Novel Interventions to Improve Cerebral and Peripheral Vascular Function

Joseph D Maxwell

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Abstract

Cardiovascular diseases (CVD) are major causes of morbidity and mortality worldwide. Complications associated with CVD encompass the entire vascular system, including blood vessels that supply brain. Impairments to cerebral blood flow (CBF) and cerebrovascular function result in increased risk of cognitive impairment, vascular dementia and stroke. Identifying interventions that can attenuate the age-related decline in CBF and enhance cerebrovascular function are essential, especially in individuals at high risk of CVD. Remote ischaemic preconditioning (rIPC), which involves cyclical periods of ischaemia-reperfusion applied noninvasively to a limb, has been shown to improve measures of peripheral vascular function and cardiovascular endpoints when applied acutely (one episode) or repeatedly (e.g. daily, 3 times per week). There is also emerging evidence that repeated rIPC elicits beneficial effects within the cerebral circulation in stroke patients. However, the mechanisms of how rIPC can improve cerebrovascular function are unknown. The overreaching aim of this thesis was to investigate whether acute and repeated rIPC could enhance cerebral and peripheral vascular function in cohorts with increased risk of CVD.

In a crossover study design, Study 1 aimed to assess the impact of a single acute bout of rIPC on cerebrovascular function. Eleven young healthy (28±4 years) and nine individuals at risk of CVD (53±7 years) underwent assessment of cerebrovascular function. Using Transcranial Doppler (TCD), markers of cerebrovascular function were assessed following either bilateral arm rIPC or sham condition. There was no change in middle cerebral artery velocity (MCAv) or blood pressure (BP) during rIPC. Application of rIPC did not alter cerebrovascular reactivity (CVR) compared to sham (0.002 MCA_{CVC}/mmHg, 95%CI= -0.001, 0.005, P=0.24), nor did it affect any parameter of dynamic cerebral autoregulation (dCA) (0.028 normalised gain%/$mmHg^{-1}$, 95%CI= -0.080, 0.137, P=0.59). This study suggested that an acute bout of rIPC does not influence cerebrovascular function in healthy young individuals and older subjects with increased risk for CVD.

In a randomised control-pilot design, Study 2 aimed to obtain estimates for the change in peripheral conduit and cerebrovascular function following a 7-day rIPC intervention. Twenty-one type 2 diabetes mellitus (T2DM) patients performed either 7-day daily rIPC or control (no rIPC). Peripheral conduit artery function was assessed using flow

mediated dilation (FMD) before and after an endothelial ischemia-reperfusion (IR) injury. Cerebrovascular function was assessed using TCD to examine dCA (0.10Hz squat stand manoeuvres). All measurements were performed at three time points; pre, immediately post intervention, and 8 days post intervention. Using pre-intervention data as a covariate, the change from pre-post in FMD was 1.3% (95%CI= 0.69, 3.80; P=0.09) and 0.23 %/mmHg^{-1} (95%CI -0.12, 0.59; P=0.18) in dCA normalised gain with rIPC versus control. The directional changes outline FMD can be enhanced by daily rIPC in patients with T2DM, whilst cerebrovascular function is unaltered.

In Study 3, in a randomised design, nineteen participants at risk of CVD were allocated into either 8 weeks of aerobic exercise training and rIPC (rIPC + Ex) or 8 weeks of rIPC only performed 3 times per week. Assessment of cerebrovascular function was performed using TCD and FMD was used to examine peripheral vascular function before and after an IR injury. Measurements were performed before (week 0) and immediately after (week 8) each intervention. Neither intervention changed resting CBFv, dCA (spontaneous or forced BP oscillations) or CVR. FMD increased by 1.6% (95% CI= 0.4, 2.8) in the rIPC + Ex intervention and by 0.3% (95% CI= -1.1, 1.5) in the rIPC only intervention, whilst no statistical difference was found between interventions (P=0.65). Data from this study suggests that combining exercise with rIPC does not result in greater changes in cerebral or peripheral vascular function.

In Study 4, dCA and baroreflex sensitivity (BRS) data collected during 0.10Hz squat stands manoeuvres was obtained from 206 individuals and analysed using transfer function analysis. Cross-sectional associations between ages were examined using linear regression adjusting for sex. Multivariable linear regression was used to adjust for sex, health status and VO_{2max}. Age, sex, CVD risk and VO_{2max} do not impact on dCA parameters normalised gain, phase or coherence (P>0.05). dCA (absolute) gain reduced with age when adjusting for sex, and CVD risk. The data from this study suggest that dCA parameters, when adjusted for BP, does not decline with age in either sex.

Collectively, the data contained within this thesis suggests rIPC interventions appear to be effective in improving peripheral endothelial function, but have little effect on cerebrovascular function. Additionally dCA, a frequently measured marker of

cerebrovascular function seems to be unaffected by aging, CVD risk factors or cardio-respiratory fitness.

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Ship?

Declaration

I declare that the work contained within this thesis is entirely my own.

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List of abbreviations

ACA	Anterior Cerebral Artery
ANOVA	Analysis Of Variance
AUC	Area Under The Curve
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CBF	Cerebral Blood Flow
CBFv	Cerebral Blood Flow Velocity
CbVC	Cerebro-Vascular Conductance
CO_2	Carbon Dioxide
CVD	Cardiovascular Disease
CVR	Cerebrovascular Reactivity
DBP	Diastolic Blood Pressure
dCA	Dynamic Cerebral Autoregulation
eNOS	Endothelial Nitric Oxide Synthase
FFA	Free Fatty Acid
FMD	Flow Mediated Dilation
HF	High Frequency
HR	Heart Rate
ICA	Internal Carotid Artery
ICP	Intracranial Pressure
IPC	Ischaemic Preconditioning
LF	Low Frequency
LMM	Linear Mixed Model
LSD	Least Significant Difference
MAP	Mean Arterial Pressure
MCA	Middle Cerebral Artery
MCAv	Middle Cerebral Artery Velocity
MCID	Minimal Clinical Importance Difference
MRI	Magnetic Resonance Imagining
nGain	Normalised Gain
NO	Nitric Oxide
NVC	Neurovascular Coupling
O_2	Oxygen
$PaCO_2$	Partial Pressure Of Carbon Dioxide
PCA	Posterior Cerebral Artery
PCAv	Posterior Cerebral Artery Velocity
$P_{et}CO_2$	Partial Pressure Of End Tidal Carbon Dioxide
rIPC	Remote Ischaemic Preconditioning
ROS	Reactive Oxygen Species
RPE	Rate Of Perceived Exertion
SBP	Systolic Blood Pressure
sCA	Static Cerebral Autoregulation
SD	Standard Deviation
SR_{AUC}	Shear Rate Under the Curve

T2DM	Type 2 Diabetes Mellitus
TCD	Transcranial Doppler
TFA	Transfer Function Analysis
VA	Vertebral Artery
VEGF	Vascular Endothelial Growth Factor
VLF	Very Low Frequency
VO_{2max}	Maximal Oxygen Uptake
VO_{2peak}	Peak Oxygen Uptake

Chapter 1: General Introduction

Cardiovascular disease (CVD) including cerebrovascular disease is the leading cause of death worldwide (Townsend et al., 2016.). Cerebrovascular disease is a general term encompassing different disturbances of the vascularisation of the brain (Truelsen et al., 2006). The human brain receives ~15% of total cardiac output and uses ~20% of oxygen available in order to maintain normal function, therefore tight regulation of blood flow and oxygen delivery is essential for survival (Willie et al., 2011). Chronic reductions in cerebral blood flow (CBF) and the mechanisms regulating stable CBF, referred to as cerebrovascular function, are associated with neurodegenerative diseases including dementia and Alzheimer's disease as well as stroke (Yonas et al., 1993, Mazza et al., 2011, Leijenaar et al., 2017). Interventions that target increasing CBF along with improving cerebrovascular function are essential in reducing the risk of cerebrovascular diseases. Ischaemic preconditioning (IPC) refers to non-lethal bouts of ischemia followed by reperfusion. IPC is a powerful technique that can protect the heart and the vasculature against prolonged ischemia (Thijssen et al., 2016a). There is emerging evidence within the last decade suggesting it can exert a positive effect on the cerebral circulation (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017).

The beneficial effects of IPC have been observed in the heart and the conduit arteries, i.e. at remote locations to where the ischemia-reperfusion stimulus was applied, which is termed *remote* IPC (rIPC) (Przyklenk et al., 1993). Importantly, application of rIPC can be performed non-invasively using a pressure cuff on limbs and still induces ischaemic protection (Kharbanda et al., 2001). Typically, a rIPC bout consists of 4 x 5 minute periods of limb cuff occlusion (ischemia) separated by 5 minutes of cuff deflation (reperfusion). Despite the frequently examined impact of rIPC on coronary

arteries, few studies have examined the impact of an acute bout of rIPC on cerebrovascular function. Therefore, aim 1 of this thesis was to identify whether an acute bout of rIPC increases CBF velocity (CBFv) or improve a marker of cerebrovascular function in young healthy and/or individuals with risk factors for CVD.

Increasing the 'dose' of the rIPC, may in theory provide longer or potent effects (Whittaker and Przyklenk, 2014) and this concept has been investigated by performing rIPC repeatedly as an intervention (Thijssen et al., 2016a), however few studies examined the human cerebrovasculature. The few studies that have employed repeated rIPC interventions and focused on cerebrovascular outcomes have identified positive clinical outcomes in stroke patients and individuals with cerebral small vessel disease following the rIPC interventions (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Whether these positive changes, which included increases in CBF and reduction in stroke reoccurrence, are related to improvements in the functional control of CBF (cerebrovascular function) is currently unknown. Individuals with Type 2 diabetes mellitus (T2DM) are at significantly higher risk of cerebrovascular disease and stroke (Chen et al., 2015) and are associated with impairments in vascular endothelial function (Hamilton and Watts, 2013). To date, one repeated rIPC intervention in T2DM proved effective in reducing the size of the diabetic foot ulcer over 6 weeks (Shaked et al., 2015), but little is known about the impact of daily rIPC on cerebrovascular function and peripheral vascular function in T2DM. Consequently, aim 2 of this thesis was to implement a 7-day daily rIPC intervention in patients with T2DM and assess changes in cerebrovascular function. .

The length of an rIPC intervention (in duration and frequency of rIPC bouts) might be related to a its effectiveness on the vascular system (Thijssen et al., 2016a). Indeed

rIPC interventions up to 1 year long have resulted in positive clinical outcomes (Meng et al., 2012, Mi et al., 2016). Recent work demonstrated that exercise has a potential preconditioning capacity. This allows for the opportunity to increase the preconditioning stimulus by combining IPC with exercise training. Incorporating an exercise training programme, alongside an rIPC intervention may add an additional, but not mutually exclusive, preconditioning stimulus for the vascular system. Accordingly, the combination of ischaemic and exercise preconditioning may increase the beneficial adaptations observed with repeated rIPC alone. Therefore, aim 3 of this thesis was to investigate whether 8 weeks of aerobic exercise combined with repeated rIPC could enhance cerebral and peripheral vascular function more than repeated rIPC alone in individuals with increased risk of CVD.

The regulation of stable CBF during changes in blood pressure (BP), termed dynamic cerebral autoregulation (dCA), represents an important functional marker of cerebral blood vessels regulating CBF (Willie et al., 2011) and is assessed in each study chapter of this thesis. Despite the growing body of literature, little is known about how age, sex and various CVD risk factors affect dCA. Similarly, there is currently conflicting evidence within the literature as to the mechanisms involved in dCA and to what extent control of BP, assessed with cardiac baroreflex sensitivity (BRS), is related to dCA. By identifying impairments in dCA, which can render the brain vulnerable to hyper- or hypoperfusion (van Beek et al., 2008), and understanding the contribution of cardiac BRS on dCA, appropriate interventions can then be developed to improving potential impairments or limitations. Therefore, aim 4 of this thesis was identify whether age, sex, cardio-respiratory fitness or CVD risk factors impact dCA and the contribution of the cardiac baroreceptors on dCA.

1.1 Aims

The specific aims of this thesis are to:

1. To assess the impact of bilateral arm rIPC on resting cerebral blood flow velocity, dynamic cerebral autoregulation and cerebrovascular carbon dioxide (CO_2) reactivity compared to a sham condition in both young healthy individuals and older individuals at risk of CVD.
2. Obtain estimates of the change in cerebrovascular function and peripheral conduit artery endothelial function before and after endothelial ischemia reperfusion (IR) injury following 7-days of daily limb rIPC in patients with type 2 diabetes.
3. Examine whether 8 weeks of exercise combined with repeated rIPC could enhance cerebrovascular and conduit artery function more than repeated rIPC alone in individuals with increased risk of cardiovascular disease.
4. Identify using squat stand manoeuvers and transfer function analysis whether age, sex, cardio-respiratory fitness or CVD risk factors impact dynamic cerebral autoregulation and the role of the cardiac baroreceptors.

1.2 Objectives

The aims outlined above will be achieved through the following objectives:

In line with **Aim 1**:

1. Utilising transcranial Doppler ultrasound, assess cerebral blood flow velocity during and following a bout of rIPC.
2. Compare the effects of acute rIPC vs a sham procedure on cerebral autoregulation and cerebrovascular CO_2 reactivity.

3. Identify whether acute rIPC can reduce the impairment to cerebral autoregulation induced by hypercapnia.
4. Identify if the effects of rIPC differ between the young healthy individuals and those at high risk of CVD.

In line with **Aim 2**:

5. Compare the effects of 7 days of daily upper arm rIPC versus non-rIPC control on conduit artery endothelial function before and after an induced temporary ischaemia reperfusion injury in patients with T2DM.
6. Compare the effects of 7 days of daily upper arm rIPC on cerebral blood flow velocity and dynamic cerebral autoregulation.
7. Establish if the effects of 7 days of daily rIPC are still present 8 days following the end of the intervention.

In line with **Aim 3**:

8. Engage individuals with increased risk of CVD in either an 8-week supervised moderate intensity aerobic exercise programme plus rIPC 3 times per week or 8 weeks of rIPC 3 times per week only.
9. Compare the effects of combining exercise training with rIPC verses rIPC alone on cardio-respiratory fitness, cerebral blood flow velocity and dynamic cerebral autoregulation and conduit artery endothelial function before and after an induced temporary ischaemia reperfusion injury.

In line with **Aim 4**:

10. Establish whether dynamic cerebral autoregulation is affected by age, sex, fitness and a number of cardiovascular disease risk factors in a large cohort of individuals aged 18-70 years.
11. Identify if there is a relationship between cardiac baroreceptor function and dynamic cerebral autoregulation.

Chapter 2: Literature Review

The focus of this literature review is two-fold; firstly, to outline cerebrovascular function measurements, the links with peripheral vascular function and the associated changes in the vasculature following interventions. Secondly, to introduce the concept of remote ischaemic preconditioning (rIPC), highlight the protective effects of rIPC on the heart and peripheral vessels and describe the potential impact of repeated rIPC as an intervention on the peripheral and cerebral vasculature.

2.1 The Cerebrovasculature and Cerebrovascular Function

2.1.1 Anatomy of the Cerebral Vasculature

Blood flow to the brain is supplied via four large extra-cranial arteries bilaterally; the internal carotid (ICA) and vertebral arteries (VA). Approximately 70% of total cerebral blood flow (CBF) is supplied through the ICA's, with the remaining ~30% of total CBF being delivered by the two VA's (Willie et al., 2014). The ICA continues upwards to the base of the brain to form the anterior (ACA) and middle (MCA) cerebral arteries bilaterally, whilst the basilar artery bifurcates to eventually form the posterior cerebral arteries (PCA) (Figure 2.1). The anterior circulation begins distal to the carotid sinus and supplies both the forebrain (frontal and parietal lobes) and midbrain (temporal lobes) on both left and right hemispheres. The ICA has a branch that feeds the ophthalmic artery, prior to trifurcating to form the MCA, ACA and posterior communicating artery. The anterior communicating artery allows the anterior circulation to supply both left and right hemispheres by joining the two ACAs. The MCA and ACA branches deliver blood throughout the brain through the smaller stem and cortical branches which feed into smaller arterioles before eventually feeding into the anterior cerebral capillary beds.

The posterior circulation arises from the two VAs joining together at the vertebro-basilar junction to form the basilar artery which supplies the hindbrain regions (brain stem and cerebellum) and the occipital cortices. The cerebral circulation is joined in a number of locations, which forms the vascular structure termed the Circle of Willis. This anatomical ring situated at the base of the brain connects the anterior and posterior circulations via the posterior communicating arteries (Figure 2.1).

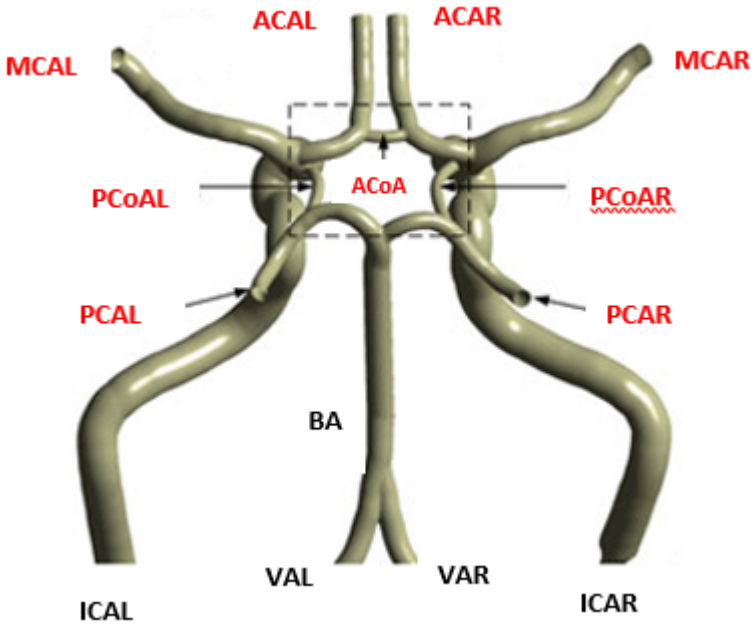

Figure 2.1: Anatomy of the cerebrovasculature including intra and extra cranial vessels. Abbreviations; Anterior cerebral artery (ACA), Middle cerebral artery (MCA), Posterior communicating artery (PCoA), Anterior communicating artery (ACoA), Posterior cerebral artery (PCA), Basilar artery (BA), Vertebral artery (VA), Internal carotid artery (ICA), left sided artery (L) & right sided artery (R). Adapted from Ren et al. (2015).

2.1.2 Regulation and control of Cerebral Blood Flow

In order to maintain normal functioning, the brain requires constant adequate nutritional flow due to its high metabolic demand. In resting conditions the brain is accountable for approximately 25% of total oxygen (O_2) consumption and receives 15-20% of total cardiac output (Q) (Franco Folino, 2007), a remarkable amount given the brain accounts for approximately 2% of total body mass. Despite the high metabolic demand, the brain has a very limited ability to store energy, therefore CBF needs to be highly regulated in order to maintain a constant supply of nutrients and O_2 (Peters et al., 2004). CBF is determined by cerebral perfusion pressure (CPP) and cerebrovascular resistance (Ainslie and Duffin, 2009, Tzeng and Ainslie, 2014). CPP is the difference in BP and intracranial pressure (ICP), with the latter formed from central venous pressure and pressures within the cerebrospinal fluid (Ainslie and Duffin, 2009). Cerebrovascular resistance refers to the resistant forces acting on blood flow through the brain. Resistance to flow occurs mostly in the cerebral arteries and capillary beds, with increasing vascular tone in turn increasing resistance (Ainslie and Duffin, 2009).

Measurement of CBF can provide information of the functional status of blood vessels supplying the brain. The non-invasive nature and high temporal resolution make transcranial Doppler (TCD) ultrasound an ideal instrument for the assessment of cerebral blood flow velocties (CBFv), in centermeters per second ($cm.s^{-1}$) as reviewed in detail by Willie et al. (2011). TCD was first used in 1982, and has since been extensively used in order to assess CBFv (Aaslid et al., 1982). The basic principle of TCD ultrasound is a transmitter emitting pulsed ultrasound waves from a Doppler probe at 2 MHz. Due to its thin acoutic window, the ultrasound waves are transmitted

through the temporal region of the cranium to assess a vessel of interest. The assessment of CBFv is derived from the Doppler shift, created by the reflection of ultrasound waves from moving erythrocytes (red blood cells) within the blood vessel that are returned to the receiver unit in the Doppler probe. Simply, the Doppler shift refers to the difference between the transmitted and received ultrasound signals (Aaslid, 1986), with faster erythrocyte movement associated with higher velocities, and by extension, blood flow. Using TCD, the ACA, MCA and PCA can be assessed, in addition to the Basillar artery and Vertebral arteries. Vessels can be insonated from four different accoustic windows; Transorbital, Transtemporal, Submandibular and Transforaminal (Figure 2.2).

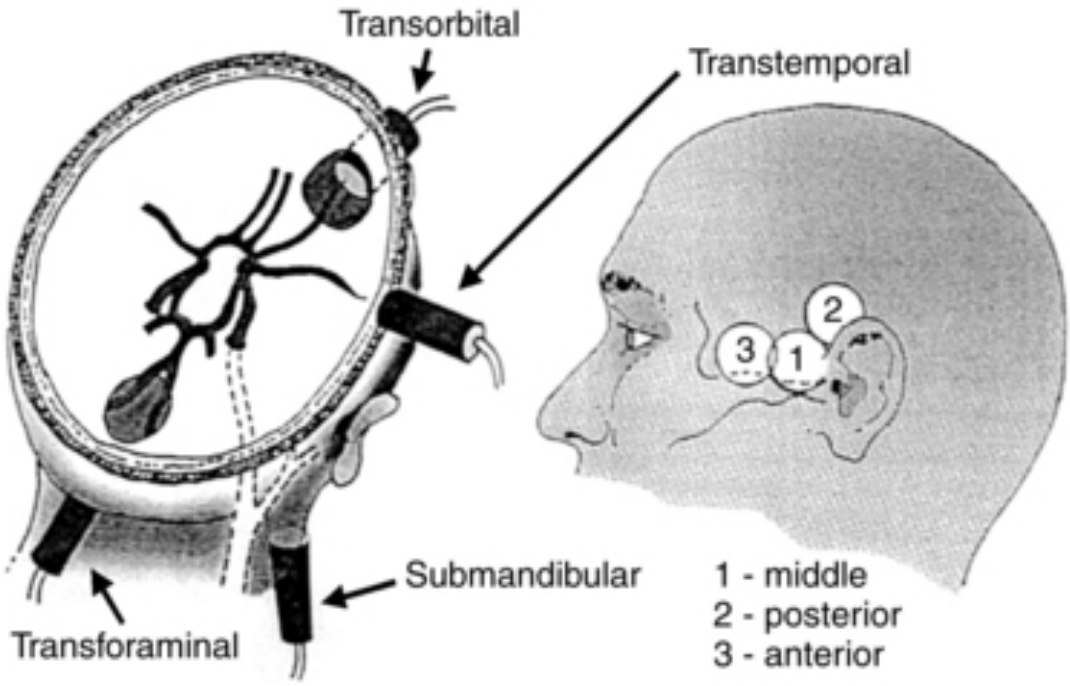

Figure 2.2: Acoustic windows used in order to insonate cerebral blood vessels.

Vessel identifcation is achieved based on knowledge of anatomical structure of the circle of Willis. (Willie et al., 2011). Understanding of isonation depths and flow

direction of the vessel of interest is vital in order to aquire reliable and valid signals (Table 1.1**)**. Additionally, certain stimuli can be applied in order to confirm correct vessel isonation including; carotid artery compression will result in reduction in MCAv, whereas PCAv will remain largerly unchanged. In addition, PCAv will increase by 15-20% with activation of occitital lobe (eyes open-eyes closed) whereas MCAv will have small response to this stimulus (<5%). Typically, the MCAs are used to examine CBF and cerebrovascular function as they account for ~80% of total CBF and have the closest proximity to the temporal window (Skow et al., 2013).

Table 1.1: Typical patterns for identification of cerebral arteries using Transcranial Doppler ultrasound

Vessel	**Probe Directon**	**Depth (mm)**	**Flow Direction**	**Ipsilateral carotid compression**	**Contralteral caroitd compression**
ACA	Anterior	60-75	Away	Flow reversal	Increased velocity
MCA	Perpendicular	35-60	Toward	Reduced velocity	No change
PCA	Posterior	55-70	Toward	No change or incresed velocity	No change

Abbreviations; Anterior cerebral artery (**ACA)**, Middle cerebral artery (**MCA**), Posterior cerebral artery (**PCA**).

The regulation of CBF is an integrative and complex process with a number of mechanisms involved including; BP (cerebral autoregulation), cerebral metabolism (neurovascular coupling), chemical control (cerebrovascular reactivity) and autonomic nervous system (Willie et al., 2011, Willie et al., 2012, Willie et al., 2014). The main regulatory features of the cerebral circulation applicable to this thesis are:

2.1.3 Blood Pressure (Cerebral Autoregulation)

Autoregulation of CBF refers to the brain's intrinsic ability to maintain a relatively constant blood flow despite changes in arterial BP. The autoregulatory response to changes in arterial BP protects against brain injury caused by potential hyper- or hypoperfusion. Indeed, evidence shows that brain injury does occur when autoregulatory mechanisms are lost (Novak et al., 1998, Euser and Cipolla, 2007). This pressure-flow relationship was noted as early as 1895, when Bayliss, Hill and Gulland concluded that '*In all physiological conditions a rise in arterial pressure accelerated the flow of blood through the brain, and a fall slackens it'* (Bayliss et al., 1895). In 1959 Lassen published a review plotting average BPs and related total CBF from seven studies, including 11 different patient groups. Lassen identified that there appears to be a plateau region, whereby CBF appears to be stable across a wide range of BPs (60-150 mmHg) (Lassen, 1959). More recent studies have established that the regulation of CBF is more pressure passive than originally suggested by Lassen (1959), with evidence now showing a 0.82 % ΔCBF/ mmHg ΔMAP^{-1} (Lucas et al., 2010). Subsequently, this so called 'autoregulatory plateau' outlined by Lassen (1959) may not exist, or in fact exists but within a considerably smaller range (± 5-10 mmHg from baseline) (Tan, 2012) (Figure 2.3).

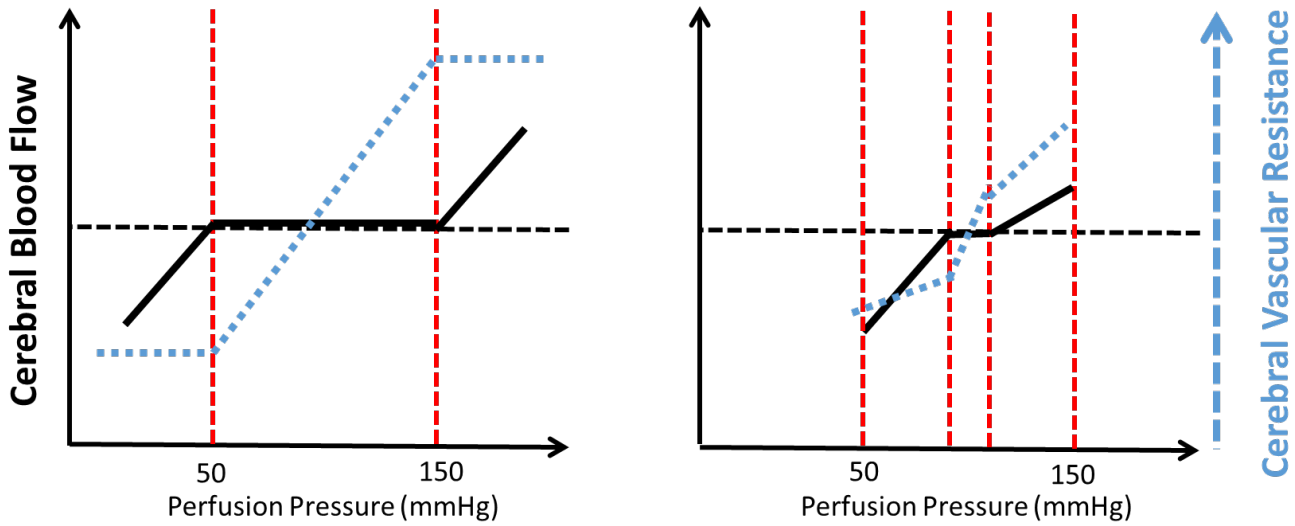

Figure 2.3: Representation of the classical (left) and modern (right) interpretation of cerebral autoregulation. Red dotted lines represent the original 'autoregulatory plateau' (left) and the modern interpretation (right). Blue dotted lines represent cerebrovascular resistance; black dotted line represents stable cerebral blood flow; black solid line represents cerebral blood changes. Adapted from (Willie et al., 2014)

Cerebral autoregulation can be classified into static (sCA) or dynamic (dCA). sCA refers CBF responses to long-term (minutes) changes in BP whereas dCA refers to acute (seconds) changes (Aaslid et al., 1989, Tiecks et al., 1995). Assessment of dCA has arisen from the ability to monitor beat-to-beat changes in cerebral blood flow velocity (CBFv) and mean arterial pressure (MAP) concurrently (Willie et al., 2014). Adequate CBF is maintained across a range of BPs due to changes in cerebrovascular resistance. Vasodilation occurs in response to decreases in BP and vasoconstriction as a result of increase in BP within the upper and lower limits of autoregulation (Figure 2.4) (Pires et al., 2013). The functions together ensure sufficient blood flow reaches in the brain (Willie et al., 2014). Fascinatingly, it appears that cerebral vascular resistance is more effective at maintaining CBF during increases in CBF compared to decreases, however this difference is lost when studies corrected for changes in $PaCO_2$ (Numan et al., 2014).

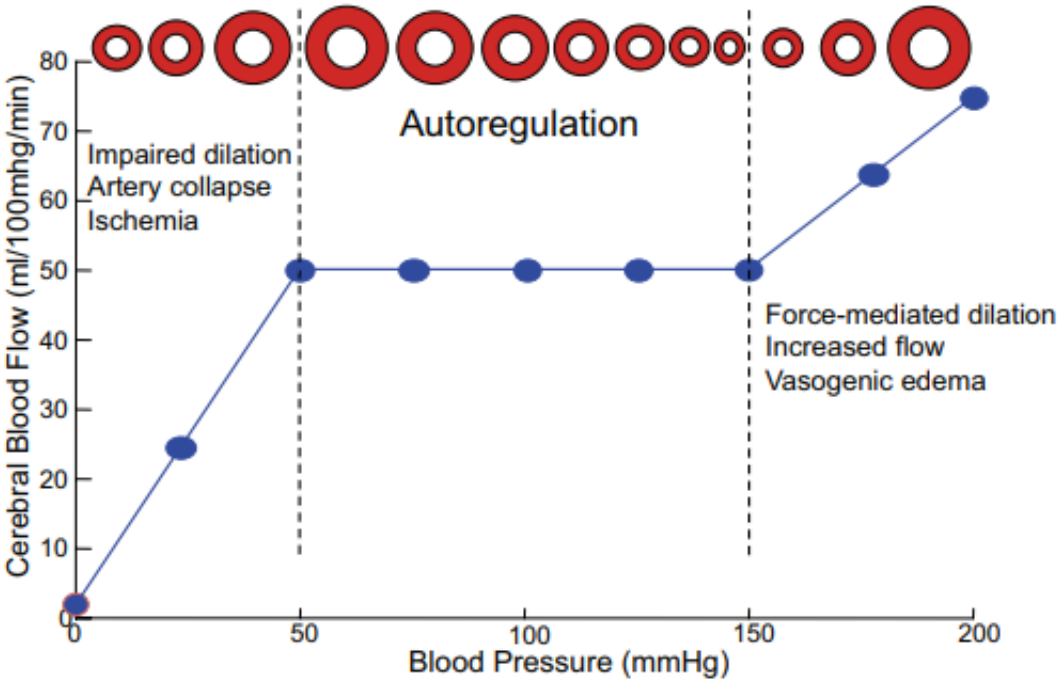

Figure 2.4: Cerebral blood flow in relation to artery lumen diameter. Dotted lines represent the lower and upper limits of cerebral autoregulation. Red circles represent the cerebral arteries, and blue line represents cerebral blood flow. Adapted from (Pires et al., 2013).

The underlying mechanism(s) controlling this pressure-flow relationship remains unclear and are likely to differ with increases vs decrease in BP. It is likely that these mechanisms are multifactorial and include neurogenic, metabolic, myogenic and endothelial factors (Tzeng and Ainslie, 2014) and impairment has been observed in some clinical conditions. A reduction in autoregulation (rate of regulation) has been reported in T2DM (Vianna et al., 2015), acute ischaemic stroke (Eames et al., 2002) obstructive sleep apena (Urbano et al., 2008) and sporadic Alzheimer's disease (den Abeelen et al., 2014). When patients display impaired cerebral autoregulation, the brain may be excessively sensitive to any changes in arterial BP and a failure to the

autoregulatory response has been associated with increased morbidity and mortality (Hu et al., 2008).

The mechanism(s) controlling dCA are still largely unknown but are believed to vary depending on the frequency range in which the changes in arterial BP occur. A number of studies have attempted to investigate the underlying mechanisms primarily using blockades and a number of interesting observations have been made. In the very low frequency (VLF: <0.07 Hz), this is thought to be under the influence of myogenic properties as shown by studies using cholinergic blockades (Hamner et al., 2012, Tan et al., 2013) and ganglion blockade (Zhang et al., 2002). Within an intermediate frequency range; low frequency (LF; 0.07-0.20 Hz), is controlled by more sympathetic influences, as demonstrated by sympathetic agonist drugs midazolam (Ogawa et al., 2010) and antagonist drugs prazosin (Purkayastha et al., 2013). The highest frequency (HF; >0.20) range, the range in which CBF is most poorly regulated is largely under the control of normal respiration rate (Reinhard et al., 2003). Nevertheless, clear and definitive answers to the mechanism controlling dCA still require significant investigations. The relationship between myogenic, sympathetic and local neuronal mechanisms and their control of dCA is uncertain and new innovative measurement methods are required to unlock the true controlling mechanisms.

Despite the growing interest in assessing dCA the question remains as to the exact location in which the regulation takes places. Dilation and constriction has been shown to occur in pial vessels in response to fluctuations in BP (Fog, 1938, Lassen, 1959). On the other hand, there is data to suggest that larger cerebral arteries respond to the changes in BP as well as the extra cranial vessels within the neck (Heistad et al., 1978, Kontos et al., 1978). Studies to investigate the main site(s) of dCA are however

extremely difficult to perform in humans. Future studies employing advanced imaging techniques are required to answer such questions.

Typical assessment of dCA is carried out with the combined use of TCD and finger photoplethysmography. In order to examine the dynamic response between CBFv and BP, transient changes in BP are induced. Whilst there is no 'gold standard' method available for the assessment of dCA (Panerai, 1998), a number of techniques have been previously used to achieve these rapid fluctuations in BP. Instant deflation of bilateral thigh cuffs after a period of inflation to supra-systolic levels was used to induce transient decreases in BP (Sorond et al., 2009), however this technique is associated with high levels of discomfort as well as a variability in the levels of BP change with each cuff deflation. More recently, a simple squat to stand (or sit to stand) procedure has been found to produce transient changes in BP, yet with less discomfort than the bilateral thigh cuffs (Lipsitz et al., 2000). Repeated squat-stand manoevers has now emerged a popular method to assess dCA and has been performed in a range of clincal and older populations (Claassen et al., 2009a, Aengevaeren et al., 2013, Oudegeest-Sander et al., 2014, Smirl et al., 2014a, Lewis et al., 2019) and is representative of BP fluctations experienced in daily life (Simpson and Claassen, 2018).

2.1.3.1 Baroreflex Sensitivity

Both dCA and baroreflex sensitivity (BRS) are key mechanisms that maintain stable CBF (de Heus et al., 2018). The baroreflex regulates BP via baroreceptors which detect changes in arterial BP. BRS reflects the complex interaction between autonomic and vascular function to manage BP fluctuations (Subramanian et al., 2019). The

baroreceptors (stretch receptors in the walls of the aorta and carotid artery) send signals via the vagus and glossopharyngeal nerves to the Nucleus Tractus Solitarius when a rise or drop in BP is detected which results in either increased vagal nerve activity and a reduction in sympathetic activity or vice versa, in order to maintain BP (Parati and Bilo, 2012). Importantly, BRS carries prognostic value in the development and progression of a number of cardiovascular diseases (CVD) including; hypertension, coronary artery disease, myocardial infarction and heart failure, with sympathetic-parasympathetic imbalances contributing to the complications (La Rovere et al., 2008). The assessment of BRS can be performed using several different techniques which have been reviewed in detail (La Rovere et al., 2008). Pharmacological techniques involve the infusion of vasoactive drugs to induce BP changes whilst monitoring heart rate. A popular none-invasive method includes the neck chamber technique which specifically manipulates the carotid baroreceptors (Fadel et al., 2003), whereas cardiac BRS can be assessed using the relationship between R-R interval and systolic BP (Zhang et al., 2009, Dutoit et al., 2010, Aengevaeren et al., 2013). Evidence suggests that cardiac BRS provides a good estimate of overall baroreflex function when measurements of sympathetic outflow are unavailable (Taylor et al., 2015) and has been validated against the Modified Oxford technique (Horsman et al., 2013) which is often referred to as the 'gold standard' method of BRS assessment (Gasch et al., 2011, Hissen et al., 2018).

Impairment to cardiac BRS may lead to orthostatic hypotension which can result in cerebral hypoperfusion or syncope (Zhang et al., 2009). Some (Tzeng et al., 2010, Witter et al., 2017), but not all (Aengevaeren et al., 2013, Xing et al., 2017) studies have observed an inverse relationship between cardiac BRS and dCA. Identifying the

relationship between BRS and dCA represents the fourth aim of this thesis and is presented in chapter 6.

2.1.4 Cerebral metabolism (neurovascular coupling)

CBF is closely linked to metabolic activity as activations of regions of the brain result in changes to local CBF (Willie et al., 2014). This relationship between neural activity and CBF is termed neurovascular coupling (NVC), and whilst the coupling between neural activity and increases in blood flow is well known, the exact underlying mechanisms are still not fully understood (Phillips et al., 2016). Cerebral blood vessels are structurally unique and different from other arteries based on their close relationship with neurons and glia. The close relationship is between the smooth muscle of the vessel walls, the neuron and the astrocyte glial cell together form what is known as the neurovascular unit. NVC is impaired in certain pathological conditions such as ischaemic stroke, Alzheimer's and hypertension (Girouard and Iadecola, 2006, Lefferts et al., 2018), resulting in the CBF response not meeting the metabolic requirement of the tissue.

Whilst the relationship between brain neural activity and CBF has been known for over a century (Roy and Sherrington, 1890), the mechanisms responsible for this response are not fully understood. When the supply of oxygen, glucose and other nutrients required by the brain are adequate, the smooth muscle cells and pericytes are thought to be in a state of basal tone (Muoio et al., 2014). Activation of the neuron sends a signal to the astrocyte via the release of glutamate, increasing calcium with the astrocyte. This causes a reaction within the astrocyte that in turn leads to the release of vasoactive substances from the end-feet causing pericytes and vascular smooth

muscle cells to hyperpolarise. The hyperpolarisation of these cells initiates either vasodilation (release or NO, adenosine or arachidonic acids) or vasoconstriction (release of endothelin or thromboxane) of the local cells (Muoio et al., 2014). The change in local vascular tone regulates a change in local vascular resistance, increasing or decreasing CBF.

2.1.5 Chemical Control (Cerebrovascular Reactivity)

The partial pressure of arterial carbon dixoide ($PaCO_2$) has a sigificant impact on CBF. An increase in $PaCO_2$ (hypercapnia) results in cerebral arteriolar vasodilation, whereas a decrease in $PaCO_2$ (hypocapnia) will cause vasocontriction reducing CBF (Ainslie and Duffin, 2009). This highly sensitive relationship between changes in $PaCO_2$ and CBF responses is termed cerebrovascular CO_2 reactivity (CVR) and is recognised as a crucial homestoatic function that helps maintain and regulate central pH and cellular function (Ainslie and Duffin, 2009). This response can be viewed as a defence mechanism, whereby elevations in $PaCO_2$ leads to an increase in CBF in an attempt to "wash out" CO_2 from brain tissue. Consequently, the increase in CBF contributes to normalising or reducing the rise in $PaCO_2$. Typically, the slope of cerebrovascular response to CO_2 is approximately 3-6% increase per mmHg in $PaCO_2$ above rest and 1-3% reduction per mmHg decrease below resting values (Willie et al., 2012). This means that the cerebrovasculature is more sensitive to a given increase in PaCO2 compared to a decrease in $PaCO_2$. It has been suggested that this lower responsiveness to hypocapnia (compared to hypercapnia) may be a protective mechanism to avoid cerebral ischaemia during reductions in $PaCO_2$, which do occur in a number of

physiological conditions (e.g. exercise or change in posture) as well as pathophysiological (syncope) (Ainslie and Duffin, 2009).

The term CVR reflects an index of the ability of the cerebral vessels to dilate or constrict in response to changes in arterial blood gases content, typically CO_2. It is believed that this serves as a control mechanims for respiration-induced changes in CO2. Within the brainstem respiratory chemoreceptors are present which are highly sensitive to alterations in pH. CVR to changes in CO_2 has been applied in clinical practice to evalute cerebrovascular function. One large scale project 'The Rotterdam Study' demonstrated in a cohort of 1695 participants, lower cerebral reactivity to 5% CO_2 stimulus was assosiated with increased risk of all-cause death (Portegies et al., 2014). In addition, lower response to a CO_2 stimulus has been observed in patients with; Alzheimers (den Abeelen et al., 2014), multiple sclerosis (Marshall et al., 2014) and heart failure (Georgiadis et al., 2000). Clinically, this impairment may represent dysfunction from a number of mechanistic inputs. The mechanims responsible for the role of CO_2 in regulating vascular tone and pH are not fully identified. Activation of potassium (K^+) channels in the vascular smooth muscle is a potential mechanism which has received attention given its ability to relax (dilate) cerebral vessels (Ainslie and Duffin, 2009). Additionally, nitric oxide (NO) and prostaglandins may play a role in the hypercapnic induced vasodilation as a result of increased shear stress (Ainslie and Duffin, 2009) and that an individuals reponse to CO_2 depends on the intergrity of the vascular endothelium (Ainslie et al., 2007). Indeed, mechanistic studies have identfitied the role of shear-stress mediated vasodilation as a result of increased CO_2 suggesting the use of such measurements to quanify cerebrovascular endothelial function (Carter et al., 2016, Hoiland et al., 2017).

The cerebrovasculature is not only sensitive to changes in CO_2, but oxygen (O_2) also plays a role in vascular tone. Hypoxia effects the cerebrovasculature, but only when PaO_2 levels drop below ~50mmHg (Willie et al., 2012, Willie et al., 2014). Acute hypoxia induces increases in CBF before plateauing (Harris et al., 2013) and the impact of hypoxia on cerebral autoregulation is still debated (Subudhi et al., 2010, Smirl et al., 2014b). The response to hypoxia however is still affected by CO_2 given that studies have shown it is the ventilatory response to the hypoxia which ultimiately determines the CBF response, as hypercapnia increase and hypocapnia decreases the sensitivity of the cerebrovasculare to hypoxia (Mardimae et al., 2012, Willie et al., 2014).

CVR has typically been assessed in one of three potential ways. The earliest technique used was breath holding, whereby participants would hold their breath in order to progressively increase $PaCO_2$. This technique has a number of limitations. First, individual variability between individuals in factors such as; gender, size of lungs, age all affected the CBF response to breathe holding (Fierstra et al., 2013). A second potential strategy relates to the rebreathing of exhaled gas, which became and remains a common technique to achieve increases in $PaCO_2$. This method requires minimal equipment with just an exhaled gas reservoir and gas sensors necessary. Limitations to this strategy is that rebreathing of exhaled gas causes a ramp-like response with each breath causing an increase in $PaCO_2$. This prevents performing a stable measurement of increased CBF. A third and final strategy is the use of external CO_2 supplied to a participant by means of a non-rebreathing face mask, with inspired CO_2 concentrations ranging from 2-7% (Fierstra et al., 2013). This method is favourable as it can induce a standardised hypercapnic stimulus (Vernieri et al., 2004) and is used throughout this thesis.

2.1.6 Cerebral and Peripheral vascular function

Functional markers of the cerebral and peripheral circulations can change simultaneously. Improvements in cerebral and peripheral vascular function are seen with exercise training (Bailey et al., 2013, Tarumi et al., 2015) and reductions in both have been observed with Alzheimer's and stroke (Silvestrini et al., 2006, Dede et al., 2007). Collectively, assessment of cerebral and peripheral vascular function is important in providing a representation for systemic vascular function. However, impairment in one artery does not seem interchangeably with changes in another artery.

The endothelium is a single layer of cells that forms the inner lining of the entire circulatory system. Once considered a passive layer of inert cells, the vascular endothelium is now known to be highly biologically active. The endothelium occupies a strategically important location between the circulating blood and vessel wall and has the ability to respond to changes in its physical, chemical or humoral environment (Rubanyi, 1993). The endothelium continuously produces the freely diffusible gas nitric oxide (NO) at a low basal rate, which helps maintain the health of the vessel wall and regulate vascular tone. NO is a potent anti-atherogenic molecule, inhibiting platelet and leukocyte adhesion and a powerful vasodilator (Green et al., 2011). Increases in blood flow, therefore increases in intimal shear stress (the fricitional drag force exerted by the blood flow on the arterial wall), represents the physioloigcal stimulus for the release of NO (Pohl et al., 1986).

Assessment of peripheral vascular function can be performed non-invasively on peripheral conduit arteries using the flow mediated dilation (FMD) technique (Celermajer et al., 1992). The dilation that occurs during an FMD can be significantly attenuated/abolished using NO blockade (Joannides et al., 1995, Lieberman et al.,

1996, Mullen et al., 2001, Kooijman et al., 2008), making the FMD a largely NO-dependent test of endothelial function, Collectively, the evidence from these physiological studies reinforces the validity of the technique as an assessment of endothelium dependent and NO-specific index of endothelial function (Thijssen et al., 2011). FMD is reduced with CVD risk factors, relates to coronary artery endothelial function, and independently predicts CVD outcomes (Thijssen et al., 2019b). Additionally, it is recognised as a reproducible technique in examining the acute and long-term impact of physiological and pharmacological interventions in humans (Thijssen et al., 2011, Thijssen et al., 2019b).

2.1.7 Interventions targeting cerebral and peripheral function

2.1.7.1 Cerebrovascular function

Reductions in cerebrovascular function are strongly associated with clinical conditions, whilst pharmacological approaches represent some of the interventional strategies to enhance CBF, CBFv and cerebrovascular function. For the purpose of this thesis, studies focusing on cardio-respiratory fitness and exercise interventions are summarised below.

2.1.7.2 Exercise training in healthy humans

Humans display a natural age-related decline in CBF, however evidence does show that maintaining high cardiorespiratory fitness does attenuate this age-related decline (Ainslie et al., 2008b). This maintenance of CBF could potentially explain lower rates of cerebrovascular disease/incidents in individuals that are physically more active (Ainslie et al., 2008b). A small number of studies have implemented structured exercise interventions in an attempt to increase CBF and improve cerebrovascular

function or attenuate the age-related decline, but the results are conflicting. Eight weeks of moderate aerobic cycling evoked an increase in CBFv in postmenopausal women (Akazawa et al., 2012), whilst others found 8-12 weeks of aerobic exercise does not induces changes in CBFv in healthy young and older individuals (Murrell et al., 2013, Lewis et al., 2019). A 6 week HIIT intervention in endurance trained men resulted in no change in CBFv and a slight reduction in dCA (Drapeau et al., 2019). This reduction in dCA with elevated cardio-respiratory fitness has been noted in a small number of other studies (Labrecque et al., 2017, Labrecque et al., 2019a), which is somewhat confusing given that elevated cardio-respiratory fitness has been linked to increased CBFv (Ainslie et al., 2008b) and better CVR (Bailey et al., 2013). Taken together, various factors, including intervention duration, exercise type and intensity, and population contribute to whether exercise training changes CBFv and cerebrovascular function. Furthermore, functional markers of the cerebrovascular system appear to respond differently to exercise training, strongly suggesting a need for further research to understand exercise training (Barnes and Corkery, 2018).

2.1.7.3 Exercise training in clinical populations

As outlined in section 2.1, reductions in CBF and cerebrovascular function often manifest in various patient cohorts. Therefore, exercise interventions, which have the potential to improve both may be of greater importance for individuals at increased CVD risk. A small number of studies have examined the impact of exercise training on CBF and cerebrovascular function in clinical groups. Eight weeks of aerobic training in patients with chronic obstructive pulmonary resulted in no change in cerebrovascular function (dCA & CVR) or CBFv (Lewis et al., 2019), whilst in individuals with mild cognitive impairment, 12 weeks of moderate aerobic training

lead to increased CBF and cognitive function (Alfini et al., 2019). Stroke survivors following 6 months of aerobic treadmill exercise improved CVR (Ivey et al., 2011) yet no such changes were evident following 18 weeks of training in congestive heart failure patients (Tanne et al., 2005). The patient population being assessed in each study may represent a critical factor as to whether the exercise intervention improves CBF or functional markers. Another factor that may influence the impact of exercise training on CBF is the duration of the intervention, especially as increase in CBF was typically found in those adopting a longer period of training. It is clear that more research is warranted to understand possible beneficial effects of exercise on cerebrovascular health in clinical and preclinical populations.

2.1.7.4 Peripheral vascular function

As outlined in section 2.1.6, peripheral vascular function is frequently used as a marker for systemic endothelial function (Thijssen et al., 2019b). Impaired peripheral vascular function (endothelial dysfunction), in the form of reduced FMD has been associated with conditions predisposing atherosclerosis and CVD (Charakida et al., 2010). The following sub section will summarise the literature that employed exercise interventions to change peripheral vascular function in healthy and clinical populations

2.1.7.5 Exercise training in healthy humans

Peripheral vascular function measured using FMD demonstrates a progressive decline with age as the result of reduced NO bioavailability (Celermajer et al., 1994, Thijssen et al., 2016b). However, a recent meta-analysis which pooled data from 14 studies

concluded that long-term aerobic exercise appears to attenuate the age related decline in FMD (Campbell et al., 2019). In young healthy individuals, a number of studies have shown that short-term exercise training increases vascular function (FMD) (Green et al., 2004, Tinken et al., 2010, Birk et al., 2012, Schreuder et al., 2015) with prolonged training inducing structural adaptations (i.e. increased artery diameter) (Green et al., 2012, Green et al., 2014b). This sequence of functional improvements preceding structural changes appears now to be a consistent observation. Studies involving 2-weekly measurements across an 8-week period of large (i.e. cycling/running exercise) (Tinken et al., 2008, Birk et al., 2012) or small muscle group (i.e. handgrip exercise) (Tinken et al., 2010) training in healthy young volunteers have consistently demonstrated that 2 weeks of exercise training is sufficient to significantly enhance vascular function. This initial rapid increase in vascular function is often normalized after 6–8 weeks of training as arterial structural remodelling occurs (Tinken et al., 2008, Green et al., 2017). These observations support the idea that exercise training leads to time-dependent adaptation in conduit artery function, which is superseded by arterial remodelling (Schreuder et al., 2015).

2.1.7.6 Exercise training in clinical populations

Vascular endothelial dysfunction is present in various clinical and pre-clinical groups such as hypertension, obesity and T2DM (Widmer and Lerman, 2014). Exercise represents an intervention that has numerous physiological benefits including improved endothelial function. Brachial artery FMD was enhanced following 8 weeks of aerobic training in patients with T2DM (Schreuder et al., 2015), with a similar 12 week intervention improving FMD in adolescents with T2DM (Naylor et al., 2016).

Numerous systematic reviews and meta-analysis have been performed concluding exercise improves peripheral vascular function in heart failure (Pearson and Smart, 2017), T2DM (Qiu et al., 2018), cancer survivor (Beaudry et al., 2018) and obese (Dias et al., 2015) patients. Different exercise types have evoked vascular improvements, with an 8-week HITT intervention increasing FMD in a cohort of obese individuals (Sawyer et al., 2016) and interestingly Boff *et al* concluding that HITT was more effective in improving FMD compared to moderate training in patients with type 1 diabetes (Boff et al., 2019).

In summary, tight regulation of CBF is essential in maintaining normal functioning and reducing the risk of cerebrovascular disease/complications. Nevertheless, the impact of exercise interventions on cerebrovascular function is unclear in both healthy and diseased groups. Unlike the changes in peripheral vascular function, measuring using FMD, that on balance suggest positive peripheral vascular functional changes with exercise training. Therefore, alternative or additional interventions might provide a larger stimulus to improve cerebrovascular function.

2.2 Ischaemic Preconditioning

In 1986, investigators discovered that brief intermittent episodes of ischaemia had protective effects on the myocardium that was later subject to a sustained bout of ischaemia (Murry et al., 1986). Murry and co were the first to identify the phenomenon known as ischaemic preconditioning (IPC) which involved 5 minutes of canine left anterior descending coronary artery occlusion, followed by 5 minutes of reperfusion repeated 4 times. Evidence of cardioprotective effects following IPC was supported when a number of follow-up studies confirmed their original observations (Li et al.,

1990, Murry et al., 1990, Murry et al., 1991, Ovize et al., 1992). These data contributed to the concept that exposure to (non-lethal) cardiac ischaemia in the period preceding coronary ischaemia may protect against the impact of reperfusion of the occluded artery on the magnitude of myocardial damage.

2.2.1 Remote IPC

Przyklenk and colleagues performed a landmark study in 1993, in which they demonstrated that cyclical ischaemia and reperfusion of the circumflex coronary artery was associated with protection of cardiac territory supplied by the left anterior descending artery (i.e. an area *remote* from the preconditioning stimulus of the circumflex coronary artery) (Przyklenk et al., 1993). These findings from Przyklenk's group provided the first evidence demonstrating the effects of what is now termed 'remote' ischaemic preconditioning (rIPC).

Preclinical studies investigating the impact of rIPC on the heart have classically collected perfusate from animals who have been subject to IPC. When naive hearts have subsequently been perfused using a Langendorff preparation, a number of studies have identified that in both the donor heart subject to the IPC and the naive recipient heart that has received the perfusate from the preconditioned donor, infarction size was significantly smaller following a prolonged ischaemic event (Dickson et al., 1999, Huffman et al., 2008). These finding demonstrated the ability of rIPC to reduce the damage induced by ischaemic injury in remote areas, potentially through a blood-borne pathway. Intriguingly, evidence has shown the rIPC can provide protection between-species, since rabbit hearts displayed protection against a prolonged bout of cardiac ischaemia when perfused with human preconditioned serum (Shimizu et al., 2009, Michelsen et al., 2012). This suggested a similarity in the factors conferring

protection across species, and that such agent/s remain conserved during such procedures, allowing binding to the recipient receptors.

Clinical human studies exploring the effects of rIPC have typically applied the rIPC cycles using a blood pressure cuff on a limb, before (planned) prolonged myocardial ischaemia. A small number of studies have investigated the use of rIPC in patients undergoing coronary artery bypass graft (CABG) or percutaneous coronary interventions (PCI), as these procedures involve global myocardial ischaemia (and subsequently cardiac damage) as shown by elevated cardiac troponins post-procedure. Consequently, strategies that can attenuate the damage as a result of global myocardial ischaemia have clinical relevance. Meta-analyses (Brevoord et al., 2012, D'Ascenzo et al., 2012) have identified lower peri-postoperative levels of troponins in patients undergoing CABG and PCI if rIPC is applied. More importantly, evidence suggests that rIPC may reduce overall perioperative myocardial infarction (Thielmann et al., 2013, Candilio et al., 2015). For example, Thielmann et al. (2013), demonstrated in 329 patients undergoing CABG proceeding a bout of rIPC, reduced post-CABG troponin concentrations and also lowered all-cause mortality after a 1.5 year follow-up. Therefore, rIPC may in turn provide long-term clinical benefits to humans.

In 2010, Bøtker and colleagues explored this concept in humans by randomizing patients with suspected acute myocardial infarction to a rIPC group or control group during transition to hospital (Botker et al., 2010). They identified that rIPC on transition along with standard treatment was associated with better myocardial salvage, assessed by myocardial perfusion imaging, compared to standard care alone. Intriguingly, a follow-up performed on the same patient group approximately 3.8 years later reported that the rIPC treated patients experienced fewer major adverse cardiac

and cerebrovascular effects (Sloth et al., 2014). Whereas it is important to present the evidence of the beneficial effects of rIPC, it is also essential to acknowledge the work that has found no positive clinical impact. In a large scale randomised control trial including 1612 patients undergoing CABG, rIPC did not improve clinical outcomes in patients (Hausenloy et al., 2015), with a similar observation in patient requiring cardiopulmonary bypass for cardiac surgery (Meybohm et al., 2015)

Whilst the beneficial effects of rIPC on the cardiovascular system has attracted attention, its effect on the cerebrovascular system has certainly received less. Nevertheless, there is a growing body of animal research that supports the notion that rIPC provides protection against ischaemic injuries in the cerebrovascular system and provides neuroprotection. This is of clinical relevance as brain ischaemia is one of the leading causes of morbidity and mortality in the world (Liu et al., 2009). Hoyte *et al*., (2006) conducted a study in mice and employed 15 minutes of MCA occlusion 72 hours prior to inducing a 45-minute injurious cerebral ischaemia. They reported an increase in regional brain blood flow during the injurious occlusion, measured via magnetic resonance perfusion and laser Doppler flowmetry in the mice that received the IPC as well as a reduction in infarct size (Hoyte *et al.*, 2006). Ren and colleagues extended this work by applying rIPC to the left femoral artery of rodents, 2 days prior to inducing cerebral ischaemia. They reported that rIPC was associated with a reduction in infarction size compared to the animals with no preconditioning (Ren et al., 2008). Jensen and colleagues also observed that pigs who received rIPC to the limb, displayed an accelerated recovery in neurological function and ECG pattern and demonstrated a lower brain lactate concentration compared to controls following ischaemic injury as a result of hypothermic circulatory arrest (Jensen et al., 2011).

These data provide evidence that (r)IPC can offer cerebrovascular and neural protection against cerebral ischaemia. Nevertheless, research in humans is warranted to be able to translate this into clinical practice. This will be the focus of study 1 within the current thesis.

2.2.1.1 Ischaemia-reperfusion injuries

When arterial blood flow is blocked, it is essential that blood flow is restored in order to prevent irreversible tissue damage. When organs become ischaemic, the deprivation of blood flow and oxygen have long been recognised as the critical factor in clinical outcomes. However, the reperfusion phase (restoring of blood flow) is not without consequence and induces even further cellular damage that is greater than the damage caused by the ischaemia itself (Eltzschig and Collard, 2004). Evidence for this was shown when 3 hours of ischaemia followed by 1 hour of reperfusion induced cellular damage that exceeded that of the damage caused by a 4 hour ischaemic protocol (Parks and Granger, 1986). Cellular damage induced by the reperfusion phase following a period of ischaemia is referred to as ischemia reperfusion injury (IRI). IRI can occur in a variety of clinical scenarios; thrombolytic therapy, organ transplant, coronary angioplasty, cardiopulmonary bypass or aortic clamping (Eltzschig and Collard, 2004) as well in cardiovascular events/disorders such as; myocardial infarction, stroke and peripheral vascular disease (Kalogeris et al., 2012). This highlights the clinical relevance to minimise IRI.

Ischaemia promotes a proinflammatory state that intensifies tissue vulnerability to any further damage during reperfusion. The extent of the injury caused by a period of ischaemia is heavily influenced by the duration of the blood flow restriction (Eltzschig and Collard, 2004) and crucially the organ in which is affected by the ischaemia, with

the brain being the most sensitive organ to reductions in blood supply (Ordy et al., 1993). When reperfusion occurs following ischaemia, levels of oxygen are rapidly restored and the extracellular pH returns to normal. Even with this restoration of oxygen and extracellular pH, the intracellular pH remains acidic and this pH gradient facilitates extrusion of H^+ from the cell in exchange for Na^+ (Murphy and Steenbergen, 2008). Additionally, increases in ROS during ischaemia and re-oxygenation is believed to be due to damage to components of the electron transport chain resulting in inefficient transfer of electrons resulting in the generation of superoxides. The significant increase in ROS production induces further oxidative damage to cellular structures (Murphy and Steenbergen, 2008). IRI occurs in a wide range of organs including the heart, gut, kidney, lung, skeletal muscle and brain and may involve not only the ischaemic organ itself but may also induce systemic damage to distant organs, potentially leading to multi-system organ failure. Reperfusion injury is a multi-factorial process resulting in extensive tissue destruction and vascular interventions to improve IRI outcomes are important. A growing body of evidence suggests that rIPC has the ability to reduce the level of cellular tissue damage following an IRI, both planned or unplanned (Heusch et al., 2015).

2.2.2 Repeated rIPC interventions

The 'traditional' doses of rIPC applied in the majority of studies have principally used 4 x 5 minutes of occlusion followed by reperfusion. Whittaker and Przyklenk outlined the potential use of repeated rIPC, referring to this traditional dose (4x5 minutes) but applied more often (e.g. daily or weekly) (Whittaker and Przyklenk, 2014). Given the potent effects of a single dose of rIPC, repeated episodes may, in theory, provide longer or more potent reduction of ischaemic myocardial damage (Whittaker and

Przyklenk, 2014). Table 2.1 provides a summary of all human studies to do date that have employed repeated rIPC interventions.

Table 2.1: Overview of all published studies to date investigating repeated (remote) ischaemic preconditioning interventions

Author (Year)	Population	rIPC or IPC	IPC protocol	Duration	Unilateral or bilateral	Control Included	Findings
Kimura et al. (2007)	Healthy young	rIPC	6 x 5 minute daily	4 weeks	Unilateral	Yes	↑ Vascular function (venous occlusion plethysmography infusion) ↑ Endothelial progenitor cells
Shimizu et al. (2010)	Healthy middle aged	rIPC	3 x 5 minute daily	10 days	Unilateral	No	↓ Neutrophil adhesion ↓ Phagocytosis
Wei et al. (2012)	Rats (myocardial infarction)	rIPC	4 x 5 minute daily	28 days	Unilateral	Sham	↓ Infarct size ↓ LV remodelling
Meng et al. (2012)	Intracranial arterial stenosis	rIPC	5 x 5 minute daily	300 days	Bilateral	Yes	↓ Stroke recurrence ↑ Cerebral perfusion
Luca et al. (2013)	Healthy young	rIPC	3 x 5 minute daily	7 days	Unilateral	No	↑ Vascular function (FMD)
Kono et al. (2014)	Healthy middle aged	rIPC	4 x 5 minute twice daily	7 days	Unilateral	No	↑ Coronary flow reserve ↔ LV end-diastolic volume
Jones et al. (2014)	Healthy young	rIPC & IPC	4 x 5 minute daily	7 days	Unilateral	No	↑ Bilateral vascular function (FMD) ↑ Bilateral skin perfusion
Kono et al. (2014)	Heart failure	rIPC	4 x 5 minute twice daily	7 days	Unilateral	No	↑ Coronary flow reserve ↔ LV end-diastolic volume
Jones et al. (2015)	Healthy young	rIPC & IPC	4 x 5 minute, three times per week	8 weeks	Unilateral	Yes	↑ Bilateral vascular function (FMD) ↔ Bilateral skin perfusion
Liang et al. (2015)	Coronary heart disease	rIPC	4 x 5 minute three times daily	20 days	Unilateral	Yes	↑ Vascular function (FMD) ↑ eNOS mRNA levels ↑ Endothelial progenitor cells

Yamaguchi et al. (2015)	Rats (myocardial infarction)	rIPC	5 x5 minute daily	4 weeks	Bilateral	Yes	↑ LV ejection fraction ↑ LV diastolic function
Meng et al. (2015)	Intracranial arterial stenosis	rIPC	5 x 5 minute twice daily	180 days	Bilateral	Sham	↓ Stroke recurrence
Shaked et al. (2015)	Type 1 and 2 diabetics	rIPC	3 x 5 minute, every 14 days	6 weeks	Bilateral	Sham	↓ Diabetic ulcer wound size
Mi et al. (2016)	Cerebral small-vessel disease	rIPC	5 x 5 minute twice daily	1 year	Bilateral	Sham	↓ White matter lesions ↑ middle cerebral artery velocity
Lindsay et al. (2017)	Healthy young	rIPC	4 x 5 minute daily	7 days	Unilateral leg	Sham	↑ Mean cycling power ↑ VO_{2peak}
Wang et al. (2017)	Cerebral small-vessel disease	rIPC	5 x 5 minute twice daily	1 year	Bilateral	Sham	↓ White matter hyperintensities ↓ Cognitive decline
Pryds et al. (2017)	Heart Failure	rIPC	4 x 5 minute daily	28 days	Unilateral	NO	↓ Blood pressure
Ahmed et al. (2018)	Claudication patients	rIPC	4 x 5 minutes every 4 days	28 days	Unilateral	Yes	↑ Improvements in pain-free walking distance ↑ Ankle-brachial pressure indices
(Jeffries et al., 2019)	Healthy young	rIPC	4 x 5 minute daily	7 days	Bilateral leg	Sham	↑ Time to exercise exhaustion ↑ Tissue oxygenation during submaximal cycling
HyTong et al. (2019)	Hypertensive patients	rIPC	3 x 5 minutes daily	30 days	Bilateral	No	↓ Blood Pressure (clinic and ambulatory) ↑ Micro-vessel endothelial function

Abbreviations: eNOS, endothelial nitric oxide synthase; FMD, flow mediated dilation, rIPC; remote ischaemic preconditioning, LV; Left ventricular, IPC; Ischaemic preconditoning Symbols: ↑ Increase, ↓ decrease, ↔ no change.

2.2.2.1 The myocardium

Only a relatively small number of studies on repeated rIPC have focused directly on the myocardium and the ability of episodic rIPC to impact the magnitude of myocardial damage. For example, 40 patients with coronary artery disease who were scheduled for CABG surgery were randomized to a 20 day period of repeated rIPC (three rIPC sessions daily, n = 20) or a control intervention before surgery (n = 20). Patients undergoing repeated rIPC demonstrated approximately 50% lower troponin expression levels after CABG compared with the control group (Liang et al., 2015). The ability of repeated rIPC to reduce postoperative cardiac troponin is in line with previous work on single rIPC scheduled before CABG. Wei et al. (2011) explored different protocols of rIPC in rats undergoing planned cardiac ischaemia, including a single episode of rIPC and repeated (every 3 days versus daily) rIPC across 28 days post-injury. Although the reduction in infarct size on days 4 and 28 was comparable across the protocols, repeated rIPC was associated with a dose-dependent protection against adverse remodelling and improved survival. In parallel with these findings, work from Yamaguchi et al. (2015) divided rats post-myocardial infarction into a 4 week repeated rIPC group and a control group and reported that repeated rIPC prevents adverse cardiac remodelling (and fibrosis in the boundary region).

A short one week rIPC intervention, consisting of twice-daily unilateral arm rIPC increased coronary flow reserve, through improvements to coronary microcirculation in both healthy participants as well as in patients with heart failure (left ventricle ejection fraction <40%) (Kono et al., 2014), suggesting the potential use of daily rIPC in the lives of patients with heart failure. Additionally, a 28 day rIPC intervention consisting of daily limb rIPC in patients with chronic ischaemic heart failure

demonstrated a significant reduction in systolic BP, the N-terminal pro-brain natriuretic peptide (as a marker of heart failure) and improved global longitudinal strain in patients with the most severe heart failure (Pryds et al., 2017)

The potential effects of repeated rIPC are not isolated to reducing the magnitude of cardiac damage following prolonged ischaemia. Repeated rIPC interventions appear to also have an effect on vascular endothelial function. Given that previous work has demonstrated that improvements in peripheral and coronary vascular function is related to lower risk for future cardiovascular events (Green et al., 2011), this is of particular importance.

2.2.2.2 The peripheral vasculature

Kimura et al. (2007) were the first to examine the effects of repeated rIPC following 28 days of rIPC on forearm resistance artery endothelial function in healthy individuals. They provided evidence that repeated rIPC improved resistance artery endothelial function via increase in NO production and numbers of endothelial progenitor cells (EPCs). Luca et al. (2013) followed this work by exploring if repeated (daily) rIPC altered the efficacy of a single bout of rIPC against an induced ischaemia-reperfusion injury. In line with their hypothesis, daily rIPC provided more sustained protection against an endothelial IRI compared to a single bout, measured with FMD. More recently, these findings of enhanced vascular endothelial function were extended when it was found that 7 days of daily rIPC improved both brachial artery FMD and skin perfusion (microvascular function), whilst these benefits were evident in the ipsilateral (exposed to IPC stimulus) and the contralateral arm (remote location to rIPC stimulus) (Jones et al., 2014). Importantly, these effects of the repeated rIPC intervention persisted for 7 days after the end of the intervention, suggesting that

improvements are related to a more prolonged adaptation rather than a transient change (Figure 2.5).

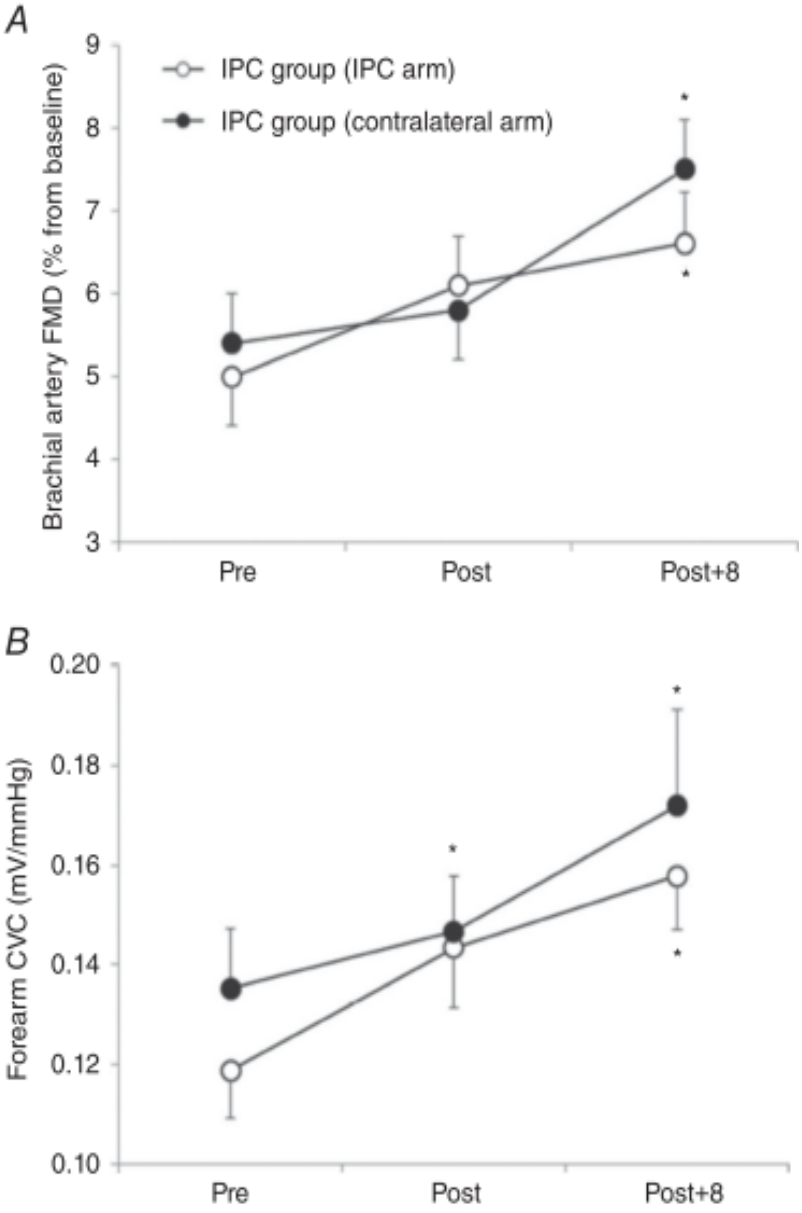

Figure 2.5: (A) Brachial artery flow-mediated dilation (FMD); expressed as a percentage and (B) resting forearm cutaneous vascular conductance before (Pre), after (Post) and 8 days after (Post+8). the 7 day daily IPC intervention in the IPC (open circles) and contralateral arm (filled circles) of healthy volunteers (n = 13). Error bars represent SEM. ∗Post hoc significantly different from day 0. Adapted from (Jones et al., 2014).

It has been suggested that infarct-sparing effects from rIPC are present for 1–2 h following an rIPC episode (early phase), whereas these protective effects return after 24 h for a period of 3–4 days (late phase) (see section 2.2.4). Although described in relation to protection of the ischaemic myocardium, a similar pattern may also be found in peripheral vessels. Therefore, repeated rIPC bouts may be timed 24–72 h apart to ensure that the tissue will be exposed simultaneously to the 'early phase' and 'late phase'. Given the relatively long duration of the 'late' phase of protection, Jones and colleagues explored the impact of less frequent and more spaced rIPC bouts by administering rIPC for 8 weeks (3 cycles per week) (Jones et al., 2015). They identified that rIPC significantly increased brachial artery FMD and that these improvements were apparent after two weeks (six rIPC bouts) in young healthy males. The impact of repeated rIPC as an intervention on vascular function, including the impact on endothelial IRI, in individuals with vascular dysfunction is currently unknown and will be investigated in patients with T2DM in study 2 and CVD risk factors in study 3 of the present thesis.

In support of repeated rIPC having a beneficial impact on those with endothelial dysfunction, patients with T2DM have complications which are vascular in nature where ischaemia plays a critical role i.e. in the development of diabetic foot ulcers. In one study, patients with diabetic foot ulcers were randomised into 6 weeks of bilateral (upper limb) rIPC or control group (no rIPC). The ratio of patients who reached complete healing of their ulcer was significantly higher in those who received repeated rIPC compared with the control group (41 versus 0%, respectively), and the remaining ulcer area was smaller (25 versus 61%, respectively) (Shaked et al., 2015). A higher

prevalence of wound healing is of major clinical significance given that 20% of patients with diabetic foot ulcers ultimately require amputation (Eldor et al., 2004).

2.2.2.3 The cerebrovasculature

Research examining the impact of repeated rIPC on cerebrovascular function and clinical outcomes in humans is limited. Two large-scale clinical trials have been conducted examining the potential benefits of repeated rIPC on human cerebrovascular function in patients with intracranial arterial stenosis. The studies employed bilateral limb rIPC twice daily for 300 days and found a significant reduction in stroke recurrence along with a significant increase in cerebral perfusion and improved modified Rankin Scale score (measurement scale for neurological disability) (Meng et al., 2012). Remarkably, the same group later observed a significant reduction in ischaemic events following a course of 180 days of bilateral limb IPC, twice daily, applied within an older population of IAS patients, with no adverse changes to heart rate or arterial BP (Meng et al., 2015). Additionally, a one-year rIPC intervention involving bilateral rIPC twice daily applied to patients with cerebral small vessel disease outlined that rIPC can help in slowing down cognitive decline as well as reduce white matter hyperintensities (Wang et al., 2017). The use of rIPC as an additional treatment for ischaemic stroke has been reviewed in detail (Landman et al., 2019), this based on preclinical and clinical evidence demonstrating that rIPC is feasible, safe and free of adverse incidents in the clinical populations studied thus far. Landman *et al* discusses how rIPC related to the brain is a few steps behind that of cardiology in terms of research and advocates future studies exploring rIPC. Whilst the current studies provide indirect evidence that rIPC may have an impact on cerebrovascular function in humans, no study to date has directly assessed

how the cerebrovasculature is changing to mediate beneficial improvement in stroke risk and cognitive function. The focus of studies 2 and 3 in the present thesis will investigate the impact of repeated rIPC on cerebrovascular function in individuals with CVD risk factors.

2.2.2.4 Phases of rIPC

rIPC has a characteristic temporal nature, involving two independent 'phases' of protection. The acute phase is believed to arise immediately after the rIPC and disappears approximately 2 hours after, and a delayed phase that appears around 24 hours after the preconditioning stimulus, lasting longer but regarded as less protective and potent (Heusch et al., 2015). The acute protection depends on immediate recruitment of signalling molecules, whereas increased expression of protective proteins is a hallmark of the delayed protection (Bolli et al., 2007). Whilst endogenous NO, generated from endothelial and inducible nitric oxide synthase does not seem to be involved for immediate protection, it is involved in the delayed protective effects of IPC (Bolli et al., 1998). The contribution of NO to the late phase of rIPC was further supported when a NO synthase inhibitors abolished the impact of rIPC in an animal model (Takano et al., 1998). Expression of other cardioprotective proteins has also been shown to be unregulated during the delayed protection (e.g. cyclo-oxygenase-2, superoxide dismutase (Zhou et al., 1996, Guo et al., 2000). These effects on the upregulation of (cardioprotective) proteins may contribute to the sustainability of the IPC stimulus and vascular adaptation (Thijssen et al., 2016a). Therefore, repetitive upregulation of these proteins, usually noted during the delayed/late protective phase, may be of relevance in understanding the effects of repeated IPC on sustainable

improvement in vascular function. Whether these effects actually translate to the peripheral and/or cerebral vascular beds, also taking into consideration the presence of potential deleterious remodelling, is currently unknown.

An alternative explanation for the local effects/adaptations to vascular function induced by rIPC may relate to the changes in haemodynamics. The repeated exposure to the ischaemia-reperfusion cycle causes elevations in blood flow (shear stress) during each reperfusion phase. Repeated episodes of increases in shear stress are recognised as an important stimulus in vascular adaptations given that such adaptations are not evident when shear stress is attenuated (Green et al., 2010, Tinken et al., 2010).

2.2.2.5 Potential mechanisms of (r)IPC and repeated rIPC

Whilst their remains no definitive mechanism identified for the cardioprotective and vascular effects of rIPC, there has been a number of potential mechanisms proposed. It is important to acknowledge that there may be differences in the mechanisms responsible for benefits of a single bout of rIPC and repeated rIPC interventions (Thijssen et al., 2016a). It is also important to acknowledge that identifying the mechanisms of rIPC is not the focus of this thesis. In-depth reviews related to potential mediating mechanisms have been published (Hausenloy and Yellon, 2008, Heusch et al., 2015, Thijssen et al., 2016a). A summary of potential mechanisms is provided below.

The classical view of the protective effects of rIPC relates to the following cascade; a trigger is released (in the occluded limb) and acts as a stimulus to activate a mediator, which in turn transmits a protective signal onto an effector that attenuates injury in

remote location (vascular and cardiac) in to response to ischaemia-reperfusion. The activation of these pathways is highly complex and is likely to involve the input of multiple signals in order to confer the protection (Hausenloy and Yellon, 2008, Heusch et al., 2015, Thijssen et al., 2016a).

The episodes of rIPC applied to the limb induce subsequent transduction of the local signal to the remote tissue (or organ), but is dependent on intact neural and humoral pathways (Lim et al., 2010). The autonomic nervous system has been shown to play a role in the protective effects of rIPC, given that infusion of Trimethaphan (autonomic ganglion blocker) abolished the effects of rIPC on vascular function pre and post IRI (Loukogeorgakis et al., 2005). Remarkably, local nerve stimulation appears to have a similar effect to rIPC on cardioprotection, whereas a nerve blocker of the peripheral nerve abolished that protection (Redington et al., 2012). With regards to cerebral protection conferred by rIPC, evidence again suggests a neural component. Several studies have demonstrated blockades of sensory inputs attenuate the beneficial/protective effects of rIPC in animal models (Malhotra et al., 2011, Wei et al., 2012).

Cardioprotection from rIPC is also mediated through circulating, blood-borne hormones that are able to protect remote (cardio)vascular regions against prolonged ischaemia (Dickson et al., 1999), with recent evidence supporting a potential role for NO, microRNA-144 and stromal-derived factor 1α (SDF-1α) (Heusch et al., 2015). In the target organ, signal transduction pathways are activated that ultimately contribute to the protection against ischaemic injury. In the heart, signal transduction of RIPC 'shares' that of local IPC, with at least significant involvement of NO (and endothelial nitric oxide synthase), protein kinase C and the RISK pathway that ultimately work on the mitochondria (Heusch et al., 2015).

The production of circulating hormones represents one logical mechanism that might contribute to the effects of repeated IPC on both the ability to ameliorate damage after prolonged ischaemia and the sustained adaptation in remote vascular function. The role of circulating hormones in the protection against myocardial injury was first highlighted by (Dickson et al., 1999) who found that coronary effluent from a preconditioned heart induced cardioprotection in a naïve acceptor heart. Subsequently, several studies adopting the Langendorff model suggested the presence of a blood-borne substance that confers protection when infused in an organ exposed to prolonged ischaemia. Despite the scientific and clinical importance, identifying the substance or substances that explain the effects of rIPC has proved challenging.

2.3 Summary

In summary, tight regulation of CBF and the mechanims controlling CBF (i.e. cerebrovascular function) is essential in reducing the risk of cerebrovascular disease. rIPC is a technique which has shown potential in improving a number of cardiovascular parameters. The majority of the research has focused on cardioprotection against IR injuries, whilst only a very small number of studies have explored the use of rIPC as intervention to improve parameters within the cerebral circulation. Nonetheless, these latter studies report remarkably positive clinical outcomes in a cohort of patients with history of cerebrovascular complications (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Yet, no study has investigated whether and/or how rIPC, applied either acutely or repeatedly, is effective in enhancing CBF via improvements in cerebrovascular function in individuals at risk of future complications.

Chapter 3: The impact of acute remote ischaemic preconditioning on cerebrovascular function

3.1 Introduction

Remote ischaemic preconditioning (rIPC) is a technique that offers enhanced hypoxic tolerance and protection to systemic organs and tissues following repeated brief periods of ischaemia and reperfusion to a remote vascular bed (Lim and Hausenloy, 2012). This phenomenon, mediated via a neural and/or humoral pathway (Shimizu et al., 2009, Jensen et al., 2012), was first described in canine hearts (Przyklenk et al., 1993) with subsequent studies demonstrating its efficacy in humans. More specifically, rIPC has been reported to reduce cardiovascular events in patients following coronary artery bypass and percutaneous coronary intervention surgeries (Thielmann et al., Davies et al., 2013a), and reduce brachial artery endothelial ischemia reperfusion damage (Kharbanda et al., 2002). Given these broad potent protective effects, it is possible that rIPC may also affect the brain and cerebral vasculature.

Animal studies have reported rIPC-mediated neuroprotection in the form of reduced infarct size and improved neurological recovery following prolonged cerebral ischaemia and hypothermic circulatory arrest (Ren et al., 2008, Jensen et al., 2011). Extending these findings to humans, a study in stroke survivors reported increased cerebral perfusion and 70% lower stroke recurrence following daily rIPC for 300 days, compared to a group of patients receiving standard care (Meng et al., 2012). This protective effect was reinforced in a recent study in acute stroke patients where repeated application of rIPC significantly improved clinical status and reduced National Institutes of Health Stroke Scale scores (England et al., 2017), while rIPC was found to significantly reduce white matter hyperintensities volume in small vessel disease patients (Wang et al., 2017). Strict regulation of brain blood flow is crucial for

the maintenance of cerebrovascular health and is impaired in numerous clinical groups, including stroke survivors. Based on previous observations that repeated rIPC improves peripheral macro- and microvascular health in humans (Kharbanda et al., 2002, Jones et al., 2015), the observed benefits of rIPC on cerebrovascular health may be related to acute improvements in cerebrovascular function *in vivo*. Assessing the impact of rIPC on cerebrovascular function would *i*) extend the fundamental understanding of the acute effects of RIPC in humans, and *ii)* may provide insight into how rIPC mediates neuroprotection and further establish it as a novel therapeutic strategy in clinically vulnerable groups.

The primary aim of this proof of principle study was to assess the impact of bilateral arm rIPC on resting CBFv, dCA and CVR to carbon dioxide (CO_2) in healthy individuals, compared to a sham condition. To examine the effectiveness of rIPC across a broader spectrum of vascular health, also included were participants at an increased risk for CVD and stroke. Finally, previous studies have reported that hypercapnia (induced by inhalation of higher concentrations of CO_2) transiently disrupts dCA and has been used as a model for impaired cerebral autoregulation (Zhang et al., 1998, Panerai et al., 1999, Ainslie et al., 2008a, Jeong et al., 2016). Therefore, the secondary aim of this study was to assess the ability of rIPC to attenuate hypercapnia-induced impairment of dCA. It was hypothesised that rIPC would improve dCA and CVR, while attenuating the hypercapnia-induced impairment in dCA, when compared to a sham condition in both young healthy individuals and those with increased cardiovascular risk

3.2 Methods

3.2.1 Participants

Twenty participants were recruited for the study (Healthy; n=11 [Females n=6] and CVD risk; n=9 [Females n=4], Table 3.1). Healthy young participants (age; 28.1±3.7yrs) were recreationally active, engaged in low to moderate intensity exercise 2-3 days per week, and were free from cardiovascular diseases, including diabetes, hypertension or hypercholesterolemia. For the second group, older individuals (52.5±6.7 yrs) with cardiovascular risk factors were recruited based on having ≥1 of the following criteria; body mass index >30kg/m^2 or a waist circumference ≥94cm (male), ≥80cm (female), blood pressure systolic >130/diastolic >85 mmHg or diagnosed with high cholesterol (total >200 mg/dL, triglycerides >150 mg/dL, LDL >100 mg/dL). Smokers, individuals with previous angina or myocardial infarction, transient ischaemic attack or stroke and thrombosis were excluded from participation. Participants were informed of the study protocol verbally and in writing before providing written informed consent. The study was approved by the University Research Ethics Committee (16/SPS/019) and adhered to the standards set out in the Declaration of Helsinki.

Table 3.1: Participant characteristics.

Characteristics	Healthy individuals	CVD risk individuals	*P* Value
	n=11; Female=6	n=9; Female=4	
Age (years)	28 ± 4	53 ± 7	<0.001
Height (cm)	173.1 ± 10.1	169.4 ± 10.3	0.44
Weight (kg)	71.7 ± 13.6	93.6 ± 23.9	0.02
BMI (kg/m^2)	24 ± 3	32 ± 6	<0.001
MAP (mmHg)	89 ± 4	104 ± 3	<0.001
$PetCO_2$ (mmHg)	37.8 ± 2.0	40.2 ± 2.9	0.10
MCAv ($cm.s^{-1}$)	70 ± 15	54 ± 8	0.02

Values are means ± SD. BMI; body mass index, MAP, mean arterial pressure; $PetCO_2$; partial pressure of end tidal carbon dioxide; MCAv, middle cerebral artery velocity.

3.2.2 Research Design

Participants attended the laboratory on two occasions (separated by a minimum of 3 days). All tests were performed at the same time of day to control for diurnal variation in cerebrovascular function (Ainslie et al., 2007). All participants arrived at the laboratory for testing following an overnight fast and had refrained from alcohol, exercise and caffeine for 24h prior to each visit. Visits were randomised and counterbalanced to receive either the bilateral upper arm rIPC or the sham condition. Each visit consisted of the bilateral assessment of MCAv during rIPC or sham. Following this cerebral autoregulation was assessed using a 5 min squat-stand protocol (0.10 Hz). This was then proceeded by a 5 minute rest period, followed by 4 minutes of hypercapnia (5% CO_2) and then another 5 minute squat-stand (0.10 Hz) protocol

but whilst breathing 5% CO_2 (Figure 3.1). The phase of menstrual cycle was not controlled for in the female participants.

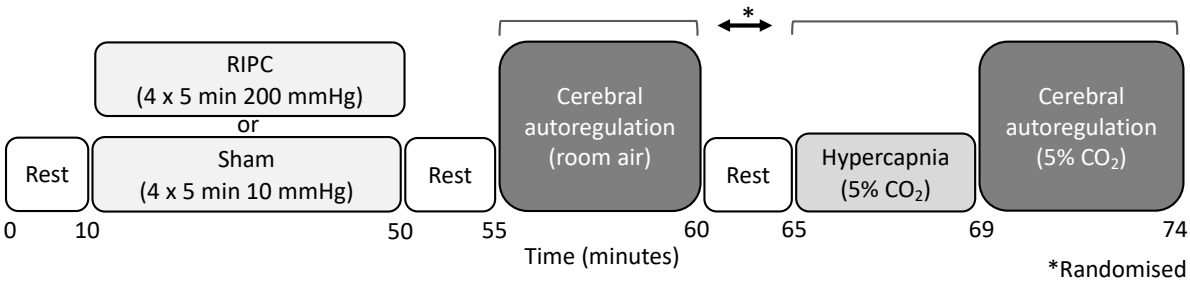

Figure 3.1: Schematic of the protocol for each testing visit.

3.2.3 Measurements

Remote ischaemic preconditioning and sham. The rIPC condition consisted of 8 bouts in total involving the inflation of a pneumatic cuff (Hokanson SC10D; USA) on the upper arm using a rapid inflator (EC-20; D.E Hokanson) to 220 mmHg for 5 minutes. Cuffs were inflated in an alternating fashion allowing for one arm to be occluded while the contralateral arm underwent reperfusion. The sham condition consisted of the identical protocol with the difference that the cuff pressure was inflated to only 10 mmHg.

Cerebral blood flow (middle cerebral artery blood velocity). Following 20 minutes rest in the supine position, bilateral MCAv's were continuously measured through the temporal window using TCD. Two 2-MHz Doppler probes (Spencer Technologies, Seattle, USA) were adjusted until an optimal signal was identified and held in place using a Marc 600 head frame (Spencer Technologies, Seattle, USA). Once the optimal MCAv signal was attained, the probe location and machine settings (depth, gain and

power) were recorded to identify the same imaging site for the second testing session. Participants were instrumented with a two-way valve mouthpiece (Hans Rudolph) from which end tidal CO_2 ($P_{ET}CO_2$) was measured using a calibrated gas analyser (ML206 ADinstruments, Colorado Springs, USA). Continuous beat-by-beat blood pressure was obtained from a digit (Finapres, Amsterdam, Netherlands) and heart rate acquired from a 3 lead electrocardiogram. All data was sampled at 50 Hz with the data acquisition system PowerLab via the interface LabChart 7 (ADinstruments, Colorado Springs, USA).

Cerebral autoregulation. Dynamic cerebral autoregulation (dCA) was assessed using a squat-to-stand procedure that induces transient changes in arterial blood pressure (Claassen et al., 2009b, Smirl et al., 2015). Participants replicated the experimenter whilst performing the manoeuvres, that involved moving from a standing upright position to squatting until the legs achieved a 90° angle. Participants performed two sets at 0.10 Hz (5 second squat - 5 second stand) while breathing normal atmospheric air, and again during hypercapnia (detailed below). The first set of squat-stands was preceded by 5 mins of seated rest while the second set immediately followed the 4 mins of hypercapnia. Squat stand manoeuvres were performed at 0.10 Hz as this falls within a frequency range whereby cerebral autoregulation is deemed to be active (Zhang et al., 1998, Smirl et al., 2015) and represents a more feasible challenge than 0.05 Hz manoeuvres, especially for those in the CVD risk factor group.

Carbon dioxide reactivity. Following a rest period of 5 mins, a baseline measurement of cerebral blood velocity, MAP and $P_{ET}CO_2$ was performed across 2 mins while participants breathed in room air. Following the baseline period, the inhaled air was

switched to a Douglas bag (100L) containing 5% CO_2, 21% oxygen and balanced nitrogen, while participants sat in a rested seated position.

Data analysis: MCAv and MAP during the 40 min rIPC and sham conditions were averaged and extracted from LabChart in 5 min intervals (n=20). MCA cerebrovascular conductance (CbVC) was calculated as MCAv/ MAP. Calculation of the CVR slopes were performed via linear regression analysis of the two time-points; baseline (MCAv, MAP, $P_{ET}CO_2$ averaged across 2 mins) and 5% CO_2 (data averaged across the last 30 secs of the 4 min hypercapnia). Two participants in the cardiovascular risk factor group were unable to complete the hypercapnic protocol, therefore data analysis for CO_2 reactivity was performed on n=18 (Healthy=11).

Cerebral autoregulation data were extracted from LabChart beat-to-beat (MAP and MCAv) before spline interpolation and were assessed via transfer function analysis (TFA) based on the Welch algorithm, using a provided script (http://www.car-net.org/). The 5 min squat-stand recordings were subdivided into five windows overlapping by 50% and passed through a Hanning window before fast Fourier transform analysis (MathWorks-Inc., Natick, Massachusetts). The cross-spectrum between MAP and MCAv was determined and divided by MAP auto-spectrum to formulate functions; normalised gain (nGain), absolute gain, phase and coherence (MAP-MCAv linearity). Gain represents the difference in amplitudes between the CBFv and BP signals, while phase describes the temporal alignment between the input (MAP) and output (MCAv). nGain refers to the same output as gain, except blood flow velocity values are normalised by dividing beat-to-beat values by the mean value. Gain, nGain and phase data were excluded from statistical analysis if coherence was

<0.4. TFA was performed in accordance with standardised guidelines from the Cerebral Autoregulation Research Network (Claassen et al., 2016). TFA parameters of the driven oscillations were band averaged across the very low (VLF; 0.02-0.07 Hz), low (LF; 0.07-0.2 Hz) and high (HF; 0.2-0.4 Hz) frequency domains. Induced BP oscillations at 0.10 Hz were employed in the current study, this falls within the ranges of the LF domain. Therefore, the low frequency (0.07-0.20 Hz) output is the most appropriate to be reported as dCA is highly active with this frequency of squats (Zhang et al., 1998). $P_{ET}CO_2$ data was averaged across each 5 min squat-stand recording. One participant in the cardiovascular risk factor group was unable to complete the cerebral autoregulation protocol during normocapnia while three participants from the same group were unable to complete the protocol during hypercapnia, therefore data was analysed on n=18 for the normocapnic and n=17 for the hypercapnic cerebral autoregulation conditions.

3.2.4 Statistical Analysis

A three-factor group*condition*time (group; healthy vs CVD risk factors, condition: rIPC vs sham, time: 5 min intervals during intervention) general linear model was employed to analyse resting MCAv and MAP during the RIPC and sham intervention. A three-factor -capnia*group*condition (capnia; normocapnic or hypercapnic, group; healthy vs CVD risk factors, condition: RIPC vs sham) general linear model was employed to analyse the cerebral autoregulation. A two way group*condition (group; healthy vs CVD risk factors, condition: RIPC vs sham) general linear model was employed to analyse the CO_2 reactivity. Statistically significant main effects and interactions were followed up with the least significant difference (LSD) approach for multiple comparisons. Statistical analysis was conducted using Statistical Package for

Social Sciences (Version 22; SPSS Inc., Chicago, IL). Statistical significance was delimited at $P<0.05$. Data are presented in the text as mean (95% confidence interval) unless otherwise stated.

3.3 Results

3.3.1 Group characteristics

Resting MCAv was significantly higher in the healthy compared to CVD risk group (Table 3.1, P=0.009), while resting MAP was significantly lower in the healthy compared to the CVD risk group (Table 3.1, P=0.001). For resting comparisons of cerebrovascular function between the groups, responses to the CO_2 reactivity and dCA tests during the sham condition are reported. No difference was evident in CO_2 reactivity slopes between the healthy and CVD risk groups at rest (2.15 [1.60, 2.70] vs 1.68 [1.13, 2.24] cm/s/mmHg, P=0.44), or for any of the dCA variables.

3.3.2 Impact of rIPC on resting cerebral blood velocity and haemodynamics

There was no impact of rIPC on MCAv across the 40 min (P=0.58, Figure 3.2). There was a group*condition interaction, with MAP being higher during rIPC compared to sham in the CVD risk group over the 40 min intervention period (P<0.005), whilst MAP was similar between conditions in the healthy group.

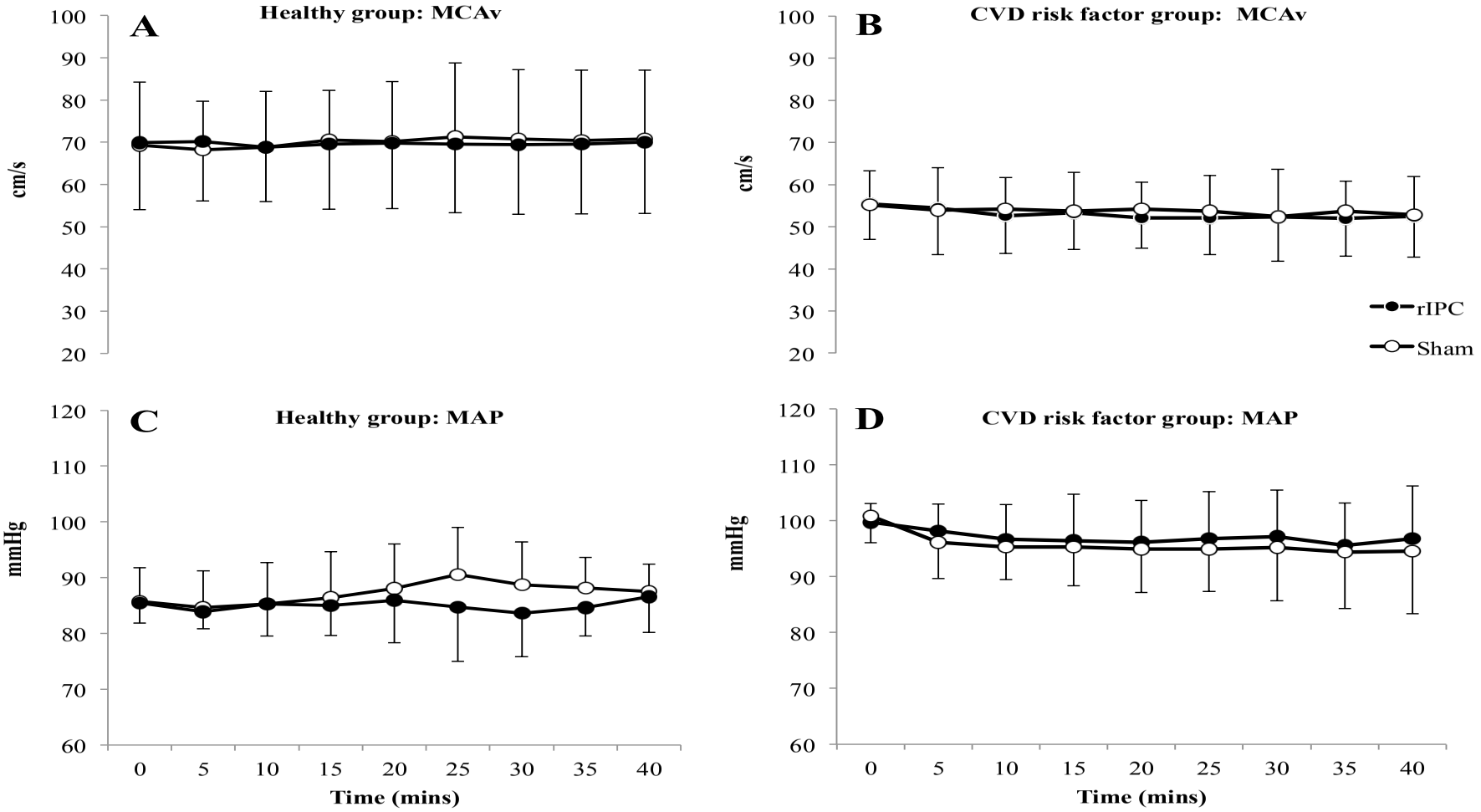

Figure 3.2: MCAv (middle cerebral artery velocity) in young healthy (A), older cardiovascular risk factor (B) individuals and MAP (mean arterial pressure) in young healthy (C) and cardiovascular risk factor (D) individuals during 40 minutes of RIPC and sham. Data is mean± SD

Table 3.2: Dynamic cerebral autoregulation analysis via transfer function using squat-stand manoeuvres. Resting comparison of healthy and CVD risk participants.

Sham condition - Normocapnia			
	Healthy	CVD Risk	***P* Value**
$P_{ET}CO_2$ (mmHg)	37.46 ± 1.89	40.28 ± 3.19	0.10
Dynamic Cerebral Autoregulation (0.10 Hz)			
MCAv power ($cm.s^2$)	88.78 ± 39.65	64.89 ± 80.16	0.16
MAP power ($mmHg^2$)	118.91 ± 50.60	87.01 ± 80.16	0.12
Normalised gain *(%.mmHg^{-1})*	1.25 ± 0.30	1.30 ± 0.20	0.87
Gain (cm/s/mmHg)	0.84 ± 0.19	0.74 ± 0.12	0.46
Phase (radians)	0.55 ± 0.37	0.60 ± 0.55	0.89
Coherence	0.61 ± 0.10	0.67 ± 0.09	0.12

Values are means ± SD

3.3.3 Impact of rIPC on normocapnic and hypercapnic cerebral autoregulation

During normocapnia, there were no main effects or interactions in the low frequency (0.10 Hz) for normalised gain (Table 3.4, P=0.46), phase (P=0.53) or coherence (P=0.59) between the sham and rIPC conditions. $PetCO_2$ values during the squat-stand procedure were not different between conditions (P=0.81). Similarly, during hypercapnia, no significant main effects or interactions in the low frequency domains for normalised gain (Table 3.4, P=0.11), phase (P=0.90) or coherence (P=0.45) were observed. $PetCO_2$ values during hypercapnia did not differ between conditions (P=0.90).

3.3.4 Effect of hypercapnia on cerebral autoregulation (comparison of sham conditions)

Hypercapnia induced a phase reduction of 0.15 radians (0.08, 0.34) when compared to normocapnic cerebral autoregulation (P=0.002) with no effect of the RIPC or Sham condition (P>0.05). Additionally, normalised gain decreased during hypercapnic cerebral autoregulation by 0.41% (0.21, 0.47) compared to normocapnic (P<0.001).

3.3.5 Impact of rIPC on cerebrovascular CO_2 reactivity

The inhalation of 5% CO_2 significantly increased $PetCO_2$ following the sham and rIPC conditions respectively (Table 3.3, both P<0.001). MCAv subsequently increased, with no difference between the sham and rIPC conditions (Table 3.3, P=0.43). There was no overall effect of RIPC on CO_2 reactivity compared to the sham condition (group x treatment x $PetCO_2$, P=0.61, Table 3.3).

Table 3.3: Cardiovascular and respiratory parameters during the carbon dioxide reactivity test.

Healthy + CVD Risk	**Sham**		**rIPC**		**Baseline vs 5% CO_2 *P* value**
	Baseline	5% CO_2	Baseline	5% CO_2	
MCAv (*cm/s*)	64 ± 12	83 ± 19	63 ± 11	83 ± 18	<0.001
$PetCO_2$ (*mmHg*)	34 ± 6	44 ± 3	34 ± 6	44 ± 3	<0.001
MAP (*mmHg*)	101 ± 8	108 ± 9	100 ± 5	106 ± 6	<0.001
					Sham vs rIPC *P* value
MCAv reactivity (*cm/s/mmHg*)	1.97 ± 0.88		2.06 ± 0.69		0.61
CbVCi ($cm/mmHg^2$)	0.034 ± 0.010		0.041 ± 0.011		0.48
MAP reactivity (*mmHg/mmHg*)	0.9 ± 0.1		0.9 ± 0.2		0.89

Values are means ± SD. MCAv, middle cerebral artery velocity; $PetCO_2$; partial pressure of end tidal carbon dioxide; MAP, mean arterial pressure.

Table 3.4: Transfer function analysis of oscillations in mean arterial pressure and middle cerebral artery velocity using squat-stand manoeuvres. Comparison between Sham and RIPC conditions with all participants grouped together.

Healthy + CVD Risk	**Normocapnia (n=19; Healthy=11)**			**Hypercapnia (n=17; Healthy=11)**		
	Sham	RIPC	*P* Value	Sham	RIPC	*P* Value
$P_{ET}CO_2$ (*mmHg*)	38.35 ± 2.65	38.15 ± 2.85	0.81	46.63 ± 2.74	46.46 ± 3.00	0.90
	Dynamic Cerebral Autoregulation (0.10 Hz)			Dynamic Cerebral Autoregulation (0.10 Hz)		
MCAv power ($cm.s^2$)	81.24 ± 54.43	80.95 ± 60.47	0.87	76.85 ± 40.04	82.82 ± 52.66	0.69
MAP power ($mmHg^2$)	108.83 ± 55.61	117.67 ± 72.05	0.73	110.81 ± 62.25	119.47 ± 88.78	0.74
Normalised gain ($\%.mmHg^{-1}$)	1.27 ± 0.25	1.22 ± 0.35	0.46	0.86 ± 0.16	0.94 ± 0.21	0.11
Gain (*cm.s/mmHg*)	0.80 ± 0.17	0.75 ± 0.17	0.86	0.75 ± 0.16	0.80 ± 0.20	0.82
Phase (radians)	0.53 ± 0.47	0.64 ± 0.39	0.53	0.40 ± 0.44	0.40 ± 0.31	0.90
Coherence	0.64 ± 0.10	0.65 ± 0.10	0.59	0.60 ± 0.20	0.58 ± 0.11	0.45

Values are means ± SD. Statistical significance was set at $P<0.05$. $PetCO_2$; partial pressure of end tidal carbon dioxide; MCAv, middle cerebral artery velocity; MAP, mean arterial pressur

3.4 Discussion

This is the first study to investigate the acute impact of rIPC on both dynamic cerebral autoregulation and cerebrovascular CO_2 reactivity in healthy humans and those at increased risk of cardiovascular disease and stroke. The principle findings from this study are i) resting cerebral blood velocity was significantly higher at baseline in the healthy group compared to the cardiovascular risk group and ii) rIPC did not impact resting cerebral perfusion, cerebrovascular CO_2 reactivity or cerebral autoregulation, in either group. These findings extend the fundamental understanding of the acute effects of rIPC in humans and reveal that a single episode of rIPC does not immediately impact cerebrovascular function in humans.

Despite the well-documented effects of rIPC on myocardial and peripheral vascular function in humans (Bøtker et al., Thielmann et al., Kharbanda et al., 2002, Loukogeorgakis et al., 2005, Davies et al., 2013b, Jones et al., 2014), the present study is the first to examine the acute impact of rIPC on CBFv, and both dCA and CVR in humans. The cerebral tests included in the present study were employed to provoke cerebral vasomotion via a number of different regulatory pathways, to better identify any specific effect rIPC may have. In response to 40 mins of upper arm rIPC (4 bouts per arm, alternated), no concurrent impact on CBFv was present. Increases in arterial diameter and blood flow to limbs and organs (heart) regional to the limb undergoing rIPC have been previously reported during the reperfusion phases of rIPC (Zhou et al., 2007, Enko et al., 2011). The findings in the present study that rIPC did not alter CBFv during the bout is an important observation in this context and suggests that rIPC does not influence blood vessel function similarly in the brain. Although it is not known

what mechanism/s are responsible for the regional changes in blood flow in the previous studies during rIPC, it cannot be discounted the possibility that rIPC did induce a change in cerebral perfusion, and that this change was counteracted by one of the numerous cerebral blood flow regulatory mechanisms (Willie et al., 2014). However, consistent with the above finding of no change in blood flow, no overall impact on cerebrovascular function was observed. Resting cerebral autoregulation, a regulatory mechanism that maintains a constant delivery of oxygenated blood to the brain despite changes in blood pressure (Aaslid et al., 1989), was unchanged by rIPC. The second aim of this study was to temporarily disturb dCA via hypercapnia to determine whether rIPC could attenuate the impairment. As expected (Birch et al., 1995, Zhang et al., 1998, Panerai et al., 1999, Ainslie et al., 2008a), hypercapnia reduced dCA phase (indicating a delayed CA response time), but did not alter absolute gain, an effect consistent with some (Ainslie et al., 2008a), but not all studies (Zhang et al., 1998, Panerai et al., 1999, Jeong et al., 2016) and interestingly decreased normalised gain, an observation which suggests reduced magnitude of BP on CBF oscillations which has also been observed during hypercapnic spontaneous oscillations (Ainslie et al., 2008a). Hypercapnia is thought to reduce the efficiency of dCA as a result of hypercapnic-induced vasodilation, preventing the ability of the blood vessels to alter their vasotone in response to blood pressure changes (Perry et al., 2014). Investigations that have examined the precise mechanism responsible for hypercapnic impaired dCA are limited. Maggio et al. (2013) outlined that metabolic pathways are likely the primary mechanism attenuating dCA from hypercapnia, as shown with significant reductions in critical closing pressure, yet these results are limited to spontaneous BP oscillations rather than driven.

In contrast to the original hypothesis, rIPC did not attenuate the hypercapnia-induced impairment in phase (temporal alignment). rIPC did not impact CVR to inhalation of 5% CO_2 compared to the sham condition. Currently, there is only one directly relevant study that assessed rIPC and cerebrovascular function via dCA in humans. Work by Guo et al. (2019) identified that dCA, assessed using spontaneous blood pressure oscillations as opposed to driven, is improved 6 hours after a rIPC bout, further supporting the idea that rIPC adaptations occur in different phases (Thijssen et al., 2016a). Additionally, Guo and colleagues identified a number of blood biomarkers (vascular endothelial growth factor and glial cell derived neurotrophic factor) that are elevated following rIPC, potentially offering an mechanistic explanation for these changes (Guo et al., 2019). An additional study using hypoxia rather than hypercapnia as a model to attenuate cerebrovascular function measured cerebral blood flow responses to acute and chronic hypoxia, and found no effect of rIPC compared to controls, findings consistent with the present study (Rieger et al., 2017). Despite this, there is increasing evidence that repeated rIPC is neuroprotective, particularly in clinical stroke and small vessels disease patients (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Meng et al. previously reported that 300 days of repeated rIPC decreased stroke recurrence and interestingly noted that cerebral perfusion was higher in the rIPC group compared to the standard care patients, potentially remedying the mismatch between perfusion and metabolism. This study raises the intriguing notion that repeated bouts of rIPC may be required in order to influence cerebral perfusion and function to a physiologically relevant extent. Additionally, the phenomenon of rIPC-mediated protection is known to be biphasic in nature, with an immediate protective period that subsides within a few hours of application, followed by a more prolonged second protective window (1-3 days)

(Koch et al., 2014). Due to the difficulties in assessing the time-course of rIPC effectiveness in humans, the vast majority of these studies have been performed in animals. Although it is unlikely, one possible explanation for the null findings is that the cerebral measures were not performed within the initial protective phase, and that the protective windows in humans may differ to that of animals, and may also be influenced by the type, number and duration of rIPC bouts.

An important aspect to this study was assessing the impact of rIPC across a spectrum of cardiovascular health, to determine if this influenced the efficacy of rIPC. Young healthy individuals typically present with unimpaired endothelial-vascular function and as the magnitude of the rIPC effect on cerebrovascular function, if any, is unknown, it is possible that a rIPC effect would not be observable in this population. Accordingly, assessment of the effects of rIPC in healthy individuals and those at increased cardio- and cerebrovascular risk. As expected, cardiovascular risk metrics were significantly different between the groups, with the young healthy individuals displaying lower mean arterial pressure and higher resting cerebral blood flows compared to the elevated risk individuals. Nonetheless, no differences in the efficacy of rIPC to improve cerebral autoregulation under normo- and hypercapnic conditions between the groups was observed.

3.5 Conclusion

The findings of this study extend the fundamental knowledge on the physiological effects of rIPC in humans by assessing for the first time the acute impact of rIPC on cerebral perfusion, dCA and CVR. Although acute rIPC has been found to increase peripheral blood flow, this study reports that this effect of rIPC does not extend to the

cerebral circulation, as no change was observed in cerebral perfusion during rIPC. Additionally, rIPC did not influence cerebral function, as measured by dCA and CVR. Despite these findings related to a single exposure to rIPC, recent clinical trials show that repeated rIPC provides neuroprotection in humans. Therefore, future studies are required to better understand whether repeated exposure to the rIPC stimulus leads to changes in cerebrovascular function and perfusion as a potential explanation for these clinical benefits.

Chapter 4: Seven-day remote ischaemic preconditioning improves endothelial function in patients with type 2 diabetes mellitus: randomised a pilot study

4.1 Introduction

Cardiovascular and cerebrovascular disease are leading causes of mortality in T2DM (Laakso, 2001). Importantly, the pathological consequences of T2DM predominately relate to vascular complications, encompassing both the macro- (e.g. cardio- and cerebrovascular disease) and microvasculature (e.g. retinopathy and nephropathy) (Orasanu and Plutzky, 2009). Clinical studies show that diabetic individuals are more susceptible to ischemia-reperfusion injuries (IRI) compared to non-diabetics (Alegria et al., 2007, Marso et al., 2007), and reduced tolerance to ischaemia has been considered responsible for the increase morbidity of ischaemic heart disease in T2DM (Haffner et al., 1998). Conventionally, the main therapeutic target in T2DM has been glucose lowering but the importance of targeting cardiovascular risk is increasingly recognised (Creager et al., 2003). Intensive glucose lowering treatment has shown limited benefits on all cause morbidity and mortality from cardiovascular causes (Boussageon et al., 2011). Lifestyle changes including improved diet and physical activity are the mainstay of management with regular exercise promoted to improve metabolic health and lower cardiovascular and cerebrovascular risk in T2DM (Chudyk and Petrella, 2011). Since a vast majority of T2DM patients do not engage in regular physical activity (Morrato et al., 2007, Hermann et al., 2014), perhaps because of disease complications (e.g. foot ulcers), alternative or adjunct interventions are required to improve cardiovascular and cerebrovascular disease risk, similar to that of exercise, in this highly vulnerable population.

rIPC is a technique whereby short periods of cyclical tissue ischaemia-reperfusion (of a limb) has been shown to have protective effects beyond the vascular bed directly

exposed to the IPC stimulus (Przyklenk et al., 1993), potentially mediated by neural and/or humoral signalling pathways (Heusch et al., 2015, Thijssen et al., 2016a), yet precise mechanisms remain elusive. When applied prior to planned ischaemia (e.g. coronary artery bypass surgery) or around spontaneous ischaemic events (e.g. myocardial infarction), studies have reported the potential beneficial and protective effects of rIPC to render remote (vascular) tissues and organs (e.g. heart) resistant to ischaemic reperfusion injuries (Heusch et al., 2015). More recently, studies have examined the impact of performing multiple rIPC episodes and explored the potential of rIPC as an intervention to improve vascular function (Thijssen et al., 2016a). Repeated rIPC interventions ranging from 1 to 8 weeks have been shown to improve vascular endothelial function before and after ischemia reperfusion injuries (Luca et al., 2013, Jones et al., 2014, Jones et al., 2015), increase the levels of endothelial progenitor cells (Kimura et al., 2007), and increase coronary flow reserve in heart failure patients (Kono et al., 2014). Some studies have also revealed a potential clinical benefit of rIPC with a 6-week intervention reducing the size of diabetic foot ulcers (Shaked et al., 2015) and lower stroke recurrence following one year of rIPC (Meng et al., 2012, Meng et al., 2015). As outlined in chapter 3 of this thesis, a single bout of rIPC applied to a limb did not induce any changes in CBFv, CVR or dCA, and these results were identified in both young healthy individuals and those deemed at high risk of CVD (see *Chapter 3*). This raises the fundamental question of whether the amount of rIPC applied is a major contributor to the effectiveness of the technique or intervention and warrants further investigation. Therefore, whether an acute intensive rIPC intervention leads to improvements in cerebrovascular function assessed measuring dCA, a key mechanism protecting the brain from fluctuations in BP, as well as peripheral endothelial function in T2DM patients is currently unknown, whilst such

benefits may have important clinical benefits, especially those with functional limitations.

The primary aim of this pilot study was to obtain estimates of the change in conduit artery endothelial function before and after endothelial IRI, a model that allows for the assessment of the efficacy of an intervention to reduce the damage that is induced by reperfusion following a period of ischaemia, succeeding a 7-day rIPC intervention. Acute intensive rIPC interventions have improved conduit artery endothelial function (Jones et al., 2014) and attenuated the injury induced by an IRI in young healthy individuals (Luca et al., 2013), yet it is not known whether rIPC offers similar benefits to individuals with T2DM whereby endothelial dysfunction is likely present (Avogaro et al., 2011).

The secondary aim was to obtain estimates of the change in cerebrovascular function after 7-days of daily limb rIPC. Given the evidence rIPC has systemic beneficial effects on vascular regulation and endothelial function (Kimura et al., 2007, Jones et al., 2015), improvements to blood vessel function may translate to enhanced responsive to blood pressure within cerebral vessels (dynamic cerebral autoregulation). Additionally, application of rIPC can regulate several vasoactive biomarkers including, nitric oxide, adenosine and bradykinin (Heusch et al., 2015, Randhawa and Jaggi, 2016) which may have the potential to enhance dCA (Takada et al., 2001, Guo et al., 2016, Guo et al., 2019).

4.2 Methods

4.2.1 Participants

Twenty-one participants (13 males, 8 females, Table 4.1) with clinically diagnosed T2DM who were managed with diet or metformin only were recruited for this randomised controlled pilot study (Figure 4.1). Participants were excluded if they had a history of stroke (including TIAs), diagnosis of chronic heart failure, were current smokers or were being treated with sulphonylureas, DPPIV, GLP-1, SGLT2 or insulin to control T2DM. Participants were informed of the study protocol verbally and in writing before providing written informed consent. The study was approved by the local NHS ethics committee (14/NW/1208) and adhered to the standards set out in the *Declaration of Helsinki (2000)*. All data collection took place at Liverpool John Moores University. Registered clinical trial at ClinicalTrials.gov NCT03598855**.** Trial is reported following CONSORT recommendations (Schulz et al., 2010)**.**

Figure 4.1: Screening, recruitment and completion of participants in the study.

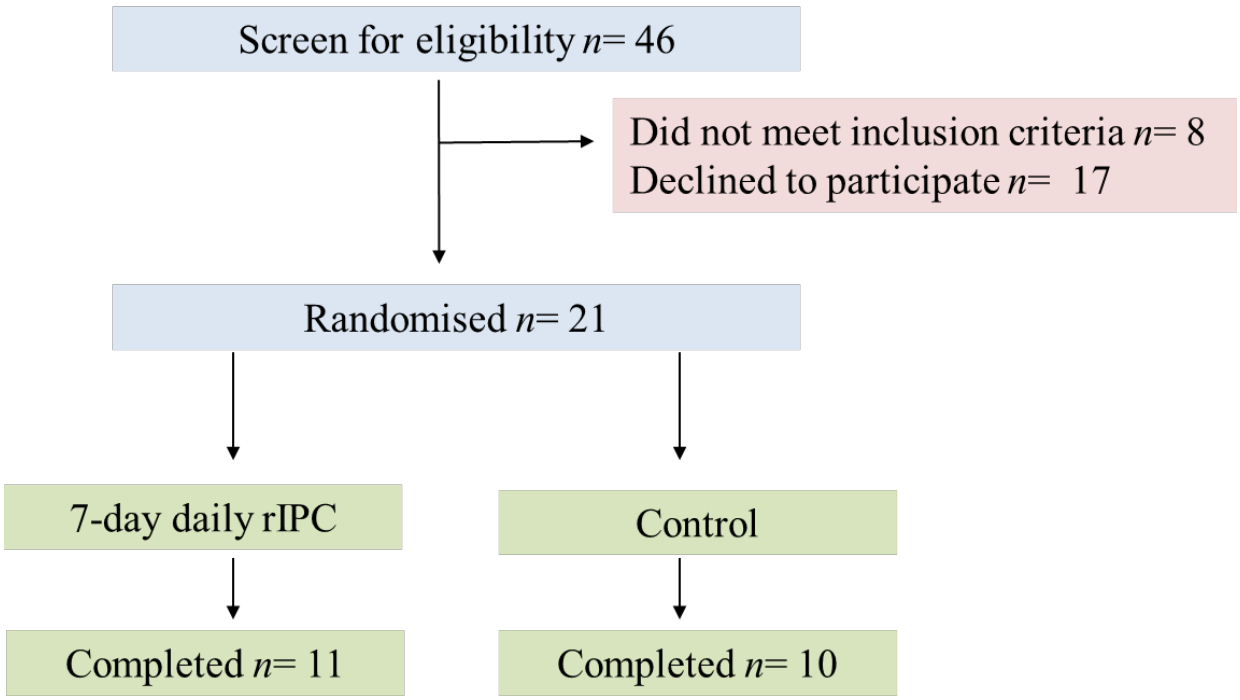

Table 4.1: Descriptive characteristics of participants in rIPC and control groups (p values reported from independent samples t-test).

	rIPC (*n*=11, 5 females)	**Control (*n*=10, 3 females)**	***P* Value**
Age (*years*)	59±7	60±10	0.72
Weight (*kg*)	92.7±18.6	101.5±32.5	0.62
BMI (kg/m^2)	32±7	34±10	0.89
MAP (mmHg)	101±14	107±11	0.37
SBP (*mmHg*)	145±16	151±19	0.57
DBP (*mmHg*)	79±9	84±10	0.31
Metformin	9/11	4/10	
Anti-hypertensive medication	4/11	0/10	
Lipid lowering medication	7/11	3/11	

Values are means ± SD. Abbreviations; BMI, Body Mass Index; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

4.2.2 Research Design

Participants attended the laboratory on three occasions, separated by seven days, having fasted overnight (12hrs), refraining from alcohol and exercise for 24hrs and caffeine for 12hrs before each visit. Each visit consisted of assessments of brachial artery function (before and after ischemia reperfusion injury) and cerebrovascular function. Assessments were performed at the same time of day for each visit (Ainslie et al., 2007, Jones et al., 2010) and occurred prior to group randomisation (computer-generated-sequence) (Pre), immediately following the cessation of the intervention (Post) and 8 days following cessation of the intervention (Post+8) (Figure 4.2).

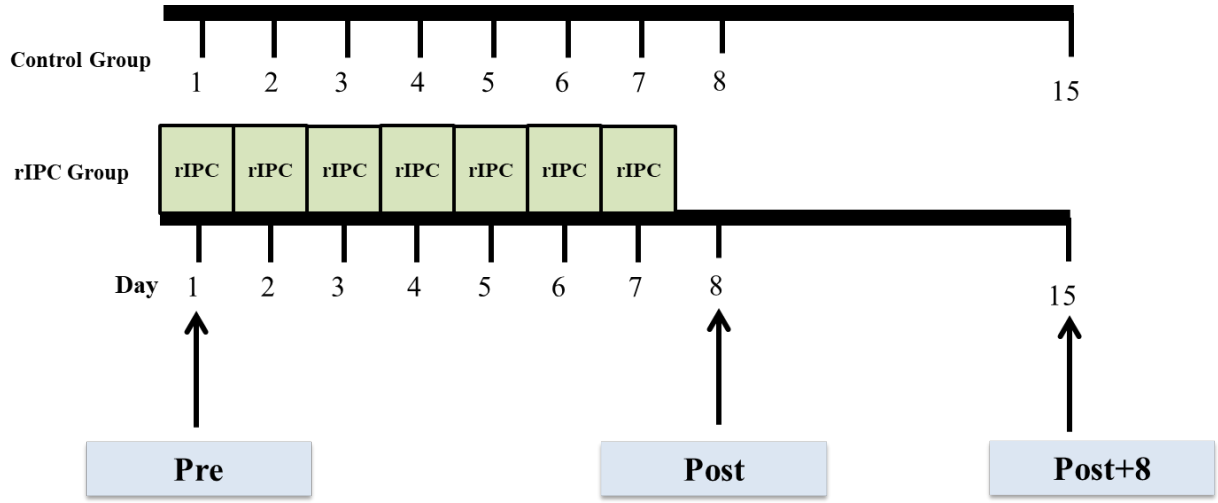

Figure 4.2: Schematic of the study design. Each rIPC consisted of 4 cycles of 5-minute ischaemia (220 mmHg) followed by 5 min reperfusion applied unilaterally. At each testing visit brachial artery flow mediated dilation, ischaemic reperfusion injury and cerebrovascular function were assessed. Abbreviations: rIPC, remote ischaemic preconditioning.

4.2.3 Measurements

Brachial artery endothelial function. Brachial artery endothelial function was assessed using the flow mediated dilation (FMD) technique following 20 min of supine rest (Thijssen et al., 2011). Images of the right brachial artery were acquired using high-resolution ultrasound (T3300; Terason, Burlington, MA). Diameter, flow and shear stress were measured prior to and following 5 minutes of forearm cuff inflation (D.E. Hokanson, Bellevue, WA). All FMD measurements were performed by the same sonographer with a day-to-day coefficient of variation in FMD% of 11% and a coefficient of variation of 3% for baseline artery diameter which is deemed good-excellent based on previous analysis (van Mil et al., 2016).

Analysis was performed using custom designed edge-detection and wall-tracking software, which is largely independent of investigator bias. Previous articles contain detailed descriptions of this analytical approach (Woodman et al., 2001, Black et al., 2008). Reproducibility of diameter measurements using this semi-automated software is significantly better than manual methods, significantly reduces observer error, and possesses within-day coefficient of variation of 6.7% (Woodman et al., 2001). Allometric scaling for baseline diameter was performed (Atkinson and Batterham, 2013). FMD analysis was performed by a researcher blinded to the group allocation using a single blinded coding-randomised procedure.

Ischaemia Reperfusion. Immediately following the baseline FMD, a temporary, endothelial IRI was induced by inflating a cuff around the upper arm to 220 mmHg for 20 min using a rapid inflation pneumatic device. This was followed by a 20 min

reperfusion period before the FMD protocol was repeated. A calculation of the relative % reduction in endothelial function following endothelial IRI was performed. The immediate decrease in FMD following temporary endothelial dysfunction induced by the 20 min cuff inflation is believed to reflect a reperfusion injury and reduced nitric oxide (NO) bioavailability (Loukogeorgakis et al., 2005, Loukogeorgakis et al., 2010, Aboo Bakkar et al., 2018). The relative % decrease in FMD following IRI was calculated by dividing the absolute change between the two FMD's by the baseline FMD *100.

Cerebrovascular function (baseline velocity & dynamic cerebral autoregulation).
Measurements of middle cerebral artery velocity (MCAv) and dynamic cerebral autoregulation (dCA) were performed as described in chapter 3, section 3.2.3.

4.2.4 Interventions

rIPC: The participants randomised into the rIPC intervention group (*n=11*) each received a hand held BP device (Welch Allyn DuraShock^{TM} DS45, New York, USA) to self-administer rIPC. The cuff was placed around the upper arm and inflated to 220 mmHg for five min, followed by five min deflation, and this cycle was repeated a further three times. This process was performed daily for seven days. The arm to which the participants applied the rIPC was randomised between the same arm the FMD's were performed (IPC arm, *n=5*) and the contra lateral arm (n=*6*). Participants were supervised for their first rIPC bout to ensure it was correctly performed and were then free to perform the rIPC at any time of day and noted this in a diary to monitor

compliance. Participants were instructed to follow their normal routine and to abstain from any new physical activity or changes in dietary habits

Control: Each participant (*n=10*) was instructed to follow their normal routine and to abstain from any new physical activity or change in dietary habits.

4.2.5 Statistical analysis

Given that this is a pilot study to obtain estimates of primary and secondary outcome variables, no *a priori* sample size was calculated. The primary outcome in the study is FMD and the primary comparison is between pre to post intervention. Using the data collected (rIPC group *n=11*, control group *n=10*) in the study we calculated post hoc power of the present study, but also calculated the sample size for a future, fully powered randomised control trial for both primary and secondary outcome variables (G*Power 3.1.5).

For exploratory purposes, we performed statistical analysis on the primary comparison (i.e. pre-to-post) to provide an estimate of the change in the primary and secondary outcome variables. Delta changes (Δ) from pre to post were calculated for each group and entered as the dependent variable in a linear mixed model (Statistical Package for the Social Sciences, Version 20: SPSS Inc., Chicago, IL) with pre-intervention data used as a covariate. Data are presented in the text as mean and 95% confidence intervals (95%CI). P-values are presented, but not interpreted. The changes in the data are described in relation to a minimally clinical important difference (MCID) of 1% for FMD, calculated based upon previous intervention studies (Jones et al., 2014, Jones et al., 2015, Schreuder et al., 2015) and from a meta-analysis indicating that 1%

improvement in brachial FMD decreases the risk of future cardiovascular events by 13% (Inaba et al., 2010). The MCID for LF gain was between 0.07 and 0.26*%cm* s^{-1} %.$mmHg^{-1}$mm Hg/%. This was based on studies showing differences between healthy and diseased populations (van Beek et al., 2012, Lewis et al., 2019) due to the limited intervention studies to date.

4.3 Results

Participants allocated to each intervention were similar in terms of age, BMI and BP status (Table 4.1). Participants randomised into the rIPC intervention group (*N=11*) demonstrated 96% compliance to the rIPC intervention. Data presented as absolute change following either intervention with statistical analysis comparing the differences between changes following rIPC or control (no rIPC).

4.3.1 Cerebrovascular function

Low frequency normalised gain changed by 0.23 *%cm* s^{-1} %.$mmHg^{-1}$mm Hg/% (-0.12, 0.59; P=0.18) following rIPC compared to control from pre to post, which was greater than the lower level of the MCID of 0.07 and 0.26. The data provided 29% power to detect a between-group difference in LF normalised gain from pre-post. Using this data a sample size of 50 in each group would provide 90% power to detect a statistically significant (P<0.05) between group difference in LF normalised gain in a future randomised control trial.

In the current study, the directional changes in any of the dCA variables were negligible between conditions (Table 4.4). The associated changes in MCAv, $P_{et}CO_2$ or CbVC were negligible between both conditions and over time from pre to post and post 8 (Table 4.3). MAP decreased by 4 mmHg (2, 6 mmHg) across both interventions.

Similarly, SBP decreased by 5 mmHg (-9, -1 mmHg) and DBP by 3 mmHg (-5, -1 mmHg).

4.3.2 Brachial artery endothelial function

Baseline FMD: Brachial artery FMD improved by 1.3% (95%CI: 0.69 to 3.80; P=0.09) with rIPC compared to control (no rIPC) from pre to post, with the change following the rIPC intervention being greater than the MCID of 1%. The data provided 65% power to detect a between-group difference in FMD from pre-post. Using this data, a sample size of 20 in each group would provide 90% power to detect a statistically significant (P<0.05) between groups in FMD in a future randomised control trial.

In the current study, FMD was 0.9 (-3.9, 2.0 %) lower in the rIPC group compared to control at pre, but 0.9 (-2.3, 4.0 %) higher than control at post, which remained higher at post+8 (0.8 (-2.3, 3.9 %), Figure 4.3). The associated changes in baseline diameter, peak diameter, shear rate or time-to-peak diameter between interventions or over time were negligible from pre to post and post 8 (Table 4.2). Within the rIPC condition, the limb to which the rIPC was administered was compared with the contralateral limb (interventions limb vs contralateral limb) and displayed no main effect of limb (P>0.05) confirming that the associated changes observed occurred as a result of both *remote* IPC and *local* IPC.

Endothelial IRI: When examining the FMD after the endothelial IRI (Table 4.2). FMD was 2.3 (-5.4, 0.8%) lower in the rIPC group compared to control at pre, but only 0.1 (-2.8, 2.6%) lower at post and 0.5 (-2.9, 2.0%;) at post+8. FMD increased over the intervention period by 0.7% (-0.1, 1.6). These directional changes were similar when the FMD data was expressed as a relative change. Prior to the intervention, the relative % decrease in FMD in response to IRI was 24.7% (-10.4, 49.7%) greater in the rIPC

group compared to control (Table 4.2). This difference was attenuated to 4.5% (-23.9, 14.9%) at post and 1.4% (-22.5, 19.6%) at post+8.

Table 4.2: Brachial artery flow mediated dilation before (Pre), immediately following (Post) and 8 days (Post+8) after the end of the intervention in both the intervention (rIPC) groups and control. Data in tables shows FMD characteristics in both before and after ischemia-reperfusion injury.

	rIPC Group (*n=11*)			**Control Group (*n=10*)**		
	Pre	**Post**	**Post+8**	**Pre**	**Post**	**Post+8**
Baseline						
Resting diameter (*mm*)	4.4±0.6	4.3±0.7	4.3±0.6	4.5±0.7	4.6±0.7	4.6±0.7
FMD%	5.5±1.7	7.2±2.4	7.2±2.6	6.8±2.9	7.0±3.0	6.9±2.5
Time to peak (*sec*)	70±30	65±24	71±25	68±23	63±22	69±20
Shear AUC (10^3)	16.9±12.5	19.3±12.2	17.1±11.9	18.3±11.4	19.6±15.4	18.2±8.1
Post-ischaemia reperfusion						
Resting diameter (*mm*)	4.5±0.8	4.5±0.8	4.5±0.8	4.7±0.7	4.8±0.7	4.9±0.7
FMD%	2.4±3.7	4.5±3.0	4.0±2.8	4.7±1.9	4.6±2.5	4.5±1.9
Time to peak (*sec*)	72±23	71±27	74±23	53±24	53±17	64±21
Shear AUC (10^3)	14.9±12.9	14.5±11.6	12.4±8.1	15.6±12.0	15.7±6.5	13.6±6.6
Ischaemia-reperfusion injury						
Relative % change following IRI	62.2±44.3	38.0±20.4	39.4±25.0	37.6±13.2	42.5±15.4	40.8±12.0

Values are means ± SD; n=11 rIPC group and n=9 control group. Abbreviations: FMD, Flow mediated dilation; IRI, ischemia-reperfusion injury; AUC, area under the curve; rIPC, remote ischaemic preconditioning

Table 4.3: Baseline hemodynamics from five minute recordings before (Pre), immediately following (Post) and 8 days (Post+8) after the end of the intervention.

	rIPC Group (*n=11*)			Control Group (*n=10*)		
	Pre	**Post**	**Post+8**	**Pre**	**Post**	**Post+8**
Resting data						
MAP (*mmHg*)	101±14	100±10	96±12	107±12	104±12	104±9
MCAv ($cm.s^{-1}$)	56.2±8.0	55.5±7.8	55.7±10.9	53.6±11.0	53.2±10.1	53.5±9.8
$P_{ET}CO_2$ (*mmHg*)	38.4±6.0	38.1±5.8	37.7±4.7	38.8±6.4	41.5±6.0	42.2±6.2
CbVC ($cm.s^{-1}.mmHg^{-1}$)	0.56±0.10	0.55±0.10	0.58±0.14	0.52±0.12	0.53±0.12	0.52±0.12
SBP (*mmHg*)	145±16	144±13	139±16	151±19	151±17	148±17
DBP (*mmHg*)	78±9	77±9	75±10	84±10	81±9	83±10

Values are means ± SD; n = 11 rIPC group and n = 10 control group. Abbreviations; MAP, mean arterial pressure;, MCAv, middle cerebral artery velocity; $P_{et}CO_2$, partial pressure of end tidal carbon dioxide; CbVC, cerebral vascular conductance; SBP, systolic blood pressure; DBP, diastolic blood pressure; rIPC, remote ischaemic preconditioning.

Table 4.4: Transfer function parameters from dynamic cerebral autoregulation before (Pre), immediately following (Post) and 8 days (Post+8) after the end of the intervention using squat-stand manoeuvres (0.10Hz).

	rIPC Group (*n=10*)			Control Group (*n=9*)		
	Pre	**Post**	**Post+8**	**Pre**	**Post**	**Post+8**
$P_{ET}CO_2$ (*mmHg*)						
	40.3±3.7	39.2±48	38.3±3.4	38.8±7.5	38.3±6.6	39.3±5.6
Coherence						
	0.65±0.10	0.60±0.12	0.60±0.21	0.61±0.17	0.59±0.18	0.60±0.22
Phase (*radians*)						
	0.44±0.12	0.48±0.28	0.48±020	0.61±0.32	0.52±0.25	0.52±0.22
Gain ($cm.s^{-1}$. $mmHg^{-1}$)						
	0.66±0.16	0.69±0.20	0.72±0.27	0.71±0.18	0.69±0.26	0.71±0.24
Normalised Gain ($\%.mmHg^{-1}$)						
	1.12±0.21	1.23±0.20	1.36±0.56	1.40±0.27	1.27±0.50	1.37±0.32

Values are means ± SD; n = 10 rIPC group and n = 9 control group. Abbreviations; rIPC, remote ischaemic preconditioning; $P_{et}CO_2$, partial pressure of end tidal carbon dioxide

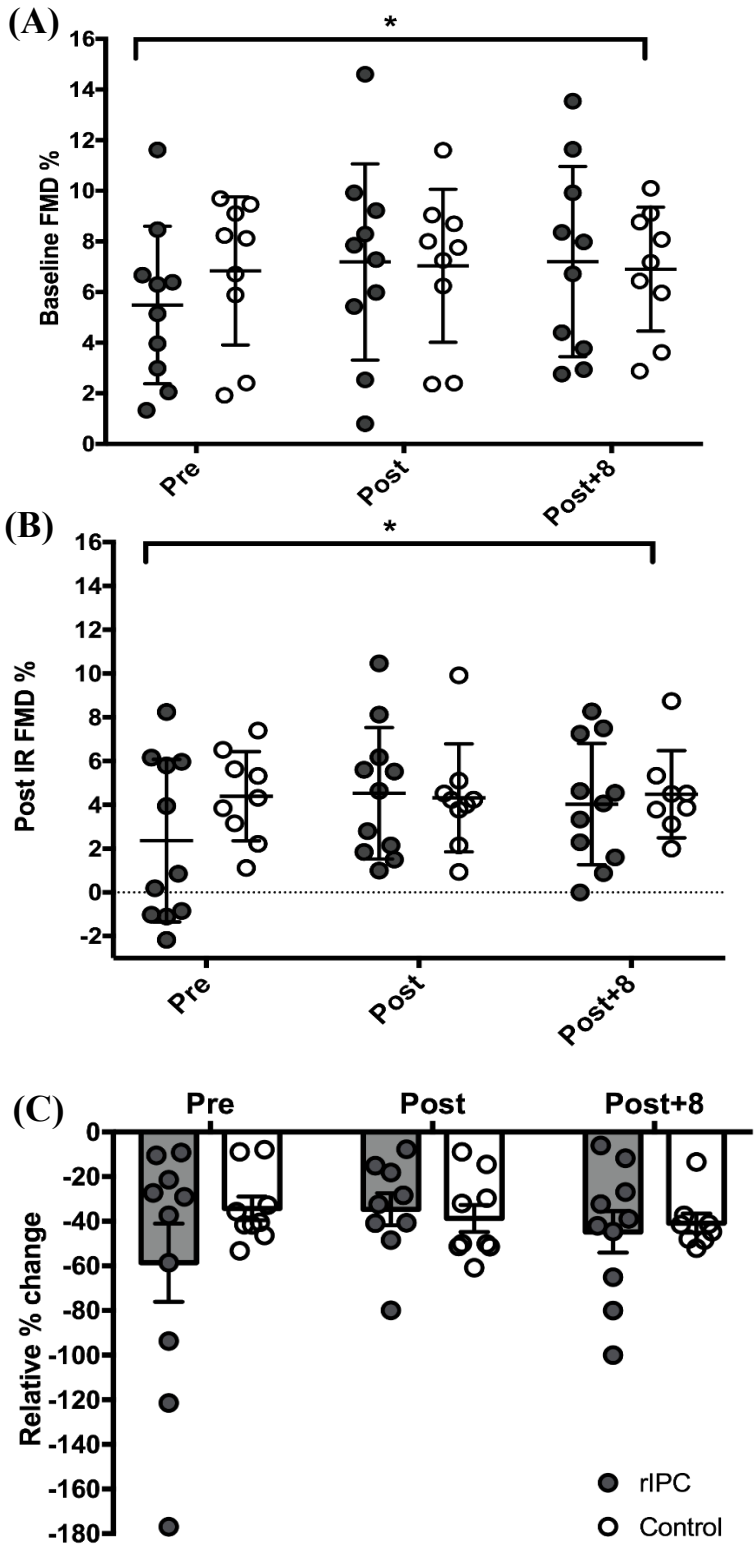

Figure 4.3: Baseline Brachial artery FMD% (A), Post IR FMD% (B) and the relative % decrease (C) before (Pre), immediately after (Post) and eight days following the intervention (Post+8) in the rIPC group (closed circles) and control group (open circles).* Denotes significant main effect from time ($P<0.05$). Abbreviations; FMD, flow mediated dilation; rIPC, remote ischaemic preconditioning; IR, ischaemia-reperfusion.

4.4 Discussion

The aim of this study was to obtain estimates of changes in peripheral conduit artery endothelial and cerebrovascular function and the response to endothelial IRI to 7-days of daily limb rIPC in T2DM. Expanding on the study presented in chapter 3, this study not only examined the cerebrovascular function response to rIPC but also incorporated the peripheral vascular bed into the research design. This study provides preliminary evidence that 7-days of daily rIPC in a representative sample of patients can enhance peripheral conduit artery endothelial function measured using FMD, and provide protection against a temporary decline in endothelial function following ischaemia reperfusion. Although the observations suggest that rIPC had little impact on cerebrovascular function, the preliminary directional findings and sample size estimations suggest the ability of a rIPC intervention to improve peripheral vasculature in T2DM. These effects should be explored further in a larger, fully powered trial.

This present study provides preliminary evidence that daily rIPC can increase conduit artery endothelial function. This is clinically important given that individuals with T2DM exhibit endothelial dysfunction (Calles-Escandon and Cipolla, 2001, Tabit et al., 2010) and are also at high risk of microvascular disease of the small vessels. Chronic hyperglycaemia limits the ability of the endothelial cells to produce nitric oxide (NO) which has important anti-atherogenic properties, contributing to the maintenance of vascular homeostasis (Sena et al., 2013). This is relevant as vascular dysfunction plays a major role in the development of cardiovascular complications (Luscher et al., 2003). Given that a meta-analysis confirmed that a 1% improvement in brachial FMD decreases the risk of future cardiovascular events by 13% (Inaba et al., 2010), strategies to improve vascular endothelial function are crucial. Numerous

clinical outcome studies have demonstrated that brachial artery FMD is a good predictor of cardiovascular risk (Cohn et al., 2004). Improvements in FMD are associated with enhanced NO production (Green et al., 2014a) and NO pathways are impaired with diabetes (Williams et al., 1996, Avogaro et al., 2011). The data suggests that vascular endothelial function can be improved in 7 days and remain elevated 8 days following the end of the intervention. Given that rIPC was administered in the arm that received the preconditioning stimulus as well as in the contralateral arm the data supports the notion that rIPC has local and systemic effects on the vascular system (Jones et al., 2014). As this present study was not designed as a mechanistic study, it is possible to only speculate on potential mechanisms involved in the change in FMD observed. Episodic increases in shear stress is likely to represent a major physiological stimulus for the local improvements in FMD (Thijssen et al., 2016a) however is unlikely to have effected contralateral arm FMD. The mechanisms mediating the systemic effects of rIPC remains elusive. Systemic stimuli or circulating markers activated by rIPC more likely explain the remote improvement in conduit artery FMD. For example, rIPC leads to an increase in vascular endothelial growth factor and endothelial progenitor cells (Kimura et al., 2007), which may improve endothelial function in remote areas (Hill et al., 2003). However, more research studies are required to gain insight into exact mediating mechanisms.

The present study provides evidence that daily rIPC can provide protection against endothelial IRI in T2DM. The endothelial IRI model performed in this study has been used by previous studies (Kharbanda et al., 2001, Luca et al., 2013) and is acknowledged as a surrogate model for myocardial reperfusion injuries. A similar model using forearm IRI identified that the decrease in FMD occurs as a result of a

decrease in plasma nitrite and plasma nitrate concentrations, indicating a reduction in NO bioavailability which is still decreased up to 50 min post reperfusion (Aboo Bakkar et al., 2018). The findings in this study agree with previous rIPC studies showing (partial) prevention of endothelial dysfunction after IRI when preceded by a bout of rIPC (Luca et al., 2013). Reduced endothelial dysfunction against IRI is of clinical significance given that patients with T2DM demonstrate more extensive injury in response to ischaemia reperfusion (Russo et al., 2017). Interestingly, a previous six-week rIPC intervention performed on patients with T2DM with foot ulcers identified an augmentation in the wound size of the foot ulcers in the patients who received the rIPC compared to a control (Shaked et al., 2015), further demonstrating the capability of a rIPC intervention to treat ischaemic induced complications in a diabetic patient group.

This study identified that a 7-day repeated rIPC intervention had little impact on resting MCAv or dCA. Despite the considerable literature on the effects of rIPC on cardiac and peripheral vascular function in humans, there are few studies on cerebrovascular function, even with stroke and cerebrovascular disease being a leading cause of death worldwide (Roger et al., 2012). Post-hoc analysis of power was performed which revealed that more participants would have been required for adequate statistical power; therefore the data should be interpreted with caution. It is likely that control of cerebral autoregulation is multifactorial encompassing neurogenic, metabolic, myogenic and endothelial factors (Tzeng and Ainslie, 2014). The exact contribution of each, including the endothelium is debated. Evidence suggests that the endothelium carries mechanoreceptor properties that allows it to actively contribute to cerebral autoregulation following changes in arterial shear stress

and transmural pressure (Peterson et al., 2011). Therefore, a healthier and more active endothelium may have translated to improved dCA, yet this was not evident in the present study. Given that dCA is controlled by highly sensitive and tight regulatory factors, it is possible that 7 days of rIPC was not a sufficient enough stimulus to result in any change/adaptation. This potential explanation is supported by the fact that the only previous studies examining repeated rIPC on human cerebrovascular markers employed daily rIPC for 300 days (Meng et al., 2012), 180 days (Meng et al., 2015) and 365 days (Wang et al., 2017) identifying increases in cerebral perfusion and reductions in stroke reoccurrence but did not assess functional markers of the cerebral circulation. Whilst there is also a strong association between T2DM and cerebrovascular dysfunction (Zhou et al., 2014), none of the participants in this study had any previous documented cerebrovascular complications unlike the aforementioned studies and were of shorter duration of T2DM.

Given the data was collected for the purposed of generating estimates for a larger trial it is important to acknowledge the small sample and limited statistical power. It is also important to acknowledge a number of other study limitations. Pre-intervention characteristics, primarily MAP, metformin and statin use were different between the intervention and control group and some evidence now suggests that certain medication used to treat risk factors of cardiovascular disease can alter the response to cardio protective interventions (Ferdinandy et al., 2014). Additionally, HbA1c data was not collected to examine clinical relevance to glucose control nor biomarkers of NO bioavailability. Stratification for medication and markers of glucose control and NO bioavailability should be incorporated into a larger fully powered future trial. Lastly, MCAv was measured using transcranial Doppler, a technique that provides a reliable surrogate for absolute cerebral blood flow providing the insonated artery

diameter remains constant across and between the study conditions (Ainslie and Hoiland, 2014). A future trial may consider assessment of extra cranial vessels (e.g. internal carotid artery) with ultrasound to assess changes in artery diameter as an indicator of changes in diameter.

4.5 Conclusion

The present study has provided estimates of sample size for a randomised control trial exploring the impact of daily rIPC for 7 days on peripheral endothelial and cerebrovascular function. The directional changes outlined from this pilot study suggest peripheral endothelial function and responses to endothelial IRI can be enhanced by daily rIPC in patients with T2DM and should be investigated in a fully powered randomised control trial. In contrast to peripheral arteries, no changes were evident in the cerebrovasculature as no change was found in MCAv or in dCA. Taken together, our results on the impact of repeated rIPC on cardio- and cerebrovascular function warrants further research.

Chapter 5: The impact of combining aerobic exercise training with repeated rIPC on peripheral and cerebrovascular function in high risk individuals

5.1 Introduction

Repeated rIPC interventions can improve cardiovascular parameters and clinical endpoints in healthy individuals and those with CVD risk factors respectively. For example, repeated rIPC interventions ranging from 1 to 8 weeks have mediated improvements in vascular endothelial function in both healthy (Jones et al., 2014, Jones et al., 2015) and T2DM (see *Chapter 4*), increases in coronary flow reserve in heart failure patients (Kono et al., 2014), increased presence of endothelial progenitor cells (Liang et al., 2015) and reduced diabetic foot ulcer wound size (Shaked et al., 2015). Collectively, suggesting that repeated rIPC interventions could be useful in preventing and reducing cardiovascular events and complications.

There is emerging evidence that repeated rIPC interventions can also have beneficial effects on the cerebrovasculature. A small number of studies have shown repeated rIPC can increase CBF in stroke patients and reduce the rate of stroke reoccurrence (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Nevertheless, Chapter 3 in this thesis attempted to understand how rIPC exerts its effects of the cerebrovasculature, demonstrating that a single bout of rIPC has negligible impact on CBFv or dCA as a marker of cerebrovascular function. Chapter 4 provided some evidence that 7-day daily repeated rIPC intervention in individuals with T2DM did not change cerebrovascular function. A number of important methodological differences are evident between experimental studies contained in this thesis (see *Chapters 3 & 4*) and the previous studies showing benefits on cerebrovascular health (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Key differences relate to participant population, length of rIPC intervention and measurement techniques. The length of the intervention and the number of rIPC bouts

performed over the intervention, which collectively can be termed the dose of rIPC, is an important aspect to consider. It is possible that a larger dose (in both duration and frequency) of rIPC might have a greater effect on cerebrovascular function.

An alternative approach to increase the rIPC stimulus is to combine the repeated rIPC with exercise training. Regular exercise bouts may potentially provide an additional, but not mutually exclusive, preconditioning stimulus for the vascular system thus combining rIPC with exercise may increase the beneficial adaptations observed with rIPC alone. The mechanisms responsible for the beneficial effects of repeated rIPC versus exercise training may differ. Where effects of rIPC may relate to both neural and humoral pathways (Anttila et al., 2016), benefits of exercise (preconditioning) may relate to repeated systemic elevations in shear stress (Hambrecht et al., 2003, Tinken et al., 2010, Thijssen et al., 2016a). It has not yet been investigated whether adding exercise to the rIPC stimulus leads to a larger adaptation in peripheral and/or any adaptation in the cerebral vasculature at all.

Therefore, the primary aim of this study was to examine whether exercise training could enhance the effects of repeated rIPC on cerebrovascular and peripheral conduit artery function more than repeated rIPC alone in individuals with increased risk of CVD. It was hypothesised that (i) exercise training would provide additional stimulus to elicit greater improvements in both cerebo- and peripheral vascular function compared to rIPC alone.

5.2 Methods

5.2.1 Participants

Nineteen participants with increased risk of cardiovascular disease were recruited (Figure 5.1). Participants were recruited on the criteria of; body mass index (BMI) >30 kg/m^2 or waist circumference ≥94cm (male), ≥80cm (female) as well as either raised blood pressure (>130 /85 mmHg) or diagnosed with hypercholesterolemia (Table 5.1). Individuals were excluded if they had a history of stroke (including TIAs), myocardial infarction, thrombosis, congenital heart disease, type 1 diabetes or currently smoking. Participants were informed of the study protocol verbally and in writing before providing written informed consent. The study was approved by the local ethics committee (approval number 17/SPS/056) and conformed to standards set out by the *Declaration of Helsinki*. Registered clinical trial at ClinicalTrials.gov NCT03624452.

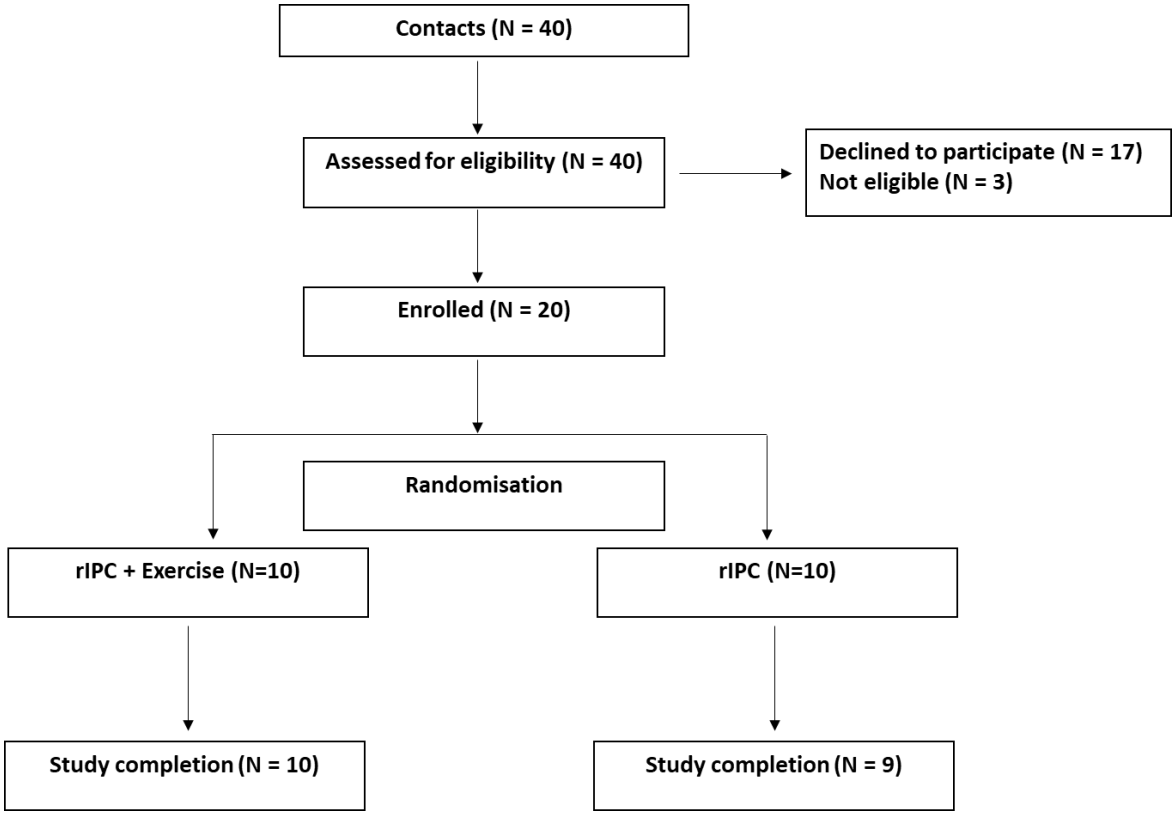

Figure 5.1: Screening, recruitment, retention and completion of the study.

5.2.2 Research Design

Participants underwent two initial visits to the laboratory. Following an overnight fast and refraining from alcohol and exercise for 24hr and caffeine for 12hr, visit 1 consisted of anthropometric measurements, fasting blood glucose, assessment of cerebrovascular function and assessment of brachial artery endothelium function before and after a temporary ischaemia reperfusion injury (IRI). Visit 2 consisted of a cardio-respiratory fitness test (VO_{2peak}). Both visits were conducted within four days of each other and all completed in a temperature-controlled laboratory (23±1°C). Participants were then randomly allocated into the rIPC + Exercise group (3 x rIPC and 3 x 50min exercise per week) or rIPC group (3 x rIPC per week). Randomisation was performed following the first testing visit using a computer-generated-sequences

to avoid bias. All measurements performed in Visit 1 were repeated following the end of the 8-week intervention. Measurements from Visit 2 were repeated again following the completion of the 8 weeks. All post intervention measurements were performed within 7 days of last exercise or rIPC bout.

5.2.3 Measurements

Cerebrovascular function: Measurement of baseline CBFv, dCA and CVR were completed as per the information in Chapter r3, section 4.2.3.

Brachial artery endothelium-dependent vasodilation: FMD was performed on the left of arm of each participant before and after an IR injury as per the information in Chapter 4, section 4.2.3 and performed by the same sonographer throughout the entire thesis.

Maximal oxygen uptake: The cardio-respiratory fitness test (VO_{2peak}) was performed on a treadmill (H/P Cosmos, Pulsar 4.0, Nussdorf-Traunstein, Germany) in order to quantify peak aerobic capacity. A modified version of the *Bruce et al. (1973) protocol* was adopted as this is frequently used protocol in sedentary/high risk populations (Pugh et al., 2013, Sprung et al., 2013). Following a 5-minute warm-up period at a self-selected speed, the protocol begins with a 2-minute stage at 2.2km/h on a flat gradient, followed by 2 minutes at 2.7km/h at a 5% gradient. Subsequently, stepwise increments in speed and gradient are applied evert minute until volitional exhaustion. Breath-by-breath expired gases were continuously monitored (Oxycon Pro, Jaeger, Hochberg Germany) for oxygen consumption (ml/kg/min) and were averaged over 15 seconds. Peak oxygen uptake was calculated from the highest consecutive 15-second period of expired gas fractions. Heart rate was measured continuously using short-

range telemetry (RS800, Polar, Finland) alongside subjective effort (RPE) using the 6-20 Borg scale. All participants reached the criteria for volitional exhaustion based upon heart rate, Borg scale and respiratory exchange ratio (Edvardsen et al., 2014).

Fasting blood glucose: Blood samples were obtained from the antecubital vein via standard venepuncture technique (Vacutainers Systems, Becton-Dickinson). All samples were collected into vacutainers containing a polymer gel for serum separation. Centrifugation for 10 minutes at 1000g at 4℃ was applied and samples were stored at -80℃ for subsequent analysis. Plasma glucose was determined spectrophotometrically using commercially available kits (Randox Laboratories, Antrim, UK) with each sample analysed in duplicate.

5.2.4 Interventions

Remote Ischaemic Preconditioning

All participants in both groups performed three bouts of rIPC per week for 8 weeks. A single bout of rIPC consisted of a pressure cuff (Welch Allyn DuraShock^{TM} DS45, New York, USA) inflated around the upper arm (220mmHg) for five minutes preceded by five minutes of reperfusion, repeated four times (total time 40 minutes). The arm in which the rIPC was applied to was randomised and participants were free to perform the rIPC bouts freely and not follow a pre-set routine. All participants were provided with an intervention diary in order to increase compliance.

Exercise intervention

Those randomly assigned to the rIPC+Exercise group performed three 50 minute exercise sessions per week for 8 weeks (98% compliance). All sessions were

performed on a cycle ergometer (Wattbike Trainer, Wattbike Limited, country) in a temperature controlled laboratory (18±2°C). The intensity of the exercise sessions was set to 70% maximum heart rate (HR_{max}) and all sessions were supervised in order to ensure target HR was achieved and maintained throughout the session.

5.2.5 Statistical analysis

Analysis was performed using Statistical Package for Social Sciences (Version 26; SPSS Inc., Chicago, IL). Baseline characteristics between conditions (Table 5.1) was analysed using an independent samples t-test. All other data were analysed using a linear mixed model, with delta changes (Δ) from week 0-week 8 calculated and added to the model as a dependent variable and pre-intervention data entered as a covariate. Linear mixed model (LMM) *P* values in results represent difference in the absolute change following each intervention. Statistical significance was delimited at $P < 0.05$ and exact *P* values are cited (*P* values of '0.000' provided by the statistics package are reported as <0.001). Significant interactions and main effects were followed up using LSD pairwise comparisons. Data are presented as mean and 95% confidence intervals.

5.3 Results

5.3.1 Resting hemodynamic

The directional changes in MAP (P=0.45), MCAv (P=0.30) and PCAv (P=0.56) were negligible between conditions (Table 5.2).

Table 5.1: Baseline characterises and medications of both groups.

Baseline characteristics	rIPC + Exercise (4 females & 6 males)	rIPC only (2 females & 7 males)	*P* value
Age (*years*)	52 ± 8	51 ± 12	0.87
Height (*m*)	1.7 ± 0.1	1.7 ± 0.1	0.26
Weight (*kg*)	97.8 ± 21.5	108.2 ± 21.7	0.31
Body mass index (kg/m^2)	34 ± 5	35 ± 5	0.56
Waist circumference (*cm*)	107 ± 17	109 ± 13	0.75
Resting heart rate (*bpm*)	69 ± 8	69 ± 6	0.96
Systolic blood pressure (*mmHg*)	137 ± 15	139 ± 12	0.75
Diastolic blood pressure (*mmHg*)	80 ± 13	84 ± 6	0.42
Mean arterial blood pressure (*mmHg*)	99 ± 12	102 ± 5	0.50
Fasting blood glucose (*mmol/L*)	5.9 ± 0.7	6.0 ± 0.7	0.70
Medications			
Statins	3 (30%)	3 (33%)	
β-blockers	1 (10%)	1 (11%)	
Calcium channel blockers	1 (10%)	0 (0%)	
alpha-1 adrenergic blockers	1 (10%)	0 (0%)	
Angiotensin-converting-enzyme inhibitors	3 (30%)	2(22%)	
Biguanides	2 (20%)	2 (22%)	

Data presented as mean±SD or as a percentage (medications).

Table 5.2: Baseline hemodynamic, cardiorespiratory fitness and fasting blood glucose data from before (week 0) and after (week 8) each intervention.

	rIPC + Exercise		rIPC only		LMM
	Week 0	**Week 8**	**Week 0**	**Week 8**	**Condition**
MCAv ($cm.s^{-1}$)	56±10	57±9	54±14	53±12	0.30
PCAv ($cm.s^{-1}$)	37±3	36±4	36±3	36±2	0.83
$P_{et}CO_2$ (mmHg)	38.5±3.1	38.0±3.7	41.6±2.7	39.6±3.5	0.35
MAP (mmHg)	99±13	95±11	102±7	99±5	0.45
Resting HR (bpm)	69±9	68±11	69±6	70±8	0.61
VO_{2peak} (ml/kg/min)	22.9±5.4	25.7±6.2	23.5±2.9	23.6±2.8	0.69
Fasting blood glucose (mmol/L)	5.9±0.7	5.9±0.7	6.4±1.6	6.5±1.4	0.27
Average cycling workload (watts)	77±27	89±25	n/a	n/a	<0.001

Data presented as means ± SD. Abbreviations; MCAv, middle cerebral artery velocity; PCAv, posterior cerebral artery velocity; $P_{et}CO_2$, partial pressure of end tidal carbon dioxide; MAP, mean arterial pressure; HR, heart rate; rIPC, remote ischaemic preconditioning; LMM, linear mixed model.

5.3.2 Cerebrovascular function

Parameters of spontaneous (Table 5.3) and dynamic (Table 5.4) cerebral autoregulation changed similarly with both interventions (P>0.05). Pararmeters of cerebral reactivity including CVR_{CO2}, $CbVCi_{Co2}$ or MAP $reactivity_{Co2}$ changed similarly with both interventions (P>0.05) (Table 5.5).

Table 5.3: Power spectral and transfer function analysis of dynamic cerebral autoregulation during spontaneous changes in BP and CBFv.

	rIPC + Exercise		rIPC only		LMM
	Week 0	Week 8	Week 0	Week 8	
Power Spectrum					
Baseline MCAv power (cm/s^2)					
VLF	2.69±1.17	2.86±1.53	2.41±2.15	2.87±1.37	0.13
LF	1.69±0.96	1.81±1.30	1.58±0.122	2.61±.1.86	0.84
HF	0.47±0.24	0.52±0.28	0.81±0.27	0.97±0.21	0.19
Baseline BP power (mmHg^2)					
VLF	4.23±1.47	3.43±1.95	4.52±2.72	4.96±3.62	0.21
LF	2.26±1.43	2.28±2.00	2.05±1.17	1.77±0.86	0.32
HF	0.44±0.13	0.40±0.22	0.44±0.31	0.43±0.23	0.69
Transfer Function					
Spontaneous Oscillations					
VLF gain (cm·s·mmHg)	0.68±0.23	0.73±0.32	0.51±0.08	0.51±0.06	0.72
VLF Ngain *(%.mmHg^{-1})*	1.15±0.22	1.34±0.62	0.98±0.25	1.35±0.75	0.69
VLF phase (radians)	0.77±0.32	0.87±0.41	1.03±0.48	1.08±0.69	0.98
VLF coherence	0.54±0.14	0.51±0.01	0.52±0.23	0.50±0.20	0.14
LF gain	0.74±0.29	0.75±0.29	0.75±0.25	0.88±0.34	0.16
LF Ngain	1.26±0.41	1.40±0.41	1.35±0.45	1.43±0.59	0.54
LF phase	0.52±0.38	0.65±0.31	0.89±0.49	0.74±0.41	0.31
LF coherence	0.56±0.09	0.55±0.12	0.50±0.11	0.51±0.21	0.31
HF gain	1.00±0.43	0.99±0.50	0.97±0.53	1.00±0.31	0.20
HF Ngain	1.77±0.42	1.81±0.70	1.47±0.32	1.84±0.45	0.43
HF phase	0.14±0.66	0.09±0.51	0.33±0.21	0.18±0.08	0.30
HF coherence	0.61±0.08	0.63±0.08	0.63±0.06	0.63±0.07	0.75

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Data presented as means ± SD. Abbreviations; PS, power spectrum; VLF, very low frequency; LF, low frequency; HF, high frequency; MCAv, middle artery cerebral velocity; BP, blood pressure; Ngain, normalised gain; LMM, linear mixed model.

Table 5.4: Power spectrum densities of forced oscillations in mean arterial pressure and cerebral blood flow velocity during squat-stand manoeuvres.

	rIPC + Exercise		**rIPC only**		**LMM**
	Week 0	Week 8	Week 0	Week 8	Condition
Power Spectrum					
BP power ($mmHg^2$)	111±80	113±87	116±108	111±99	0.91
MCAv power (cm/sec^2)	43.1±36	40.3±33	45.6±29	41.5±32	0.98
Transfer Function					
Gain ($cm \cdot s \cdot mmHg$)	0.66±0.15	0.68±0.13	0.62±0.24	0.63±0.20	0.85
Ngain *(%.mmHg^{-1})*	1.18±0.30	1.34±0.23	1.25±0.29	1.23±0.17	0.40
Phase (radians)	0.40±0.14	0.42±0.19	0.49±0.17	0.46±0.18	0.52
Coherence	0.72±0.07	0.72±0.08	0.72±0.04	0.71±0.08	0.91

Data presented as means ± SD. Abbreviations; rIPC, remote ischaemic preconditioning;; BP, blood pressure; MCAv, middle cerebral artery velocity; Ngain, normalised gain; LMM, linear mixed model.

Table 5.5: Cerebrovascular reactivity to 5% carbon dioxide

	rIPC + Exercise		rIPC only		LMM
	Week 0	**Week 8**	**Week 0**	**Week 8**	**Condition**
MCAv reactivity (cm.s/mmHg)	1.85±0.90	1.90±0.59	1.65±0.70	1.58±0.69	0.41
CbVCi reactivity (cm.s/mmHg^2)	0.018±0.011	0.013±0.005	0.009±0.006	0.017±0.011	0.26
MAP reactivity (mmHg/mmHg)	0.5±0.4	0.9±0.6	1.1±0.7	1.5±0.7	0.37

Data presented as means ± SD. Abbreviations; MCAv, middle cerebral artery velocity; CvVCi, cerebrovascular conductance index; MAP reactivity, mean arterial pressure; rIPC, remote ischaemic preconditioning; LMM, linear mixed model.

5.3.3 Brachial artery endothelium-dependent vasodilation

Brachial artery FMD% increased by 1.6% (95%CI;0.4, 2.8) in the rIPC+Ex intervention and by 0.3% (-1.1, 1.5) in rIPC only intervention but there was no main effect of condition (P=0.65, Figure 5.2). No intervention-mediated differences were evident in baseline diameter, peak diameter, SRAUC or time to peak (P>0.05, Table 5.6).

The directional changes in brachial artery FMD following an IR injury after both interventions were similar (P=0.50). FMD reduced by of 0.5% (-2.2, 1.3) following rIPC + Ex and by 0.2% (-1.7, 1.6) following the rIPC only. No intervention-mediated differences were evident in baseline diamter, peak diameter, SRAUC or time to peak (P>0.05, Table 5.6).

5.3.4 Cardiorespiratory fitness

VO_{2peak} increased by 2.8 ml/kg/min (1.7, 3.9) following the rIPC + Ex intervention and increased by 0.1 ml/kg/min (-1.0, 1.4) following the rIPC only intervention there was no main effect of condition (P=0.69).

5.3.5 Blood glucose

There was no main effect of condition between rIPC + Ex and rIPC only in changes in fasting blood glucose (P=0.27).

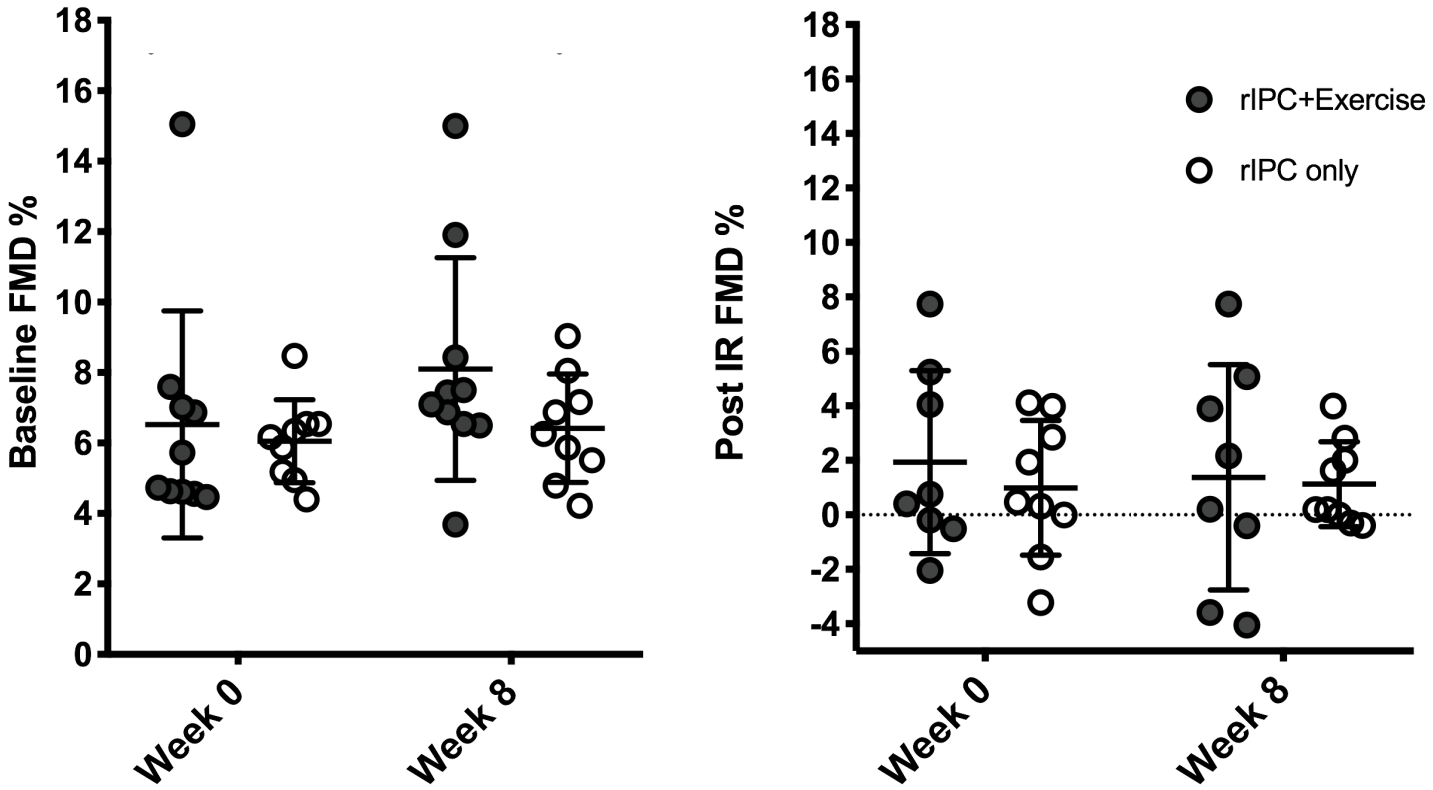

Figure 5.2: Individual data points with means±SD for baseline flow mediated dilation (FMD, left panel) and post ischaemic reperfusion (IR) injury flow mediated dilation (right panel).

Table 5.6: Brachial artery characteristics before and after an ischemia reperfusion injury

	rIPC + Exercise		rIPC only		LMM
	Week 0	**Week 8**	**Week 0**	**Week 8**	**Condition**
Baseline					
Resting diameter (*mm*)	4.0±0.9	4.1±1.0	4.2±0.7	4.1±0.8	0.40
FMD%	6.5±3.2	8.1±3.2	6.1±1.2	6.4±1.6	0.65
Scaled FMD%	6.5±2.6	7.9±1.3	6.1±2.4	6.3±2.3	0.35
Time to peak (*sec*)	77±32	67±29	72±24	63±26	0.83
Shear AUC (10^3)	28.2±15.0	23.3±14.8	22.2±14.9	22.0±14.2	0.64
Post Ischemia Reperfusion Injury					
Resting diameter (*mm*)	4.4±1.0	4.4±1.0	4.3±0.7	4.6±0.9	0.95
FMD%	1.9±3.3	1.4±4.1	1.0±2.5	0.8±1.2	0.50
Scaled FMD%	1.9±2.9	1.3±3.7	1.0±3.0	1.0±3.0	0.56
Time to peak (*sec*)	79±35	73±32	63±39	62±33	0.87
Shear AUC (10^3)	21.2±7.1	19.0±5.7	13.8±10.4	12.1±7.1	0.24

Data presented as means ± SD. Abbreviations; FMD, flow mediated dilation; AUC, area under the curve: LMM, linear mixed model

5.4 Discussion

The present study aimed to examine whether combining aerobic exercise training with rIPC resulted in greater improvements in the cerebral and peripheral vasculature compared to repeated rIPC alone in individuals with increased risk of CVD. The results of this study suggest that increasing the preconditioning stimulus by combining rIPC intervention with exercise training does not mediate greater changes in the cerebral or peripheral vasculature when compared directly to rIPC alone.

Despite increasing the dose of rIPC (compared to previous 7-day rIPC study, see *Chapter 4*) and increasing the rIPC stimulus (by adding exercise training) negligible changes were observed in markers of cerebrovascular function, including CBFv following both interventions. Studies that examined repeated rIPC and found increases in CBF and CBFv typically adopted longer interventions and in patient groups with overt cerebrovascular disease (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017). Nevertheless, the aforementioned studies had not examined cerebrovascular function to understand how the positive changes in CBF and risk of stroke occurrence. The markers of cerebrovascular function dCA and CVR are recognised as independent predictors of ischaemic stroke (Markus and Cullinane, 2001) and provide in-depth information about mechanoreceptor and chemoreceptor, control respectively (Rubanyi et al., 1990, Hoiland et al., 2019). Given the impact of repeated rIPC in stroke patients, it was hypothesised that the repeated rIPC intervention in the present study would mediate positive effects on the cerebral vasculature. Moreover, it was expected the addition of exercise would mediate a greater response, given that some previous exercise training studies have shown improvement in CVR and CBFv with exercise training interventions in a number of

ages and disease groups (Zhu et al., 2011, Akazawa et al., 2012, Murrell et al., 2013). Whilst it cannot be discounted that a longer intervention may have mediated positive changes, or that rIPC and exercise might have had an impact on cerebrovascular function in individuals with overt disease, the data in the present study suggest that repeated rIPC intervention has negligible impact on cerebrovascular function. Within this present study, further investigations into dCA were performed through assessing cerebral autoregulation during both spontaneous and driven BP oscillations, whereas the studies presented in chapter 3 and 4 focus primarily on driven BP oscillations. By extending the scope of the investigation into multiple different methods of assessing dCA, this allows us to identify if similar response are observed in both spontaneous and driven oscillations. Indeed, it is currently debated as to which is the 'best' method of dCA assessment using TCD and TFA (Simpson and Claassen, 2018, Tzeng and Panerai, 2018). In this present study, and in a number of recent studies (Labrecque et al., 2017, Labrecque et al., 2019a), a multifactorial approach to the assessment of dCA was utilised in order to account for potential mechanistic differences in cerebral autoregulation from spontaneous or driven BP oscillations.

The data from this study also shows that combining exercise training with rIPC mediates a 1.6% increase in FMD. Given that a 1% increase in FMD is indicative of a 8-13% reduced risk of cardiovascular events (Thijssen et al., 2019b), the increase observed following the rIPC combined with exercise in the current study is of clinical importance. Intriguingly, a 0.3% increase in FMD was observed in the rIPC only group. This is somewhat smaller than a previous study employing the same repeated rIPC intervention in young healthy individuals (Jones et al., 2015). The differences in responses to the repeated rIPC intervention between the two studies may be explained

by differences in participant groups. Indeed, there is evidence to suggest differences in the time course of changes in FMD are apparent depending on health status following exercise interventions (Schreuder et al., 2015). Similarly, an acute bout of rIPC has attenuated efficacy in older and diseased individuals in response to FMD following an endothelial reperfusion injury (van den Munckhof et al., 2013, Seeger et al., 2016). Collectively, the data presented in this study suggests that either; (i) rIPC, with or without exercise is unable to evoke improvements in cerebral vascular function or, (ii) individuals at risk of CVD may require large 'doses' of rIPC and exercise in order to mediate improvements compared to a healthier population. Additionally, the 0.3% increase in FMD% following the rIPC only intervention is also surprising given the data presented in chapter 4 showed a 1.3% increase in FMD% following 7 days of daily rIPC. Collectively, this evidence suggests that the intensity and frequency in which the rIPC is applied may alter the effectiveness of the outcome measurements, rather than it being attributed to the 'dose' of the rIPC. Such differences in results outlines the importance of future studies identifying an optimal 'dose' of rIPC in order to maximise adaptations as to date, no such study has been published.

Interestingly, no protective effect from either intervention on post IR injury FMD was observed. Numerous studies have acknowledged that rIPC provides protection against the IR injury model adopted in the current study by means of attenuated differences between pre and post IR injury FMD's (Luca et al., 2013, van den Munckhof et al., 2013) (see *Chapter 4*). This inconsistent finding may be attributed to rIPC intervention intensity, given that those aforementioned studies applied either acute (one bout) or daily rIPC in the lead up to the IR injury, building a more substantial preconditioning effect. Additionally, and again some-what surprising was the inability of the exercise

to elicit any protection, especially given the suggestion that exercise does pose a preconditioning-like effect (Thijssen et al., 2018). Both lifelong exercise (Maessen et al., 2017) and short-term exercise interventions (Thijssen et al., 2019a) have resulted in increased tolerance to endothelial IR injury. Given a clinically important change in fitness was observed in the rIPC combined with exercise intervention, it might be more plausible that exercise type may represent an explanation for differences in post IR injury results. However, Thijssen et al found that the protective effects were present and not different following moderate and high intensity exercise (Thijssen et al., 2019a). Assessing further vascular parameters may provide additional information in understanding the effects of an IR injury, with some studies measuring low flow mediated constriction in order to explore a different mechanistic view (Rakobowchuk et al., 2013, Carter et al., 2014b). Alternatively, methodological differences may explain differences in post IR injury FMD results, with the present study adopting a 15 minute- ischemia-15 minute-reperfusion model whereas Thijssen et al. (2019a) used 5 minutes of ischaemic handgrip exercise followed by 15 minutes reperfusion. Further research is warranted to understand the potential preconditioning effect of exercise across a wide population.

5.5 Conclusion

In conclusion, combining 8 weeks of rIPC with exercise does not result in greater changes in cerebrovascular function and peripheral endothelial function compared to a rIPC only intervention in individuals at increased risk of CVD. Therefore, based on this data, careful consideration and further investigation is recommended exloring whether rIPC offers a beneficial short-term intervention for improvement of systemic vascular health in at risk individuals.

Chapter 6: Dynamic cerebral autoregulation assessed using squat stand manoeuvres and transfer function analysis is not influenced by age, sex, cardiovascular disease risk factors or fitness

6.1 Introduction

Cardiovascular and cerebrovascular disease is the leading cause of morbidity and mortality worldwide (Townsend et al., 2016). The age related decline is CBF is well established (Ainslie et al., 2008b, Tarumi and Zhang, 2018), but the age related changes in cerebrovascular function and haemodynamic responses contributing to CBF regulation are not well described. The delivery of oxygenated blood to the brain over a wide range of systemic BP levels by neurogenic, myogenic and endothelial factors is essential in order to maintain normal brain functioning (Tzeng and Ainslie, 2014). dCA refers to the intrinsic ability of the brain to maintain adequate CBF in the presence of transient changes in arterial BP that occur over a number of seconds (Aaslid et al., 1989). dCA acts as a defensive mechanism protecting the brain from potential damage as a result of high or low BP (van Beek et al., 2008). At the same time, BP is controlled neurally by the baroreflex (Monahan, 2007). Both of these regulatory systems are essential in maintaining stable CBF. Impairment in either of these regulatory mechanisms are early markers of cerebrovascular dysfunction and thus associated with increased the risk of cognitive impairment, dementia and stroke (Jordan and Powers, 2012, Laosiripisan et al., 2015).

Over the past few years, it has been established in the literature that currently, the best way to assess dCA is to employ forced BP oscillations by performing repeated squat-stand manoeuvres (Claassen *et al.*, 2009b; Smirl *et al.*, 2015; Simpson & Claassen, 2018) and analyse the data using transfer function analysis (TFA) thus adhering to international recommended guidelines (Claassen et al., 2016). Following the guidelines studies found that, despite the age-related decline in CBF and increase in BP (Ainslie et al., 2008b), there is little evidence of impairment in dCA between young

and old (mean age 23 *vs* 66 years) healthy individuals (Smirl et al., 2015) or within clinical populations (e.g. Alzheimer's) (Claassen et al., 2009a, Smirl et al., 2014a, Lewis et al., 2019) *Chapter 3*). There is some evidence of sex differences for efficiency of dCA but the directions of effect conflict (Xing et al., 2017, Favre and Serrador, 2019, Labrecque et al., 2019a).

In the largest such study to date, Xing et al. (2017) conducted a study (n=136) in healthy individuals aged 21 to 80 years to characterise the age and sex alterations in dCA. They reported dCA was not different across the lifespan but suggested women had a better dCA phase compared to men using 0.05Hz squat stand manoeuvres. Nevertheless, subsequent studies have suggested that cardiorespiratory fitness is an important factor when assessing sex differences in dCA (Labrecque et al., 2017, Labrecque et al., 2019a, Labrecque et al., 2019b). Moreover, Xing et al. (2017) also explored age and sex differences in cardiac baroreflex sensitivity (BRS) (quantified using BP and R-R intervals throughout the same squat-stand protocol). BRS was reduced in the older group but no sex differences were observed. BRS was positively correlated with dCA in young but not middle aged or older healthy participants. However, the fundamental relationship between dCA and cardiac BRS is unclear as other evidence suggests an inverse relationship in young healthy individuals participants (Tzeng et al., 2010), and no relationship in older endurance trained athletes (Aengevaeren et al., 2013) or in heart transplant recipients (Smirl et al., 2014a). The aim of the current study was 2 fold in order to build upon previous work (Xing *et al*., 2017); (i) to examine the impact of sex, cardiorespiratory fitness and the presence of CVD risk factors on dCA and cardiac BRS over the life span; and (ii) to explore the relationships between cardiac BRS and dCA whilst controlling for age and sex. By expanding upon the work by Xing *et al*., (2017) by incorporating individuals with

CVD risk factors and quantifying cardio-respiratory fitness, this will allow us to present findings from a demographically varied population.

6.2 Methods

6.2.1 Participants

Data from eleven studies collected at Liverpool John Moores University, Research Institute for Sport and Exercise Science were examined for eligibility. Data were included if: (i) all measurements were performed with strict adherence to cerebral autoregulation network (CARNET) guidelines (Claassen et al., 2016), (ii), data for individual participants was provided (minimum; age, sex, BMI and resting BP), (iii) data was collected in studies that adhered to the Declaration of Helsinki. Of the data included, 146 participants are from four previously published studies (Carter et al., 2018, Maxwell et al., 2019, Brislane et al., 2020, Carter et al., 2020)

dCA and cardiac BRS recordings were complied with corresponding participant characteristics and medical history (where available) from eleven studies. When studies adopted a repeated measurements design, only baseline data were included. Participant data was excluded if the duration of recordings was < 5 minutes, and if the coherence value was < 0.4. Based on these criteria, 206 participants were with 83 males and 123 females aged between 18-70 years. All participants were non-smokers, with no previous myocardial infarction, stroke or thrombosis. Individuals clinically diagnosed with T2DM were treated with Metformin (n=18) or diet (n=8) at the time of data collection. Additional medications taken by participants included anti-hypertensive (n=15) and lipid lowering (n=16) medication. Participants that had a

body mass index (BMI) >30kg/m^2, diagnosed with hypercholesterolemia or type 2 diabetes, as well treated or untreated ≥ stage 1 hypertension were stratified to a CVD risk group. In total 45 of the individuals who were included with this study met the criteria for increased risk of CVD. Out of the 123 females included within the study 58 were post-menopausal. Post-menopausal women were classified based on having no menstrual cycle for at least 12 consecutive months and were not previously or currently taking any form of hormone therapy (Moreau et al., 2012).

6.2.2 Measurements

dCA: MCAv was measured using TCD, continuous blood pressure was monitored using Finapres and $P_{et}CO_2$ was recorded using a calibrated gas analyser throughout 0.10 Hz squat stand manoeuvres to quantify dCA, as described in detail in Chapter 3.

Baroreflex Sensitivity: Using the same 5-minute recording window during the 0.10 Hz squats-stand manoeuvres, continuous cardiac BRS was assessed. The cardiac BRS was determined by applying TFA to systolic BP (SBP) and R-R interval (pressure-cardiac interval) at the point frequency (low frequency) of the squat-stand manoeuvres (0.10 Hz). Data analysis was performed using a commercially available software Ensemble (Version 1.0.0.28, Elucimed, Wellington, New Zealand). Mean gain (magnitude of relationship between SBP and R-R intervals changes), phase (temporal displacement of R-R intervals and SBP) and coherence (linear correlation between changes in R-R interval and SBP changes) along with spectral power of systolic BP and R-R interval were calculated in the low frequency range (0.10 Hz).

6.3 Statistical Analysis

Statistical analysis was performed using IBM SPSS version 26 (SPSS Inc., Chicago, IL). Firstly, participants were divided into three age categories: young (18 – 35 years, n= 93), middle age (36 – 55 years, n=62) and old age (56 - 70 years, n=51). Between age-category differences in baseline characteristics and power spectrum densities during squat stand manoeuvres were explored using one-way ANOVA. To examine the influence of age, sex, CVD risk and VO_{2max} linear regression was employed. Cross sectional associations between age and measures of dCA and cardiac BRS were examined using linear regression adjusting for sex (Model 1). Multivariable linear regression was used to further adjust for health status (model 2) as well as VO_{2max} (model 3).

Relationship between cardiac BRS and dCA: The linear relationship between cardiac BRS and dCA was determined using R^2 . For the models, each parameter of cardiac BRS was independently used as a predictor variable and each parameter of dCA an outcome variable with adjustments for age and sex. Multicollinearity was investigated using variance inflation factor. Statistical significance was set a $P<0.05$.

6.4 Results

Participant characteristics: There was an increase in SBP, DBP and BMI (P <0.001) and decrease in MCAv and VO_{2max} (P <0.001) with age (Table 6.1), with a trend towards an inverted-U relationship between $P_{ET}CO_2$ and age (P=0.08)

Table 6.1: Participant characteristics when divided into age categories

Characteristics	**Age categories**			**ANOVA**
	18-35 yrs (young) N=93	**36-55 yrs (middle age) N=62**	**56-70 yrs (old age) N=51**	***P* Value**
Age (*years*)	26±5	47±6	61±4	
Male/Female	45/48	18/44	20/31	
SBP (*mmHg*)	115±11	120±15	138±18	<0.001
DBP (*mmHg*)	67±11	73±10	78±10	<0.001
VO_{2max} (ml.kg.min)	42.2±10.8	28.6±7.4	23.7±5.2	<0.001
BMI (kg/m^2)	24±3	27±6	29±5	<0.001
MCAv (*cm.s*)	67±13	64±13	56±13	<0.001
$P_{ET}CO_2$ (*mmHg*)	36.8±4.3	37.9±4.8	35.9±4.9	0.08

Data presented as mean±SD. Abbreviations; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; MCAv, middle cerebral artery velocity; $PETCO_2$, partial pressure of end tidal carbon dioxide; ANOVA, analysis of variance

dCA: Age, sex, CVD risk factors and VO_{2max} do not impact on the dCA parameters normalised gain, phase or coherence with minimal change (β) compared to the young aged reference group (18-35 yrs) in all statistical models (P>0.05, Table 6.2). There was a significant reduction in dCA gain with age, which was apparent when adjusted

for sex and CVD risk factors (young – middle age; β = -0.09, P = 0.02 and young - old age; β = -0.18, P <0.001, model 2) but not when adjusted for VO_{2max} (model 3).

BRS: Cardiac BRS gain was attenuated with age when adjusted for sex and CVD risk factors (young – middle age; β = -2.18, P <0.001 and young - old age; β = -2.86 P <0.001, model 2) along with cardiac BRS phase (young – middle age; β = -0.31, P <0.001 and young - old age; β = -0.44 P <0.001, model 2) but not adjusted for VO_{2max} (model 3).

Power spectral analysis: When divided into age categories, dCA BP power, MCAv power and cardiac BRS R-R interval power all decreased with age (P<0.001) with no difference in SBP power (P=0.55, Table 6.3).

Relationship between cardiac BRS and dCA: There was no correlation between dCA normalised gain and dCA phase with either parameter of cardiac BRS (P >0.05; Figure 6.1), but dCA gain shows a significant inverse relationship. dCA gain was correlated with cardiac BRS gain (R^2 = 0.19, P <0.001) and with cardiac BRS phase (R^2 = 0.18, P <0.001). However, the total variance explained in these significant outcomes is small, meaning that other factors are likely to be important, whether independent or as interacting variables.

Table 6.2: Cross sectional associations between age and both dCA and cardiac BRS during 0.10 Hz squat stand manoeuvres.

	Mean±SD	Model 1		Model 2		Model 3	
		β (95% CI)	*P* value	*β* (95% CI)	*P* Value	*β* (95% CI)	*P* Value
dCA Normalised Gain *(%.mmHg^{-1})*							
18-35 yrs	1.34±0.28	reference		reference		reference	
36-55 yrs	1.31±0.30	-0.04 (-0.14,0.06)	0.42	-0.03 (-0.14,0.07)	0.55	0.00 (-0.18,0.18)	0.96
56-70 yrs	1.29±0.34	-0.06 (-0.16,0.05)	0.29	-0.04 (-0.16, 0.08)	0.55	0.01 (-0.20,0.21)	0.96
dCA Gain (*cm.s^{-1}. mmHg^{-1}*)							
18-35 yrs	0.89±0.23	reference		reference			
36-55 yrs	0.82±0.22	-0.09 (-0.16,-0.02)	0.01	-0.09 (-0.16,-0.01)	0.02	-0.05 (-0.17,0.08)	0.45
56-70 yrs	0.70±0.18	-0.20 (-0.28,-0.13)	<0.001	-0.18 (-0.27,-0.10)	<0.001	-0.22 (-0.36,-0.08)	0.002
dCA Phase (*radians*)							
18-35 yrs	0.39±0.28	reference		reference		reference	
36-55 yrs	0.35±0.32	-0.007 (-0.10,0.08)	0.88	-0.001 (-0.09,0.09)	0.98	-0.004 (-0.18,0.17)	0.96
56-70 yrs	0.39±0.24	0.01 (-0.08,0.11)	0.78	0.02 (-0.09,0.14)	0.67	0.10 (-0.09,0.29)	0.29
dCA Coherence							
18-35 yrs	0.67±0.1	reference		reference		reference	
36-55 yrs	0.65±0.1	-0.02 (-0.05,0.02)	0.34	-0.02 (-0.06,0.01)	0.20	-0.02 (-0.07,0.04)	0.55
56-70 yrs	0.70±0.1	0.03 (-0.01,0.06)	0.18	0.01 (-0.03,0.05)	0.59	0.02 (-0.05,0.08)	0.58
BRS Gain *(ms/mmHg)*							
18-35 yrs	5.99±2.96	reference		reference		reference	
36-55 yrs	3.57±2.27	-2.18 (-3.00,-1.36)	<0.001	-1.85 (-2.70,-0.99)	<0.001	-0.54 (-1.67,0.58)	0.34
56-70 yrs	3.01±2.06	-2.86 (-3.72,-1.99)	<0.001	-2.21 (-3.20,-1.22)	<0.001	-0.60 (-1.87,0.67)	0.35
BRS Phase (*radians*)							
18-35 yrs	-0.78±0.42	reference		reference		ref reference	
36-55 yrs	-1.11±0.56	-0.31 (-0.48,-0.14)	<0.001	-0.31 (-0.49,-0.13)	0.001	-0.20 (-0.50,0.09)	0.18
56-70 yrs	-1.22±0.61	-0.44 (-0.60,-0.25)	<0.001	-0.43 (-0.63,-0.22)	<0.001	-0.28 (-0.61,0.06)	0.10
BRS Coherence							
18-35 yrs	0.70±0.13	reference		reference		reference	
36-55 yrs	0.64±0.11	-0.06 (-0.09,-0.02)	0.004	-0.06 (-0.10,-0.02)	0.003	-0.05 (-0.11,0.14)	0.13
56-70 yrs	0.67±0.11	-0.03 (-0.07,0.01)	0.13	-0.04 (-0.09,-0.01)	0.11	0.02 (-0.05,0.09)	0.60

The regression coefficient β represents the change in the parameter from either young (15-35 yrs)-middle (36-55 yrs) aged or from young-old (56-70 yrs) aged when accounting for model covariates. Model 1: Adjusted for sex. Model 2: Adjusted for sex and health status (healthy or CVD risk). Model 3: Adjusted for sex, health status and VO_{2max}.. Abbreviations; dCA, dynamic cerebral autoregulation; BRS, baroreflex sensitivity.

Table 6.3: Power spectral analysis of both dynamic cerebral autoregulation and baroreflex sensitivity during 0.10 Hz squat stand manoeuvers.

	Age categories			ANOVA
	18-35 yrs (young) N=93	**36-55 yrs (middle age) N=62**	**56-70 yrs (old age) N=51**	
Dynamic cerebral autoregulation				
BP power *(mmHg^2)*	215±128	172±105	140±124	0.001
MCAv power (cm/sec^2)	166±97	132±91	60±41	<0.001
Baroreflex sensitivity				
R-R interval power (*ms^2*)	8916±6932	4390±5190	3146±3991	<0.001
SBP power (*mmHg^2*)	474±368	412±299	470±429	0.55

Values are mean±SD. Abbreviations; BP, blood pressure; MCAv, middle cerebral artery velocity; SBP, systolic blood pressure.

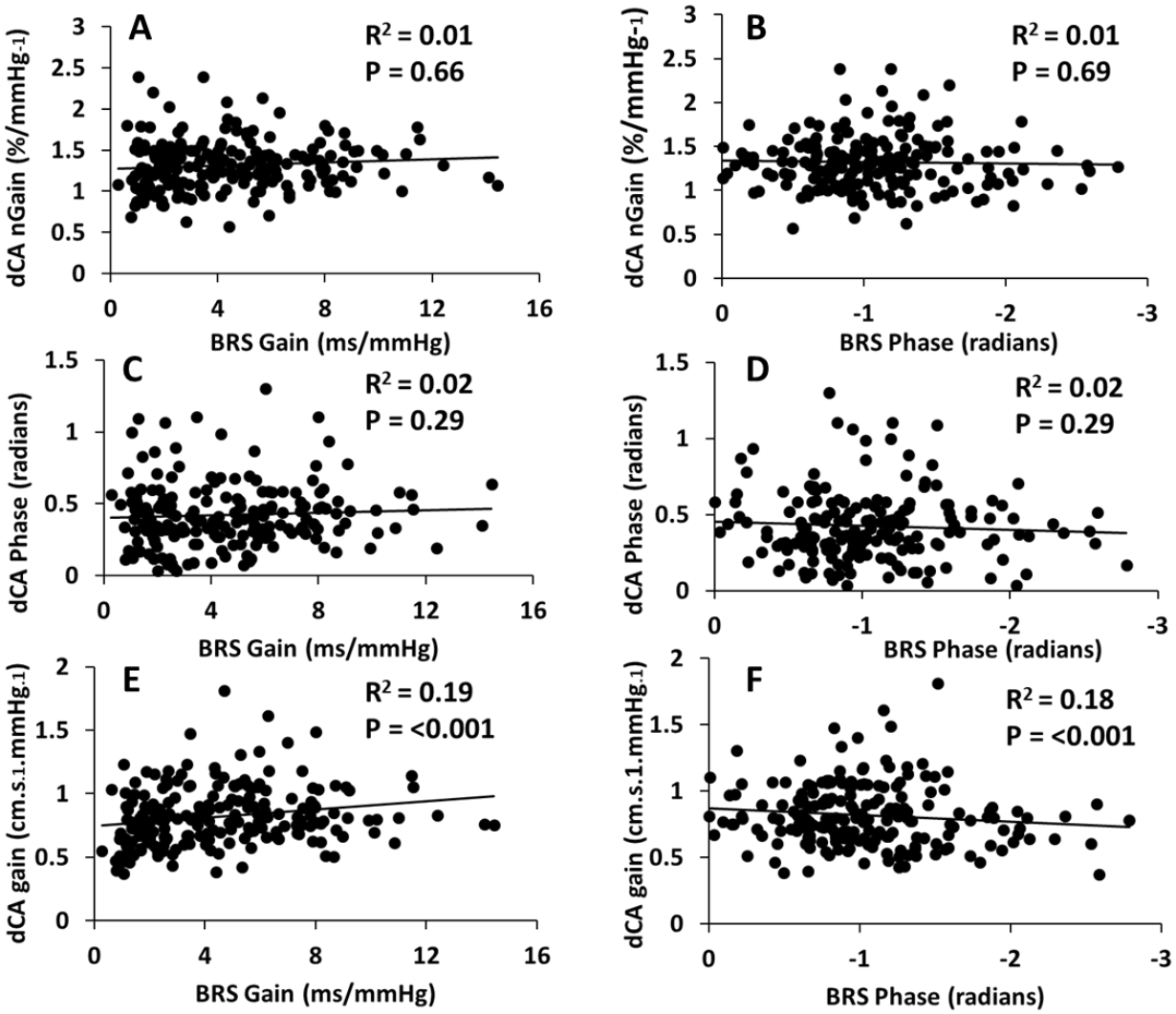

Figure 6.1: Relationship between dynamic cerebral autoregulation and baroreflex sensitivity during 0.10 Hz squat stand manoeuvres. Data presented as individual data points with R^2 and P values.

6.5 Discussion

The aims of the current study were to (i) examine the impact of sex, cardiorespiratory fitness and the presence of CVD risk factors on dCA and cardiac BRS over the life span; and (ii) explore the relationships between cardiac BRS and dCA whilst controlling for age and sex. The data indicates that dCA, measured using repeated squat stand manoeuvres, is not impaired with age, and not impacted by sex, fitness or the presence of CVD risk factors. Cardiac BRS was reduced with aging, and displayed an inverse relationship with TFA gain, but no relationship with any other parameter of dCA.

Ageing is a well-established principle risk factor for cerebrovascular disease and complications. A number of cerebral hemodynamic parameters have been shown to change with age, including reductions in CBF volume and CBFv (Krejza et al., 1999, Ainslie et al., 2008b), yet the data from the current study suggest that the intrinsic ability of cerebral vessels to maintain stable flow despite changes in BP appears to be unaffected by ageing. The ability of the cerebrovasculature to buffer these changes in BP represents a vital defence mechanism protecting the brain (Claassen and Zhang, 2011), and data from this present study is in agreement with that of smaller previous studies which identified no reduction in dCA with ageing using both squat stand manoeuvres (Oudegeest-Sander et al., 2014, Smirl et al., 2014a, Xing et al., 2017) or other dCA techniques (Carey et al., 2000, Yam et al., 2005, Dineen et al., 2011). The data presented in this study extends these findings of preserved dCA with ageing, showing that dCA is not different between sexes when age is considered, and is also unaffected by CVD risk factors during low frequency squat stand manoeuvres. With the cohort of participants and based on the risk factors included within this study, no

evidence of any decline in dCA with increased risk of CVD was observed. Central obesity, hypertension, hypercholesterolemia and T2DM represent major risk factors in the development of systemic vascular disease and complications (Seven, 2015) including significantly increased risk of cerebrovascular disease (Pinto et al., 2004, Kivipelto et al., 2005, Law et al., 2009). Each risk factor, individually or collectively is associated with endothelial dysfunction, increased arterial stiffness, alongside a range of other vascular abnormalities (Stapleton et al., 2008). Despite these vascular changes, no reduction in dCA was identified when using forced BP oscillations induced by squat stand manoeuvres. In chapter 3, a small sample of individuals with increased CVD risk had a similar dCA to young healthy individuals, with this present study confirming the original observation but on a larger scale. To date, no other studies have assessed dCA in a population at high risk of CVD. Studies utilising the same dCA methods have observed no difference in patients with chronic obstructive lung disease (Lewis et al., 2019) or in early stage Alzheimer's (Claassen et al., 2009a). Collectively, the data from this study suggests that despite the vascular maladaptation's that are associated with CVD risk factors, the intrinsic ability of the cerebral blood vessels to maintain stable flow is persevered.

Elevated cardio-respiratory fitness is associated with increased resting CBFv values (Ainslie et al., 2008b) and better CVR (Bailey et al., 2013), but its association with dCA is less conclusive. We have provided evidence that indicates that those individuals with increased cardio-respiratory fitness (VO_{2max}) do not have an improved ability to counteract acute, large changes in BP. Interestingly, two previous studies concluded higher VO_{2max} was related to attenuated dCA (Lind-Holst et al., 2011, Labrecque et al., 2017) whereas, Aengevaeren et al. (2013) identified no effect of VO_{2max} on dCA. Disparities in results are likely down to differences in dCA

assessment methods, further stressing the importance of following previously published guidelines (Claassen et al., 2016). This current study has provided evidence in a demographically varied cohort, using a single method of dCA assessment with TFA, suggesting that better VO_{2max} does not translate to enhanced dCA. The physiological connection between VO_{2max} and dCA is unknown but likely to be a complex multifactorial relationship. Indeed, increased VO_{2max} is associated with lower stroke risk (Lee and Blair, 2002), improved cognition (Brown et al., 2010) and all round lower mortality risk from CVD (Kodama et al., 2009), therefore raising the concern that either dCA mechanisms are unrelated to such clinical events, or the use of TCD and TFA parameters are not sufficient enough to categorically understand dCA.

Data from this study further supports a wealth of research that shows cardiac BRS declines with age (Monahan, 2007, Smirl et al., 2014a, Xing et al., 2017). Similarly, the data in this present study provides additional evidence that CVD risk factors are linked to reduced cardiac BRS (Skrapari et al., 2007, Madden et al., 2010, Sakamoto et al., 2019). The relationship between dCA and cardiac BRS however, has proven complex. Understanding whether enhanced BP control leads to better control of CBF or vice visa is of importance in understanding how these regulatory mechanisms operate, and whether they should be the focus of interventions. The maintenance of BP and CBF with postural changes, and thus the avoidance of dizziness or syncope, depends on the action of baroreflexes to compensate (James and Potter, 1999). Additionally, having an efficient and fast acting BP control (cardiac BRS) could potentially limit BP fluctuations that are thus transferred into the cerebral vascular system. This present study provides evidence showing that cardiac BRS parameters show no relationship with dCA normalised gain and dCA phase during forced BP

oscillations, but does appear to have a relationship with dCA (absolute) gain. This relationship demonstrates that a reduced cardiac BRS gain and BRS phase is associated with a reduced dCA (absolute) gain. This implies that the lower an individual's BRS (i.e. reduced BP control), the more efficient their dCA is at counteracting large fluctuations in BP. As previously mentioned, it is important to consider that the parameter of gain reflects the absolute change in CBFv, whereas when normalised to account for baseline CBFv and BP (normalised gain) (van Beek et al., 2008) no relationship was present. Therefore, the data in this study outlines that despite having a significantly greater BP control at a younger age, this does not alter how well the cerebral vessels regulate blood flow during BP challenges. Nevertheless, an inverse relationship between dCA (absolute) gain and cardiac BRS is not completely surprising, given that one previous study using TCD to measure rate of regulation and autoregulation index for dCA, and the modified Oxford technique to estimate BRS identified an inverse relationship between the two processes (Tzeng et al., 2010). On the other hand, previous studies utilising the same methods adopted in this present chapter concluded no relationship between dCA and BRS parameters (Aengevaeren et al., 2013, Smirl et al., 2014a). Referring back to the original Lassen curve of CA, CBF remained largely constant independent of BP changes between 60 to 150 mmHg (Lassen, 1959), although this is an often challenged observation (Lucas et al., 2010, Tan, 2012). To what extent a plateau region of CBF over a given change in BP remains elusive. However, if a plateau region does exists or is similar to that outlined by Lassen (1959), this indicates that the baroreflex, as an important mechanism controlling short-term BP regulation may have negligible input in regulation short term CBF (Tzeng et al., 2010). Identifying the fundamental relationship between this functional role of the cardiac baroreceptors and dCA is of

clinical significance, primarily because baroreflex impairment is an adverse prognostic indicator for both cardiac and cerebrovascular disease (Tzeng et al., 2010).

6.6 Conclusion

In conclusion, this study has provided data from a wide cohort of participants, which shows that dCA remains intact during the ageing process of 18-70 years. The data presented in this study suggests that common risk factors for CVD are not impairing dCA and that having increased cardio-respiratory fitness does not translate to more efficient dCA. Finally, this present study shows that there is a relationship between cardiac BRS and dCA (absolute) gain, and that this relationship implies that a reduced cardiac BRS is associated with an enhanced dCA, but this relationship is not present when normalised for baseline values (normalised gain).

Chapter 7: Synthesis of Findings

7.1 Aims of the thesis

The work described in the present thesis was designed to investigate the impact of rIPC, using acute (one bout) of rIPC as well as repeated bouts for 7 days daily and 8-week (3 x per week), on both peripheral and cerebrovascular function in individuals at risk of CVD. Given the lack of change in cerebral autoregulation as an early marker of cerebrovascular function with the interventions implemented within this thesis, the final study was designed to investigate whether age, sex, cardiorespiratory fitness or CVD risk factors affected cerebral autoregulation.

7.2 Summary of Major Findings

The novel work undertaken in this thesis has generated new knowledge for the literature and for clinical practice. The main findings of this thesis are:

1. In chapter 3, a single bout of rIPC did not influence CBFv or cerebrovascular function acutely in healthy individuals, or those at increased CVD risk.

2. Chapter 4 demonstrated that in a randomised pilot study, the directional changes suggest peripheral vascular endothelial function can be enhanced with 7-day daily rIPC in T2DM.

3. Chapter 5 identified that combining aerobic exercise with rIPC for 8 weeks does not mediate greater changes in cerebral and peripheral vascular function compared to a rIPC only intervention.

4. In chapter 6, dCA measured using squat stand manoeuvres and TFA was unaffected by age, CVD risk factors and cardio-respiratory fitness and operates independent of the baroreceptors.

7.3 General discussion of major findings

7.3.1 The Dose of rIPC

rIPC can be administered as a single dose (i.e. 4 x 5 min bouts of forearm ischemia and reperfusion) and has shown cardioprotective benefits in the myocardium and the peripheral vasculature (Botker et al., 2010, van den Munckhof et al., 2013). Increasing the 'dose' of rIPC by simply increasing the amount of times the ischemia-reperfusion cycles are applied was investigated throughout the current thesis. The data from this thesis suggests that increasing the dose of rIPC by either increasing the number of bouts (chapters 4 and 5) and/or or combing with a potentially mutually beneficial intervention of exercise training (chapter 5) has little impact on improving peripheral vasculature or cerebrovascular outcomes in individuals with CVD risk factors.

One of the novel aspects of this thesis was to examine the impact of rIPC on the cerebrovasculature. Chapter 3 suggested that an acute bout of rIPC had no immediate (i.e. during the first window of protection) effect on cerebrovascular function. Whilst an acute bout of rIPC has been shown previously to have a positive impact on endothelial IR injuries in older individuals and those with congestive heart failure (van den Munckhof et al., 2013, Kono et al., 2014, Thijssen et al., 2016a), there is evidence to suggest that there is also little impact on vascular function measured using the FMD technique (Enko et al., 2011, Moro et al., 2011), so no acute effect may not be surprising. Nevertheless, increasing the dose by performing more bouts of rIPC over days (chapter 4) or weeks (chapter 5) also had little impact on cerebrovascular function. In the present thesis the largest dose was administered in chapter 5, the dose of rIPC was 3 x per week, thus 24 bouts in total over an 8 week period. In comparison to the previous studies which have observed improvements in cerebrovascular markers and

stroke reoccurrence with daily and/or twice daily rIPC bouts over a longer intervention period (~ 300 individual bouts) (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017) the dose applied in the current thesis is still a relatively small. Therefore, research studies exploring the impact of larger rIPC doses (duration of intervention and frequency of bouts) or investigating the optimal dose are required to further explore the impact of rIPC of cerebrovascular health.

Large doses of rIPC have typically been employed to improve parameters of the cerebral circulation but smaller doses (duration of intervention and frequency of bouts) have been shown to be effective in the peripheral vasculature. Chapter 5 in the present thesis supports that of two previous studies which show that FMD can be improved in as little as 7 days (7 rIPC bouts) (Luca et al., 2013, Jones et al., 2014) in individuals with T2DM that likely have vascular dysfunction. Smaller doses of rIPC have also elicited improvements in FMD with in healthy individuals, Jones et al. (2015) demonstrating that 6 bouts spread over two weeks as well as 24 bouts (3 x per week for 8 weeks) of rIPC also results in enhanced FMD. It is plausible that individuals with vascular dysfunction may require a larger dose of rIPC to show changes in vascular function and further research studies examining this concept or warranted. Interestingly, the ability of rIPC to provide protection against a temporary endothelial IR injury was not consistent within this thesis, whereas a small number of studies have observed sustained protection from rIPC to the same IR injury model used in this thesis (Kharbanda et al., 2001, Kharbanda et al., 2002, Luca et al., 2013). In Chapter 4, following 1 week of daily rIPC, the dysfunction following an IR injury was reduced compared to that of pre intervention values. However, in study 5, with 3 rIPC bouts per week for 8 weeks, post IRI FMD was unchanged following both interventions.

Such findings further support the call for future research to target identifying the optimal dose of rIPC.

To what extend the proximity of the stimulus to the target area is unknown. Whether rIPC applied to a limb, as was the case throughout this thesis, is too far away from target area (cerebral blood vessels) may be a potential explanation to the lack of adaptation from rIPC in chapters 3, 4 and 5 of this thesis. On the other hand, given the growing body of evidence suggesting that rIPC induces humoral and neural factors that translate into systemic changes/adaptations (Heusch et al., 2015, Thijssen et al., 2016a), the site/location in which the rIPC is applied should not necessarily determine the extent of protection or adaption in the target area. Intriguingly, evidence from a rodent model showed that direct IPC was less effective and providing protection against renal IR compared to rIPC (Oral et al., 2018). Furthermore, human studies, including the data from chapter 4 of this thesis, have shown no difference between remote and local IPC (Jones et al., 2014) in the peripheral vascular beds but to do what extent this also applied to the cerebral vascular bed in unknown, and is likely to be limited to animal models. Alternative strategies to induce IPC-*like* stimulus in terms of the fluctuations blood flow and shear stress patterns may be of potential interest including; cold exposure (Brown et al., 2003), hypoxia (Harris et al., 2013) and water immersion (Carter et al., 2014a) certainly warrant further investigation.

7.3.2 rIPC in clinical populations

An important consideration when executing any intervention is the population who are targeted. Numerous rIPC interventions have focused on individuals who are deemed fit and healthy with no current health risk (Kimura et al., 2007, Luca et al., 2013, Jones et al., 2014, Jones et al., 2015, Lindsay et al., 2017) or with various

diseases (Meng et al., 2012, Liang et al., 2015, Meng et al., 2015, Pryds et al., 2017, Wang et al., 2017) with few studies aimed at those individuals deemed at risk. Whether the effectiveness of rIPC is influenced by the health status of an individual, or the health of the endothelium is unclear. Therefore, the individuals primarily targeted within the current thesis were those who are deemed at risk of CVD, therefore examining the impact of rIPC as an early intervention to improve vascular health and reduce disease risk was a primary focus. A recent study in hypertensive patients suggested that 30 days of daily rIPC (30 bouts) was successful in reducing BP and also improved micro-vessel endothelial function, thus reducing their risk of hypertension complications (HyTong et al., 2019). Interestingly such reductions in BP were not evident in chapters 4 and 5 of this thesis, but in chapters 4 and 5 the total dose and rIPC was lower and not all individuals were hypertensive nor had any know cardiovascular or cerebrovascular disease. Taken together, it is possible that those individuals diagnosed with a CV disease (e.g. hypertension) or post CV event (e.g. stroke) may benefit the most from a rIPC intervention (of a big enough dose). Future studies examining the effectiveness of rIPC interventions in healthy, at risk of CVD, with overt risk factors and with CVD or cerebrovascular disease are required.

7.3.3 Interventions and cerebrovascular function

Interventions that target CBF and cerebrovascular function are of paramount importance in preventing and treating cerebrovascular health, cognitive decline, vascular dementia and stroke (Kalaria et al., 2016). Interventions, such as exercise but also pharmacological and lifestyle interventions have been implemented targeting the improvement of cerebrovascular function but findings on the impact of such interventions have been inconsistent as to whether they can improve CBF or

cerebrovascular function (Akazawa et al., 2012, Murrell et al., 2013, Drapeau et al., 2019, Lewis et al., 2019). In the present thesis the addition of exercise to the rIPC intervention in chapter 5 did not mediate any changes in the cerebrovascular function. Exercise improves systemic cardiovascular function, but specific to the brain exercise is thought to increase shear stress and signalling cascades as well as enhances neural activation and metabolism (Lucas et al., 2015). As outlined in earlier chapters, to the best of our knowledge no study to date has reported an improved dCA measured using TFA following an exercise training intervention. Cross-sectional studies have identified no beneficial adaptations to dCA with habitual exercise training (Aengevaeren et al., 2013, Perry et al., 2019). Acute exercise elicits increases in CBF up to around 70% VO_{2max} before hyperventilation induced hypocapnia either plateaus or reduces CBF (Lucas et al., 2015, Smith and Ainslie, 2017). These increases in blood flow and shear stress patterns may contribute to enhanced cerebrovascular function through improved endothelium function (Smith et al., 2019), but the extent to which this can contribute to enhanced dCA remains unanswered.

An additional factor which must be considered when assessing cerebrovascular function using dCA is what exactly an improved dCA presents itself as. Classically, the consensus has focused on improved dCA would be represented by TFA as reduced gain and/or improved phase (van Beek et al., 2008, Claassen et al., 2016), however given the lack of published data that shows any improvements in dCA using similar techniques (squat-stand manoeuvres and TFA) adopted throughout this thesis, a lot of questions remained unanswered. Theoretically, the argument could be made that for dCA to truly be improved both parameters (gain and phase) should display improvements and physiologically they may be true. If an individual improves the temporal alignment of BP-CBF oscillations (phase) – this could in turn result in a

reduced BP-CBF magnitude (gain). Nevertheless, this all remains speculative and future work is required to accurately understand the metrics of dCA with TFA, outlining if the metrics are associated with each other, and if one parameter of dCA is more physiologically 'important' than another.

Given the lack of consistent changes in exercise and lifestyle interventions on cerebrovascular function it might not be surprising that a rIPC intervention did not mediate a beneficial effect. One the other hand, given the previous remarkable finding in stroke patients of increased CBF, reduced stroke recurrence and decreased white matter hyperintensities (Meng et al., 2012, Meng et al., 2015, Mi et al., 2016, Wang et al., 2017), then one might expect some functional cerebrovascular changes. The experimental studies in this thesis attempted to extend the understanding of rIPC by investigating functional parameters of the cerebral circulation, to try and understand how rIPC may elicit such adaptations. The data from these chapters show that rIPC did not alter CBFv or any of the aspects of cerebrovascular function. As discussed above this could be explained by rIPC dose or evidence of disease but one other potential explanation could be related to how cerebrovascular function was assessed in the current thesis.

7.3.4 Measurements of cerebrovascular function

TCD offers a non-invasive and high temporal resolution measurement technique for the assessment of CBFv and together with measurements of BP, expired CO_2 and neural activity can provide information on cerebrovascular function. Throughout this thesis, TCD was utilised to assess cerebrovascular function using a number of assessment techniques set out by Willie et al. (2011). The measurement of dCA was performed throughout all experimental studies in this thesis as well as being the

primary focus in chapter 6. dCA represents an essential protective mechanism ensuring stable blood flow to the brain (van Beek et al., 2008), therefore maintaining good or even improving dCA is likely to be of benefit. Measurement and assessment of dCA however is not straightforward and whilst attempts have been made in order to standardise measurement techniques when using TCD together with BP to understand cerebral autoregulation and to improve repeatability (Claassen et al., 2009b, Meel-van den Abeelen et al., 2014, Claassen et al., 2016, Simpson and Claassen, 2018), there still appears to be a range of measurement technique in the literature.. It is possible that these techniques or protocols are not sensitive enough to assess changes in dCA. It is noteworthy that in an attempt to obtain a more rounded dCA profile, in chapter 5 assessment of MCAv in response to spontaneous BP fluctuations was also included in the testing protocol alongside the forced BP oscillation in the squat stand manoeuvres. Nevertheless as seen in chapter 5, neither measurements of autoregulation were changed following either intervention.

In chapters 3, 4 and 5, none of the interventions used mediated any change in dCA. Whilst this could be as a direct consequence of the intervention, it is also possible that either the physiological variable being targeted is not responsive to the intervention and /or the measurement technique is insensitive. As previously mentioned throughout the thesis, there is consistent evidence of impairment in peripheral vascular function with CVD risk, yet no such evidence is available regarding dCA, a key homeostatic mechanism. The results from chapter 6 suggest that CVD risk factors are not influencing dCA when assessed using squat stand and TFA and that ageing does not alter dCA from 18-70 years. Based on the findings from this chapter, should dCA be a target for interventions if it is not impaired with ageing (unlike CBF). There are also studies that have utilised the same squat stand protocol with TFA as employed within

the current thesis that show no impairment in dCA in heart transplant recipients (Smirl et al., 2014a), chronic obstructive lung disease (Lewis et al., 2019) or stage 2 Alzheimer's (Heus et al., 2018). Future research studies are required to examine the sensitivity of these techniques in assessing dCA or alternative measurement techniques or protocols need to be developed to understand changes in cerebral autoregulation with age and/or intervention.

7.4 Methodological Considerations and Limitations

There are a number of strengths in the methodology of this thesis. The participants recruited for each of the experimental studies in this study give a good representation of the general population in the UK. Chapters 3, 4, 5, and 6 all contain individuals with established risk factors for CVD, chapter 4 focuses on individuals diagnosed with T2DM and chapters 3 and 6 also included a cohort of both young and old healthy and active individuals. Based on this, the findings presented from this thesis are applicable to a wide cohort and not limited to one participant group.

Each study of this thesis ensured strict inclusion and exclusion criteria in addition to the control of diet and exercise prior to and during laboratory visits. Importantly, each measurement applied within this thesis was performed adhering to the most recent published guidelines for that specific measurement. For example, FMD assessments were undertaken according to the latest peer-reviewed consensus guidelines (Thijssen et al., 2011a), together with the use of custom-designed edge-detection and wall-tracking analysis software, the accuracy, validity and prognostic value of FMD outcomes were maximised. dCA measurements were all performed following cerebral autoregulation network recommendations (Claassen et al., 2016). Another major strength to the work contained within this thesis was the research design of the

experimental studies. Chapters 3 and 5 include intervention trials including an 8 week intervention, and chapter 4 included a randomised control trial. Lastly, chapter 6 included a relatively large sample size, incorporating a demographically varied cohort of individuals in an in order to describe changes in dCA and BRS.

Despite these methodological strengths, there are a number of limitations contained. The most notable limitation within this thesis is the use of TCD for the measurement of CBF and cerebrovascular function. As is frequently discussed in a number of reviews (Willie et al., 2011, Ainslie and Hoiland, 2014), TCD provides measurement of CBFv as an index of CBF, as TCD does not provide feedback regarding vessel diameter. Changes in vessel diameter can affect measurement accuracy of CBF with TCD (Heus et al., 2018). CBFv is an adequate surrogate of absolute flow only if the insonated vessel maintains constant vessel diameter across time and experimental conditions (Ainslie and Hoiland, 2014), and it is unlikely that acute rIPC induced a vessel diameter change, but chronic diameter changes connect be excluded. The use of additional imaging techniques such as MR arterial spin labeling, SPECT, and Xenon-CT would overcome such limitations, however their temporal resolution is currently too low to assess the dynamics of dCA (Heus et al., 2018) and they are either expensive or invasive techniques. The MCA diameter is unlikely to change under resting conditions or during moderate changes in BP (Serrador et al., 2000), similar to that induced during repeated squat stand manoeuvres. MCA diameter alterations in responses to changes in blood CO_2 content remains a highly controversial topic (Brothers and Zhang, 2016, Hoiland and Ainslie, 2016). Previous work utilising MRI identified that with increases of $P_{ET}CO_2$ of ≈8 mmHg MCA diameter remains unchanged (Serrador et al., 2000), however more recent studies using higher resolution MRI (3 and 7 Tesla) have shown both dilation and constriction of the MCA during

hyper- and hypocapnia (Coverdale et al., 2014, Verbree et al., 2014, Coverdale et al., 2015). Certain studies have highlighted that MCA diameter may remain constant during modest changes in $P_{ET}CO_2$ but no definitive threshold has yet been described (Verbree et al., 2014). Based on this uncertainty it is important to acknowledge that the MCAv data during hypercapnia may have underestimated flow as a result of potential MCA diameter changes.

The model employed to induce a temporary endothelial IR injury in chapters 4 and 5 is used only as a surrogate index to cardiac tissue. Nonetheless, this is a frequently used model to assess endothelial ischemia reperfusion injuries in research studies (van den Munckhof et al., 2013, Carter et al., 2014b, Thijssen et al., 2019a). Moreover, applying this technique significantly decreases plasma nitrite and plasma nitrate concentrations, indicating that any change in endothelial IR injury is due to a reduction in NO bioavailability (Aboo Bakkar et al., 2018) and thus provides some mechanistic insight.

In chapter 6, whilst the study sample size was the largest to describe dCA to date, there are still some weaknesses/limitations of the study. Cross sectional study designs are limited in their ability to confirm causality/mechanisms. Additionally, despite the sample size being relatively large, it is still possible that the data lacked the statistical power to detect differences due to effect sizes of the measurements included.

7.5 Recommendations for clinical practise and future studies

Whilst the phenomenon of IPC has come a long way since it was first identified by Murry *et al* in 1986, it still has some way before it is implemented clinically. This thesis aimed to answer questions regarding the impact of rIPC as an intervention in

individuals at risk of CVD and explore its effects on systemic vascular function. rIPC has the potential to offer a relatively low cost, non-invasive and easily applicable intervention to improve a range of parameters of cardiovascular health. Given the improvements in peripheral vascular function in chapter 4 and because endothelial dysfunction represents a significant event in the atherosclerotic cascade and predicts cardiovascular and cerebrovascular events (Inaba et al., 2010). The findings from chapter 4 suggest that rIPC has the potential to improve endothelial function in a patient group with likely endothelial dysfunction and at higher risk of vascular complications.

It is too soon to recommend rIPC interventions for routine clinical practice based on the research evidence form this thesis and literature to date. Numerous, large scale phase II, III and IV clinical trials are required in a variety of clinical cohorts to truly understand the effects of the interventions and to gain better insight into the mechanisms mediating such changes. Nevertheless, based on the evidence presented in this thesis, below are the key areas in which future studies can build upon the work presented here.

1. Aim to identify the optimal dose of rIPC in terms of duration (e.g. weeks or months) and frequency (e.g. daily or weekly). By designing large scale trials with a range of different rIPC intervention protocols, this should in turn identify the most effective rIPC protocol which is a crucial step should rIPC be implemented clinically.
2. Establish whether similar to exercise, rIPC exerts it effects differently depending on health status. Expanding on the work contained within this thesis, future research should open up to exploring the rIPC effects on a broad range

of clinical groups, and gain an understanding of how different clinical conditions respond differently.

3. Attempt to further understand the mechanism mediating the rIPC adaptations. By designing mechanistic focused studies, future work should aim to identify the mechanisms involved in the protective effects of rIPC as well as its association with enhanced vascular function.
4. Building on chapter 4, future trials with adequate statistical power are required to identify if rIPC has the ability to improve vascular outcomes in T2DM. Chapter 4 has laid the foundations for a large scale clinical trial by demonstrating the positive directional changes in vascular function in a pilot study. Future randomised control trials, which a sufficiently powered statistically should focus on rIPC in T2DM.
5. Based on the findings in chapter 6, further understanding of factors that can influence dCA and identify in what disease states dCA is impaired or attenuated. By exploring dCA in a range of clinical groups, not just limited to CVD risk as in this thesis, researchers will be able to identify when it is impaired, which may then potentially assist in understanding the exact mechanisms contributing to dCA,

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