nd vs 5th contraction changes.

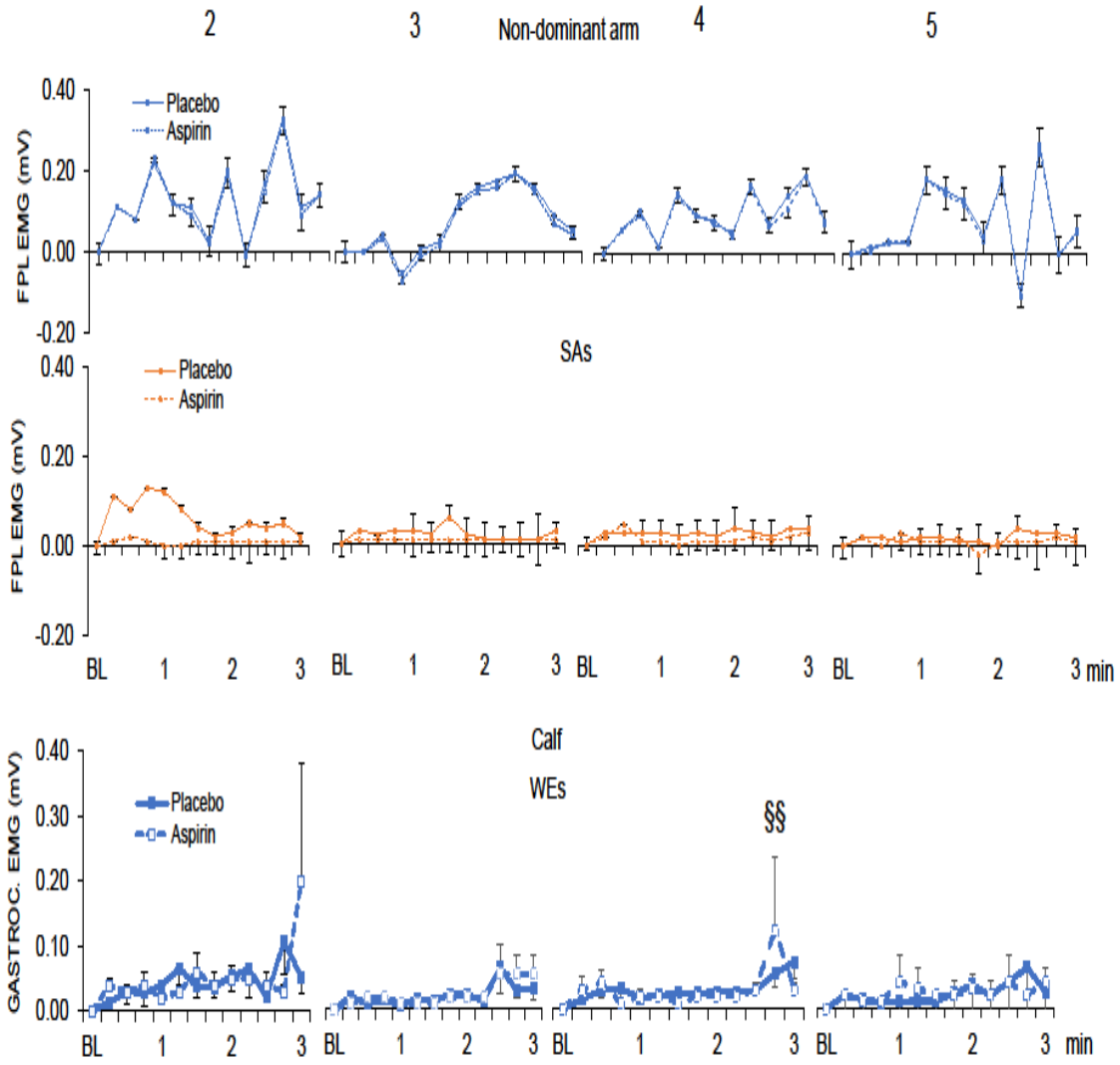


Figure 6.10 **EMG activity recorded in contralateral forearm of WEs and SAs (top and middle respectively) and ipsilateral calf of WEs (bottom) by successive forearm contractions**. EMG activity recorded from Flexor Pollicus Longus (FPL) and gastrocnemius muscle. Data are shown after placebo and after COX inhibition with aspirin.

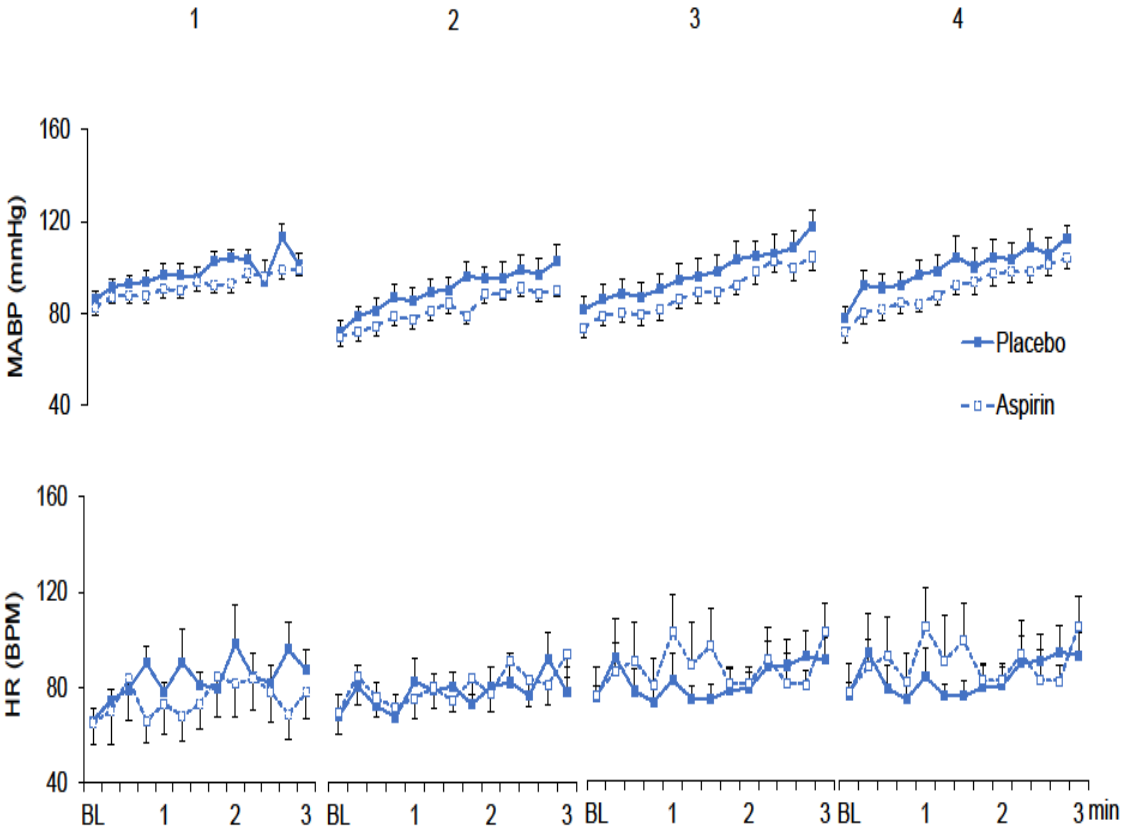


Figure 6.11 **Responses evoked in mean ABP (mABP) and heart rate (HR) by successive forearm contractions in Protocol B in which recordings were made from the ipsilateral leg**. Data are shown after placebo and after COX inhibition with aspirin as mean and SEM.

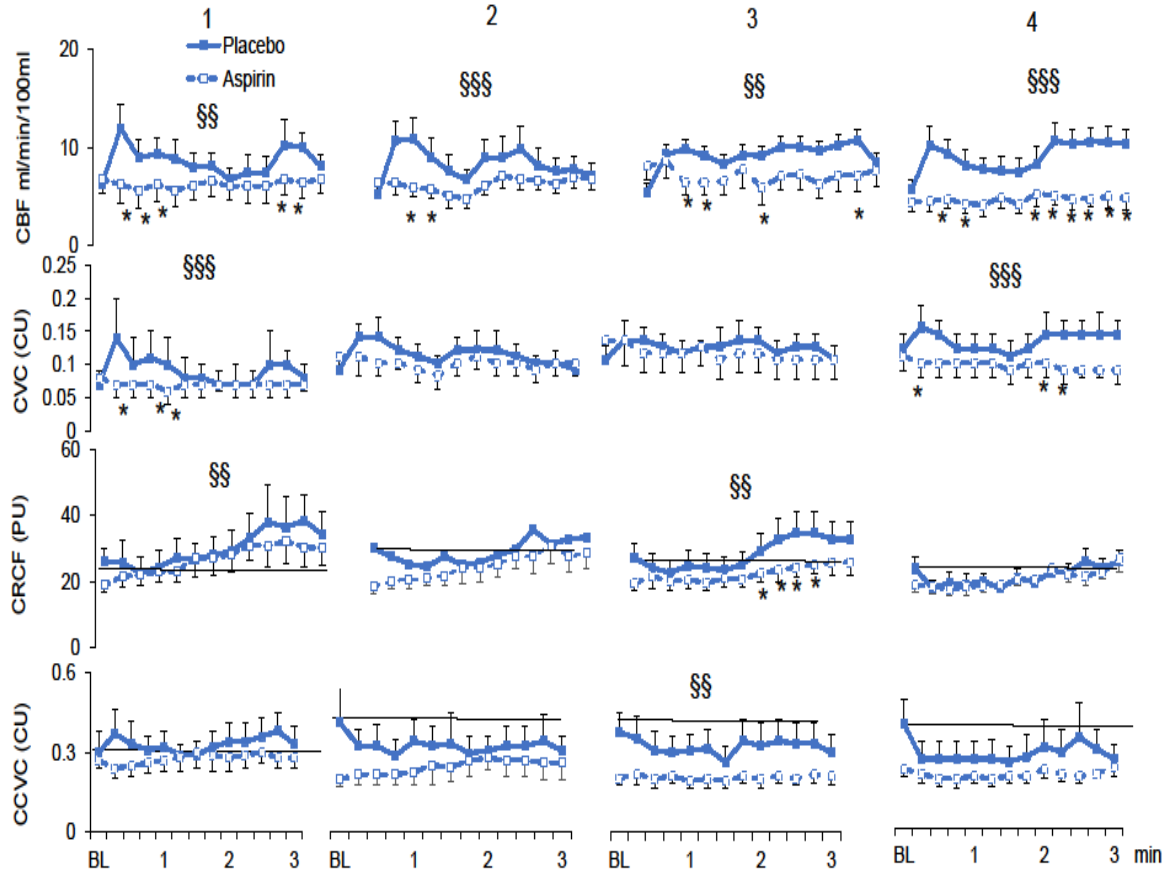


Figure 6.12 **Responses evoked in calf vasculature by successive contractions of ipsilateral forearm**. Data are shown after placebo and after COX inhibition with aspirin. §: p<0.05, §§: p<0.01, §§§: p<0.001 placebo vs COX inhibition; *: p<0.05 at time point shown.

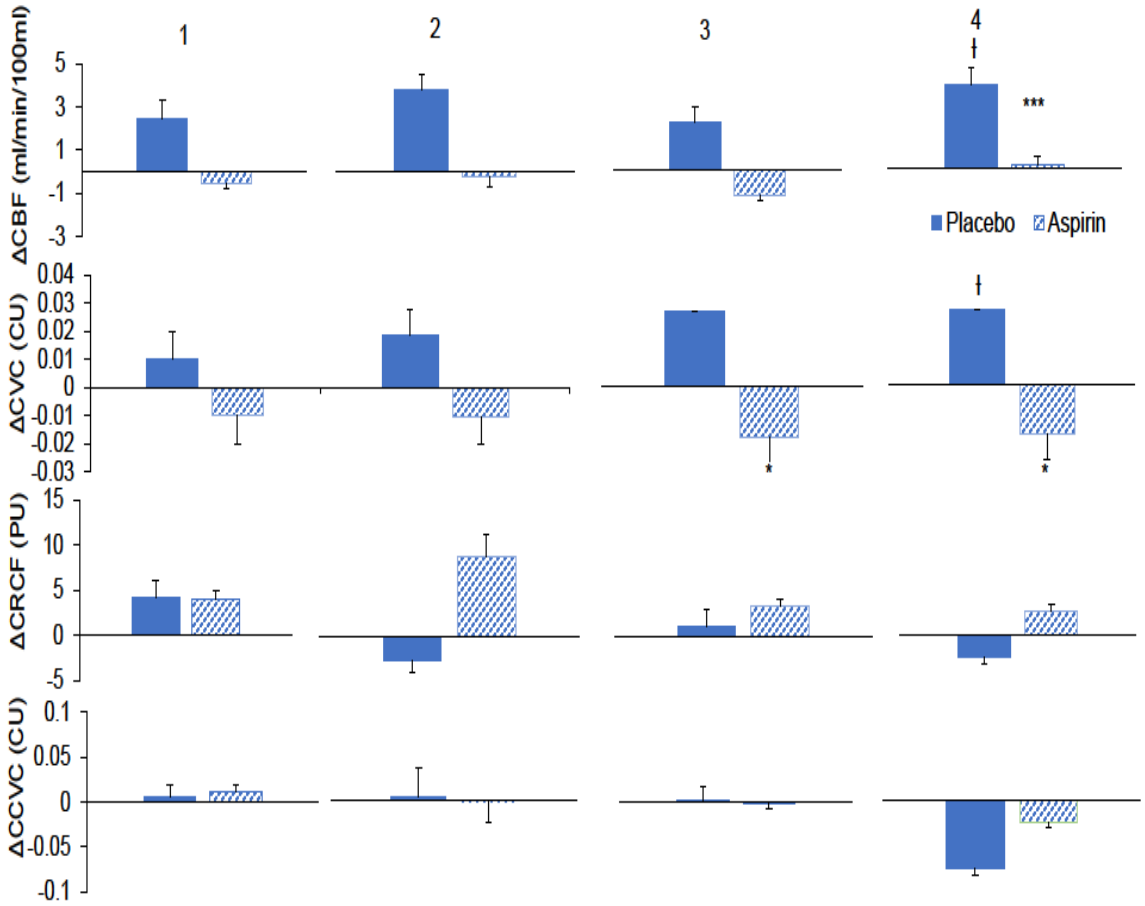


Figure 6.13 **Calf vascular responses evoked in WEs before (blue bars) and after aspirin (stripped blue bars) by successive forearm contractions expressed as Δ (change) from baseline**. Vascular responses: ΔCBF: calf blood flow, ΔCVC: calf vascular conductance, cutaneous responses: ΔRCF: red cell flux, ΔCCVC: cutaneous calf vascular conductance, PU: perfusion units, CU: conductance units. Data are shown as mean and SEM. *: $p<0.05$, ***:$p<0.001$ effect of aspirin. Ł, Ħ: $p<0.05$, $p<0.001$ 2nd vs 5th contraction.

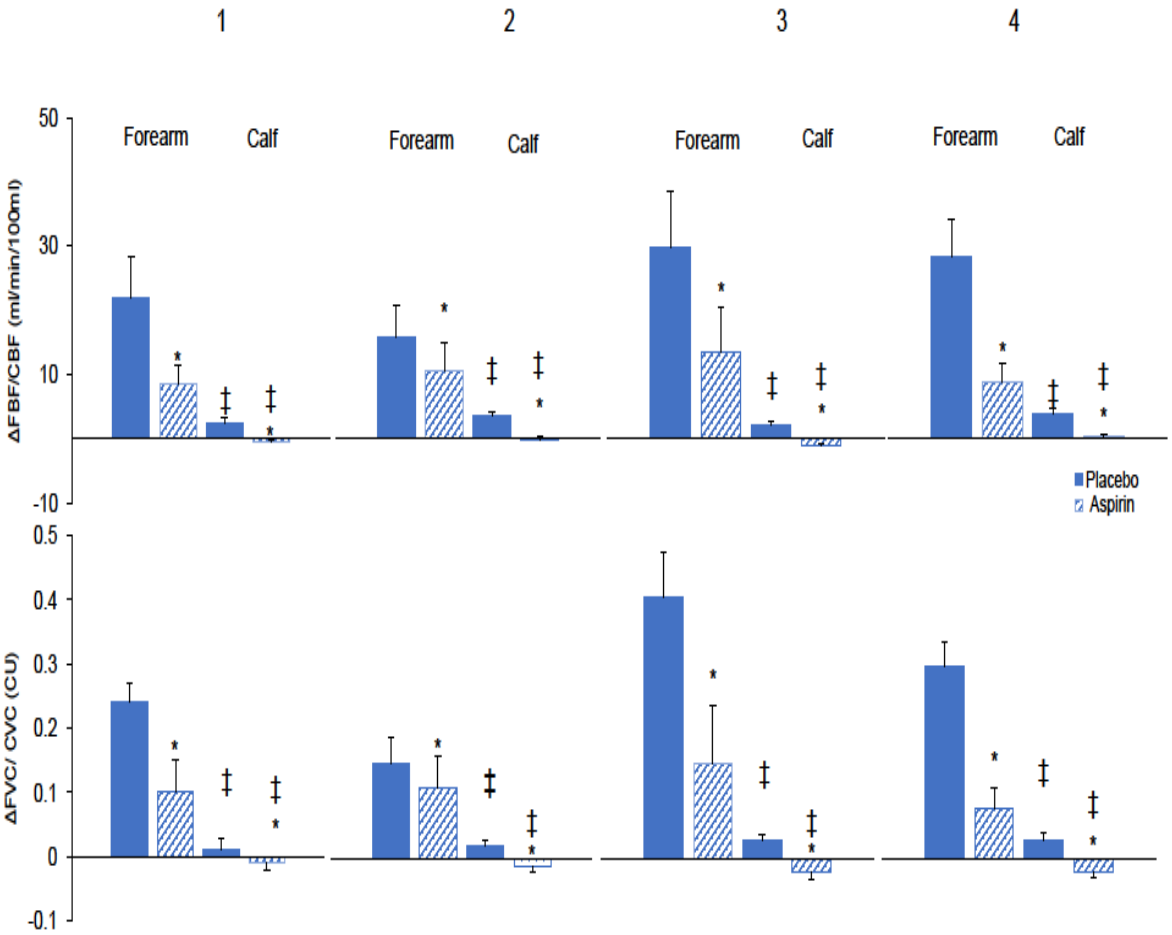


Figure 6.14 **Comparisons between contralateral Forearm and ipsilateral Calf for ΔFBF/CBF (upper panel) and ΔFVC/CVC (lower panel) evoked by successive isometric contractions of forearm**. Data are shown as mean and SEM. *: p<0.05, effect of aspirin ‡:p<0.05 forearm vs calf before or after aspirin.

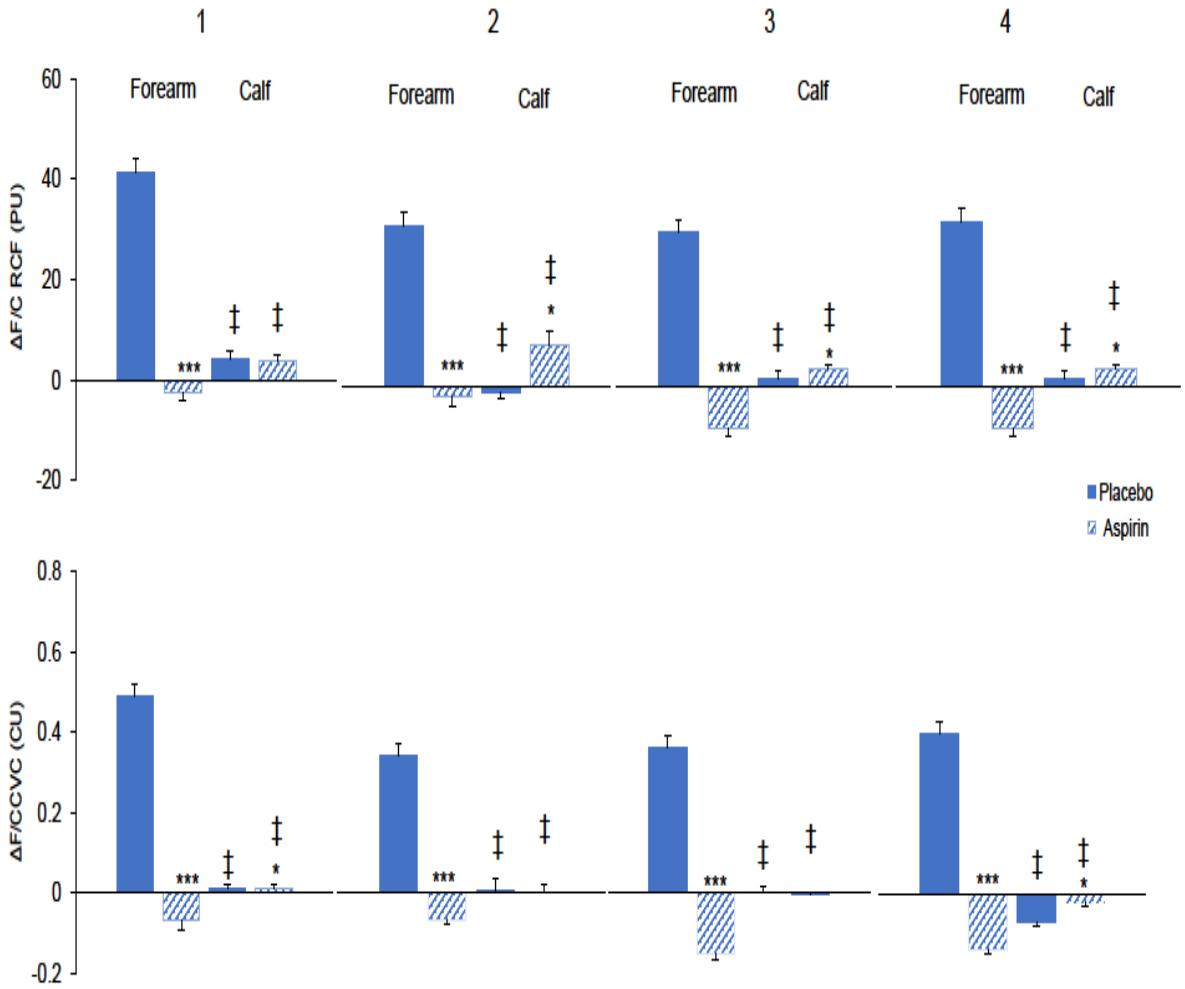


Figure 6.15 **Comparisons between contralateral Forearm and ipsilateral Calf ΔRCF (upper panel) and ΔCVC (lower panel) evoked by successive isometric contractions of forearm**. Data are shown as mean and SEM. *: p<0.05, effect of aspirin ‡: p<0.0001 forearm vs calf before or after aspirin.

6.5 DISCUSSION

The main objectives for the study described in this Chapter were to investigate the vascular responses that occur in the contralateral arm during a series of isometric handgrip contractions at 30% MVC for 3 minutes comparable to those of the IHG training protocol used for studies described in Chapter 3 and 4 and to test the potential involvement of COX products in these responses. The present study demonstrated that FBF and FVC in the contralateral arm were progressively increased during each of the 4 successive isometric forearm contractions in both young WE and SA men. These substantial increases in FBF and FVC occurred with little EMG activity in contralateral forearm muscle. Increases in forearm RCF (FRCF) and skin vascular conductance (FCVC) also occurred during the isometric contractions, particularly in WEs. Further, the COX inhibitor aspirin attenuated the increases in FBF and FVC in WEs, but augmented them in SAs, whereas increases in cutaneous perfusion were attenuated or reversed in WEs *and* SAs.

Another main objective was to investigate vascular responses in the ipsilateral leg during successive isometric handgrip contractions. In WEs, in whom this was tested, CBF increased during each contraction as did CVC, with little apparent EMG activity in calf muscles; these vascular responses were smaller than those evoked in the forearm. CBF and CVC reached lower values during contractions after aspirin than after placebo, this effect being most pronounced in the 1st min and again, in the 3rd min of contractions 3 and 4.

Overall, these results are consistent with the hypothesis of increases in blood flow and/or increases in shear stress occurring in the contralateral arm and the ipsilateral leg during each bout of forearm contraction that could lead to improved endothelial dilator function during IHG training, and they are consistent with COX products contributing to these effects. To summarize the findings of this study, a list is presented below:

Contralateral arm findings:

- FBF and FVC in the contralateral arm were progressively increased during each of the 4 successive isometric forearm contractions in both young WE and SA men.
- These substantial increases in FBF and FVC occurred with little EMG activity in contralateral forearm muscle.

- Increases in forearm RCF (FRCF) and skin vascular conductance (FCVC) also occurred during the isometric contractions, particularly in WEs.
- Further, the COX inhibitor aspirin attenuated the increases in FBF and FVC in WEs, but augmented them in SAs,
- whereas increases in cutaneous perfusion were attenuated or reversed in WEs and SAs.

Ipsilateral leg findings:

- In WEs, in whom this was tested, CBF increased during each contraction as did CVC, with little apparent EMG activity in calf muscles.
- These vascular responses were smaller than those evoked in the forearm.
- CBF and CVC reached lower values during contractions after aspirin than after placebo.
- This effect being most pronounced in the 1st min and again, in the 3rd min of contractions 3 and 4.

However, before accepting these hypotheses, the findings must be considered in more detail.

Subject characteristics

Baseline characteristics were generally similar between WEs and SAs of Protocol A, apart from age, SAs being slightly younger, but baseline FBF and FVC, were also lower in SAs than WEs. This contrasts with the findings of Chapter 3 for baseline FBF and FVC in the non-dominant arm were not different between the young WE and SA groups nor was their mean age. It may be noted that in the WE and SA groups of Chapter 3, the number of subjects with hypertensive parents were 4/10 and 2/10 respectively, whereas in the present study it was 5/10 and 6/10 respectively. Young men with hypertensive parents have higher risk of developing CVD than those with normotensive parents whether they are WE or SA (Wang et al., 2008, Ranasinghe et al., 2015) and they already show evidence of endothelial dysfunction in forearm (Taddei et al., 1996). Further, in groups of young WE and SA men matched for the proportions with hypertensive parents, SAs showed blunted endothelium-dependent dilatation relative to WEs, but in both ethnicities, those with hypertensive parents showed blunted endothelium-dependent dilatation relative to those with normotensive parents (Hirst

and Marshall, 2018). Thus, it may be that the lower baseline FBF and FVC in the SAs of the present study reflects depressed *tonic* endothelium-dependent dilatation in young SA men, particularly in those with hypertensive parents. This interpretation is consistent with the evidence that healthy young SA men had lower flow-mediated dilatation (FMD) than matched WEs, while NO synthase inhibition decreased baseline FVC less in SAs than WEs, consistent with depressed NO bioavailability (Murphy et al., 2007)(see below for further discussion).

The anthropometric characteristics and cardiovascular baselines of the WE group who took part in the ipsilateral leg experiments (Protocol B) were comparable to those who performed the experiments of the contralateral arm. However, the baseline CBF and CVC were lower when expressed per 100 g tissue than FBF and FVC even though steps were taken to keep the calf and forearm at heart level. In contrast, in previous studies, calf and forearm blood flow measured with VOP in subjects with both limbs at heart level were very similar (Stewart and Montgomery, 2004, Essandoh et al., 1987). In retrospect, it seems likely the present discrepancy is explained by the fact that although the site of measurement of CBF was at heart level, the calf was inclined so the ankle was below level: the leg shows pronounced myogenic vasoconstriction when in the dependent position (Imadojemu et al., 2001).

Hemodynamic responses in the contralateral arm during IHG contractions

As mentioned in the Methods section of this Chapter, ABP recordings were taken during the 1st contraction only in Protocol A, while VOP, cutaneous and EMG recordings were made in during 4 contractions (2-5) to replicate as far as possible the IHG training protocol used in Chapter 3 and 4. It was impossible to make ABP recordings and vascular recordings simultaneously because the other arm needed to be used for contraction, and yet, ABP values were required in order to be able to calculate the hemodynamic indices FVC and FCVC. It was hypothesized based on previous evidence of Cotzias and Marshall (1993) that ABP would show similar changes throughout all contractions. In addition, in the calf protocol (Protocol B), it was possible to make ABP recordings during all contractions and these showed similar ABP responses throughout. Thus, it can be concluded it was justifiable to use ABP recordings made during the 1st contraction of Protocol A to calculate FVC and FCVC during all other contractions.

Increases in FBF occurred during handgrip of the contralateral arm at 30% MVC in Protocol A in both ethnic groups during each contraction, reaching their maxima in the 3rd min. Although previous studies have made no mention of the ethnicity of the subjects, they also reported a marked increase in FBF in the contralateral arm during the first 30 s of 2-min isometric contraction at 30% MVC, that either reached a plateau at the end of the 1st min (Eklund, 1974), or increased gradually over the 2 min when they gave no instructions to subjects to avoid contracting this arm (Cotzias and Marshall, 1993, Sanders et al., 1989). Since FVC also increased gradually and progressively during contractions 2-5 of the contralateral arm in both WEs and in SAs, the increases in FBF can be partially attributed to vasodilation in the forearm and not just to the increases in ABP, as reported previously (Cotzias and Marshall, 1993, Eklund, 1974, Sanders et al., 1989). In WEs, FVC was higher than in SAs during each contraction when expressed in absolute values, but, when expressed as change from baseline there was no significant difference between the ethnicities reflecting the higher baseline FVC in WEs. Thus, the magnitude of the vasodilation in the contralateral forearm was not different between ethnicities for any contraction.

Interestingly, in WEs, the increases in contralateral FBF were augmented during the 4th and 5th contractions relative to the 2nd, while in SAs, the increases in FBF in the 3rd, 4th and 5th were all augmented relative to the 2nd contraction, FBF reaching significantly higher values in WEs than SAs during contraction 4. Indeed, when the responses were expressed as a compacted mean during each contraction, this revealed that the increases in both FBF and FVC were augmented in the 5th contraction compared with the second contraction in SAs, whereas in WEs, changes in FBF and FVC were similar across all contractions. This suggests that if increased FBF, or increased shear stress occurring during the period of isometric contraction in IHG training does act as a stimulus for augmented endothelium-dependent dilatation in the contralateral arm as proposed, then this stimulus is consistent across all contractions in WEs but augments with repetition, in SAs.

Hemodynamic responses in the ipsilateral leg during IHG contractions

During the 3 min forearm contractions in Protocol B, increases in ipsilateral CBF and modest increases in CVC in the WEs were seen during each contraction. These observations agree with those of Eklund et al (1974) who also indicated CBF was

increased during a 2 min period of handgrip at 30% MVC, while calf vascular resistance tended to fall indicating modest vasodilation. In some contrast, Fisher and White (2003) reported that CVC initially increased during plantar flexion of the contralateral dominant leg followed by a gradual decrease below baseline levels indicating initial calf vasodilation followed by progressive vasoconstriction. There is no obvious reason for this apparent disparity: it may simply reflect differences in the protocols: forearm vs calf contractions.

Importantly, in relation to our hypothesis, the compacted mean values of the present study showed that on repetition of the IHGs, the mean changes in CBF and CVC were significantly *augmented* in the 4th contraction compared to the 1st contraction. Thus, the present results provide novel evidence that in WEs, repeated isometric contraction of the forearm during IHG training induces progressive increases in calf blood flow with successive contractions that could potentially provide a stimulus for increased shear stress-induced endothelial dilator function in the calf. These increases in CBF at least partly reflected gradually augmenting vasodilation (CVC) in the calf. Unfortunately, we do not know whether SAs show similar changes in the calf: this should be tested in future studies.

Cutaneous responses in contralateral arm and ipsilateral leg during IHG contractions

Although absolute forearm RCF (FRCF) increased gradually during each contraction in both WEs and SAs, the levels of FRCF were very attenuated in SAs compared to WEs. This was demonstrated similarly for absolute FCVC in both WEs and SAs. The compacted mean values showing change in FRCF and FCVC from baseline demonstrated these effects more clearly. Therefore, repeated isometric forearm contractions for 3 min at 30% MVC evokes more substantial increases in cutaneous perfusion and cutaneous vasodilation in WEs, than SAs. These findings contrast with those of Cotzias and Marshall (1992) who reported that a 2 min period of isometric hand grip at 25% or 50% MVC evoked a significant decrease in RCF and a rise in cutaneous vascular resistance in the contralateral forearm, suggesting reflex vasoconstriction. There is no obvious reason why these results differ. In the present study, it is reasonable to assume that increases in cutaneous blood flow and FCVC contributed to the increases in FBF and FVC recorded in the whole forearm in both WEs and SAs. However, the magnitude of the increases in FBF and FVC in both

ethnicities suggests these changes largely reflect increases in blood flow and vasodilation in the *muscles* of the forearm.

In the *calf* cutaneous circulation of WEs, any increases in CRCF were modest and developed gradually during each period of contraction after an initial decrease in CRCF, such that the compacted mean change in CRCF values showed a net increase in contraction 1 only and net *decreases* from baseline in contractions 2 and 4. Thus, the present results suggest that even if increased cutaneous flow or shear stress might provide a stimulus for increasing endothelium-dependent dilator function in cutaneous circulation of the calf during isometric forearm contractions, this stimulus would be lost in successive contractions of an IHG training session. The CCVC attenuating responses in contractions 2nd-4th, seems as a baseline alteration in CCVC. This is gradually augmenting vasoconstriction, which was responsible for the modest increase in cutaneous perfusion. Indeed, as the changes in calf cutaneous perfusion and CCVC were small and sometimes in the opposite direction (as shown on contraction 4) from the increases in whole calf CBF and CVC, it is reasonable to conclude that the responses in the whole calf were predominantly due to responses in the *muscles* of the calf.

Mechanisms underlying vascular responses

EMG activity in the contralateral arm and calf

Regarding electrical activity of the muscles (EMG) during isometric forearm contractions in Protocol A, the subjects, showed minor levels of EMG activity in the contralateral forearm during all contractions in both WEs and in SAs. The EMG activity occurred in both the placebo and aspirin protocols and tended to increase gradually during each contraction, particularly in the WEs. This is consistent with previous evidence that isometric contraction of finger muscles of one hand at 20-40% MVC evoke inadvertent contraction and EMG responses at <5% MVC in the muscles of the contralateral hand which gradually increased during a maintained contraction, often in a saw-tooth manner (Zijdewind and Kernell, 2001, Shinohara et al., 2003) as seen in the present study. However, while the *vascular* responses in the contralateral forearm increased with repetition over the 4 contractions in both WEs and SAs, as discussed above, this was not the case for the EMG activity. Further, as discussed below, the vascular responses were affected in opposite directions by aspirin in WEs and SAs, whereas the EMG activity was not altered at all. Thus, the present results provide

further evidence for conclusions drawn in previous studies: that the EMG activity and vascular responses in the "resting arm" are not interconnected during isometric contraction of the contralateral arm. In other words, the vasodilation in the "resting" arm is not simply exercise hyperaemia. As far as the present results are concerned, the increases in FBF and FVC were far larger than would be expected, in that FBF in the non-dominant arm increased to as much as ~50 and ~53 ml/min/100 ml tissue in WEs and SAs respectively concomitant with contractions in that arm which were too small to be visibly detected, whereas in the experiments of Chapter 3, contraction of the non -dominant forearm contractions as great as 60% MVC increased FBF in that same arm to 75 and 86 ml/min/100 ml tissue in WEs and SAs, respectively. Further, in previous studies, it was shown that the vasodilator response peaked during the first minute of isometric contraction of the contralateral arm, even though little or no EMG activity was detected in this minute (Lind et al., 1964, Sanders et al., 1989), while the time course of EMG and FBF responses did not correlate with each other Eklund et al. (1974).

The very fact that EMG activity occurred in the "resting" forearm during repeat contractions of the contralateral arm at 30%MVC is consistent with the cross-transfer effect proposed in Chapter 3 to explain the increase in muscle performance and reduction in fatigue that occurred in the non-trained arm during 4-5 weeks of IHG training. Thus, the small EMG activity in the "resting" arm and increase in motor neuron activity it must reflect, would be expected if there were 'spill-over' of neural drive from the intentionally contracting arm to the neural control system of the contralateral arm (Carroll et al., 2006).

Similar arguments can be made for the ipsilateral calf of WEs in Protocol B. For, the calf also showed only minor EMG activity, which tended to increase during each forearm contraction but was not accompanied by any visible muscle contraction, even though CBF and CVC increased in the 1st min and again in the 3rd min of each contraction, and the mean changes in CBF and CVC augmented over the 4 contractions. Thus, it seems reasonable to conclude for the calf as for the forearm, that the increases in CBF and CVC were *not* exercise hyperaemia secondary to inadvertent muscle contraction. It was reported previously that isometric forearm contraction for 2 min produced an increase in calf blood flow within the first minute, with no obvious muscle contraction (Eklund et al, 1974). It seems the present study is the first to show that CBF

and CVC increase again when forearm contraction is prolonged for 3 min, and that these changes augment with repeated contractions.

Effects of COX inhibition

Forearm haemodynamic responses to COX inhibition

Firstly, the fact that FBF and FVC baselines were reduced after aspirin in WEs, but not in SAs, suggests that SAs lack a tonic vasodilator influence of PGs in forearm muscle which WEs have. This is consistent with previous evidence that the tonic dilator influence of NO was greater in young SA, than WE men (Murphy et al., 2007) and provides additional evidence of endothelial dysfunction in young SA men.

Secondly, aspirin, reduced the FBF and FVC responses seen in the contralateral forearm during all four IHG contractions in WEs, but augmented them in SAs. Further, aspirin reduced the CBF and CVC responses seen in the ipsilateral calf in the WEs. The simplest explanation for these results is that in WEs these vasodilator responses are mediated partially by vasodilator PGs while in SAs, vasoconstrictor COX products normally limit the vasodilation that occurs in the forearm during the isometric contraction of the contralateral forearm under placebo conditions. Thus, in SAs, COX inhibition allows the vasodilator influence of another vasodilator/s to become manifest. This interpretation raises the question of *how* isometric contraction of the forearm might act as a stimulus for increased synthesis of COX products in the contralateral arm and ipsilateral leg.

It has also been shown by using Doppler ultrasound, that leg exercise acts as a stimulus to cause endothelial-dependent dilatation in the forearm by increasing anterograde blood flow and increasing shear stress in the brachial artery, and this dilatation was NO-dependent (Tinken et al., 2009, Green et al., 2002). Further, it was shown in single muscle arterioles that increases in pressure and flow alone and in combination caused arteriolar dilatation, which was shown to be endothelium- and NO-dependent and to involve stretch-activated calcium channels (Koller and Bagi, 2002, Tagawa et al., 1994). There is also evidence that endothelial cells release PGs in response to increased blood flow and shear stress (Bhagyalakshmi and Frangos, 1989, Berthiaume and Frangos, 1992). Thus, it seems reasonable to propose that in WEs, increased shear stress caused by isometric contraction of one arm and the increase in ABP and HR this evokes, act as a stimulus for release of vasodilator PGs and vasodilation in the

contralateral forearm and ipsilateral calf as we hypothesized. The evidence that vasodilation in the "resting" limb during contraction of another limb is mediated by ACh which is released by endothelial cells and acts on endothelial ACh receptors to cause NO-mediated dilatation (Wilson et al., 2016, Dietz et al., 1997) see Chapter 1 - General Introduction, sections 1.2.1 Nitric Oxide, 1.13.1 Responses reported in resting limbs), is also consistent with this proposal, given that ACh releases PGs as well as NO from endothelial cells (Hellsten et al., 2012).

On the other hand, given there is evidence of endothelial dysfunction in SAs, it may be that a similar shear stress stimulus triggered the COX pathway to release vasoconstrictor COX products such as TXA_2 or PGH_2, or that PGI_2 acts on the TP receptors that mediate the constrictor responses to TXA_2 or PGH_2 (Vanhoutte et al., 2009) as discussed in Chapter 3. Whether isometric contraction of one forearm for 3 min *does* increase anterograde flow and shear stress in the contralateral forearm and ipsilateral calf, needs to be tested in future studies by recording blood velocity and diameter in brachial and femoral artery by using Doppler ultrasound.

If these interpretations are correct , then the present finding that COX inhibition in WEs greatly reduced the vasodilator responses in the forearm and virtually abolished them in the calf leaves no obvious role for NO, which as indicated above, has been shown to be a major contributor to shear stress-induced vasodilation (Tinken et al., 2009, Green et al., 2002). It could be that removal of one of the dilator influences of increased shear stress allowed the effects of exercise-induced increases in MSNA to forearm and calf muscle (Murphy et al., 2011) to exert greater vasoconstrictor influences, so counteracting the effect of NO. It could also be that COX inhibition unbalanced the known interactions between the COX and NOS pathways, such that inhibition of one attenuated or prevented manifestation of the dilator influence of the other (Holowatz et al., 2005, Lopez et al., 2013).

On the other hand, it could be that in SAs, inhibition of the vasoconstrictor effects of COX products by aspirin removed an inhibitory influence on NOS such that NO and/or other vasodilators were able to contribute to shear-stress related vasodilation (Osanai et al., 2000). However, it seems rather unlikely that the contribution of NO would increase so much, following acute blockade of the COX pathway given the evidence presented in this thesis and elsewhere (Murphy et al., 2007, Hirst and Marshall, 2018,

Ormshaw et al., 2018) of endothelial dysfunction in SAs. It seems more likely that blocking COX revealed a shear-stress related effect of EDHFs and that their effects are augmented in SAs because they have endothelial dysfunction (Luksha et al., 2009). These proposals are consistent with the findings of Chapter 5 that reactive hyperaemia, which is a shear-stress induced dilatation was accompanied by significant efflux of PGI_2 in WEs but not SAs and a smaller efflux of NO in SAs than WEs.

Irrespective of the mechanisms, the present finding that following COX inhibition, SAs showed augmentation with repetition of the increases in FVC that occurred in the "resting" forearm during each isometric contraction of the contralateral arm raises the possibility that SAs could gain more benefits of the effects of IHG training on NO-dependent endothelial dilator function if they performed each bout of training after a dose of aspirin.

These interpretations will all require further assays of venous efflux from the resting limbs during isometric forearm contraction before and after aspirin to uncover the effects of release on factors such as PGI_2, PGH_2, TXA_2, NO, and K^+ and/or EETs.

Cutaneous circulation in forearm and calf

The finding that in WEs, aspirin had no effect on baseline FRCF or FCVC in Protocol A, whereas in SAs, there was a clear trend for baseline FRCF and FCVC to increase after aspirin even though this did not reach statistical significance suggests there was no tonic influence of COX products on forearm cutaneous circulation of WEs, but a trend for a tonic vasoconstrictor influence of PGs in SAs as has been reported before in the skin of older subjects (Holowatz et al., 2005) as might be expected in individuals with endothelial dysfunction (Feletou et al., 2010, Holowatz et al., 2005). The finding that after COX blockade, FRCF and FCVC tended to be lower during all four contractions in WEs and that the increases in FRCF and FCVC evoked by each contraction were converted to no change or decreases from baseline indicates these responses were mediated by vasodilator PGs. Similarly, the finding that from the higher baseline values after COX blockade in SAs, the small increases in FRCF and FCVC during each contraction were converted to decreases although the effect on ΔFRCF and ΔFCVC values did not reach statistical significance suggests that vasodilator COX products may have also contributed to the forearm cutaneous responses to isometric contraction of the contralateral arm in SAs. When the influences of COX products were removed, cutaneous vasoconstrictor responses to isometric contraction were revealed in both ethnicities, particularly in SAs. These responses could be attributed to the increase in sympathetic nerve activity that is evoked in forearm skin by isometric contraction (Vissing et al., 1991).

Any effects of aspirin on the small and variable responses evoked by isometric forearm contractions in cutaneous circulation of the calf in WEs in Protocol B, were variable. Thus, it seems unwise to make any proposals about the contributions COX products may have made to them.

Since the increases in cutaneous blood flow evoked in the forearm of WEs by repeated isometric contraction of the contralateral forearm can be attributed to vasodilator COX products, it seems reasonable to propose, as suggested for forearm muscle above, that this may affect a shear stress-mediated effect on endothelial synthesis of vasodilator PGs. By contrast, it also seems reasonable to propose, that any similar contribution of shear stress on the release of COX products in forearm cutaneous circulation of SAs

was small. Clearly, the greater variability of the responses evoked in cutaneous circulation and in the effects of COX blockade indicates that larger group sizes as well as bioassays of potential mediators of the vascular responses are required in order to improve clarity of these observations.

Limitations and future experiments

Firstly, for Protocol A, it is an obvious limitation of the present study that it was impossible to gain continuous recordings of ABP during the 4 contractions in which vascular responses in the contralateral arm were recorded. Although the findings of (Cotzias and Marshall, 1993) and our own findings in Protocol B indicate that changes in ABP during IHG contractions are consistent upon repetition, in future studies, ABP could be recorded continuously during repeated forearm contraction by intra-arterial catheter insertion. Alternatively, FBF could be recorded by a technique other than venous occlusion plethysmography which requires inflation and deflation of cuffs on the experimental arm, for example by applying a Doppler ultrasound probe to the brachial artery to allow blood velocity and vessel diameter to be used to compute FBF. Then, the Finapres could be used to record ABP from that same arm, without being disturbed by the FBF recordings. Doppler ultrasound recordings of diameter and velocity could also be used to calculate shear stress in brachial artery before and after aspirin administration. This would be an advantage given that we were interested in potential effects of shear stress in improving endothelial dilator function during IHG training. However, it should be noted that estimations of shear stress in brachial artery would not give us any indication of shear stress more distally at the level of the resistance vessels where shear stress may affect the endothelial function of the arterial and arteriolar resistance vessels that contribute to changes in vascular conductance.

Secondly, EMG activity in the contralateral arm (Protocol A) was recorded from the flexor Pollicus longus muscle only, which is the major flexor muscle of the thumb. Therefore, any possible EMG activity from the major flexor muscles of the four digits was not detected. Future studies should also measure EMG activity from Flexor Digitorum Profundus muscle. Similarly, for the ipsilateral leg (Protocol B), EMG activity recordings were only taken from the Gastrocnemius muscle, which runs from the upper lateral tibia to the calcaneus (heel bone) and talus bone which form the subtalar joint of the foot and is responsible for dorsiflexion and inversion of the foot.

In future experiments it would be good to record activity from other leg muscles such as soleus that are plantar flexors.

Similar studies to those of the present Chapter could also be made midway and at the end of a 4-week IHG training period, to test whether the changes in FBF, CBF and cutaneous RCF in forearm and calf are altered during the IHG training protocol. It might be expected that if isometric contraction of forearm does increase anterograde flow and shear stress the contralateral arm and ipsilateral calf in the acute experiments as proposed above, that these effects would be abolished after 2 weeks of IHG training as a consequence of vascular remodelling and structural increases in vessel diameter (Tinken et al., 2009, Tinken et al., 2010, Green et al., 2005). If so, then future studies should also be conducted to establish whether IHG training of one arm with an inflated sphygmomanometer placed on the contralateral arm so as to prevent the increases in FBF seen during training sessions can prevent the improvements in vasodilator responses during exercise and reactive hyperaemia presented in Chapter 3. This would be particularly worthwhile because Tinken et al. (2010) showed that when an inflated cuff was used to prevent the increase in blood flow and shear stress that normally occurs in the contracting forearm during repeated *rhythmic* handgrip training, this prevented the improvements in vasodilator function seen in the trained arm.

Conclusion

The findings of the present study indicate that substantial increases in FBF occur in the contralateral arm during a single bout of IHG training in young WE and SA men, that cannot be attributed to the minor EMG activity and that augmented with repeated contractions in SAs, but not WEs. FVC was also increased during each contraction and was augmented on repetition in SAs, suggesting the increases in FBF were largely due to vasodilation in the contralateral arm that became larger with repetition in SAs. The finding that CBF and CVC also increased in WEs demonstrates that the increases in limb blood flow and vasodilation evoked by isometric contraction of one forearm occurred at the whole-body level. Further, the finding that cutaneous blood flow and FCVC also occur in the contralateral arm also increased during repeated isometric contraction of the forearm, particularly in WEs indicates these effects extend to cutaneous circulation as well as muscle.

The effects of COX inhibition on these responses indicated that dilator PGs contribute to the forearm and calf vasodilation and to the cutaneous vasodilation induced in the "resting" limbs of WEs, but that vasoconstrictor PGs contribute in the "resting" forearm of SAs.

These results accord with our hypothesis that blood flow does increase in the contralateral arm of young WE and SA men and in the ipsilateral leg during each 3 min period of isometric forearm contraction at 30% MVC, when these are repeated 4 times as during the IHG training protocol, but indicate that in SAs, more repetitions are required for the increases in blood flow to fully develop. They are also consistent with the hypothesis that increases in limb flow may act as a stimulus for shear stress to improve endothelial dilator function during IHG training and importantly, are consistent with the evidence presented in Chapter 3 and 5 that the improvements in endothelial dilator function are greater in WEs than SAs. In WEs, the fact that COX-inhibition attenuated the increases in muscle blood flow and cutaneous circulation suggests that undertaking IHG training after aspirin or other COX inhibitor may limit the ability of shear stress to augment endothelial dilator function. By contrast, in SAs the larger increases in muscle blood flow during each contraction of the contralateral arm after COX-inhibition raises the possibility that aspirin may improve the benefits of IHG training on endothelial function.

7. **General Discussion**

7.1 Introduction

The aim of the present study was to investigate the effects of a 4-5 week training programme of unilateral IHG at 30 % MVC on the vasculature of young normotensive men of WE and SA ethnicities and older normotensive WE men with the intention of gaining greater understanding of how IHG training reduces resting ABP. The major hypotheses were that unilateral IHG training would improve EDD remotely, in the non-trained arm and leg, that the effects would be greater in young SA men and older WE men as they were likely to have endothelial dysfunction and that improved EDD would lead to decreased ABP.

The major novel findings were that even though 4 weeks of IHG training did not affect ABP in young WEs and SAs, resting ABP was significantly reduced in older WE men. Secondly, and unexpectedly, IHG training substantially improved muscle performance during fatiguing contraction in all 3 groups, as well as increasing peak exercise hyperaemia and reactive hyperaemia in the *non*-trained arm of young and older WE men, but not SA men. Thirdly, we showed reactive hyperaemia in forearm is associated with increased release of K^+, PGI_2, NO metabolites and 8-isoprostane in young WEs, SAs and older WEs, but IHG training of the contralateral forearm caused a *decrease* in efflux of NO metabolites at baseline and in reactive hyperaemia in young WEs and SAs, while increasing NO efflux in older WEs, and increasing PGI_2 efflux in SAs. A final, major finding was that during a single bout of IHG training, FBF and FVC in the contralateral arm increased progressively with repeated IHGs in young WEs and SAs, but these responses were smaller in SAs than WEs, and were augmented after COX inhibition in SAs, but attenuated in WEs.

These findings are consistent with the proposal that IHG training improves EDD and gives some indications as to how this might happen. However, there are limitations to the experiments performed and several issues require further investigation. These are discussed in brief below.

7.2 Arterial blood pressure and Heart rate

The findings of Chapters 3 and 4 that in sedentary older WE men, there was a significant reduction in SBP, DBP and MABP following 4 weeks of IHG training, but no significant changes in SBP, DBP or MABP in young WEs or SAs is consistent with previous reports. For, in normotensives of mixed ages, ABP decreased gradually over 8 weeks of IHG training and was generally *not* decreased at 4 weeks (Badrov et al.,

2016, McGowan et al., 2007). However, it was also found that individuals with higher ABP initially showed the largest decrease in ABP (Millar and McCartney, 2007), consistent with the higher absolute ABP in the older WE men of the present study. The lack of change in HR with IHG training in all 3 groups is also in concordance with previous evidence (Taylor et al., 2003, Millar et al., 2009b).

Considering the mechanisms underlying the fall in ABP in older WEs, baseline FVC did not decrease following IHG training in older subjects, suggesting that tonic vasodilation in skeletal muscle, did not contribute to a reduction in TPR and therefore in ABP. However, the finding that baseline efflux of NO metabolites was increased by IHG training in older subjects raises the possibility that there may have been a generalized NO-induced tonic vasodilation that was not detected by measuring baseline FVC. In future studies, it would be interesting to assess cardiac output before and after IHG training in older normotensive subjects so that TPR can be calculated and to test the effect of NOS inhibition on TPR before and after IHG training. Stroke volume can be calculated by analysing the pulsatile pressure when using photoplethysmography to record ABP (Wesseling et al., 1993), as in the present study; cardiac output is then calculated beat-by-beat as the product of HR and stroke volume.

Since aging leads to stiffening of the arteries which can decrease carotid baroreceptor sensitivity (BRS) (Mattace-Raso et al., 2007) and thereby to an overall increase in the sympathetic activity (Okada et al., 2012), it would be interesting to test whether IHG training reduced arterial stiffness in older WE men by improving endothelial function. A longitudinal study on men and women of 36-45 over 6 years showed that endothelial dysfunction and low-grade inflammation were directly associated with increased arterial stiffness, providing evidence they are causally related (van Bussel et al., 2011). Thus, future studies could test the effect of IHG training on Pulse Wave Velocity in carotid, femoral and brachial arteries, an established marker of arterial stiffness (Ring et al., 2014).

By contrast with older WE men, IHG training *did* increase baseline FVC in the trained and non-trained arm of young WE men and in the trained, but not the non-trained arm of young SA men. This raises the question of whether a reduction in vascular resistance in skeletal muscles with IHG training decreases TPR in WE, but not SA men. This could be tested as indicated above by assessing CO and calculating TPR.

If TPR is reduced in young WE men, by IHG training, then an opposing effect on CO might explain the lack of effect on resting ABP. Alternatively, tonic vasodilation in skeletal muscle of young WE men might be counterbalanced by tonic vasoconstriction elsewhere.

7.3 Maximum Voluntary Contraction in the trained arm

Enhanced MVC in the trained arm after 4 weeks of IHG training as demonstrated in young WEs and SAs in Chapter 4 has been reported in many studies involving resistance training, irrespective of ethnic background or age. As discussed in Chapter 4, the main contributors to enhanced muscle power with training are considered to be the physiological and morphological adaptations of motor neurons and the properties of spinal motor neurons, rather than muscle hypertrophy or psychological factors (Yue and Cole, 1992, Gabriel et al., 2006).

As far as we are aware, the finding that IHG at 30% MVC improves muscle *performance* of the trained arm during the 3-min period of *rhythmic* contractions at 60% MVC in both young WEs and SAs in that they fatigued less, is novel. It may be explained in part, by improved motor neuron firing rate and synchronization (Gabriel et al., 2006), but it seems more likely improved vasodilation and muscle blood flow as demonstrated by the augmented post-contraction hyperaemia in the trained arm of young WEs and SAs are important: this requires investigation as considered below.

The finding of Chapter 3 that muscle performance improved in the *non-trained* arm of young WEs and SAs during *rhythmic* contractions at 60% MVC is also novel. As discussed in Chapter 3, there is established evidence that training of one limb can produce a small increase in muscle power in the contralateral limb by "cross-transfer", involving activation of contralateral motor pathways and re-modelling on repeated activation of the trained limb. However, there seems to have been no previous investigation of muscle performance. This particular outcome could be of great potential significance in sport and clinically. For example, training of a healthy limb may provide a way of maintaining muscle performance in a contralateral limb while it is immobilised to allow repair of a fracture, or tendon damage.

That older WE men, who were sedentary apart from the activity required for daily living, not only showed increased MVC in the trained arm following IHG training, but also improved MVC in the *non-trained* arm (Chapter 4), demonstrates that with age,

there is still capacity for the neuronal remodelling that allows more effective motor neurone activation and "cross-transfer" to the contralateral limb (Gabriel et al., 2006). The fact that IHG training also greatly improved muscle performance during rhythmic contractions at 60% MVC in the trained *and* non-trained arm of older WE men, is particularly exciting. It suggests that relatively light IHG training for just 4-5 weeks could considerably improve the ability to carry out physical activities involving repeated muscle contractions with the arms. In future studies, it will be important to establish how long such improvements persist after cessation of training and how often IHG needs to be repeated to sustain the improvement. It would also be interesting to establish whether IHG training affects muscle performance in the legs.

7.4 Exercise hyperaemia and reactive hyperaemia in the trained and non-trained arms

Trained arm. The observations of Chapter 3 that the post-contraction hyperaemia and increase in FVC evoked by rhythmic contractions at 60% MVC were improved in the trained arm after IHG training in both young WE and SA men is consistent with evidence that isometric training and other exercise modalities are effective in augmenting exercise hyperaemia in the trained limb (McGowan et al., 2006A, McGowan et al., 2006B, Green et al., 2004, Tinken et al., 2010, Badrov et al., 2013A, Hellsten, 2016) . As far as we are aware, ours is the first evidence that *isometric* IHG training augments exercise hyperaemia evoked by *rhythmic* contractions and the first evidence that the improvement is similar in young WEs and SAs. The finding of Chapter 4, that there was pronounced augmentation of the blunted post-contraction hyperaemia in the trained arm of older WE men is noteworthy and indicates there is still capacity for blood flow to increase during rhythmic exercise in older men, even with light IHG training of that limb.

As discussed in Chapters 3 and 4, improved peak FVC during exercise hyperaemia with training has been attributed to increases in dilator responses in individual arteriolar resistance vessels in muscle (O'Leary, 1991, Lautt, 1989, Brown, 2002) and/or to angiogenesis and vasculogenesis which increase the density of vascular bed at the level of capillaries and arterioles respectively (Brown and Hudlicka, 2003, Hellsten et al., 2008, Prior et al., 2004). Although *rhythmic* handgrip training increased peak vascular conductance in the trained arm after only 2 weeks, suggesting angiogenesis/vasculogenenesis (Tinken et al., 2010), the time course of the effects of

isometric/resistance training has received far less attention. Young men who performed high intensity resistance exercise comprising four sets of leg press and leg extension, plus two sets of chest or shoulder press starting at 70% of maximal workload showed only a modest increase in capillary density at 4 weeks (Holloway et al., 2018), while a similar study on older men showed a substantial increase in capillary angiogenesis at 12 weeks, but did not investigate effects earlier than that (Verdijk et al., 2016). The time course of effects of rhythmic or isometric training on vasculogenesis has not been investigated (Hellsten, 2016). We cannot exclude the possibility that the increase in peak exercise hyperaemia in the trained arm of young SAs and WEs, and older WE men following light IHG training was partly due to angiogenesis, but it is also likely it reflected augmented endothelium-dependent arteriolar dilatation as seen in young and older sedentary rats following exercise training (Sindler et al., 2009). These issues should be tested by assessing maximal vascular conductance in the trained arm of young WE, SA, and older WE men following IHG training (see (Tinken et al., 2010).

Seen against this background, the findings that IHG training augmented the peak FVC of *reactive* hyperaemia in the trained arm of young and older WE men but not in young SAs, taken together with the fact that peak reactive hyperaemia largely reflects EDD (Carlsson et al., 1987, Tagawa et al., 1994, Crecelius et al., 2013), is consistent with EDD being augmented in the trained limb of both young and older WE men, but not young SA men. Such an outcome is contrary to the working hypothesis of this project, but consistent with evidence that EDD is limited in young SA men (Hirst and Marshall, 2018, Murphy et al., 2007, Ormshaw et al., 2018) and suggests this blunted EDD is less readily up-regulated by stimuli associated with IHG training in young SA, than WE men. In previous studies involving 8 weeks of IHG training, peak reactive hyperaemia was *reduced* in the trained limb of young women (McGowan et al., 2007) but was increased in the trained limb of a mixed group of young men and women at 4 and 8 weeks (Badrov et al., 2013A). Neither study indicated the ethnicity of the subjects.

Non-trained arm. Training-induced angiogenesis/vasculogenesis has been attributed to increased shear stress, as well as to metabolic stressors associated with muscle contraction (e.g., increased ATP turnover, or reduced PO_2), which up-regulate hypoxia-inducible factor 1α (HIF1α), vascular endothelial growth factor (VEGF) and other angiogenic factors (Hellsten, 2016, Prior et al., 2004, Egginton, 2009). Thus, the

increase in baseline FVC recorded in the *non-trained* arm of young WE men and the augmented exercise hyperaemia recorded in the non-trained arm of both young and older WE men, following IHG training could have reflected vasculogenesis/angiogenesis, as well as augmented dilatation in individual arterioles. On the other hand, the finding that young SA men did *not* show augmented baseline FVC, or peak FVC in the non-trained arm during exercise hyperaemia suggests they either do not experience an adequate shear stimulus remotely during IHG training to induce vascular remodelling or improve EDD, or their impaired endothelium (Murphy et al., 2007) is unable to respond. That IHG training also increased the peak FVC of reactive hyperaemia in the whole circulation of the non-trained arm (mainly skeletal muscle) and cutaneous circulation of both young *and* older WE men but had no effect in young SA men is fully consistent with these proposals. The results obtained in Chapter 6 shed further light on these issues as discussed below.

7.5 Contribution of vasodilator mediators

In Chapters 3, 4 and 6, the effects of COX inhibition on the non-trained arm indicated that before IHG training, COX dilator products have a tonic dilator influence on forearm cutaneous circulation and whole forearm circulation of young WE men, but not young SA men, or older WE men. Since a tonic forearm vasodilator influence of NO was also present in young WE, but missing in young SA men (Murphy et al., 2007), we suggest the endothelial dysfunction that occurs in apparently healthy young SA men affects tonic, shear stress-induced release of dilator PGs as well as NO, and that a similar situation may exist for older sedentary WE men.

The effects of COX inhibition also indicated that the tonic vasodilator effect of COX products was lost in cutaneous circulation of young WE men following IHG training and continued to be lacking in young SA, and older WE men. Clearly these results cannot be fully interpreted in the absence of effects of COX inhibition on FVC in whole forearm after IHG training. However, interpretation is also difficult because even though Chapter 5 showed baseline venous efflux of PGI_2 metabolite from the non-trained limb was *not* affected by IHG training in young WE, SA, or older WE men, if there were vasculogenesis in the forearm as discussed above, *no* change in net efflux may actually represent *decreased* release of PGI_2 from individual arterioles. This proposal is important given evidence that rhythmic handgrip training causes a decrease

in shear stress-induced release of NO in the trained arm concurrent with vascular remodelling (Green et al., 2004, Tinken et al., 2010). Indeed, the finding of Chapter 6 that IHG training decreased baseline efflux of NO metabolites from the non-trained arm of young WE and SA men is consistent with that evidence and extends it to the non-trained arm. On the other hand, the observation that baseline efflux of NO metabolites was increased in the non-trained arm of older WE men following IHG training, suggests any effect of vasculogenesis in reducing shear stress-induced NO release from individual arterioles may have been offset in older WE men by increased eNOS activity associated with training (Hellsten, 2016, Sindler et al., 2009, Verdijk et al., 2016).

In future studies, it will be important to test these ideas in young WE and SA men and older WE men by recording baseline FVC and FBF before and after COX and NOS inhibition, whilst also assaying NO and PG metabolites in the venous efflux and to do this with knowledge of the effects of IHG training on maximum vascular conductance as an index of vasculogenesis.

Importantly, Chapter 5 also provided the very first demonstration that metabolites of PGI_2 and NO, plus K^+, all of which have been implicated in reactive hyperaemia by use of selective antagonists (Kilbom and Wennmalm, 1976, Tagawa et al., 1994, Engelke et al., 1996, Crecelius et al., 2013) are actually released during reactive hyperaemia in young WE, SA and older WE men. They also showed that 8-isoprostane is released during reactive hyperaemia in all 3 groups. The possibility is raised it may contribute to the vasodilation: although 8-isoprostane is a recognised marker of oxidative stress (Janssen, 2002), it is released by increased shear stress (Fong et al., 2010) as a consequence of activation of NADPH oxidase (De Keulenaer et al., 1998). Its contribution to reactive hyperaemia could be tested with isoprostane receptor antagonists. Importantly, as 8-isoprostane efflux was not increased by IHG training it seems this training regime did not increase oxidative stress.

The finding of Chapter 5 that efflux of PGI_2 metabolite was significantly increased during reactive hyperaemia in young SA men after IHG training but not before, is consistent with the evidence discussed above that release of dilator COX products is normally depressed in young SAs and suggests that IHG training may increase COX activity and/or the generation of PGI_2 in the non-trained arm. The fact that peak reactive hyperaemia was not augmented in the non-trained arm of SAs

following IHG training despite increased PGI_2 efflux is difficult to interpret given the evidence presented in Chapter 6 that COX inhibition augments forearm vasodilation in young SAs before IHG training implying removal of a vasoconstrictor product of COX, and the established evidence that in conditions of endothelial dysfunction, PGI_2 acts on TP receptors that bind PGH_2/TXA_2 and mediate vasoconstriction (Feletou et al., 2011). In future studies it will be important to follow efflux of PGH_2 and TXA_2 during reactive hyperaemia before and after IHG training, test the effects of COX inhibition and TP receptor antagonism on reactive hyperaemia and possibly, to measure COX expression in biopsies.

On the other hand, the finding presented in Chapter 5 that efflux of NO during reactive hyperaemia was enhanced in the non-trained arm of older WE men after IHG training raises the possibility that NO contributed to the augmented peak of their reactive hyperaemia even though it has generally been concluded NO contributes to the recovery phase (Tagawa et al., 1994). This could be tested in future studies by using NOS inhibitors.

However, the overarching problem associated with estimating the contributions of different mediators to a given dilator response from the effects of antagonists and release of mediators is the interactions between the endothelium-dependent mediators NO, COX products and EDHFs. This problem appeared to arise in Chapters 3 and 4, for COX inhibition had no effect on peak reactive hyperaemia in cutaneous circulation of the non-trained arm of young WE or SA men, before or after IHG training, no effect on cutaneous dilator responses evoked by ACh before IHG training in any group, but *enhanced* peak hyperaemia in older WEs and ACh responses in young and older WEs *after* IHG training. Since IHG training generally improved EDD in young and older WE men as discussed above, it seemed very unlikely these effects of COX inhibition reflected *increased* influence of vasoconstrictor COX products. Rather, it seemed more likely COX inhibition removed an inhibitory influence on NOS activity (Medow et al., 2007) and/or revealed the effects of EDHF. It was also noted in Chapter 3 that in young WE and SA men, COX inhibition attenuated reactive hyperaemia and ACh-induced dilatation in cutaneous circulation in ON but not in OH (those with normotensive/hypertensive parents respectively; (Hirst and Marshall, 2018)). Thus, variability between OH and ON may have accounted for lack of effect of COX inhibition before IHG training. Looking forwards, the effects of IHG training on the

contributions of NO, COX products and EDHF can only be unravelled by using NOS and COX inhibitors sequentially and together, while assaying release of mediators and for young WEs and SAs, there should be subgroups of ON and OH men.

7.6 Mechanisms underlying effects of IHG training

The experiments of Chapter 6 were performed with the aim of gaining evidence on whether IHG training of one arm increased shear, or flow rate in the contralateral arm and ipsilateral calf, i.e., in non-trained limbs. The results were consistent with the hypothesis for, during the equivalent of a single session of repeated IHG at 30% MVC, substantial increases in FBF occurred in the contralateral arm of both young WE and SA men and there were larger increases in forearm RCF in WE than SA men. Further, these increases in FBF and RCF were consistent over 4 repeated IHGs in WEs whereas the increases in FBF were progressively augmented on repetition in SAs to equal that of WEs. Thus, the relative magnitude of the changes in FBF/RCF were consistent with these influences being more effective in young WE than SA men in enhancing exercise and reactive hyperaemia in the non-trained arm.

Tinken et al. (2010) found during training studies involving bilateral rhythmic handgrip contractions that external compression of one arm with a sphygmomanometer cuff inflated to 60mmHg, which prevented the increase in shear stress occurring in the brachial artery during each bout of rhythmic exercise not only prevented the increase in FMD, but also the progressive increase in maximum vascular conductance. They therefore proposed the stimulus for these effects was increased shear stress. It would therefore be interesting in future studies on young WE men initially, to use the Doppler ultrasound technique to record blood velocity in the brachial artery and brachial artery diameter and to test whether using external compression to prevent an increase in shear rate in the non-trained arm during IHG training can prevent the augmented exercise and reactive hyperaemia and improvement in muscle performance seen in the present study. However, shear stress in the brachial artery gives no indication of shear rate in the resistance vessels responsible for exercise and reactive hyperaemia and where stimuli act to improve EDD and induce vasculogenesis.

Considering the mechanisms that might be responsible for increasing FBF in the contralateral arm during acute repetition of IHGs, it is established that isometric exercise causes reflex vasoconstriction in non-exercising limbs as part of the exercise

reflex (Murphy et al., 2011). The gradual increases in FBF recorded in all 3 groups in the present study could not be attributed to the minimal EMG and therefore to exercise hyperaemia. The fact that the increases in FBF and RCF were associated with increases in FVC and FCVC, suggests they were partly due to vasodilation. However, the fact that ABP and HR increased from the onset of each IHG means that the increase in FBF and RCF must have partly reflected an increase in perfusion pressure. Thus, it is a reasonable hypothesis that at very onset of each IHG a sudden increase in perfusion through the contralateral arm acted as a stimulus for the arteriolar endothelium to release NO and PGI_2 and to cause dilatation as shown in isolated arterioles (Koller et al., 1993). The vasodilation would further increase FBF and provide further stimulus to the arterioles for release of NO and PGI_2 and so on, until limited by reflex vasoconstriction of the metaboreflex.

This hypothesis is consistent with evidence of Chapter 6 that COX inhibition attenuated the repeated increases in FBF, FVC, RCF and FCVC in the young WE men. By contrast, since COX inhibition augmented the increases in FBF, FVC, RCF and FCVC in young SA men this indicated their dilator responses were limited by vasoconstrictor COX products, or by COX activity limiting vasodilation induced by NO or EDHFs as discussed above. If blunted EDD occurring acutely in the non-trained arm during each session of IHG training explains why young SA men showed limited improvement in exercise and reactive hyperaemia following 4 weeks of IHG training, the question arises as to whether they would benefit from training after COX inhibition. This could be tested.

Finally, the finding of Chapter 6 that CBF and CVC in the calf of young WE men also increased during a single session of repeated IHGs in the absence of EMG activity suggests that the effects of increased perfusion and arteriolar dilatation described above occurred in the lower limbs as well, but to a much smaller extend than in the contralateral forearm, with little dilatation in cutaneous circulation. The increases in CBF and CVC were also attenuated by COX inhibition implicating vasodilator COX products. In view of these findings, it would be interesting in future studies to establish whether IHG training might increase muscle performance and EDD in the legs of young and older WE men.

7.7 Conclusion

This study has left many questions unanswered. However, the notable outcomes are that in young healthy WE men, just 4 weeks IHG training with one arm at only 30% MVC (four 3-minute contractions at 5-minute intervals 4 times/week) caused a substantial increase in muscle performance together with augmented endothelium-dependent responses in both skeletal muscle and cutaneous circulations of the non-trained arm. Furthermore, qualitatively similar effects occurred in older normotensive WE men who were sedentary, except that they showed reduction in resting ABP as well as a marked increase in muscle performance and substantial improvement in their blunted endothelium-dependent dilator responses in the non-trained arm. Remote effects of IHG training on vasodilator function and muscle performance have never been shown before. Thus, these new findings support the prescription of IHG training recommended by the ACC/AHA (2017) and CHEP (2017) for delaying development of HTN and indicate that a similar practice should be adopted by NICE in the UK, not only to reduce ABP in the older population, but more specifically to limit or restore endothelial dysfunction - the forerunner of CVD. An additional new finding that a single session of IHG training in young WE men caused progressive EDD in the leg raises the possibility that similar training adaptations might occur in the leg as in the arm. Thus, a very important extension of the present study would be to implement IHG training in a community setting to establish whether it can improve physical conditioning and EDD in older sedentary individuals and so improve their wellbeing and ability to cope with daily physical activities.

Another major outcome of the present study was that the same IHG training programme produced similar improvements in muscle performance in young SA men, but much less pronounced effects on endothelium-dependent dilator responses in the non-trained arm. This is consistent with evidence that apparently healthy young SA men have endothelial dysfunction and greater risk of CVD than WE men (Murphy et al., 2007). Moreover, the findings made during a single session of IHG training indicated vasoconstrictor COX products may limit the beneficial effects in the non-trained limb. Thus, if IHG training is to be recommended more generally as a way of improving EDD and reducing CVD, it is clearly important to establish whether greater effects can be produced in SA men by increasing the frequency or length of IHG training, or potentially by undertaking IHG training after a proprietary COX inhibitor.

8. APPENDIX

Approved Ethics Application (ERN_14-1395)

UNIVERSITY OF BIRMINGHAM

APPLICATION FOR ETHICAL REVIEW –

REQUEST FOR AMENDMENTS

Who should use this form:

- This form is to be completed by PIs or supervisors (for PGR student research) who are requesting ethical approval for amendments to research projects that have previously received ethical approval from the University of Birmingham.

  Please be aware that all new research projects undertaken by postgraduate research (PGR) students first registered as from 1st September 2008 will be subject to the University's Ethical Review Process. PGR students first registered before 1st September 2008 should refer to their Department/School/College for further advice.

- What constitutes an amendment?

  Amendments requiring approval may include, but are not limited to, additions to the research protocol, study population, recruitment of participants, access to personal records, research instruments, or participant information and consent documentation. Amendments must be approved before they are implemented.

NOTES:

- Answers to questions must be entered in the space provided
- An electronic version of the completed form should be submitted to the Research Ethics Officer, at the following email address: aer-ethics@contacts.bham.ac.uk. Please **do not** submit paper copies.

- If, in any section, you find that you have insufficient space, or you wish to supply additional material not specifically requested by the form, please submit it in a separate file, clearly marked and attached to the submission email.
- If you have any queries about the form, please address them to the Research Ethics Team.

UNIVERSITY OF BIRMINGHAM APPLICATION FOR ETHICAL REVIEW - REQUEST FOR AMENDMENTS	*OFFICE USE ONLY:* Application No: Date Received:

1. **TITLE OF PROJECT**

The roles of oxygen-dependent mechanisms in the cardiovascular changes associated with exercise in health and disease"

2. **APPROVAL DETAILS**
   What is the Ethical Review Number (ERN) for the project?

ERN_14-1395

3. **THIS PROJECT IS:**
   University of Birmingham Staff Research project ☐
   University of Birmingham Postgraduate Research (PGR) student project ☐
   Other ☐ (Please specify):

4. **INVESTIGATORS**

   a) PLEASE GIVE DETAILS OF THE PRINCIPAL INVESTIGATORS OR SUPERVISORS (FOR PGR STUDENT PROJECTS)

Name: Title / first name / family name	Professor Janice Marshall
Highest qualification & position held:	PhD, DSc, Deputy Head of School
School/Department	Clinical & Experimental Medicine
Telephone:	[redacted]
Email address:	[redacted]

Name: Title / first name / family name	
Highest qualification & position held:	
School/Department	
Telephone:	
Email address:	

b) PLEASE GIVE DETAILS OF ANY CO-INVESTIGATORS OR CO-SUPERVISORS (FOR PGR STUDENT PROJECTS)

Name: Title / first name / family name	Professor Una Martin
Highest qualification & position held:	PhD, FRCP, Reader / Consultant Physician
School/Department	Clinical & Experimental Medicine
Telephone:	
Email address:	

c) In the case of PGR student projects, please give details of the student

Name of student:	Kyriakos Tsitoglou	Student No:	
Course of study:	PhD Physiology Lab FT	Email address:	
Principal supervisor:	Professor Janice Marshall		

Name of student:		Student No:	
Course of study:			
Principal supervisor:			

5. **ESTIMATED START OF PROJECT** Date: January 2015

ESTIMATED END OF PROJECT Date: October 2018

Title and reference number of application or amendment **ERN_14-1395**	Key points of application and/or changes made by amendment (include: aims of study, participant details, how participants were recruited and methodology)	Ethical considerations arising from these key points (e.g. gaining consent, risks to participants and/or researcher, points raised by Ethical Review Committee during review)	How were the ethical considerations addressed? (e.g. consent form, participant information, adhering to relevant procedures/clearance required)
Original application The effects of handgrip exercise training on blood pressure and vascular responses in Asians and Caucasians	**Aims**: to test in Asians and Caucasians whether handgrip exercise training of one arm for 4-5 weeks improves the increase in blood flow that occurs (i) in the trained and untrained arm during exercise (ii) by release of substances from the endothelial lining of blood vessels and (iii) whether prostaglandins are involved. **Methods**: (i) recording forearm muscle blood flow by using venous occlusion plethysmography, which involves inflating and deflating blood pressure cuffs on wrist and upper arm, following handgrip exercise and following deflation of a blood pressure cuff placed on the arm and repeating this in dominant and non-dominant arm (ii) recording forearm skin blood flow via a laser Doppler probe on skin following deflation of a blood pressure cuff and during minute application of a dilator substance (acetylcholine) to the skin before and after oral aspirin, on 2 different days, before training and after training. **Recruitment:** Posters in social spaces to recruit young subjects from the UG and PG populations displayed in lecture theatres and appropriate social spaces. In addition, by word of mouth. **Characteristics:** Young men, 18-25 **Exclusion criteria**: history of cardiovascular or respiratory disease, diabetes, smoking, alcohol consumption over 21 units per week and BMI over 29kg/m^2.	All interested volunteers receive a **participant information leaflet (PIL)**. Those who indicate willingness to participate are contacted by the researcher and asked simple screening questions to ensure they have no excluding medical condition. If they are suitable and continue to show interest the remaining questions on the questionnaire are completed. After a familiarisation visit to the lab, and on arrival for their first study, written, **informed consent** is obtained. The PIL informs them they have the right to withdraw from the study at any time without giving reason, this will not affect them in the future in any way. They are reminded of this when written consent is obtained. Participants are allocated a unique study code and **all data are held and used anonymously** when the study results are presented at conferences etc. **Risks:** We already had >5 years experience with essentially similar protocols for the physiological recording sessions; all the techniques are non-invasive. We had no previous experience of handgrip training but could foresee no obvious risks. No adverse effects had occurred at the time of application or since. The dose of aspirin is the recommended dose for a headache. Participants are asked about any	We were asked to: include a statement explaining that in the event that any abnormal/unusual test results are found, we will tell them and provide guidance (e.g. to visit their G.P.). It is difficult for us to imagine what we could notice that would be abnormal in our test results, but we recognised the participants might be concerned. make clear that if participants were concerned or experience symptoms after taking part, they should notify us. Again it was difficult for us to imagine a side-effect that will not be apparent at the time. make it clear on the PIL that those carrying out the procedures have been fully trained. make the language used in the PIL easier to read and understand. We were also asked to sort of minor points of confusion and minor discrepancies between different sections of the form. All of these points were accomplished to the satisfaction of the committee

	Inclusion criterion recreationally active rather than trained.	previous adverse reactions to aspirin and are excluded on this basis.	
Title and reference number of application or amendment **ERN_14-1395** ***Subsequent amendment 1***	**a.** We were granted approval to extend the age range to men of 55-70	No additional ethical considerations were raised by us or the ERC; the study specifically excludes those taking any medication	

6. **ORIGINAL APPLICATION FOR ETHICAL REVIEW AND ANY SUBSEQUENT APPROVED AMENDMENTS:**

Please complete the table below for the original application and any subsequent amendments submitted

Title and reference number of application or amendment **ERN_14-1395**	Key points of application and/or changes made by amendment (include: aims of study, participant details, how participants were recruited and methodology)	Ethical considerations arising from these key points (e.g. gaining consent, risks to participants and/or researcher, points raised by Ethical Review Committee during review)	How were the ethical considerations addressed? (e.g. consent form, participant information, adhering to relevant procedures/clearance required)
	b. We were granted approval to recruit from older University staff, recently retired staff and through the Thousand Elders data-base held by the Centre for Aging Research, via other groups concerned research on healthy aging on ampus (eg SportEx), via Aston Research Centre for Healthy Aging, Aston University, Clinical/Community Engagement Lead) and from Sports Clubs and Fitness/Leisure Centres by using posters and personal contacts. **c.** We were granted approval to record an ECG in parallel with other physiological variables, by attaching 2 standard electrodes to the arms or chest. **d.** We were granted approval to insert a needle attached to tubing into the vein on one arm before the study proper and at the end, to allow withdrawal of 10-15ml blood for analysis. This was to allow us to assay substances like glucose and cholesterol to help assess the health status of the subject	No additional ethical considerations were raised by us, or the ERC; the study specifically excludes those taking any medication from our studies and the activities they undertake as part of the study are of very low risk. This raised no ethical considerations at all We acknowledged there was a possibility the person may experience mild bruising at the sample site. We also stated the	Appropriate amendments were made to the PIP Appropriate amendments were made to the PIP Appropriate amendments were made to the PIP

		assays may give an indication that the individual has diabetes or high cholesterol in which case, he would not fit the inclusion criteria for our study and we would suggest that he visits his GP.	We were asked by the ERC if we intended to store whole blood samples. We stated that we would, at most, retain plasma for analysis at a later date and that all cells would be safely discarded on the day of collection

7. DETAILS OF PROPOSED NEW AMENDMENT

Provide details of the proposed new amendment, and clearly and explicitly state how the proposed new amendment will differ from the details of the study as already approved (see Q6 above).

Amendment 1. We currently perform 2 experimental sessions on the subjects, (see above), show them how to undertake the handgrip training (4x 3 minute handgrips at 30% of their maximum force, 4 days/ week for 4-5 weeks) and then repeat these sessions.

We now wish to recruit additional groups of 10-12 young Asian and Caucasian men who fit the criteria already approved to attend a familiarisation session and a new session at which we record blood flow changes in the resting arm and leg during a single bout of the hand grip training protocol with the dominant arm, This will involve simultaneously recording in the resting arm, forearm muscle blood flow by venous occlusion plethysmography and skin blood flow via a laser Doppler probe on forearm skin before and at intervals following each handgrip. We would also monitor arterial blood pressure and heart rate non-invasively via a finger cuff. We now wish to repeat this protocol in the same subjects on another day after the subject has taken an oral dose of aspirin to inhibit the enzyme cyclooxygenase (COX), which synthesises prostaglandins. The techniques and methodology are ones for which we already have approval in the pre- and post-training studies. We already have approval to use aspirin within a session to block COX, both pre- and post-training. In these experiments we do not wish record blood flow from the contracting forearm, or use iontophoresis, or take blood samples, and the subjects will not undertake 4-5 week training (**see new PIP 2**).

Amendment 2. As indicated above, we already have approval to use a laser Doppler probe to record skin blood flow in the forearm before and after deflation of a blood pressure cuff and during iontophoresis of small quantities of Acetylcholine (ACh) which increases blood flow locally in the skin by releasing dilator substances from the endothelial lining of blood vessels. Iontophoresis entails application of a small electric current to deliver ACh into the skin. We currently have approval to do these tests before and 30 minutes after an oral aspirin to block COX. We now wish to test in the same session, the involvement of the other major endothelium-dependent dilator nitric oxide (NO) and test interactions between the COX and NO pathways. Others have shown that the NO synthesis inhibitor L-NAME can be topically applied to the skin by simply allowing diffusion from the iontophoresis well. In order to do this without lengthening the session substantially, we will ask them to come to two sessions in which the antagonists are given in opposite orders.

As indicated above, we have approval to take blood samples through a needle and tubing at recruitment and at the beginning and one end of the training period. We now wish to take blood samples from subgroups of the main groups before and immediately after release of the blood pressure cuff.

Thus, we wish to recruit additional groups of 10-12 young Asian and Caucasian men who fit the criteria already approved, to attend a familiarisation session followed by two sessions at which we will use our approved protocol for testing responses evoked in skin by release of a blood pressure cuff and iontophoresis of ACh except that:

(i) In one session, we wish to perform both of these tests before and after L-NAME has been applied in the iontophoresis well for 20-25 minutes, and then again, 30 minutes after aspirin.
(ii) In the other session, we wish to perform both tests before and after aspirin, and then before and after application of L-NAME.

At the beginning of one sessions, in a subgroup of 6-7 young men of each ethnic group who agree to undertake the handgrip training for 4-5 weeks, we

(iii) wish to insert a needle attached to tubing into the vein of the non-dominant arm so that we can withdraw small samples of blood (10-15ml) for analysis before and immediately after the blood pressure cuff has been inflated on the non-dominant arm for 3 minutes.
(iv) In a similar manner to the approved protocols, we wish to perform both experimental sessions after they have undertaken the handgrip training protocol for 4-5 weeks.
(v) As in our approved project, we wish to perform comparable experimental sessions on older men of Caucasian and South Asian ethnicity who fit our criteria: **see new PIP 3**

Amendment 3. As indicated above, we have approval to withdraw a small sample of blood (10-15ml) for analysis at recruitment and after training. In the approved study on older men of Asian and Caucasian ethnicity, we now wish to take blood samples from 6-7 men from each group, in one sessions before and in another after they undertake handgrip training. We will take blood before and immediately following rhythmic handgrips and immediately before and after release of arterial occlusion. In order to do this, we have added rhythmic handgrip contractions to the experimental session in which we are recording skin blood flow. This avoids us taking blood samples from the same site on two different sessions a few days apart: **see amended PIP 1**.

7. **JUSTIFICATION FOR PROPOSED <u>NEW AMENDMENT</u>**

The overall aim of this project is to establish whether handgrip training can be of benefit in improving cardiovascular function in Asians who are at much greater risk than Caucasians of developing cardiovascular disease in middle and older age. Having now completed our initial study on young Asian and Caucasian men, we have shown for the first time that there are improvements in vasodilator function in the *untrained* arm, as well as the trained arm in both ethnic groups. This has very important implications for potential use of handgrip training as a means of

improving general cardiovascular health and we now wish to explore the mechanisms involved. Firstly, we do not know ***how*** handgrip training of one arm might act as a stimulus for improvement in dilator functioning in the other, resting arm.

Amendment 1 above would test the changes in blood flow and blood pressure that occur in the resting forearm and leg during one day's worth of the handgrip training protocol. There is evidence in the literature that increased shear stress associated with increased blood flow and/or pressure, can provide a long-term stimulus that up-regulates COX and NO synthesis, so this may be how handgrip training works. However, there is also evidence that contraction of one limb produces reflex vasoconstriction and decreased blood flow in the contralateral resting limb. This response is modulated by prostaglandins, and their synthesis is changed on repetition. This is why we want to compare the effects of the training protocol with and without COX inhibition.

Amendment 2 The COX pathway generates both dilator and vasoconstrictor prostaglandin, and there is evidence that the COX pathways and nitric oxide (NO) pathway interact with one another. Our results already show that in the non-trained arm, the effects of COX inhibition on the vasodilator responses are different in young Asians and Caucasians and that the effects of COX inhibition are differently affected by training in the two ethnicities. Notably, training produces greater improvement in the dilator responses evoked by forearm contraction in the untrained arm of Asians than Caucasians. This suggests there may be inherent differences between Asians and Caucasians in the contributions of vasoconstrictor and vasodilator prostaglandins and NO and that training differentially affects the balance between them in the two ethnicities. This is why we wish to test the effect of COX blockade with aspirin before and after inhibition of NO synthesis in the main groups. It also explains why we wish to repeat this in a subgroup of each ethnicity after handgrip training. The blood samples we take in these subgroups will allow us to analyse the plasma for prostaglandin and NO metabolites to complement our analyses of the vascular responses. Statistical power calculations using data we have collected in a different study **ERN_12-1377** indicate that we need to recruit 6-7 for the training phase of the study.

Amendment 3. We initially planned to use blood samples taken before and after training to assay for indicators of general health status such as blood cholesterol, lipids, blood sugar, antioxidants and oxidants and to see how these are changed by the handgrip training. We are still interested in this information,but having obtained the novel results mentioned above on the young men, we realise that it would be very valuable in the approved study on the older men, to assay for prostaglandins and NO released into the venous drainage of the arm following the experimental stimuli and to establish how they are affected by handgrip training. These results will complement the data we collect on vascular responses.

8. **ETHICAL CONSIDERATIONS**

What ethical considerations, if any, are raised by the proposed new amendment?

Amendment 1. There are no obvious additional ethical considerations arising from monitoring blood flow responses in the resting arm during one day's worth of the handgrip training protocol, nor of monitoring these responses on another day after a dose of aspirin. All the methodology is approved. The dose of aspirin is the recommended dose for a headache. Participants are asked about any previous adverse reactions to aspirin and are excluded on this basis.

Amendment 2. There are no obvious additional ethical considerations arising from monitoring blood flow responses to release of arterial occlusion, or ACh before and after topical application of L-NAME, an NO synthesis inhibitor to a small area of skin (1.5cm in diameter). The dose is minute and could have no systemic effects; there are no recorded local adverse effects.

We have previously acknowledged that when taking blood samples, there is a possibility the person may experience mild bruising at the sample site. We also stated the assays may give an indication that the individual has diabetes or high cholesterol in which case, he would not fit the inclusion criteria for our study and we would suggest that he visits his GP. No additional ethical issues are raised by assaying for plasma levels of prostaglandins or NO in the sample: there are no recognised normal values, or ranges for these substances.

Amendment 3. We are now requesting approval to take 8 samples in total from older men, 4 at one experimental session before training and then to repeat this 4-5 weeks later when they have completed the handgrip training. On each occasion, we would take a sample of 10-15 mls, which amounts to 2-3 teaspoonfuls. Thus, the total amount taken is very small. There is a risk of bruising as we have already acknowledged, but this should clear up in a few days.

The staff member who will be involved in all experimental studies (Kyriakos Tsitoglou) has been trained in phlebotomy and has completed the competency training. Should a medical emergency arise, we have several staff qualified in First Aid who work in the department and are accessible by phone: the phone numbers are listed in the lab. Should a serious emergency arise, the Queen Elizabeth hospital is within 200 metres of the laboratory and has ready access via an external door.

9. DECLARATION BY APPLICANTS

I make this application on the basis that the information it contains is confidential and will be used by the

University of Birmingham for the purposes of ethical review and monitoring of the research project described

herein, and to satisfy reporting requirements to regulatory bodies. The information will not be used for any

other purpose without my prior consent.

I declare that:

- The information in this form together with any accompanying information is complete and correct to the best of my knowledge and belief and I take full responsibility for it.
- I undertake to abide by University Code of Conduct for Research (http://www.birmingham.ac.uk/Documents/university/legal/research.pdf) alongside any other relevant professional bodies' codes of conduct and/or ethical guidelines.
- I will report any changes affecting the ethical aspects of the project to the University of Birmingham Research Ethics Officer.
- I will report any adverse or unforeseen events which occur to the relevant Ethics Committee project to the University of Birmingham Research Ethics Officer.

Signature of Principal investigator/project supervisor:	
Date:	5th December 2015

8.1 Information Sheet

Cardiovascular-Respiratory Integration and Control
School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
University of Birmingham

Investigation of vasodilator responses and the effects of handgrip training in Caucasian and Asians

Investigators: *Kyriakos I Tsitoglou and Prof. Janice M Marshall*

Participant Information Sheet

You are invited to take part in a non invasive research study that will compare the effects of handgrip exercise training on blood pressure and vasodilator responses in young and older Caucasian and Asian men. All the investigators carrying out the study are fully trained.

Background – What is already known?

It has been established that handgrip exercise training for a few weeks can reduce blood pressure in people who have high blood pressure. There is also published evidence that the reduction in blood pressure is accompanied by improved vasodilator responses in the trained arm. It has been suggested this reflects enhanced release of vasodilator substances from the endothelial cell lining of the blood vessels caused by the repeated handgrip exercise. In our recent studies, we have shown that in young men of Caucasian and Asian ethnicity, exercise training not only increases dilator responses in the trained arm, but in the untrained arm as well. This is particularly exciting because it raises the possibility there is a generalised improvement in endothelial function throughout the body. This is important because a generalised improvement in endothelial function is known to reduce the risk of cardiovascular disease (CVD).

The prevalence of high blood pressure and diabetes is particularly high in Asians. These are diseases that usually become apparent in middle age, and they are associated with endothelial dysfunction. Thus, the overall aim of our project is to establish whether localised handgrip training could reduce blood pressure, improve vasodilator and endothelial function and reduce the risk of CVD in middle aged - elderly people who may not be easily persuaded to do whole body exercise. We anticipate that handgrip training could be particularly valuable in those of Asian ethnicity. However, in order to properly assess this possibility, we need a greater understanding of how handgrip training might achieve its effects in young and older people.

The substances that are released by the endothelium include nitric oxide (NO) and prostaglandins, which are produced by the cyclooxygenase (COX) enzyme. Some prostaglandins are vasodilator and help to increase blood flow, but others are vasoconstrictor; these are associated with endothelial dysfunction and high blood pressure. The dilator influence of NO is also impaired in endothelial dysfunction. In the experiments we have carried out so

far, we found that in young Asian men with no evidence of CVD, there is already evidence that vasoconstrictor prostaglandins are released from the endothelium, whereas this is not the case in young Caucasians. In Asians, handgrip training switches the balance towards vasodilator prostaglandins so becoming similar to the Caucasians. So far, we have not tested the contribution of NO, but we anticipate its dilator influence may be less in Asians than Caucasians, particularly because vasoconstrictor prostaglandins are known to inhibit synthesis of NO. We anticipate that handgrip training promotes endothelial release of NO in both ethnicities.

With this background, we now want to test the relative contributions of NO and prostaglandins to vasodilation that involves the endothelium in young Caucasians and Asians and to do this before any training.

In addition, we want to test in some people how handgrip training for 4-5 weeks affects the contributions of NO and prostaglandins to vasodilator responses in the *untrained* forearm and to see whether they are affected differently in Asians and Caucasians.

We would like to perform similar studies on older Asians and Caucasians because aging is known to be associated with endothelial dysfunction, but there is no evidence as to whether the processes are different in different ethnicities.

Who can take part?

You are invited to take part because you are a healthy young Caucasian or Asian (18-25 years old) man, or a healthy older Caucasian or Asian (55-70 years old) who is free from any cardiovascular, respiratory disease or metabolic disease (diabetes, etc). You will also need to be recreationally active, non-obese, non-smoking individual who does not consume over 21 units/week of alcohol and not taking any medication (see below). We intend to recruit 10-12 people men people of each ethnicity and each age group who will be tested before and after undertaking 4-5 weeks handgrip training. In these men we wish to take small blood samples for analysis of prostaglandins and NO before and after training

Benefits – What do we want to know?

Even though there may be no long-lasting beneficial effects for you, the results of the study will give us new understanding of vascular responses involving the endothelium in Asians and Caucasians. It will also give us new understanding of the effects of handgrip training on blood pressure and endothelium-dependent vascular responses in young Asian and Caucasian men and tell us whether these are accentuated in older Asians and Caucasians.

What will the study involve?

You will be asked to come for a first visit to the laboratory in the Medical School, in order to complete a routine health questionnaire. You will also have the opportunity to ask any questions that you might have regarding the study. If for any reason you are not suitable for the study, you will be told. However, if you are suitable and willing to participate, this visit will be used to familiarize you with the laboratory equipment and the procedures. We will also run through the techniques we use to record blood flow and test your vascular responses (see below) so that you can experience them before the experimental sessions.

If you have agreed to undertake handgrip training, we will record your maximum handgrip force (100% MVC) with your dominant hand and your non-dominant hand.

Following this first visit, you will be requested to visit the laboratory for 2 different experimental sessions if you are *not* doing the training and 5 different sessions if you are.

If you agree to the handgrip training, after the second experimental session and for a period of 4-5 weeks, we will ask you to perform handgrip exercise training four times a week (see below). You will be asked to come back after 2-weeks for a short visit and then twice after 4-weeks of training so that we can monitor the effects of training.

We will endeavour to make the time of each session as convenient for you as possible.

The Experimental sessions will involve the following:

- Recording blood pressure by using a standard blood pressure monitor.
- Recording Heart Rate and blood pressure continuously by using a small cuff placed on a finger of your hand (non-invasive procedure).
- Recording dilatation from blood vessels in the skin. This will involve delivering a small amount of a vasodilator substance into your skin from a small Perspex ring, by using a technique called iontophoresis. The ring is attached to the skin by double-sided adhesive discs and a probe will be placed into the ring to measure blood flow. Iontophoresis involves applying 8 tiny electrical pulses, at 1-minute intervals to the fluid in the ring. Each pulse pushes ions into a tiny area of skin which is no more than 1mm in depth and which dilates the blood vessels in this area. Iontophoresis is a routine procedure in our lab and in many other laboratories. (It is a non-invasive procedure).
- We will also inflate a blood pressure cuff on your arm and then record your skin blood flow when the cuff is deflated (non-invasive).
- Consuming an orange flavoured drink containing aspirin near the beginning, or midway through each of the visits: aspirin inhibits prostaglandin formation for 2-3 hours.
- Recording forearm muscle blood flow by using venous occlusion plethysmography, which involves inflating and deflating blood pressure cuffs on wrist and upper arm or calf and ankle, following handgrip exercise and following deflation of a blood pressure cuff placed on the arm and repeating this in dominant and non-dominant arm
- **If you agreed to the handgrip training, you will be asked to give two blood samples** during one of the experimental session. A needle with a plastic tube (cannula) will be placed in the vein in the crook of your arm so that we can collect small volumes of blood into tubes for analysis of substances like cholesterol, blood sugar, prostaglandins, nitric oxide. You may have seen these as they are commonly used in hospitals and GP practices. If you undertake the handgrip training, we would like to take two samples before training and two after training,

Handgrip training. If you consent to undertake handgrip training, you will be required to use your dominant hand to perform 4 3-minute periods of sustained handgrip at 30%MVC separated by 5-minute rest periods on 4 different days per week for 4-5 weeks by using a handgrip dynamometer. We will show you how to do this either at the familiarisation visit, or on one of the first two sessions whichever is most convenient for you. After 2 weeks handgrip training, we will ask you to come back for a short visit so that we can record your resting blood pressure and check your maximum handgrip. Your grip strength may improve and so we may adjust the handgrip you use for training.

What will you have to do before the visits for experiments?
You will be asked not to consume any non-steroidal anti-inflammatory drugs (such as aspirin,

ibufrofen, nurofen, paracetamol, panadol, etc) for 1 week before your visit, or consume any alcohol or exercise vigorously for at least 24 hours prior to your visit. You will also be asked not to consume any caffeinated drinks/caffeinated medication (tea, coffee, Red Bull, flu medicine, etc) or heavy meals for at least 12 hours prior to each visit.

What will you have to do during the 2 visits for experiments?

- The first 2 visits will take place within a few days of one another.
- On each of these visits, we will record your resting blood pressure from your non-dominant arm three times, by using a standard blood pressure monitor.
- We will then put a finger cuff on your dominant arm and use this to continuously record blood pressure and heart rate.
- We will record blood flow in the skin of your non-dominant arm by using the Perspex ring into which a probe is placed, as described above.
- When the recordings have settled down, we will inflate the blood pressure cuff wrapped around the upper arm for 3 minutes.
- Following this, we will deliver a vasodilator substance into the skin from the Perspex ring by iontophoresis as described above.
- We will then move the Perspex ring and probe to a different site on your forearm and put a small volume of a nitric oxide (NO) synthesis inhibitor into the well. This will be left in the well for 20-30 minutes so that it inhibits NO synthesis in the area of skin underlying the well.
- We will then test your skin blood flow responses to inflation and deflation of the blood pressure cuff and to the vasodilator substance as described above.
- We will then ask you to consume an orange drink containing aspirin.
- Approximately 25 minutes later, we will again test skin blood flow responses to inflation and deflation the cuff and to the vasodilator.
- In the second experimental session, we will perform the same protocol except that we will ask you to take the orange drink containing aspirin first and will apply the NO synthesis inhibitor second.
- **If you have agreed to give blood samples,** we will take one just before the blood pressure cuff is inflated the first time and a second immediately the cuff is deflated. We will do this in one visit only.

- **If you have agreed to do the handgrip training,** after 2 weeks of training, we will ask you to visit the laboratory again. On this occasion, we will measure your resting blood pressure 3 times by using a standard monitor.
- We will then ask you to do a maximum handgrip with your dominant hand as you did on your familiarisation visit. If this has changed significantly, we will ask you to adjust the handgrip you use during your training sessions.

- **After a further 2 weeks of training,** we will ask you to return to the laboratory for two further visits within 5 days of one another. On these occasions we will repeat the recordings we made on the first two experimental sessions. If there are more than 3 days between these visits, we will ask you to do one further handgrip training session.

Can you change your mind about participation?

It is entirely up to you to decide whether or not you take part in the study. If you decide to take part, but later change your mind you can pull out at anytime. If you have completed either of the first two experimental sessions, we may ask your permission to retain your data so that we can enter it into the grouped results. Otherwise, your data will be destroyed following your withdrawal. All of the data collected and retained with your permission may be held by the University for research purposes on a secure database for up to 10 years. This is in accordance with University's research guidelines.

Are there any risks involved?

Any risks attached to this study are minimal. As you know, aspirin is a widely used. It is regularly prescribed by doctors for a number of medical conditions and is available over the counter in pharmacies. **However, it is important you let us know if you have ever had side effects from using aspirin, as we would then not be able to use you for the study.**

The process of iontophoresis is entirely safe. All you may feel is a slight prickling sensation when the tiny current is delivered – it feels rather like a nettle sting. Your skin may show a little red spot due to the dilatation of blood vessels when the probe is removed. This is very normal and expected and will go away within an hour or so. There are usually no lasting sensations.

The procedure of applying a NO inhibitor to the skin is entirely safe. The dose used is minute and it produces no sensation. The skin may be paler under the iontophoresis well when it is removed, but this will disappear very quickly.

The needle insertion for sampling blood may cause minor soreness or discomfort, but these usually subside within a few days.

If you do think you have experienced any side effects during the course of study, please do not hesitate to report them. However, the likelihood of any adverse effects is minimal. Nonetheless, you can always contact us through email or telephone.

What will happen to the results of the research study?

The results of the study will be used to contribute to publication of a PhD thesis and in papers published in medical science journals. All the data collected will be stored on a password protected database. All data will be strictly confidential and will be made anonymous so that you cannot be recognized from it.

Are you interested?

Having read though this information, if you are interested in participating in this study, please contact;

Kyriakos I Tsitoglou
School of Clinical and Experimental Medicine
University of Birmingham
Mobile:
Email:

Supervisor: Professor Janice Marshall
School of Clinical and Experimental Medicine
University of Birmingham
Email:

8.2 Consent Form

Cardio-respiratory Integration and Control
School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
University of Birmingham

Investigation of localised exercise training between young Caucasian and Asian males

Investigators: *Kyriakos I Tsitoglou and Prof. Janice M Marshall.*

Healthy Volunteer's Consent Form

Please read this form carefully and sign it once one of the above named, has explained the aims and procedures of the study to you.

- I have voluntarily agreed to take part in this study.
- I confirm that I have been given a full explanation by at least one of those named above and that I have read and understood the information sheet given to me.
- I have been given the opportunity to ask questions and discuss the study with one of the above investigators on all aspects of the study and have understood the advice and information given as a result.
- I agree to comply with the reasonable instructions of the investigators and will notify them immediately of any unexpected unusual symptoms.
- I understand that any data and/or samples collected will be strictly used for scientific research/study.
- I authorize the investigators to disclose the results of my participation in the study but not any details which could lead to my identification (e.g. name, contact details).
- I understand that all of the information about me recorded during the study will be kept secure.
- I understand that the data collected and/or results of the study may be published in a scientific journal and Thesis and that if published, my identity will be protected.

- I authorize the investigators to disclose to me any abnormal test results.
- I understand that I can ask for further instructions and/or explanations at any time.
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. I also understand that any data/samples collected during the course of my participation will not be discarded due to my withdrawal from the study.
- I confirm that I have disclosed all the relevant medical information before the study.

Please note that a copy of this form will be provided for you to keep. If you have any questions, require further instructions and/or explanations, please do not hesitate to contact Kyriakos I Tsitoglou at [redacted]

Volunteer's Name: _____

Signature: _____

Date: _____

Investigator's Name: _____

Signature: _____

Date: _____

Subject Number: _____

PLEASE, DO NOT FORGET TO BRING THE CONSENT FORM WITH YOU ON THE PREARRANGED DATE OF EXPERIMENT.

8.3 Screening Questionnaire & Handgrip Exercise Schedule

UNIVERSITY OF BIRMINGHAM

Cardiovascular-Respiratory Integration and Control

School of Clinical and Experimental Medicine

College of Medical and Dental Sciences

University of Birmingham

THE EFFECTS OF HANDGRIP EXERCISE TRAINING ON BLOOD PRESSURE AND VASCULAR RESPONSES IN ASIANS AND CAUCASIANS

Investigators: *Kyriakos I. Tsitoglou, Dr. Una Martin and Prof. Janice M Marshall*

Healthy Volunteer Screening Questionnaire

Please remember, all the information will be treated in the strictest of confidence.

VOLUNTEER INFORMATION

Subject No (for researcher only):_____

Name:_____

Date of Birth:_____ BMI:_____ Kg/m^2

Address:_____ Resting Heart Rate:_____ B/min

_____ Resting Heart Rate (4wks):___ B/min

_____ Resting Blood Pressure:_____ mm Hg

_____ Resting Blood Pressure (4wks):_____

mm Hg_____ Dominant Arm: _____(R or L)

_____ Arm Circumference (Dominant):___cm

Phone No:_____ Arm Circumference (Non-dominant):___cm

Email Address:_____ Max. Contraction (Dominant):_____Kg

Height:_____cm Max. Contraction (Non-dominant): Kg

Weight:_____Kg Max. Contraction (Dominant) (4wks):____Kg

WHR:_____(W/H) Max. Contraction (Non-dominant) (4wks)___Kg

MEDICAL HISTORY

Do you currently smoke? **Yes / No**

Have you ever smoked? **Yes / No**

If yes, please provide details of when you quit?

_____

Do you, or have you ever used ANY drugs for recreational purposes? **Yes / No**

Are you taking ANY medication? **Yes / No**

If yes, please give details:

_____

_____

Are you allergic to non steroidal anti-inflammatory drugs (NSAIDs) e.g. Aspirin, Nurofen, Ibuprofen, Naproxen, etc?

Yes/ No

If yes, please give details:

_____

_____

Do you, or have you ever suffered from any of the following?

- Cardiovascular Disease **Yes / No**
- Hemodynamic / Clotting Disorder **Yes / No**
- Metabolic Disease (e.g. Diabetes) **Yes / No**
- Respiratory Disease **Yes / No**
- Liver Disease **Yes / No**
- Kidney Disease **Yes / No**
- Ulcers (e.g. Stomach) **Yes / No**
- Any Other Chronic Medical Condition **Yes / No**

If yes to any of the above, please give details:

_____

_____

_____

_____

Does your family have a history of these diseases? **Yes / No**

If yes, please give details:

_____

_____

Have you ever fainted (*e.g. on standing, following fasting, during exercise)*? **Yes / No**

If yes, please give details: _____

Do you consume alcohol? **Yes / No**

If yes, how many units of alcohol do you consume per week?

_____ Units

Note: *Half a pint of ordinary strength larger/beer/cider, or one small glass of wine, or one pub measure of spirits (e.g. a shot of tequila), or half a bottle of alcopop (e.g. Smirnoff Ice) is equal to **1 Unit.***

Roughly, how many cups of coffee, tea, cola or any other caffeinated/energy drink (e.g. Red Bull) do you drink in a day?

_____ Cups

Do you take dietary supplementation (Vitamins, Botanicals, Amino acids, etc)? **Yes / No**

If yes, what do you take and how often?

_____

_____

How often do you eat citrus fruits?

_____

How many portions of fruit and vegetable do you have daily?

_____

How often do you eat fish rich in oil (Omega 3), eg mackerel, tuna, herring, salmon, sardines, or consume Fish oil supplements?

_____

Have you taken part, or do you intend to take part, in any other trial within the three months prior to and following this particular study (e.g. other studies, blood donor)?

If yes, please give details? **Yes / No**

_____

_____

ETHNICITY

WHAT IS YOUR ETHNIC GROUP? Choose one section from (a) to (e) and tick the appropriate box to indicate your cultural background	
(a) WHITE ☐ British ☐ Irish ☐ Other White background ……	(b) BLACK or BLACK BRITISH ☐ Caribbean ☐ African ☐ Other Black background …….
(c) ASIAN or ASIAN BRITISH ☐ Indian ☐ Pakistani ☐ Bangladeshi ☐ Other Asian background ……..	(d) MIXED ☐ White and Black Caribbean ☐ White and Black African ☐ White and Asian ☐ Oher mixed background ……
(e) CHINESE or OTHER ETHNIC GROUP ☐ Chinese	

☐ Any other mixed background *please write in opposite*	

Country of origin of your

Parents:

_____

Grandparents:

_____

_____

Have you lived in the UK since birth?

If not, please, indicate the time period residing in UK: (yrs.)

SPORTS & PHYSICAL ACTIVITY STATUS

How would you assess your present level of fitness? **POOR / AVERAGE / HIGH**

Do you exercise regularly? **Yes / No**

If yes, how regularly do you take part in physical activity/sport? Please state the type of physical activity and how often? If you have multiple exercise schedules, please do not forget to list them all._____

_____

Approximately, how long do you exercise for on each occasion?

_____

How long have you been exercising regularly?

_____

On a Scale of 1 to 10, with 10 being highest, how strenuous would you say your each exercise routine is?

_____

Do you take part in other activities? Please tick the appropriate one from the list provided. If the activity is not present, please specify.

- Rowing / Canoeing / Kayaking
- Corps training
- Cycling
- Long distance running
- Walking/hiking
- Dancing
- Martial arts
- Mountaineering
- Rock climbing
- Skiing / Snowboarding
- Swimming
- Golf
- Gym

Other_____

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken, we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible, please consider making your translated version of

IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an International Physical Activity Prevalence Study is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active **in the last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise, or sport. Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include

unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

Yes

No Skip to **PART 2: TRANSPORTATION**

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2**.** During the **last 7 days**, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work?

Think about only those physical activities that you did for at least 10 minutes at a time.

_____ days per week

No vigorous job-related physical activity **Skip to question 4**

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?

_____ hours per day

_____ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.

_____ days per week

No moderate job-related physical activity **Skip to question 6**

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ hours per day

_____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

_____ days per week

No job-related walking **Skip to PART 2: TRANSPORTATION**

7. How much time did you usually spend on one of those days walking as part of your work?

_____ hours per day

_____ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days,** on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

_____ days per week

No traveling in a motor vehicle **Skip to question 10**

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

_____ hours per day

_____ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

_____ days per week

No bicycling from place to place **Skip to question 12**

11. How much time did you usually spend on one of those days to bicycle from place to place?

_____ hours per day

_____ minutes per day

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?

_____ days per week

No walking from place-to-place **Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY**

13. How much time did you usually spend on one of those days walking from place to place?

_____ hours per day

_____ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shovelling snow, or digging in the garden or yard?

_____ days per week

No vigorous activity in garden or yard Skip to question 16

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?

_____ days per week

No moderate activity in garden or yard Skip to question 18

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

_____ days per week

No moderate activity inside home Skip to PART 4: RECREATION,

SPORT AND LEISURE-TIME

PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

_____ hours per day

_____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during **the last 7 days**, on how many days did you walk for at least 10 minutes at a time in your leisure time?

_____ days per week

No walking in leisure time **Skip to question 22**

21. How much time did you usually spend on one of those days walking in your leisure

time?

_____ hours per day

_____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During **the last 7 days**, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

_____ days per week

No vigorous activity in leisure time **Skip to question 24**

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?

_____ hours per day

_____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During **the last 7 days,** on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

_____ days per week

No moderate activity in leisure time **Skip to PART 5: TIME SPENT SITTING**

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

_____ hours per day

_____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

_____ hours per day

_____ minutes per day

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

_____ hours per day

_____ minutes per day

This is the end of the questionnaire, thank you for participating.

Handgrip Exercise Schedule for 4-5 weeks

Participant Name& No:……

Week 1

	Intensity	Duration	Rest Periods	Frequency	**Tick achievement**
DomArm(….)	30% MVC	4 x 3 min.	4 x 5 min.	4x week	
30%MVC(kgs)					

Date:……

*Suggested Frequency of the exercise: Monday-Wednesday-Friday-Saturday or Sunday.

Week 2

Intensity	Duration	Rest Periods	Frequency	**Tick achievement**
30% MVC	4 x 3 min.	4 x 5 min.	4x week	

Date:……

*Suggested Frequency of the exercise: Monday-Wednesday-Friday-Saturday or Sunday.

Exercise Training Compliance visit (after 2wks)			**Date**
100% MVC (Avg.):		Blood Pressure (Avg.):	

Week 3

Intensity	Duration	Rest Periods	Frequency	**Tick achievement**
30% MVC: (Adj):	4 x 3 min.	4 x 5 min.	4x week	

Date:……

*Suggested Frequency of the exercise: Monday-Wednesday-Friday-Saturday or Sunday.

Week 4

Intensity	Duration	Rest Periods	Frequency	**Tick achievement**
30% MVC	4 x 3 min.	4 x 5 min.	4x week	

Date:……

*Suggested Frequency of the exercise: Monday-Wednesday-Friday-Saturday or Sunday.

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