Remote Ischaemic Conditioning: Individual Patient Data Meta-Analysis of Acute Stroke Trials and Prospective MRI Evaluation in Healthy Adults

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ABSTRACT

Background: Remote ischaemic conditioning (RIC) is a promising neuroprotective method, with preclinical studies showing improved neurological outcome following acute stroke. However, early phase randomised controlled trials (RCTs) have given rise to conflicting results. Furthermore, the optimal strategy to apply RIC in humans is unknown. Clinical studies have utilised a variety of doses and methods, ranging from a single 'dose' using one limb, to daily application for 300 days using two limbs, decisions that are not based on meaningful pre-clinical data. A reliable way to directly measure end-organ effects of RIC would allow optimal methods of application and 'dose' to be determined, thereby informing clinical trial design.

Aims: To prospectively pool and analyse individual patient data (IPD) from RCTs on the effects of RIC on safety and outcomes in acute stroke. The aim was to also test a small group of healthy young adults to establish the feasibility and tolerability of administering RIC during a non-invasive magnetic resonance imaging (MRI) scan, and to collect preliminary data on using Phase-contrast (PC) and Arterial Spin Labelling (ASL) for detecting RIC-induced cerebral haemodynamic changes.

Methods: For the individual patient data meta-analysis, we systematically searched electronic databases up to December 2020, including PubMed/MEDLINE, EMBASE, Cochrane, and clinical trial registries using pre-specified keywords. RCTs were included when RIC (intervention) and sham therapy (control) were administered within 24 hours of stroke. We extracted individual patient data obtained from the invited trial investigators. The primary functional outcome (mRS) was assessed by ordinal analysis at the end of the trials. The secondary outcomes included early and late neurological deterioration based on the National Institute of Health Stroke

Severity (NIHSS) scores, adverse vascular events, death at 90 days, and changes in infarct volume, NIHSS scores (between baseline and end-of-trial) and serum biomarkers. Unadjusted and multivariable regression analysis adjusted for age, sex, baseline NIHSS and systolic blood pressure, and time-to-treatment were conducted.

For the healthy human volunteer feasibility study, we recruited 6 young (18-40 years) healthy males to undergo a single 'dose' of 4 cycles of intermittent arm ischaemia - alternating 5 minutes inflation (200 mmHg) followed by 5 minutes deflation of an upper arm blood pressure cuff during an MRI scan. ASL & Phase contrast MRI of the brain were performed before and after RIC, as well as 24 hours later. Measurements of blood flow and perfusion were compared to baseline pre-RIC values.

Results: Seven RCTs from four countries comprising 556 patients (281 RIC, 275 sham) were included: age 66.3years (SD 13.9), 61% male, NIHSS 7 (IQR 5-12) and 43% randomised ≤3 hours. Final NIHSS scores significantly improved following RIC therapy (OR=-0.85, 95%CI -1.55 to -0.16, p=0.01), but there was no statistical difference in the mRS scores (OR=0.97, 95%CI 0.71-1.31,p=0.83), overall major cardiovascular adverse events (OR=0.76, 95%CI 0.42-1.39,p=0.38), or mortality (OR=1.44, 95%CI 0.69-2.98,p=0.33). A possible treatment interaction (p=0.08) occurred with time-to-treatment (mRS: ≤3 hours, OR 0.71, 95%CI 0.44-1.16; >3 hours, OR 1.23, 95%CI 0.82-1.85). Study heterogeneity and risk of bias (except one study) were low.

In the healthy human volunteer pilot study, RIC administration during the MRI scan appeared feasible, safe, and was well tolerated by the study participants. There were no significant changes observed in the blood flow velocity of the major intracranial vessels (bilateral internal carotid arteries and basilar artery). Although ASL analysis

was planned in this cohort, due to extenuating circumstances caused by the COVID-19 pandemic, no formal analysis could be performed by the time of the thesis submission.

Conclusions: RIC appeared safe and significantly improved the final NIHSS, but did not improve the functional outcome (mRS) in acute stroke. RIC therapy may be beneficial when administered ≤3 hours from stroke onset.

The preliminary findings of the human volunteer pilot study showed RIC administration during MRI scanning appeared to be feasible, safe, and well tolerated. It also allowed quantitative haemodynamic measurements during RIC, though no significant change in blood flow velocity was demonstrated in this small study sample. Future work could exploit this method further, in a larger sample size and potentially assess the treatment 'dose' and administration parameters in target populations.

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AF = Atrial fibrillation

AIS = Acute ischaemic stroke

ASL = Arterial Spin Labelling

BA = Basilar artery

CABG = Coronary artery bypass graft

CBF = Cerebral blood flow

DCA = Dynamic cerebral autoregulation

DCE = Dynamic contrast enhanced

DM = Diabetes mellitus

DSC = Dynamic susceptibility contrast

DWI = Diffusion weighted imaging

FDG-PET = Fluoro-deoxyglucose positron emission tomography

FLAIR = Fluid attenuated inversion recovery

HSP = Heat shock protein

ICA = Internal carotid artery

ICH = Intracerebral haemorrhage

MCA = Middle cerebral artery

MMP9 = Matrix metallopeptidase-9

MRI = Magnetic Resonance Imaging

mRS = Modified Rankin Scale

NIHSS = National Institute of Health Stroke Scale

ND = Neurological deterioration

PC = Phase Contrast

PWI = Perfusion weighted imaging

RISC = Remote ischaemic conditioning in stroke collaboration

RCT = Randomised controlled trial

RIC = Remote ischaemic conditioning

SPECT = Single photon emission computed tomography

TCD = Transcranial Doppler

TOF = Time of flight

TIA = Transient ischaemic attack

VEGF = Vascular endothelial growth factor

1. INTRODUCTION

1.1 Remote Ischaemic Conditioning (RIC)

Remote ischaemic conditioning (RIC) is an inexpensive, promising tissue-protective method that has been shown to reduce the infarct volume and decrease morbidity in a variety of pre-clinical experimental settings (1, 2). In particular, RIC can reduce the risk of ischaemia-reperfusion injury in multiple organs, including in the heart, brain, liver, intestine and kidneys by inducing 'ischaemic tolerance' or a form of 'cellular protection' in various organs (2, 3).

In 1986, Murry et al first described the potential of ischaemic conditioning by observing a decrease in the myocardial infarct volume following a sustained period of arterial occlusion after intermittently interrupting the blood supply to the left anterior descending artery of the heart in dogs (4). Later in 1993, regional ischaemic conditioning was hypothesized when similar tissue protective effects were seen in the left anterior descending arterial territory of the heart after transiently occluding the circumflex artery (5). Further early animal model studies experimented the use of ischaemic conditioning in facilitating cardioprotection by transiently disrupting/clamping the direct blood supply to remote organs such as the kidneys (renal artery), intestine (mesenteric artery), and the hindlimb (femoral artery), all of which showed promising results (3, 6, 7). Eventually, a non-invasive method of RIC applied to a limb was successfully trialled in humans in 2002 by reducing endothelial injury (8). The authors demonstrated the prevention of endothelial dysfunction caused by ischaemic-reperfusion injury at a site distal to the application of RIC, which implied potential systemic effects of RIC (8). More recently in 2010, RIC was also shown to confer cardioprotection when applied prior to primary coronary intervention

during acute myocardial infarction (9). Since then, there have been numerous clinical trials investigating the safety and efficacy of RIC in cardiovascular, renal and neurological settings.

RIC typically involves the use of repeated cycles of transient limb ischaemia, for example by applying a blood pressure (BP) cuff above the systolic BP or a tourniquet to the arm or leg for a few minutes at a time (2, 3). This simple, non-invasive intervention can be performed either before (pre-conditioning), during (per-conditioning) or after (post-conditioning) the end-organ ischaemic event.

Isolated studies have also attempted to extend the application of RIC in preventing the severity of vascular events following interventions such as coronary artery bypass graft, organ transplantation or carotid stenting (10, 11), as well as decreasing the risk of vascular injury in chronic disease states including chronic kidney disease or vascular dementia (3, 12). Meng et al also demonstrated the potential of long term remote ischaemic post-conditioning use (> 6 months) in preventing recurrent vascular events such as stroke or transient ischaemic attack (TIA) in patients with symptomatic intracranial atherosclerosis (13).

1.1.1 RIC Mechanisms

The overall/combined effect RIC aims to decrease the risk of cell damage conferred in ischaemia-reperfusion injury, through the reduction in inflammation and improvement of blood flow. Periods of ischaemia or hypoperfusion commonly underlie various conditions such as stroke, myocardial infarction and sepsis. The resultant anaerobic state is caused by a dysfunction of the mitochondrial electron transport chain, which

in turn disrupts the adenosine triphosphate (ATP) production and the sodium-potassium (Na+ K+) pump (14). Accumulation of intracellular ions including sodium then causes cellular swelling and impairment of cellular activity, referred to as the 'primary cellular injury'.

Once perfusion is restored to the injured or ischaemic tissue bed, the oxygen supply promotes the generation of reactive oxygen species (free radicals), which increases the oxidative stress and inflammatory response, precipitating further endothelial dysfunction and cellular damage (15, 16). This secondary cellular injury, particularly if prolonged (hours or days), may lead to cell death. However, if the total cellular damage is limited, cellular repair mechanisms are activated, promoting cell survival (15). The aforementioned processes typically occur within the 'ischaemic penumbra', an area that remains metabolically active during a period of ischaemia (reversible damage), but may further develop into an ischaemic core (irreversible damage) following prolonged ischaemic-reperfusion injury.

The idea of a naturally occurring 'ischaemic tolerance' or preconditioning of tissues has been demonstrated in previous studies. These studies have suggested that episodes of angina or transient ischaemic attacks (TIA) in patients could have a protective effect on any ensuing myocardial infarction or ischaemic stroke respectively, by decreasing the size of the infarct volume and subsequent morbidity and mortality (17-20).

Several mechanisms conferring the protective effects of RIC in the early phase (develops rapidly and lasts for minutes to hours) and late 'booster' phase (develops

within hours and may last for days or weeks) have been postulated (3, 21). Both phases of protection can be seen when applied pre- or per-conditioning, whilst a chronic protective phase can be induced with repeated RIC interventions over weeks or months.

Early cardiology based studies have hypothesized the release of underlying endogenous blood-borne chemicals and activation of the inter-linked humoral and neuronal pathways by the conditioned tissue in conferring remote protection to the end-organ at ischaemic risk (22).

Neuronal pathway

The stimulation of the autonomic nervous system has been identified as a potential mechanism of inducing protective effects on end organs. Indirect effects of this conditioning pathway are evidenced following the use of topical capsaicin, which activates the pain fibres, which in turn stimulates the spinal and sympathetic cardiac nerves and induces cardio-protection (23). Similarly, no cardio-protective effects of RIC have been identified following transection of the thoracic spinal cord or femoral nerves (24). The stimulation of the autonomic nervous system is beneficial in increasing the end organ blood flow and circulating protective factors, particularly to the threatened tissues (25).

The neural pathway is also believed to be involved in activating the humoral pathway after the application of RIC. For example, following RIC, dialysate from the blood of diabetic patients with peripheral neuropathy conferred less cardio-protection in isolated hearts compared to the dialysate from the blood of diabetic patients without

peripheral neuropathy (26). The dialysate obtained from blood of individuals treated with RIC has also showed cardiac tissue protective effects when transferred to isolated or transplanted denervated hearts, which signifies the importance of endogenous or humoral factors (27).

Humoral pathway

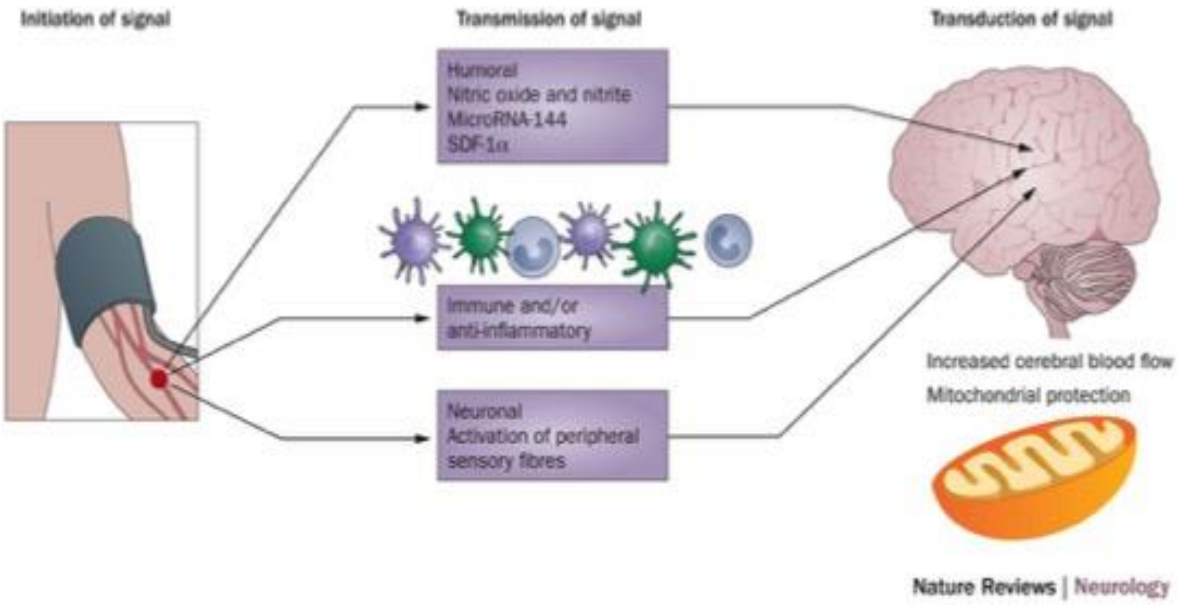
One of the underlying mechanisms of RIC postulated in acute ischaemic stroke (AIS) is the reduction in cerebral inflammation by activating chemical messengers such as anti-oxidants, bradykinin, nitric oxide, adenosine, vascular endothelial growth factor (VEGF), endocannabinoids and heat shock protein 70 (HSP-70) (3, 15). In pre-clinical studies, various markers of oxidative stress were investigated, such as including malondialdehyde (MDA), tumour necrosis factor alpha (TNF-a), interleukin-6 (IL-6) (15, 28, 29). These markers were found to be markedly reduced following remote ischaemic per- and post-conditioning, thereby conferring tissue protective effects from the oxidative stress damage induced during the ischaemic-reperfusion injury (30, 31). Inflammatory pathways have also been shown to be down-regulated in animal models following RIC. A decrease in select chemokines including the monocyte-chemoattractant protein-1 (MCP-1), responsible for the recruitment of inflammatory cells such as neutrophils, monocytes and lymphocytes, have been identified in these models (28). Other endogenous substances such as adenosine and its receptors are overexpressed in myocardial and cerebral tissues following the application of RIC, and work by preventing migration of these inflammatory cells to the affected tissue (32, 33).

Another underlying pathophysiological entity implicated in ischaemic stroke is cerebral oedema (cytotoxic-intracellular, and vasogenic-extracellular). In addition to the disruption of the Na+K+ pump, dysregulation of aquaporin-4 (AQP-4), a water channel protein that regulates the transport of water along astrocytes, has also been implicated in cytotoxic oedema, leading to neuronal damage during AIS (34). On the other hand, upregulation of matrix metallopeptidase-9 (MMP-9) has been thought to disrupt the tight junctions forming the blood brain barrier, causing vasogenic oedema (35). However, both molecules have been shown to be down-regulated in experimental models following RIC, leading to a reduction in cerebral oedema and infarct size (34).

Other mechanisms include decreasing cell death by inhibition of the opening of the mitochondrial permeability transition pore receptor and improvement of cerebral blood flow (3, 36, 37). Endothelial nitric oxide synthase is responsible for the derivation of nitric oxide from the endothelium, which in turn acts as an effective vasodilator remote (38). Other chemical messengers involved in this complex pathway vasodilation include bradykinin, and endocannabinoids (39, 40). Vasodilation of the microcirculation has an important role in regulating the bodily haemodynamics and increasing blood flow to the threatened distal tissues, thereby inducing protection against the effects of reactive oxygen species and ischaemic-reperfusion injury. The upregulation of endocannabinoids and its receptors (CB_1 and CB_2) have also been implicated in the reduction of inflammatory pathway activation and signalling (39, 41). This effect has been demonstrated by Leker et al. in animal models treated with RIC, resulting in a decrease in the cerebral infarct volume (41). Interestingly, HSP-70, particularly in its phosphorylated form, has been shown to be

upregulated in the ischaemic penumbra of cerebral and myocardial tissues after the application of RIC (38, 42). HSP-70 works as an anti-apoptotic chaperone in protecting cells from harmful insults and preventing cell death during a period of ischaemia (2, 3, 15).

Figure 1-1: Summary of the postulated humoral and neuronal pathways of the underlying mechanism of remote ischaemic conditioning administered to an arm and the transduction of the signal to the brain to increase blood flow and its effect on mitochondrial protection (3). Diagram reproduced from *Hess, D. C. et al. (2015) Remote ischaemic conditioning—a new paradigm of self-protection in the brain. Nat. Rev. Neurol. doi:10.1038/nrneurol.2015.223.*



1.2 Clinical Applications in RIC

RIC has been utilised in various clinical conditions, in both animal and human studies. Most of the initial studies in RIC were described in patients with cardiac,

renal and neurological deficits. A brief overview of the current literature in these fields are explored below.

1.2.1 RIC in Myocardial Infarction and Cardiac Surgery

Many studies employing RIC were initially reported in the field of Cardiology. In 2006, Schmidt et al. demonstrated a reduction in the myocardial infarct size using a pig model to study the effects of pre-RIC in a hind limb with four cycles of RIC (43). Subsequently, ischaemic post-conditioning was attempted in patients who presented with acute ST-elevation myocardial infarction that required primary percutaneous coronary intervention. Results from this study revealed a 36% decrease in the creatine kinase levels after 3 days (44). Similarly, other early trials showed lower levels of serum troponin (marker of myocardial tissue injury) and smaller myocardial infarct sizes after 6 months (45).

Ischaemic pre- and per-conditioning were also studied in patients undergoing coronary artery bypass graft for ischaemic heart disease, by clamping the aorta for brief periods during the surgery. A pooled analysis of such studies revealed an improvement in the requirements of inotrope support, rate of arrhythmias and length of stay in the intensive care unit post operatively (46). Further studies also applied RIC using a BP cuff to the upper limb for short cycles (e.g. 3 x 5 minutes) before the coronary artery bypass graft surgery. Patients in the RIC group had significantly lower levels of troponin T compared to the control group after 3 days (47).

However, translation from pre-clinical and early clinical studies to larger trials has proved challenging. Various reasons postulated for the contradictory results and lack of reproducibility include patient related factors or co-morbidities, medications or

anaesthetic agents used, methods and duration of RIC used (48). Natural ischaemic pre-conditioning in patients with a history of angina (or transient ischaemic attack) may also lead to lower efficacy in patients treated with RIC in an acute setting of myocardial infarction or stroke respectively (2, 48).

1.2.2 RIC in Renal Dysfunction

Benefits of RIC have also been demonstrated in peri-operative acute kidney injury, for example in cardiac surgery or renal transplantation, as well as in haemodialysis (49). In 2013, Deftereos et al reported a significant improvement in the incidence of acute kidney injury, measured by the serum creatinine level, in the RIC group of patients that underwent primary coronary intervention for non-ST elevation myocardial infarction (50). The mortality level was also lower in the RIC group (12.4% vs 22.3% in the sham group). In another group of patients that received RIC prior to PCI for ST elevation myocardial infarction, the occurrence of acute kidney injury post-operatively was significantly lower than the control group (8.7% vs 18.5% respectively, p=0.03) (51). RIC was also protective against intravenous contrast-induced AKI when patients received a single 'dose' 3 x 5 minute cycles of RIC (52). Additionally, Crowley et al demonstrated the protective effects of RIC prior to haemodialysis in mitigating haemodialysis-induced cardiac injury by decreasing the number of cardiac events seen on the electrocardiography (ECG) monitoring within 28 days post intervention (49).

However, these promising results were not extrapolated to larger randomised controlled trials involving patients that underwent cardiac surgery (ERRICA study by Haunseloy et al, RIPHeart by Meybohm et al and RIPC by Hong et al) (11, 53, 54). No significant differences in the incidence of post-procedural acute kidney injury or

renal failure were identified between groups that underwent RIC (pre- or post-conditioning) or sham. Even meta-analyses that investigated the effects of RIC on AKI revealed mixed results (55, 56).

As with the Cardiology literature, multiple potential confounding factors have been identified and there has been much criticism of the current trials' design and conduct. At present, the ideal approach in investigating the effects of RIC in renal dysfunction remains unclear and further well-designed trials are warranted.

1.2.3 RIC in Ischaemic Stroke

Acute ischaemic stroke (AIS) is the commonest cause of neurological disability and one of the leading causes of death worldwide (57). Furthermore, the collective healthcare and societal costs on individuals and cares in the National Health Service (NHS) are estimated at ~£26 billion per year (58). To date, systemic thrombolysis and endovascular thrombectomy are the standard of care for eligible patients presenting with AIS (59). However, many patients are not eligible to undergo the aforementioned reperfusion therapies whist more than half of patients presenting with AIS continue to have functional dependence or disability at 3 months despite best medical treatment (59). Hence, there is an increasing need for additional or concomitant neuroprotective therapies to help improve clinical outcomes by increasing the 'ischaemic tolerance' of neural tissue and minimising the effects of ischaemic-reperfusion injury following revascularisation.

In 1990, pre-clinical experiments of 2-minute repeated ischaemic pre-conditioning prevented ischaemic injury in the hippocampal region of animal brain after a 5 minute

period of cerebral ischaemia (60). Later in 2008, Ren et al demonstrated the reduction in the middle cerebral arterial territory infarct size in rats following application of pre-conditioning with varying cycles of 5- or 15 minute controlled limb ischaemia, immediately before the stroke onset (61). Ren et al postulated a brief protective effect of approximately 2 hours occurring immediately after RIC application, whilst a 'late' protective effect could emerge after 12 hours and lasts up to 72 hours (61). Subsequently, many other pre-clinical studies also confirmed the protective effects of RIC when applied immediately before the ischaemic insult (3). However, pre-conditioning stimuli is not a viable option in AIS due to its unpredictable occurrence. Fortunately, early studies also demonstrated promising results of infarct size reduction when a single dose of RIC was applied during or immediately after an ischaemic insult. A recent pre-clinical meta-analysis (1) of 57 studies also demonstrated a greater effect of per- or post-ischaemic conditioning on the infarct size compared to pre-conditioning in an AIS model (standardised mean difference SMD -2.00, [95%CI -2.38 to -1.61] versus -1.54 [95%CI -2.07, -1.01], $p<0.00001$). Furthermore, when repeated doses of RIC were applied daily for up to 14 days following a provoked stroke in animal models, improvement in the neurological deficit and infarct size were further potentiated compared to a single bout of RIC application (62, 63).

More recently, there have been early phase clinical trials that investigated the safety and efficacy of RIC in stroke, transient ischaemic attack, cerebrovascular small vessel disease, intracranial arterial stenosis and following carotid artery stenting (10, 13, 64, 65). Whilst these studies have confirmed its safety and tolerability, Hougaard et al found no overall effect on the infarct volume in patients with AIS who received a single 'dose' of pre-hospital RIC (66). However, almost one-fifth of these patients received

an incomplete ‘dose’ of RIC due to short ambulance transit times. Despite this limitation, additional tissue survival analysis on the affected brain region demonstrated a potential protective effect of RIC (66). In 2017, England et al. demonstrated a significant improvement in the neurological outcome based on the National Institute of Health Stroke Scale (NIHSS) scores (median NIHSS 1 vs 3 in the control group; p=0.04) at 90 days in a small cohort of patients who received a single dose of RIC between 6 to 24 hours of AIS onset (67).

Other studies have also investigated the effectiveness of repeated applications or ‘doses’ of RIC in patients with stroke. Guo et al reported a significant increase in the number of patients with an excellent neurological outcome based on the modified Rankin Scale (mRS) scores at 90 days in patients who received repeated RIC doses from the onset of AIS up to their discharge date (compared to the sham group) (68). Meng et al. also demonstrated improvement in the functional outcome at 90 days and a reduction in the incidence of recurrent stroke when RIC was applied twice daily on the upper limb for 300 consecutive days after the incidence of a TIA or AIS (13). The same research group also reported similar results in patients with intracranial arterial stenosis following 180 consecutive days of twice daily RIC application (69). Furthermore, Mi and Wang et al respectively showed a decrease in the white matter hyperintensities or lesions after a year of repeated RIC applied twice daily on both arms in patients with intracranial small vessel ischaemic disease (65, 70). However, no clear improvement in the neurological outcome was identified by England et al when RIC was delivered twice daily for up to four days on a single arm, when initiated within 6 hours of AIS (71).

Whilst some of the protective effects of RIC in AIS have been alluded to, the overall promising results seen in the pre-clinical studies have yet to be re-produced in the early phase clinical trials, dubbed as a 'translational road block'. Reasons for this are multi-factorial, which include heterogeneity of the co-morbidities in patients compared to animal models may preclude generalisability of this application. Additionally, there are variations in the methods employed in the application of RIC as well as the various methods of measuring the outcomes.

1.3 RIC methods

The optimal way to apply RIC in patients remains unknown. Thus far, RIC design methods have involved inducing brief periods of a controlled ischaemia, either directly clamping the main arterial supply to an end organ intra-operatively, or remotely on the upper or lower limb. Applying a tourniquet or blood pressure cuff on an arm and/or leg and transiently inflating it until the distal portion of the involved limb is rendered ischaemic is non-invasive, and is therefore the more common and preferred method in numerous studies of applying RIC. The set of complete inflation and deflation cycles which can be repeated multiple times in a single sitting is typically considered a single 'dose'.

Clinical studies have utilised an array of doses and methods, mostly adapted from early studies from Cardiology literature. The heterogenous nature of the duration of the controlled ischaemia, the number of cycles of cuff inflation/deflation, the number limbs used for the RIC application, and the time point when RIC is applied in relation to the onset of stroke, decisions that are not based on meaningful pre-clinical data. For example, the methodologies range from a single 'dose' using one or two limbs (e.g. 4

x 5 minute cycles cuff inflation/deflation (72, 73), twice daily for 4 days, (71) to daily application for 300 days using two limbs (13). At present, most studies have preferentially used between 3 to 5 x 5 minute cycles of cuff inflation and 5 minutes of deflation or reperfusion (as a single 'dose') on the upper arm. This technique has been shown to be safe and well tolerated among participants (74).

The three main timings of the RIC application have all demonstrated beneficial effects by reducing the ischaemic-reperfusion injury in patients with (i) planned surgical intervention (pre-conditioning applied hours or days before the surgery) evidenced by the reduction in the incidence of cerebrovascular events following carotid stenting, (ii) acute stroke (per-conditioning applied within a few hours of the ischaemic insult) by improving neurological outcome (mRS or NIHSS score) at 90 days, thought to be due to the increase of cerebral blood flow, reduction of cerebral inflammation and oedema, as well as (iii) post stroke treatment (post-conditioning applied repeatedly over many days or weeks) by reducing the recurrence of stroke, number of white matter and diffusion weighted imaging (DWI) lesions, and improving clinical outcomes (10, 13, 65, 71, 74). However, it is yet to be determined which group(s) of patients are more likely to benefit from the application of RIC before, during and/or after an ischaemic event.

Furthermore, the upper-end of the systolic pressures when a BP cuff is used have varied between studies. For example, England et al described cuff inflation pressures to 20mmHg above the systolic BP (74), whilst Guo et al and Carter et al employed 200mmHg (72) and 220mmHG (73) respectively. It is unclear if these differences have a confounding role in the results of the studies. There are also variations in the time of

the day RIC is applied. This has implications on the potential effect on the haemodynamics as there are reports of diurnal variation of mean arterial pressures and cerebral blood flow (72, 73, 75). Interactions of RIC and established treatment pathways such as intravenous thrombolysis or endovascular thrombectomy may also be factor in the lack of reproducibility of the results in patients.

Finally, the duration of the protective effects of RIC (which may be related to the duration and method of RIC application), as well as the optimal time to measure the specified outcomes remain unclear. Ideally, a reliable and reproducible way to directly measure end-organ effects of RIC would allow optimal methods of application and 'doses' to be determined, thereby informing clinical trial design.

1.4. Measurement of the Effects of RIC

Studies that investigated the effects of RIC have utilised different outcome measures, partly in an exploratory fashion, to further understand the potential effects of RIC in specific end organs. The outcome measures include clinical markers such as disability or neurological deterioration, recurrent cardiovascular events, mortality, as well as blood biomarkers including Troponin levels in myocardial infarction, S100ß, matrix mettaloproteinase-9 (MMP9); heat shock protein-27 (HSP-27), and vascular endothelial growth factor (VEGF). According to the studied end organ, other measures such as the indirect measures of cerebral blood flow/velocity using transcranial Doppler, resistive index in renal arteries using Ultrasound Doppler, and the infarct size using single photon emission computed tomography (SPECT) or magnetic resonance imaging (MRI). The different time points chosen to measure

these outcome effects have also been variable across the studies, ranging from within a few hours following RIC, to a few days or at the end of the specified trial. These outcome measures are summarised and further discussed below.

1.4.1 Clinical and Blood Markers

There are multiple clinical or blood biomarkers used in studies to assess the response of RIC on end organs. In RIC-based studies on patients with cerebrovascular disease, the functional outcome has been most commonly measured using the modified Rankin Scale (mRS) score that ranges from 0 (functional independence) to 6 (death) at 90 days. For example, whilst Guo et al. demonstrated a significant improvement in the excellent functional outcome (mRS 0-1 and 0-2) at 90 days in the RIC group, no significant difference was observed in Pico et al's, Hougaard et al's, He et al's or England et al's 2019 studies (66, 68, 71, 76, 77).

Alternatively, the Barthel Index can also be used to identify the degree of independence with 10 variables of activities of daily living using an ordinal scale. England et al 2019 reported no significant difference in the Barthel Index scores between patients with AIS that were treated with RIC and sham (71). Similar results between groups were also observed in the Zung Depression Scale, quality of life and cognitive impairment (using the mini-mental state examination, MMSE) measures. Mi et al and Wang et al reported no difference in the cognitive impairment MMSE outcome measures in patients with cerebral small vessel disease, corroborating the findings of England et al and An et al (65, 68, 70, 71).

The stroke severity and neurological impairment or improvement are also identified using the National Institutes of Health Stroke Scale (NIHSS) score that is composed

of 11 items with item scores of between 0 to 4, and the overall score range from 0 (no disability) to 42 (severe disability). England et al and He et al reported no significant difference in the 3-month and 30-day (respectively) follow-up NIHSS scores between the RIC and control groups (74, 77). However, An et al found significantly improved NIHSS scores of 6 or more at 90 days in the RIC group (68).

In certain studies, the incidence of recurrent stroke or TIA have been used to quantify differences in the outcome measures between the intervention and control groups (10, 13). Although Meng et al found a statistically significant difference between groups, Zhao et al reported no significant difference between patients who were treated with RIC and sham therapy (10, 13).

Other clinical markers of morbidity, including serious vascular adverse events such as myocardial infarction, venous thromboembolism, intracranial haemorrhage and mortality rates have been studied. Meng et al and Zhao et al reported no serious adverse events in their studies even at the 1-year follow-up (10, 13). However, there were a few reports of self-limiting sporadic petechiae and discomfort involving the upper arm used for administering RIC. A recent meta-analysis by England et al demonstrated a significant reduction in the rate of recurrent adverse vascular events, including strokes following pre- per- and post-RIC in patients with acute stroke, which adds strength to the safety profile of the administration of RIC (71). Hougaard et al and Pico et al also reported no significant differences in the mortality rates between groups (66, 76).

Clinical characteristics such as the heart rate and arterial blood pressure readings have also been analysed in various studies, without revealing any significant changes between groups (67, 73, 77).

A study by Carter et al, 2020 assessed the carbon dioxide reactivity as a marker of cerebral endothelial and cerebrovascular function, a method previously described by Lavi et al and Hoiland et al (73, 78, 79). The authors reported no alteration in the carbon dioxide reactivity, and therefore cerebral perfusion (indirectly), during/immediately after both groups of healthy volunteers and those at risk of cerebrovascular disease underwent a single '4-cycle dose' of RIC.

There have been a vast array of blood biomarkers investigated as indirect markers of neuroprotection and inflammation. Many of these blood markers are up- or down-regulated in the body during or after RIC and are thought to induce contributory effects on the vascular health and dynamics. Some of the blood markers reported in previous studies include S100ß, matrix metalloproteinase-9 (MMP9), vascular endothelial growth factor (VEGF), heat shock protein 27 (HSP-27), brain-derived neutrotophic factor (BDNF), basic fibroblast growth factor (bFGF) and heme oxygenase-1 (HO-1) (71, 72, 77). In particular, the S100ß has been found to be significantly reduced whilst the VEGF and HSP-27 levels have been shown to be significantly increased in patients that received RIC treatment. The increase in the total and phosphorylated HSP-27 levels are thought to be neuroprotective and may enhance infarct volume reduction (74). S100ß, found in perivascular astrocytes, play a role in inducing and recruiting inflammatory cytokines (80, 81). It is also a marker of acute brain tissue injury, for example in acute stroke or intracranial trauma. Hence, it indexes glial injury as well as the intracranial inflammatory response (81). On the other hand, VEGF is involved in angiogenesis and can have a neuroprotective effect in increased levels (82, 83).

1.4.2 Transcranial Doppler (TCD)

Dynamic cerebral autoregulation (DCA) is a reflection of the cerebrovascular health and can be impaired in cardio- or cerebrovascular disease states (84). DCA is therefore frequently measured in clinical studies identifying effects of an intervention on cerebrovascular haemodynamics. Evaluation of the DCA is obtained using a transfer function analysis algorithm, which can be calculated using the arterial BP reading and cerebral blood flow velocity (CBFV). Arterial BP is commonly measured at the brachial artery using manual or automated BP monitors whilst the CBFV is usually measured using a Trans-cranial Doppler (TCD), assuming the arterial diameter remains constant during the TCD readings (85). Specifically, the CBFV is usually measured using the middle cerebral arteries (MCA), at a depth of 45 to 60mm, by placing a 2MHz probe against the temporal bone until a strong and steady pulse reading is obtained (73). Thereafter, the probe is held in place using a head frame whilst the patient lies in a supine position.

The phase difference, gain and coherence function within a low frequency range, around 0.06 to 0.12Hz, can be derived from the transfer function analysis to reflect the DCA (73). In particular, the phase difference (PD) value indicates the relationship between the CBFV and ABP changes. For example, a low PD value suggests that CBFV changes are in line with that of the arterial BP, but a high PD value suggests that the CBFV is altered to oppose the arterial BP fluctuations, which indicates active cerebral autoregulation.

In Guo et al's study, the PD value showed a significant increase from 6 hours (but not prior to that), until at least 24 hours after healthy volunteers underwent RIC (72).

Similar findings were also demonstrated in Meng et al's study (13). However, no difference was observed in the MCA velocity and PD value in Carter et al's study which was measured during RIC (73). This finding was also not reproduced in England et al's study,(74) which attempted to use TCD as a measure during the RIC cycles.

Ultimately, a high degree of user subjectivity and operator skill significantly limits the use of TCD as a reliable biomarker.

1.4.3 Single Photon Emission Computed Tomography (SPECT)

Single photon emission computed tomography (SPECT) is a form of nuclear imaging scan that combines the use of a radionuclide which emits gamma rays which are detected by a detector array allowing reconstruction of cross sectional images shows sites in the body where the radioactive tracer has accumulated. A study by Meng et al investigated the use of SPECT as a secondary semi-quantitative measure of cerebral perfusion or cerebral blood flow (13). The authors used Technetium-99m ethylene cysteine dimer (^{99m}Tc-ECD) as the radionuclide tracer to reflect the perfusion status. The radionuclide uptake index in regional areas of the brain represents the cellular metabolic status and cerebral perfusion. In Meng et al's study, patients underwent twice daily RIC application on both arms for 300 days and SPECT readings were obtained at 90 and 300 days (13). The authors found statistically significant cerebral blood flow (CBF) augmentation (and improved cerebral perfusion) in the RIC group (in 31.6% of cases at 90 days and 76.3% at 300 days) compared to the control group (in 6.7% of cases at 90 days and 53.3% at 300 days)(13). However, using this method of assessment is largely limited by exposure to ionizing radiation, radioactive material and radiotracer availability. Furthermore, this method also precludes the ability to

evaluate the cerebral haemodynamic changes that may occur in parallel during the administration of RIC.

1.4.4 Magnetic Resonance Imaging (MRI)

MRI techniques have been utilised in estimating the outcomes measures in previous animal-based studies that revealed promising results in significant reduction of the cerebral infarct volumes in the group treated with RIC. Diffusion weighted imaging (DWI) is a specific MRI sequence commonly used in clinical practice to identify areas of restricted diffusion of water molecules that can be demonstrated in cytotoxic oedema which occurs during cerebral infarction.

Recently, two randomised clinical trials conducted utilised MRI sequences to investigate the intracranial effects following RIC in patients presenting with AIS (66, 76). Specifically in Hougaard et al's study, DWI and fluid attenuated inversion recovery (FLAIR) were used to identify the change in the final infarct volume in the RIC and sham groups between baseline (DWI measure) and at the 1-month follow-up (FLAIR measure) (66). T2* gradient-recalled echo and perfusion weighted imaging (PWI) in the form of dynamic susceptibility contrast (DSC) MRI were also used in their MRI protocol at baseline. The PWI and DWI at baseline were used to quantify the penumbral salvage, outlined by the area that did not proceed to the final infarcted area at the final follow-up. Pico et al instead investigated the change in the infarct growth/volume using DWI measurements at 24 hours (76). Both studies reported no significant differences between groups in the final infarct volume, infarct growth between baseline and follow-up, and in the penumbral salvage. This lack of difference may be due to the possibility that the additive neuroprotective effect of RIC in AIS

may have been mitigated in patients who received reperfusion therapy by intravenous thrombolysis or endovascular thrombectomy in both studies.

In a study conducted by Zhao et al that evaluated the incidence of recurrent stroke following the application of RIC versus standard medical treatment for two weeks prior to carotid artery stenting, the authors found fewer new DWI lesions and smaller DWI lesion (infarct) volumes in the RIC group after 48 hours and at 6 months (10). In two further studies by Wang et al and Mi et al, RIC as applied to a cohort of patients with cerebral small vessel ischaemic disease twice daily for one year (65, 70). Interestingly, the authors reported a reduction in the number of lacunar infarctions or recurrent stroke events and the white matter hyperintensities volume after one year (65, 70).

1.5 MRI Techniques in Cerebral Blood Flow

Cerebral blood flow (CBF) is an important measure of the amount of blood delivered to tissues and is directly associated with the exchange of oxygen through the blood brain barrier at the capillary level. CBF is responsible for providing sufficient nutrients (glucose) and oxygen to the tissues, and is regulated according to the metabolic demand of the neuronal activity in either physiological (for example increased during exercise and normal brain development or aging) or pathological states (for example altered in acute ischaemic stroke or neurodegenerative disorders).

The initial perfusion measure was first described in 1945 by Kety and Schmidt, using the Fick principle that involved the quantification of nitrous oxide tissue extraction,

reported in units of millilitres per 100g of brain tissue per minute (ml/100g brain tissue/min) (86).

Various imaging techniques have been described in estimating cerebral blood flow and perfusion, including TCD, contrast-enhanced computed tomography (CT) perfusion, SPECT, fluoro-deoxyglucose positron emission tomography (FDG-PET) and MRI sequences such as arterial spin labelling (ASL) and dynamic susceptibility contrast MRI (DSC-MRI). The advantages of MRI techniques in quantifying cerebral perfusion in particular are primarily its non-ionizing radiation requirements and its ability to measure the blood velocity in multiple vessels and tissue perfusion of various organs in the same sitting.

1.5.1 Arterial Spin Labelling (ASL)

ASL is an advanced perfusion MRI technique, which uses blood as an endogenous contrast agent and therefore has no ionizing radiation requirement, and is non-invasive, with no IV contrast (Gadolinium) required. Hence, there is no risk of Gadolinium-related side effects such as nephrogenic systemic fibrosis. Other than CT perfusion, the relative speed in the acquisition of ASL measurement (<5 minutes) is another advantage over other forms of perfusion imaging.

ASL imaging essentially ‘labels’ a column of the feeding arterial intrinsic blood/proton spins as a ‘contrast medium’ that decays with the longitudinal relaxation (T1) of blood (87, 88). These ‘labelled’ (inversely magnetised) proton molecules are then exchanged with water molecules in the ‘unlabelled’ distal brain tissue through the blood brain barrier at the capillary level. The acquisition of imaging is performed

distally at the brain tissue level after a short delay following the labelling period. The perfusion matrix is obtained following the subtraction of two successively acquired images, with and without the labelling. A rapid imaging technique is required, such as echo planar imaging (EPI), though more recently, 3-dimensional gradient and spin echo (3D-GRASE) is increasingly used (89). As opposed to a standard multi-slice 2D image acquisition, 3D-GRASE allows the whole region of interest to be imaged in a single shot and has a higher signal-to-noise ratio (90). However, T2 blurring or ghosting artefacts along the phase-encoding direction may appear in the reconstructed image, particularly for single shot 2D imaging.

When combined with a few variables such as the transit time of the labelled arterial protons, equilibrium magnetisation of blood and longitudinal relaxation times of blood and water, quantitative ASL perfusion maps can be calculated (91). Furthermore, ASL imaging also enables selective cerebral perfusion territorial maps by selectively labelling feeding arteries.

There are various ASL techniques that include continuous, pulsed, pseudo-continuous and velocity-selective ASL (88, 91). Continuous ASL (CASL) and pulsed ASL (PASL) differ based on the volume of labelled protons of the feeding artery and the radio-frequency pulses. CASL applies a continuous or long RF pulse and magnetic gradients within a thin labelling plane in the direction of blood flow whilst PASL applies a short RF pulse within a large labelling plane to cause inverse magnetisation of the blood water molecules/protons (92). Pseudo-continuous ASL was also used as a combination of both CASL and PASL whilst velocity-selective ASL, which was later discovered, labels protons according to the blood velocity.

ASL has been employed in the study of various neurological disease states, including cerebrovascular and neurodegenerative diseases, epilepsy, and brain tumour work-up (87). Specifically in acute ischaemic stroke imaging, differentiating the ischaemic core and penumbra (brain tissue-at-risk) regions according to the variations in the perfusion and cerebral blood flow can be used as an aid to inform treatment decisions of mechanical thrombectomy in suitable/eligible patients (93). For example, the reference perfusion levels at an ischaemic core and penumbra are estimated to be <10ml/100g/min and 10-17ml/100g/min respectively (94).

ASL imaging is also increasingly used in clinical studies investigating treatment effects, for example in carotid endarterectomy/stenting and extracranial-intracranial bypass, as well as and underlying disease mechanisms secondary to perfusion changes (87).

ASL had initially been reported to overestimate cerebral hypoperfusion (95, 96). This has been postulated to be due to the significantly lower image quality of ASL (when compared to DSC-MRI), which causes a larger inter-rater variation when reviewing the type and location of the perfusion abnormality (95). Reasons for the poorer image quality include a low signal-to-noise ratio inherent in ASL imaging (only 1-2% of the derived tissue signal is from the labelled blood signal) and its susceptibility to motion artefact (90). This can be largely mitigated by using measurements at various inflow times and using the summation of the acquisitions acquired with background suppression, resulting in an increased signal-to-noise ratio in ASL (91). Hence, the improved ASL imaging techniques are now validated against DSC-MRI (97).

It should also be noted that the CBF in cerebral white matter is up to 2.4 times lower than that of grey matter, resulting in reduced of signal-to-noise ratio (98). Hence, perfusion changes demonstrated in small vessel white matter ischaemic disease is less reliable than cortical ischaemia. Additionally, voxel sizes of ASL are larger than that of the mapped or overlaid T1-weighted structural images (lower ASL spatial resolution obtained to improve signal-to-noise ratio). This discrepancy causes a partial volume effect, as the voxels may contain a combination of the tissue of interest and cerebrospinal fluid in the brain, made worse with cortical atrophy (90). The partial volume effect can be modulated using partial volume correction during the post processing of the acquired images.

Figure 1-2: Schematic representation of inverted magnetisation of arterial water molecules 'labelled' (red circles) moving from the arteries through blood brain barrier into tissues at the capillary bed (white arrow). These are exchanged with the 'unlabelled' magnetised tissue water molecules (blue circles), which enter the capillary bed and diffuse out through the veins (black arrow) with any remaining un-absorbed inverted magnetised 'labelled' molecules. The overall loss of intensity or signal of the inversely magnetised 'labelled' arterial molecules in the tissue bed (compared to the signal in the arterial tree), becomes a direct measure of the perfusion.

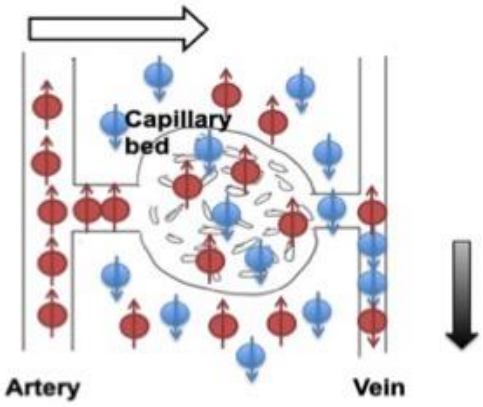
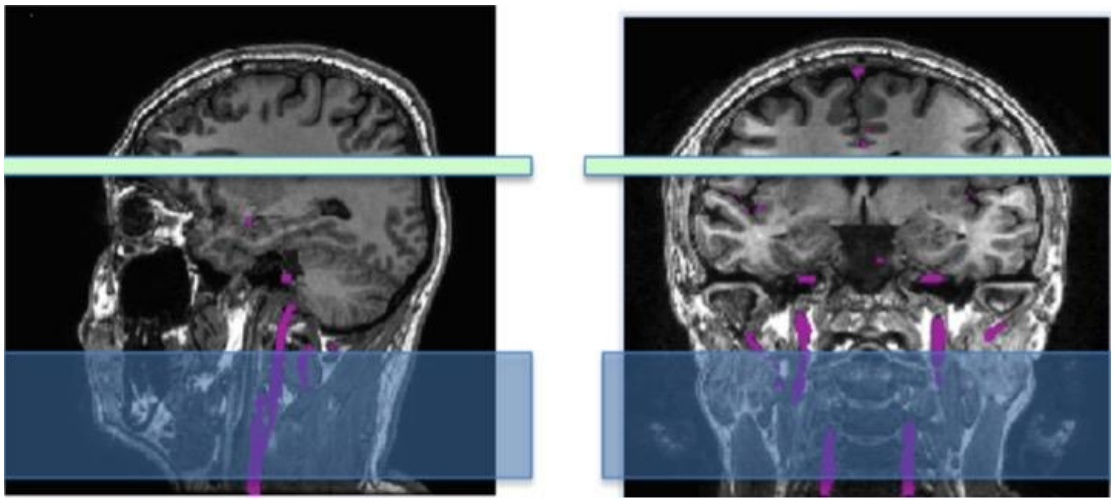


Figure 1-3: Example of a labelled slide (blue bar) and feeding carotid and vertebral arteries (both in purple) in both sagittal and coronal planes for pulsed arterial spin labelling (90). Diagram reproduced from *Grade. et al. (2015) A neuroradiologist's guide to arterial spin labeling MRI in clinical practice. Neuroradiology. doi:10.1007/s00234-015-1571-z.*



1.5.2 Phase Contrast (PC)

Phase contrast is a relatively simple, validated, non-invasive MRI technique routinely used to provide a quick quantitative estimation of the overall blood flow and velocity within an artery supplying a specific organ. This is in contrast to the ASL technique, which maps the regional blood flow and perfusion within a tissue bed. More commonly now, both techniques are used concomitantly to provide a more accurate representation of the blood flow and perfusion.

Phase angle of an MRI image correlates with the flow velocity within a vessel based on the encoding velocity of the moving water molecule spins within a vessel.

The main supplying arteries in an organ are usually selected for flow velocity quantification, including the internal cerebral and basilar arteries in the brain and the renal artery in the kidney. MIP images of the time of flight (TOF) angiogram are used to select the image slice location for the relevant feeding artery and an acquisition plane is set perpendicular to the vessel. A saturation slab is also applied on the TOF angiogram to suppress the venous signal. Furthermore the scan acquisition duration for PC-MRI is generally very short and is easily employed.

Whilst PC-MRI is widely used, there are a few factors that can affect its accuracy and reliability, and should be taken into account when interpreting the results. These factors include the encoding velocity, eddy current field inhomogeneity (caused by changes in the magnetic flux in the gradient coils which in turn causes spatial phase errors), intra-voxel dephasing, complex regions of interest and partial volume effects, image slice orientation, gradient non-linearity and concomitant gradients (both of which also cause phase errors that can be rectified when reconstructing the image). The inherent phase errors due to the factors described above are influenced by the vessel diameter and flow rates. The phase errors are most pronounced in vessels with smaller diameters and slower flow whereby the flow velocities are commonly overestimated by up to 35%, whilst these errors are largely mitigated in larger calibre vessels and higher flow rates, including the internal carotid, basilar or renal arteries (99).

In comparison to ASL and DSC-MRI, PC-MRI is not affected by factors including the labelling efficiency and arterial input function that are required for the quantification of the CBF.

1.5.3 Dynamic susceptibility contrast (DSC) and Dynamic contrast enhanced perfusion weighted (DCE-PW) MRI

Both DSC- and DCE-MRI technique utilize gadolinium-chelates as an IV contrast agent. The contrast medium, which has paramagnetic properties, causes a decrease in the T2* or gradient-echo T2 signal (100). The larger the amount of local contrast medium in the region of interest, the larger the T2* signal deficit is observed. The amount of local contrast medium is dependent on many factors, some of which include the blood flow, the degree of vascularisation, the blood vessels' surface area product and blood vessel permeability (101). The signal-time curve obtained from DSC-MRI is used to measure the cerebral blood flow, cerebral blood volume, and mean transit time of blood to the tissue as an estimate of the overall perfusion to the region of interest.

DCE-MRI instead utilises the paramagnetic properties of the extravasated contrast medium into the extra-vascular space in tissues that shorten the T1 and T2 relaxation times (102). Hence, DCE-MRI estimates the vascular permeability and transfer rate contrast (K-trans) of the area of interest. High vascular permeability allows increased extra-vascular contrast accumulation and a shortened T1 relaxation time increases the T1-weighted imaging signal identified (103). Hence, in addition to the parameters obtained through DSC-MRI, DCE-MRI also allows quantitative measurements of the permeability of the blood brain barrier and microcirculation (104).
Compared to ASL imaging which is non-invasive, repeated image acquisition and measurements cannot be performed to improve the signal-to-noise ratio due to the long half-life of the contrast agent in DSC-MRI. However, DSC-PW imaging is

advantageous in measuring smaller perfusion changes compared to ASL due to its superior signal-to-noise ratio.

2. AIMS

2.1 Individual Patient Data Meta-Analysis of Randomised Controlled Trials

Recent early phase clinical trials utilising RIC in AIS have shown that RIC is well tolerated and feasible in the hyper-acute setting, with suggestions of improving neurological outcome and reducing recurrence of stroke (64, 66-68, 71, 76, 77). Whilst it appears that RIC is safe, the number of randomised patients in the individual trials are currently too small to comment on its efficacy, and the heterogeneous methods and duration of the intervention across the studies have possibly contributed to the conflicting results.

The Remote Ischaemic Conditioning in Stroke Collaboration (RISC) is an international collaboration, which aims to systematically review, prospectively pool and analyse individual patient data (IPD) from randomised controlled trials on the effects of RIC on safety and outcomes in acute stroke.

Primary Aim/Objective(s)

- To determine the safety and efficacy of remote ischaemic conditioning in treating acute stroke based on meta-analysis of individual patient data from existing randomised controlled trials

Secondary Aim/Objectives

- To meta-analyse individual patient data from existing randomised controlled trials to assess:
    - The efficacy of RIC administered after acute stroke with respect to the patient characteristics, method, timing, dosing and duration of the intervention
    - The association between RIC and the radiological outcome, measured by the infarct size and growth
    - The safety of RIC administered after acute stroke with respect to the neurological deterioration and major vascular events

2.2 Healthy Volunteer Remote Ischaemic Conditioning Pilot Study

Primary Aim/Objective(s)

- To establish feasibility and tolerability of administering remote ischaemic conditioning (RIC) and, in parallel, measuring cerebral perfusion, and vessel flow velocities using MRI arterial spin labelling (ASL) and Phase contrast (PC) respectively.

Secondary Aim/Objectives

- To establish the magnitude of change in cerebral perfusion and flow velocities using MRI ASL and PC in parallel with a single dose of RIC in healthy young adults.
- To test whether mechanistic RIC serum biomarkers correlate with cerebral perfusion changes induced by RIC in young healthy adults.
- To test whether changes in cerebral perfusion induced by RIC persistent at 24 hours post-RIC in young healthy adults.

Rationale for undertaking this feasibility study:

Optimisation of administration and dosing of RIC in different target groups would be made easier by having a well-tolerated comprehensive platform for non-invasive *in-vivo* measurement of organ level haemodynamic parameters during RIC.
In this proposal we will assess blood flow and perfusion in the brain using MRI during RIC. Based on previous experience of MRI (3T Ingenia scanner) measurement of brain perfusion using ASL in this institution, we will establish and optimise an MRI protocol for use in participants before, during and after RIC. ASL does not use any contrast agent but instead uses arterial water protons labelled by radiofrequency pulses as an endogenous tracer, thus allowing repeat measures of ASL perfusion.

We will test the protocol in a small group of healthy young adults to establish feasibility/tolerability of the protocol, and collect preliminary data on the use of the protocol for detecting RIC-induced cerebral haemodynamic changes. If successful, this will pave the way for testing different RIC protocols to identify which regime produces the optimal increase in the brain perfusion in different patient groups.

3. METHODS

3.1 Individual Patient Data Meta-Analysis of Randomised Controlled Trials

Search Strategy

The study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The protocol was registered with the international prospective register of systematic reviews (PROSPERO), (CRD42020197351). We systematically searched electronic databases up to December 2020, including PubMed/MEDLINE, EMBASE, Cochrane, and clinical trial registries including ClinicalTrials.gov, ISRCTN registry and the World Health Organisation (WHO) International Clinical Trials Registry Platform. The reference lists of included publications were be hand-searched for additional relevant studies on RIC for stroke. Trial investigators in the field were also contacted directly to identify ongoing or unpublished studies.

The following keywords were used in combination or individually by using the Boolean operators "OR" and "AND": (stroke or cerebrovascular disease or brain infarction or brain ischemia or carotid artery disease or cerebral artery disease or cerebrovascular accident or (isch?emi$ adj6 (stroke$ or apoplex$ or cerebral vasc$ or cerebrovasc$ or CVA)) or poststroke) AND (remote isch?emic conditioning or

(remote adj3 (preconditioning or perconditioning or postconditioning)) or RIC or RIPerC or RIPostC or RIP or RIPC or RPC or IPerC or rIPC).

Study selection

The articles were selected in two stages. First, the titles and abstracts were screened for relevant studies, and duplicates excluded. Second, the full texts were downloaded and assessed for eligibility according to the pre-specified criteria. This process was carried out independently by two assessors (PD, TE). Any differences were resolved by consensus, and if necessary, a third assessor was consulted. The chief trial investigators of the included studies were invited to participate in the collaboration.

Eligibility criteria

Studies evaluating one or more clinical outcomes, including the safety and efficacy of RIC in acute stroke (ischaemic or haemorrhagic) compared to sham or standard medical management alone were included. We included trials comparing different protocols (different number of intervention or sham cycles and duration of conditioned ischaemia).

Inclusion criteria

Randomized controlled trials (RCTs) were included when an intervention group (RIC) and a control group (standard medical management alone or sham) were administered in the hyper-acute (<6 hours) or acute (<24 hours) phases of stroke (ischaemic or haemorrhagic).

Exclusion criteria

We excluded studies that include interventions or surgeries with the risk of subsequent ischaemic stroke, review articles and meta-analyses, guidelines, technical notes and studies in animals. No restrictions were applied to publication status (published, unpublished, in press, in progress, preliminary results or abstracts), sample size or language.

Participants

We included adult participants (aged 18 years and above) with any of the following: acute ischaemic stroke diagnosed on the basis of a combination of clinical examination findings and diagnostic test results or according to definitions for acute stroke used by researchers to enrol patients in their RCTs.

Intervention(s)

RIC may consist of remote ischaemic pre-conditioning (RIPreC; before), remote ischaemic per-conditioning (RIPerC; during) and remote ischaemic post-conditioning (RIPostC; after) in relation to the ischaemic event. A remote stimulus may be applied to any organ (e.g. either upper, lower or both limbs). The intervention may consist of one or more cycles of controlled ischaemia of varying duration.

Comparator(s)/Control

The control may consist of standard medical management alone or sham RIC, which requires application of a blood pressure cuff, tourniquet or other occlusive device without complete interruption of blood flow.

Data sharing and management

Trial investigators of the eligible studies were invited to join the Collaboration and share their anonymised individual patient data. Sharing involved a formal contract between the Collaboration and the sharing organisation to ensure transparency and defined an appropriate use of the data according to the protocol.

Data checking and merging

Initial analysis involved the comparison of data of each study with the published results, including the baseline variables and outcome measures, to ensure the transferred data is accurate. Any queries were resolved with the trial investigators. The data was merged into a new master dataset on Microsoft Excel 2016 and the trial identifier numbers were recorded accordingly.

Data Extraction

A minimal dataset of baseline variables, study design and outcome measures, of individual patient data, were requested from each participating trial investigators.

Outcome measures

The primary outcome was the modified Rankin Scale (mRS) scores at the end of the trials, measured by shift ordinal analysis.

The secondary outcomes include:

- Change in the NIHSS scores between baseline and final scores at the end of the trials

- Early neurological deterioration (END), defined as worsening of the NIHSS score of 4 or more between the baseline and early (within 24 hours to hospital discharge, whichever is earlier) NIHSS scores.
- Late neurological deterioration (LND) defined as worsening of the NIHSS score of 4 or more between the early and late (either 30- or 90-day, whichever is later) NIHSS scores.
- Any ND (composite of END and LND)
- Recurrent or extension of ischaemic stroke and haemorrhagic transformation (HT) or intracranial haemorrhage (ICH),
- All stroke (composite of recurrent or extension of ischaemic stroke, HT and ICH, and any ND)
- Barthel Index at 90 days
- Death at 90 days
- Transient ischaemic attack (TIA), myocardial infarction (MI), venous thromboembolism (VTE), acute kidney injury (AKI), neurovascular limb complications or soft tissue injury
- Total adverse cardiovascular events (composite of all stroke, TIA and MI)
- Serum biomarkers of efficacy [S100ß, matrix mettaloproteinase-9 (MMP9), Heat shock protein 27 (HSP-27)]
- Infarct growth and volume based on diffusion weighted imaging (DWI) between baseline and 24 hours

Statistical analysis

Study characteristics and extracted variables were summarized using standard descriptive statistics to identify any similarities and differences between studies.

Continuous variables were expressed as means and standard deviation (SD) or medians and interquartile ranges (IQR), and categorical variables were expressed as frequencies or percentages. Comparisons of baseline variables were made using the Chi-square, Fisher's exact test or Student's t-test, wherever applicable. An intention-to-treat analysis was performed. Any missing outcome data was imputed into the statistical software without any value.

Univariable analyses of the outcome measures were performed as follows: 1) Ordinal logistic regression for ordinal outcomes of the full-scale mRS; 2) Binary regression analysis for dichotomised end-of-trial mRS scores (good functional outcome; mRS $\leq$2 and excellent functional outcome; mRS $\leq$1), death, ND, recurrent or extension of ischaemic stroke/HT/ICH, all stroke events and adverse vascular events; 3) Multiple regression analysis for change in NIHSS scores, Barthel Index scores, infarct volume and serum biomarkers of efficacy. Multivariable analysis was also conducted, adjusted for: age, sex, stroke severity (NIHSS), systolic blood pressure and time to treatment. Trial and trial*treatment as random effects variables in a mixed effects model was also included for the primary outcome measure.

Analyses of binary and ordinal outcomes were expressed as an odds ratio (OR) with a 95% confidence interval (CI), and continuous variables as a weighted mean difference (MD) or beta coefficient with a 95%CI. All analyses were analysed using a mixed-effects model as appropriate.

Two-tailed P-value of <0.05 was considered statistically significant. All analyses were conducted using StataSE 16.1 and figures created with the R software (64-bit).

Subgroup analysis

We conducted sub-group analyses for the safety and efficacy according to the following variables:

- Age
- Sex
- Ethnicity
- Co-treatment with recombinant tissue plasminogen activator (rt-PA) or mechanical thrombectomy
- Dose, duration and method of RIC administration (upper vs lower limb, single vs bilateral limb, automated vs manual device, number of RIC cycles and 'doses')
- Time-to-treatment/intervention (categorised into <3 hours, 3 to 6 hours and >6 hours, and dichotomised into ≤3 or >3 hours, as well as pre-hospital vs hospital RIC administration).

Assessment of heterogeneity

Trials were tabulated according to their study design. Tests of heterogeneity was conducted with the Cochrane Q statistic distributed as a Chi-square variate (assumption of homogeneity of effect sizes). The extent of between-study heterogeneity was assessed based on the final mRS outcome with the I^2 statistic. Study heterogeneity I^2 values >50% were considered substantial and >75% deemed considerable heterogeneity.

Risk of bias assessment

Two review authors (PD and TE) independently assessed the risk of bias for each study using the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions (105). Any disagreements were resolved by discussion or by assessment of a third review author, whenever required. The risk of bias of each study was assessed in the following categories: selection, performance, detection, attrition, reporting and ‘other’ biases. Each domain was assessed as presenting 'low risk', 'high risk', or 'unclear risk' of bias accordingly, along with a justification for the judgement in the 'Risk of bias' table. If required, further information was requested from the trial investigators. Publication bias was assessed using funnel plots and Egger's statistic for the primary outcome.

Ethics

This study is an pseudonymised individual patient data meta-analysis, and no new patient data or human participant procedure is involved. Informed consent and ethical approval are not essential for this study and data sharing was granted by the trials’ ethical approvals. Data was transferred using institutionally approved secure email and stored on a secure password protected NHS workstation. The General Data Protection Regulation (GDPR) 2018 principles were adhered to.

3.2 Healthy Volunteer Remote Ischaemic Conditioning Pilot Study

Selection and Recruitment of Participants

Participants were recruited by advertisement by posters in the University of Nottingham and in other collaborating organisations (Nottingham University Hospitals NHS Trust). Volunteers were be initially contacted by one of the investigators who sent the volunteer a copy of the participant information sheet and consent form, checked inclusion and exclusion criteria, and if the volunteer was suitable and willing to participate, arrangements were made for the volunteer to attend for the scan (after undergoing the informed consent process).

It was explained to the potential participant that entry into the trial was entirely voluntary. It was also explained that they could withdraw at any time but attempts would be made to avoid this occurrence. In the event of their withdrawal it was be explained that their data collected could not be erased and consent would be sought to use the data in the final analyses where appropriate.

Eligibility criteria

Inclusion criteria

- Adult (age ≥ 18 years but <40 years)

- Healthy adult people (taking no regular prescribed medication or recreational drugs within the last month)
- Male
- Non-smoker/never smoked
- Body Mass Index (BMI<30)

Exclusion criteria

- Inability to complete MRI safety questionnaire and / or informed consent process
- Known contraindication to MRI scanning (for example pacemaker / implanted defibrillator, intracranial vascular clip, implanted programmable device, intra-ocular metallic fragment, etc)
- Current or previous neurological, neurosurgical, psychiatric, cognitive or mood disorder
- Any major illness, including (myocardial infarction, stroke, intracranial bleed, transient ischaemic attack, peripheral vascular disease or limb claudication, chronic kidney disease, diabetes, hypertension, cancer) or surgery
- Any acute minor illness within the 2-weeks prior to participation
- Claustrophobia

All participants provided written informed consent before they entered the study.

Study Treatment and Regimen

Prospective, blinded-endpoint study of remote ischaemic conditioning (RIC) in healthy human volunteers (n = 6) to receive RIC via a single left arm (Figure 3.1).

Sample size

6 volunteers were included in this pilot feasibility study. As this work has never been previously performed, no sample size was calculated. The data gained obtained will help determine population size for future dose-finding trials.

Interventions

RIC arm group: 4 cycles of intermittent arm ischaemia - alternating 5 minutes inflation (200 mmHg) followed by 5 minutes deflation of an upper limb blood pressure cuff during the MRI scan.

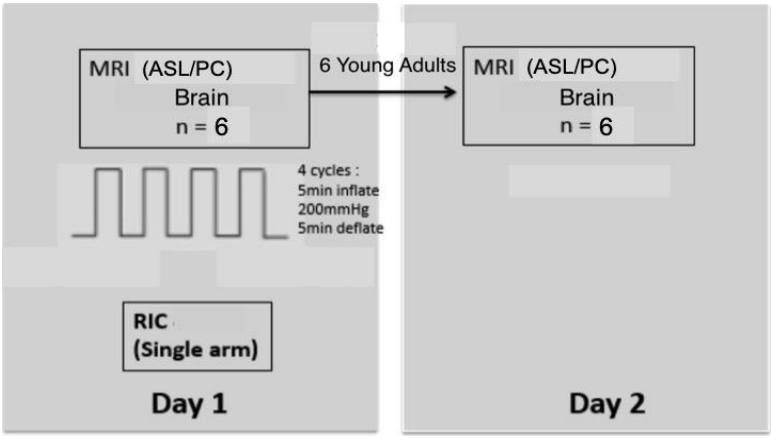
Control: There was no control group per se. Measurements of blood flow and perfusion were compared to baseline pre-RIC values.

Duration of RIC and MRI scan**:** A single dose of 40 minutes to complete 4 cycles of limb ischaemia and reperfusion. PC- and ASL-MRI (brain) were performed before, and immediately after RIC. A further PC- and ASL-MRI reading was performed at 24 hours after the treatment with RIC.

Serum biomarkers: Blood taken (2xEDTA tubes; max 5ml each), centrifuged and frozen and 3 time-points: pre and post RIC on Days 1 and 2. Approximately 20ml urine samples were also collected on Day 1 and Day 2. These will be processed, frozen and batch analysed at the end of the trial. Mechanistic serum biomarkers which include heat-shock proteins (HSP-27), vascular endothelial growth factor (VEGF), MMP-9 and S100ß will be tested, (22, 74) though other biomarkers may be

considered at a later point. Blood samples (venepuncture) were be taken by a trained clinician.

Figure 3-1: Experimental design for the remote ischaemic conditioning (RIC) intervention and MRI scanning using arterial spin labelling (ASL) and phase contrast (PC).



Statistics

Repeated measures ANOVA was used to compare changes in organ perfusion and serum biomarkers over time.

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS) statistical software (version 18, Chicago, IL, USA). Continuous variables were expressed as means ± standard deviations or median and interquartile range and categorical variables were expressed as frequencies (n) or percentages (%). The P-values were two-tailed and statistical significance was set at a value of <0.05.

<u>MRI Acquisition & Analysis</u>

Acquisition

Blood flow measures

Phase-contrast (PC)-MRI was used to quantify vessel lumen cross-sectional area, velocity and bulk blood flow in vessels within each system. A TFE technique (TFE factor 4–6 dependent on subjects' heart rate) was used with a single slice perpendicular to the vessel of interest. A total of 30 phases (carotid arteries) were collected across the cardiac cycle using specified velocity encoding for each vessel (Both Carotid arteries and Basilar artery 90 cm/s). The Carotid arteries and Basilar artery were acquired free breathing.

Perfusion Measures

Respiratory-triggered Flow Alternating Inversion-Recovery Arterial Spin Labelling (FAIR-ASL) (106, 107) (post-labelling delay 1,500 ms, balanced fast field echo [bFFE] readout) was used to measure tissue perfusion. Perfusion data was collected in five contiguous slices (15-20 ASL pairs in 5 mins). An equilibrium base magnetisation M_0 and T_1 image were acquired at baseline for perfusion quantification.

Analysis

Blood Flow Measures

'Q-flow' software (Philips Medical Systems) was used to analyse PC-MRI data. For each vessel, a region-of-interest (ROI) was drawn to estimate flow by averaging the flow velocity values within the ROI and multiplying by vessel lumen cross sectional area. This was performed separately for the Right Internal Carotid, Left Internal Carotid and Basilar arteries Mean flow was calculated by averaging the flow rates for each cardiac phase across the cardiac cycle.

Perfusion Measures

Each ASL label/control image will be motion corrected to the base magnetisation M0 image using in-house software. Individual perfusion-weighted images (control-label)

were calculated, inspected for motion (exclude >1 voxel movement) and averaged to create a single perfusion-weighted image (DM). DM, M_0 and T_1 maps will be used in a kinetic model to compute tissue perfusion maps. A binary mask of cerebral tissue was formed from the T_1 map and used to calculate the mean cerebral perfusion.

4. RESULTS

4.1. Individual Patient Data Meta-Analysis of Randomised Controlled Trials

Literature search results

We screened 127 non-duplicate titles and abstracts, from which 25 full-text articles were evaluated (Figure 4.1). Out of those, seven eligible studies were identified and trial investigators from each trial was contacted to join and contribute their data to the Collaboration (RISC) (64, 66, 68, 71, 74, 76, 77).

Characteristics of Included Studies

We pooled individual participant data from 7 RCTs from 4 countries published between 2010-2020 comprising 561 participants, of which 5 were excluded due to missing randomisation or any outcome data. In total, 556 participants were included (281 randomised to RIC therapy and 275 to sham therapy) for acute stroke. All included participants were treated for acute ischaemic stroke (randomisation occurred within 24 hours). The overall mean age was 66.3 years (SD 13.9), 61% were male, mean systolic blood pressure was 150.4 mmHg (SD 21.7) and mean baseline NIHSS score was 9 (SD 5.7). All baseline variables were equally balanced across both groups. The largest study cohort had 188 participants (93 received RIC and 95 received Sham therapy) whilst the smallest study had 26 participants (13 RIC and 13 Sham). The studies are summarised in Table 4.1 and 4.2. The detailed baseline characteristics are presented in Tables 4.3. The study heterogeneity was low (I^2=15%)**.**

Clinical outcomes

Compared to sham therapy, participants randomised to RIC did not significantly change the odds of disability based on the primary outcome measure of the end-of-trial mRS shift from the pooled data (adjusted common OR=0.97, 95%CI 0.71-1.31, p=0.83). The end-of-trial mRS score distribution by treatment groups is detailed in Figure 4.2. When adjusted for trial as a random effects variable in the mixed model, no significant difference remained (cOR=0.96, 95%CI 0.70-1.31, p=0.81).

There was no significant difference between groups in the good clinical outcome (mRS≤2; cOR=0.93, 95%CI 0.59-1.45, p=0.74). However, there was an improvement in the final NIHSS score by 1 point (95%CI -1.55, -0.16, p=0.016) in patients that received RIC. RIC showed some improvement in the final BI scores (p=0.07). All stroke and ND were non-significantly reduced by 39% (RIC 6.4%, control 10.3%, cOR=0.65, 95%CI 0.35-1.23, p=0.19). There was no significant effect on death (p=0.32), infarct volume (p=1.00) and serum biomarkers [S100ß (p=0.79), MMP9 (p=0.77) and HSP27 (p=0.46)]. The detailed results are summarised in Table 4.4.

Sub-group analysis

In the planned subgroup analysis of RIC vs sham therapy, a possible treatment interaction (p=0.08) occurred with time-to-treatment, favouring treatment initiation of RIC within 3 hours of stroke onset (mRS: ≤3hrs, OR 0.71, 95%CI 0.44-1.16; >3hrs OR 1.23, 95%CI 0.82-1.85). However, no treatment interaction was observed with age (≥70 vs <70 years; p=0.26), sex (p=0.16), thrombolysis (p=0.34), thrombectomy (p=0.31) or any of the RIC delivery methods (Table 4.5, Figure 4.4).

Risk of bias

All studies had an overall low risk of bias, except Hougaard et al's study which had a high risk of bias (Figure 4.3).

Figure 4-1: PRISMA flow chart of study selection

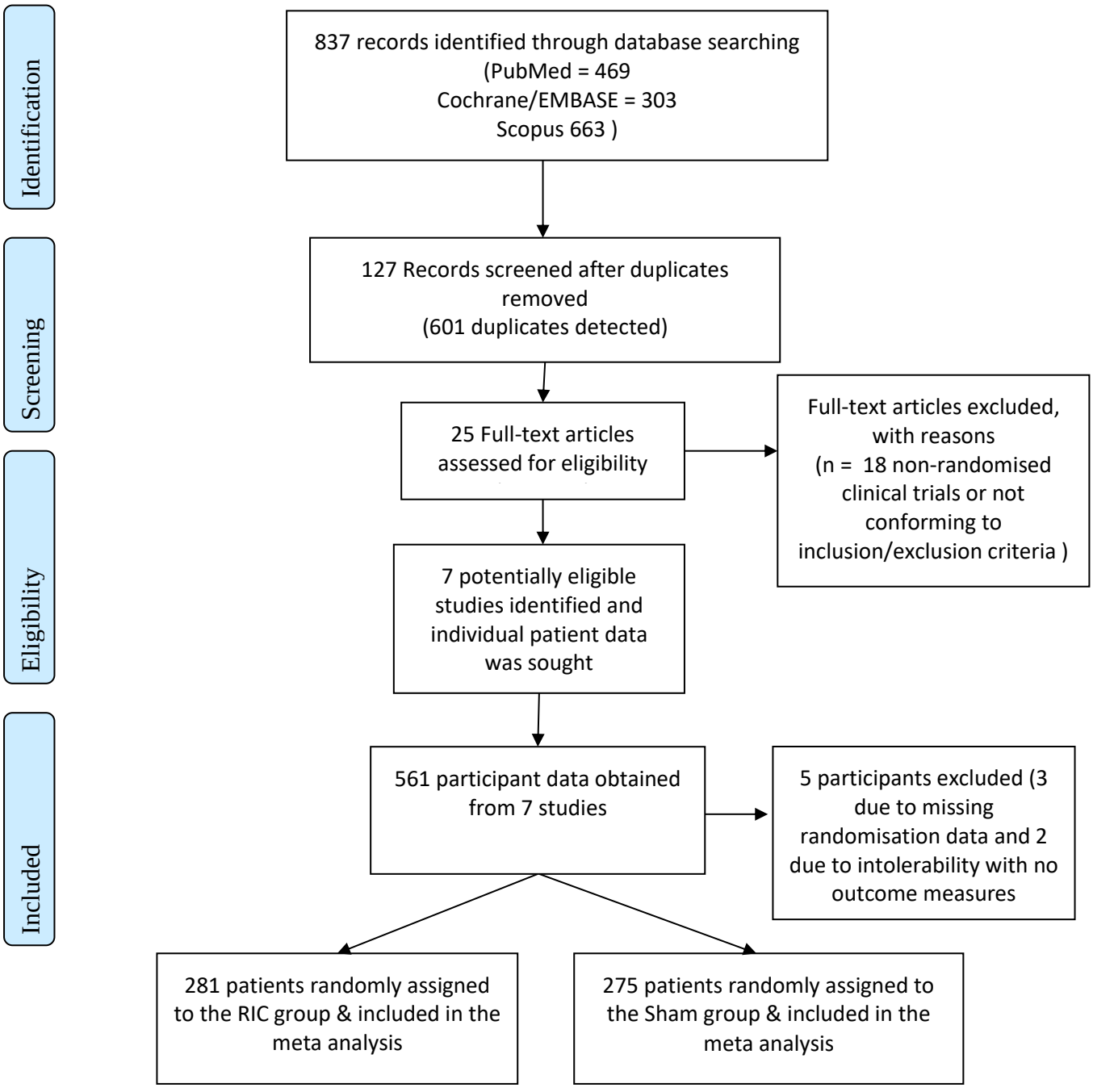


Table 4-1: Baseline characteristics and trial design of each included trial.

Author, Year of Study	Country	Study recruitment period	Study Design	RIC method	Sample size, n	Risk of bias
He et al, 2019	China	2019	Randomized computer generated and sealed envelope; Outcome blinded to group	Twice within 6-24hrs	Total 49. IVT within 4.5hrs, NIHSS >4 & <25	Low
An et al, 2020	China	2017-2018	Randomized using hospital ID numbers; Outcome blinded to group	Twice daily during hospital stay	Total 68 (2 excluded). IVT within 4.5hrs, NIHSS <25, mRS ≤2	Low
Hougaard et al, 2013	Denmark	2009-2011	Randomized using sealed envelope; Outcome blinded to group	Pre-hospital only	Total 140 (3 excluded). IVT within 4.5hrs	High (high exclusion and drop-out rate, unclear randomization)
Pico et al, 2020	France	2015-2018	Randomized computer generated; Outcome blinded to group	Once within 6 hours of onset	Total 188. IVT within 4.5hrs, NIHSS >4 & <25	Low
England et al 2017	United Kingdom	2013-2015	Randomized computer generated; Outcome blinded to group	Once after 6 hours of onset	Total 26. No IVT	Low
England et al 2019	United Kingdom	2016-2018	Randomized computer generated; Outcome blinded to group	Up to twice daily for 4 days	Total 60. IVT within 4.5hrs	Low
Che et al, 2019	China	2017	Randomized computer generated and sealed envelope; Outcome blinded to group	Once within 4.5hrs then twice daily for 6 days	Total 30. IVT within 2hrs	Low

Key studies excluded from individual patient data meta analysis:

1. Li Y, Liang K, Zhang L, et al. Upper limb ischemic postconditioning as adjunct therapy in acute stroke patients: a randomized pilot. J Stroke Cerebrovasc Dis 2018;27:3328–3335. (108)

Recruited acute stroke patients within 72 hours (not 24 hours from onset as per inclusion criteria).

2. Li Y, Liang K, Zhang L, et al. Remote ischemic post-conditioning may improve post-stroke cognitive impairment: a pilot single centre randomized controlled trial. J Stroke Cerebrovasc Dis 2020;29:105217. (109)

Recruited acute stroke patients within 72 hours (not 24 hours from onset as per inclusion criteria).

3. Zhao W, Che R, Li S, Ren C, Li C, Wu C, et al. Remote ischemic conditioning for acute stroke patients treated with thrombectomy. Ann Clin Transl Neurol. 2018;5:850–856. doi: 10.1002/acn3.588. (110)

Single arm study only.

Table 4-2: Trial design and outcomes of each included trial.in patients with acute stroke.

Study	Patient/Disease type	Randomisation	Location of RIC	Number of Cycles; Inflation/Deflation	Time of RIC	Effect on Neurological outcome	Effect on Infarct size	Serious Adverse Events	Effect on Blood markers
He et al, 2020	AIS with IVT only (n=49)	RIC = 24 Sham = 25	Upper limb; 200mmHg RIC, 60mmHg Sham	4 cycles, 5min x 5min	Twice within 6-24hrs of IVT (first given 6hrs after IVT)	No significant difference in mRS at 90days, NIHSS at 1,7 & 30 days	N/A	No significant difference in ICH/HT, mortality or SAE	High sensitivity CRP lower in RIC at 24hrs
An et al, 2020	AIS with IVT only (n=68)	RIC = 34 (2 excluded) Sham = 34	Both Upper Limbs; Auto device; 180mmHg RIC, 0mmHg Sham	5 cycles, 5min x 3min	Twice daily during hospital stay 8-14days (first within 3hrs of IVT)	Improved mRS 0-1, mRS 0-2 and NIHSS scores at 90days.	N/A	No significant difference in ICH/HT, mortality or SAE	Lower plasma S100ß & higher plasma VEGF in RIC. No significant change in MMP9, BDNF, bFGF, HO1.

Hougaard et al, 2013	AIS with IVT (n=443)	RIC = 73 Sham = 64 (3 excluded in total, missing randomisation data)	Upper Limb; 200mmHg or 25mmHG above SBP in RIC, 0mmHg in Sham	4 cycles; 5min x 5min	Pre-hospital (once)	No significant difference in mRS at 90days	No significant difference in the final infarct size, infarct growth and penumbral salvage at 1 month	No significant difference in ICH/HT, mortality or SAE	N/A
Pico et al, 2020	AIS with/without IVT (n=188)	RIC = 93 Sham = 95	Lower limb; Auto device; 110mmHg above SBP in RIC, 0mmHg in Sham	4 cycles; 5min x 5min	Once within 6 hours of onset	No significant difference in mRS at 90days	No significant difference in the final infarct size and infarct growth at 24 hours	No significant difference in ICH/HT, mortality or SAE	N/A
England et al 2017	AIS without IVT (n=26)	RIC = 13 Sham = 13	Upper Limb; 20mmHG above SBP in RIC, 30mmHg in Sham	4 cycles; 5min x 5min	Once after 6hrs but within 24 hours of onset	Improved NIHSS scores at 90days. No significant difference in	N/A	No significant difference in ICH/HT, mortality or SAE	Raised HSP-27 in RIC group after 4 days

						mRS at 90 days			
England et al 2019	AIS with/with out IVT (n=60)	RIC = 31 Sham = 29	Upper Limb; 20mmHG above SBP in RIC, 30mmHg in Sham	4 cycles; 5min x 5min	Once within 6hrs of onset then up to twice daily for 4 days: (20pts once only), 20pts twice for 1 day, 20pts twice daily for 4 days)	No significant difference in mRS, BI at 90days & NIHSS at 4 days	N/A	No significant difference in ICH/HT, mortality or SAE	Raised S100ß in Sham but not in RIC group. No significant change in MMP9.
Che et al, 2019	AIS with IVT (n=30)	RIC = 15 Sham = 15	Both Upper Limbs; Auto device; 200mmHg RIC, 0mmHg Sham	5 cycles; 5min x 5min	Once within 2 hours of IVT (IVT within 4.5hrs of onset) then twice daily for 6 days	Significant reduction in NIHSS on day 30 in RIC. No significant difference in 90-day mRS, BI & NIHSS	N/A	No significant difference in ICH/HT, mortality or SAE	N/A

Table 4-3: Baseline variables according to the intervention or control groups.

Feature	RIC, n/N (%) or mean±SD/N	Control, n/N (%) or mean±SD/N	P value
Socio-demographics			
Sample size	281	275	-
Sex (male)	164 (58.4)	175 (63.6)	0.203
Age (years)	66.5±13.6	66.8±14.1	0.756
Baseline characteristics			
NIHSS	8.7±5.5	9.4±5.9	0.153
rt-PA	242 (86.1)	237 (86.2)	0.983
MT	38 / 281 (13.5)	42 / 275 (15.2)	0.517
SBP	149.0±21.2	151.7±22.1	0.146
DBP	83.5±14.4	83.9±13.8	0.708
Infarct volume	17.8±31.1 / 165	19.9±36.4 / 159	0.567
Co-morbidities			
HTN	150 / 281 (53.4)	150 / 275 (54.5)	0.783
DM	49 / 281 (17.4)	52 / 275 (18.9)	0.653
AF	63 / 281 (22.4)	56 / 275 (20.4)	0.554
Prior Stroke/TIA	62 / 281 (22.1)	50 / 275 (18.2)	0.254
Prior IHD	35 / 281 (12.5)	35 / 275 (12.7)	0.923
Smoking	72 / 213 (33.8)	93 / 208 (44.7)	0.096
Stroke Aetiology			
Cardioembolic	39 / 138 (28.3)	34 / 129 (26.4)	Combined 0.914
LVD	29 / 138 (21.0)	24 / 129 (18.6)	
SVD	40 / 138 (29.0)	41 / 129 (31.8)	
Unknown/other	30 / 138 (21.7)	30 / 129 (23.3)	
Time to Treatment			
0-3 hours	120 / 281 (42.7)	119 / 275 (43.3)	Combined 0.99
3-6 hours	124 / 281 (44.1)	120 / 275 (43.6)	
> 6 hours	37 / 281 (13.2)	36 / 275 (13.1)	

Footnote: HTN hypertension; DM diabetes mellitus; AF atrial fibrillation; TIA transient ischaemic attack; IHD ischemic heart disease; NIHSS National Institutes of Health Stroke Scale; RIC remote ischaemic conditioning; rt-PA recombinant tissue plasminogen activator; MT mechanical thrombectomy; SBP systolic blood pressure; DBP diastolic blood pressure; TACS total anterior circulation stroke; PACS partial anterior circulation stroke; LACS lacunar stroke; POCS posterior circulation stroke; LVD large vessel disease; SVD small vessel disease; SD standard deviation

Table 4-4: Clinical and safety outcomes of patients with acute ischaemic stroke treated with remote ischaemic conditioning or sham/control.

Outcome measures, N	RIC (n= 281); Mean (SD) or N (%)	Control (n= 275); Mean (SD) or N (%)	Unadjusted		Adjusted**	
			OR / Beta coefficient (95% CI)	P value	OR / Beta coefficient (95% CI)	P value
mRS (Ordinal)	N = 273	N = 265	0.92 (0.68 – 1.24)	0.601	0.97 (0.71 – 1.31)	0.834
mRS 0	82 (30.0%)	77 (29.1%)	-	-	-	-
mRS 1	73 (26.7%)	69 (26.0%)	-	-	-	-
mRS 2	41 (15.0%)	40 (15.1%)	-	-	-	-
mRS 3	35 (12.8%)	29 (10.9%)	-	-	-	-
mRS 4	18 (6.6%)	19 (7.2%)	-	-	-	-
mRS 5	4 (1.5%)	14 (5.3%)	-	-	-	-
mRS 6	20 (7.3%)	17 (6.4%)	-	-	-	-
mRS≤2 at 90 days	196 / 273 (71.8)	186 / 265 (70.2)	1.08 (0.74 – 1.56)	0.681	0.93 (0.59 – 1.45)	0.745
mRS≤1 at 90 days	155 / 273 (5.7)	146 / 265 (5.5)	1.07 (0.76 – 1.50)	0.694	0.97 (0.66 – 1.44)	0.903
BI at 90 days / n=307, Mean (SD)	N = 153 89.25 (21.4)	N = 152 84.80 (27.1)	4.45 (-1.04 to 9.93)	0.112	4.27 (-0.43 to 8.98)	0.075
Early ND / n=543, (%)	11 / 279 (3.9)	16 / 264 (6.1)	0.63 (0.28 – 1.39)	0.260	0.66 (0.29 – 1.48)	0.322
Late ND / n=294	0 / 152 (0)	2 / 142 (1.4)	1	1	1	1
Change in NIHSS (Baseline to end of trial)/ N=296 ; mean (SD)	N = 152 -5.88 (4.11)	N = 144 -4.95 (5.28)	-0.94 (-2.02 to 0.14)	0.089	-0.85 (-1.55 to -0.16)	**0.016***
Any ND / n=542	11 / 279 (3.9)	17 / 273 (6.2)	0.59 (0.27 – 1.29)	0.189	0.62 (0.28 – 1.37)	0.241
Recurrent Stroke / n=490	9 / 249 (3.6)	16 / 251 (6.4)	0.52 (0.22 – 1.21)	0.134	0.55 (0.23 – 1.31)	0.182
All stroke / n=553	18 / 281 (6.4)	28 / 272 (10.3)	0.59 (0.32 – 1.10)	0.101	0.65 (0.35 – 1.23)	0.191

Any adverse vascular event / n=553	21 / 281 (7.5)	28 / 272 (10.3)	0.70 (0.38 – 1.27)	0.245	0.76 (0.42 – 1.39)	0.383
Death / n=556	20 / 281 (7.1)	17 / 275 (6.2)	1.09 (0.56 – 2.11)	0.789	1.44 (0.69 – 2.98)	0.326
Change in infarct volume / n=316, Mean (SD)	N = 149 11.8 (35.3)	N = 150 14.1 (35.7)	-2.08 (-9.94 to 5.76)	0.600	0.00 (-7.79 to 7.79)	1.000
Change in serum biomarkers:						
S100ß (pico) / n=126, Mean (SD)	N = 59 80.2 (831.4)	N = 67 83.0 (877.8)	-2.85 (-305.48 to 299.78)	0.985	41.45 (-269.23 to 3452.14)	0.792
MMP9 (pico) / n=126, Mean (SD)	N = 59 -1576.7 (39531.2)	N = 67 -1012.7 (17378.4)	-563.97 (-11116.04 to 9988.09)	0.916	-1580.18 (-12564.63 to 9404.27)	0.776
HSP-27 (%) / n=88, Mean (SD)	N = 39 9.0 (47.5)	N = 44 39.9 (212.8)	-30.94 (-100.10 to 38.20)	0.376	-26.23 (-97.68 to 45.23)	0.467

Footnote: mRS modified Rankin Scale; ND neurological deterioration; MMP9 matrix mettaloproteinase-9; RIC remote ischaemic conditioning; HSP-27 heat shock protein-27; BI Barthel Index; OR odds ratio; MD mean difference; CI confidence interval; *indicates statistical significance of P<0.05; **analysis adjusted for: age, sex, stroke severity (NIHSS), systolic blood pressure and time to treatment;

Figure 4-2: Shift plot of end-of-trial mRS by treatment group; remote ischaemic conditioning (RIC) or sham therapy.

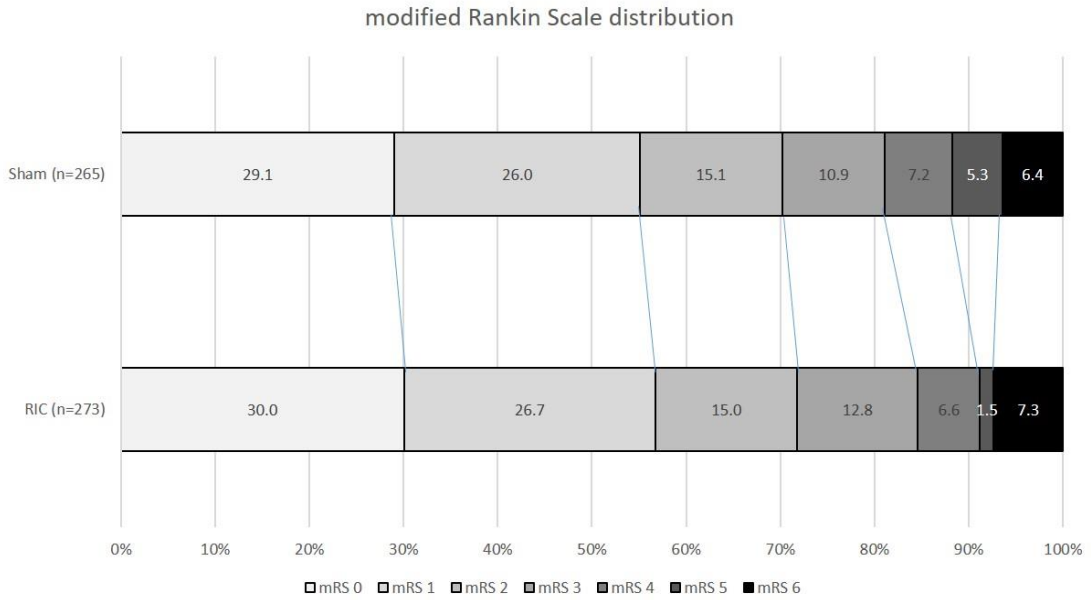


Figure 4-3: Risk of bias table for the included studies.

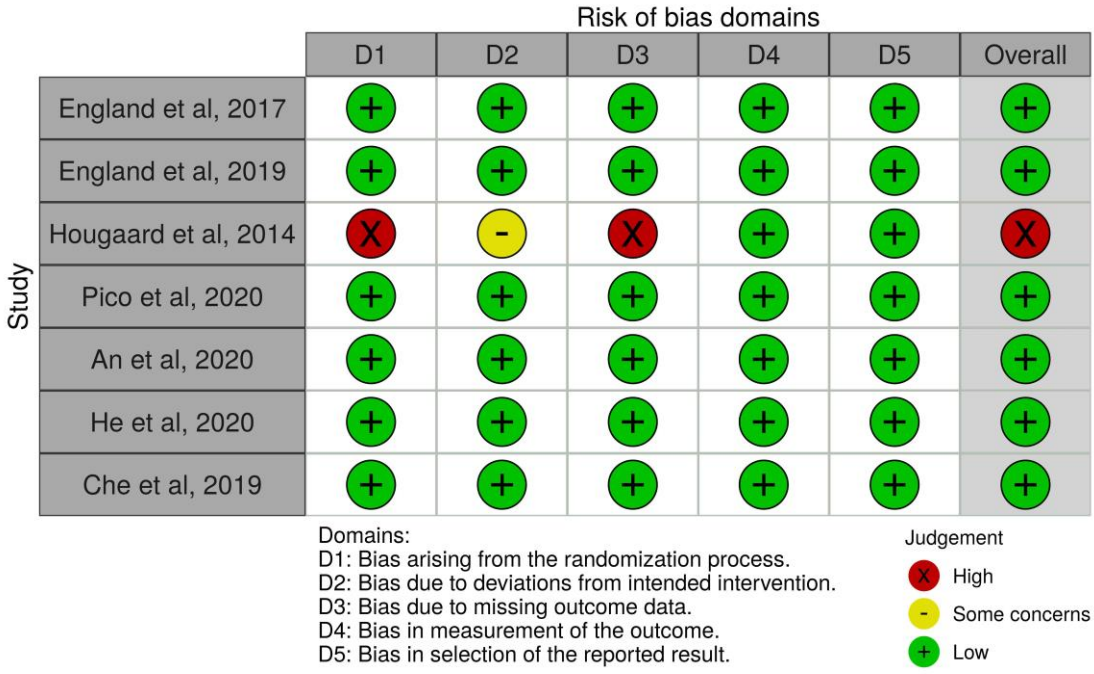
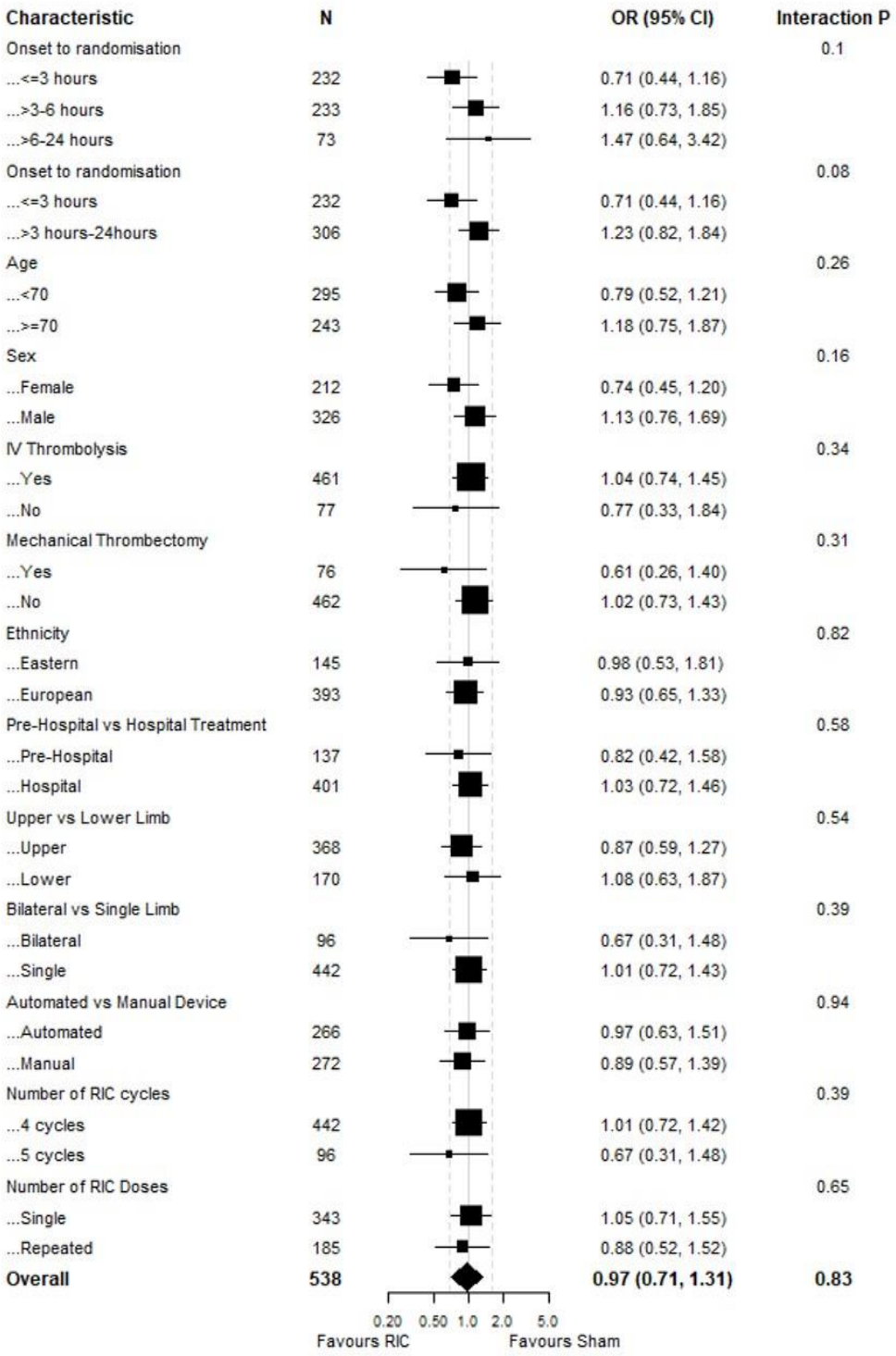


Table 4-5: Subgroup analysis based on the mRS ordinal outcome of patients with acute ischaemic stroke treated with remote ischaemic conditioning or sham/control.

Variable	N	RIC	Sham	aOR (95% CI)	P value for Interaction
Sample size	556	281	275		
Available final mRS	538	273	265		
Onset to randomisation					0.101
≤3 hours	232	117	115	0.71 (0.44 – 1.16)	
3 to 6 hours	233	119	114	1.16 (0.73 – 1.85)	
6 to 24 hours	73	37	36	1.47 (0.64 – 3.42)	
Onset to randomisation					0.08
≤3 hours	232	117	115	0.71 (0.44 – 1.16)	
3-24 hours	306	156	150	1.23 (0.82 – 1.84)	
Age					0.264
<70	295	152	143	0.79 (0.52 – 1.21)	
≥ 70	243	121	122	1.18 (0.75 – 1.87)	
Sex					0.159
Female	212	114	98	0.74 (0.45 – 1.20)	
Male	326	159	167	1.13 (0.76 – 1.69)	
rT-PA					0.343
Yes	461	234	227	1.04 (0.74 – 1.45)	
No	77	39	38	0.77 (0.33 – 1.84)	
MT					0.314
Yes	76	36	40	0.61 (0.26 – 1.40)	
No	462	237	225	1.02 (0.73 – 1.43)	
Ethnicity					0.826
Eastern	145	71	74	0.98 (0.53 – 1.81)	
European	393	202	191	0.93 (0.65 – 1.33)	
Pre-vs Hospital Treatment					0.587
Pre-hospital	137	73	64	0.82 (0.42 – 1.58)	
Hospital	401	200	201	1.03 (0.72 – 1.46)	
Upper vs Lower limb					0.543
Upper	368	188	180	0.87 (0.59 – 1.27)	
Lower	170	85	85	1.08 (0.63 – 1.87)	
Bilateral vs Single limb					0.392
Bilateral	96	47	49	0.67 (0.31 – 1.48)	
Single	442	226	216	1.01 (0.72 – 1.43)	
Automated vs Manual device					0.948
Auto	266	132	134	0.97 (0.63 – 1.51)	
Manual	272	141	131	0.89 (0.57 – 1.39)	
Number of RIC Cycles					0.392
4 cycles	442	226	216	1.01 (0.72 – 1.42)	
5 cycles	96	47	49	0.67 (0.31 – 1.48)	
Number of RIC Doses					0.651
Single	343	161	182	1.05 (0.71 – 1.55)	
Repeated	185	92	93	0.88 (0.52 – 1.52)	

Figure 4-4: Forest plot according to the subgroup analysis (based on the mRS ordinal outcome) of patients with acute ischaemic stroke treated with remote ischaemic conditioning or sham/control.



4.2. Healthy Volunteer Remote Ischaemic Conditioning Pilot Study

The summary characteristics of the participants are summarised in Table 4.6.

Table 4-6: Summary characteristics of the participants

Feature	RIC, n/N or mean±SD
Sample size	6
Gender (male)	6
Age (years)	27.7±2.8
BMI	22.1±2.4
Non-smoker	6

Tolerability Data:

All participants were able to tolerate the entire duration of the RIC intervention. Three participants reported only mild discomfort and the remaining three reported no discomfort during the intervention. There were no reports of petechiae at the cuff site.

ASL Analysis

No analysis on the ASL data has been performed at the time of submission due to unforeseen circumstances of the COVID-19 pandemic.

Phase Contrast Analysis

There was no significant difference in the flow velocities measured between baseline, immediately after RIC and at 24 hours post-RIC across both Internal Carotid arteries (Right ICA; p=0.23, Left ICA; p=0.18) and Basilar artery (p=0.62) based on repeated measures ANOVA analysis (Tables 4.7 to 4.10 and Figures 4.5 to 4.7). Similarly, no significant difference was identified in each of the aforementioned vessels (Right ICA, Left ICA, and BA) according to the paired *t-test* analysis between three groups

(pre-RIC and post-RIC; pre-RIC and 24 hours after RIC; post-RIC and 24 hours after RIC) (Tables 4.11 to 4.13).

Table 4-7: Mean velocities of the right internal carotid artery at the different time points in relation to the remote ischaemic conditioning (RIC) application (pre-intervention, immediately post-intervention, and 24 hours after intervention).

Subject	Mean velocity (cm/s)		
	Pre-RIC	Post-RIC	24hr RIC
1	29.5	30.4	29.5
2	22.4	20.9	22.7
3	17.4	21.2	11.9
4	15.7	15.1	14.2
5	23.2	22.1	19.1
6	23.8	26.1	25.4

Table 4-8: Mean velocities of the left internal carotid artery at the different time points in relation to the remote ischaemic conditioning (RIC) application (pre-intervention, immediately post-intervention, and 24 hours after intervention).

Subject	Mean velocity (cm/s)		
	Pre-RIC	Post-RIC	24hr RIC
1	32.8	35.0	21.6
2	25.5	23.7	24.4
3	30.8	16.8	27.1
4	24.0	19.9	23.8
5	24.5	26.5	19.1
6	24.9	22.7	26.7

Table 4-9: Mean velocities of the basilar artery at the different time points in relation to the remote ischaemic conditioning (RIC) application (pre-intervention, immediately post-intervention, and 24 hours after intervention).

Subject	Mean velocity (cm/s)		
	Pre-RIC	Post-RIC	24hr RIC
1	30.1	32.6	26.7
2	26.9	23.0	24.4
3	32.2	33.0	29.7
4	23.6	21.1	22.1
5	21.4	18.0	19.8
6	25.2	28.9	31.9

Figure 4-5: Line chart of the mean velocities (cm/s) and standard deviations between subjects of the right internal carotid artery.

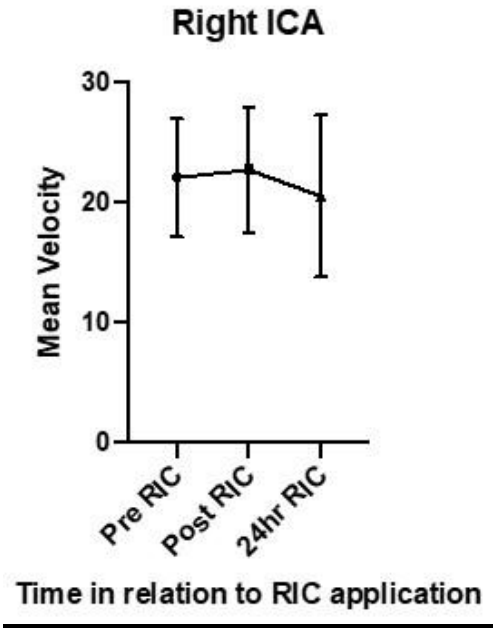


Figure 4-6: Line chart of the mean velocities (cm/s) and standard deviations between subjects of the left internal carotid artery.

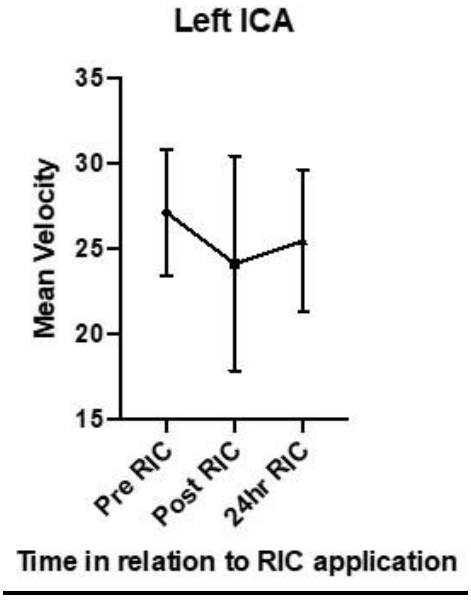


Figure 4-7: Line chart of the mean velocities (cm/s) and standard deviations between subjects of the basilar artery.

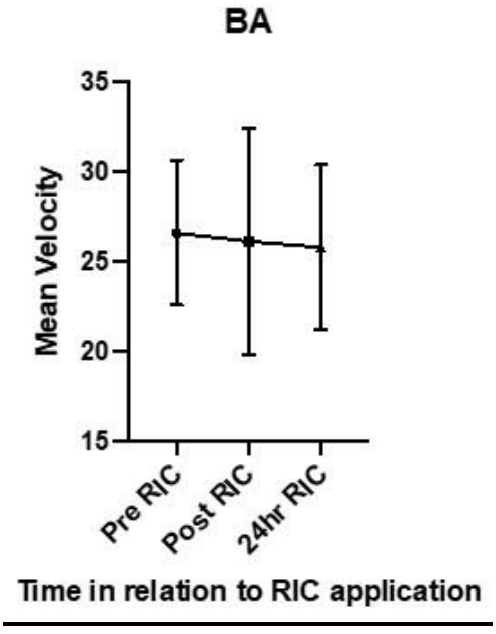


Table 4-10: Statistical significance based on two-tailed P values using the repeated measures ANOVA analysis according to each intracranial blood vessel. ICA = internal carotid artery

Blood Vessel	P value
Right ICA	0.23
Left ICA	0.18
Basilar artery	0.62

Table 4-11: Mean difference of the right internal carotid artery between different time points in relation to the remote ischaemic conditioning (RIC) application based on paired t-test analysis. Pre-RIC = Pre-intervention; Post-RIC = immediately post-intervention; 24hr RIC = 24 hours after intervention.

Comparison	Mean difference	95% CI (lower)	95% CI (higher)	P value
Pre RIC - Post RIC	-0.65	-2.85	1.55	0.48
Post RIC – 24hr RIC	2.18	-1.81	6.18	0.21
Pre-RIC – 24hr RIC	1.53	-1.35	4.43	0.23

Table 4-12: Mean difference of the Left internal carotid artery between different time points in relation to the remote ischaemic conditioning (RIC) application based on paired t-test analysis. Pre-RIC = Pre-intervention; Post-RIC = immediately post-intervention; 24hr RIC = 24 hours after intervention.

Comparison	Mean difference	95% CI (lower)	95% CI (higher)	P value
Pre RIC - Post RIC	2.99	-3.23	9.22	0.27
Post RIC – 24hr RIC	-1.34	-7.89	5.20	0.62
Pre-RIC – 24hr RIC	1.64	-1.06	4.36	0.17

Table 4-13: Mean difference of the Basilar artery between different time points in relation to the remote ischaemic conditioning (RIC) application based on paired t-test analysis. Pre-RIC = Pre-intervention; Post-RIC = immediately post-intervention; 24hr RIC = 24 hours after intervention.

Comparison	Mean difference	95% CI (lower)	95% CI (higher)	P value
Pre RIC - Post RIC	0.46	-2.93	3.86	0.74
Post RIC – 24hr RIC	0.34	-3.29	3.97	0.81
Pre-RIC – 24hr RIC	0.80	-3.12	4.73	0.62

5. DISCUSSION

5.1 Individual Patient Data Meta-Analysis of Randomised Controlled Trials

In this individual patient data meta-analysis of RCTs investigating the safety and efficacy of RIC compared to a sham control group in acute ischaemic stroke, patients treated with RIC had significantly increased odds of improvement in the final NIHSS scores (p=0.01). However, this did not translate to a significant improvement in the functional outcomes (ordinal mRS shift analysis, mRS$\leq$2 or $\leq$1). Similarly, RIC did not significantly improve the final Barthel Index scores (p=0.07) nor reduce the likelihood of recurrent stroke or neurological deterioration (p=0.19). There was no between-group statistical difference in the rates of mortality, END, or change in the infarct volume, and serum biomarkers (S100ß, MMP9, HSP27).

Pre-clinical findings of the use of RIC in AIS have been promising with evidence of reduction in the infarct volume compared to the sham group (1). However, translation of this intervention to early phase clinical trials has led to equivocal results, with no significant difference identified in the infarct growth and clinical outcomes (66-68, 76). Whilst the safety profile of RIC when administered during AIS has been demonstrated in previous studies, there remains a lack of evidence of clear efficacy in the neurological improvement following intervention, reflected by our pooled analysis of patient data. This may be due to the heterogeneity in the study design, including the number of RIC cycles or doses administered, duration of the intervention, and time to treatment initiation. These factors may have precluded the full therapeutic effect of RIC in the acute setting.

Furthermore, initial pre-clinical research largely consisted of single centre studies, which failed to meet the recommendations of Stroke Treatment Academic Industry Roundtable (STAIR) for effective translational research of neuroprotective treatments (111, 112). Some of the identified issues by a recent pre-clinical meta analysis included a low level of study quality and a significant publication bias amongst 57 studies, thus limiting comparisons with the available evidence (1). Pre-clinical multi-centre RCTs are also underway to more accurately investigate and predict the efficacy of the RIC in an acute stroke setting (113).

There have been comparisons to the lack of efficacy of RIC in previous cardiology studies, which found no significant differences in the clinical outcome amongst patients that underwent coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI) (11, 114). However, there is a possibility that RIC is less efficacious in patients with a history of angina preceding coronary intervention, which may have resulted in prior natural tissue conditioning (115). There are also suggestions of potential interactions with comorbidities, anaesthetic agents or other medications that may have mitigated the therapeutic effects of RIC (116, 117). Similarly, there is a possibility of patients with a history of previous TIA or diabetes mellitus may elicit a reduced response to the conditioning effect of RIC (2, 118).

There was a smaller proportion of patients that experienced recurrent stroke and neurological deterioration in the RIC group (6.4%) compared to the sham group (10.3%), although not reaching statistical significance. The fewer adverse events observed following perconditioning is also in line with the results of two previous studies by Meng et al (13, 69). The authors described a lower incidence of recurrent

stroke in a small group of patients with intracranial arterial stenosis following twice daily postconditioning treatment for 300 days. These findings would support a possible underlying RIC-induced protective humoral and neuronal mechanism in reducing the ischaemic-reperfusion injury that may last for days after an acute stroke, as well as the development of a tolerance against further ischaemic insults.

In the pre-specified subgroup analysis, there was a tendency towards a potential interaction in the time-to-treatment for RIC, without reaching statistical significance. When initiated within 3 hours, there were slightly higher odds of achieving a favourable outcome in patients who received RIC (p=0.08). This observation of borderline significance could be explained by the increased likelihood of the effect of RIC in protecting the ischaemic penumbra in the hyper-acute setting. Future trials may consider a shorter time to treatment (≤3 hours from stroke onset) to exploit a potentially larger therapeutic effect.

Whilst there was no between-group difference when RIC was initiated in a pre-hospital setting, this may largely be due to the inclusion of patient data from a single study (Hougaard et al) in the pre-hospital group (66). Due to short ambulance transfer times, up to 18% of the randomised patients in this study were unable to complete all cycles of the intended RIC therapy, thereby potentially delivering a sub-therapeutic dose. Furthermore, up to 22 patients were lost to follow-up and there was a high risk of bias in the randomization and concealment process, which may have confounded the final outcomes of the study.

No significant differences were identified in the remaining subgroups comparing the use of rT-PA or mechanical thrombectomy (MT), number of 'doses' or cycles of therapy administered, the limbs used during the therapy, type of device used, and the age and sex of patients. A possible explanation for the lack of difference observed in patients who received reperfusion therapies (rT-PA or MT) could be due to the successful recanalization of the vessel occlusion, leading to inherent improvements in the functional outcomes and hence may make any potential effect of RIC less conspicuous. Another consideration is the indeterminate optimal RIC 'dose' adopted in the included studies. An et al's study which described favourable mRS outcomes at 3 months in the RIC group, was the only study that employed twice daily RIC treatment throughout the hospital admission (between 8 to 14 days), whilst other studies with neutral findings were limited to a single 'dose' or repeated 'doses' of up to 7 days (64, 66, 68, 71, 77). It is unclear if this difference may have contributed to the overall outcome and the ideal location, length and number of RIC cycles remain unknown. Although the frequently used method of 3 to 5 cycles of 5-minute intervals of cuff inflation/deflation appears safe and pragmatic, the therapeutic effect of this protocol compared to other administrations is undetermined and requires further research.

A previous systematic review and meta analysis of RIC for treating AIS concluded that there was insufficient evidence to suggest that RIC had the potential to influence early neurological improvement compared to the sham group (21). The authors suggested that there was a higher odds of death in the treatment group, contrary to our findings. However, only two studies of low quality evidence and with relatively small numbers of participants were included in Zhao et al's analysis (21, 66, 74).

Furthermore, the varied study characteristics including, but not limited to the baseline variables, time to randomisation/treatment, severity of stroke, use of rt-PA and mechanical thrombectomy were not investigated or accounted for.

Our analysis includes several limitations. First, the heterogeneity in study design of the included studies as well as a high degree of bias in one of the included studies may have confounded the overall outcomes. Despite this, there was a low level of heterogeneity between studies (I^2=15%). Additionally, the slight variation in the methodology and timing of the RIC administration, and the diversity of the patient cohort in terms of the baseline characteristics and ethnicity between trials allowed distinctive exploratory subgroup analyses that may inform the design of future larger RCTs. Second, only 2 of the included studies investigated the infarct volume at 24 hours, limiting conclusions that can be drawn from this cohort. Furthermore, it is possible that changes in the infarct size within 24 hours may be too early to detect any meaningful difference. Third, paucity of data on the outcomes according to the stroke location and aetiology precluded this planned sub-group analysis. Fourth, although the overall sample size was large (n=556), the treatment effect estimate for the subgroups was limited by the available number of participants in each subgroup (n=538). Fifth, all analyses were only performed on an intention-to-treat basis; thus the definitive treatment may be different from the group allocated, which may have influenced the results.

5.2 Healthy Volunteer Remote Ischaemic Conditioning Pilot Study

In this feasibility study of 6 healthy volunteers receiving 4 repeated cycles of RIC during an MRI scan of the brain, RIC administration was feasible, safe, and was well tolerated by the study participants. Blood flow velocity measurements were successfully obtained from all three target vessels at all three time-points in all seix participants. Although there were no significant changes observed in the blood flow velocity of the major intracranial vessels (bilateral ICAs and BA) in response to RIC in this small healthy adult group, the success in obtaining haemodynamic measurements while RIC was administered in the MRI scanner shows that this protocol could be used for evaluating the cerebral haemodynamic effects of different RIC administrations in target populations. ASL data was acquired and analysis was planned in this cohort, but due to extenuating circumstances caused by the COVID-19 pandemic unfortunately, no formal analysis could be performed by the time of the thesis submission.

A probable reason for the lack of significance in the observed results could be due to this feasibility study not being powered to investigate potential significant differences between pre- and post-RIC intervention. Furthermore, only young healthy adults were included in this study, where the effect of RIC on the cerebral blood flow may be difficult to influence. It remains to be seen if a greater response can be observed in an older population with vascular risk factors, although a potential treatment effect (not reaching significance) was observed in participants below 70 years in the meta-analysis. The effect of RIC on the smaller cerebral blood vessels is also of interest

given the RIC effects on pial arteries and collateral circulation in animal models (119, 120). Such effects may be demonstrated in the planned ASL analysis.

At present, underlying mechanisms of RIC have remained speculative. One of the proposed mechanisms (implicated in the neural and humoral pathways) is a positive haemodynamic effect of increased blood flow and perfusion to a distal tissue bed. Surrogate serum markers including increased VEGF and nitric oxide (NO) expression in patients treated with RIC have supported these claims (121, 122). Both cell factors have important roles in mediating angiogenesis and increasing vascular permeability. Additionally, a recent study by Rytter et al demonstrated improved microvascular endothelial function as well as an increased endogenous prostacyclin production (implicated in the VEGF expression pathway) remote to the site of RIC administration (123). The activation of the aforementioned pathways, as well as the Notch signalling pathway, which involves arterial and venous differentiation of endothelial cells, play a key role promoting intracranial leptomeningeal and pial anastomoses and collateral circulation (12, 124). Overall, the growing body of evidence suggests the effects of RIC administration may be similar to the beneficial effects of exercise in stimulating improved cerebrovascular blood flow and perfusion (123, 125, 126). However, it remains to be seen if such changes at the tissue level can be observed using non-invasive modern day MRI techniques.

Previous RIC-based investigating the effects of RIC have utilised TCD to assess potential haemodynamic changes by obtaining readings of the velocities within the middle cerebral artery (MCA) (13, 72, 73). However, these studies have revealed conflicting results. In Guo et al's study, the PD value showed a significant increase

from 6 hours (but not prior to that), until at least 24 hours after healthy volunteers underwent RIC (72). However, in Carter et al's study, no difference was observed in the MCA velocity and PD value, which were measured during a single 'dose' of the RIC administration (73).

There are many reasons that may have contributed to the disparity in results, including the study design (number of RIC doses and cycles, number of limbs used for treatment), method and timing of the outcome assessment. Furthermore, TCD itself is a crude method of assessment of blood flow velocities, largely due to a high degree of user subjectivity and operator skill in obtaining the readings, which may lead to spurious results and limits the use of TCD as a reliable biomarker.

Well conducted PC-MRI acquisition and analysis on the other hand may offer a more sensitive and reproducible measurement of the blood flow velocities, giving rise to a more reliable outcome measure in future studies. PC-MRI provides the ability to detect changes in the vessel diameter when determining the blood flow velocity. This is an important factor that needs to be taken into account during measurements of the velocity, due to constant regulatory effects of the cerebral haemodynamic system (causing vasodilatation or vasoconstriction) to maintain a stable cerebral perfusion pressure. However, measurements of the MCA velocity using TCD do not take into account the vessel diameter, with assumptions made of a constant diameter of the MCA throughout the outcome measure (85).

Another factor to consider between are the variations between the intracranial vessel(s) selected for flow velocity measurement in previous studies (MCA) and this

feasibility study (ICA and BA). A segment of the ICA is bound by the carotid canal, which limits its ability to vasodilate and constrict, unlike the MCA, which is suspended in the cerebrospinal fluid space of the Sylvian fissure. Hence, the degree of dynamic cerebrovascular regulation incurred within these vessels may differ during or after the administration of RIC, with more marked changes in the diameters potentially occurring in the more distal vasculature.

ASL-MRI is also a relatively new method of assessing tissue level perfusion, which can be more sensitive in detecting haemodynamic changes within the brain. Whilst larger haemodynamic changes might not be observed in the major intracranial blood vessels, small changes at the capillary level may be detected using the ASL quantification method. SPECT imaging is an alternative in demonstrating cerebral perfusion. However, compared to ASL-MRI, SPECT utilises ionizing radiation which precludes its repeated use in assessing perfusion outcome measures at various time points.

This study contains a few limitations. First, the sample size of this feasibility study is small, limiting any conclusions to be drawn from the available results regarding the haemodynamic measurements. Second, although feasibility/tolerability of RIC administration during an MRI scan has been shown, this was limited to a young group of healthy adults. It remains to be seen if similar tolerability can be demonstrated in an older population with multiple comorbidities that may affect the compliance (for example musculoskeletal health issues that impact on the ability to lie comfortably for an extended period. Furthermore, it would be important to investigate the reproducibility of the haemodynamic measurements in the older population, which is

more representative of patients presenting with an acute stroke. Third, only male participants were included, limiting generalisability of the findings. Fourth. whilst discomfort/pain related to the RIC administration may have confounded the regional cerebral perfusion, no formal assessment of pain was obtained in this study (127). Fifth, no formal analysis of the ASL data was performed. Last, any correlation with the obtained serum biomarkers remains to be investigated.

6. CONCLUSIONS

The meta-analysis of pooled individual data from early phase RCTs assessing the safety and efficacy of RIC versus sham therapy in acute ischaemic stroke showed a significant improvement in the final NIHSS scores, but no difference in the functional outcome (mRS scores). RIC appeared safe with no significant differences in the overall major cardiovascular adverse events or mortality. Subgroup analysis suggest a possible effect favouring RIC therapy when administered less than 3 hours from stroke onset. The findings of ongoing well-designed multi-centre randomised clinical trials including RECAST-3, REMOTE-CAT, RESIST and REPOST are eagerly awaited (128-131).

The results from the healthy volunteer feasibility study showed RIC administration during MRI scanning appeared to be feasible, safe, and well tolerated. An MRI protocol incorporating PC and ASL measurement of cerebral blood flow (and perfusion) has been established for use in participants before, during and after RIC in different target populations. The results provide pilot data that could inform the sample size and design of future clinical trials in RIC by allowing optimal methods of RIC application and 'dose' to be determined in a principled way. It may also allow dose-finding studies to use MRI as a non-invasive, reliable surrogate measure of end-organ effects of RIC.

Overall, the findings of the individual patient data meta analysis and healthy volunteer feasibility study suggest there are many unanswered questions in determining the efficacy of the administration of RIC, in both health individuals and disease states. However, there remains much potential for further investigations into understanding

the underlying mechanisms and exploiting its therapeutic effect to a targeted patient cohort or disease entity, such as acute stroke. In the future, larger well-designed studies that incorporate the optimal RIC dosing strategy and utilise potentially reproducible outcome measures using MRI techniques such as ASL and PC, which may translate to a meaningful clinical outcome, are warranted.

7. FUTURE DIRECTIONS

In the near future, we will aim to complete the ASL analysis on this cohort of participants, and correlate the imaging findings with the obtained key serum biomarkers of efficacy such as HSP-27 and VEGF.

We will proceed to seek further funding to increase the sample of our pilot cohort, test feedback from older participants on tolerability of the protocol and measure any potential haemodynamic changes in comparison to the young cohort of participants involved in this feasibility study.

A series of studies will ultimately be developed to test the effect of different RIC interventions on cerebral perfusion (varying in terms of duration, number of cycles, limb number/site), to identify the optimal RIC dosing strategy that induces the maximal and/or most sustained changes in cerebral perfusion. These data will then support dose-finding trials, using MRI as a surrogate measure for *in vivo* organ-level modelling of the haemodynamic effects of RIC, in the stroke population. Specifically, reducing risk of vascular events in those with recent TIA or stroke using remote ischaemic post-conditioning is likely to require repeated doses, balanced with RIC acceptability and tolerability.

8. REFLECTIONS ON THE IMPACT OF THE COVID-19 PANDEMIC

During the course of this study period, there were extenuating circumstances due to the interruptions caused by the COVID-19 pandemic which limited access to the data processing and analysis involving the healthy volunteer pilot study. Nonetheless, I have performed and learnt various transferrable skills as follows:

1) Individual patient data meta analysis:

- Critical appraisal of the literature
- Data amalgamation/synthesis
- Trial design and conduct,
- Comprehensive method of systematically reviewing and meta-analysing current literature
- Detailed statistical analyses required within a trial setting
- Drafting a manuscript for potential peer-reviewed publication

2) Healthy volunteer remote ischaemic conditioning pilot study:

- Research Ethics Committee application
- Participant recruitment & enrolment
- Scanning participants
- Blood & urine sample collection and processing
- Data acquisition & analysis
- Statistical analysis
- Future funding application for potential PhD work (reached interview stage with excellent feedback but unsuccessful in securing the funding).

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10. APPENDIX

1. PROSPERO Registration form for Individual Patient Data Meta-Analysis of Randomised Controlled Trials
2. Healthy Volunteer Remote Ischaemic Conditioning Pilot Study Participant Information Sheet
3. Healthy Volunteer Remote Ischaemic Conditioning Pilot Study Participant Consent Form