Remote Ischaemic Preconditioning and its Effect on Coronary Physiology and Platelet Activation

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy (PhD).

I, Jerrett Lau, declare that the work presented in this thesis is original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

The Human Ethics Committee of the Sydney Local Health District Human Research Ethics Committee – Concord Repatriation General Hospital granted approval for the conduct of the studies presented herein. Subjects were provided with verbal and written information regarding the study and gave informed written consent prior to their participation.

Signature: …… Date: ……..

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- Pasalic L, Wing-Lun E, **Lau JK**, Campbell H, Pennings GJ, Lau E, Connor D, Liang HP, Muller D, Kritharides L, Hogg PJ, Chen VM. Novel assay demonstrates that coronary artery disease patients have heightened procoagulant platelet response. J Thromb Haemost 2018;16:1198-1210.

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- **Lau JK**, Alcock RF, Brieger D, Lowe HC. Complex, diffuse in-stent atherosclerosis over a decade following bare metal stenting. Coron Artery Dis 2015;26 Suppl 1:e69-70.
- **Lau JK**, Anastasius MO, Hyun KK, Dabin B, Coverdale S, Ferry C, Hung J, Antonis P, Chew DP, Aliprandi-Costa B, Cass A, Brieger DB. Evidence-based care in a population with chronic kidney disease and acute coronary syndrome. Findings from the Australian Cooperative National Registry of Acute Coronary Care, Guideline Adherence and Clinical Events (CONCORDANCE). Am Heart J 2015;170:566-72.e1.
- **Lau JK**, Sy RW, Corbett A, Kritharides L. Myotonic dystrophy and the heart: A systematic review of evaluation and management. Int J Cardiol 2015;184:600-8.
- **Lau JK**, Yong AS. Use of fractional flow reserve in different anatomical subsets. Coron Artery Dis 2015;26 Suppl 1:e2-7.
- Bing R, Chow V, **Lau JK**, Thomas L, Kritharides L, Ng AC. Prevalence of Echocardiography Use in Patients Hospitalized with Confirmed Acute Pulmonary Embolism: A Real-World Observational Multicenter Study. PLoS One 2016;11:e0168554.

- **Lau JK**, Grogan J, Chan C, Yiannikas J. An unusual case of amyloidosis leading to death. Intern Med J 2016;46:236-8.
- Ng AC, Adikari D, Yuan D, **Lau JK**, Yong AS, Chow V, Kritharides L. The Prevalence and Incidence of Atrial Fibrillation in Patients with Acute Pulmonary Embolism. PLoS One 2016;11:e0150448.
- Wong CC, Ng AC, **Lau JK**, Chow V, Chen V, Ng AC, Yong AS, Sindone AP, Marwick TH, Kritharides L. High mortality in patients presenting with acute pulmonary embolism and elevated INR not on anticoagulant therapy. Thromb Haemost 2016;115:1191-9.
- Wong CC, Ng AC, **Lau JK**, Chow V, Sindone AP, Kritharides L. The prognostic impact of chest pain in 1306 patients presenting with confirmed acute pulmonary embolism. Int J Cardiol 2016;221:794-9.
- Anastasius M, **Lau JK**, Hyun K, D'Souza M, Patel A, Rankin J, Walters D, Juergens C, Aliprandi-Costa B, Yan AT, Goodman SG, Chew D, Brieger D. The underutilisation of dual antiplatelet therapy in acute coronary syndrome. Int J Cardiol 2017;240:30-36.
- **Lau JK**, Chow V, Brown A, Kritharides L, Ng ACC. Predicting in-hospital death during acute presentation with pulmonary embolism to facilitate early discharge and outpatient management. PLoS One 2017;12:e0179755.
- **Lau JK**, Roy P, Bing R, Bannon PG, Lowe HC. A Time to Act and a Time to Watch: Severe Guide-Catheter Induced Proximal Coronary Dissection With Extensive Ascending Aorta and Arch Dissection, Managed by Immediate Coronary Stenting and Watchful Waiting. J Invasive Cardiol 2017;29:E99-e100.

- Yong ASC, Javadzadegan A, Fearon WF, Moshfegh A, **Lau JK**, Nicholls S, Ng MKC, Kritharides L. The relationship between coronary artery distensibility and fractional flow reserve. PLoS One 2017;12:e0181824.
- Chow WWK, Bing R, Kanawati J, **Lau JK**, Sheriff J, D'Souza M, Brieger D. A Comparison of Image Quality Using Radial vs Femoral Approaches in Patients Undergoing Diagnostic Coronary Angiography. J Invasive Cardiol 2018;30:411-415.
- Ng ACC, **Lau JK**, Chow V, Adikari D, Brieger D, Kritharides L. Outcomes of 4838 patients requiring temporary transvenous cardiac pacing: A statewide cohort study. Int J Cardiol 2018;271:98-104.
- Wong CCY, Chow WWK, **Lau JK**, Chow V, Ng ACC, Kritharides L. Red blood cell transfusion and outcomes in acute pulmonary embolism. Respirology 2018;23:935-941.

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Summary

Background

Remote ischaemic preconditioning (RIPC) is the phenomenon where brief non-harmful ischaemia to a remote organ can confer protection to the heart against a future insult. Most commonly delivered with a sphygmomanometer on the arm, RIPC has been shown to confer protection when delivered prior to primary or elective percutaneous coronary intervention (PCI). The mechanism by which this occurs is not clearly defined.

Coronary microcirculatory function is an important determinant of patient prognosis at the time of PCI. Platelets play an important role in the pathophysiology of coronary atherosclerosis related thrombotic events, with platelet inhibition an integral component of the clinical management of patients with coronary artery disease (CAD) including those undergoing PCI. Given the important role of both the microcirculation and platelet inhibition at the time of PCI, this project was designed to investigate the effect of RIPC on these two prognostically important factors.

Methods and results

This thesis has examined 1) the effect of RIPC on coronary artery physiology and 2) the effect of RIPC on platelet activation in response to platelet agonists and the interaction between these effects and antiplatelet medications.

To study the effect of RIPC on coronary artery physiology and microcirculatory function, a randomised blinded placebo controlled trial was performed. Patients with a clinical indication for fractional flow reserve (FFR) measurement (n=30) were randomised to receive RIPC or sham treatment while on the catheterisation table. A comprehensive coronary physiology study was performed with a temperature-pressure sensor coronary guidewire before and immediately after the allocated treatment. The index of microcirculatory resistance (IMR) and coronary flow reserve (CFR) were measured as markers of microcirculatory function. RIPC was associated with a significant reduction in the calculated IMR (median (interquartile range), 22.6 (17.9-25.6) vs 17.5 (14.5-21.3), P=0.007) and a significant increase in the CFR (mean ± standard deviation, 2.6 ± 0.9 vs 3.8 ± 1.7, P=0.001). These changes were associated with a reduction in hyperaemic transit time (0.33 (0.26-0.40) vs 0.25 (0.20-0.30), P=0.01) indicating an increase in coronary blood flow. There was no effect on FFR. Sham treatment had no effect on any coronary physiology parameter. Analysis of plasma stored from blood collected before and after RIPC/sham treatment found no changes in plasma nitrite, cyclic guanosine monophosphate (cGMP) or adrenomedullin with RIPC. There was a reduction in plasma 6-keto-PGF1α (the metabolite of prostacyclin) with RIPC.

The effect of RIPC on platelet activation was studied in a separate randomised controlled trial where patients referred for coronary angiography (n=60) were randomised prior to their procedure to RIPC or sham treatment. Venous blood was collected from the contralateral arm before and immediately after their allocated treatment, prior to cardiac catheterisation. Platelets were stimulated in whole blood with adenosine diphosphate, protease activated receptor-1 agonist (SFLLRN), thrombin with and without collagen, and analysed by flow cytometry for expression of CD62P

(marker of α-granule release), CD63 (marker of dense granule and lysosome release) and conformationally active glycoprotein IIb-IIIa (GPIIb-IIIa) (PAC-1 binding). RIPC was associated with decreased platelet PAC-1 binding in response to SFLLRN (50.4% (31.3-73.2) vs 49.3% (23.0-67.7), P=0.002) and thrombin and collagen (79.5 ± 11.9% vs 68.9 ± 22.5%, P<0.001). Similar effects were seen in patients on both aspirin and a $P2Y_{12}$ receptor inhibitor. Despite their role in regulating GPIIb-IIIa activation, there was no effect of RIPC on plasma cyclic adenosine monophosphate and cGMP levels or intra-platelet phosphorylated vasodilator stimulated phosphoprotein levels.

RIPC was also assessed for its effects on the generation of procoagulant platelets (n=60), identified by the uptake of 4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid (GSAO, an intracellular cell death marker) and staining for CD62P ($GSAO^+/CD62P^+$). RIPC decreased circulating levels of procoagulant platelets in the circulation (2.0% (1.3-2.4) vs 1.3% (1.1-2.1), P=0.01), whereas sham had no effect. In the subgroup of patients on aspirin monotherapy, RIPC was associated with a reduction in procoagulant platelet formation in response to SFLLRN (11.4±5.2% vs 8.9±2.8%, P=0.04) and thrombin (12.9±5.9% vs 8.7±3.2%, P=0.04). In order to determine the mechanism behind the RIPC-mediated attenuation of procoagulant platelet formation, further patients were studied to assess for the effects of RIPC on platelet mitochondrial membrane potential using a fluorescent potential-sensitive probe (TMRE). RIPC was associated with a significant reduction in platelet mitochondrial membrane depolarisation in response to a range of thrombin (P=0.001) and thrombin and collagen (P=0.01) concentrations.

To investigate whether RIPC modifies the plasma microRNA profile, plasma stored from blood collected before and after RIPC/sham in the platelet cohort was analysed. Plasma microRNA was extracted from paired plasma samples collected from 4 patients

treated with RIPC. The plasma microRNA profile before and after RIPC was compared with a card based quantitative polymerase chain reaction (qPCR) array system and identified 6 candidate species which appeared to be increased with RIPC. A validation study was performed in the entire randomised platelet cohort (n=60) by extracting microRNA from paired plasma samples collected from patients before and after treatment with either RIPC or sham, and measuring the levels of the 6 candidate species with specific reverse transcription and qPCR reactions. This demonstrated no definite difference with RIPC over sham treatment in any of the 6 microRNA species.

Conclusion

RIPC leads to rapid improvements in coronary microcirculatory function as demonstrated by validated catheterisation based coronary physiology measurements. Additionally, RIPC attenuated platelet GPIIb-IIIa activation in response to potent platelet agonists which are present at the site of complicated atherosclerotic plaques. Attenuation of GPIIb-IIIa activation was evident in patients on contemporary antiplatelet therapy, suggesting benefit additional to these medications. RIPC also reduced the level of circulating procoagulant platelets. Reductions in procoagulant platelet activity appeared to be due to RIPC-mediated reduction in platelet mitochondrial membrane depolarisation. These novel actions of RIPC are likely to contribute to the beneficial effects of RIPC during elective and primary PCI.

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List of abbreviations

ACS – Acute coronary syndrome

ADP – Adenosine diphosphate

ALDH-2 – Aldehyde dehydrogenase-2

ATP – Adenosine triphosphate

AUC – Area under the curve

CABG – Coronary artery bypass graft surgery

CAD – Coronary artery disease

cAMP – Cyclic adenosine monophosphate

cDNA – Complementary deoxyribonucleic acid

CFR – Coronary flow reserve

cGMP – Cyclic guanosine monophosphate

Ct – Cycle threshold

DAPT – Dual antiplatelet therapy

DPBS - Dulbecco's modified phosphate buffered saline

ECG – Electrocardiogram

ELISA – Enzyme linked immunosorbent assay

ERK – Extracellular signal regulated kinases

FACS – Fluorescence-activated cell sorting

FFR – Fractional flow reserve

GPIIb-IIIa – Glycoprotein IIb-IIIa

GSAO - 4-[N-(S-glutathionylacetyl)amino]phenylarsonous acid

HBSS - Hank's balanced salt solution

HAS – Human serum albumin

HSP-70 – Heat shock protein-70

IMR – Index of microcirculatory resistance

IPC – Ischaemic preconditioning

IQR – Interquartile range

IR – Ischaemia reperfusion

IV - Intravenous

MACCE – Major adverse cardiac and cerebrovascular events

MAPK – Mitogen activated protein kinases

MI – Myocardial infarction

mRNA – Messenger ribonucleic acid

miRNA – Micro ribonucleic acid

MPTP - Mitochondrial permeability transition pore

P_a – Mean aortic pressure

P_d – Mean distal coronary pressure

P_w – Mean coronary wedge pressure

PAR-1 – Protease activated receptor-1

PCI – Percutaneous coronary intervention

PCR – Polymerase chain reaction

PKC – Protein kinase C

PLA – Platelet-leukocyte aggregates

PRP – Platelet rich plasma

PS - Phosphatidylserine

pVASP – Phosphorylated vasodilated stimulated phosphoprotein

QCA – Quantitative coronary angiography

qPCR – Quantitative polymerase chain reaction

RIPC – Remote ischaemic preconditioning

RIPerC – Remote ischaemic preconditioning

RISK - Reperfusion injury salvage kinase

RNA – Ribonucleic acid

RT – Reverse transcription

SAFE - Survival activating factor enhancement

SD – Standard deviation

STAT 5 – Signal transducer and activator of transcription 5

STEMI – ST elevation myocardial infarction

T_{mn} – Transit time

TMR – True microcirculatory resistance

TMRE - Tetramethylrhodamine ethyl ester

TRAP6 – Thrombin related activating peptide 6

VASP – Vasodilator stimulated phosphoprotein

Chapter 1: General introduction

On a global scale, of the non-communicable diseases, cardiovascular disease is the leading cause of mortality and morbidity (1,2), and coronary artery disease (CAD) is responsible for more than half of cardiovascular deaths. Guideline recommended therapy includes antiplatelet medications and lipid lowering therapy (3,4) as well as revascularisation with percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG) for selected individuals (5).

Despite optimal medical therapy and revascularisation with both PCI and CABG, patients with CAD are at ongoing risk of cardiovascular death, myocardial infarction (MI) and need for repeat revascularisation (6). Additionally, PCI and CABG are associated with myocardial injury, which includes ischaemia reperfusion (IR) injury, which is detectable as an elevation in serum troponin after the procedure (7,8). Post procedural myocardial injury is associated with a worse prognosis (9-11).

Given the significant impact that CAD has on patient quality of life and longevity, the search for strategies that provide clinical benefit additional to current treatment paradigms are active areas of investigation. While most treatment modalities focus on preventing progression of CAD and preventing or minimising myocardial ischaemia through prevention of coronary thrombus formation and revascularisation, there is currently a paucity of therapies to protect the heart from ischaemic insult. Remote ischaemic preconditioning (RIPC) has been proposed to protect the myocardium against injury, including at the time of primary PCI for ST elevation myocardial infarction (STEMI), or elective PCI for management of refractory angina. However, clinical trials of RIPC have yielded inconsistent results. Consideration of the mechanisms implicated in RIPC may help explain discrepant results and outline future studies to identify which

patient populations may benefit from this simple, low-cost, non-invasive intervention. Additionally, novel therapies which recapitulate the favourable effects of RIPC could be developed in their own right once mechanisms are elucidated.

The primary aim of this thesis is to explore mechanisms by which RIPC may contribute to reduced myocardial injury, especially in patients undergoing PCI for CAD. The main focus will be on coronary artery physiology and platelet activation, factors that significantly influence the outcome of patients undergoing PCI.

1.1 Remote ischaemic preconditioning

First described in 1993 (12), RIPC describes the phenomenon whereby brief non-harmful insults to remote tissues can protect against future IR injury in a tissue or organ of interest such as the heart. Whilst many of the early studies of RIPC were performed in animal models, the observation that RIPC can confer cardiac protection through an ischaemic stimulus to the limbs has facilitated its clinical application in humans including its use prior to PCI and CABG.

RIPC involves the delivery of repeated non-harmful ischaemia to a tissue remote from the heart. In contemporary clinical studies, RIPC is most commonly delivered with a sphygmomanometer inflated on the upper limb to 200 mmHg (or a supra-systolic pressure) for 5 min, followed by deflation for 5 min with the cycle performed three to four times (13-23). Delivery of ischaemia to upper and lower limbs may differ in relation to RIPC (24) and variations in the interval between RIPC and subsequent cardiac procedure may also influence the effectiveness of RIPC (25).

While RIPC describes delivery of intermittent limb ischaemia prior to the onset of myocardial ischaemia, remote ischaemic perconditioning (RIPerC) refers to intermittent

limb ischaemia occurring after onset of myocardial ischaemia such as is the case during efforts to condition the myocardium during acute myocardial infarction, prior to primary PCI. Remote ischaemic postconditioning refers to intermittent limb ischaemia occurring after myocardial reperfusion. Figure 1.1 demonstrates the temporal association between the types of conditioning and the onset of myocardial ischaemia.

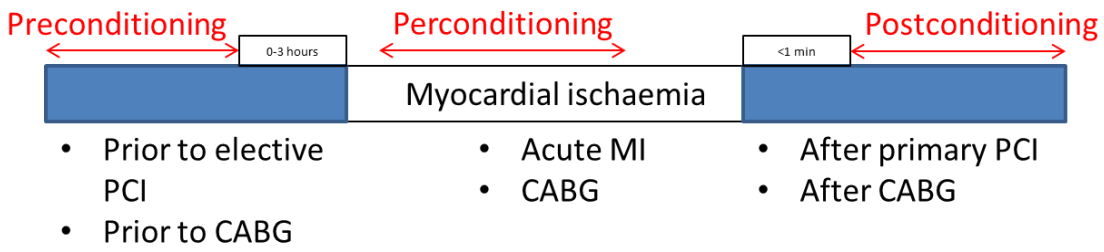


Figure 1.1 Cardiac ischaemic conditioning

Temporal relationship between preconditioning, perconditioning and postconditioning and the onset of myocardial ischaemia with examples of clinical scenarios where the conditioning stimulus may be used. CABG, coronary artery bypass grafting; MI, myocardial infarction; PCI, percutaneous coronary intervention;

Adapted from Hausenloy D, 2016 (26).

1.2 Potential mechanisms

1.2.1 Neural pathways

There are two predominant theories explaining the cardioprotective effects of RIPC. The humoral hypothesis proposes that a substance is released from the remote organ or tissue after RIPC and this is transferred to the heart in the circulation. Conversely, advocates of the less widely studied neural hypothesis suggest that RIPC protection is

transduced from the remote organ or tissue via somatosensory nerves to the spinal cord and then to the heart via autonomic nerves (27) (Figure 1.2).

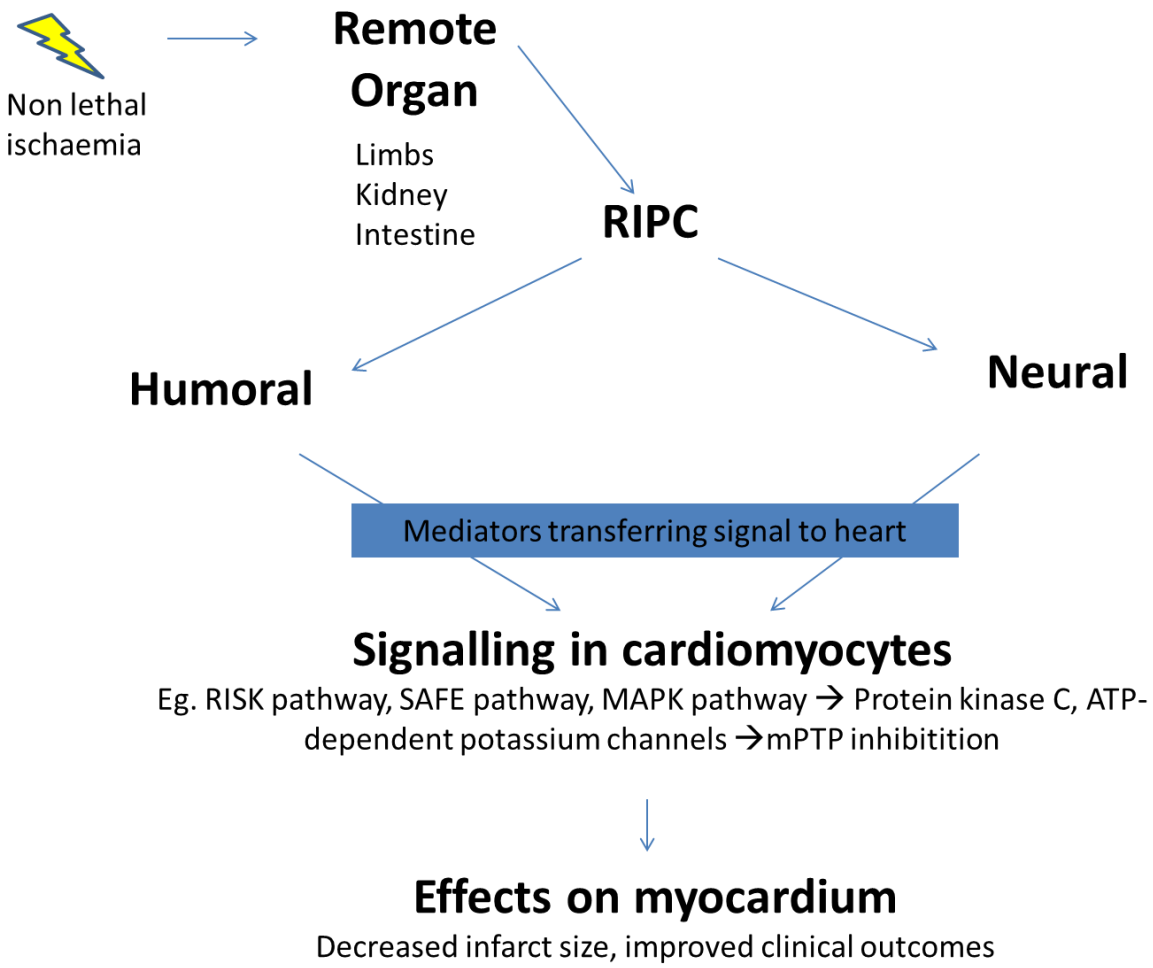


Figure 1.2 Mechanisms potentially involved in remote ischaemic preconditioning

ATP, adenosine triphosphate; MAPK, mitogen activated protein kinases; mPTP, mitochondrial permeability transition pore; RIPC; remote ischaemic preconditioning; RISK, reperfusion injury salvage kinase; SAFE, survival activating factor enhancement;

The neural RIPC hypothesis is supported by several animal studies. For example, transection of the femoral nerve prior to RIPC delivered to the hind limb of a rabbit abolished the protection RIPC conferred against cardiac IR injury (28). Further to this,

administration of the nicotinic acetyl choline ganglion blocker hexamethonium to rats attenuated RIPC-mediated reduction in infarct size after cardiac IR injury (29). It has also been suggested that opioid receptors may be involved in RIPC related neural pathways, as demonstrated by attenuation of RIPC-mediated protection with the use of specific opioid receptor antagonists (30-32).

1.2.2 Circulating mediators

There is evidence that RIPC induces a protective circulating factor that travels to the target organ in the blood. Plasma from animals subjected to RIPC may be used to transfer cardioprotection to other animals and between species suggesting a protective factor in the blood (30,31,33-35). The circulating mediator(s) of RIPC appears to be hydrophobic, resistant to freezing and thawing with a molecular weight between 15 and 30 kDa (30,36). A number of molecules have been implicated in RIPC-mediated cardioprotection. Most studies have used specific inhibitors to elucidate the pathways involved in RIPC. Most, if not all of these pathways relate to myocyte biology, but some relate to vascular tone.

The human plasma proteomic profile is significantly altered by RIPC. One study found 51 proteins found to be differentially expressed in response to RIPC, including upregulation of albumin, α1-antitrypsin, apolipoprotein A1, haptoglobin, lipoprotein B100 and transferrin (37). In the same study, antithrombin III complex, complement C1r and immunoglobulin M, amongst other proteins, were found to be down-regulated after RIPC. The changes in plasma proteins due to RIPC were apparent immediately and increased further up to 24 h after the treatment. Similarly, a study of RIPC in children undergoing cardiac surgery found that RIPC was associated with upregulation of 48 peptides corresponding to 6 proteins compared to control (38). In contrast to the

previously described study, the difference in the plasma proteome between the RIPC and control groups was most marked at 6 h after surgery and returned towards baseline at 24 h. There is animal data suggesting that metabolic disturbances such as diabetes mellitus can affect humoral signalling (39).

The results of these proteomic studies demonstrate the wide spectrum of changes induced by RIPC and help explain the difficulty in identifying a single mediator. It is likely that multiple pathways are involved to provide protection to the heart and other organs against IR injury. Some of the more extensively studied candidate circulating mediators of RIPC are outlined in Table 1.1.

Table 1.1: Potential circulating mediators of remote ischaemic preconditioning

Mediator	Species	Effect in RIPC	References
Adenosine	Rabbits	Adenosine in dialysate from rabbits treated with RIPC involved in protection of cardiomyocytes against simulated IR injury. Adenosine also reduced infarct size after cardiac IR injury. The adenosine A1 receptor appears to be involved in RIPC.	(31,34)
Aldehyde dehydrogenase-2	Humans and rabbits	RIPC associated attenuation of endothelial dysfunction after IR injury to the forearm not seen with genetic polymorphism of ALDH-2 gene or with Disulfiram (ALDH-2 inhibitor). ALDH-2 inhibitor abolished RIPC induced cardioprotection against IR injury.	(40)
Apolipoprotein A1	Rats	Upregulated by RIPC and administration mimicked RIPC cardioprotection. Human HDL administered to mice reduced myocardial infarction size.	(41)
Nitrite	Mice	Upregulated by RIPC and administration mimicked RIPC cardioprotection. Dependent on eNOS and myoglobin to reduce nitrite to NO.	(42)
Stromal cell derived factor-1α	Rats	RIPC increased levels in rat plasma with cardioprotection blocked by SDF-1α receptor antagonist and mimicked by SDF-1α administration.	(43)

Exosomes	Humans and rats	Plasma exosomes (containing CD63, CD81 and HSP-70) increased by RIPC and confer protection against cardiac IR injury in recipient rats.	(44)
MicroRNA-144	Humans and mice	RIPC increased plasma microRNA-144. An antagomir of microRNA-144 and administration of microRNA-144 abolished and mimicked RIPC induced cardioprotection respectively.	(45)
MicroRNA-24 in exosomes	Rats	Expression of microRNA-24 associated with exosomes increased with RIPC, associated with reduced infarct size and reduced cellular oxidative stress. Effects abolished by microRNA-24 antagomirs or inhibitors.	(46)

ALDH-2, Aldehyde dehydrogenase-2; eNOS, endothelial nitric oxide synthase; HSP-70, heat shock protein-70; IPC, ischaemic preconditioning; IR, ischaemia reperfusion; NO, nitric oxide; RIPC, remote ischaemic preconditioning; SDF-1α, Stromal cell derived factor-1α**;**

RIPC appears to increase the levels of factors such as adenosine, apolipoprotein A1, bradykinin, nitrite and stromal cell derived factor-1α in both human and animal studies. Pretreatment of experimental animals with these factors mimicked RIPC induced cardioprotection against IR injury (34,41,43). Additionally, the enzyme aldehyde dehydrogenase-2 (ALDH-2) is involved in RIPC mediated cardiac protection in rabbits and in attenuation of endothelial dysfunction in human volunteers (40). This raises the potential issue of variable effect of RIPC in populations where ALDH-2 polymorphisms are relatively common (47).

Exosomes are a subpopulation of cell-derived microparticles or extracellular vesicles which are 50-100 nm in size and have also been proposed to be mediators of RIPC (48). Plasma exosomes containing heat shock protein-70 (HSP-70) are increased by RIPC and can transfer RIPC-mediated cardioprotection from one animal to another (44). The addition of protective exosomes to cardiomyocytes *in vitro* leads to rapid activation of cellular kinases such as ERK 1/2 (extracellular signal regulated kinases) demonstrated by the attenuation of myocyte protection against IR injury by pharmacological inhibitors of ERK 1/2 (44).

Micro ribonucleic acid (miRNA) are short (~22 nucleotides) evolutionary conserved segments of RNA, which regulate protein translation and that have important and emerging regulatory roles in cell biology and cardiovascular medicine (49). MicroRNA, and in particular miRNA-144 has been shown to be involved in RIPC probably through down regulation of mammalian target of rapamycin in the mouse heart, promoting improved cell survival (45). RIPC, local ischaemic preconditioning (IPC) and ischaemic postconditioning appear to have different effects on miRNA. While local ischaemic preconditioning is associated with a rise in myocardial miRNA-1 and miRNA-21 in the rat heart, RIPC and ischaemic postconditioning reduced miRNA-1 in the myocardium and had no effect on miRNA-21 (50). In patients undergoing CABG, miRNA-338-3p levels in right atrial tissue samples were found to be higher in patients who received RIPC compared to control (51).

1.2.3 The role of platelets in remote ischaemic preconditioning

Platelet-derived microparticles have been proposed to be potential mediators of RIPC. Platelet microparticles isolated from rats that had received RIPC reduced the extent of cerebral infarction when transfused into recipient rats (52). In a mouse model of hepatic

IR injury, the protective effect of RIPC was abolished when mouse platelets were depleted using anti-CD41 antibodies or when platelet activation was inhibited by a platelet activating factor inhibitor (53). The results of these studies suggest that platelets have a direct role in mediating the protective effects of RIPC.

1.2.4 The effect of remote ischaemic preconditioning on platelets

RIPC has been shown to attenuate platelet activation in both human and animal studies. In a rat model of femoral artery injury, RIPC was associated with a lower rate of thrombus formation and reduced distal embolisation (54). Additionally, in 19 patients undergoing ablation for atrial fibrillation, RIPC reduced CD41 expression, CD62P and monocyte-platelet aggregate formation in response to the platelet agonist adenosine diphosphate (ADP) and the ablation procedure (55). CD62P is a marker signifying platelet α granule release and CD41 is a component of the glycoprotein IIb-IIIa complex. In another study of patients with stable CAD, RIPC attenuated the rise in ADP induced platelet-monocyte aggregates and CD41 expression occurring after an exercise stress test (56). RIPC has also been demonstrated to attenuate platelet-monocyte aggregate formation and CD41 and CD62 expression which increased in response to cardiac catheterisation (57). In another study of healthy volunteers, upper limb ischaemia reperfusion injury caused an increase in platelet-monocyte aggregate formation which was attenuated by RIPC (18).

A discussion regarding the importance of platelet inhibition in patients with CAD and the evidence to suggest that attenuation of platelet activation may be a contributor to RIPC mediated benefits can be found in chapter 4.

1.2.5 Cellular mechanisms

The cellular mechanisms underpinning IPC have been extensively studied and efforts to define the mechanisms involved in RIPC have stemmed from what is known about IPC. It appears that the cellular signalling patterns, while demonstrating some similarities, differ between RIPC and IPC.

HSP-70, which is markedly elevated in the myocardium after infarction, stimulates inflammatory cytokine release and the innate immune response. HSP-70 was downregulated by both RIPC and IPC in a rat model (50). Conversely, Bcl-2, a factor promoting cell survival, was not altered by RIPC in this study.

Hypoxia inducible factor-1 (HIF-1) is a central molecule in mediating the response to IR injury. Under conditions of hypoxia, HIF-1 and its subunit HIF-1α regulate the transcription of genes leading to protective cellular responses. The delivery of RIPC prior to cardiopulmonary bypass was associated with higher levels of HIF-1α in right atrial tissue (58). RIPC was also associated with an increase in IL-1b, IL-8 and TNF-α levels in this study, suggesting that RIPC may modulate systemic inflammation. Supporting an effect of RIPC on inflammatory cytokines, a study of patients undergoing cardiac surgery found an association between conditioning and a rise in serum interleukin-1α (59).

In a rat model of cardiac IR injury, IPC was more efficacious than RIPC in protecting against cardiac injury and they differed in their cellular effects (60). While IPC led to increased myocardial phosphorylation of protein kinase C (PKC) and other MAPK in animal models, RIPC did not (60,61). Contrary to these findings, in a rabbit model, RIPC was shown to increase phosphorylation of ERK 1/2 and activate PKC-ε within left ventricular tissues (30). PKC-ε has been suggested to be an integral component of the pathway underpinning both IPC and RIPC (62).

The reperfusion injury salvage kinase (RISK) and survival activating factor enhancement pathway (SAFE) are intracellular pro-survival pathways which have also been studied in the setting of RIPC. The SAFE pathway has been shown to be more important than the RISK pathway in cardioprotection conferred by RIPC (33). Signal transducer and activator of transcription 5 (STAT 5) phosphorylation, a component of the SAFE pathway, has been shown to be associated with RIPC-mediated reduction in myocardial injury in patients undergoing CABG (63). The STAT 5 pathway appears to be affected by the use of propofol, which may be important when RIPC is utilised in the setting of CABG (64).

The upregulation of the vasoactive factors nitrite, the major metabolite of nitric oxide, and adenosine by RIPC (31,34,42) raises the possibility of vascular effects of RIPC. In a study involving healthy volunteers and patients with stable coronary artery disease, using flow mediated dilatation of the brachial artery as a measure of endothelial function, RIPC attenuated endothelial dysfunction caused by IR injury to the upper limb (24). This protection was abolished by administration of the glibenclamide, a non-selective potassium-ATP channel inhibitor, suggesting that potassium-ATP channels were involved. The contribution of potassium-ATP channels to RIPC-mediated cardiac protection has also been suggested by animal data (65). These data are complementary to evidence of an important interaction between cGMP dependent pathways and mitochondrial BK potassium channels in cardiac protection induced by ischaemic preconditioning (66).

1.2.6 The effect of remote ischaemic preconditioning on coronary physiology

RIPC has been shown to augment coronary flow and endothelial function. The coronary endothelium is primarily responsible for regulating vascular tone and coronary flow and

endothelial dysfunction associated with coronary artery disease (67). RIPC increased peak diastolic coronary flow velocity in the LAD as assessed by echocardiography in patients attending cardiac rehabilitation (68). Similarly, in patients with heart failure and in healthy volunteers, there was an increase in coronary flow reserve (CFR) with RIPC as assessed by flow in the distal left anterior descending artery by echocardiography (69). Additionally, RIPC reduced coronary vasoconstriction in response to acetylcholine (70), a marker of endothelial dysfunction (71).

The importance of the coronary microcirculation in patients with coronary artery disease including those undergoing PCI is discussed in chapter 3.

1.3 Remote ischaemic preconditioning in percutaneous coronary intervention

PCI can cause myocardial injury, measured as increased serum troponin after the procedure (7), and this predicts future cardiovascular events and the need for revascularization (9,10,72). RIPC prior to PCI has reduced post PCI troponin levels in most studies, and there was a suggestion of improved clinical outcomes in some studies. Randomised trials investigating the effect of RIPC and RIPerC on cardiac outcomes when delivered prior to PCI are included in Table 1.2.

1.3.1 Remote ischaemic perconditioning during ST elevation myocardial infarction

The results of the randomised controlled trials of RIPerC in the setting of STEMI suggest a cardioprotective role with a reduction in infarct size and increase in myocardial salvage (73) (Table 1.2).

RIPerC increased myocardial salvage index, reduced infarct size, reduced cardiac biomarker levels and has been associated with more rapid resolution of ST elevation in

the setting of STEMI treated with primary PCI (32,74-77). RIPerC also reduced major adverse cardiac and cerebrovascular events (MACCE) and was associated with lowered all-cause mortality during long term follow up in one study (78). In the setting of thrombolysis, RIPerC has also been shown to be associated with reduced infarct-related biomarker levels in patients with STEMI (79). Intermittent limb ischaemia commenced at the time of primary PCI (remote postconditioning) has been associated with reduced levels of myocardial injury biomarkers and reduced myocardial oedema on cardiac MRI (80).

Whether the effect of RIPerC on surrogate markers of myocardial protection will translate to clinical benefits may be answered by the ongoing CONDI-2 and ERIC-PPCI studies (81). These large, randomised multicentre studies will assess the effect of RIPerC on clinical outcomes after STEMI.

1.3.2 Remote ischaemic preconditioning during elective percutaneous coronary intervention

The results of RIPC in elective PCI have been predominantly favourable, with some exceptions (Table 1.2). RIPC has been associated with reduced post PCI troponin (14,17,21) and a reduction in MACCE, predominantly due to a reduction in acute coronary syndromes, during long term follow up (13). In those studies where there was no benefit of RIPC (16,25), PCI was performed immediately after RIPC, suggesting that there is an optimal interval (1-2 h) between the end of RIPC and PCI. An optimal window of benefit was also suggested by the CRISP study (13).

One of the studies demonstrating no benefit of RIPC was a study of 95 patients undergoing elective PCI (25). Methodological differences compared to studies demonstrating a benefit of RIPC, such as sphygmomanometer inflation for only 3 min

intervals instead of 5 min and delivery of treatment immediately prior to PCI instead of 1-2 h before, are noteworthy. Other studies have demonstrated a dose-response relationship is evident with RIPC (24) and 3 min of limb ischaemia may be insufficient for cardiac preconditioning. Contrary to this, a separate study found benefit with only one cycle of arm ischaemia delivered immediately prior to elective PCI (82). In this study, a single 5 min inflation of a sphygmomanometer on the upper limb, with 1 min of reperfusion before commencement of PCI was associated with a significantly lower post PCI troponin level than control. This study is differs from other studies which suggest a dose effect and an optimal delivery time between RIPC and PCI of 1-2 h.

Systematic reviews and meta-analyses support the findings of a reduction in post PCI myocardial infarction with RIPC (83,84). However, there is difficulty in synthesising the data due to the heterogeneous nature of the individual studies and patient subgroups. Whether specific subgroups, such as patients with diabetes need separate consideration is unclear. In a study of diabetic patients, there was no benefit of RIPC demonstrated despite a seemingly adequate ‘dose’ and interval between RIPC and PCI, raising the possibility of reduced effectiveness in diabetic patients (85).

Table 1.2: Randomised studies involving remote ischaemic preconditioning in primary percutaneous coronary intervention and elective percutaneous coronary intervention

Study	Design	Primary outcome	Results
Studies in primary PCI			
Eitel 2015 (75)	n=696, STEMI; RIPerC[a] + IPostC vs IPostC alone vs control, on admission to hospital.	Myocardial salvage index (CMRI).	Higher myocardial salvage index with RIPerC + IPostC. No effect of IPostC alone.
Botker 2010(74) Sloth 2014 (78)	n=333, STEMI; RIPerC[b] vs control, during transport to hospital.	Myocardial salvage index (SPECT).	Higher myocardial salvage index, reduced MACCE at 3.8 yr.
Munk 2010 (86)	n=242, STEMI; RIPerC[b] vs control, during transport to hospital.	LVEF and infarct size (SPECT).	Increased LVEF, smaller infarct size for anterior infarcts.
White 2015 (76)	n=197, STEMI; RIPerC[b] vs control, on admission to hospital.	Infarct size (CMRI).	Smaller infarct size and lower troponin.
Prunier 2014 (77)	n=151, STEMI; RIPerC [a] vs RIPerC + IPostC vs control, on arrival to catheterisation laboratory.	CK-MB level.	Lower CK-MB in both treatment groups.
Rentoukas 2010 (32)	n=96, STEMI; RIPerC (3 x 4 min	ST segment resolution,	More frequent ST segment resolution lower troponin

	cycles, 20 mmHg above systolic pressure to arm), vs RIPerC + morphine infusion vs control, 10 min before coronary balloon inflation.	troponin level.	in both treatment groups.
<u>Positive studies in elective PCI</u>			
Miyoshi 2017 (87), Ejiri 2018 (88)	n=391; RIPC [a] vs intravenous nicorandil vs control, at least 1 h before PCI.	Periprocedural myocardial infarction.	Periprocedural myocardial infarction lower with RIPC but did not reach significance. Significantly lower in subgroup with complex PCI.
Hoole 2009 (14), Davies 2013 (13)	n=242; RIPC [a] vs control, 1h before PCI.	Troponin 24 h.	Lower post PCI troponin and less chest pain and ST segment change during balloon inflation. Reduced MACCE at 6 months and 6 yr.
Luo 2013 (17)	n=205; RIPC [a] vs control, <2 h before PCI.	Troponin 16 h and type 4a MI at 16 h.	Reduced troponin and type 4a MI.
Liu 2014 (89)	n=200; RIPC [a] vs control, 18-24 h before PCI.	Not specified.	Lower troponin and creatine kinase, less chest pain and ST segment change during PCI. Lower MACCE at 6 months.
Zhou 2017 (90)	n=184; RIPC[b] vs control, 1 h before	Not specified.	Lower troponin and creatine kinase 1 day after

	PCI.		PCI in RIPC group.
Ahmad 2013 (21)	n=149; RIPC [a] vs control, immediately before PCI.	Troponin level at 16 h.	Lower post PCI troponin.
Zografos 2014 (82)	n=96; RIPC (1 x 5 min cycle, 200 mmHg to arm) vs control, immediately prior to PCI.	Change from pre PCI troponin at 24 h.	Lower post PCI troponin.
Ghaemian 2012 (91)	n=80; RIPC (2 x 5 min cycles, above systolic blood pressure to thigh) vs control, 1 h prior to PCI.	Troponin at 12 and 24 h.	Smaller increase in troponin with PCI.
<u>Negative studies in elective PCI</u>			
Xu 2014 (85)	n=200 diabetic patients; RIPC [a] vs control, <2 h prior to PCI.	Not specified.	No effect on 16 h troponin.
Prasad 2013 (25)	n=95; RIPC (3 x 3 min cycles, 200 mmHg to arm) vs sham (10 mmHg), immediately prior to PCI.	Post PCI troponin.	No effect on troponin levels.
Iliodromitis 2006 (16)	n=41; RIPC [a] (both arms) vs control, immediately prior to PCI.	Not specified.	Increased troponin and creatinine kinase-MB with RIPC.

All treatment effects refer to effect of RIPerC or RIPC unless otherwise stated.

[a] 3 x 5 min cycles, 200 mmHg to arm.

[b] 4 x 5 min cycles, 200 mmHg to arm.

AUC, area under the curve; CK-MB, creatine kinase MB fraction; IPostC, ischaemic postconditioning; LVEF, left ventricular ejection fraction; MACCE, major adverse cardiovascular and cerebrovascular events; MI, myocardial infarction; CMRI, cardiac magnetic resonance imaging; PCI, percutaneous coronary intervention; RIPC, remote ischaemic preconditioning; RIPerC, remote ischaemic perconditioning; SPECT, single photon emission computed tomography, STEMI- ST elevation myocardial infarction.

1.4 Remote ischaemic preconditioning in coronary artery bypass grafting surgery

Despite improvements in surgical and anaesthetic techniques, IR injury is still a significant concern in patients undergoing CABG. Myocardial injury is reflected by an increase in serum troponin levels postoperatively, which is very commonly observed after CABG (8). Elevated postoperative troponin is associated with a worse prognosis (11,92). Randomized controlled trials of at least 100 patients (50 patients in RIPC and control arms) investigating RIPC prior to on-pump CABG are included in Table 1.3. As is demonstrated in Table 1.3, the results of early studies suggested that RIPC reduced myocardial injury and MACCE when delivered prior to CABG and this was supported by early meta-analyses (84,93). There also appeared to be secondary beneficial effects of RIPC in the perioperative setting such as a reduction in postoperative atrial fibrillation, acute kidney injury and reduction in length of intensive care stay (94). However several larger studies, including the two most recent multi-centre studies (ERICCA and RIPHeart) have failed to show clinical benefit or any definite reduction in postoperative cardiac biomarker levels as a result of RIPC (22,23,95). It is however important to note that in the ERICCA (23) and RIPHeart (22)

studies a significant proportion of patients underwent concomitant valve surgery which is associated with myocardial injury unrelated to IR.

Table 1.3 details the anaesthetic agents used in the clinical studies. Propofol is known to be a scavenger of free radicals (96), and has been proposed to interfere with the protective effects of RIPC (97). However, any interaction between propofol and RIPC is inconsistent, with some studies demonstrating a benefit of RIPC in patients undergoing CABG under propofol anaesthesia. Conversely, it has also been proposed that volatile anaesthetic agents, which are known preconditioning agents, may mask or attenuate the effect of RIPC (84,93,98).

Whether the duration of RIPC or anaesthetic agents used are relevant to the discrepancies in clinical outcomes after CABG is unclear. At this time, RIPC cannot be advocated for use in on pump CABG surgery. In the absence of studies in patients receiving off pump surgery no specific recommendation can be made for these patients.

Table 1.3: Randomised studies involving remote ischaemic preconditioning in coronary artery bypass grafting surgery

Study	Design	Anaesthetic regimen	Primary outcome	Results
Positive studies				
Thielmann 2013 (19)	n=329, on-pump CABG; RIPC[a] vs control.	Not standardized, propofol in 79 patients.	Postoperative troponin (AUC in first 72 h).	Reduced troponin, all-cause mortality and MAACE (4 yr). No effect in sub-group receiving propofol.
Candilio 2015 (94)	n=180, on-pump CABG ± valve surgery; RIPC (2 x 5 min cycles, 200 mmHg to arm and leg) vs control, after induction of anaesthesia and prior to surgical incision.	All patients received propofol.	Postoperative troponin (AUC in first 72 h).	Reduced troponin.
Ali 2010 (99)	n=100, on-pump CABG; RIPC[a] vs control.	Not standardized.	Postoperative CK-MB.	Reduced CK-MB.
Negative studies				
Hausenloy 2015 (23)	n=1612, on-pump CABG ± valve surgery; RIPC[b] vs sham.	90% received propofol.	Cardiovascular death, MI, coronary revascularisation and stroke at 12 months.	No difference in combined primary or individual clinical endpoints. Lower postoperative troponin in patients

				with complete data.
Meybohm 2015 (22), Meybohm 2018 (100)	n=1403, on-pump CABG ±/or valve surgery; RIPC[b] vs RIPC to dummy arm.	All received propofol.	Death, MI, stroke or acute renal failure up to 14 days postoperatively.	No difference in combined primary or individual clinical endpoints. No difference at 1 yr follow up.
Hong 2013 (95)	n=1280, on-pump (62.7%) and off-pump (37.3%) CABG ±/or valve surgery; RIPC[b] + remote ischaemic postconditioning vs control.	All received propofol.	Major clinical adverse events.	No difference.
Rahman 2010 (98)	n=162, on-pump CABG; RIPC[a] vs RIPC to dummy arm.	Most patients received enflurane and sevoflurane.	Postoperative troponin (AUC in first 48 h).	No difference.

All treatment effects refer to effect of RIPC unless otherwise stated.

[a] 3 x 5 min cycles, 200 mmHg to arm, delivered after induction of anaesthesia and prior to surgical incision.

[b] 4 x 5 min cycles, 200 mmHg to arm, delivered after induction of anaesthesia and prior to surgical incision.

AF, atrial fibrillation; AUC, area under the curve; CABG, coronary artery bypass grafting surgery; CK-MB, creatine kinase MB isoenzyme; GI, gastrointestinal; ICU, intensive care unit; MACCE, major adverse cardiovascular and cerebrovascular events; RIPC, remote ischaemic preconditioning;

1.5 Summary

RIPC remains a potential treatment modality for the reduction of myocardial injury in defined settings. It is supported by extensive mechanistic data in animals and variable data in humans. Mechanistic studies indicate that there are differences between RIPC, local ischaemic preconditioning, and postconditioning which should be considered in the design of future clinical studies. Differences in the delivery of RIPC such as the interval between the end of RIPC and the onset of myocardial IR injury may relate to the release of soluble mediators and their downstream effects on target tissues and should also be considered in future study design. While there appears an effect of the number of cycles of RIPC and the interval between delivery and PCI on the cardioprotection conferred, the optimal treatment protocol has yet to be determined. Given the results of recent high quality randomised trials, RIPC cannot be advocated for use in the setting of on-pump CABG. The results of RIPC during the setting of PCI are promising, both elective and primary, but require further investigation before incorporation into routine clinical practice, which is also suggested in the 2014 ESC/EACTS guidelines on myocardial revascularization (5). Clarification of the benefits of RIPC prior to PCI delivered to specific patient groups, particularly in patients with diabetes, and in those receiving optimal medical therapy including dual antiplatelet therapy and statins in larger multicentre studies are clearly needed.
There are multiple clinical trials ongoing or recently completed, assessing the utility of RIPC in the management of not only coronary artery disease, but also other disease processes such as vascular disease, stroke, contrast induced nephropathy and non-cardiac surgery. The results of these studies may further clarify which patient populations may benefit from this simple, low-cost, non-invasive intervention.

Given the benefits of RIPC in the setting of coronary artery stenting, the focus of the studies presented in this thesis is the investigation of the effects of RIPC on factors that contribute significantly to patient prognosis during PCI. The coronary microcirculation predicts the prognosis of patients undergoing both elective and primary PCI. Chapter 3 describes the effects of RIPC on invasively measured markers of microcirculatory function. Chapters 4 and 5 describe the effect of RIPC on platelet activation. Although previously performed studies have described an attenuation of platelet activation in response to platelet agonists, the studies presented in this thesis investigated the effect of RIPC on expression of a larger range of markers of platelet activation in response to a range of platelet agonists. Additionally, given the importance of dual antiplatelet therapy (DAPT) at the time of PCI, the interaction between antiplatelet medications and the platelet effects of RIPC were also explored. Finally, chapter 6 describes the effect of RIPC on plasma miRNA profile. As miRNA have been shown to contribute to RIPC-mediated cardiac protection, the effect of RIPC on plasma miRNA in patients with suspected CAD was explored. Given the modulation of protein transcription, miRNAs could potentially play a part in the long standing effects of RIPC seen in some studies.

Chapter 2: Methods

2.1 Remote ischaemic preconditioning

RIPC or sham treatment was delivered to patients in order to investigate for mechanisms by which conditioning may provide benefit to patients undergoing PCI. RIPC was delivered by inflation of a sphygmomanometer (Welch Allyn, Skaneateles Falls, New York, USA) on the left upper limb to 200 mmHg or 50 mmHg greater than systolic blood pressure (whichever was greater) for 5 min, followed by deflation for 5 min. This cycle was performed a total of 3 times as previously published with the total protocol lasting 30 min (14,17,89). Patients allocated to sham treatment had the sphygmomanometer inflated to 10 mmHg but otherwise the protocol was identical to the RIPC protocol. To ensure that the sphygmomanometer pressure was adequate to occlude the brachial artery, the radial pulse was examined in each patient ensuring that it was impalpable during RIPC and unaffected during sham treatment. Effective ischaemia was established in a pilot study (section 2.1.2) before commencing the randomised study.

2.1.1 Patient population

Patients who were referred for invasive coronary angiography at Concord Repatriation General Hospital, Sydney, Australia, were invited to participate in the study. All patients were suspected of having CAD based on symptoms or the results of non-invasive investigations. Exclusion criteria included the need for emergent coronary angiography, contra-indication to inflation of a sphygmomanometer on the left arm (e.g. arterio-venous fistula for haemodialysis, peripheral vascular disease involving the upper

limbs) and existing neuropathy or myopathy which may predispose to nerve or muscle injury from upper limb ischaemia.

All patients received an information sheet detailing the background, requirements and potential risks involved with participation in the study. Informed consent was obtained from each patient prior to commencement of the study protocol. Patients were recruited for inclusion into one of two study cohorts. The cardiac catheterisation group received RIPC or sham treatment during their cardiac catheterisation procedure, while on the catheterisation laboratory table. The non-invasive group received RIPC or sham treatment prior to their cardiac catheterisation procedure.

Unless otherwise stated, patients were randomised in a 1:1 fashion to RIPC or sham through a closed envelope system. Patients were blinded to treatment allocation. The operator performing the cardiac catheterisation and coronary physiology measurements in the cardiac catheterisation group and the investigators processing the blood samples in the non-invasive group were also blinded to patient treatment allocation.

Ethics approval was granted by the Sydney Local Health District Human Research Ethics Committee, Sydney, Australia (CH62/6/2014-016). This study was registered with the Australia and New Zealand Clinical Trials Registry (ACTRN12616000486426).

2.1.2 Blood lactate with remote ischaemic preconditioning

To ensure that the RIPC protocol was effectively inducing ischaemia to the treated upper limb a pilot study was performed. Consecutive patients underwent RIPC or sham treatment and had venous blood collected immediately before and after their treatment from the ipsilateral cubital fossa, into tubes containing lithium heparin (77 IU) (BD, Franklin Lakes, New Jersey, USA). Whole blood lactate was measured in these samples

were within 20 min of collection(RapidLab 1260 blood gas analyser, Siemens, Munich, Germany).

In 10 patients who received RIPC, there was a significant increase in blood lactate (1.4 ± 0.6 mmol/L before RIPC vs 1.7 ± 0.6 mmol/L after RIPC, P=0.004) while there was no effect on blood lactate in 6 patients who received sham treatment (1.4 (1.1-1.6) mmol/L before sham vs 1.5 (1.2-1.7) mmol/L after sham, P=0.50) (Figure 2.1) confirming effective ischaemia after the RIPC protocol.

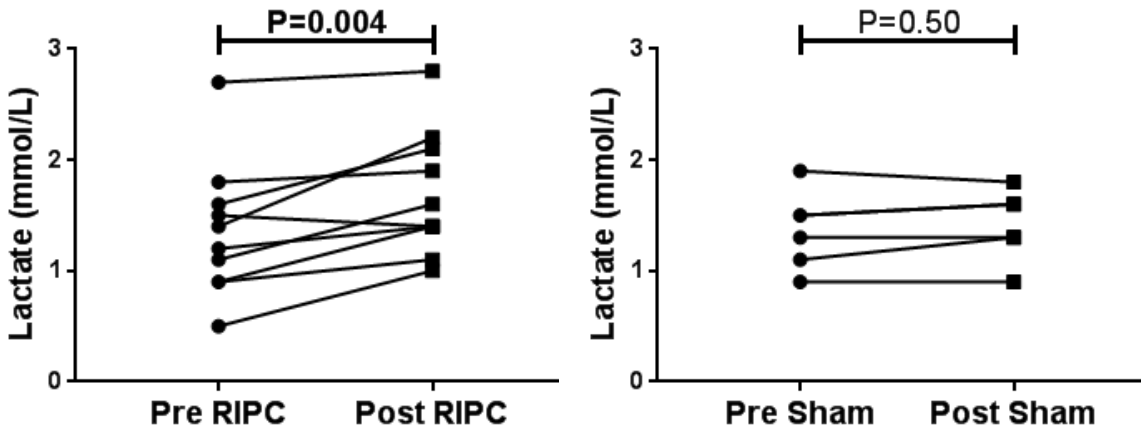


Figure 2.1 Blood lactate concentration pre and post remote ischaemic preconditioning or sham treatment

Blood lactate concentration was significantly higher post RIPC than pre RIPC. There was no effect of sham treatment on blood lactate. RIPC, n=10; sham, n=6. P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

RIPC, remote ischaemic preconditioning;

2.2 Coronary physiology measurements

Coronary physiology studies were performed in patients from the cardiac catheterisation cohort during their procedure. Established techniques were used to calculate the fractional flow reserve (FFR), CFR and index of microcirculatory resistance (IMR) (101-106). The FFR is a measurement used to help determine the haemodynamic significance of an epicardial coronary lesion and to help guide revascularisation (107,108). The IMR assesses the resistance of the microcirculation and has been shown to correlate well with true microcirculatory resistance, and to be minimally affected by haemodynamic changes in the patient (105,109). While the FFR assesses the epicardial coronary artery and the IMR assesses the coronary microcirculation, the CFR is a ratio of maximal hyperaemic flow to resting flow and thus assesses flow across both the epicardial coronary artery and the microcirculation. The CFR therefore is unable to discriminate between pathology in the epicardial coronary artery or the microcirculation (110).

2.2.1 Measurement of the fractional flow reserve

To perform cardiac catheterisation, a 6F radial or femoral arterial sheath was inserted and coronary angiography was performed by standard techniques. Unfractionated heparin was administered at a dose of 70 U/kg. Patients were included in the cardiac catheterisation cohort if after coronary angiography the decision was made to interrogate a lesion in a major epicardial coronary artery with measurement of the FFR. A 4F femoral venous sheath was inserted for administration of intravenous (IV) adenosine. In order to perform the coronary physiology study, a 6F guiding catheter without side holes was used to engage either the left main coronary artery or the right

coronary artery. A pressure-temperature sensor guidewire (Certus; St Jude Medical, St Paul, Minnesota, USA) was used in conjunction with the associated analysis console (RadiAnalyzer Xpress; St Jude Medical, St Paul, Minnesota, USA). The wire was advanced to the tip of the guiding catheter for pressure equalisation before being advanced to the distal segment of the target artery ensuring that the sensor position was at least 30 mm distal to the lesion in question. Intracoronary glyceryl trinitrate was administered (100 μg). The mean proximal/aortic pressure and mean distal pressure were recorded at rest.

Hyperaemia was induced with an IV infusion of adenosine (140 μg/kg/min) via the femoral venous sheath. The mean aortic and distal pressures during hyperaemia were also recorded and as previously described, the fractional flow reserve was calculated during hyperaemia by (101,102):

$$FFR = P_d/P_a$$

Where P_d is mean distal coronary pressure and P_a is mean aortic pressure, both measured during hyperaemia.

2.2.2 Thermodilution to calculate the coronary flow reserve

In addition to recording the pressure measurements to calculate FFR, the pressure-temperature sensor wire was also used to measure the transit time (T_{mn}) which is inversely proportional to flow. With the pressure-temperature sensor wire in the same position, the T_{mn} was measured by producing thermodilution curves in triplicate by brisk injection of 3 mL of room temperature saline down the coronary artery as previously described (103,104). An average of the three measurements was recorded as the resting transit time (T_{mnR}). This procedure was repeated during IV adenosine

infusion to record the hyperaemic transit time (T_{mnH}). The coronary flow reserve was calculated by (103,104):

$$CFR = T_{mnR} / T_{mnH}$$

Where T_{mnR} is the transit time during rest and T_{mnH} is the transit time during hyperaemia.

2.2.3 Calculation of the index of microcirculatory resistance

True microcirculatory resistance (TMR) can be calculated with a pressure-temperature sensor wire with the formula below:

$$TMR = P_a \times T_{mnH} \times [(P_d - P_w)/(P_a - P_w)]$$

Where P_a is the mean aortic pressure during hyperaemia, T_{mnH} is the transit time during hyperaemia, P_d is the mean distal coronary pressure during hyperaemia and P_w is the mean distal coronary pressure during proximal occlusive balloon inflation.

TMR is independent of disease in the epicardial coronary artery as the wedge pressure (P_w) accounts for flow in collateral arteries (111). The wedge pressure requires coronary balloon inflation, which is not convenient to measure if it is uncertain whether the patient is undergoing PCI. Additionally, balloon inflation may cause local ischaemic preconditioning confounding studies of RIPC.

The IMR correlates well with true microcirculatory resistance but overestimates the degree of resistance in the microcirculation when there is haemodynamically significant disease in the epicardial coronary artery due to increasing collateral flow (105,111). The IMR was calculated by two methods. In patients where there was no haemodynamically significant epicardial coronary artery disease (FFR >0.80), the apparent IMR (IMR_{app}) was calculated by (105):

$IMR_{app} = P_d \times T_{mnH}$

Where P_d is the mean distal coronary pressure during hyperaemia and T_{mnH} is the transit time during hyperaemia.

In all patients, regardless of the FFR, the calculated IMR (IMR_{calc}) was calculated by the formula derived by Yong et al. (106) negating the need to measure P_w:

$IMR_{calc} = P_a \times T_{mnH} \times ((1.34 \times P_d/P_a) - 0.32)$

Where P_a is the mean aortic pressure during hyperaemia, T_{mnH} is the transit time during hyperaemia and P_d is the mean distal coronary pressure during hyperaemia.

The TMR was not calculated during this study as not all patients underwent coronary balloon inflation for PCI. Coronary balloon inflation solely to measure P_w could not be justified due to the associated coronary endothelial injury, increasing the risk procedure related complications.

2.3 Quantitative coronary angiography

Coronary angiography was performed on each epicardial coronary artery in at least two orthogonal views at a frame rate of 15/s (Artis, Siemens, Munich, Germany). Two dimensional quantitative coronary angiography (QCA) was performed after the procedure with commercially available software (Artis, Siemens) to determine the diameter stenosis, lesion length and reference vessel diameter, as previously described (112). Pixel size calibration was performed using the contrast filled, non-tapered part of the guiding catheter. Measurements were performed during end diastole based on the electrocardiogram (ECG) in views which demonstrated the lesion well with the least

amount of foreshortening. The automatically detected lumen edge was adjusted if necessary. Patients with a diameter stenosis >50% in at least one epicardial coronary artery were deemed to have significant CAD. Additionally, patients with prior PCI or CABG were also deemed to have significant CAD.

2.3.1 Three dimensional quantitative coronary angiography

Three dimensional QCA was performed post-procedure with 3D reconstruction software on a Leonardo workstation (IC3D, Siemens) as previously described (112). Pixel size calibration was performed using the contrast filled, non-tapered part of the guiding catheter. Two orthogonal angiographic views of the coronary artery were used for three dimensional QCA reconstructions in both the end diastolic and end systolic frames based on the ECG. The lesion, proximal arterial segment and distal arterial segment were manually identified in each angiographic projection and a three dimensional reconstruction of the arterial segment of interest was produced by the software. The minimum luminal area and diameter were recorded based on the three dimensional reconstruction allowing compliance and distensibility to be calculated at the site of the lesion as follows (113-115):

$$\text{Compliance} = (A_S - A_D)/A_D$$

$$\text{Distensibility} = 1000 \times [(A_S - A_D)/A_D]/\Delta P$$

Where A_S is the minimum lumen area at end-systole, A_D is the minimum lumen area at end-diastole and ΔP is the difference between systolic and diastolic intracoronary pressure.

2.4 Blood sampling

Blood was collected from study participants via a 20G cannula inserted into a right cubital fossa vein (contralateral to arm subjected to RIPC or sham treatment) as previously described (116). The first 3 mL of blood was discarded to avoid collection of activated platelets. Blood was drawn slowly through the cannula, without a tourniquet or haemostatic valve, at a rate of 0.5 mL/s into a syringe and gently transferred into tubes containing 3.2% (v/v) buffered sodium citrate (BD, Franklin Lakes, New Jersey, USA), 15 μg/mL hirudin (Roche, Basel, Switzerland) and K_2EDTA (BD, Franklin Lakes, New Jersey, USA). Blood collection was performed before and after RIPC or sham treatment and transported carefully at room temperature for processing within 20 min of collection.

2.5 Storage of patient plasma

Patient blood collected before and after RIPC/sham into tubes containing sodium citrate and K_2EDTA were centrifuged for plasma storage. Blood was centrifuged at 2500 g for 15 min at room temperature with the resultant plasma from the K_2EDTA containing blood tubes aliquoted for storage. The plasma from the sodium citrate containing tubes was centrifuged again at 2500 g for 15 min at room temperature and aliquoted for storage. Plasma was stored at -80 °C for later analysis. Unless otherwise specified, analysis of stored plasma was performed on plasma aliquots which had not previously been thawed.

2.6 Platelet activation

Platelet activation plays an important role in the pathophysiology of complications of CAD. This importance is reflected by the emphasis placed on antiplatelet therapy in CAD clinical management guidelines (3,4). Platelets contain intracellular granules, the contents of which are released by fusion with the surface membrane upon activation, exposing antigens on the platelet surface which can be detected by flow cytometry (117,118). Glycoprotein IIb-IIIa (GPIIb-IIIa) is a platelet surface receptor which upon activation, changes conformation exposing a fibrinogen binding site, allowing cross-linking between platelets and aggregation to occur (118). Conformationally activate GPIIb-IIIa was detected on the platelet surface with the monoclonal antibody PAC-1 utilising flow cytometry techniques (117,119).

Upon activation, a subpopulation known as procoagulant platelets develops, which have an emerging role in cardiovascular disease and pathological thrombosis (120-122). Platelet uptake of a trivalent arsenical 4-[N-(S-glutathionylacetyl)amino]phenylarsonous acid (GSAO), cell death marker, can allow identification of these platelets. Platelets which take up GSAO and express P-selectin ($GSAO^+/CD62P^+$) were defined as procoagulant platelets by flow cytometry (120) and have been shown to be increased in patients with CAD (123). Platelets may also form aggregates with leukocytes upon activation and these aggregates may also have important roles in thrombosis (124).

2.6.1 Surface markers of platelet activation

Platelet CD62P expression (signifying α-granule release), CD63 expression (signifying dense granule and lysosome release) and PAC-1 binding (signifying GPIIb-IIIa active

conformational change) were quantified as markers of platelet activation (119,125). Quantification of these markers was performed using whole blood by flow cytometry as previously described (116,126).

Citrated blood was added to reaction mixes of Dulbecco's modified phosphate buffered saline (DPBS, Invitrogen, Carlsbad, California, USA) with and without agonists ADP (5 μM, final concentration) (Helena Laboratories, Mt Waverley, Victoria, Australia), protease-activated receptor-1 (PAR-1) agonist (SFLLRN, 20 μM) (AUSPEP, Tullamarine, Victoria, Australia), thrombin (2 U/mL) (Sigma-Aldrich, St Louis, Missouri, USA) ± collagen (10 μg/mL) (Takeda, Osaka, Osaka Prefecture, Japan). H-Gly-Pro-Arg-Pro-OH (173 μg/mL) (Calbiochem, Merck Group, Frankfurter, Darmstadt, Germany) was added to thrombin containing reaction mixes to prevent platelet aggregation. Reaction mixes (50 μL) were added to fluorescence-activated cell sorting (FACS) tubes (Falcon, Tewksbury, Massachusetts, USA) containing isotype control IgG (MOPC-21) or IgM (G155-228) and antibodies to CD61 (VI-PL2), CD62P (AK-4), CD63 (H5C6) or conformationally active GPIIb-IIIa (PAC-1). These were incubated at room temperature for 20 min then fixed with 1 mL of 0.16% (v/v) paraformaldehyde (ProSciTech, Kirwan, Queensland, Australia) saline solution before immediate analysis by flow cytometry. All antibodies were sourced from BD Bioscience (Franklin Lakes, New Jersey, USA).

Flow cytometry was performed on a FACSCalibur (BD, Franklin Lakes, New Jersey, USA) with 10,000 platelet-gated events acquired for each analysis. Platelet events were selected based on forward and side scatter. The isotype control was used to determine positive events on the basis of a 0.5% background expression (Figure 2.2). Levels of CD62P and CD63 expression and PAC-1 binding were then quantified in a similar

manner as previously published with results expressed as a percentage of total platelets (116,126). Data were analysed with FlowJo (FlowJo, LLC, Ashland, Oregon, USA).

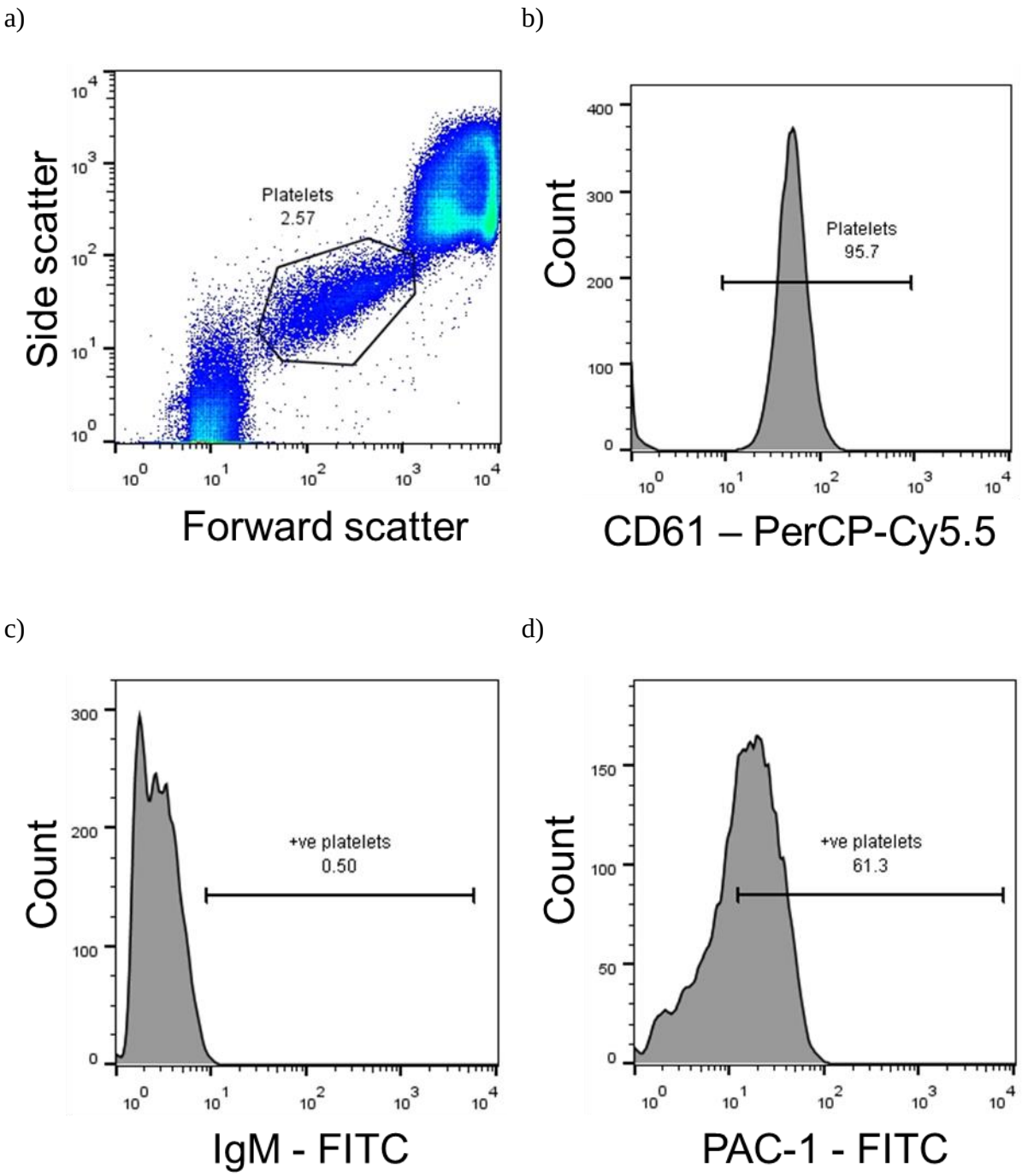


Figure 2.2 Selection of platelets and determination of positive events by flow cytometry

Representative example of analysis of flow cytometry data with FlowJo software. Platelets were selected by their light scatter characteristics (forward and side scatter) (a). The platelet population was confirmed to be CD61 positive (b). PAC-1 positive events were determined based on 0.5% background expression using the isotype control (c). The PAC-1 positive events were calculated as a proportion of the total platelet events (d).

2.6.2 The stability of platelet activation markers over time

Platelet activation experiments as described in 2.6.1 were performed on pre and post RIPC/sham blood samples approximately 60 min apart. After delivery of the pre RIPC/sham samples, approximately 60 min was required to walk back to the patient, deliver RIPC/sham and transport post samples from the cardiac catheterisation laboratory to the platelet laboratory.

To determine the effect of delay in analysing fixed platelet reaction mixtures by flow cytometry, venous blood was collected from the cubital fossa of healthy volunteers into citrate tubes, without a tourniquet. Platelet activation experiments were performed as described in 2.6.1 with some alterations. Blood from citrate tubes was added to reaction mixes of DPBS with and without agonists ADP (2.5 μM and 5 μM) or collagen (5 μg/mL). Reaction mixes (50 μL) were added to FACS tubes containing isotype control IgG or IgM, or antibodies to CD61, CD62P, CD63 or PAC-1. These were incubated at room temperature for 20 min then fixed with 1 mL of 0.16% paraformaldehyde saline solution. The samples were analysed by flow cytometry with a FACSCalibur, as described in section 2.6.1, immediately after fixation and then at 30 min, 60 min, 90 min and 120 min after fixation. The samples were stored at room temperature, in the dark, between flow cytometry analyses.

Variations in the measurements of platelet activation caused by delay in analysis after addition of the platelet fixative was observed (Figure 2.3). Therefore platelet samples were analysed immediately after fixation during the randomised study.

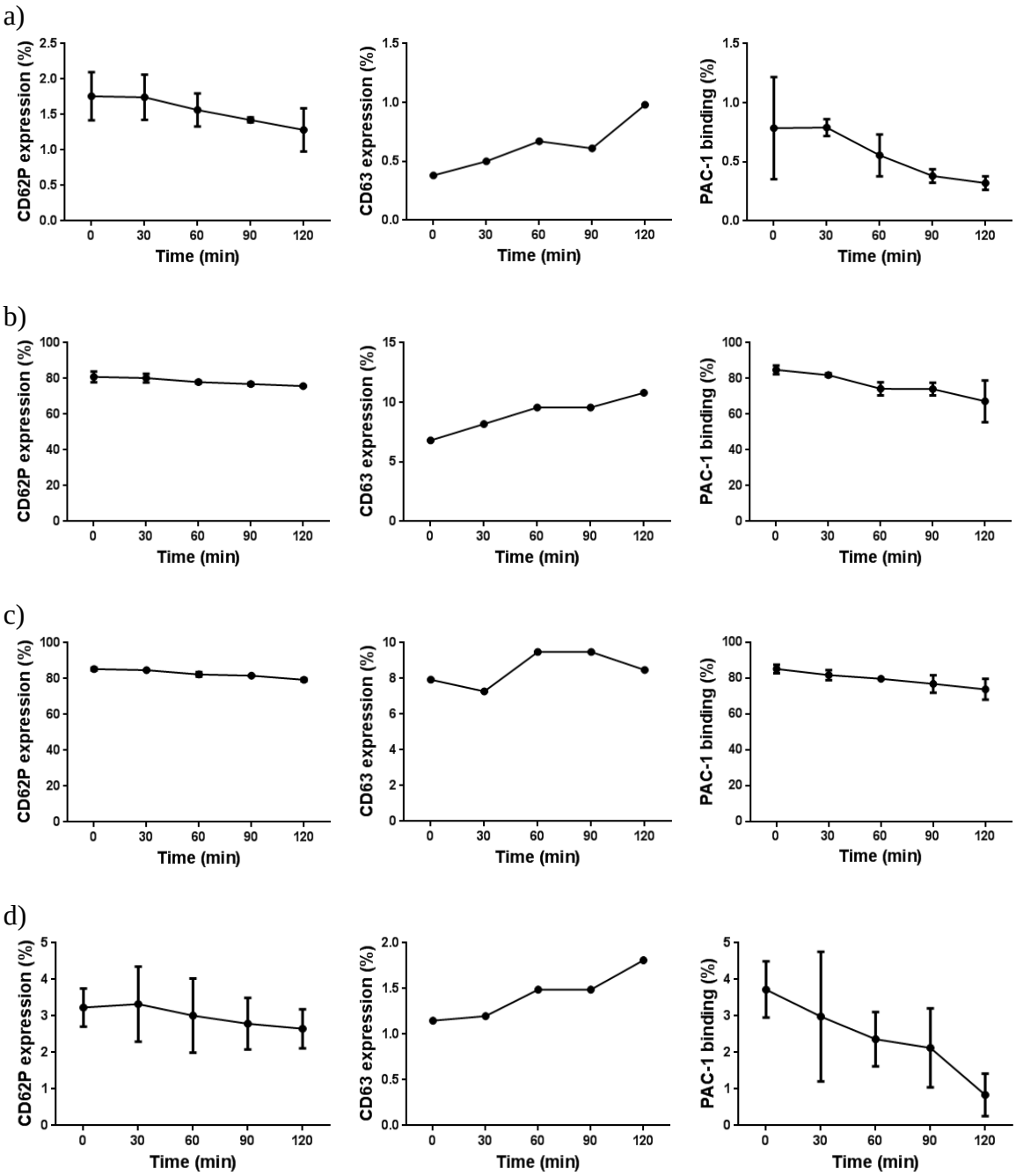


Figure 2.3 Stability of platelet activation markers on flow cytometry analysis with delay after fixation

Healthy volunteer platelet CD62P expression, CD63 expression and PAC-1 binding under basal/ unstimulated conditions (a), 2.5 μM ADP (b), 5 μM ADP (c) and 5 μg/mL collagen (d). Time intervals on x-axis denote time after platelet reaction mixture fixation with 0.16% paraformaldehyde saline solution. CD62P and PAC-1, n=2; CD63, n=1. Symbols and bars represent mean ± SD.

ADP, adenosine diphosphate;

2.6.3 The effect of fixative concentration on platelet activation marker measurement

Given the apparent lack of efficacy of 0.16% paraformaldehyde saline solution in stabilising platelet activation marker expression as demonstrated by Figure 2.3, a comparison with a 1% (v/v) paraformaldehyde saline was performed. Venous blood was collected from the cubital fossa of heathy volunteers into citrate tubes, without a tourniquet. Whole blood was added to reaction mixes of DPBS with and without agonists ADP (0.1 μM and 5 μM). Reaction mixes (50 μL) were added to FACS tubes containing isotype control IgG or IgM, or antibodies to CD61, CD62P, CD63 or PAC-1 in duplicate. These were incubated at room temperature for 20 min then fixed with either 1 mL of 0.16% paraformaldehyde saline solution or 1 mL of 1% paraformaldehyde saline solution. The samples were analysed by flow cytometry with a FACSCalibur, as described in section 2.6.1, immediately after fixation, 30 min, 60 min, 90 min and 120 min after fixation. The samples were stored at room temperature, in the dark, between flow cytometry analyses.

The effect of time delay from fixation to analysis by flow cytometry was not corrected by the higher concentration of paraformaldehyde saline and thus 0.16% paraformaldehyde saline was used to fix the platelet samples (Figure 2.4). The platelets were analysed by flow cytometry immediately after fixation as described in 2.6.1, and always within 30 minutes of activation.

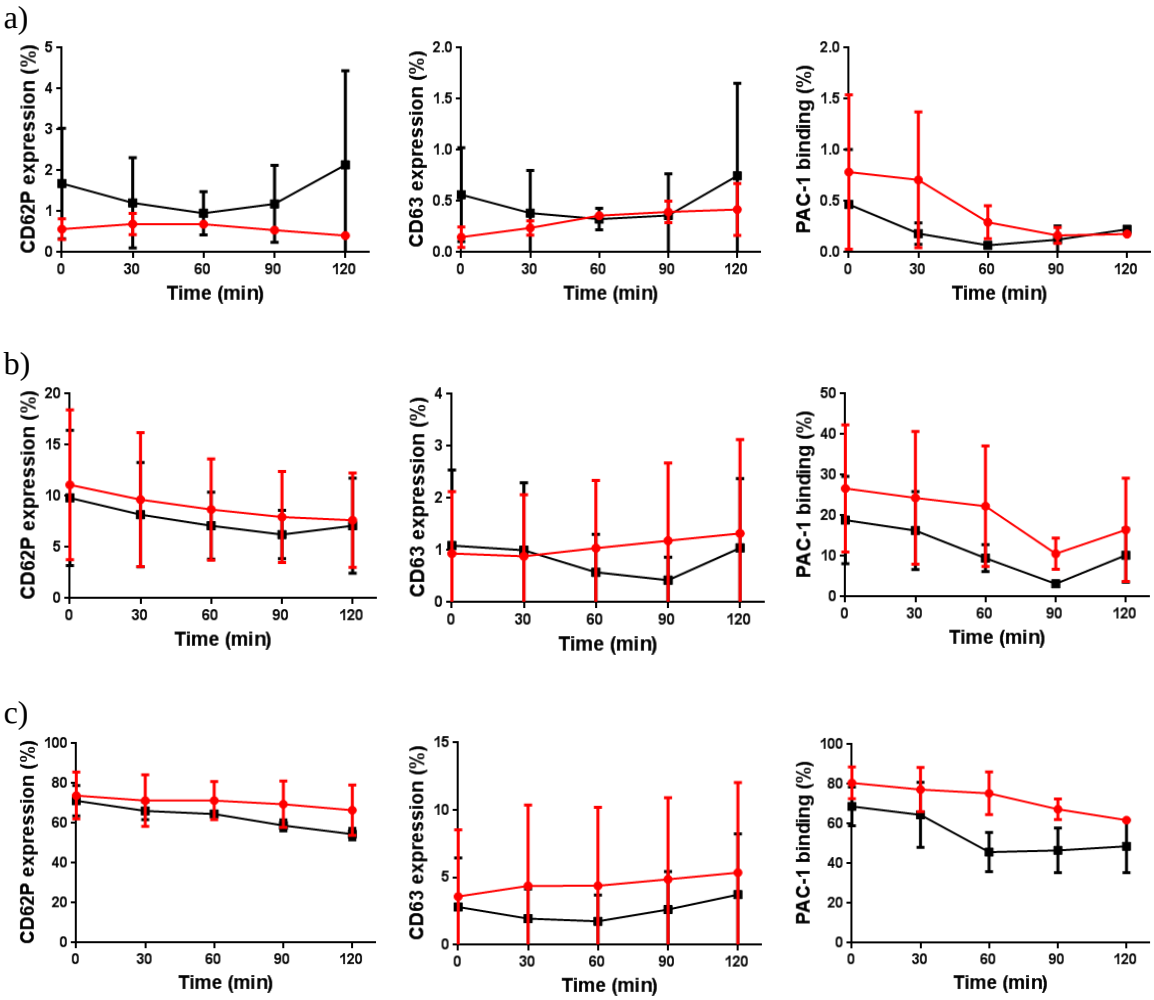


Figure 2.4 Stability of platelet activation markers on flow cytometry analysis with delay after fixation and two concentrations of paraformaldehyde saline

Healthy volunteer platelet CD62P expression, CD63 expression and PAC-1 binding under basal/ unstimulated conditions (a), 0.1 µM ADP (b), 5 µM ADP (c). Time intervals on x-axis denote time after platelet reaction mixture fixation with 0.16% or 1% paraformaldehyde saline solution. n=2. Symbols and bars represent mean ± SD. Red and black symbols represent data from samples fixed with 0.16% and 1% paraformaldehyde saline respectively.

ADP, adenosine diphosphate;

2.6.4 Platelet aggregometry

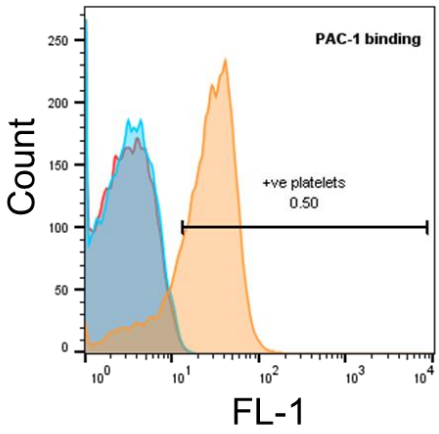
Impedance aggregometry was performed in whole blood collected before and after RIPC/sham into hirudin tubes. Aggregometry was performed with a Multiplate^{TM} analyser (Roche) as per the manufacturer's instructions. ADP (6.67 μM) and arachidonic acid (1 mM) (Helena Laboratories, Mt Waverley, Victoria, Australia) were used as agonists. The Multiplate analyser reaction cuvettes have two pairs of electrodes which act as an internal quality control mechanism and allow the measurements to be performed in duplicate.

2.6.5 Platelet-leukocyte aggregates

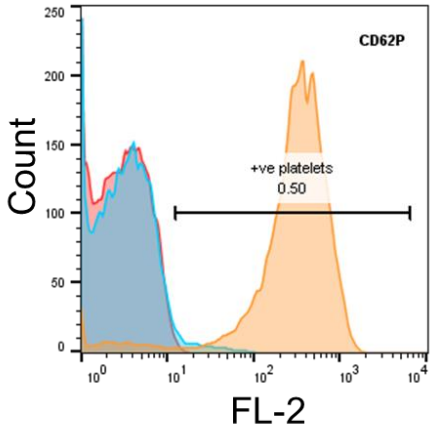
The reaction mixtures of citrated whole blood with DPBS without agonists and with 5 μM ADP, 20 μM SFLLRN, 2 U/mL Thrombin, with H-Gly-Pro-Arg-Pro-OH (5 mg/mL) added to the thrombin reaction mix, were used to assess platelet-leukocyte aggregates (PLA). Reaction mixes (200 μL) were added to FACS tubes containing isotype control IgG (MOPC-21) and antibodies to CD45 (2D1) or CD42b (HIP1). All antibodies were sourced from BD Bioscience (Franklin Lakes, New Jersey, USA). The samples were then left to incubate for 20 min at room temperature before being fixed with a combination of paraformaldehyde, Hank's buffered salt solution and water. After a further 10 min, water (840 μL) was added to restabilise the osmotic balance and lyse the erythrocytes. The samples were analysed by flow cytometry (FACSCalibur) with 1000 monocyte gated events, based on light scatter characteristics and CD45 expression, acquired for each analysis. The isotype control was used to determine positive events based on 0.5% background expression. The proportion of monocyte and granulocyte events, based on light scatter characteristics and the leukocyte marker CD45, which expressed the platelet marker CD42b were quantified with FlowJo. Figure

2.5 shows flow cytometry histograms demonstrating increased PAC-1 binding, CD62P expression, CD63 expression and PLA formation after stimulation with platelet agonists.

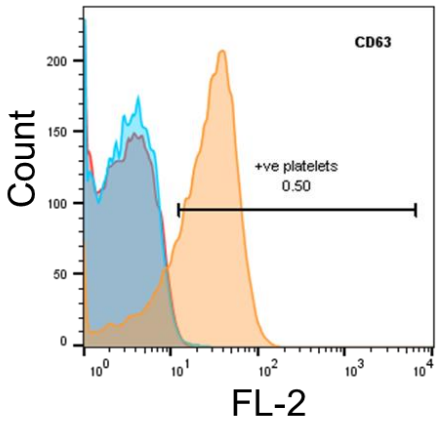
a)



b)



c)



d)

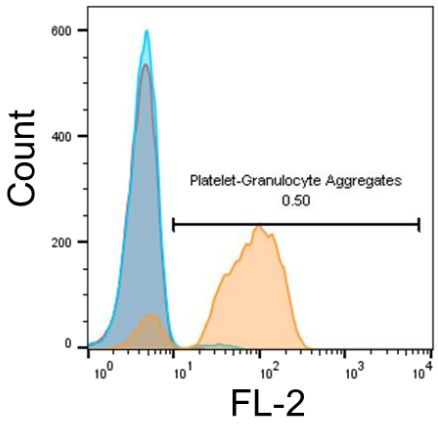


Figure 2.5 Flow cytometry histograms demonstrating platelet activation by agonist stimulation

Representative examples of platelet activation as demonstrated by flow cytometry in a patient with coronary artery disease demonstrating increased PAC-1 binding (a), CD62P expression (b), CD63 expression (c) and platelet-granulocyte aggregate formation (d) in response to thrombin and collagen (a,b and c) and SFLLRN (d). In each histogram platelet binding of the isotype control (red) and binding of PAC-1(a), CD62P antibody (b), CD63 antibody (c) or CD42b antibody (d) under unstimulated (blue) or stimulated conditions (orange) is displayed. Increased positive events in stimulated sample demonstrated by shift of curve to the right indicating increased numbers of events with greater fluorescence intensity relative to the isotype control.

SFLLRN, PAR-1 agonist;

2.6.6 Procoagulant platelets

Procoagulant platelets were quantified in whole blood with incubation and staining steps performed in a sterile 96-well polypropylene plate. Citrated whole blood was diluted with Hank's balanced salt solution (HBSS, pH adjusted to 7.35, Life Technologies, Carlsbad, California, USA), Gly-Pro-Arg-Pro amide (2.5 mM) (Sigma-Aldrich, St Louis, Missouri, USA) and calcium (2.5 mM), with and without agonists (as in section 2.6.1). Reactions were stopped after 10 min of incubation at room temperature by a 20- fold dilution with HBSS. Aliquots of agonist or non-stimulated blood were labelled for 15 min with antibodies to CD62P (Psel.KO2.3) (eBioscience, Waltham, Massachusetts, USA), CD41a (HIP8) (BD Biosciences, Franklin Lakes, New Jersey, USA), CD45 (HI30) (StemCell Technologies, Vancouver, British Colombia, Canada) and with GSAO conjugated to AlexaFluor 647. Samples were then fixed with two volumes of PAMFix (Platelet Solutions Ltd, Nottingham, United Kingdom). Samples were then transferred to FACS tubes and washed once with 10 volumes of HBSS with 0.35% (v/v) human serum albumin (HSA) (CSL, Melbourne, Victoria, Australia). The cells were then pelleted (1,500 g for 8 min) and then re-suspended in 1 mL of HBSS/HSA before being stored at room temperature in the dark for 1 h prior to analysis. Flow cytometry was performed on a BD FACS Canto II or BD Fortessa (BD, Franklin Lakes, New Jersey, USA) with 10,000 platelet events acquired for each analysis. Procoagulant platelets were defined as being CD62P and GSAO positive (CD62P+/GSAO+) and represented as a percentage of total platelet events. Data were analysed with FlowJo.

2.7 MicroRNA extraction from plasma

In chapter 6, microRNA was extracted from plasma to assess for the effects of RIPC. Methods relevant to this work are described below.

2.7.1 Determination of degree of haemolysis in plasma

Specific miRNA species are abundant in erythrocytes and hence, haemolysis during plasma preparation can have a significant impact on the miRNA profile (127,128). In particular, the levels of miRNA-16 and miRNA-451 are elevated in plasma samples with significant degrees of haemolysis. In order to assess for the degree of haemolysis, once plasma samples were thawed, 100 μL was pipetted into a 96-well plate and assessed by spectrophotometry on an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, Massachusetts, USA). The optical density at 414 nm, which is the absorbance peak of free haemoglobin, was used to determine the degree of haemolysis with $A_{414}<0.2$ interpreted as an insignificant amount of haemolysis, as described by Kirschner et al (127).

A comparison of stored citrated plasma which was centrifuged twice and stored EDTA plasma which was centrifuged once, from a patient with coronary artery disease, demonstrated a lower optical density at 414 nm indicating that there was less haemolysis in double-centrifuged plasma samples (Figure 2.6). Therefore twice-centrifuged citrated plasma was used for isolation of miRNA from randomised patient samples.

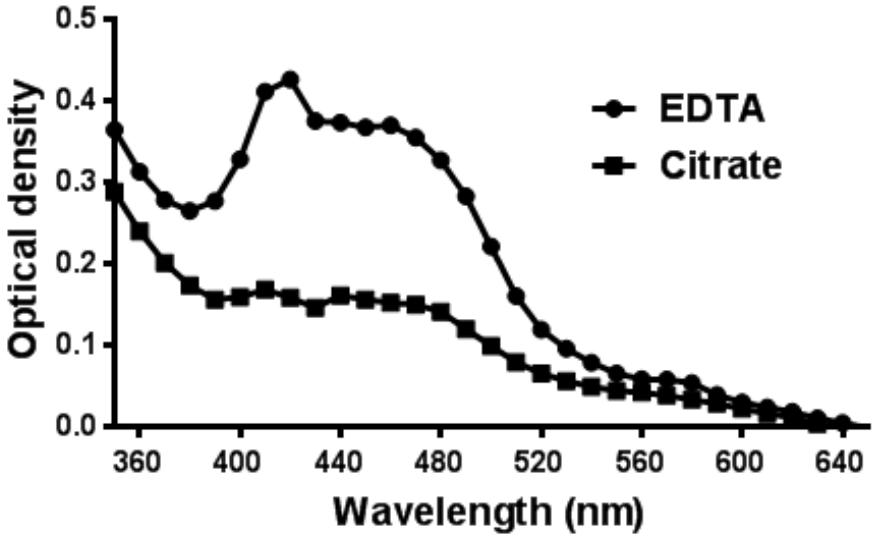


Figure 2.6 Comparison of the degree of haemolysis in single-centrifuged and double-centrifuged plasma from the same patient

Plasma in EDTA (centrifuged once) and in citrate (centrifuged twice) from the same patient was stored at -80 °C. After thawing absorption spectra between 350 nm and 640 nm were determined. The OD at 414nm was used to determine the degree of haemolysis.

2.7.2 Comparison of microRNA extraction kits and method optimisation

In order to determine which commercially available miRNA extraction kit was most suitable to isolate miRNA from patient plasma, several extraction kits were compared (Table 2.1). All kits extract miRNA from plasma based on differential binding to silica or glass-fibre columns and elution of miRNA from the column using water or an elution buffer. Kits differed in amount of plasma used for extraction and elution volumes. All experiments were carried out with RNase-free equipment in areas of the laboratory dedicated to RNA-based work.

2.7.2.1 Comparison of miRNeasy, mirVana and Isolate II kits

The miRNeasy serum/plasma kit (Qiagen, Hilden, Germany), mirVana PARIS miRNA isolation kit (Thermofisher, Waltham, Massachusetts, USA) and Isolate II miRNA kit (Bioline, Taunton, Massachusetts, USA) were used to extract miRNA from plasma prepared from a healthy volunteer as described in section 2.6, as per the manufacturers guidelines. All extractions were performed in duplicate. The plasma was stored at -80 °C in RNase free tubes and was not thawed until analysis.

The miRNeasy kit involves phenol/guanidine-based lysis of samples followed by silica membrane based miRNA extraction. The mirVana kit involves an acid-phenol chloroform solution followed by glass-fibre filter based extraction with both miRNA and 'large' RNA extracted separately on different columns. Conversely, the Isolate II kit uses a high affinity RNA resin to extract RNA from the sample without phenol or chloroform. This kit also allows extraction of 'large' RNA from the sample followed by miRNA extraction using separate columns. A phenol-free method was also assessed as concerns have been raised about phenol contamination being responsible for variable yields and quality of the various different extraction methods (129). Differences in starting plasma volume and final elution volume are listed in Table 2.1. The isolated small RNA species were analysed and quantified using a Bioanalyzer (Agilent, Santa Clara, California, USA), with 1 μL of sample loaded onto a small RNA chip, as per the manufacturer's instructions.

Table 2.1 Comparison of the input plasma volume and final elution volumes of the miRNeasy, mirVana and Isolate II kits

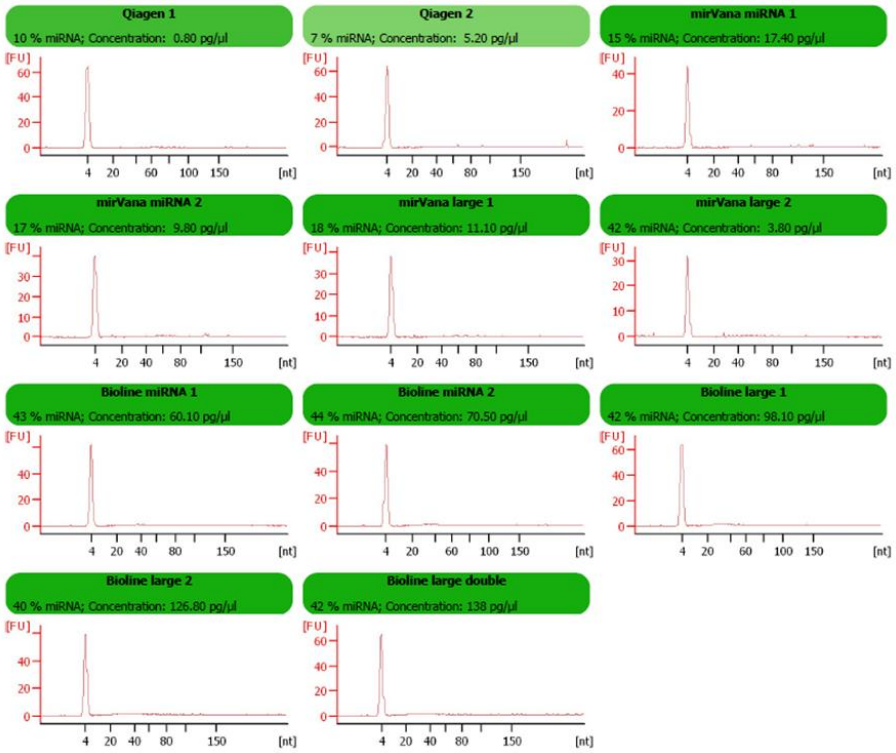
Kit	Plasma volume (µL)	Final elution volume (µL)
miRNeasy	200	14
mirVana*	450	100
Isolate II	100	20

* Up to 625 µL permitted.

miRNA, micro ribonucleic acid;

Quantification of miRNA concentration by Bioanalyzer analysis indicated large differences between duplicate samples (Figure 2.7 and Table 2.2). It also suggested the greatest concentration of miRNA in Isolate II samples despite requiring the smallest input plasma volume (Figure 2.7 and Table 2.2). Interestingly, the Isolate II large RNA samples also seemed to have relatively large amounts of small miRNA. However, the concentrations obtained in these isolations were significantly lower than described in the literature. The yield of miRNA from frozen human plasma has been reported to be greater than 105 ng/100 µL input plasma (130). Additionally, there were no discernible peaks in the electropherogram from any of the samples, suggesting very low concentrations of miRNA, raising concerns regarding the accuracy of the miRNA quantification. The manufacturer recommended range for small RNA detection in samples analysed using a Bioanalyzer with a small RNA chip was 1-20 ng/µL, significantly higher than most of the concentrations that were achieved with any of the three kits trialled. This suggested that these concentrations most likely were not an accurate reflection of the true quantity of miRNA in the samples.

a)



b)

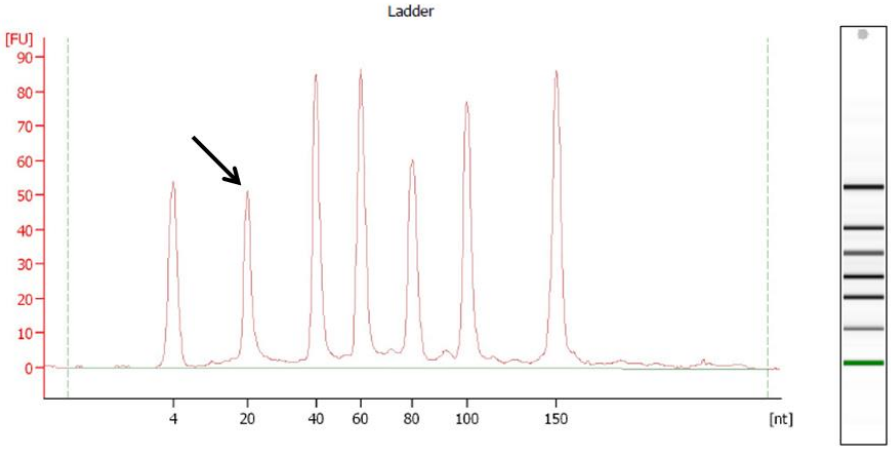


Figure 2.7 Comparison of miRNeasy, mirVana and Isolate II kits with Bioanalyzer

Bioanalyzer software output after microfluidics electrophoresis of miRNA samples run with a small RNA chip to determine miRNA concentration (a). As per the manufacturer's instructions, the gel-dye mix, small RNA conditioning solution, small RNA marker, ladder and 1 µL of each miRNA sample was loaded into the appropriate wells of the small RNA chip before analysis. Electropherogram of standard/ladder with arrow indicating expected location of miRNA peak (b).

miRNA, micro ribonucleic acid; RNA, ribonucleic acid;

Table 2.2 Comparison of microRNA concentrations of extractions with miRNeasy, mirVana and Isolate II kits

Kit/sample	miRNA concentration (pg/µL)	Total miRNA per input plasma volume (pg/100 µL)*
miRNeasy-1	0.8	5.6
miRNeasy-2	5.2	36.4
mirVana miRNA-1	17.4	386.7
mirVana miRNA-2	9.8	217.8
mirVana large-1	11.1	246.7
mirVana large-2	3.8	84.4
Isolate II miRNA-1	60.1	1202.0
Isolate II miRNA-2	70.5	1410.0
Isolate II large-1	98.1	1962.0
Isolate II large-2	126.8	2536.0

Concentrations based on Bioanalyzer analysis with a small RNA chip. Plasma from the same heathy subject was isolated in duplicate using the indicated extraction kits ("-1" and "-2" respectively). For mirVana and Isolate II kits the quantity for both the 'large' and miRNA isolations are shown.

* Calculated by multiplying concentration by elution volume and dividing by input plasma volume (x 100 µL).

miRNA, micro ribonucleic acid;

Given the low miRNA concentrations, potential methods to increase the miRNA yield were explored, with the Isolate II kit used for extraction as this kit appeared to provide the greatest and most consistent miRNA yield without the use of phenol containing reagents.

2.7.2.2 Assessment of microRNA from extracellular vesicle-enriched plasma

Extracellular vesicles are known to be carriers of miRNA in the circulation (48). In an attempt to increase the yield of miRNA or determine whether extracellular vesicles were a better source of miRNA to investigate, isolating miRNA from the microparticle fraction of plasma was compared to miRNA isolated from whole plasma. Frozen plasma (500 μL), which had not been previously thawed, from a patient with significant CAD, was thawed and centrifuged at 164,000 g for 60 min (Beckman Coulter TLA-110 rotor) to concentrate extracellular vesicles (131). The bottom fraction (100 μL) containing the extracellular vesicles, and whole plasma from the same patient, were processed with the Isolate II kit, in duplicate, to extract miRNA. The resultant miRNA was stored at -80 °C until analysed with a Bioanalyzer.

Each miRNA extraction from microparticle concentrated plasma was derived from 500 μL of whole plasma compared with 100 μL of plasma with the Isolate II kit. Despite this, as is demonstrated in Table 2.3, miRNA extraction from microparticle-enriched plasma did not result in a consistent increase in miRNA yield.

Table 2.3 Comparison of microRNA concentrations of extractions from whole plasma and microparticle concentrated plasma

Kit/sample	miRNA concentration (pg/µL)	Total miRNA per 100 µL plasma volume (pg)
Isolate II miRNA-1	17.3	346
Isolate II miRNA-2	32.5	650
Isolate II miRNA-1 (M)	28.9	115.6
Isolate II miRNA-2 (M)	7.3	29.2
Isolate II large-1	37	740
Isolate II large-2	23.6	472
Isolate II large-1 (M)	2.2	8.8
Isolate II large-2 (M)	2.5	10

Concentrations based on Bioanalyzer analysis with small RNA chip.

* Calculated by multiplying concentration by elution volume and dividing by total plasma volume (x 100 µL). 100 µL of whole or microparticle-enriched plasma (derived from 500 µL of whole plasma) was loaded onto the Isolate II kit.

M, microparticle-enriched plasma; miRNA, micro ribonucleic acid;

This result was not completely unexpected as most miRNA in the circulation is found independent of microvesicles (132,133). There were significant differences between duplicate samples. All concentrations were below the recommended range for the Bioanalyzer and this may have led to error in quantification.

2.7.2.3 Assessment of microRNA extracted from lipoprotein fraction of plasma and efficacy of concentration with SpeedVac

Lipoprotein particles such as high density lipoprotein are also known to be carries of miRNA in the circulation (134). Hence, a comparison of miRNA extracted from the lipoprotein fraction of plasma and from whole plasma was performed. Plasma (850 µL)

from the same patient described in section 2.7.2.2 was thawed and subjected to density ultracentrifugation to isolate total plasma lipoproteins. Potassium bromide was added to the plasma to adjust the density to 1.21 g/mL and the sample was centrifuged at 100,000 rpm (Optima TLX Ultracentrifuge, TLA-110 rotor, Beckman Coulter, Brea, California, USA) for 16 h at 4 °C. Following centrifugation, total lipoproteins (>1.21 g/mL) were concentrated at the top of the solution and were visible as a white band. The lipoprotein fraction was removed, saline was added to a total volume of 100 µL and miRNA was extracted using the Isolate II kit. MicroRNA was also extracted from 100 µL of whole plasma from the same patient for comparison.

As all previous Bioanalyzer results indicated very low RNA concentrations, which hampered accurate determination of the miRNA quantity, the miRNA samples were concentrated using a SpeedVac (Savant, Thermofisher). The SpeedVac is a centrifuge which applies a negative pressure to the samples to promote evaporation of water. The samples, as described in section 2.7.2.2 (microparticle-associated miRNA) were also concentrated with a SpeedVac. The samples were placed in the SpeedVac until approximately half the volume had evaporated.

The results of analysis of the samples with a Bioanalyzer are displayed in Table 2.4. While concentration by SpeedVac of the lipoprotein fraction of plasma appeared to provide the greatest concentration of miRNA, it was also below the recommended range for the Bioanalyzer. This miRNA was derived from a larger volume of plasma (850 µL) than the samples extracted from whole plasma (100 µL). The 8.5 fold greater volume of plasma did not translate to a measurable quantity of miRNA.

Table 2.4 Comparison of microRNA concentrations of extractions from whole plasma and lipoprotein fraction of plasma and the effect of concentration by SpeedVac

Kit/sample	miRNA concentration before SpeedVac (pg/µL)	miRNA concentration after SpeedVac (pg/ µL)
Isolate II miRNA-1	17.3	5.2
Isolate II large-2	23.6	25.3
Isolate II miRNA-1 (M)	28.9	36.7
Isolate II large-2 (M)	2.5	40.2
Isolate II miRNA-3	-	3.9
Isolate II large-3	-	74.1
Isolate II miRNA-3 (L)	-	127.2
Isolate II large-3 (L)	-	107.3

Concentrations based on Bioanalyzer analysis with small RNA chip. Samples 1 and 2 were prepared as described in section 2.7.2.2 and reported in Table 2.3. Sample 3 was prepared in parallel from whole plasma and the lipoprotein fraction. Samples were centrifuged in a SpeedVac until the volume had reduced by approximately 50% to increase the concentration of miRNA.

L, lipoprotein fraction of plasma; M, microparticle-enriched; miRNA, micro ribonucleic acid;

2.7.2.4 Combining microRNA and large RNA output from Isolate II extraction kit

As it appeared that there was a significant amount of miRNA in the Isolate II large RNA column (sections 2.7.2.1, 2.7.2.2 and 2.7.2.3), pooling the miRNA and large RNA isolate II kit outputs, followed by SpeedVac based concentration, was tested. MicroRNA and large RNA Isolate II outputs from the plasma of a patient with significant CAD were pooled and an aliquot was stored for analysis with a Bioanalyzer. The remaining sample was concentrated with a SpeedVac, aiming for a reduction in volume by half. After 25 min in the SpeedVac, one sample (A) had almost completely evaporated while the other sample (B) volume had decreased by approximately 50%,

indicating variable evaporation using this method. Sample A had 5 µL water added (total volume 10 µL) and the total volume of sample B was 25 µL after SpeedVac centrifugation. The results of Bioanalyzer analysis of these samples are displayed in Table 2.5. The results demonstrated discrepant concentrations between the duplicate samples (between A and B) prior to SpeedVac concentration and inconsistent apparent increases in miRNA concentration after evaporation.

Additionally, the concentration of miRNA was still below the recommended range of the Bioanalyzer and given the un-predictable rate of evaporation of samples during centrifugation in the SpeedVac, it was decided that the method of pooling of the miRNA and large RNA outputs from the Isolate II kit and using the SpeedVac was inappropriate to use with randomised cohort miRNA samples. During these experiments it also became apparent that due to a lack of a protein precipitation step in the protocol, the Isolate II isolation columns had the potential to become occluded, resulting in loss of miRNA. As this issue could not be resolved, the use of Isolate II columns to isolate miRNA from patient plasma samples was abandoned as was the use of the Bioanalyzer to assess miRNA concentration.

Table 2.5 MicroRNA concentrations after combining outputs of Isolate II kit and the effect of concentration by SpeedVac

Kit/sample	miRNA concentration before SpeedVac (pg/µL)	miRNA concentration after SpeedVac (pg/ µL)
Isolate II-A	12.3	164.6
Isolate II-B	147.4	79.4

Concentrations based on Bioanalyzer analysis with small RNA chip. Samples A and B were duplicate extractions from the same plasma with miRNA and large RNA outputs of the Isolate II kit pooled prior to analysis. Samples were centrifuged in a SpeedVac until the volume had reduced by approximately 50% to increase the concentration of miRNA.

miRNA, micro ribonucleic acid;

2.7.2.5 Comparison of NucleoSpin and mirVana Phenol Free microRNA extraction kits and analysis for microRNA-16 by reverse transcription and quantitative polymerase chain reaction

Given the inconsistencies described in earlier sections, the clogging of the Isolate II columns and difficulty increasing and quantifying the concentration of the miRNA extracted from plasma, the approach was changed to adopt a published method based on consistent volumes of miRNA rather than absolute quantities (127). Additionally, two additional commercially available phenol-free miRNA extraction kits were trialled. Instead of analysis with a Bioanalyzer, specific quantitative polymerase chain reactions (qPCR) for miRNA-16 were used to assess isolation and relative quantity of miRNA. MicroRNA-16 is a stable and abundant species in plasma and is commonly used for normalisation (135,136).

Plasma from a patient with significant CAD underwent miRNA extraction with the NucleoSpin miRNA Plasma kit (Macherey-Nagel, Duren, Germany) and the mirVana Phenol Free miRNA kit (Thermofisher) in triplicate and duplicate extractions

respectively, as per the manufacturer's instructions. Mussel glycogen (Roche) was added as a carrier protein as previously described, to increase miRNA yield (127,130,137). As the NucleoSpin and mirVana Phenol Free kits differed in the final elution volume (Table 2.6), the miRNA isolated by the mirVana kit was precipitated and finally diluted in a volume of water to match the sample volume produced by the NucleoSpin kit (20 µL).

Precipitation was performed as previously described (127). The mirVana miRNA samples (100 µL) were mixed with 0.1 volumes of 3 M sodium acetate (pH 5.2), 2.5 ng mussel glycogen and 2.5 volumes ice cold 100% ethanol (Sigma-Aldrich). This mixture was incubated overnight at -80 °C. After thawing, the sample was centrifuged at 17000 g for 20 min at 4 °C. The pellet was washed with 1 mL 80% (v/v) ice cold ethanol and re- centrifuged at 17000 g for 10 min at 4 °C. The supernatant was carefully removed and the pellet air dried at room temperature for 10 min before being resuspended in RNase free water (20 µL).

Table 2.6 Comparison of the input plasma volume and final elution volumes of the NucleoSpin and mirVana Phenol Free kits

Kit	Plasma volume (µL)	Final elution volume (µL)
NucleoSpin	300	20-30
mirVana Phenol Free	450	100

Volume of plasma loaded onto and final volume for elution of miRNA from each kit as recommended by the manufacturer.

miRNA, micro ribonucleic acid;

After sample preparation, a reverse transcription (RT) reaction was performed as described previously (127). In short, 0.5 µL RNA was mixed with 0.5 nM dNTPs, 16.5

U MultiScribe reverse transcriptase, 0.5 µL 10x RT buffer, 1.2 U RNAse inhibitor, 1 µL miRNA-16 primer (5x) in a total volume of 5ul. The RT reagents were sourced from Applied Biosystems (Thermofisher). The RT reaction was performed in a Multigene thermal cycler (Labnet International, Edison, New Jersey, USA) with reaction conditions of 16 °C for 30 min, then 42 °C for 30 min then 85 °C for 5 min. After the RT reaction, 5 µL of water was added and samples were either frozen or used for qPCR analysis.

To perform the qPCR, 2.25 µL of complementary deoxyribonucleic acid (cDNA) was added to 1x KAPA fast probe universal mix, 1x Rox low and 0.25 µL miRNA-16 probe (20x) in a total volume of 10 µL. The qPCR reagents were sourced from Kapa Biosystems (Wilmington, Massachusetts, USA). The qPCR reactions were performed in duplicate on a Viia 7 system (Thermofisher) under the following reaction conditions: 95 °C for 20 sec followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec. The amplification curves and number of PCR cycles required for fluorescence to reach a threshold value (Ct values) were determined by using PCR analysis software (QuantStudio, Applied Biosystems, Foster City, California, USA). The threshold was automatically determined by the software and the reported Ct values are the mean of the duplicate qPCR reactions.

Figure 2.8 demonstrates the Ct values, indicating comparable miR-16 content of the samples prepared with the NucleoSpin and mirVana Phenol Free kits, with lower Ct values indicating a greater concentration of the species in the sample. The results indicated a similar yield of miRNA-16 with both extraction kits, despite the mirVana kit having a greater input plasma volume. Therefore the NucleoSpin kit was used to isolate plasma miRNA from the plasma stored from the randomised patient cohort. The NucleoSpin kit also included a protein precipitation step, which was deemed likely to

reduce clogging of the isolation columns by plasma proteins. Additionally, as all RNA is isolated with one column in the NucleoSpin kit, as opposed to a miRNA and 'large' RNA isolation, the reduced number of steps was anticipated to result in reduced variability between samples.

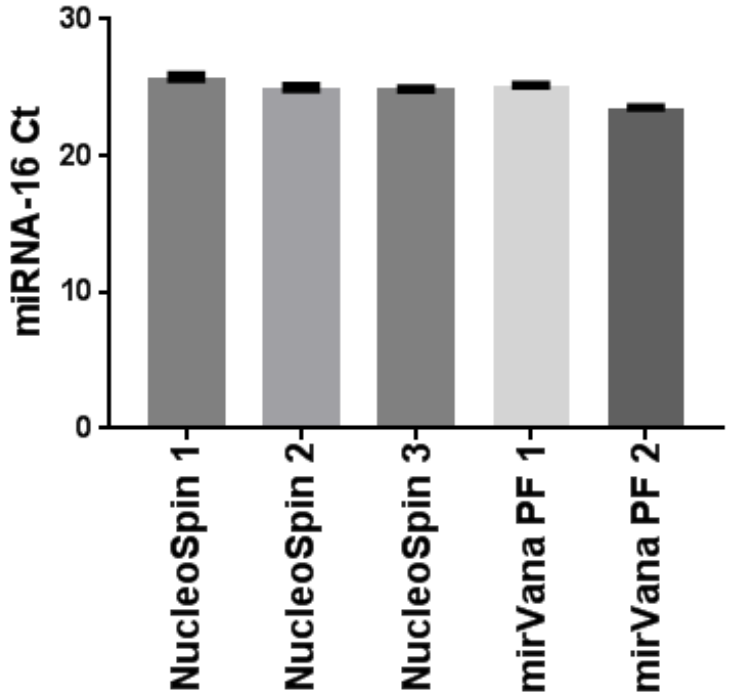


Figure 2.8 Quantitative polymerase chain reaction for microRNA-16 of samples prepared with NucleoSpin and mirVana Phenol Free kits

Values represent the mean Ct value of duplicate reactions for each sample, with lower values indicating greater a greater concentration of miRNA-16 in the sample. Quantitative PCR was performed as described in section 2.7.2.5. Bars represent range.

miRNA, micro ribonucleic acid; PF, phenol free;

2.7.2.6 Summary and conclusions regarding microRNA extraction

The experiments performed to compare various commercially available miRNA extraction kits and methods to optimise miRNA recovery revealed inconsistencies in

isolations and that achieving the recommended concentration for analysis with the Bioanalyzer was difficult. Several methods to increase the concentration of miRNA were tested with all methods resulting in a concentration below the recommended range for Bioanalyzer analysis. It was therefore decided to follow a reported protocol for investigation of plasma miRNAs that uses minimal handling steps, rather than based on miRNA yield. The NucleoSpin isolation kit was deemed most suitable and specific RT and qPCR reactions for miRNA-16, an abundant species in plasma which is commonly used as a reference species, indicated successful isolation of miRNA with comparable miRNA-16 levels between samples indicating consistency in miRNA isolation. MicroRNA isolation using this kit with subsequent determination of miRNA-16 levels was therefore chosen to investigate changes in plasma miRNA levels with RIPC in chapter 6.

2.8 Storage of platelet lysates

To study the effect of RIPC on intracellular platelet pathways, platelet pellets were stored from patients who were treated with RIPC. In particular, phosphorylation of Vasodilator Stimulated Phosphoprotein (VASP), cyclic Adenosine Monophosphate (cAMP) and cyclic Guanosine Monophosphate (cGMP) were to be studied as these factors play a role in pathways mediating platelet activation. Blood collected in sodium citrate containing tubes was centrifuged at 200 g for 10 min at room temperature. The resultant platelet rich plasma (PRP) (2 x 500 μL) was either centrifuged immediately at 2500 g for 15 min at room temperature in a 15 mL centrifuge tube (Greiner, Kremsmünster, Austria) to study platelet lysates under basal conditions or stimulated with SFLLRN (20 μM) for 20 min at room temperature prior to centrifugation. The

platelet count of the PRP was measured (Sysmex, Roche). The plasma supernatant was discarded and the platelet pellet was resuspended in 1 mL of ice-cold DPBS before being centrifuged at 2600 g for 10 min at 4 °C. The supernatant was discarded and the platelet pellet was washed with another 1 mL of ice-cold DPBS before being centrifuged again. The platelet pellets were then further handled in one of two manners:

- The supernatant was discarded and 100 μL of a mix of 1 mL Mammalian Protein Extraction Reagent (Thermofisher), 40 μL Complete Protease Inhibitor Mix (Roche) and 50 uL PhosSTOP Phosphatase Inhibitors (Roche), before being stored at -80 °C.
- A second platelet pellet was washed a third time with 1 mL of ice-cold DPBS before being centrifuged at 2600 g for 10 min at 4 °C. The supernatant was discarded and resuspended in 1 mL (if PRP platelet count <200 x 10^9/L) or 1.5 mL (if PRP platelet count >200 x 10^9/L) of cell lysis buffer (cAMP/cGMP ELISA kit, R&D Systems, Minneapolis, Minnesota, USA). The suspension was then frozen at -80 °C, then thawed with gentle mixing. The sample was then re-frozen and thawed as previously before being centrifuged at 600 g for 10 min at 4 °C to remove cellular debris. The supernatant was stored at -80 °C either undiluted or diluted with cell lysis buffer aiming for a cell count of 1 x 10^7/mL, as per the manufacturer's instructions and to maintain consistency in protein mass across patients samples, with the dilution factor calculated based on the platelet count of the PRP.

2.9 Statistical analysis

The primary analysis in the majority of the studies presented is the difference between a specific measurement pre and post RIPC to determine the effect of conditioning on that particular marker. Similar analyses of pre and post sham were performed to ensure that the effect demonstrated was specific to RIPC.

In the following chapters, data distribution was assessed with the D'Agostino and Pearson normality test. Categorical variables are presented as frequency and percentage while continuous variables are expressed as mean ± SD for parametrically distributed data and median (IQR) for non-parametrically distributed data. Categorical data were compared using χ^2 tests or Fisher's exact test as appropriate while comparisons between continuous variables were performed using paired or unpaired t tests as appropriate for parametrically distributed data, and Wilcoxon signed rank or Mann-Whitney U tests as appropriate for non-parametric data. Where performed, correlations between variables was assessed by Pearson's correlation or Spearman rank correlation for parametric and non-parametric data respectively.

The percentage change in a particular marker or measurement in response to RIPC or sham was calculated by:

Percentage change = ((post – pre)/pre) x 100

Where post and pre are the specific measurements post or pre RIPC/sham respectively. A negative value indicates a decrease with RIPC/sham while a positive value indicates an increase with RIPC/sham.

The percentage change between RIPC and sham groups was compared with the Mann-Whitney U test in analyses where the paired comparison demonstrated an effect of

RIPC. This was performed to assess the robustness of the results. Specific statistical methods relevant to each study are described in the respective chapters.

Chapter 3: The effect of remote ischaemic preconditioning on coronary physiology

3.1 Introduction

As discussed in chapter 1, most studies of RIPC in the setting of PCI have demonstrated protection against procedure related myocardial injury or a reduction in myocardial infarct size (138). While the main focus of treatment of patients with CAD is often the epicardial atherosclerotic disease, the coronary microcirculation confers an important prognostic influence. The coronary flow reserve (CFR) and the index of microcirculatory resistance (IMR) have been shown to predict outcome in patients with epicardial coronary artery disease (CAD) and in patients without obvious obstructive coronary lesions (139-144). In the setting of elective or primary PCI, patients with a lower IMR have a superior prognosis (143,144). As discussed in section 1.2.6, there is some evidence that RIPC may augment coronary microcirculatory function. Given the importance of coronary physiology markers and the microcirculation in predicting the prognosis of patients with CAD, including those undergoing PCI, it was hypothesised that RIPC may confer a beneficial effect on microcirculatory function. In order to determine the effect of RIPC on coronary physiology, a randomised, blinded, placebo controlled trial to investigate the effect of RIPC on invasively measured markers of microcirculatory function was conducted.

3.2 Methods

3.2.1 Coronary physiology measurements and remote ischaemic preconditioning

Clinically stable patients, who were referred for non-urgent coronary angiography at Concord Hospital, Sydney, Australia, with symptoms or non-invasive investigations suggestive of significant CAD, were invited to participate as described in chapter 2. Coronary physiology measurements, coronary angiography and RIPC were performed as described in chapter 2. Methods specific to this chapter not described elsewhere are described below.

Patients were approached prior to their planned procedure and provided with written and verbal information about the study before informed consent was obtained. Coronary angiography was performed, as planned, and patients were only included if they required FFR assessment of an angiographically equivocal lesion in a major epicardial coronary artery based on clinical grounds. The need for FFR was determined by the interventional cardiologist performing the procedure. The decision to only include patients who required FFR measurement on clinical grounds was to justify the additional risk associated with coronary artery wiring. Consecutive patients who met these inclusion criteria were included in the study.

Patients with prior myocardial infarction in the target artery territory were excluded as were patients with coronary anatomy which would impact on accurate coronary physiology assessment, such as left or right coronary ostial disease leading to guide pressure ‘damping’. Patients in atrial fibrillation were also excluded as irregularity of the cardiac cycle could affect thermodilution measurements. Finally, patients with severe asthma were not invited to participate as adenosine, required for the coronary

physiology study, can exacerbate airways disease. Data regarding patient demographics, co-morbidities, medications and pre-procedure investigations were collected. Participants were randomised to either RIPC or sham treatment by way of a closed envelope system. RIPC/sham was delivered as described in section 2.1 by an assistant who did not communicate with the operator. Patients and the investigator performing the cardiac catheterisation procedure were blinded to the treatment allocation, with randomisation performed by the assistant. The patients were warned of possible discomfort from sphygmomanometer inflation but were asked not to express discomfort unless it was intolerable. All patients complied with this request. To maintain blinding of the operator, the sphygmomanometer was obscured from view and music was played throughout the laboratory to mask the sound of sphygmomanometer inflation. Coronary physiology measurements, including IMR_{calc}, IMR, CFR and FFR, were recorded pre and post RIPC or sham ensuring that the pressure-temperature sensor position remained constant. Two and three dimensional QCA was performed off-line in two orthogonal views in patients where there was adequate image quality to allow analysis.

3.2.2 Assessment of circulating regulators of microcirculatory function

In order to study whether RIPC-mediated changes in microcirculatory function were associated with changes in circulating factors previously reported to affect vascular function, blood was collected from the femoral venous sheath after each coronary physiology study. After the first 3 mL was discarded, blood was drawn slowly into syringes at a rate of 0.5 mL/sec and transferred to tubes containing K_2EDTA. Plasma was prepared and stored as described in section 2.6 by centrifugation at 2500 g for 15 min.

The stored plasma was analysed with the following commercially available kits as per the manufacturer's instructions: Total Nitric Oxide and Nitrate/Nitrite (all from R&D Systems), 6-keto-PGF1α (Abcam, Cambridge, United Kingdom) and Adrenomedullin (MyBioSource). Nitric oxide, adrenomedullin and prostacyclin (metabolised to 6-keto-PGF1α) are factors that contribute to regulation of the human microcirculation (145). Nitric oxide is metabolised to nitrite and nitrate. Adrenomedullin is a peptide hormone which plays a role in the regulation of vascular tone by regulating vasodilation in a cAMP and cAMP-independent manner (146). Adrenomedullin has been shown to be involved in RIPC mediated protection against IR injury (147). Hence these factors were measured in the plasma to explore the mechanism behind RIPC induced changes in coronary microcirculatory function.

3.2.3 Statistical analysis

Categorical variables are presented as frequency and percentage while continuous variables are expressed as mean ± SD for parametrically distributed data and median (IQR) for non-parametrically distributed data. Categorical data were compared using χ^2 tests or Fisher's exact test as appropriate. Comparisons between continuous variables were performed using paired or unpaired t tests as appropriate for parametrically distributed data, and Wilcoxon signed rank or Mann-Whitney U tests as appropriate for non-parametric data. Correlations between variables were assessed by Pearson r correlation or Spearman rank correlation for parametrically and non-parametrically distributed data respectively.

Pre treatment and post-treatment measurements of markers of microcirculatory function were compared. The primary analysis was change in the index of microcirculatory resistance with RIPC. Based on the first 4 patients who received RIPC as part of the

randomised study, an absolute decrease in IMR_{calc} with RIPC was expected to be 5.5 ± 7.0. With a power of 80% and a two-sided α value of 0.05, it was estimated that 15 patients would need to be studied for paired comparison to detect a change in IMR_{calc} with RIPC. Therefore 30 patients, randomised in a 1:1 manner to RIPC or sham treatment, were recruited. The secondary analyses included changes in IMR, CFR, T_{mnR}, T_{mnH} and FFR. The percentage change (section 2.9) with RIPC was compared with the percentage change with sham using the Mann-Whitney U test in measurements where the paired comparison demonstrated an effect of RIPC.

All analyses were performed with the use of SPSS version 22 (IBM, Armonk, New York, USA) or GraphPad, Prism (GraphPad Software Inc., La Jolla, California, USA) software. A two-tailed probability value < 0.05 was considered statistically significant.

3.3 Results

A total of 65 patients underwent coronary angiography as part of screening for inclusion into the study. Figure 3.1 summarises patient recruitment and randomisation. Thirty four patients were excluded after coronary angiography as there was no clinical indication for FFR measurement. One additional patient was excluded as coronary wiring precipitated significant coronary spasm (which was successfully treated with appropriate vasodilators and withdrawal of the wire). Thirty patients underwent randomization and the full study protocol. There were no complications of RIPC or sham treatment.

3.3.1 Baseline clinical characteristics

Of the 30 patients who underwent randomisation to either RIPC or sham treatment, the mean age was 63.1 ± 10.0 yr and 26 (87%) were male. Paired coronary physiology measurements were performed before and after the allocated treatment in all patients. There were 15 patients randomised to RIPC and 15 patients randomised to sham treatment. The baseline characteristics, lesion characteristics and medications, of the RIPC and sham groups are displayed in Table 3.1. Although there were some numeric differences between the groups, the differences did not meet statistical significant for any of the characteristics, acknowledging the relatively small numbers.

Twenty two (73%) of the lesions assessed were in the left anterior descending artery, 12 in the RIPC cohort and 10 in the sham cohort (P=0.41). The mean pre treatment (RIPC/sham) FFR in the RIPC and sham cohorts were not significantly different (0.83 ± 0.06 vs 0.82 ± 0.08, P=0.76). There were 3 (20%) and 6 (40%) pre treatment FFR measurements ≤0.80 in the RIPC and sham groups respectively (P=0.43).

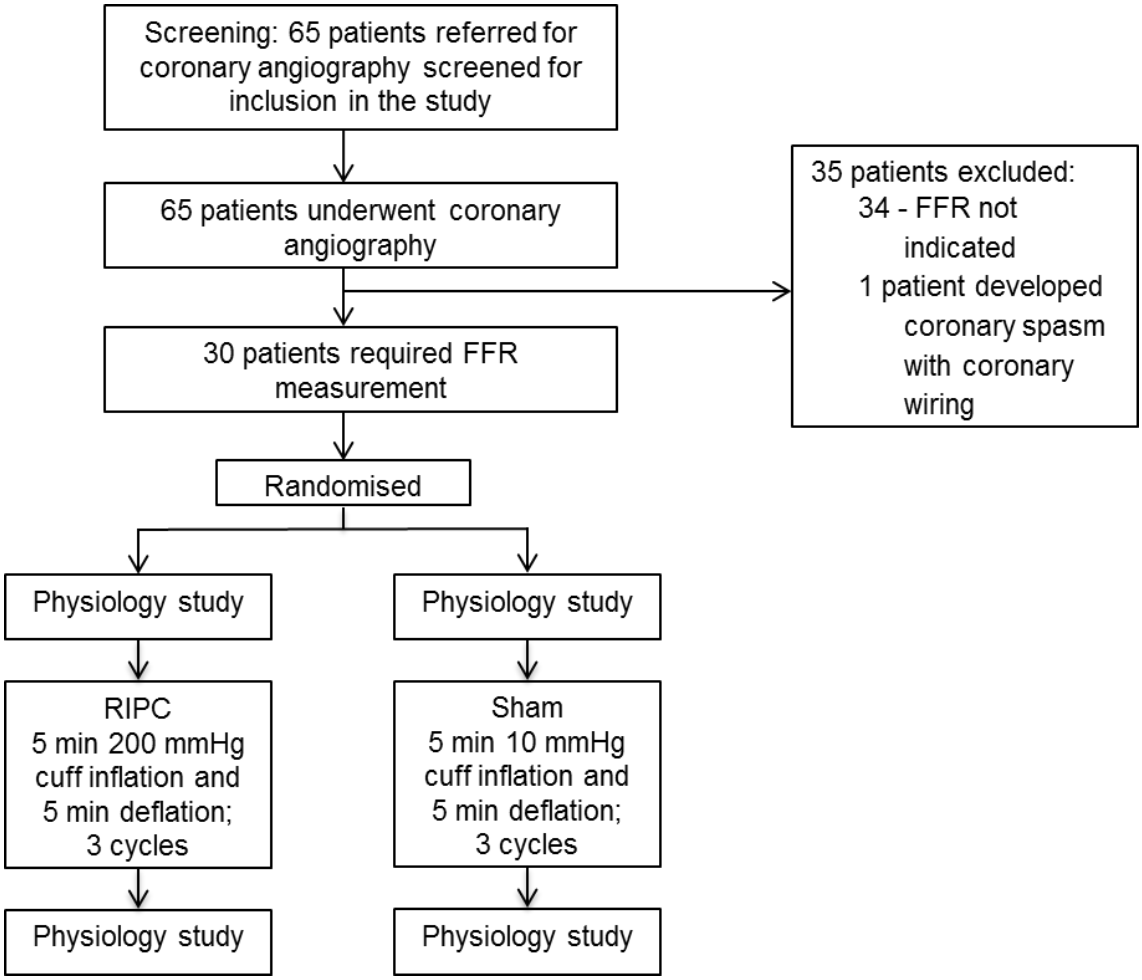


Figure 3.1 Patient recruitment and randomisation – Coronary physiology cohort

Patients with suspected CAD, referred for cardiac catheterisation, were approached prior to their planned procedure. After informed consent was obtained, patients underwent cardiac catheterisation as planned and randomised to RIPC or sham treatment if there was a clinical indication for FFR. In 34 patients, there was no coronary artery lesion with a diameter stenosis between 40-70% by visual assessment and hence FFR was not indicated. A coronary physiology study was performed before and after RIPC/sham while on the catheterisation table. Venous blood was collected after each coronary physiology study, after the cessation of the adenosine intravenous infusion. After the study protocol, the procedure proceeded as clinically indicated.

CAD, coronary artery disease; FFR, fractional flow reserve; RIPC, remote ischaemic preconditioning;

Table 3.1 Baseline characteristics of randomised coronary physiology cohort

Characteristic	RIPC (n=15)	Sham (n=15)	P value
Age - years	64.5 ± 8.8	61.7 ± 11.2	0.47
Male – no. (%)	13 (87)	13 (87)	1.00
Clinically stable – no. (%)	14 (93)	14 (93)	1.00
Prior myocardial infarction – no. (%)	1 (7)	4 (27)	0.33
Prior PCI – no. (%)	2 (13)	6 (40)	0.22
Prior CABG- no. (%)	0 (0)	0 (0)	-
Heart failure – no. (%)	0 (0)	1 (7)	1.00
Prior stroke – no. (%)	1 (7)	2 (13)	1.00
Peripheral vascular disease – no. (%)	0 (0)	2 (13)	0.48
Hypertension – no. (%)	11 (73)	10 (67)	0.69
Diabetes – no. (%)	7 (47)	3 (20)	0.25
Dyslipidaemia – no. (%)	13 (87)	9 (60)	0.22
Current smoking – no. (%)	1 (7)	3 (20)	0.60
Normal left ventricular contractility – no. (%)	14 (93)	14 (93)	1.00
Left ventricular hypertrophy – no. (%)	2 (13)	1 (7)	1.00
Medications			
Aspirin – no. (%)	14 (93)	15 (100)	0.31
P2Y12 antagonist – no. (%)	12 (80)	10 (67)	0.41
Warfarin/ NOAC – no. (%)	0 (0)	0 (0)	-
Statin – no. (%)	14 (93)	14 (93)	1.00
Beta blocker – no. (%)	5 (33)	8 (53)	0.27
ACEi or ARB – no. (%)	11 (73)	7 (47)	0.14
Nitrate – no. (%)	1 (7)	1 (7)	1.00
Coronary assessment			
LAD assessed – no. (%)	12 (80)	10 (67)	0.41
Lesion diameter stenosis - %*	38.7 ± 10.0	39.5 ± 6.4	0.78
Vessel reference diameter – mm*	2.9 ± 0.5	2.6 ± 0.5	0.05
Lesion length – mm*	9.4 ± 4.3	10.1 ± 4.2	0.65
Parameters during admission			
Systolic blood pressure – mmHg	128.5 ± 12.8	136.1 ± 12.3	0.11
Heart rate – beats/min	68.4 ± 11.0	67.9 ± 10.5	0.89
Haemoglobin concentration – g/L	138.1 ± 19.9	130.9 ± 15.2	0.28
eGFR - mL/min/1.73m^2	83.1 ± 11.1	84.9 ± 13.4	0.68

P values compare characteristics from RIPC and sham treatment groups using unpaired t tests for continuous data. Categorical data were compared using χ^2 tests or Fisher's exact test as appropriate.

* Average value from quantitative coronary angiographic assessment of 2 views of each lesion per patient.

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; eGFR, estimated glomerular filtration rate; NOAC, novel oral anticoagulant; PCI, percutaneous coronary intervention;

3.3.2 The effect of remote ischaemic preconditioning on the index of microcirculatory resistance

An example of the coronary physiology data obtained from one patient is presented in the Figure 3.2. This patient was randomised to receive RIPC on the catheterisation laboratory table during the procedure. Figure 3.2a displays the coronary physiology indices pre RIPC and Figure 3.2b displays the same indices post RIPC. In this case, RIPC was associated with a decrease in IMR and an increase in CFR.

a)

b)

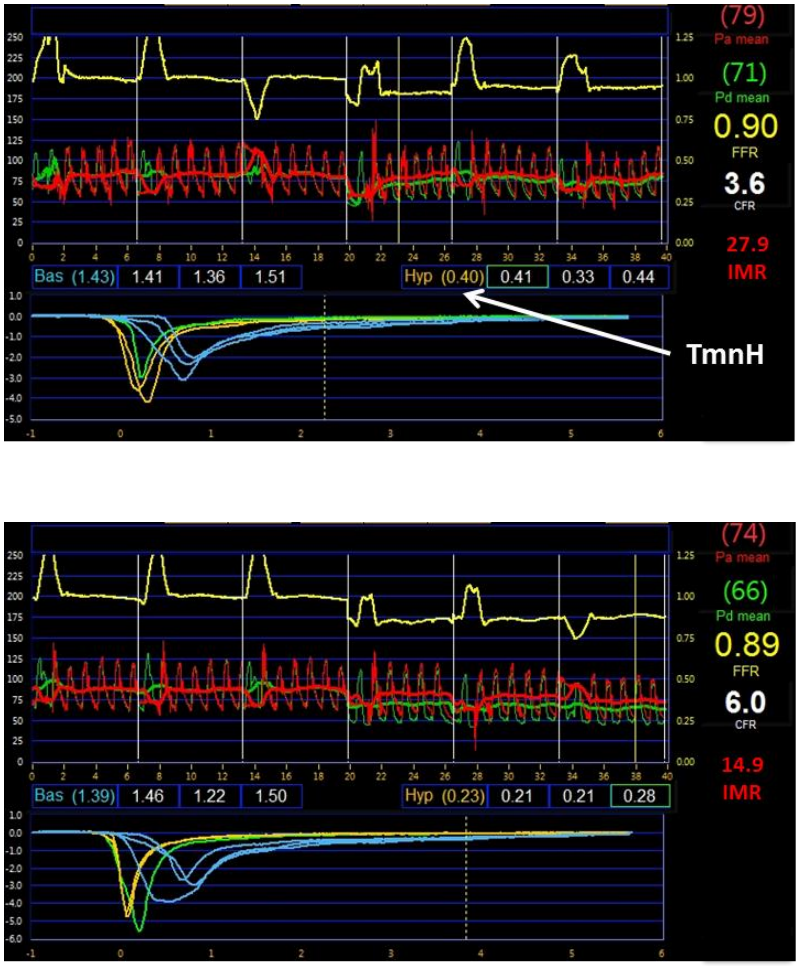


Figure 3.2 An example of coronary physiology measurements from one patient randomised to remote ischaemic preconditioning

Coronary physiology measurements obtained from one patient who was randomised to RIPC. The data in (a) was obtained pre RIPC and the data in (b) was obtained post RIPC. There was a reduction in IMR (calculated) and T_{mnH} with RIPC, while the CFR increased. The green and red pressure traces were recorded from the pressure sensor on the coronary wire and tip of the guiding catheter respectively. The yellow line (top of each figure) represents the ratio of distal to proximal pressure. The figures in the rectangular boxes represent the transit times with the average displayed to the left of the triplicate recordings. The measurements in the left half of each figure were recorded in the resting state whereas the measurements in the right half were taken during hyperaemia induced by administration of intravenous adenosine. IMR (calculated) = $P_a \times T_{mnH} \times (1.34 \times P_d/P_a - 0.32)$, calculated during hyperaemia.

CFR, coronary flow reserve; IMR, index of microcirculatory resistance; RIPC, Remote ischaemic preconditioning; TmnH, hyperaemic transit time;

Considering the entire randomised cohort, RIPC resulted in a significant reduction in IMR_{calc} and a significant reduction in IMR in patients with a pre treatment FFR >0.80 (Table 3.2 and Figure 3.3). There was no significant change in IMR_{calc} or IMR with sham treatment.

Table 3.2 The effect of remote ischaemic preconditioning and sham on coronary physiology measurements

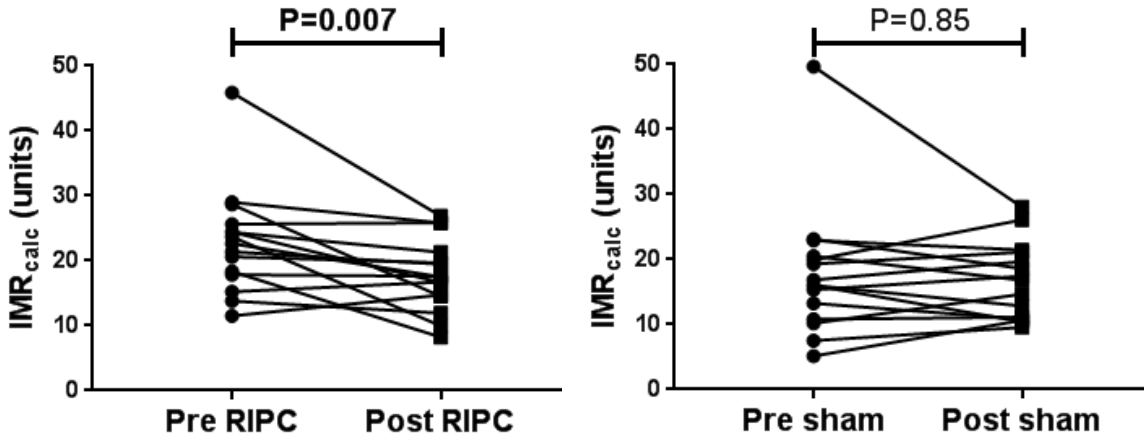
Marker	**RIPC (n=15)**			**Sham (n=15)**		
	Pre	**Post**	**P†**	**Pre**	**Post**	**P †**
IMR_{calc}	22.6	17.5	**0.007**	16.0	16.8	0.85
	(17.9-25.6)	(14.5-21.3)		(10.8-20.5)	(10.8-21.2)	
IMR*	24.3	17.7	**0.005**	16.1	11.4	0.83
	(18.5-26.1)	(13.2-21.7)		(9.3-22.8)	(10.6-24.7)	
CFR	2.6 ± 0.9	3.8 ± 1.7	**0.001**	3.1 ± 1.5	3.1 ± 1.6	0.97
T_{mnH} (s)	0.33	0.25	**0.01**	0.27	0.31	0.64
	(0.26-0.40)	(0.20-0.30)		(0.23-0.41)	(0.16-0.39)	
T_{mnR} (s)	0.95 ± 0.43	1.03 ± 0.55	0.29	0.88 ± 0.51	0.82 ± 0.47	0.19
FFR	0.83 ± 0.06	0.83 ± 0.07	0.99	0.82 ± 0.08	0.81 ± 0.09	0.052

P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

* Patients with pre treatment FFR >0.80: RIPC, n=12; Sham, n=9.

FFR, fractional flow reserve; CFR, coronary flow reserve; IMR_{calc}, calculated index of microcirculatory resistance; IMR, index of microcirculatory resistance; RIPC, remote ischaemic preconditioning; TmnR, transit time at rest; TmnH, transit time during hyperaemia;

a) IMR_{calc}



b) IMR

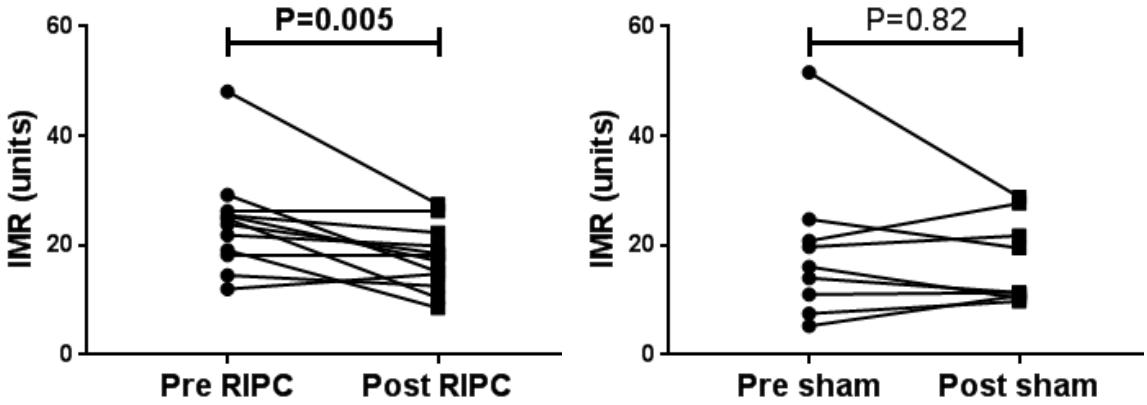


Figure 3.3 The effect of remote ischaemic preconditioning on the index of microcirculatory resistance

Effect of RIPC (left panels) or sham (right panels) on IMR_{calc} (a) and IMR in patients with an FFR>0.8 (b). IMR_{calc}: RIPC, n=15; Sham, n=15. IMR: RIPC, n=12; Sham, n=9. P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

IMR_{calc}, calculated index of microcirculatory resistance; IMR, index of microcirculatory resistance; FFR, fractional flow reserve; RIPC, remote ischaemic preconditioning;

There was one patient in each of the RIPC and sham groups that exhibited a very large reduction in IMR_{calc} and IMR with their respective treatments. When this patient was removed, the RIPC-mediated decrease in IMR_{calc} and IMR remained significant (Figure 3.4).

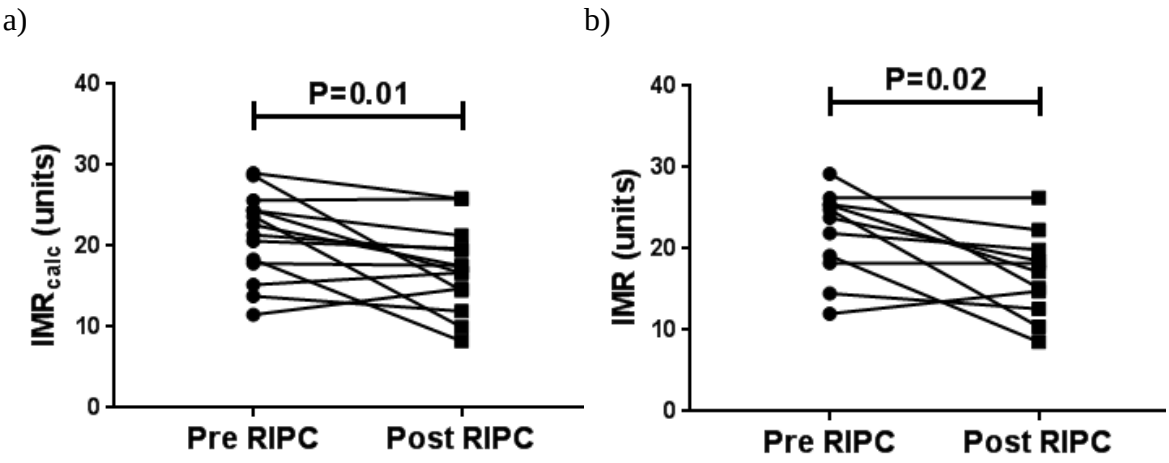


Figure 3.4 The effect of remote ischaemic preconditioning on the index of microcirculatory resistance – outlier removed

Effect of RIPC on IMR_{calc} (a) and IMR (b) with the patient exhibiting a large drop in IMR_{calc} and IMR removed. IMR_{calc}, n=14; IMR, n=11; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

IMR_{calc}, calculated index of microcirculatory resistance; IMR, index of microcirculatory resistance; RIPC, remote ischaemic preconditioning;

There was a larger percentage reduction in IMR_{calc} with RIPC than sham (-12.6% (-41.5 - -1.5) vs +2.8% (-19.7 - +27.1), P=0.09) but this did not reach statistical significance. A similar result was seen in the percentage change in IMR (-17.7% (-46.7 - -2.5) vs 2.9% (-28.4 - +2.9), P=0.11).

3.3.3 The effect of remote ischaemic preconditioning on coronary flow reserve

In the 15 patients randomised to RIPC, there was a significant increase in CFR as demonstrated in Figure 3.5 and Table 3.2. There was no effect of sham treatment on CFR. There was a significant increase in CFR in the subgroup of patients treated with RIPC who had an FFR>0.8 (2.8 ± 0.9 vs 4.1 ± 1.8, P=0.002, n=12).

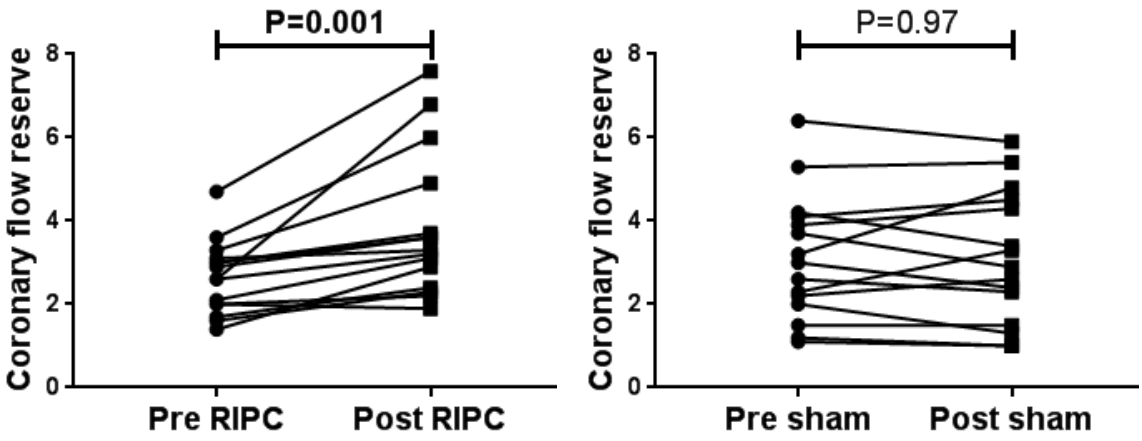


Figure 3.5 The effect of remote ischaemic preconditioning on coronary flow reserve

Effect of RIPC (left panel) or sham (right panel) on CFR. RIPC, n=15; Sham, n=15. P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.
CFR, coronary flow reserve; RIPC, remote ischaemic preconditioning;

There was a statistically significant greater percentage increase in CFR with RIPC than sham (+41.2% (+20.0 - +61.7) vs (-7.8% (-19.1 - +10.3), P<0.001).

3.3.4 The effect of remote ischaemic preconditioning on other coronary physiology measurements

The changes in IMR_{calc}, IMR and CFR with RIPC were associated with a significant decrease in T_{mnH} signifying an increase in hyperaemic coronary flow (Figure 3.6). Conversely, there was no change in T_{mnR} with RIPC, suggesting no change in resting coronary flow (Figure 3.6).

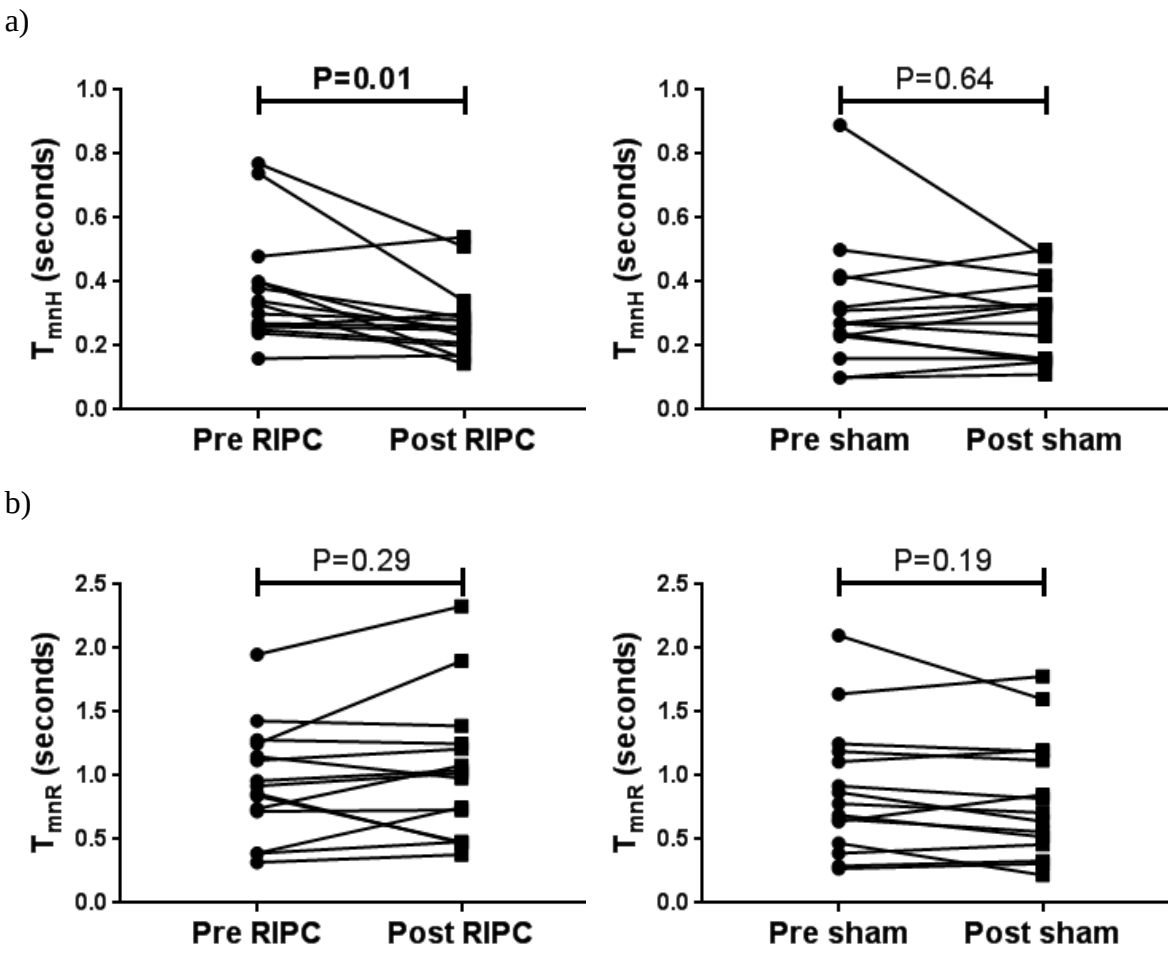


Figure 3.6 The effect of remote ischaemic preconditioning on transit time

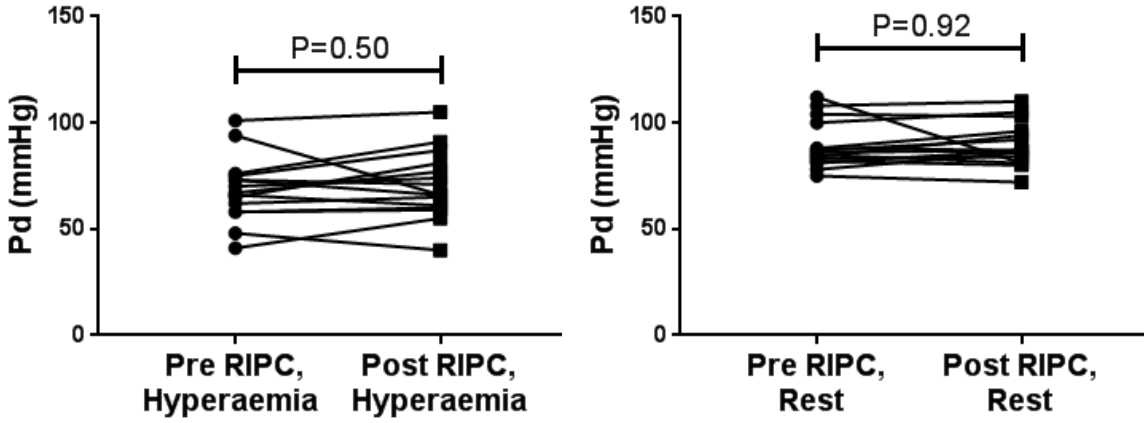
Effect of RIPC (left panels) or sham (right panels) on T_{mnH} (a) and T_{mnR} (b). Lower values indicate an increase in coronary flow. RIPC, n=15; Sham, n=15. P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

T_{mnH}, transit time during hyperaemia; T_{mnR}, transit time during rest; RIPC, remote ischaemic preconditioning;

Considering the other components of the IMR_{calc} formula, there was no effect of RIPC on P_d or P_a during hyperaemia or rest (Figure 3.7). The trans-lesion pressure gradient (P_a – P_d) did not change with RIPC either during hyperaemia (13 mmHg (10-14) vs 13

mmHg (11-19), P=0.37) or at rest (6 ± 4 mmHg vs 6 ± 4 mmHg, P=0.85). Associated with this, there was no effect of RIPC on FFR (Table 3.2).

a)



b)

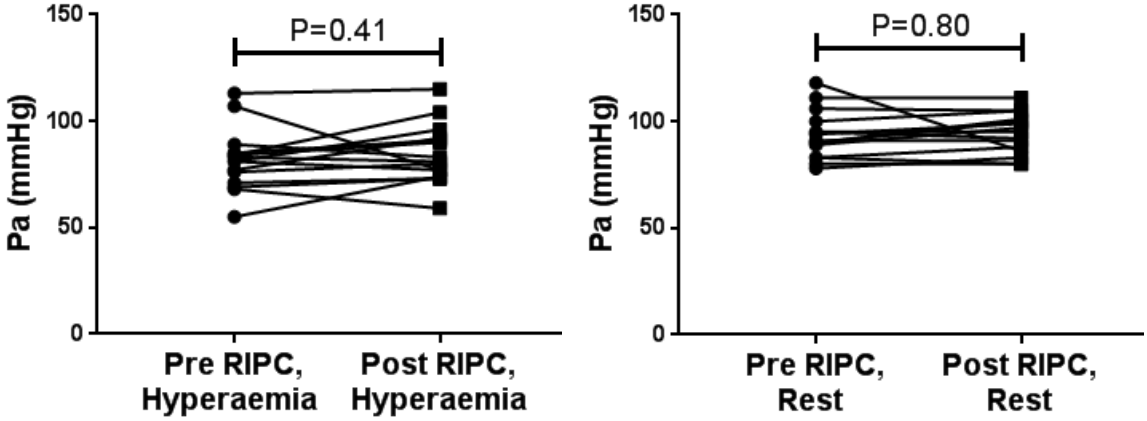


Figure 3.7 The effect of remote ischaemic preconditioning on distal and proximal mean coronary pressures

Effect of RIPC on P_d (a) and P_a (b) measured during hyperaemia (left panels) or rest (right panels). n=15; P values compare measurements from patients pre and post RIPC using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

P_a, mean aortic (proximal coronary) pressure; P_d, mean distal coronary pressure; RIPC, remote ischaemic preconditioning;

With regards to the pulse pressure (systolic – diastolic pressure), RIPC resulted in no difference in the distal coronary pulse pressure or proximal coronary pulse pressure, either during hyperaemia or at rest (Figure 3.8).

a)

b)

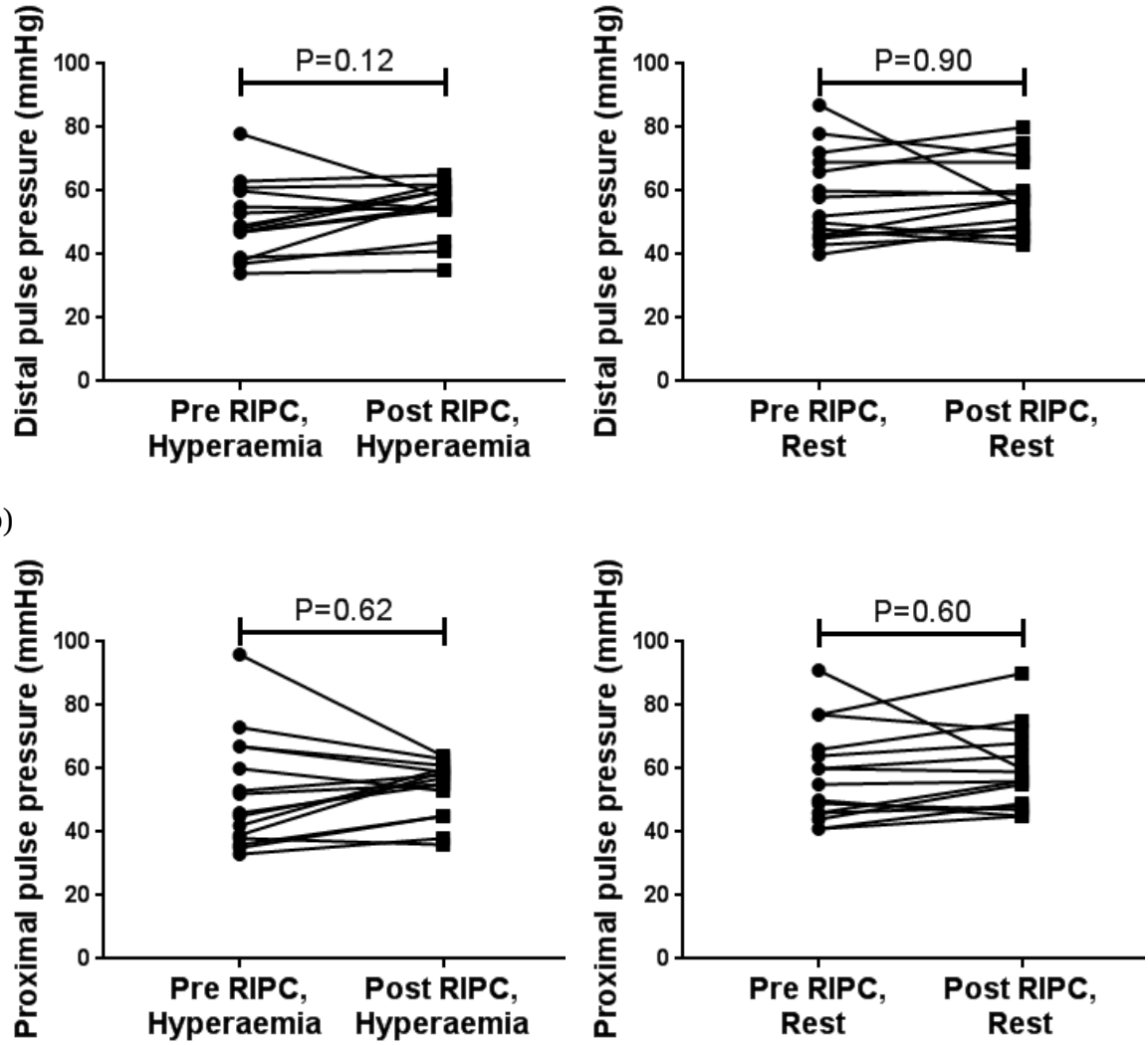


Figure 3.8 The effect of remote ischaemic preconditioning on distal and proximal pulse pressures

Effect of RIPC on the distal (a) and proximal (b) coronary pulse pressure measured during hyperaemia (left panels) or rest (right panels). n=15; P values compare measurements from patients pre and post RIPC using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

RIPC, remote ischaemic preconditioning;

3.3.5 The effect of remote ischaemic preconditioning on lesion characteristics as assessed by three dimensional quantitative coronary angiography

There were adequate angiogram images both pre and post RIPC to perform three dimensional QCA in 10 patients. An example of a three dimensional reconstruction of a coronary artery lesion during end diastole is displayed in Figure 3.9.

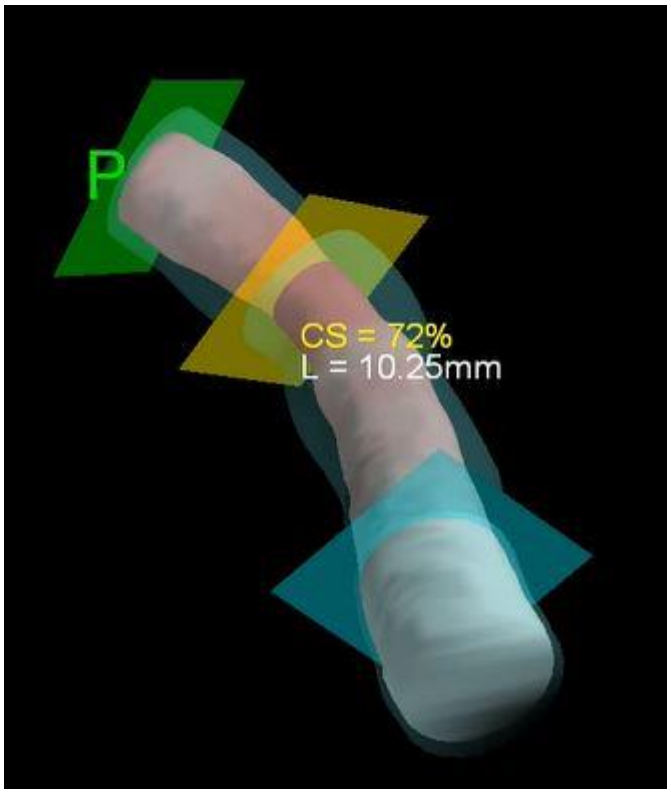


Figure 3.9 Example of a three dimensional quantitative coronary angiogram reconstruction of a coronary artery lesion

An example of a three dimensional reconstruction of a coronary artery lesion at end diastole. The IC3D software provides measurements including the lumen area and diameter at the site of the lesion, cross-sectional area stenosis (CS) and length of the lesion (L).

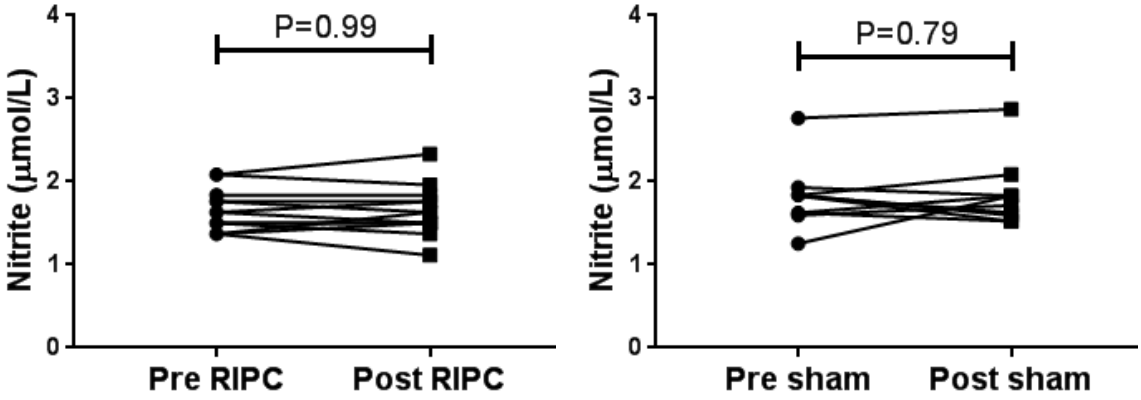
Given the change in hyperaemic coronary flow with no change in trans-lesional pressure gradient or FFR, a change in the lesion characteristics which may give rise to a reduction in resistance at the site of the lesion was sought. The compliance and

distensibility at the site of the lesion was calculated with lesion measurements taken at end-diastole and end-systole before and after RIPC. There was no change in compliance (0.35 ± 0.34 vs 0.18 ± 0.27, P=0.27, n=10) or distensibility (7.49 ± 7.06 $mmHg^{-1}$ vs 2.64 ± 4.43 $mmHg^{-1}$, P=0.14, n=10) as a result of RIPC. Indeed, contrary to the expected changes in these parameters, there was a trend towards a reduction in compliance and distensibility with RIPC.

3.3.6 The effect of remote ischaemic preconditioning on circulating regulators of microcirculatory function

To investigate potential mediators of the reduction in microcirculatory resistance and increase in coronary flow reserve, the levels of known regulators of the microcirculation were measured in stored plasma. There was no change in plasma nitrite with either RIPC or sham (Figure 3.10a). There was a significant reduction in plasma nitrate with RIPC (22.8 μmol/L (14.5-25.2) vs 21.9 μmol/L (13.7-24.3), P=0.006, n=13), however, this was also the case with sham treatment (23.0 ± 16.5 μmol/L vs 21.6 ± 15.5 μmol/L, P=0.009, n=10) (Figure 3.10b).

a)



b)

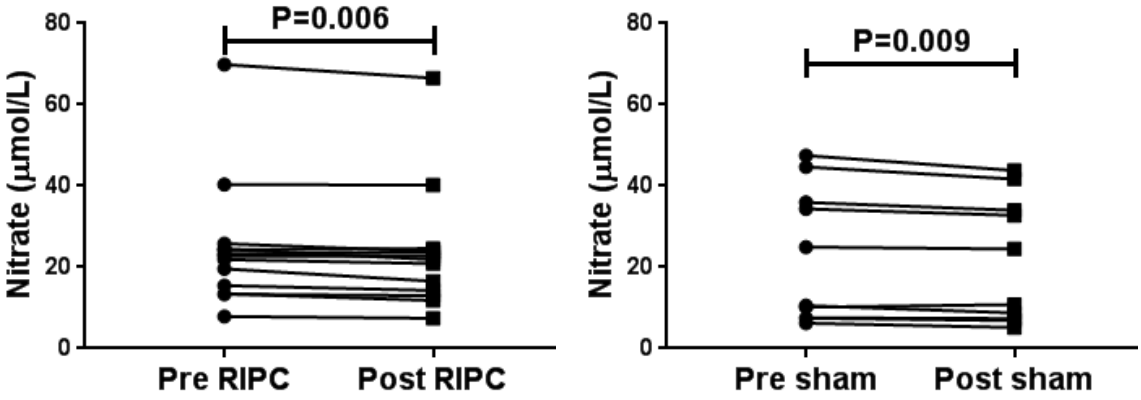


Figure 3.10 The effect of remote ischaemic preconditioning on plasma nitrite and nitrate

Plasma nitrite (a) and nitrate (b) concentrations in stored plasma pre and post RIPC (left panels) and sham (right panels) measured with a commercially available ELISA kit as per manufacturer guidelines. RIPC, n=13; Sham, n=10; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ELISA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

Measurement of other factors, which are known to induce a vasodilatory response in the microcirculation, in stored plasma samples demonstrated a significant decrease in in 6-keto-PGF1α with RIPC (845.9 pg/mL (298.4-1374.0) vs 576.9 pg/mL (254.5-1282.0),

P=0.02, n=11) with no effect of sham treatment (Figure 3.11). Plasma adrenomedullin levels were unchanged by either RIPC or sham treatment (Figure 3.12).

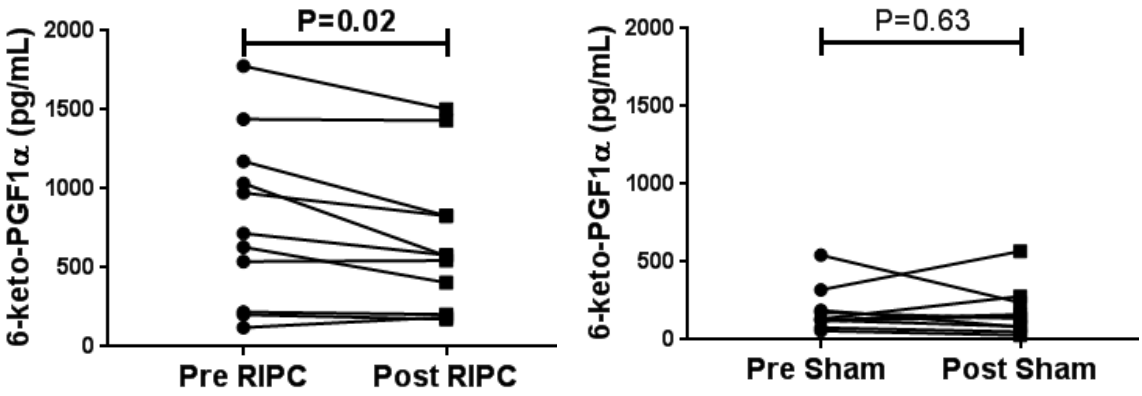


Figure 3.11 The effect of remote ischaemic preconditioning on plasma 6-keto-PGF1α

Plasma 6-keto-PGF1α concentrations in stored plasma pre and post RIPC (left panel) or sham (right panel) measured with a commercially available ELISA kit as per manufacturer guidelines. RIPC, n=11; Sham, n=10; P values compare measurements from patients pre and post RIPC or sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ELISA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

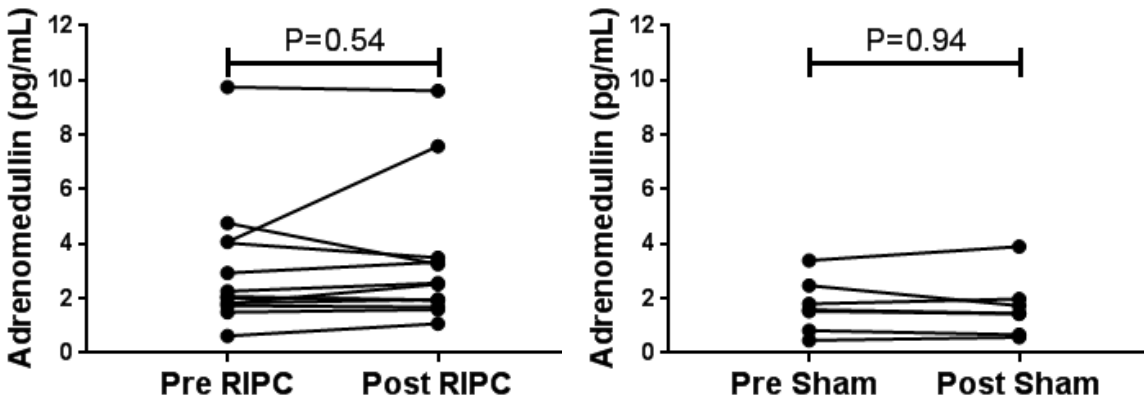


Figure 3.12 The effect of remote ischaemic preconditioning on plasma adrenomedullin

Plasma adrenomedullin concentrations in stored plasma pre and post RIPC (left panel) or sham (right panel) measured with a commercially available ELISA kit as per manufacturer guidelines. RIPC, n=13; Sham, n=7; P values compare measurements from patients pre and post RIPC or sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ELISA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

3.4 Discussion

RIPC has been shown to confer cardioprotection to patients undergoing elective and primary PCI with reductions in post PCI troponin and infarct size (14,74,76) yet the mechanism remains unknown (148). This study has demonstrated that RIPC acutely improves coronary microcirculatory function as assessed by validated coronary pressure/temperature sensor wire based techniques. In this cohort of patients with coronary artery disease, RIPC was associated with a significant reduction in microcirculatory resistance as measured by the IMR and an increase in coronary flow reserve, both contributed to by a reduction in hyperaemic transit time indicating an increase in coronary flow.

The coronary microcirculation is recognised as an important determinant of prognosis in patients with CAD. Multiple studies have shown the utility of the CFR to predict cardiac events and may be of greater importance than the degree of disease in the epicardial coronary arteries in determining prognosis (140,142,149-151). The IMR predicts post PCI myocardial infarction after elective PCI and the occurrence of death and heart failure hospitalisation after primary PCI (143,144). Given the importance of these markers of microcirculatory function in predicting prognosis in patients with CAD, including those undergoing PCI, and the benefits of RIPC in patients undergoing PCI, a randomised blinded placebo controlled trial was conducted to determine the effect of RIPC on the IMR and CFR. RIPC led to a rapid reduction in the index of microcirculatory resistance and an increase in coronary flow reserve with no effect of sham treatment. This suggests that beneficial effects on the coronary microcirculation contribute to RIPC mediated cardioprotection during PCI. These results were supported by the percentage change analysis between RIPC and sham groups. However, the difference in percentage change in IMR_{calc} and IMR did not reach statistical significance likely due to the study being underpowered to demonstrate a difference with these measures.

The results of this study raise the possibility that RIPC may be beneficial in other clinical settings where microcirculatory dysfunction is important such as microvascular angina, congestive heart failure and aortic stenosis (152,153).

There is data supporting the role of the coronary microcirculation as a target of RIPC mediated cardioprotection (154). In a study by Kono et al (69), 10 healthy volunteers and 10 patients with heart failure who received RIPC twice per day for 1 week demonstrated an increase in CFR as assessed by echocardiographic spectral Doppler analysis of flow in the distal left anterior descending artery. Additionally, RIPC has

been shown to attenuate vasoconstriction after acetylcholine administration, which is a marker of endothelial dysfunction (70). The endothelium is an important component of the microcirculation and plays a central role in the regulation of flow and resistance (145).

However, there is conflicting evidence regarding the effects of RIPC on the coronary microcirculation. Studies of RIPC in the setting of primary PCI, despite demonstrating reduction in myocardial injury, have reported no changes in surrogate markers of microcirculatory function such as the TIMI frame count and the appearance of microvascular obstruction on magnetic resonance imaging (74,76). Additionally, a study by Hoole et al found that RIPC had no effect on coronary microvascular resistance in 11 patients assessed by a Doppler/pressure wire based technique requiring coronary balloon inflation during cardiac catheterisation (15). The null result in this study may have been due to local preconditioning, or distal embolisation, induced by the coronary balloon inflation. There was a small numerical increase in the microcirculatory resistance after coronary balloon inflation in patients who did not undergo RIPC in this study, but the study may have been underpowered to detect a statistically significant difference.

In the present study, there was no change in T_{mnR}, suggesting that RIPC does not affect resting coronary flow. However there was an increase in hyperaemic flow, indicated by a reduction in T_{mnH}, in the absence of change in distal or proximal coronary pressures or a change in FFR. This increase in hyperaemic flow supports the interpretation that RIPC reduces coronary microcirculatory resistance. The increase in coronary flow in the absence of a change in trans-lesional gradient is interesting as an increase in the gradient might have been expected. A possible explanation for this is the modest cohort size, possibly leading to the study being underpowered to detect a change in gradient.

Alternatively, it was hypothesised that there may have been a change in the lesion characteristics, leading to a reduction in resistance to flow at the site of the lesion. Hence the angiograms were reconstructed with three dimensional QCA to calculate the compliance and distensibility of the vessel at the site of the lesion, which may have been expected to increase with RIPC. A comparison of measurements before and after RIPC did not demonstrate any significant change in either of these measurements. This result should be interpreted in the context of the modest cohort size and also the fact that the angiogram images, which were used to perform the three dimensional reconstructions, were taken during resting conditions. The changes in flow were observed during hyperaemia and changes in lesion characteristics during hyperaemia may not be reflected in angiogram images obtained at rest.

Considering the mechanism behind the rapid enhancement of microcirculatory function caused by RIPC, given evidence in the literature for circulating mediators, factors known to control the human microcirculation were studied in stored plasma taken before and after RIPC/sham. Adenosine and nitric oxide (metabolised to nitrite and nitrate) contribute to the coronary microcirculatory tone, and as they have been implicated in RIPC mediated cardiac protection (31,34,42,155), are candidate mediators of the effects of RIPC on microcirculatory resistance and flow that were observed. However, given the high doses of adenosine and intracoronary glyceryl trinitrate that were given at the time of each coronary physiology study to achieve hyperaemia, it is unlikely that these mediators are primarily responsible for the effects that were identified. The lack of change in plasma nitrite levels, the major metabolite of nitric oxide, with RIPC supports this conclusion with respect to nitric oxide. Although the measured level of nitrate in plasma was reduced after RIPC, there was a similar effect with sham treatment and hence this result is likely to be an artefact.

Prostacyclin is known to lead to vasodilation in the coronary vasculature, with 6-keto-PGF1α a stable metabolite (156,157). We hypothesised an increase in prostacyclin may contribute to the effect of RIPC, however there was a reduction in plasma6-keto-PGF1α with RIPC without a change with sham. This result suggests reduced prostacyclin the in the circulation which is unexpected given the increase in coronary flow demonstrated. Adrenomedullin is another hormone that is known to enhance microcirculatory function and has been identified as a potential mediator of RIPC induced protection in an animal model of IR injury (147,158). Adrenomedullin augments vascular tone, vasodilation and endothelial function through the regulating of cAMP, cGMP and intracellular calcium mobilisation (159). Contrary to animal data suggesting an upregulation of adrenomedullin in response to RIPC (147), there was no change in plasma adrenomedullin levels with RIPC in the present study suggesting that this hormone is not contributing to the changes in microcirculatory function that were observed.

A range of other candidate mediators remain unexplored in this study. Other mediators and pathways implicated in cardiac preconditioning include bradykinin, potassium ATP channels and calcium activated potassium channels of the BK type (24,66,160). As potassium ATP channels are involved in RIPC mediated protection against IR injury associated endothelial dysfunction (24), they may play a role in the improved microcirculatory function observed and warrant future investigation.

There are some limitations with the present study. Due to the relatively small numbers, it was not possible to investigate for clinical characteristics that predict an improvement in coronary microcirculatory function in response to RIPC. Additionally, due to the modest numbers, despite randomisation, there were some minor differences in the baseline characteristics of the study cohorts. There were numerically more patients with diabetes mellitus in the RIPC cohort. However, there is a suggestion of reduced efficacy

of RIPC in patients with diabetes (85,161) and hence, this difference between the cohorts, if anything, may have biased the study towards a null result. These minor differences along with the relatively small cohort size may have also led to differences in the pre treatment levels of IMR_{calc}, IMR and CFR, as well as plasma factors such as 6-keto-PGF1α. The lesion reference vessel diameter was also numerically larger in the RIPC group than the sham group. In a subgroup analysis, matched for vessel reference diameter, the effect of RIPC on IMR and CFR were still apparent (data not shown). These minor difference in pre treatment measures are unlikely to have influence the overall results as the primary analysis was a paired comparison of coronary physiology measurements or plasma marker taken before and after RIPC/sham, however it is not possible to exclude that this confounded the results.

It is difficult to comment on the durability of the RIPC effect and to be certain that the maximal effect on microcirculatory resistance and flow reserve has been identified. Performing a third coronary physiology study at a later time point was ethically difficult to justify given the invasive nature of the measurements. Due to the administration of glyceryl trinitrate, additional assessments of endothelial function, such as response to acetylcholine administration, could not be performed in this study. Finally, as this was a mechanistic study with only small numbers of patients undergoing PCI, clinical outcomes and the correlation of microcirculatory change with change to post PCI troponin were not assessed. The results of this study indicate the need for a study correlating change in coronary microcirculatory status and clinical outcomes with RIPC.

3.5 Conclusions

Remote ischaemic preconditioning rapidly decreases the index of microcirculatory resistance and increases the coronary flow reserve, suggesting an enhancement of microcirculatory function. This suggests that RIPC confers cardioprotection during PCI as a result of an improvement in coronary microcirculatory function. The application of RIPC to augment the coronary microcirculation in other clinical settings demonstrating microcirculatory dysfunction also warrants investigation.

Chapter 4: The effect of remote ischaemic preconditioning on traditional markers of platelet activation

4.1 Introduction

As discussed in chapter 1, RIPC at the time of primary and elective PCI has been shown to be associated with reduced myocardial injury and reduced thrombotic events during follow up (13,14,74,78). Platelets play an important pathological role in the occlusion of the coronary artery lumen, obstructing coronary blood flow and leading to acute coronary syndromes (ACS) with associated morbidity and mortality. Hence, inhibition of platelet activity is an important treatment strategy (3,4,162). Upon this basis, a study was conducted to explore the effects of RIPC on platelet activity, in a population being investigated and treated for potential CAD.

As summarised in section 1.2.4, RIPC has been shown to reduce platelet activity as determined by flow cytometry based analysis of surface markers of activation. In the previously performed studies, with the exception of the study by Lanza et al. (57), participants were either taking aspirin (most studies) or no antiplatelet medications, and few have investigated the effects of RIPC in patients taking $P2Y_{12}$ inhibitors. The effect of RIPC on the activation of GPIIb-IIIa has not previously been investigated. Additionally, the effects of RIPC after exposure of platelets to agonists other than ADP, such as thrombin and collagen, which are particularly relevant at the site of coronary atherosclerotic plaques, has not been investigated. Given the proposed benefits of RIPC in the setting of elective and primary PCI, where DAPT is commonly used, establishing the effects of RIPC on platelet activation in the presence of DAPT is important and has not been established.

The aims of the studies presented in this chapter were to investigate the effect of RIPC on specific pathways of platelet activation under basal conditions and in response to ADP, SFLLRN (thrombin receptor PAR-1 agonist), thrombin and a combination of thrombin and collagen. Given the contextual importance of antiplatelet therapy in the clinical studies demonstrating a benefit of RIPC, the present studies investigated the effects of RIPC in patients recruited from the cardiac catheterisation laboratory, with suspected CAD, taking contemporary antiplatelet therapy, with a significant number of patients on DAPT. As the aim of this study was to investigate resting and soluble agonist-mediated effects on platelet activation, all platelet studies were performed on samples taken prior to cardiac catheterisation so that there was no confounding by the procedure itself.

4.2 Methods

4.2.1 Remote ischaemic preconditioning and markers of platelet activation

Patients referred by their physician for non-urgent coronary angiography at Concord Hospital, Sydney, Australia, with symptoms or non-invasive investigations suggestive of significant CAD, were invited to participate as described in chapter 2. Patients were randomised in a 1:1 ratio to receive RIPC or sham treatment prior to their planned procedure as described in section 2.1 (randomised platelet cohort). Venous blood was collected before and immediately after the allocated treatment as described in section 2.4. The blood samples were processed within 20 min of collection. Patients and investigators processing the blood samples were blinded to patient treatment allocation. The study design is summarised in Figure 4.1. This cohort was separate from the cohort described in chapter 3.

An additional cohort of patients presenting for coronary angiography were recruited to investigate the changes in intracellular phosphorylated-VASP, cAMP and cGMP (intracellular platelet cohort). These patients all received RIPC with blood collected before and after treatment as in the randomised cohort.

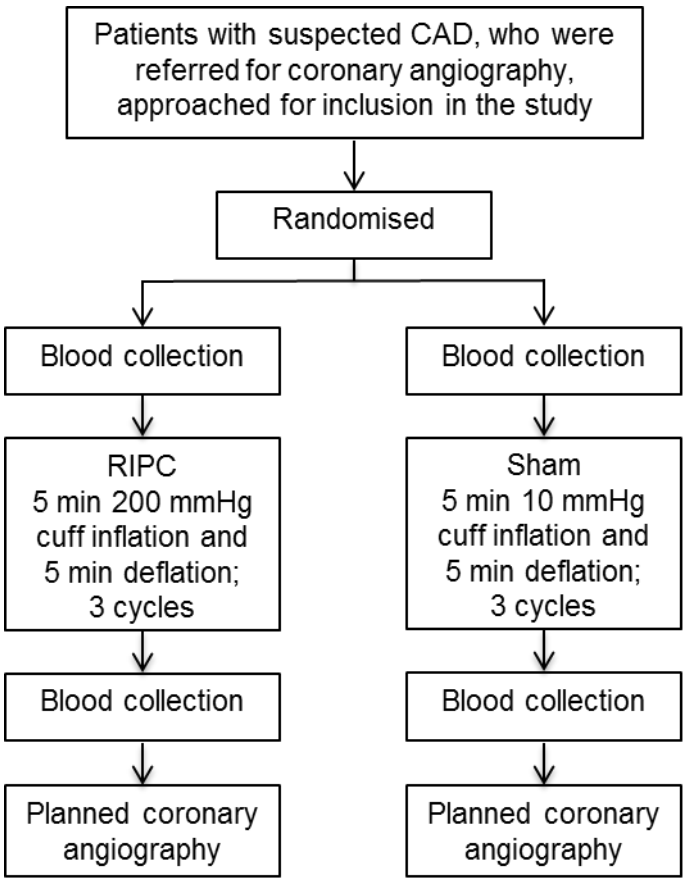


Figure 4.1 Patient recruitment and randomisation – Randomised platelet cohort

Patients with suspected CAD, referred for cardiac catheterisation, were approached prior to their planned procedure. After informed consent was obtained, patients were randomised to RIPC or sham treatment with blood collected before and after the allocated treatment. All blood samples were collected prior to cardiac catheterisation.

CAD, coronary artery disease; RIPC, remote ischaemic preconditioning;

4.2.2 Assessment of platelet activation

Unless otherwise stated, the assessment of platelet activation was performed in whole blood collected into tubes containing either sodium citrate or hirudin. Blood samples collected before and after RIPC/sham were processed in the same manner.

4.2.2.1 Surface markers

As described in detail in section 2.5.1, platelet CD62P expression, CD63 expression and PAC-1 binding (conformationally active GPIIb-IIIa) were quantified in whole blood collected with sodium citrate as the anticoagulant (119,125). The expression of these markers was quantified by flow cytometry as previously described (116,126). In brief, whole blood was added to reaction mixes of DPBS with and without agonists ADP (final concentration, 5 μM), protease-activated receptor-1 (PAR-1) agonist (SFLLRN, 20 μM), thrombin (2 U/mL) ± collagen (10 μg/mL). H-Gly-Pro-Arg-Pro-OH (173 μg/mL) was added to thrombin containing reaction mixes to minimise fibrinogen aggregation and fibrin polymerisation. Reaction mixes were immediately added to FACS tubes containing isotype control antibodies and antibodies to CD61, CD62P, CD63 or PAC-1. After incubation at room temperature for 20 min, the samples were fixed with 0.16 % paraformaldehyde in saline before immediate analysis by flow cytometry on a BD FACSCalibur with 10,000 platelet gated events, based on light scatter characteristics, acquired for each analysis. The isotype control was used to determine positive events on the basis of a 0.5% background expression. Levels of CD62P and CD63 expression and PAC-1 binding were then quantified as a percentage of total platelets.

4.2.2.2 Platelet aggregometry

Platelet aggregometry was performed with blood collected into hirudin containing tubes with a Multiplate™ analyser. ADP (6.67 μM) and arachidonic acid (1 mM) were used as agonists for aggregometry and results are presented as a mean of the duplicate measurements expressed as area under the curve (AUC).

4.2.2.3 Assessment of platelet-leukocyte aggregates

As described in section 2.5.5, the reaction mixtures of whole blood containing 5 μM ADP, 20 μM SFLLRN, 2 U/mL Thrombin, with H-Gly-Pro-Arg-Pro-OH (173 μg/mL) added to the thrombin reaction mix, were used to assess PLAs. These were added to FACS tubes containing an isotype control antibody and antibodies to CD45 and CD42b. After incubation at room temperature for 20 min, the samples were fixed with a combination of paraformaldehyde, Hank's buffered salt solution and water. After a further 10 min, water was added to stabilise the osmotic balance and lyse the erythrocytes. The samples were analysed by flow cytometry with 1000 monocyte gated events, based on light scatter characteristics and CD45 expression, acquired for each analysis. The isotype control was used to determine positive events based on 0.5% background expression. The proportion of monocyte and granulocyte events, based on light scatter characteristics and the leukocyte marker CD45, which expressed the platelet marker CD42b were quantified. The percentage of platelet-leukocyte aggregates was expressed as a percentage of total events.

4.2.3 Assessment of circulating regulators of platelet function

Stored plasma from the randomised platelet cohort was used to assess the effect of RIPC on circulating regulators of platelet function. The plasma was analysed with the

following commercially available kits as per the manufacturers' instructions: cAMP Parameter Assay, cGMP Parameter Assay and Total Nitric Oxide and Nitrate/Nitrite (R&D Systems), Prostacyclin (MyBioSource, San Diego, California, USA), and 6-keto-PGF1α (Abcam, Cambridge, United Kingdom). The ELISA plates were analysed with an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, Massachusetts, USA) with the concentration of each sample determined from a standard curve derived according to the manufacturer's guidelines.

4.2.4 Intracellular VASP and phosphorylated-VASP assessment

The intracellular regulatory protein VASP, is known to inhibit GPIIb-IIIa activation in its phosphorylated form (163,164). Platelet pellet lysates stored with a mixture of Mammalian Protein Extraction Reagent, protease inhibitor mix and PhosSTOP, prepared from PRP collected before and after RIPC/sham as described in section 2.8, were used to assess intracellular VASP and phosphorylated-VASP. Lysates prepared from platelets under basal conditions and after SFLLRN stimulation were analysed in the same manner. The bicinchoninic acid (BCA) protein assay (Thermofisher) was used to measure protein concentrations in the platelet lysates with this data used to load equal quantities of protein onto 4–12% bis–Tris gels (Thermofisher). The iBlot transfer apparatus (Thermofisher) was used to transfer proteins to nitrocellulose membranes. The membranes were blocked by incubation in Tris-buffered saline containing 0.1% (v/v) Tween-20 and 4% (w/v) bovine serum albumin for 60 min. Membranes were probed with antibodies against VASP (Cell Signalling Technology, Danvers, Massachusetts, USA) or phosphorylated-VASP (Cell Signalling Technology) overnight at 4 °C, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) for 1 h. Blots were

visualised using enhanced chemiluminescence reagents and the Gel Doc XR + CCD imaging system (BioRad, Hercules, California, USA). Images were acquired before signal saturation occurred. Densitometric quantitation and estimation of apparent molecular mass of bands were performed using the ImageLab v4.1 software (BioRad).

4.2.5 Intracellular cAMP and cGMP assessment

Platelet pellet lysates prepared from blood collected before and after RIPC were stored with a cell lysis buffer from the cAMP and cGMP Parameter Assay Kits (R&D Systems) as described in section 2.8. These platelet lysates were used to assess changes in cAMP and cGMP with RIPC treatment. The platelet lysates were analysed with cAMP and cGMP ELISA kits as per the manufacturers' instructions. The samples were diluted aiming for a platelet count of 1 x 10^7/mL as recommended by the manufacturer. The optical absorbance of each well in the ELISA plates was assessed with an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, Massachusetts, USA) with the concentration of each sample determined from a standard curve.

4.2.6 Statistical analysis

Categorical variables are presented as frequency and percentage while continuous variables are expressed as mean ± SD for parametrically distributed data and median (IQR) for non-parametrically distributed data. Data distribution was assessed with the D'Agostino and Pearson normality test. Categorical data were compared using χ^2 tests or Fisher's exact test as appropriate. Comparisons between continuous variables were performed using paired or unpaired t tests as appropriate for parametrically distributed data, and Wilcoxon signed rank or Mann-Whitney U tests as appropriate for non-parametric data. Correlations between variables were assessed by Pearson's correlation

or Spearman rank correlation for parametric and non-parametric data respectively. Ratios of post-treatment to pre treatment parameters were assessed with a one sample t test. The pre- and post-treatment expression of markers of platelet activation were compared in patients randomised to RIPC or sham treatment. The percentage change (section 2.9) in the RIPC cohort was compared with the percentage change with sham using the Mann-Whitney U test in analyses where the paired comparison demonstrated an effect of RIPC.

Based on preliminary data collected from 10 patients who underwent the RIPC study protocol in a non-randomised fashion prior to commencement of the randomised study, an absolute reduction in PAC-1 binding after agonist stimulation of 7.4% ± 9.8% (mean ± SD) was expected with RIPC. With a power of 80% and a two-sided α value of 0.05, it was estimated that 27 patients would need to be studied for paired comparison. Hence, in the randomised platelet cohort, 60 patients were recruited and randomised in a 1:1 fashion to receive either RIPC or sham treatment.

4.3 Results

There were 60 patients recruited into the randomised platelet cohort with 31 randomised to the RIPC group and 29 to the sham group. The intracellular platelet cohort consisted of another 18 patients who were all treated with RIPC to study mechanistic aspects of intracellular signalling pathways. There were no complications of RIPC. All patients tolerated RIPC well with no patients requesting cessation of the treatment protocol.

4.3.1 Baseline clinical characteristics

The randomised cohort had a mean age of 63.3 ± 13.2 yr and consisted of 51 (85%) male patients. The cohort had 52 (87%) patients who were clinically stable, 43 (72%) were on dual antiplatelet therapy with aspirin and a $P2Y_{12}$ inhibitor (DAPT), 6 (10%) were on intravenous heparin and 48 (80%) were found to have significant coronary artery disease based on their coronary angiogram. This was determined by the presence of at least 50% stenosis in a major epicardial coronary artery based on 2 dimensional QCA. There was no significant difference between the RIPC and sham groups in terms of baseline characteristics and medications (Table 4.1). There was a trend towards lower haemoglobin concentration in the sham group but this is unlikely to have affected the results given the primary analysis was a paired comparison of platelet activation markers before and after treatment within each group.

Table 4.1 Baseline characteristics of randomised platelet cohort

Characteristic	RIPC (n=31)	Sham (n=29)	P value
Age - years	64.0 ± 6.9	62.6 ± 17.8	0.70
Male – no. (%)	28 (90)	23 (79)	0.23
Clinically stable – no. (%)	27 (87)	25 (86)	0.92
Significant CAD – no. (%)	25 (81)	23 (79)	0.90
Prior myocardial infarction – no. (%)	8 (26)	8 (28)	0.88
Prior PCI – no. (%)	7 (23)	8 (28)	0.66
Prior CABG- no. (%)	1 (3)	1 (3)	0.96
Heart failure – no. (%)	2 (7)	2 (7)	0.95
Prior stroke – no. (%)	1 (3)	3 (10)	0.27
Peripheral vascular disease – no. (%)	2 (7)	2 (7)	0.95
Hypertension – no. (%)	20 (65)	21 (72)	0.51
Diabetes – no. (%)	11 (36)	11 (38)	0.84
Dyslipidaemia – no. (%)	20 (65)	22 (76)	0.34
Current smoking – no. (%)	5 (16)	7 (24)	0.44
Medications*			
Aspirin – no. (%)	30 (97)	27 (93)	0.51
$P2Y_{12}$ antagonist – no. (%)	21 (68)	23 (79)	0.31
Clopidogrel – no. (%)	14 (45)	18 (62)	0.19
Ticagrelor – no. (%)	6 (19)	4 (14)	0.56
Prasugrel – no. (%)	1 (3)	1 (3)	0.96
Dual antiplatelet therapy – no. (%)	21 (68)	22 (76)	0.49
Warfarin/ NOAC – no. (%)	0 (0)	0 (0)	-
Heparin – no. (%)	3 (10)	3 (10)	0.93
Statin – no. (%)	22 (71)	25 (86)	0.15
Beta blocker – no. (%)	17 (55)	16 (55)	0.98
ACEi or ARB – no. (%)	19 (61)	18 (62)	0.95
Nitrate – no. (%)	7 (23)	5 (17)	0.61
Parameters during admission			
Systolic blood pressure – mmHg	141.5 ± 21.2	142.3 ± 21.1	0.89
Heart rate – beats/min	67.9 ± 10.4	71.7 ± 8.3	0.13
Haemoglobin concentration – g/L	141.9 ± 14.7	133.5 ± 18.0	0.052
Platelet count – 10^9/L	223.6 ± 58.7	229.5 ± 71.2	0.73
eGFR - mL/min/1.73m^2	81.5 ± 10.8	76.2 ± 15.4	0.13

P values compare characteristics from RIPC and sham treatment groups using unpaired t tests for continuous data. Categorical data were compared using χ^2 tests or Fisher's exact test as appropriate.

* The number of patients on dual antiplatelet therapy, aspirin monotherapy, $P2Y_{12}$ monotherapy and no antiplatelet therapy in the RIPC and sham groups were 21, 9, 0, 1 and 22, 5, 1, 1 respectively.

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; NOAC, novel oral anticoagulant; PCI, percutaneous coronary intervention;

4.3.2 Patients undergoing coronary angiography demonstrate substantial platelet activation despite antiplatelet therapy

Examining the baseline (pre RIPC/sham) data in the whole randomised cohort (n=60), it was evident that there was substantial residual platelet activation in response to all agonists despite the administration of DAPT (Figure 4.2). Patients receiving DAPT (n=43, 31 clopidogrel, 10 ticagrelor, 2 prasugrel) had significantly less PAC-1 binding to unstimulated circulating platelets (basal), and less PAC-1 binding in response to stimulation with ADP and SFLLRN, compared to patients receiving aspirin alone (n=14). However, there were similarly high levels of PAC-1 binding in response to thrombin alone and to thrombin and collagen in patients on DAPT and in patients on aspirin alone, indicating significant levels of GPIIb-IIIa activation (Figure 4.2a). Similarly, DAPT therapy was also associated with reduced CD62P and CD63 expression after stimulation with ADP, however CD62P and CD63 expression after thrombin and thrombin and collagen appeared to be unaffected by the administration of DAPT (Figure 4.2b and 4.2c). Analysis of the pre RIPC/sham platelet aggregometry results demonstrated that patients on DAPT had significantly lower levels of platelet aggregation after stimulation with ADP. A similar trend was seen in response to arachidonic acid, but this did not reach significance (Figure 4.3).

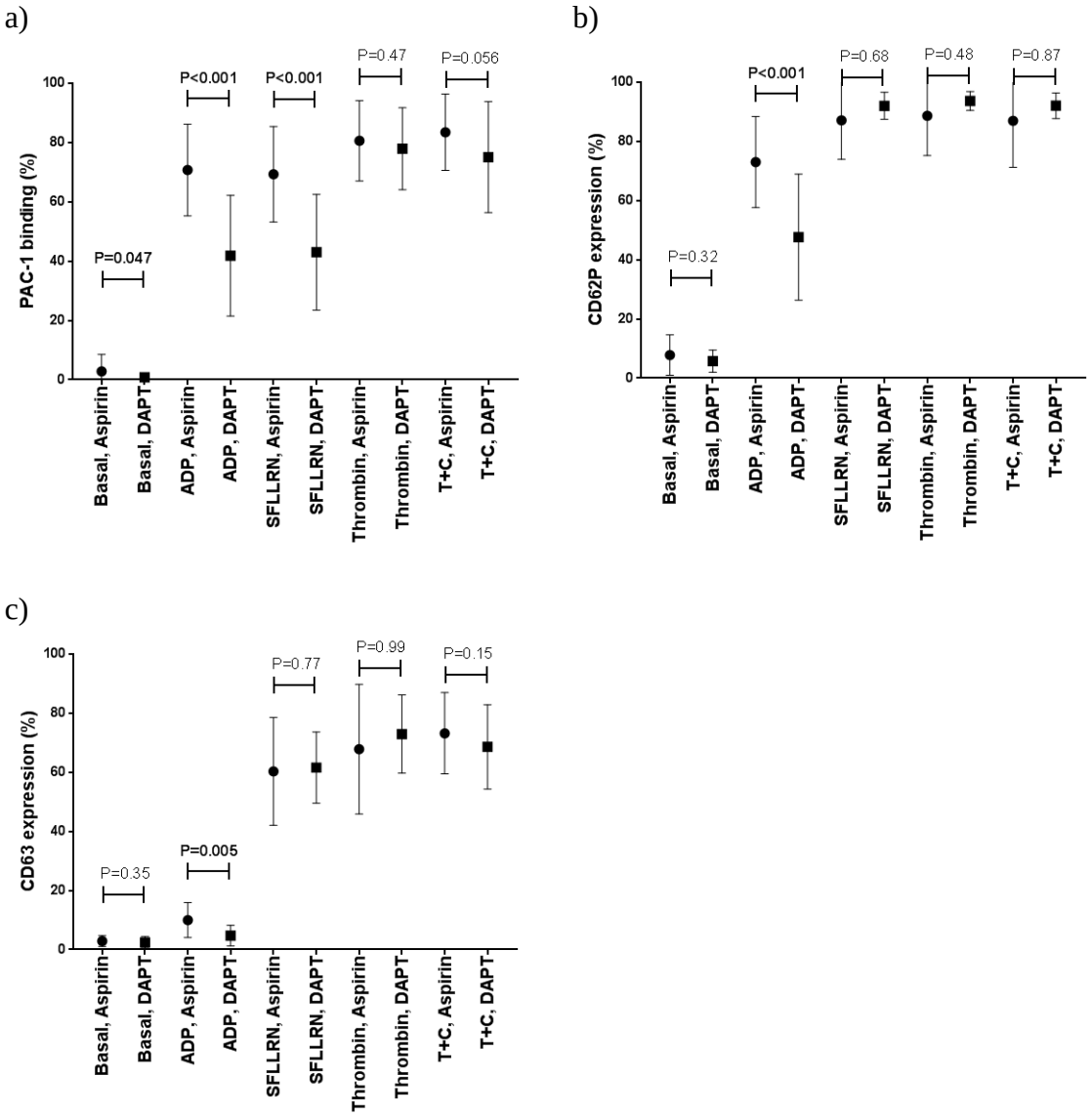


Figure 4.2 Platelet activation in response to agonist stimulation, stratified by antiplatelet therapy

Data from pre RIPC/sham platelet analysis. Comparison of platelet activation markers, PAC-1 binding (a), CD62P expression (b) and CD63 expression (c), without agonist stimulation (basal) and with agonist stimulation in patients on aspirin monotherapy or DAPT. Aspirin monotherapy, n=14; DAPT, n=43; Symbol and bars represent mean ± SD. P values compare measurements from patients on aspirin or DAPT using the unpaired t test for parametrically distributed data or the Mann-Whitney U test for non-parametrically distributed data.

ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

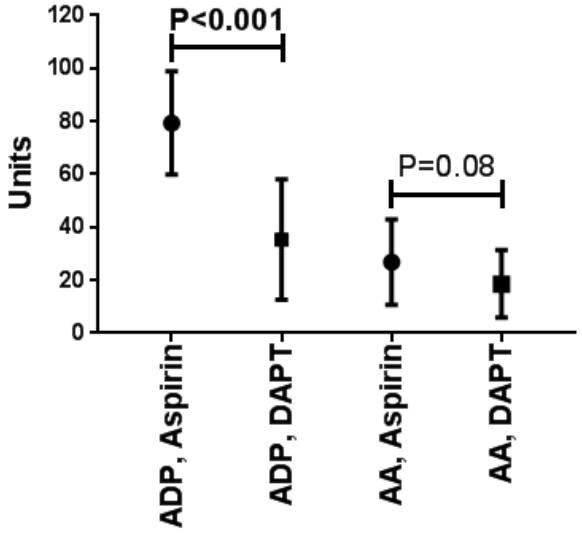


Figure 4.3 Platelet aggregometry in response to agonist stimulation, stratified by antiplatelet therapy

Data from pre RIPC/sham platelet analysis. Comparison of platelet aggregometry in response to ADP or AA in patients on aspirin monotherapy or DAPT. Aspirin monotherapy, n=14; DAPT, n=43; Symbol and bars represent mean ± SD. P values compare measurements from patients on aspirin or DAPT using the unpaired t test for parametrically distributed data or the Mann-Whitney U test for non-parametrically distributed data.

AA, arachidonic acid; ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy;

4.3.3 The effect of remote ischaemic preconditioning on resting and agonist-induced binding of PAC-1 and expression of CD62P and CD63

The results of the flow cytometry analyses of paired blood samples collected from the RIPC-and sham-treated groups are displayed in Table 4.2.

Table 4.2 The effect of remote ischaemic preconditioning and sham treatment on surface platelet activation markers in response to agonists

Marker*	**RIPC (n=31)**			**Sham (n=29)**		
	Pre	**Post**	**P**	**Pre**	**Post**	**P**
Basal						
PAC-1	0.6 (0.3-1.4)	0.6 (0.4-1.1)	0.56	0.6 (0.4-1.2)	0.8 (0.3-1.9)	0.38
CD62P	3.9 (3.4-7.7)	5.0 (3.6-7.4)	0.22	5.9 (3.4-8.2)	5.6 (3.9-8.6)	0.63
CD63	2.0 (1.1-2.8)	1.7 (1.3-3.2)	0.29	2.7 (1.3-3.6)	2.4 (1.5-3.6)	0.90
5 µM ADP						
PAC-1	49.1 (31.5-74.6)	45.8 (22.8-69.8)	0.14	49.1 ± 23.1	49.9 ± 20.7	0.67
CD62P	51.8 (28.8-71.6)	53.2 (35.9-74.1)	0.26	56.6 ± 22.9	55.9 ± 24.0	0.59
CD63	3.5 (1.6-8.5)	4.7 (2.3-10.8)	**0.01**	6.2 (3.2-8.8)	5.2 (2.2-10.8)	0.80
20 µM SFLLRN						
PAC-1	50.4 (31.3-73.2)	49.3 (23.0-67.7)	**0.002**	49.1 ± 22.5	47.5 ± 21.7	0.38
CD62P	93.7 (87.5-95.9)	94.2 (87.9-95.6)	0.22	93.9 (91.3-95.3)	94.2 (89.5-95.1)	0.65
CD63	65.4 (50.9-70.8)	67.1 (50.1-72.8)	0.14	65.8 (55.0-69.2)	66.4 (59.7-71.9)	0.11
2 U/mL thrombin						
PAC-1	78.7 (70.3-89.7)	83.5 (63.8-90.2)	0.92	82.2 (76.2-88.2)	86.0 (72.1-89.7)	0.81
CD62P	94.6 (91.8-96.7)	95.5 (92.0-96.5)	0.36	95.0 (90.5-96.0)	94.6 (92.0-96.4)	0.16
CD63	77.8 (66.1-82.8)	74.7 (63.5-83.7)	0.61	77.2 (70.6-83.1)	78.1 (74.3-83.6)	0.26
2 U/mL thrombin and 10 µg/mL collagen						
PAC-1	79.5 ± 11.9	68.9 ± 22.5	**<0.001**	82.9 (68.6-87.0)	85.5 (65.9-87.9)	0.80
CD62P	94.0 (89.5-96.1)	93.7 (91.2-95.6)	0.88	94.1 (87.5-95.3)	92.4 (89.1-95.4)	0.32
CD63	75.8 (63.6-80.7)	74.7 (52.3-79.9)	0.35	74.4 (66.4-79.1)	73.1 (64.0-82.6)	0.98

P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test or the Wilcoxon signed rank test for parametrically and non-parametrically distributed continuous data respectively.

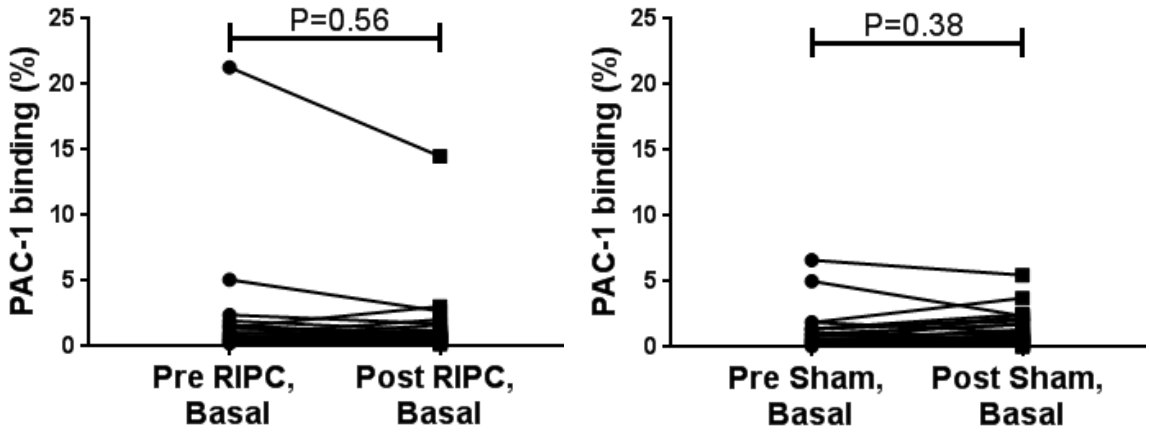
* Values represent percentage (%) PAC-1 binding or percentage CD62P and CD63 expression.

ADP, adenosine diphosphate; RIPC, Remote ischaemic preconditioning; SFLLRN, PAR-1 agonist;

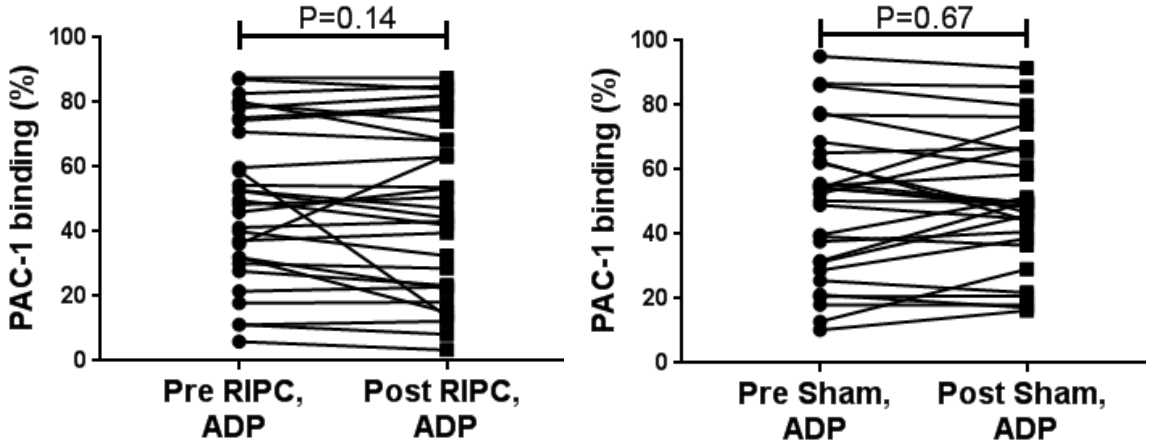
4.3.3.1 PAC-1

RIPC treatment had no significant effect on the percentage of unstimulated platelets expressing the conformationally active form of GPIIb-IIIa (PAC-1 binding). There was also no effect of RIPC on PAC-1 binding in response to ADP. However, RIPC did decrease GPIIb-IIIa active conformational change in response to stimulation with SFLLRN (50.4% (31.3-73.2) vs 49.3% (23.0-67.7), P=0.002) and after stimulation with thrombin and collagen (79.5 ± 11.9% vs 68.9 ± 22.5%, P<0.001). In contrast, there was no effect of sham treatment on PAC-1 binding in unstimulated conditions or in response to agonists (Figure 4.4). Interestingly, despite the attenuation of GPIIb-IIIa activation in response to SFLLRN and thrombin and collagen, RIPC had no effect on PAC-1 binding induced by thrombin alone.

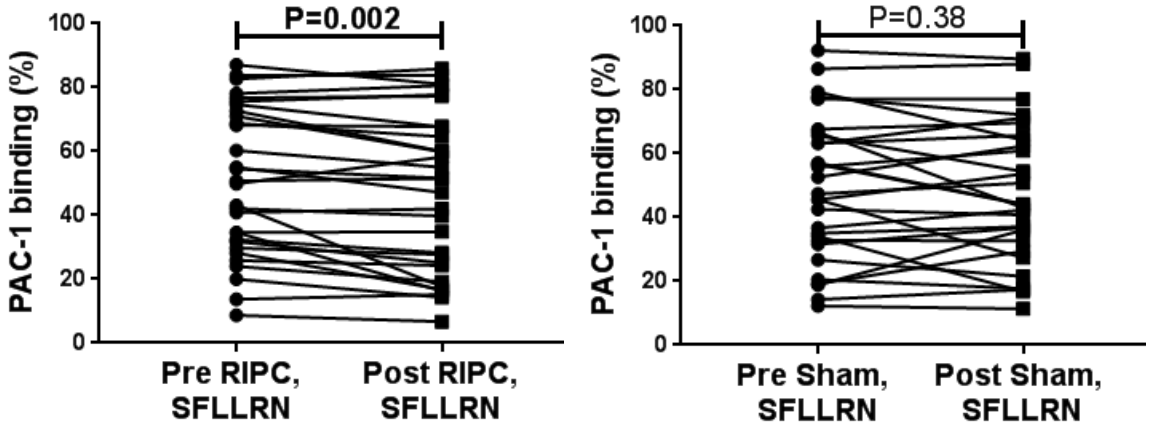
a)


P=0.56
PAC-1 binding (%)
25
20
15
10
5
0
Pre RIPC, Basal
Post RIPC, Basal
P=0.38
PAC-1 binding (%)
25
20
15
10
5
0
Pre Sham, Basal
Post Sham, Basal

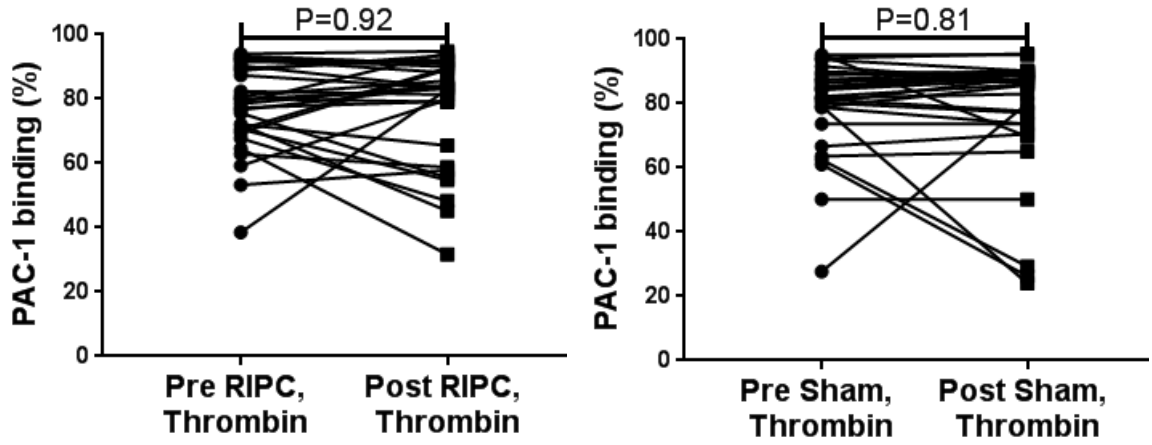
b)


P=0.14
PAC-1 binding (%)
100
80
60
40
20
0
Pre RIPC, ADP
Post RIPC, ADP
P=0.67
PAC-1 binding (%)
100
80
60
40
20
0
Pre Sham, ADP
Post Sham, ADP

c)


P=0.002
PAC-1 binding (%)
100
80
60
40
20
0
Pre RIPC, SFLLRN
Post RIPC, SFLLRN
P=0.38
PAC-1 binding (%)
100
80
60
40
20
0
Pre Sham, SFLLRN
Post Sham, SFLLRN

d)



e)

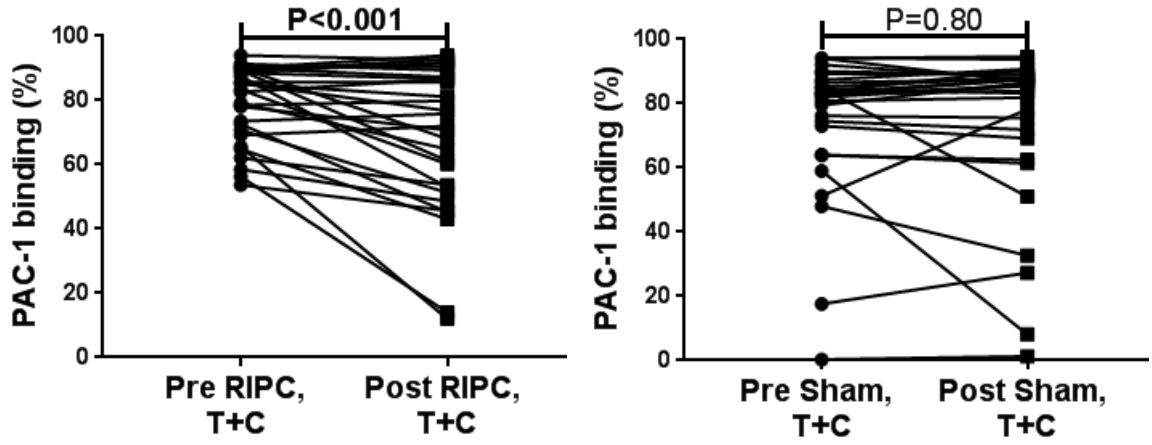


Figure 4.4 The effect of remote ischaemic preconditioning on glycoprotein IIb-IIIa conformational activation

Effect of RIPC (left panels) or sham (right panels) on PAC-1 binding without stimulation (basal) (a) and in response to ADP (b), SFLLRN (c), thrombin (d) and thrombin and collagen (e). RIPC, n=31 patients; Sham, n=29 patients; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine di-phosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

There was a significantly greater percentage reduction in PAC-1 binding in response to thrombin and collagen with RIPC than sham (-7.1% (-27.6 - +1.9) vs +0.5% (-3.9 - +4.8), P=0.009). Similarly there was a greater percentage reduction in PAC-1 binding in response to SFLLRN with RIPC than sham (-6.6% (-18.1 - +1.0) vs -0.1% (-18.1 - +14.1), P=0.07) but this did not reach statistical significance.

The attenuation of GPIIb-IIIa activation by RIPC was also observed in the subgroup of patients receiving DAPT (n=21). In these patients there was a significant reduction in PAC-1 binding in response to SFLLRN (43.0 ± 20.9% vs 38.1 ± 21.6%, P=0.008) and thrombin and collagen (78.8 ± 10.9% vs 67.8 ± 24.1%, P=0.005) by RIPC (Figure 4.5).

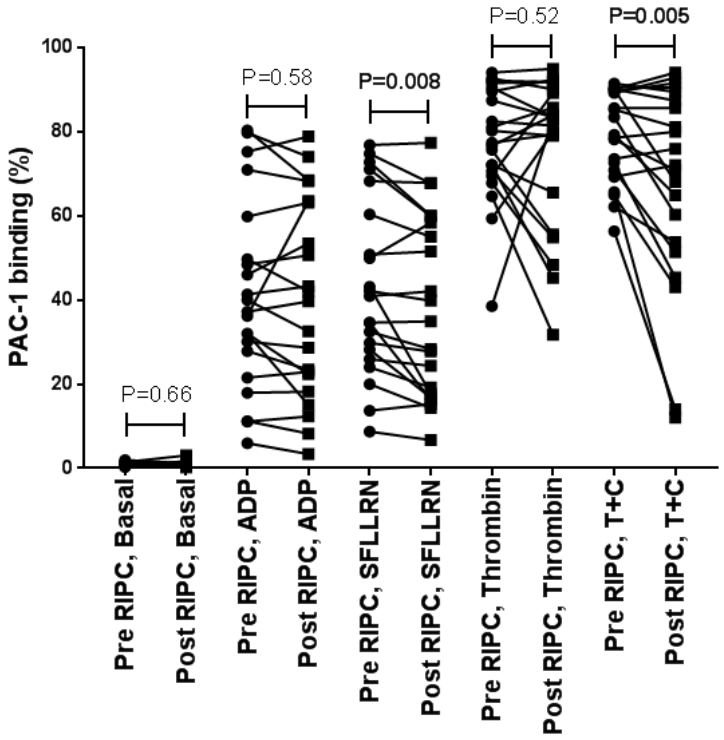


Figure 4.5 The effect of remote ischaemic preconditioning on glycoprotein IIb-IIIa conformational activation in patients on dual antiplatelet therapy

The effect of RIPC on platelet PAC-1 binding under basal conditions and in response to stimulation with ADP, SFLLRN, thrombin, and thrombin and collagen in patients on dual antiplatelet therapy. n=21. P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data. ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

In the 8 patients on aspirin monotherapy, RIPC was also associated with a significant reduction in PAC-1 binding after stimulation with thrombin and collagen (80.7 ± 15.5% vs 69.5 ± 19.7%, P=0.048) and a trend towards a decrease after stimulation with SFLLRN (66.9 ± 18.6% vs 64.0 ± 21.0%, P=0.08).

Coronary angiography demonstrated >50% luminal stenosis in at least one epicardial coronary artery in 25 (81%) patients in the RIPC cohort. In these patients with

significant CAD, there was an attenuation of GPIIb-IIIa activation in response to SFLLRN and thrombin and collagen (Figure 4.6). In the remaining 6 patients treated with RIPC, there was a trend towards reduced PAC-1 binding in response to SFLLRN (71.8% (23.5-83.8) vs 66.2 (15.0-82.2), P=0.09) and thrombin and collagen (87.0% (78.2-90.2) vs 81.7% (61.8-87.6), P=0.16).

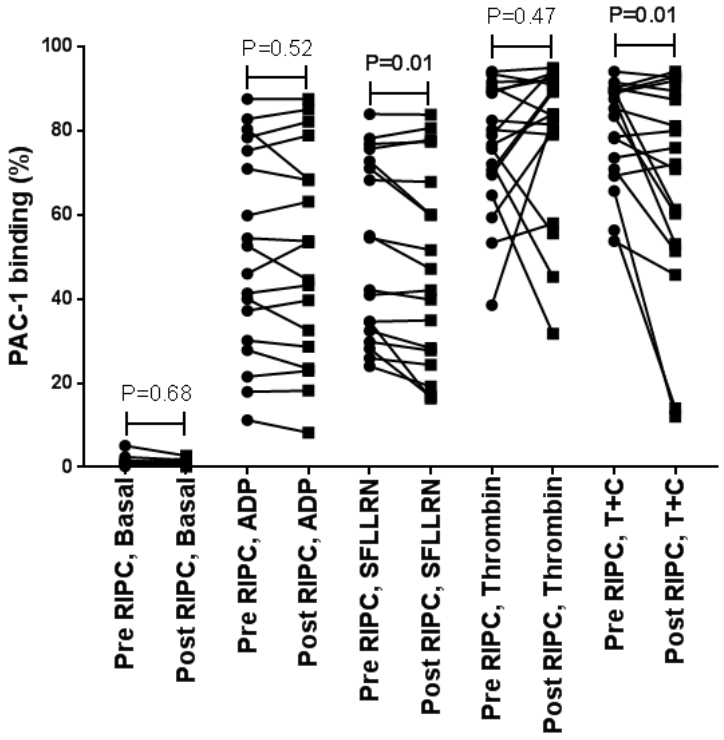


Figure 4.6 The effect of remote ischaemic preconditioning on glycoprotein IIb-IIIa conformational activation in patients with significant coronary artery disease

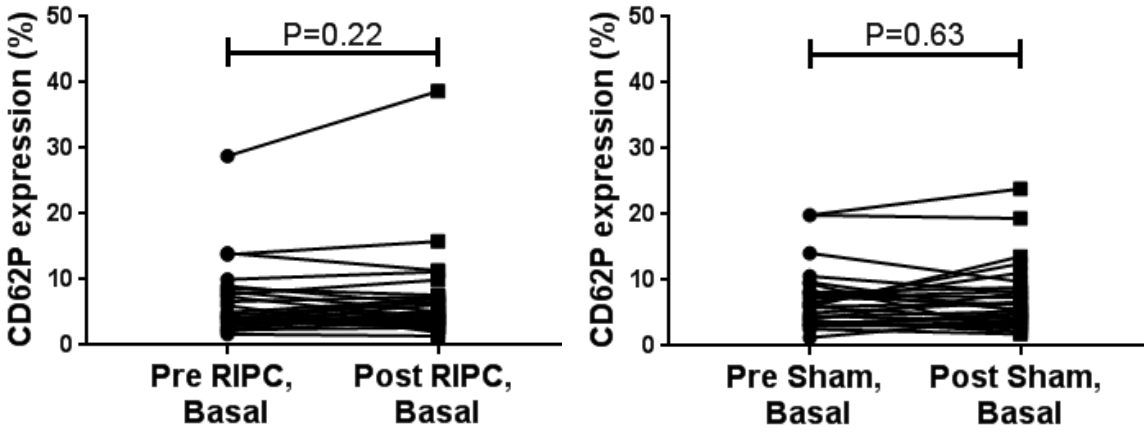
The effect of RIPC on platelet PAC-1 binding under basal conditions and in response to stimulation with ADP, SFLLRN, thrombin, and thrombin and collagen in patients with significant coronary disease demonstrated by coronary angiography. n=25. P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

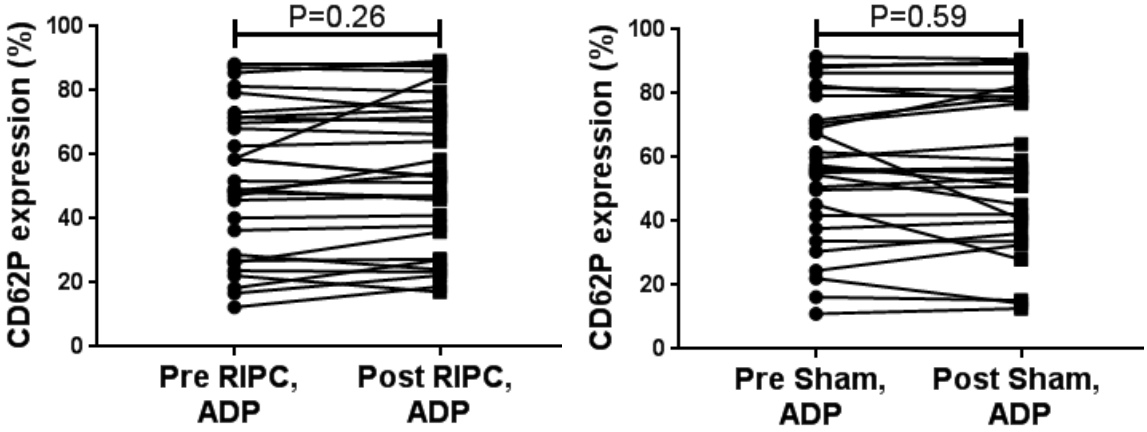
4.3.3.2 CD62P

There was no effect of either RIPC or sham treatment on CD62P expression in unstimulated samples or in response to platelet agonists (Figure 4.7) indicating selectivity for GPIIb-IIIa activation.

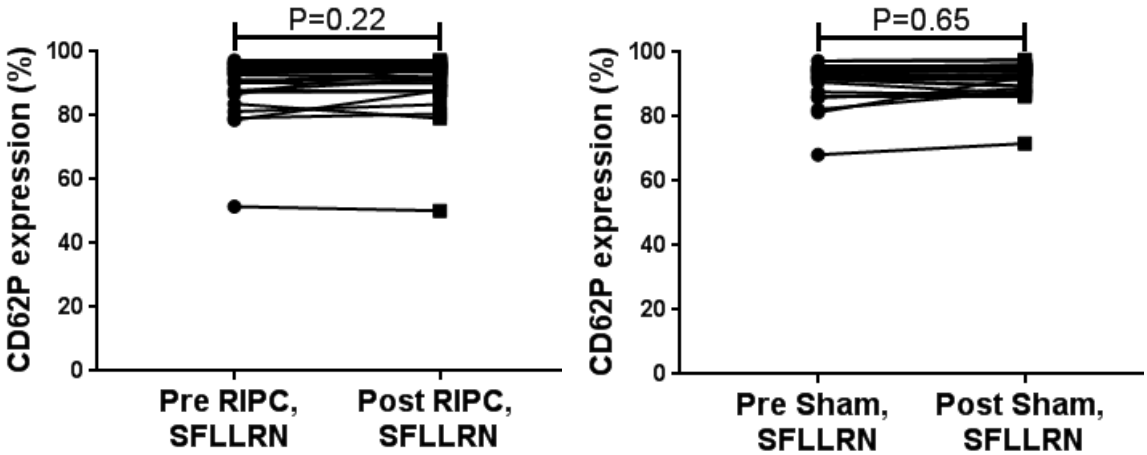
a)


CD62P expression (%)
P=0.22
Pre RIPC, Basal
Post RIPC, Basal
P=0.63
Pre Sham, Basal
Post Sham, Basal

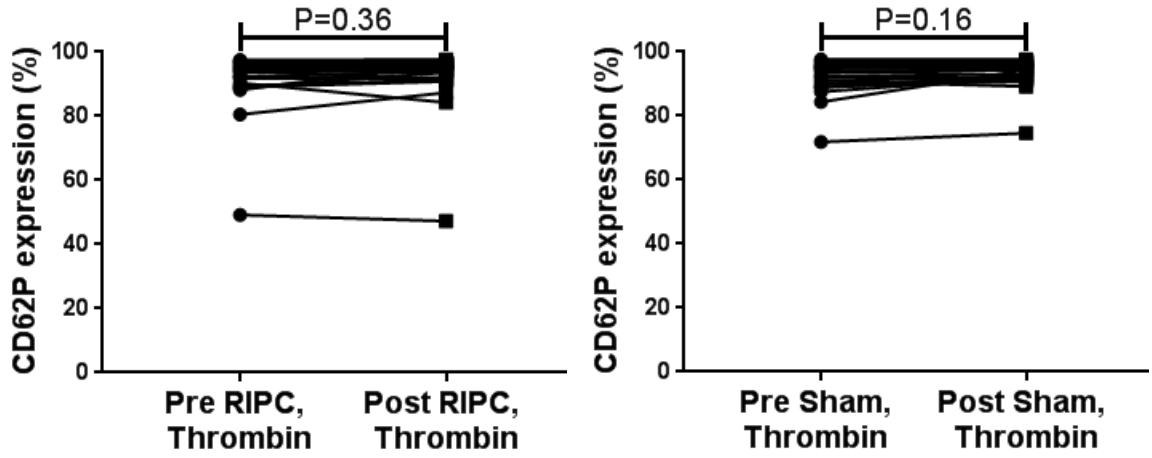
b)


CD62P expression (%)
P=0.26
Pre RIPC, ADP
Post RIPC, ADP
P=0.59
Pre Sham, ADP
Post Sham, ADP

c)


CD62P expression (%)
P=0.22
Pre RIPC, SFLLRN
Post RIPC, SFLLRN
P=0.65
Pre Sham, SFLLRN
Post Sham, SFLLRN

d)



e)

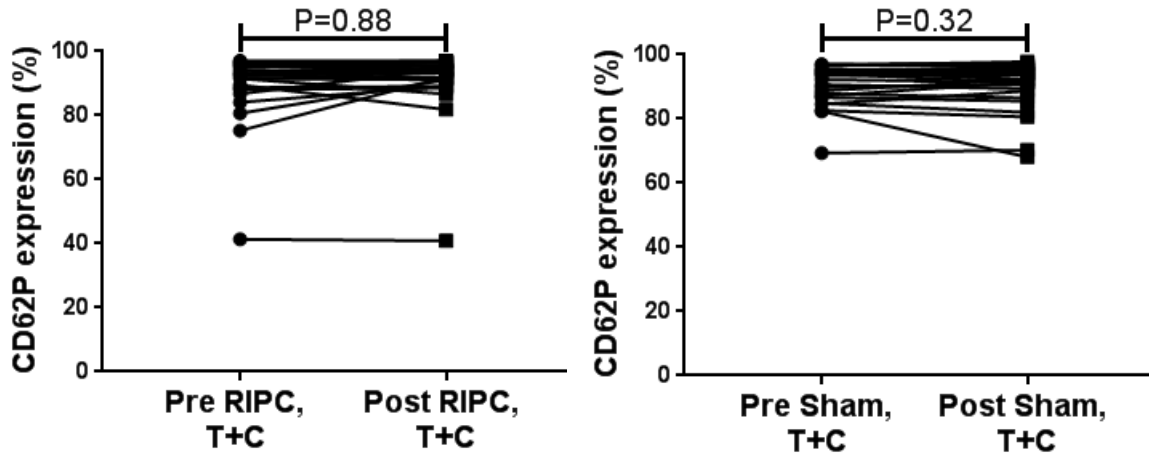


Figure 4.7 The effect of remote ischaemic preconditioning on CD62P expression

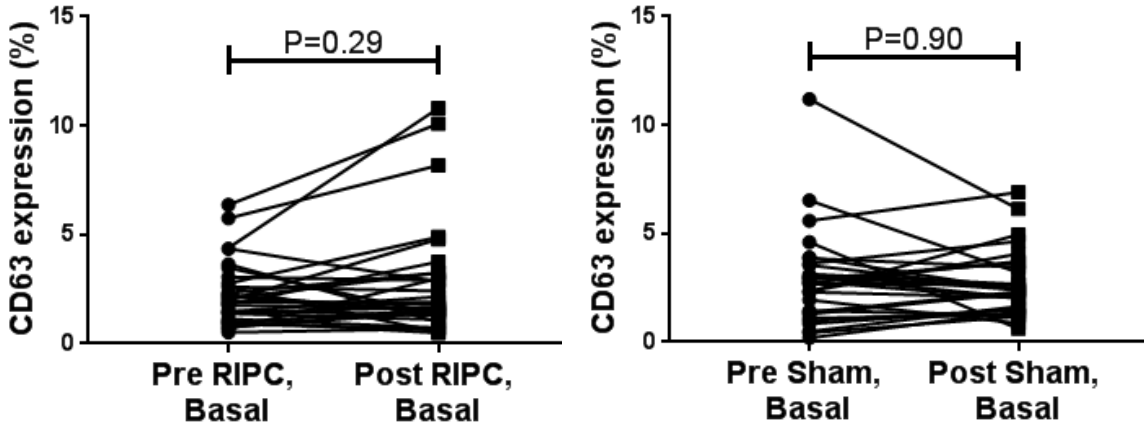
Effect of RIPC (left panels) or sham (right panels) on CD62P expression without stimulation (basal) (a) and in response to ADP (b), SFLLRN (c), thrombin (d) and thrombin and collagen (e). RIPC, n=31; Sham, n=29; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine di-phosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

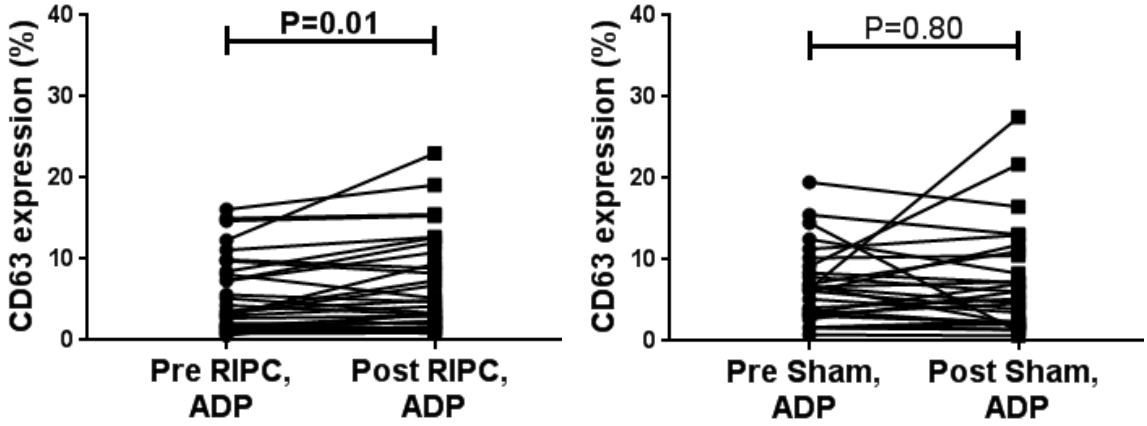
4.3.3.3 CD63

Interestingly there was a small but statistically significant increase in CD63 expression in response to ADP after RIPC (Figure 4.8b). There was no other effect of either RIPC or sham on CD63 expression either in the unstimulated samples or in response to other agonists (Figure 4.8).

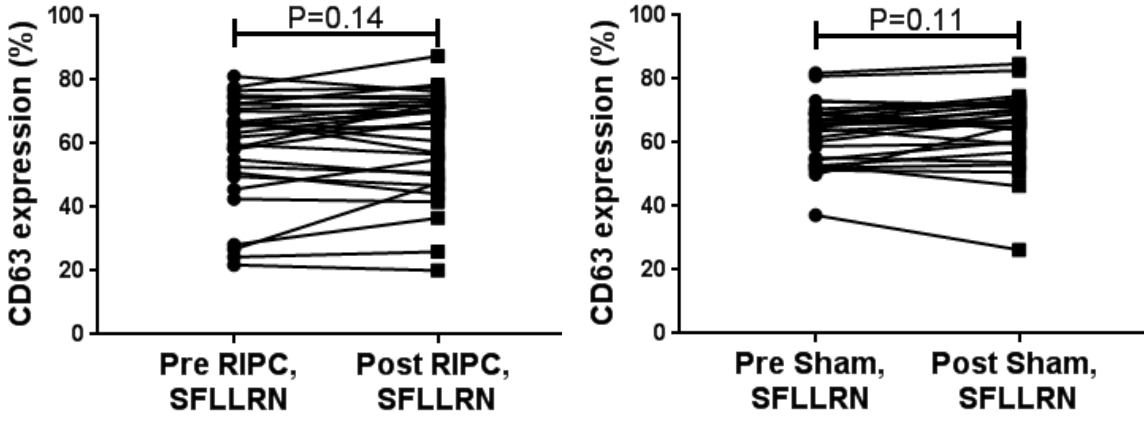
a)


CD63 expression (%)
15
10
5
0
P=0.29
Pre RIPC, Basal
Post RIPC, Basal
P=0.90
Pre Sham, Basal
Post Sham, Basal

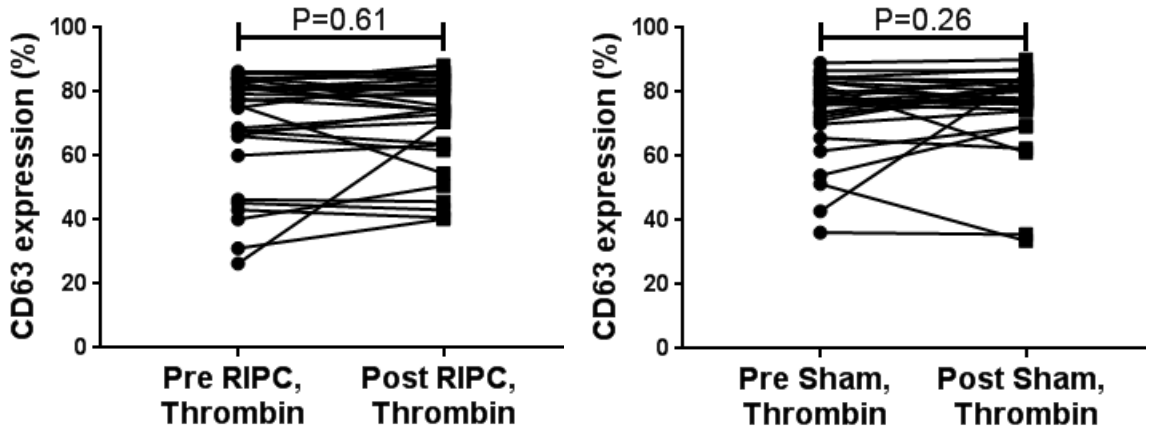
b)


CD63 expression (%)
40
30
20
10
0
P=0.01
Pre RIPC, ADP
Post RIPC, ADP
P=0.80
Pre Sham, ADP
Post Sham, ADP

c)


CD63 expression (%)
100
80
60
40
20
0
P=0.14
Pre RIPC, SFLLRN
Post RIPC, SFLLRN
P=0.11
Pre Sham, SFLLRN
Post Sham, SFLLRN

d)



e)

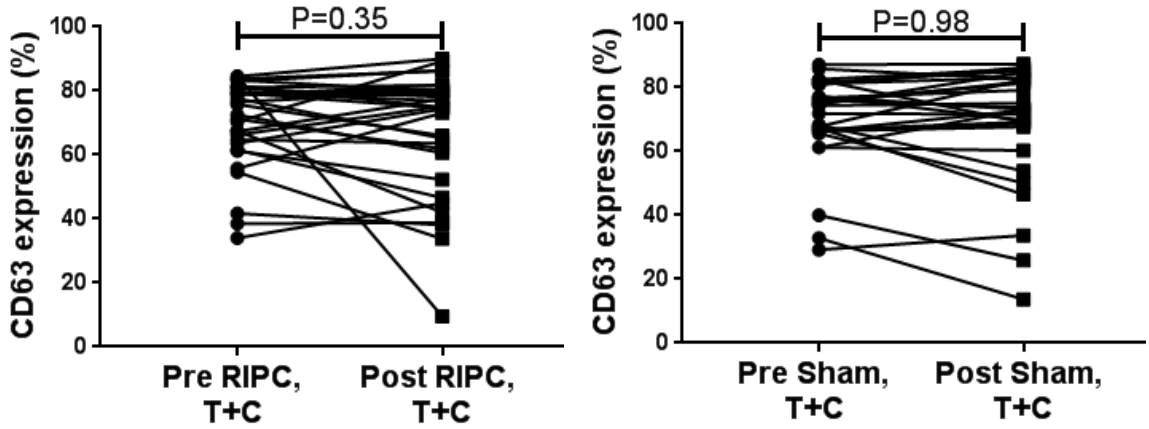


Figure 4.8 The effect of remote ischaemic preconditioning on CD63 expression

Effect of RIPC (left panels) or sham (right panels) on CD63 expression without stimulation (basal) (a) and in response to ADP (b), SFLLRN (c), thrombin (d) and thrombin and collagen (e). RIPC, n=31; Sham, n=29; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

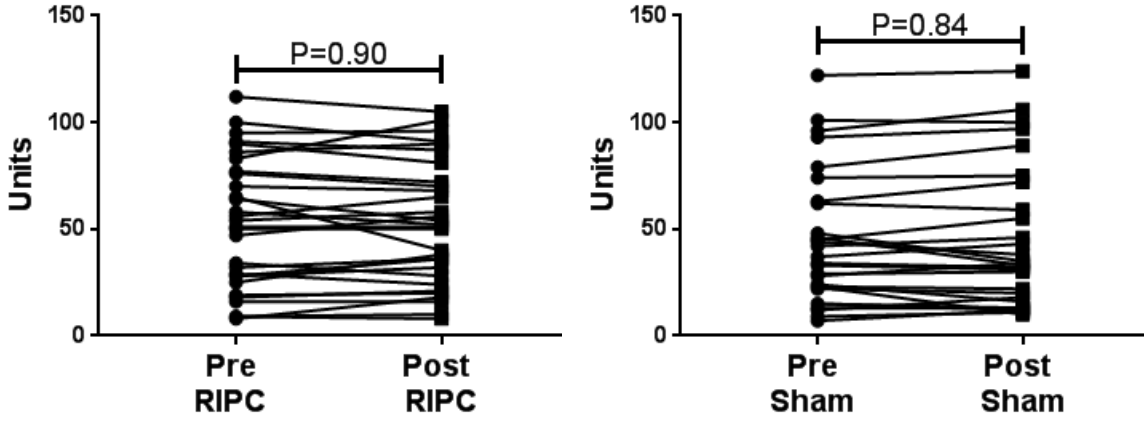
ADP, adenosine di-phosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

There was a greater percentage increase in CD63 expression in response to ADP with RIPC than sham (+14.4% (-10.5 - +54.0) vs -14.1% (-29.7 - +42.2), P=0.07) but this did not reach statistical significance.

4.3.5 The effect of remote ischaemic preconditioning on platelet aggregation

Impedance aggregometry demonstrated no effect of RIPC on platelet aggregation in response to ADP (52.7 ± 30.5 U vs 52.5 ± 28.9 U, P=0.90) or arachidonic acid (15.5 (9.5-38.3) U vs 18.5 (11.5-30.0) U, P=0.78) (Figure 4.9).

a) ADP



b) Arachidonic acid

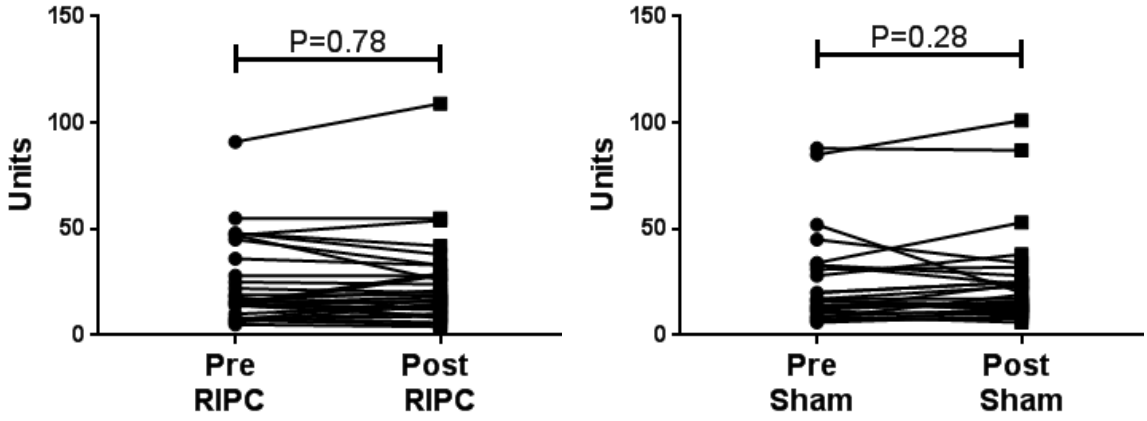


Figure 4.9 The effect of remote ischaemic preconditioning on platelet aggregation

Effect of RIPC (left panels) or sham (right panels) on platelet aggregation, as assessed by impedance aggregometry, in response to a) ADP or b) arachidonic acid. RIPC, n=30; Sham, n=28. P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine di-phosphate; RIPC, remote ischaemic preconditioning.

4.3.6 The effect of remote ischaemic preconditioning on platelet-leukocyte aggregates

PLA were measured in 40 patients from the randomised cohort (20 RIPC, 20 sham). The baseline characteristics of the patients who had PLA assessed did not differ significantly from those who did not. The results of PLA measurement is summarised in Figure 4.10. There was a significant increase in platelet-granulocyte aggregates in response to SFLLRN stimulation with RIPC (62.2 ± 21.6% vs 68.2 ± 19.4%, P=0.008). There was also a significant increase in platelet-monocyte aggregates under basal unstimulated conditions with RIPC (6.9% (5.1-9.1) vs 8.7% (7.4-10.5), P=0.006). RIPC was associated with a small but statistically significant decrease in platelet-monocyte aggregates in response to thrombin stimulation (96.0% (93.1-97.9) vs 95.0% (88.9-96.0), P=0.007).

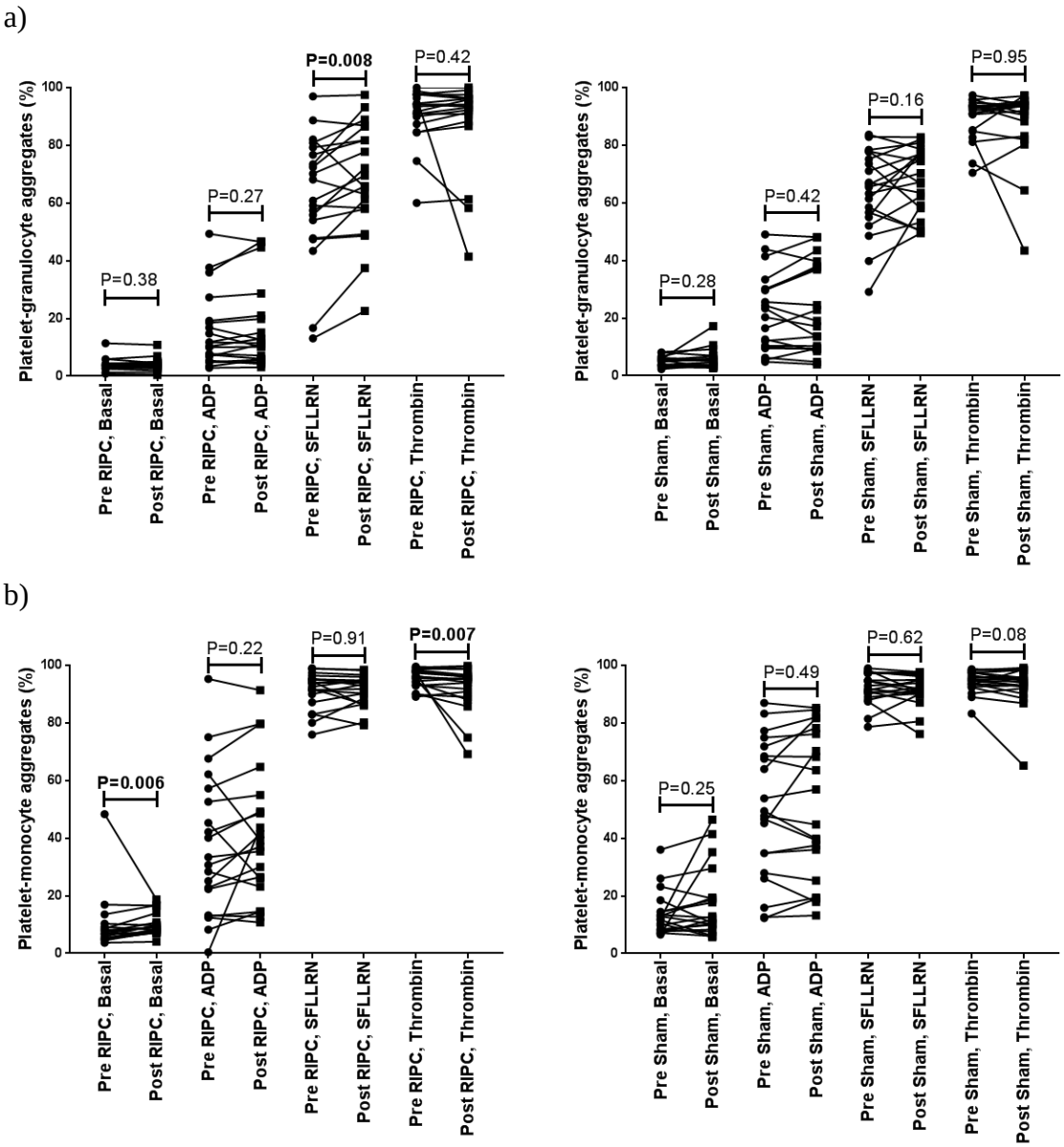


Figure 4.10 The effect of remote ischaemic preconditioning on platelet-leukocyte aggregate formation

Effect of RIPC (left panels) or sham (right panels) on platelet-granulocyte aggregates (a) and platelet-monocyte aggregates (b) formation under basal conditions or in response to stimulation with ADP, SFLLRN or thrombin. RIPC, n=20; Sham, n=20; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; SFLLRN, PAR-1 agonist;

4.3.7 The effect of remote ischaemic preconditioning on circulating regulators of platelet function

Platelet activity is modulated by multiple factors in the circulation, which lead to downstream modulation of intracellular pathways, resulting in changes in the receptor profile on the platelet surface. Despite their predominant intracellular role in intracellular signalling, the circulating levels of cAMP and cGMP were measured by ELISA. There was no effect of RIPC on circulating levels of cAMP or cGMP as assessed by ELISA analysis of the stored plasma (Figure 4.11).

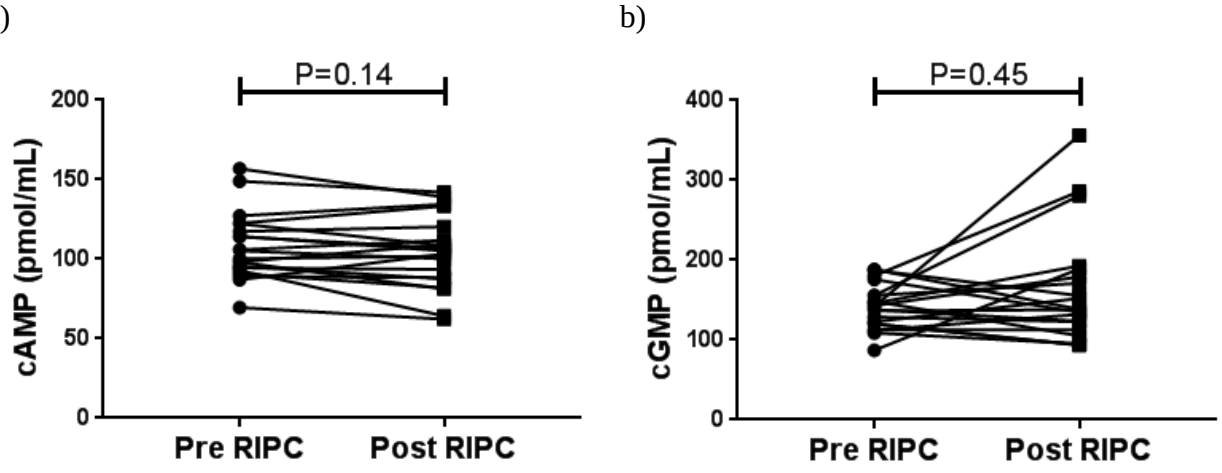


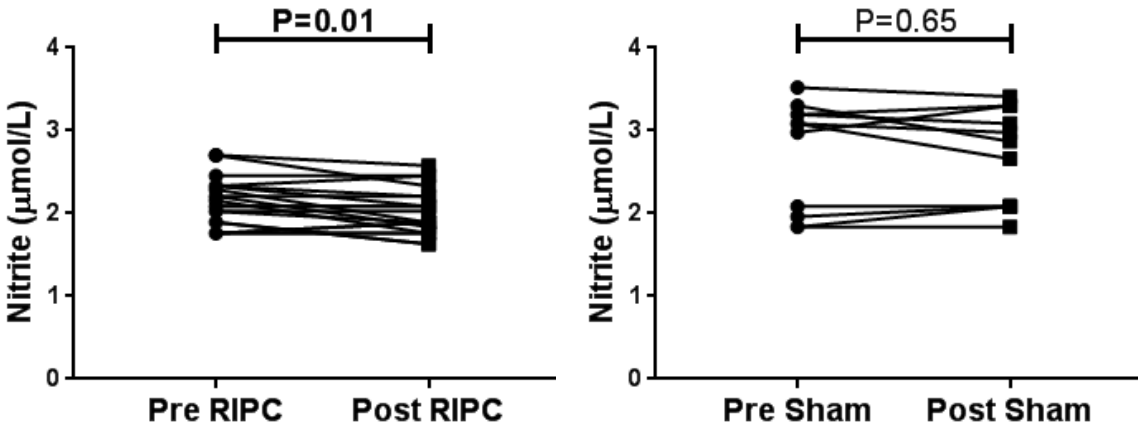
Figure 4.11 The effect of remote ischaemic preconditioning on plasma cAMP and cGMP

Plasma cAMP (a) and cGMP (b) concentrations pre and post RIPC measured with commercially available ELISA kits as per manufacturer guidelines. n=20. P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ELSIA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

Nitric oxide is an important suppressor of platelet activation. Nitric oxide is metabolised to nitrite, which is then metabolised to nitrate. Given the short half-life of nitric oxide, the levels of the metabolites nitrite and nitrate were assessed to evaluate for changes in response to RIPC. RIPC was associated with a reduction in plasma nitrite concentration (2.1 ± 0.3 μmol/L vs 2.0 ± 0.3 μmol/L, P=0.01, n=20) while there was no change in patients randomised to sham (Figure 4.12). Analysis of stored plasma demonstrated a significant reduction in plasma nitrate concentration with RIPC (21.0 μmol/L (17.9-30.5) vs 19.8 μmol/L (15.9-27.5), P=0.001, n=20). There was also a reduction in the measured plasma nitrate concentration in patients randomised to sham treatment (17.1 ± 6.0 μmol/L vs 16.2 ± 5.8 μmol/L, P=0.006, n=11). There was no correlation between the change in plasma nitrite concentration with RIPC and the change in PAC-1 binding in response to SFLLRN. However, there was a moderate association between the change in plasma nitrite with RIPC and the change in PAC-1 binding in response to thrombin and collagen with RIPC, which approached statistical significance (Figure 4.13).

a)



b)

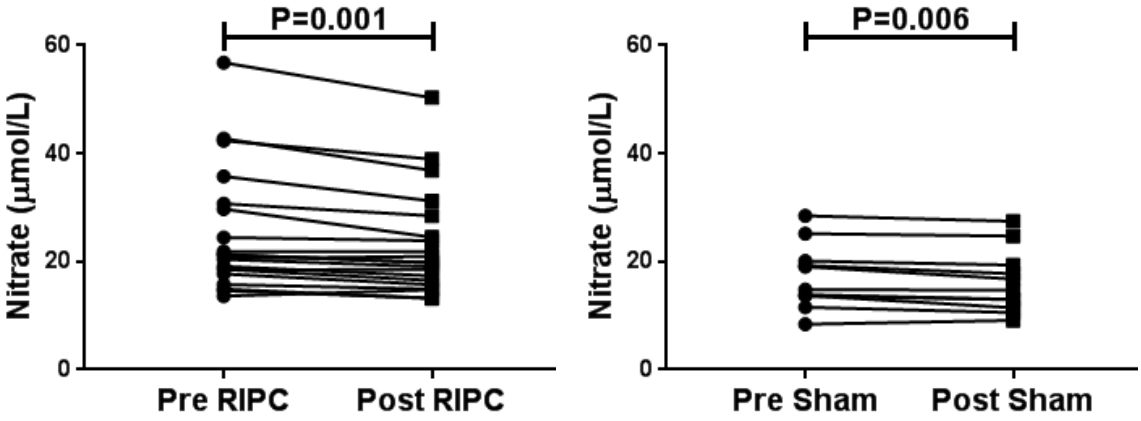


Figure 4.12 The effect of remote ischaemic preconditioning on plasma nitrate and nitrite concentrations

Plasma nitrite (a) and nitrate (b) concentrations in stored plasma pre and post RIPC and sham measured with a commercially available ELISA kit as per manufacturer guidelines. RIPC, n=20; Sham, n=11; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data. ELSIA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

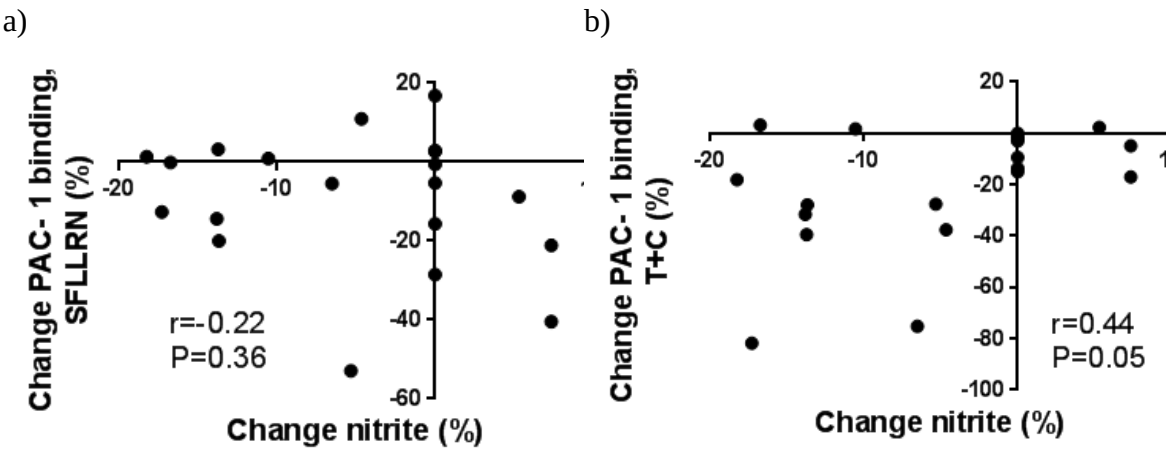


Figure 4.13 Correlation between change in plasma nitrite and change in PAC-1 binding with remote ischaemic preconditioning

Correlation between changes in plasma nitrite with RIPC and changes in PAC-1 binding in response to SFLLRN (a) and thrombin and collagen (b) measured by ELISA and flow cytometry respectively. Values determined by calculating the difference in post and pre values and representing the difference as a percentage of the pre value. A negative value suggests a reduction with RIPC. n=20. Correlations between variables were assessed by Pearson's correlation or Spearman rank correlation for parametrically and non-parametrically distributed data respectively.

ELSIA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

The prostaglandin, prostacyclin, is another inhibitor of platelet activation. Prostacyclin is metabolised to 6-keto-PGF1α. To assess whether the prostacyclin pathway was involved in the observed attenuation of platelet activation by RIPC, prostacyclin and 6-keto-PGF1α levels were measured in stored plasma. There was no change in plasma prostacyclin or 6-keto-PGF1α concentrations with RIPC (Figure 4.14).

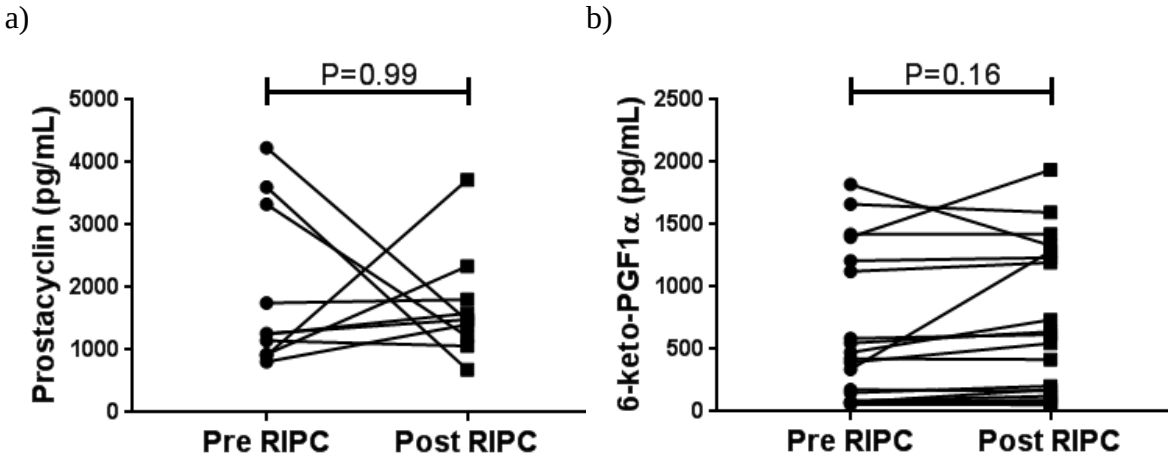


Figure 4.14 Plasma prostacyclin and 6-keto-PGF1α concentrations with remote ischaemic preconditioning

Plasma prostacyclin (a) and 6-keto-PGF1α (b) concentrations before and after RIPC measured with commercially available ELISA kits as per manufacturer guidelines. Prostacyclin, n=10; 6-keto-PGF1α, n=20; P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ELISA, enzyme-linked immunosorbent assay; RIPC, remote ischaemic preconditioning;

4.3.8 The effect of remote ischaemic preconditioning on platelet intracellular VASP and phosphorylated-VASP

In order to explore the possibility that RIPC was associated with an increase in VASP phosphorylation in platelets, the effect of RIPC was carefully determined in a separate group of 18 patients undergoing coronary angiography. The baseline characteristics of this second patient cohort are shown in Table 4.3. The cohort consisted of predominantly male patients (94%), with significant CAD demonstrated in 13 (72%) of patients. The number of patients on DAPT was 6 (33%) with all of these patients on aspirin and clopidogrel.

Table 4.3 Baseline characteristics of intracellular platelet cohort

Characteristic	**RIPC treated (n=18)**
Age - years	64.5 ± 9.9
Male – no. (%)	17 (94)
Clinically stable – no. (%)	18 (100)
Significant CAD – no. (%)	13 (72)
Prior myocardial infarction – no. (%)	3 (17)
Prior PCI – no. (%)	2 (11)
Prior CABG- no. (%)	3 (17)
Heart failure – no. (%)	0 (0)
Prior stroke – no. (%)	4 (22)
Peripheral vascular disease – no. (%)	1 (6)
Hypertension – no. (%)	10 (56)
Diabetes – no. (%)	8 (44)
Dyslipidaemia – no. (%)	14 (78)
Current smoking – no. (%)	3 (17)
Medications	
Aspirin – no. (%)	17 (94)
$P2Y_{12}$ antagonist – no. (%)	6 (33)
Clopidogrel – no. (%)	6 (33)
Ticagrelor – no. (%)	0 (0)
Prasugrel – no. (%)	0 (0)
Dual antiplatelet therapy – no. (%)	6 (33)
Warfarin/ NOAC – no. (%)	0 (0)
Heparin – no. (%)	0 (0)
Statin – no. (%)	14 (78)
Beta blocker – no. (%)	6 (33)
ACEi or ARB – no. (%)	7 (39)
Nitrate – no. (%)	2 (11)
Parameters during admission	
Systolic blood pressure – mmHg	143.9 ± 22.2
Heart rate – beats/min	70.5 ± 11.2
Haemoglobin concentration – g/L	141.7 ± 16.1
Platelet count – 10^9/L	232.1 ± 79.9
eGFR - mL/min/1.73m^2	84.7 ± 13.3

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; NOAC, novel oral anticoagulant; PCI, percutaneous coronary intervention;

From the 18 patients who had blood collected before and after RIPC and had platelet lysate samples stored, there were 15 paired lysates stored under basal unstimulated conditions and 5 pairs of lysates stored after stimulation with SFLLRN. A

representative western blot of the platelet lysates stored under basal conditions is shown in Figure 4.15. The pixel volume after interrogation of each sample with a phosphorylated-VASP antibody was corrected for total VASP and GAPDH. A post RIPC: pre RIPC ratio was calculated with values >1 indicating an increase in phosphorylated-VASP with RIPC and values <1 suggesting a reduction with RIPC. The corrected and uncorrected post RIPC: pre RIPC phosphorylated-VASP ratios for all the patients are displayed in Table 4.4. The mean phosphorylated-VASP post:pre ratio, corrected for total VASP and GAPDH, was 1.0 ± 0.5 (P=0.98) in unstimulated platelet lysates and 1.1 ± 0.5 (P=0.65) in SFLLRN stimulated platelet lysates.

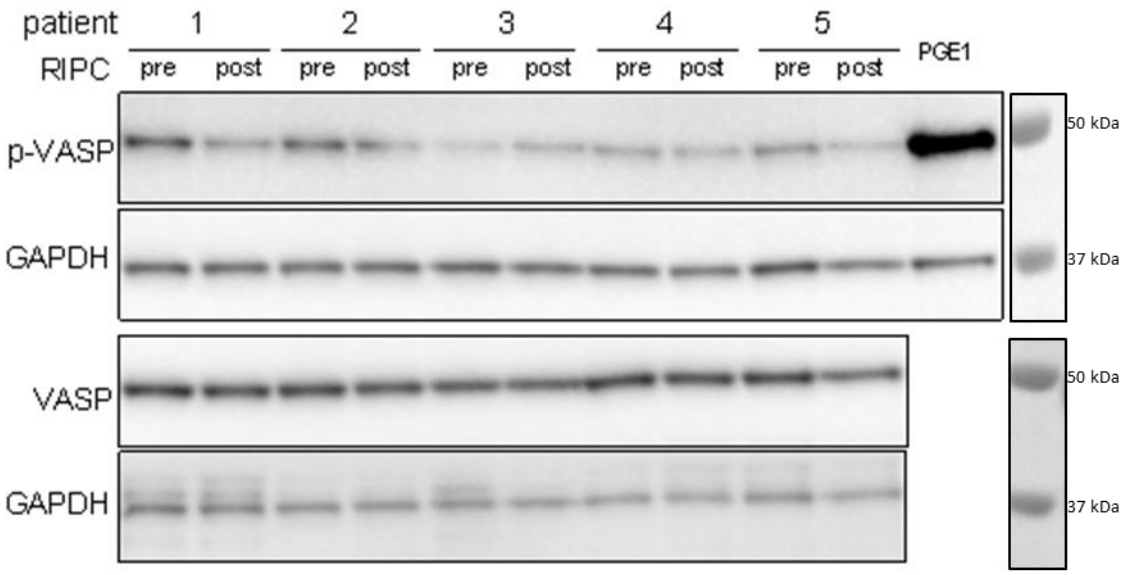


Figure 4.15 Western blot of platelet lysates before and after remote ischaemic preconditioning

Representative western blot of stored platelet lysates pre and post RIPC under unstimulated conditions and of a positive control sample (platelets stimulated with prostaglandin E1, only loaded onto the p-VASP blot). Each blot probed with antibodies to VASP, p-VASP and GAPDH. The predicted molecular weight of VASP and p-VASP is 46 kDa and the predicted molecular weight of GAPDH is 37 kDa. n=5. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; p-VASP, phosphorylated vasodilator stimulated phosphoprotein; RIPC, remote ischaemic preconditioning; VASP, vasodilator stimulated phosphoprotein;

Table 4.4 Changes in phosphorylated-VASP with remote ischaemic preconditioning

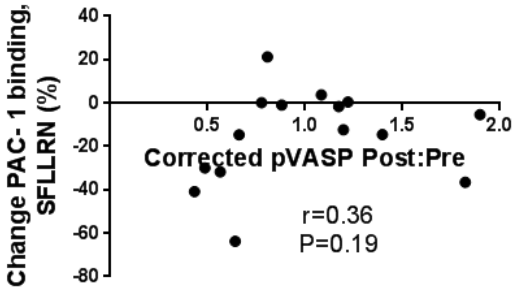
	Basal		SFLLRN	
Patient number	**Uncorrected**	**Corrected**	**Uncorrected**	**Corrected**
1	0.73	0.66		
2	0.48	0.43		
3	0.56	0.57		
4	1.60	1.90		
5	0.93	1.40		
6	0.59	0.88		
7	0.82	0.78		
8	1.32	1.82		
9	0.33	0.49		
10	1.37	1.08		
11	0.79	0.64		
12	1.86	1.17		
13	1.23	1.20		
14	0.76	0.81	0.50	0.67
15	1.21	1.22	0.88	0.89
16			1.10	1.94
17			0.55	1.10
18			0.85	0.93

Ratio of phosphorylated-VASP in post RIPC platelet lysates to pre RIPC platelet lysates stored under unstimulated (basal) conditions or after SFLLRN stimulation. Ratios without and with correction for total VASP and GAPDH presented. A ratio >1 suggests an increase in phosphorylated-VASP with RIPC. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; RIPC, remote ischaemic preconditioning; SFLLRN, PAR-1 agonist; VASP, vasodilator stimulated phosphoprotein;

In the intracellular platelet cohort, treatment with RIPC reduced GPIIb-IIIa activation (PAC-1 binding) in response to SFLLRN (77.7% (62.2-84.7) vs 63.0% (42.6-79.4),

P=0.002) and thrombin and collagen (87.8% (82.1-90.8) vs 84.8% (66.2-91.2), P=0.006) as observed in the main study cohort, establishing the robustness of the key observation. There was a significant correlation between pre RIPC corrected phosphorylated-VASP pixel volumes and PAC-1 binding in response to SFLLRN (r=-0.80, P=0.0004) and in response to thrombin and collagen (r=-0.64, P=0.01). Similarly there was a significant correlation between post RIPC corrected phosphorylated-VASP pixel volumes and PAC-1 binding in response to SFLLRN (r=-0.57, P=0.03) and in response to thrombin and collagen (r=-0.67, P=0.008). However, there was no correlation between changes in PAC-1 binding in response to SFLLRN and thrombin and collagen and the phosphorylated-VASP post:pre ratio in unstimulated samples (Figure 4.16).

a)



b)

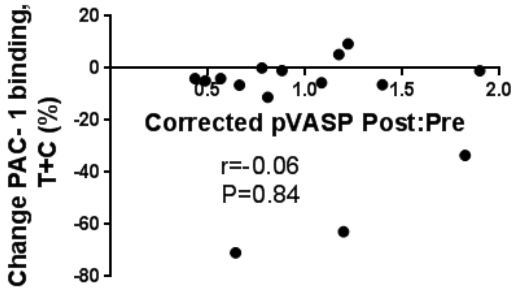


Figure 4.16 Correlation between change in PAC-1 binding and phosphorylated-VASP with remote ischaemic preconditioning

Change in PAC-1 binding determined by calculating the difference in PAC-1 binding post and pre RIPC as a percentage of PAC-1 binding pre RIPC, with a negative value representing a reduction with RIPC. Phosphorylated-VASP post: pre ratio in unstimulated platelet lysates corrected for total VASP and GAPDH with a ratio <1 suggesting a reduction with RIPC. n=15. Correlations between variables were assessed by Pearson's correlation or Spearman rank correlation for parametrically and non-parametrically distributed data respectively.

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; RIPC, remote ischaemic preconditioning; VASP, vasodilator stimulated phosphoprotein; T+C, thrombin and collagen; SFLLRN, PAR-1 agonist;

4.3.9 The effect of remote ischaemic preconditioning on platelet intracellular cAMP and cGMP

The cAMP and cGMP concentrations of 15 pairs of platelet lysates under unstimulated conditions and 3 pairs after SFLLRN stimulation was determined by ELISA. The cAMP concentrations were below the detection limit on the standard curve in all but one pair. There were 11 pairs of unstimulated lysates and one pair of SFLLRN stimulated platelet lysates with cGMP concentrations within the range of the standard curve with the remaining samples below this range. There was no significant change in cGMP levels in the unstimulated platelet lysates with RIPC (5.8 pmol/mL (4.8-10.7) vs 6.6 (4.6-7.7), p=0.83, n=11).

4.4 Discussion

In this chapter, the effect of RIPC on pathway-specific platelet activation in response to defined agonists was investigated in a randomised controlled trial. The results demonstrate that RIPC attenuates the induction of GPIIb-IIIa conformational activation in response to SFLLRN and in response to combined thrombin and collagen stimulation. There were also other unexpected effects on CD63 expression and platelet-leucocyte aggregate formation. Importantly, these effects were not seen in sham-treated patients indicating that these are RIPC-specific and were supported by the analysis of percentage change in these markers with RIPC or sham.

RIPC attenuated platelet GPIIb-IIIa conformational activation in response to the PAR-1 agonist, SFLLRN, and in response to combined stimulation with thrombin and collagen, potent platelet agonists that are present at the site of atherosclerosis related thrombosis. *In vivo*, reduced exposure of the fibrinogen binding site on platelet surface GPIIb-IIIa may reduce platelet aggregation at the site of complicated atherosclerotic coronary plaques and may help explain the benefit conferred by RIPC in acute myocardial infarction and in reducing peri-procedural events in the context of primary and elective PCI (13,14,74,76,165). The effects on GPIIb-IIIa conformational activation appear to be specific. As there was no effect on GPIIb-IIIa activation in circulating platelets before stimulation, it is likely that the preconditioning influences a pathway which attenuates activation by subsequent agonist stimulation rather than directly affecting PAC-1 binding sites.

There was no effect of RIPC on CD62P or CD63 expression apart from a small but statistically significant increase in CD63 expression in response to ADP after RIPC. There was also an increase in platelet-granulocyte aggregates in response to SFLLRN

and platelet-monocyte aggregates under unstimulated conditions with a reduction in platelet-monocyte aggregates in response to thrombin. These results suggest an increase in dense granule or lysosomal release in response to a relatively weak agonist and varied effects on PLA formation. The potential clinical significance of these findings is unclear but suggests that RIPC may exert complex effects on platelet biology, not all of which inhibit platelet activation.

The observed effects of RIPC on PLA formation are in contrast to prior studies which have demonstrated RIPC-induced reduction in platelet-monocyte aggregate formation with ADP, exercise or cardiac catheterisation (56,57). Whilst one of these studies studied patients undergoing cardiac catheterisation on DAPT (57), there were differences in the flow cytometry methods, range of agonists and timing of blood collection compared to this present study. In the previous study, blood was collected before RIPC, conditioning was then delivered and blood was collected again after the cardiac catheterisation procedure was completed whereas in our study blood was collected before angiography. Thus the former study may be identifying RIPC related decreases in the induction of PLA by the coronary catheterisation procedure. These methodological differences may account for the discrepant results.

There was no observed effect of RIPC on platelet aggregation in response to ADP or arachidonic acid, as assessed by platelet impedance aggregometry. This is in keeping with another study which investigated the effect of long term RIPC on platelet aggregation (166).

The effects of RIPC on platelet activation have been studied in other patient populations and unlike our study, have principally concentrated on the effect of RIPC on procedure-related platelet activation augmented by ADP stimulation (55,57). This study is unique in that pathway-specific and agonist-specific effects of RIPC on platelet activation have

been identified and it is the first to investigate the effect of RIPC on GPIIb-IIIa conformational activation induced by thrombin-related pathways. Furthermore, the inhibitory effects of RIPC on platelet activation have been shown to be additional to the inhibitory effects of contemporary DAPT. Although DAPT was effective in inhibiting platelet activation in response to ADP compared to aspirin alone, the addition of a $P2Y_{12}$ ADP receptor inhibitor had relatively little inhibitory effect on the activation of platelets by thrombin and the potent combination of thrombin and collagen. This is of interest as thrombin and collagen are abundant at the site of complicated atherosclerotic plaques and are involved in the development of ACS.

Thrombin is understood to activate platelets via the protease activated receptor (PAR) family of G Protein coupled receptors, PAR-1 and PAR-4 being expressed in human platelets (167). Cleavage of the N-terminus of PAR-1 and PAR-4 by serine proteases exposes a new terminus which acts as a tethered ligand, and this can be replicated by synthetic ligands for each PAR receptor. Activation of PARs activates the β isoforms of phospholipase C, in turn activating IP3 and inducing calcium release to cause a cascade of effects including activation of Rap1, promoting its binding to talin on the C-terminus of GPIIb-IIIa to promote its conformational change (168,169). In our study, SFLLRN, which activates the PAR-1 receptor, effectively activated platelets despite DAPT, but did so less effectively than did thrombin, suggesting added effects arising from PAR-4 activation by thrombin. In a sub-group of 10 patients, the effect of RIPC on PAC-1 binding in response to a PAR-4 agonist (AYPGFK, 300 μM) was assessed. This demonstrated no effect of RIPC (data not shown), highlighting the importance of the PAR-1 thrombin activation pathway in the RIPC induced attenuation of GPIIb-IIIa activation.

SFLLRN-mediated conformational activation of GPIIb-IIIa was inhibited by RIPC whereas SFLLRN-mediated expression of CD62P and CD63 were unaffected, indicating that alpha and dense granule release are not inhibited by RIPC, whereas inside-out signalling regulating GPIIb-IIIa conformational change is specifically attenuated. This suggests that downstream effectors of conformational change, rather than generalised effects on platelet activation, are targets of RIPC. However, given that the power calculation was based upon PAC-1 binding, it is possible that the study was underpowered to detect an effect of RIPC on these other markers of platelet activation. RIPC also attenuated GPIIb-IIIa conformational change after stimulation with thrombin and collagen in combination, but, surprisingly, did not inhibit activation after thrombin alone, suggesting that collagen facilitates the inhibitory effects of RIPC. Collagen activates platelets through direct interaction with GPVI and the integrin $\alpha_2\beta_1$, and by indirect interaction via vWF-mediated binding to GPIb-IX-V complex and GPIIb-IIIa (167). Under the *ex vivo* conditions in this study, without circulatory flow, interaction via GPVI and the integrin $\alpha_2\beta_1$ are likely to be involved. After activation, clustering of GPVI results in phosphorylation of tyrosine residues in the FcRγ cytoplasmic domain causes downstream activation of Syk, which leads to Rap1 activation and initiation of inside-out activation of integrin GPIIb-IIIa. Similarly, activation of the integrin $\alpha_2\beta_1$ promotes activation of Syk. At this stage it can only be speculated that clustering of GPVI after exposure to collagen or binding to integrin $\alpha_2\beta_1$ induce novel inhibitors of GPIIb-IIIa conformational change that work synergistically with the inhibitory pathways induced by RIPC. Whether RIPC also inhibits conformational activation of integrin $\alpha_2\beta_1$ and whether these changes are a direct consequence of collagen binding will require further investigation.

Nitric oxide has important functions in human biology (170). It is known to have inhibitory actions on platelet activation with nitrite being the major metabolite and cGMP its major intracellular messenger (170-172). In an animal model, nitric oxide and nitrite were demonstrated to be mediators of RIPC mediated cardiac protection (42). On this basis, it was hypothesised that RIPC mediated modulation of nitric oxide metabolism may contribute to the attenuation of platelet activation that was observed. Hence, an analysis of stored plasma was undertaken which demonstrated a reduction in plasma nitrite with RIPC. There was also a reduction in plasma nitrate with RIPC, but this may have been a result of artefact after blood collection and storage as there was also a reduction in the patients randomised to sham. There was a moderate association between the change in plasma nitrite and reduction in PAC-1 binding, which approached statistical significance. Further investigation will be required to characterise this in more detail.

An alternative explanation for the observed reduction in plasma nitrite levels with RIPC is consumption during reduction to nitric oxide under hypoxic conditions. This pathway of reduction of nitrite to form nitric oxide by molecules such as haemoglobin and myoglobin allows maintenance of nitric oxide levels during hypoxia (170). Forearm ischaemia during RIPC may have led to promotion of this pathway of nitric oxide production, resulting in the reduced levels of systemic nitrite observed during plasma analysis. Thus, decreased plasma nitrite may be a surrogate marker for effectiveness of remote ischaemia rather than directly biologically responsible for the inhibition of GPIIb-IIIa activation effects. It does remain possible that the modest stimulatory effects of RIPC seen on platelet-leukocyte aggregate formation and CD63 release could be contributed to by NO consumption during RIPC.

Prostacyclin also has important platelet inhibitory actions mediated through cAMP (173-176). Prostacyclin is metabolised to 6-keto-PGF1α, which is more stable in the circulation. RIPC in an animal model appeared to upregulate prostacyclin production in the heart, demonstrated by an increase in 6-keot-PGF1α (177). In this study however, there was no correlation between prostacyclin levels and cardiac protection against tachyarrhythmia after IR injury. In this present study, there was no change in plasma prostacyclin or 6-keto-PGF1α levels with RIPC. This suggests that either 1) RIPC has no effect on these circulating factors and that the prostacyclin pathway is not primarily responsible for the attenuation of platelet activation that was observed or 2) that the methods utilised were not sensitive enough to detect such a change.

While there was no significant change in the platelet cohort, there was a small but statistically significant reduction in 6-keto-PGF1-alpha in the coronary physiology group. While the two cohorts have similar baseline characteristics and were both comprised of patients undergoing coronary angiography for investigation of CAD, the samples were collected under different conditions. In the coronary physiology cohort, blood samples were collected after administration of IV adenosine, other drugs administered during the procedure such as heparin, midazolam and fentanyl and after the physical stimulus of cardiac catheterisation. In the platelet cohort, the blood samples were collected prior to the patient's procedure. These multiple differences may help explain the discrepant results.

GPIIb-IIIa conformational activation is the result of inside-out signalling through several different pathways as discussed above. A well described regulator of GPIIb-IIIa conformational activation is the intracellular phosphoprotein VASP. Phosphorylation of VASP by cAMP and cGMP dependent kinases results in inhibition of GPIIb-IIIa and is the pathway involved in inhibition of platelet activity by substances such as nitric oxide

and prostacyclin (164). It was hypothesised that RIPC may mediate its platelet inhibitory effects via an increase in VASP phosphorylation. As such, a separate cohort of 18 patients were recruited and treated with RIPC to specifically study these intracellular pathways. This second cohort of patients also demonstrated a significant reduction in GPIIb-IIIa conformational activation in response to SFLLRN and thrombin and collagen after RIPC confirming robustness of our primary observation. Western blot analysis of platelet lysates demonstrated heterogeneity between patients, with no significant effect of RIPC overall after appropriate adjustments for total VASP and GAPDH levels. In addition, there was no correlation between changes in phosphorylated-VASP and changes in PAC-1 binding in response to either SFLLRN or thrombin and collagen. Thus, the effect of RIPC on GPIIb-IIIa activation does not appear to be explained by increased VASP phosphorylation.

To investigate the effect of RIPC on the integral intracellular messengers cAMP and cGMP, platelet lysates were also stored for analysis with ELISA. All but one pair of platelet lysates had cAMP levels below the lowest concentration on the standard curve and thus were interpretable. There were also some pairs of platelet lysate samples which had concentrations below the standard cGMP curve. There appeared to be no change in cGMP levels with RIPC in the samples with concentrations within the standard curve range. Despite following the manufacturer's instructions, intracellular cAMP levels were not detectable with the method used to store platelet lysates. Given the important role of cAMP in intracellular platelet signalling, analysis of intracellular cAMP levels could provide interesting insights into the effects of RIPC on platelet biology. This is an area for potential future investigation but will require adjustment of the current method or an alternative method to measure intracellular cAMP. Analysis of stored plasma cAMP and cGMP levels demonstrated no change with either RIPC or sham treatment.

Due to the mechanistic focus of this study, and the small numbers of patients that subsequently had PCI, correlation between changes in PAC-1 binding and clinical outcomes such as troponin rise post PCI could not be performed. Additionally, due to the relatively small patient numbers, clinical characteristics that predict a response to RIPC could not be sought.

4.5 Conclusions

RIPC attenuates platelet GPIIb-IIIa conformational activation in response to SFLLRN and thrombin and collagen. This response is additional to the effects of contemporary antiplatelet therapy in patients with CAD. The greatest effect of RIPC appeared to be in response to thrombin and collagen, a combination of agonists against which DAPT appears to be relatively ineffective and which are abundant at the site of complicated coronary atherosclerotic plaques. Attenuation of pathological platelet activation may contribute to the beneficial effects of RIPC at the time of primary and elective PCI. The mechanism behind the RIPC mediated attenuation of platelet activation remains uncertain.

Chapter 5: The effect of remote ischaemic preconditioning on procoagulant platelet formation

5.1 Introduction

The significant role of platelets in in both physiological haemostasis and pathological thrombosis was discussed in chapter 4. Procoagulant platelets are a subset of platelets that provide a surface for assembly of coagulation complexes and are characterised by externalisation of phosphatidylserine (PS) (121,122,178-180). They can be found at the site of collagen exposure in the vessel wall and are most readily formed *ex vivo* by stimulation with a combination of thrombin and collagen (181). Procoagulant platelets have been shown to have low levels of GPIIb-IIIa activation and thus may have a greater or more specific role in thrombus formation than in platelet aggregation and adhesion (181). Within thrombus, platelets with a procoagulant phenotype appear to interact with aggregatory platelets expressing activated GPIIb-IIIa (182). Consequently, inhibiting the formation of procoagulant platelets could have additional consequential secondary effects via their interaction with other platelets.

Platelet PS externalisation has been linked to cyclophilin D-dependent inner mitochondrial membrane disruption through formation of the mitochondrial permeability transition pore (MPTP), leading to membrane depolarisation (178,182). Another pivotal step is an increase in cytosolic calcium (183). Cyclosporin A, which inhibits cyclophilin D, inhibits PS exposure, mitochondrial membrane depolarisation and the increase in cytoplasmic calcium induced by platelet agonists (183). High cytoplasmic calcium and mitochondrial membrane depolarisation distinguishes

procoagulant platelets from aggregating platelets with active GPIIb-IIIa on their surface (184).

As described in section 2.6.6, a novel flow cytometry assay to detect procoagulant platelets has been developed using the uptake of a trivalent arsenical 4-[N-(S-glutathionylacetyl)amino]phenylarsonous acid (GSAO) and co-staining with CD62P (GSAO+/CD62P+ platelets) (120). GSAO is tagged with the fluorophore AlexaFluor 647 and is retained in the platelet cytoplasm by covalently binding to proteins containing closely spaced di-thiol bonds (185). Data using the above method demonstrates that compared to healthy controls, patients with CAD have increased levels of circulating procoagulant platelets and hyper-reactivity to thrombin, mimicking thrombin and collagen stimulation of platelets from healthy volunteers, indicating that this platelet population may be relevant to patients with coronary disease (123).

It was hypothesised that RIPC may confer protective effects against coronary events by inhibiting the formation of procoagulant ($GSAO^{+}/CD62P^{+}$) platelets. This was investigated under unstimulated conditions and in response to platelet agonists in this chapter.

5.2 Methods

The data presented in this chapter was collected concurrently with the data presented in chapter 4. Hence, the patient population, blood collection methods and delivery of RIPC were identical.

5.2.1 Patient population

Patients who were recruited to study the effects of RIPC on platelet activation, described in chapter 4, also had procoagulant platelet activity studied. These patients were referred for coronary angiography by their treating physician due to a suspicion of CAD. Participants provided informed consent and all blood samples were collected prior to the cardiac catheterisation procedure. Medication prescription, including the administration of antiplatelet therapy, was at the discretion of the patient's treating physician.

5.2.2 Blood collection

Blood collection was performed as described in chapters 2 and 4.

5.2.3 Remote ischaemic preconditioning

Remote ischaemic preconditioning and sham treatment was delivered as described in chapters 2 and 4. A closed envelope system was used to randomise 60 patients, in a 1: 1 manner, to receive either RIPC or sham treatment. Patients and investigators processing the blood samples were both blinded to patient treatment allocation. The study design is described in Figure 4.1. A separate cohort of patients (n=25) had blood samples taken to further explore the effect of RIPC on agonist-induced procoagulant platelet formation and to investigate changes in platelet mitochondrial membrane potential in response to RIPC (exploratory cohort). All of these patients received RIPC with blood collected before and after treatment.

5.2.4 Assessment of procoagulant platelet formation

As described in section 2.6.6, procoagulant platelets were quantified in whole blood with reactions performed in a sterile 96-well polypropylene plate. Whole blood collected into sodium citrate tubes was diluted with HBSS, Gly-Pro-Arg-Pro amide and calcium with and without agonists. The agonist panel mirrored the study of surface markers of platelet activation (chapter 4), ADP (5 μM), SFLLRN (20 μM), thrombin (2 U/mL) and thrombin (2 U/mL) + collagen (10 μg/mL). In the exploratory cohort, procoagulant platelet formation was assessed additionally with the PAR-4 agonist AYPGFK (300 μM) and also with 0.5 U/mL thrombin, 1 U/mL thrombin, 2 U/mL thrombin, 5 U/mL thrombin each with and without 10 μg/mL collagen and 10 μg/mL collagen alone. A 20- fold dilution with HBSS was used to stop reactions after 10 min at room temperature. Agonist or non-stimulated blood were labelled for 15 min with antibodies to CD62P, CD41a, CD45 and with GSAO conjugated to AlexaFluor 647 before being fixed with two volumes of PAMFix. Samples were then transferred to FACS tubes and washed with 10 volumes of HBSS/HSA. The cells were then pelleted and then re-suspended in 1 mL of HBSS/HSA before being stored at room temperature in the dark for 1 h prior to analysis. Flow cytometry was performed on a BD FACS Canto II or BD Fortessa with 10,000 platelet events acquired for each analysis. Procoagulant platelets were defined by CD62P and GSAO co-positivity (CD62P+/GSAO+). Preliminary data with this assay demonstrated that heparin inhibits formation of procoagulant platelets in response to thrombin and thrombin + collagen, but not TRAP. Consequently patients receiving heparin were excluded from this part of the analysis. Additionally, data was excluded when clot was noted during in-vitro agonist stimulation (4 patients in thrombin and thrombin + collagen stimulated samples). Data were analysed with FlowJo.

5.2.5 Platelet mitochondrial membrane potential

The mitochondrial membrane potential of platelets was quantified in PRP with incubation and staining steps performed in a sterile 96-well polypropylene plate. Citrated whole blood was centrifuged at 200g for 10 min at room temperature with low brake to obtain PRP. Citrated PRP was then diluted with HBSS, Gly-Pro-Arg-Pro amide (2.5 mM) and calcium (2.5 mM), with and without agonists. The agonists used were 0.5 U/mL thrombin, 1 U/mL thrombin and 2 U/mL thrombin, each with and without 10 μg/mL collagen and 10 μg/mL collagen alone. Reactions were stopped after 10 min of incubation at room temperature by a 20- fold dilution with HBSS. Aliquots of agonist- or non-stimulated PRP were labelled for 15 min with antibodies to CD41a (HIP8) and CD45 (HI30) (BD Biosciences), and with tetramethylrhodamine ethyl ester perchlorate (TMRE), a fluorescent potential-sensitive probe for mitochondria (Sigma-Aldrich). Samples were then transferred to in 1 mL of HBSS with 0.35% HSA in FACS tubes and analysed immediately. Flow cytometry was performed on a BD Fortessa with 10,000 platelet events acquired for each analysis. Mitochondrial membrane disruption and depolarisation was indicated by absence of TMRE signal (TMRE negative, TMRE-). Platelets with intact mitochondrial membranes retain TMRE (TMRE+) and were defined based on fluorescence. The proportion of TMRE- events was determined by 100% - TMRE+.

5.2.6 Statistical analysis

The statistical analysis was performed as in chapter 4 with the primary outcome being pre treatment vs post-treatment expression of procoagulant platelets. TMRE agonist-response curves were determined with linear regression with differences between the curves determined with statistical analysis software (GraphPad, Prism).

5.3 Results

5.3.1 Baseline clinical characteristics

The baseline characteristics of the 60 patients who were recruited to study the effects of RIPC on procoagulant platelet activity were described in section 4.3.1 and Table 4.1. There were 6 patients, 3 in the RIPC cohort and 3 in the sham cohort, who were on intravenous heparin at the time of the study. The data regarding thrombin and thrombin + collagen induced procoagulant platelet formation from these patients were excluded from analysis as heparin inhibits thrombin-induced formation of procoagulant platelets.

5.3.2 The effect of remote ischaemic preconditioning on circulating procoagulant platelets

The effect of RIPC or sham treatment on the formation of procoagulant platelets in the unstimulated basal sample and in response to platelet agonists is summarised in Table 5.1.

Table 5.1 The effect of remote ischaemic preconditioning and sham treatment on procoagulant platelet formation

Agonist*	**RIPC**			**Sham**		
	Pre	**Post**	**P**	**Pre**	**Post**	**P**
Basal	2.0 (1.3-2.4)	1.3 (1.1-2.1)	**0.01**	2.0 (1.3-2.9)	1.9 (1.1-2.5)	0.91
ADP	4.6 ± 2.6	4.2 ± 2.5	0.25	3.7 (2.5-6.4)	3.6 (2.4-5.3)	0.36
SFLLRN	9.2 (7.0-11.7)	7.4 (6.0-10.5)	**0.04**	10.6 (8.0-12.8)	8.7 (6.9-12.0)	0.06
Thrombin	9.8 (7.9-12.3)	9.5 (6.2-13.0)	0.43	10.2 (7.1-15.0)	10.4 (6.3-14.9)	0.63
T+C	14.7 (11.3-17.0)	14.2 (11.1-18.8)	0.75	16.6 (12.6-23.4)	16.7 (12.4-20.5)	0.68

P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

* Values represent percentage (%) procoagulant platelets.

Basal, ADP, SFLLRN, Thrombin, T+C: RIPC, n=26, 24, 26, 23 and 23 respectively; Sham, n=27, 26, 28, 22 and 22 respectively.

ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

There was a significant reduction in the proportion of unstimulated procoagulant platelets in the circulation with RIPC, from 2.0% to 1.3% (p=0.01), whereas there was no effect of sham treatment (Figure 5.1). This remained significant after repeating the analysis excluding the one outlier with a dramatic reduction in procoagulant platelets in the RIPC group (1.9 ± 0.8% vs 1.6 ± 0.9%, P=0.03).

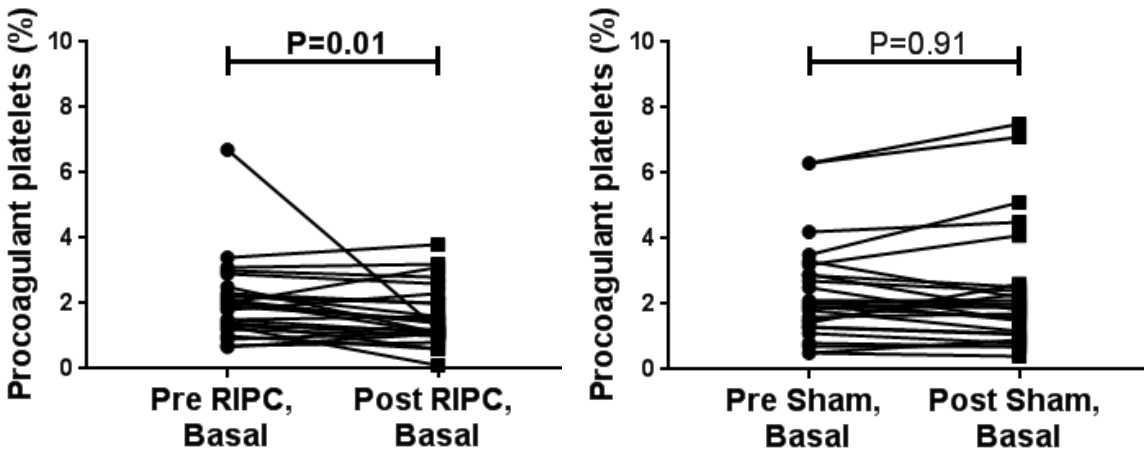


Figure 5.1 The effect of remote ischaemic preconditioning on circulating procoagulant platelets

Effect of RIPC (left panel) or sham (right panel) on procoagulant platelet formation without stimulation (basal). RIPC, n=26; Sham, n=27; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

RIPC, remote ischaemic preconditioning;

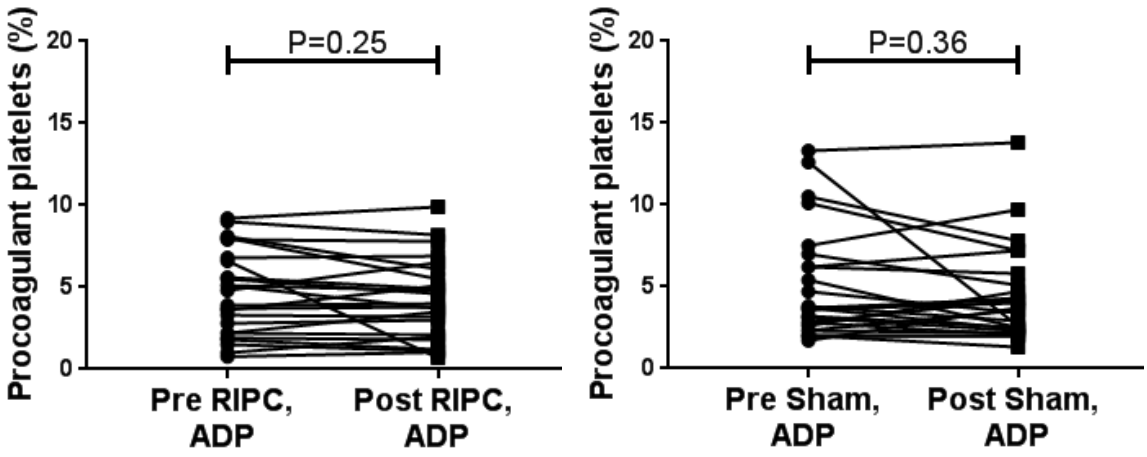
There was also a greater percentage reduction in unstimulated procoagulant platelets with RIPC than sham (-14.9% (-35.0 - +7.9) vs -5.0% (-20.0 - +12.7), P=0.15) but this did not reach statistical significance.

5.3.3 The effect of remote ischaemic preconditioning on procoagulant platelets in response to platelet agonists

Examining the pre RIPC/sham data in the entire cohort, the median level of procoagulant platelets increased from 2.0% (1.3-2.8) (n=53) without agonist stimulation, to 3.7% (2.3-6.7) (n=50) after ADP, 10.1 (7.5-12.3) (n=54) after SFLLRN, 10.0% (7.6-14.7) (n=45) after thrombin, and 15.4% (11.8-19.9) (n=45) after the

combination of thrombin and collagen, indicating that there were substantial differences in the extent to which procoagulant platelets were generated by the different agonists. Under agonist stimulation, there was a small decrease in procoagulant platelet formation after SFLLRN stimulation which just reached statistical significance (Figure 5.2). There was no effect of RIPC on procoagulant platelet formation in response to any other agonists.

a)



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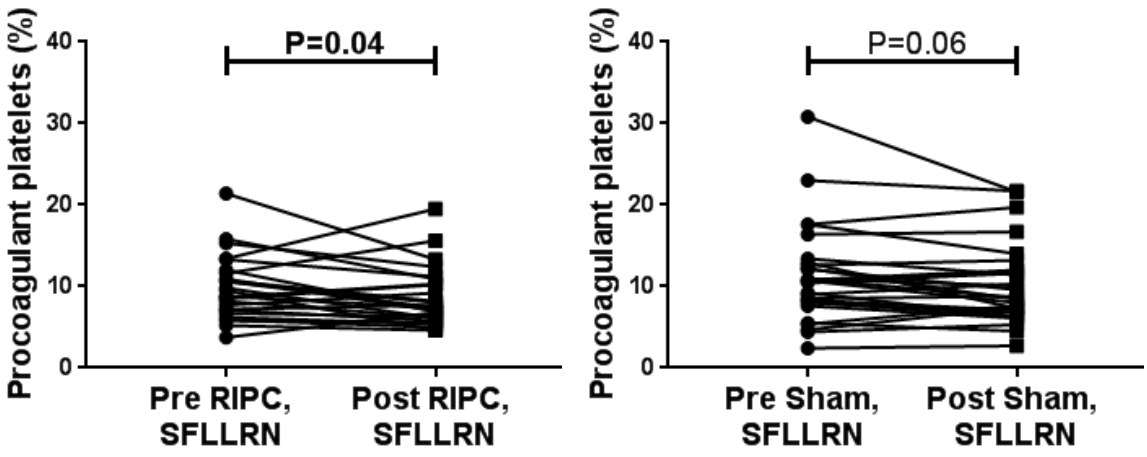

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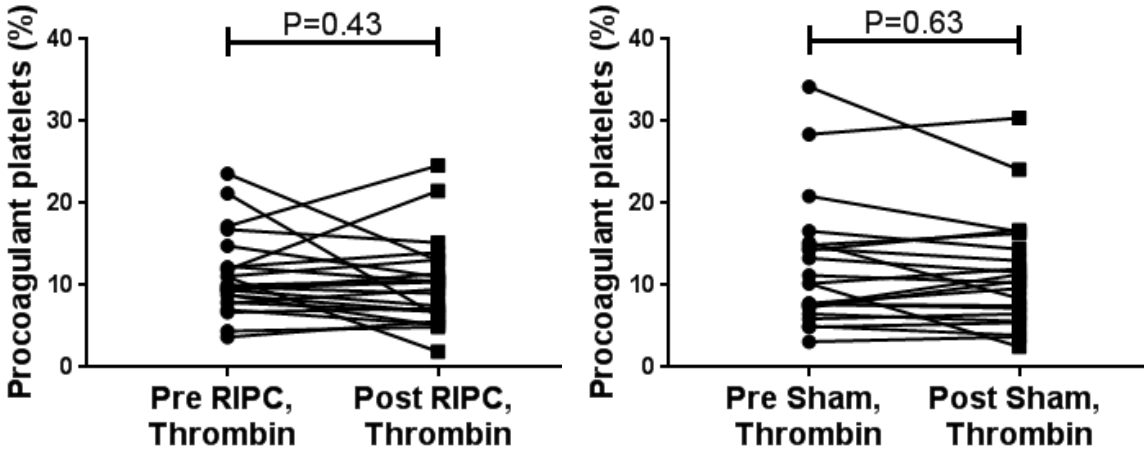


d)

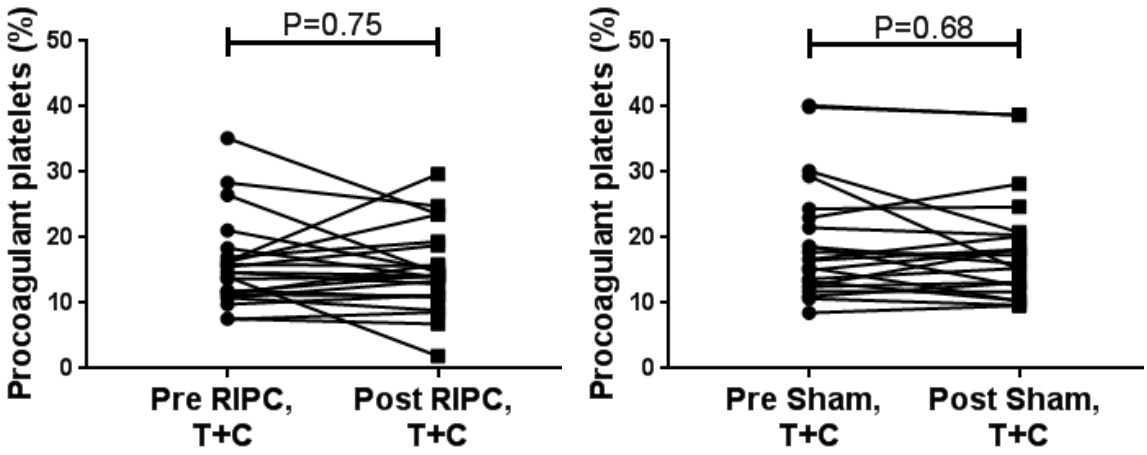


Figure 5.2 The effect of remote ischaemic preconditioning on procoagulant platelets in response to agonists

Effect of RIPC (left panels) or sham (right panels) on procoagulant platelet formation in response to ADP (a), SFLLRN (b), thrombin (c) and thrombin + collagen (d). ADP, SFLLRN, Thrombin, T+C: RIPC, n=24, 26, 23 and 23 respectively; Sham, n=26, 28, 22 and 22 respectively; P values compare measurements from patients pre and post RIPC/sham treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

Analysis of these results according to the use of antiplatelet agents revealed that in the subgroup of patients receiving aspirin monotherapy there was a significant reduction in procoagulant platelet formation in response to SFLLRN (11.4 ± 5.2% vs 8.9 ± 2.8%, P=0.04) and thrombin (12.9 ± 5.9% vs 8.7 ± 3.2%, P=0.04) with a trend towards a reduction in response to thrombin + collagen (19.3 ± 8.7% vs 15.5 ± 5.3%, P=0.052). However there was no apparent effect of RIPC on procoagulant platelet formation in patients on DAPT (Figure 5.3 and Table 5.2). These reductions were not seen with sham treatment.

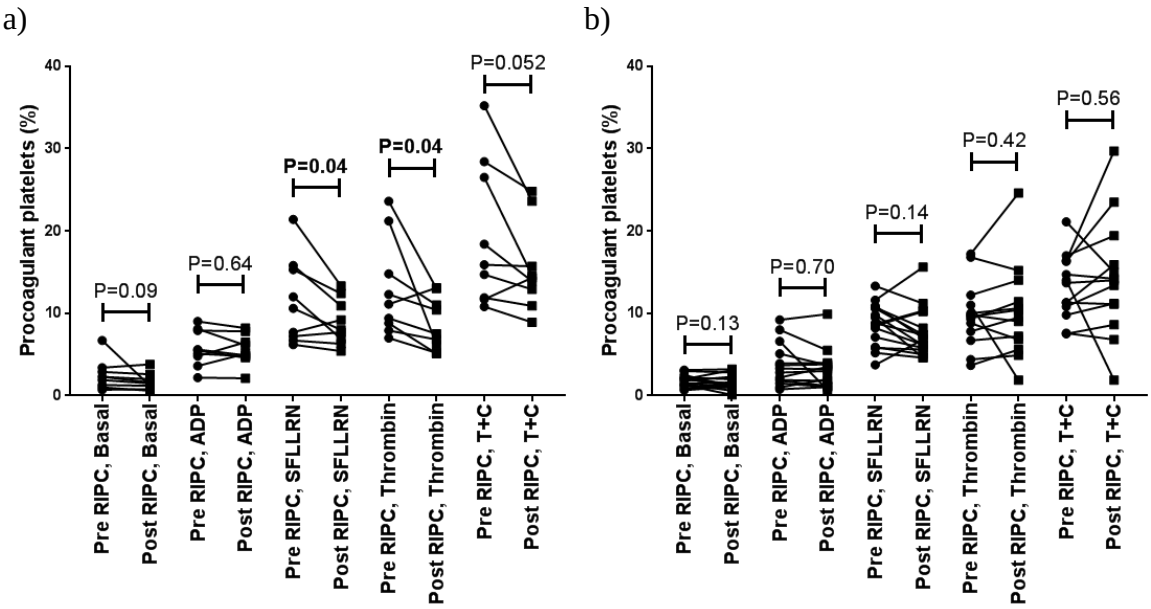


Figure 5.3 The effect of remote ischaemic preconditioning on procoagulant platelet formation in patients on aspirin monotherapy and dual antiplatelet therapy

Effect of RIPC on procoagulant platelets in unstimulated basal samples or in response to ADP, SFLLRN, thrombin and thrombin + collagen in patients on aspirin monotherapy (a) and DAPT (b). Aspirin, n=9; DAPT: basal, ADP, SFLLRN, thrombin, T+C, n=16, 14, 16, 13 and 13 respectively; P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy; RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

Table 5.2 The effect of remote ischaemic preconditioning on procoagulant platelet formation in response to agonists, stratified by antiplatelet therapy

Agonist*	Aspirin monotherapy			DAPT		
	Pre RIPC	**Post RIPC**	**P**	**Pre RIPC**	**Post RIPC**	**P**
Basal	2.3 (1.2-3.2)	1.5 (1.0-2.3)	0.09	1.8 ± 0.7	1.6 ± 0.9	0.13
ADP	5.8 ± 2.2	5.7 ± 1.8	0.64	3.1 (1.7-5.5)	3.1 (1.2-3.9)	0.70
TRAP6	11.4 ± 5.2	8.9 ± 2.8	**0.04**	8.8 (6.2-10.7)	6.9 (5.7-9.7)	0.14
Thrombin	12.9 ± 5.9	8.7 ± 3.2	**0.04**	9.6 (7.3-11.6)	9.5 (6.2-12.7)	0.42
T+C	19.3 ± 8.7	15.5 ± 5.3	0.052	13.2 ± 4.0	14.2 ± 7.2	0.56

P values compare measurements from patients pre and post RIPC using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

* Values represent percentage (%) procoagulant platelets.

Basal, ADP, TRAP6, Thrombin, T+C: aspirin monotherapy, n=9, 9, 9, 9 and 9 respectively; DAPT, n=16, 14, 16, 13 and 13 respectively.

ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy; T+C, thrombin + collagen; TRAP6, thrombin related activating peptide 6;

In the subgroup of patients with significant CAD based on QCA of the coronary angiogram, there was a significant reduction in procoagulant platelet formation under unstimulated circulating conditions (Figure 5.4). There was no effect of sham on the formation of procoagulant platelets in response to any of the agonists or in the 6 patients treated with RIPC without significant CAD (data not shown).

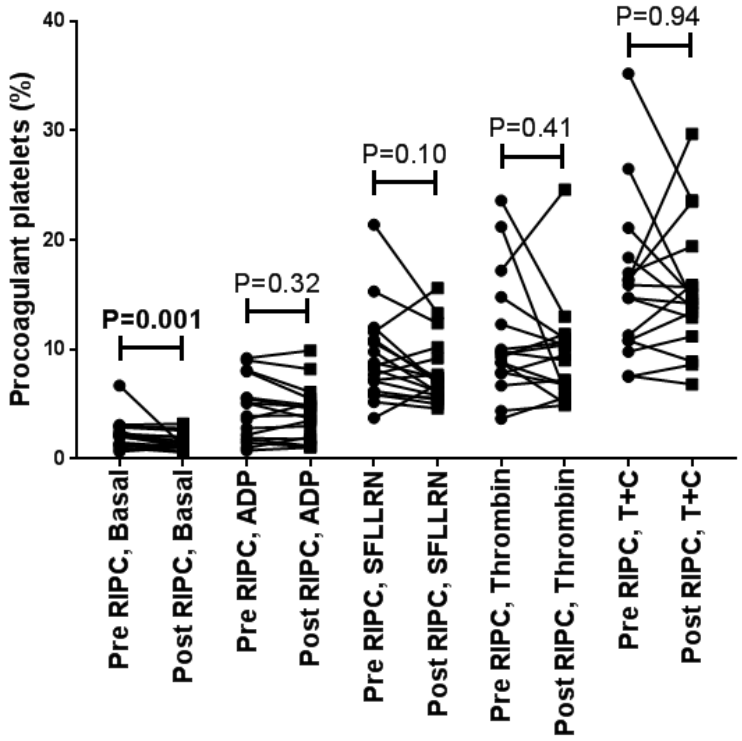


Figure 5.4 The effect of remote ischaemic preconditioning on procoagulant platelet formation in patients with significant coronary artery disease

Effect of RIPC on procoagulant platelets in unstimulated basal samples or in response to ADP, SFLLRN, thrombin and thrombin + collagen in patients with significant coronary artery disease as demonstrated by coronary angiography. Basal, ADP, SFLLRN, thrombin, T+C, n=18, 17, 18, 16 and 16 respectively; P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data. ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

5.3.4 Correlation between remote ischaemic preconditioning induced reduction in circulating procoagulant platelet formation and agonist induced PAC-1 binding

There was no significant correlation between the change in procoagulant platelets under unstimulated conditions with RIPC and the changes in PAC-1 binding with either SFLLRN or thrombin + collagen as described in chapter 4 (Figure 5.5). This suggests

that RIPC acts differentially on these two pathways of platelet activation in individual patients and inhibition of one platelet activation pathway does not predict effect on the other.

a) b)

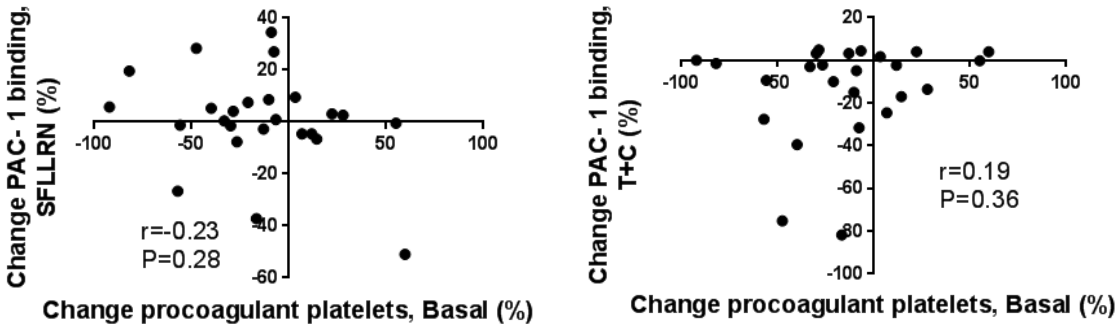


Figure 5.5 Correlation between change in procoagulant platelets under unstimulated conditions and change in PAC-1 binding with remote ischaemic preconditioning

Correlation between changes in procoagulant platelet formation under unstimulated conditions with RIPC and changes in PAC-1 binding in response to SFLLRN (a) and thrombin + collagen (b). Values determined by calculating the difference in post and pre values and representing the difference as a percentage of the pre value. A negative value suggests a reduction with RIPC. n=26. Correlations between variables were assessed by Pearson r correlation or Spearman rank correlation for parametrically and non-parametrically distributed data respectively.

RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

5.3.5 Further exploration into the effect of remote ischaemic preconditioning on procoagulant platelet formation in response to agonist stimulation

Despite the relatively small size of this subgroup in the randomised cohort, there was a RIPC-mediated reduction of procoagulant platelet formation in response to SFLLRN and thrombin in patients on aspirin monotherapy. To explore the effect of RIPC on

agonist induced procoagulant platelet formation further, a separate cohort of patients was recruited focusing on patients on aspirin monotherapy. All these patients had blood collected before and after RIPC. In total 25 patients were recruited with a mean age of 66.0 ± 9.5 yr, 23 (92%) were male, 20 (80%) were found to have significant CAD. Fifteen (60%) of these patients were on aspirin monotherapy and 9 (36%) were on aspirin and clopidogrel. The baseline characteristics of this exploratory cohort are displayed in Table 5.3.

As observed in the randomized cohort, there was a significant reduction in unstimulated circulating procoagulant platelets with RIPC (Figure 5.6). Additionally, RIPC decreased agonist-induced procoagulant platelet formation, showing significant effects with all agonists used in the randomised cohort (Figure 5.6). There was also a significant reduction in procoagulant platelet formation in response to the PAR-4 agonist AYPGFK (6.4% (5.3-8.2) vs 5.1 (4.2-6.5), P=0.006, n=24).

Table 5.3 Baseline characteristics of exploratory procoagulant platelet cohort

Characteristic	RIPC treated (n=25)
Age - years	66.0 ± 9.6
Male – no. (%)	23 (92)
Clinically stable – no. (%)	25 (100)
Significant CAD – no. (%)	20 (80)
Prior myocardial infarction – no. (%)	3 (12)
Prior PCI – no. (%)	3 (12)
Prior CABG- no. (%)	3 (12)
Heart failure – no. (%)	0 (0)
Prior stroke – no. (%)	6 (24)
Peripheral vascular disease – no. (%)	2 (8)
Hypertension – no. (%)	14 (56)
Diabetes – no. (%)	11 (44)
Dyslipidaemia – no. (%)	20 (80)
Current smoking – no. (%)	3 (12)
Medications*	
Aspirin – no. (%)	24 (96)
$P2Y_{12}$ antagonist – no. (%)	9 (36)
Clopidogrel – no. (%)	9 (36)
Ticagrelor – no. (%)	0 (0)
Prasugrel – no. (%)	0 (0)
Aspirin monotherapy – no. (%)	15 (60)
Dual antiplatelet therapy – no. (%)	9 (36)
Warfarin/ NOAC – no. (%)	0 (0)
Heparin – no. (%)	0 (0)
Statin – no. (%)	19 (76)
Beta blocker – no. (%)	10 (40)
ACEi or ARB – no. (%)	10 (40)
Nitrate – no. (%)	3 (12)
Parameters during admission	
Systolic blood pressure – mmHg	142.3 ± 20.5
Heart rate – beats/min	67.7 ± 10.9
Haemoglobin concentration – g/L	131.0 ± 15.5
Platelet count – 10^9/L	219.4 ± 72.1
eGFR - mL/min/1.73m^2	81.7 ± 13.5

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; NOAC, novel oral anticoagulant; PCI, percutaneous coronary intervention;

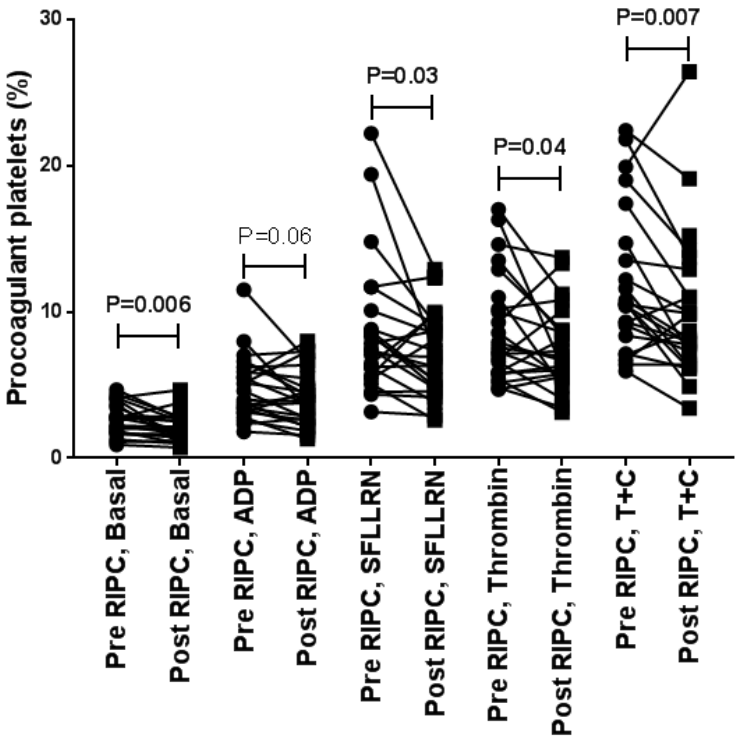


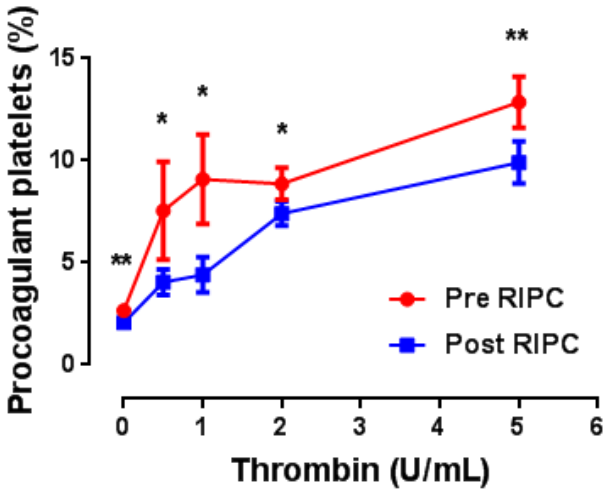
Figure 5.6 The effect of remote ischaemic preconditioning on procoagulant platelet formation in exploratory platelet cohort

Effect of RIPC on procoagulant platelets in unstimulated basal samples or in response to ADP, SFLLRN, thrombin and thrombin + collagen in exploratory platelet cohort. Basal, ADP, SFLLRN, thrombin, T+C, n=24, 24, 23, 23 and 23 respectively; P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

ADP, adenosine diphosphate; RIPC, remote ischaemic preconditioning; T+C, thrombin + collagen; SFLLRN, PAR-1 agonist;

RIPC was also associated with a significant reduction in procoagulant platelet formation in response to a range of thrombin and thrombin + collagen concentrations (Figure 5.7). The effect of RIPC commenced at low doses and was maximal at 1 U/mL thrombin and between 0.5 – 1 U/mL thrombin + 10 μg/mL collagen.

a)



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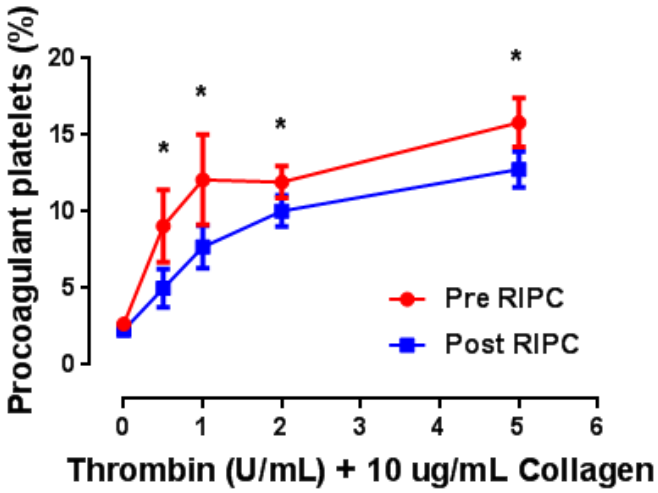


Figure 5.7 The effect of remote ischaemic preconditioning on procoagulant platelet formation in response to a range of thrombin and thrombin + collagen concentrations

The effect of RIPC on procoagulant platelet formation in response to a range of thrombin (a) and thrombin + 10 µg/mL collagen (b) doses. Symbol and bars represent mean ± SEM. * - P<0.05; ** - P<0.01; Thrombin 0 U/mL, 0.5 U/mL, 1 U/mL, 2 U/mL, 5 U/mL: n=24, 7, 7, 23, 16 respectively; Collagen 10 µg/mL + Thrombin 0 U/mL, 0.5 U/mL, 1 U/mL, 2 U/mL, 5 U/mL: n=24, 7, 7, 23, 16 respectively; P values compare measurements from patients pre and post RIPC treatment using the paired t test for parametrically distributed data or the Wilcoxon signed rank test for non-parametrically distributed data.

RIPC, remote ischaemic preconditioning;

5.3.6 The effect of remote ischaemic preconditioning on platelet mitochondrial membrane potential

Procoagulant platelet formation is dependent on cyclophilin D dependent regulated cell death and characterised by opening of the MPTP. Thus, a marker of mitochondrial membrane potential, TMRE, was used to investigate whether stabilisation of mitochondrial membrane potential is mechanistically related to the RIPC-mediated attenuation of platelet procoagulant activity. Patients with significant CAD on aspirin monotherapy (n=5) were treated with RIPC to assess mitochondrial membrane potential in response to agonist stimulation and procoagulant platelet formation in parallel over a range of thrombin and thrombin + collagen concentrations. There was a positive correlation between CD62P+/GSAO+ platelets and TMRE- events in this cohort (Figure 5.8).

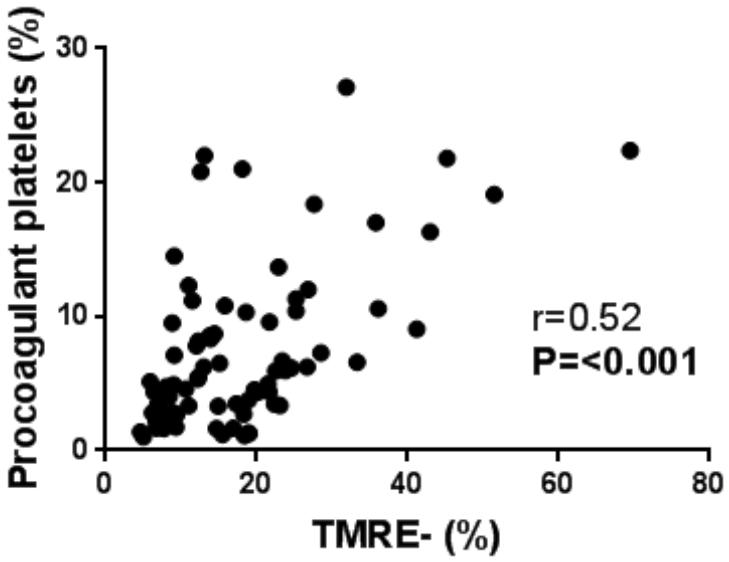


Figure 5.8 Correlation between procoagulant platelets and TMRE-negative events

Correlation between CD62P+/GSAO+ (procoagulant) platelets and TMRE- events based on flow cytometry analysis of platelets after stimulation with a range of agonists in the exploratory cohort. n=78 procoagulant platelet/ TMRE- pairs; Correlations between variables was assessed by Spearman rank correlation.

TMRE, tetramethylrhodamine ethyl ester;

A representative example of the RIPC-induced change in TMRE- and TMRE+ platelet populations after thrombin + collagen stimulation as measured by flow cytometry in one patient is displayed in Figure 5.9a. RIPC was associated with a significant reduction in TMRE- platelets in response to increasing doses of thrombin (Figure 5.9b) and also the strong agonist combination of thrombin + collagen (Figure 5.9c).

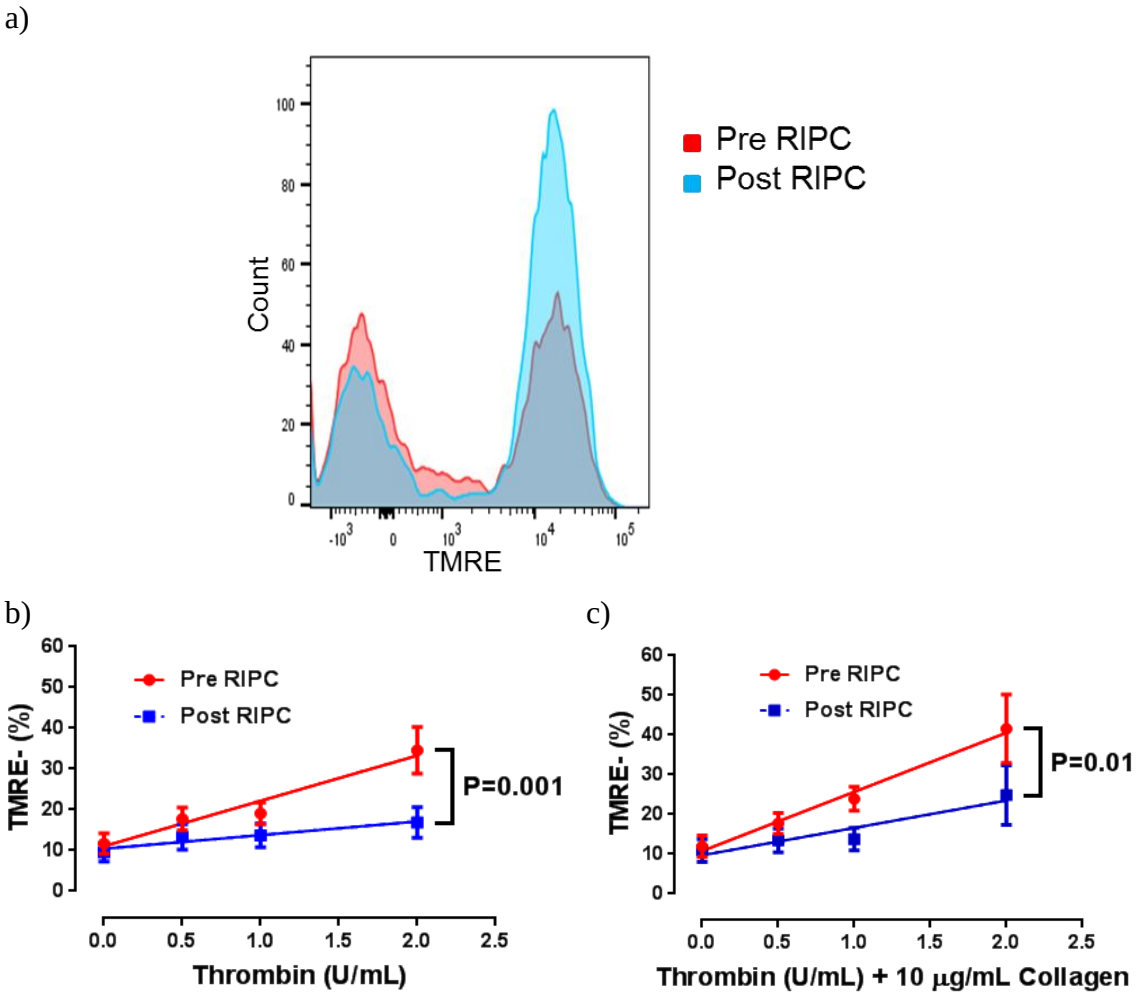


Figure 5.9 The effect of remote ischaemic preconditioning on platelet mitochondrial membrane depolarisation in response to agonists

Representative example of RIPC-induced shift in TMRE- and TMRE+ populations in one patient. Histogram demonstrating absence of staining (left peaks, TMRE-) and staining (right peaks, TMRE+) by TMRE in platelets, from the same patient with CAD before RIPC (red) and after RIPC (blue) after stimulation with thrombin + collagen (a). The effect of RIPC on TMRE- platelet population in response to thrombin (b) and thrombin + collagen (c). Red and blue symbols and bars represent mean ± SEM with lines representing linear regression of data points. n=5; P values represent comparison of dose curves pre and post RIPC treatment using curve analysis software.

CAD, coronary artery disease; RIPC, remote ischaemic preconditioning; TMRE, tetramethylrhodamine ethyl ester perchlorate;

In these same 5 patients, there was a significant reduction in procoagulant platelet formation in response to the range of thrombin concentrations and also to the range of thrombin + collagen concentrations which approached statistical significance (Figure 5.10). There was a significant correlation between the change in TMRE- platelets and the change in procoagulant platelets (r=0.35, P=0.03, n=39 pairs). In support of the previously demonstrated reduction in circulating procoagulant platelets with RIPC, there was a RIPC-mediated reduction in TMRE- platelets under unstimulated conditions which approached statistical significance (9.3% (6.8-17.9) vs 6.7% (5.4-15.1), P=0.06).

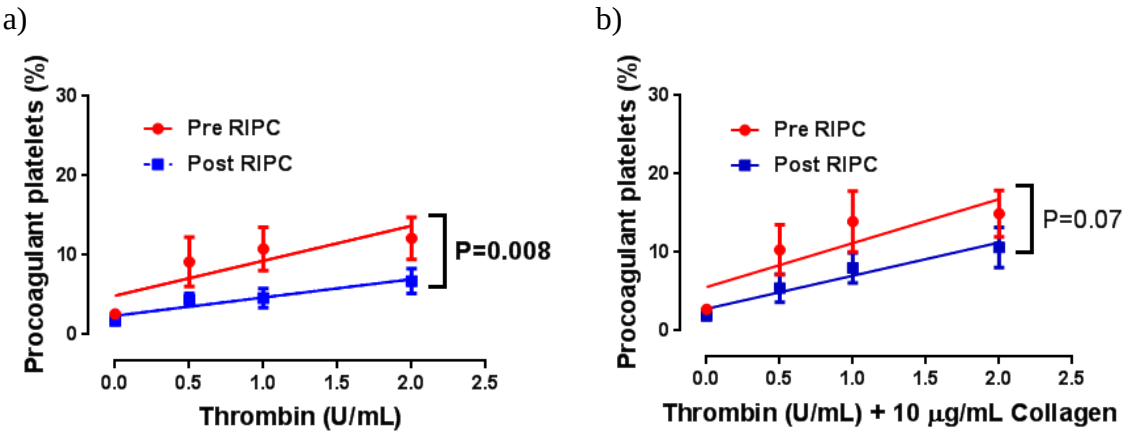


Figure 5.10 The effect of remote ischaemic preconditioning on procoagulant platelet formation in patients with conditioning-mediated reduction in platelet mitochondrial membrane depolarisation

The effect of RIPC on procoagulant platelet formation in response to a range of thrombin (a) and thrombin + 10µg/mL collagen (b) doses in patients assessed with platelet TMRE flow cytometry assay. Red and blue symbols and bars represent mean ± SEM with lines representing linear regression of data points. Thrombin 0 U/mL, 0.5 U/mL, 1 U/mL, 2 U/mL: n=5, 5, 5, 4 respectively; Collagen 10 µg/mL + Thrombin 0 U/mL, 0.5 U/mL, 1 U/mL, 2 U/mL: n=5, 5, 5, 5 respectively; n=5 patients; P values represent comparison of dose curves pre and post RIPC treatment using curve analysis software.

RIPC, remote ischaemic preconditioning; TMRE, tetramethylrhodamine ethyl ester perchlorate;

5.4 Discussion

Platelets have sub-populations with different functions. Classical activation is required for haemostasis as well as promoting thrombosis, thus standard anti-platelets agents inevitably increase bleeding risk. It is now well recognised that a subset of highly activated platelets, procoagulant platelets, can change their membrane surface to assemble the coagulation complexes tenase and prothrombinase, which generate thrombin and localise fibrin formation to the platelet thrombus (122,179). Excess procoagulant platelets are implicated in pathological thrombosis, however, previous studies on hyperactive platelets and cardiovascular risk have concentrated on classical platelet activation rather than procoagulant platelets (186). Recent studies show that strategies to selectively reduce procoagulant platelets including targeting intracellular calcium (Orai1 deficiency) (187), ATP regulation (14-3-3ζ,) (188) and membrane water channels (aquaporin) protect against murine models of arterial thrombosis (121) without an increase in bleeding. The study of procoagulant platelets is particularly exciting as specific inhibition of procoagulant platelet formation could reduce pathological thrombosis in CVD without increasing bleeding.

Procoagulant platelets are characterized by externalization of PS in a process involving Cyclophilin D-regulated formation of the MPTP (121,178). Clinical studies of the role of procoagulant platelets in CVD have been hampered by the lack of a sensitive and specific assay. It is known that PS exposure is a prerequisite for procoagulant platelets, but there is an imperfect correlation between PS and procoagulant function. It was recently established that a labelled cell death marker GSAO [(4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid] can detect procoagulant platelets ex vivo and in vivo (120) with very good correlation with activated factor binding (r^2

=0.98, p <0.0001) (120,123). This subset of activated platelets, which take up GSAO, undergo cyclophilin D-mediated necrosis and provide a procoagulant surface (120). A recent major review contrasting varying methods to identify procoagulant platelets, including PS exposure and formation of the MPTP identified GSAO as an important means of evaluating the role of procoagulant platelets in disease and mechanism studies (189). A flow cytometry assay to detect procoagulant platelets using the platelet uptake of a GSAO and co-staining with CD62P (GSAO+/CD62P+ platelets) was adapted to identify procoagulant platelets in whole blood and was used in this study (120,123). Patients with CAD have increased circulating procoagulant platelets and an increased propensity to form procoagulant platelets in response to soluble agonists (123). This hyper-reactivity, compared to healthy volunteers, is greatest in response to soluble agonist thrombin (8.8±2 vs 18.9±11%, P<0.0001) unaffected by aspirin and was not associated with other markers of platelet function including aggregation, dense granule release and activation of GPIIb-IIIa (123).

In the present study, RIPC reduced the number of circulating procoagulant platelets, and attenuated the formation of procoagulant platelets in response to a range of thrombin and thrombin + collagen agonist concentrations. This effect on procoagulant platelet formation appeared to be due to an effect of RIPC on platelet mitochondrial membrane depolarisation as demonstrated by a flow cytometry assay utilising the mitochondrial potential sensitive dye TMRE. The effect of RIPC on circulating procoagulant platelets is supported by the greater reduction in percentage change with RIPC than sham, which may have been statistically significant had the cohort sizes been larger.

It is traditionally believed only strong agonists activate platelet MPTP and procoagulant platelet formation. The presence of procoagulant platelets in the circulation of patients with CAD suggests that a low level of agonist stimulation, possibly as a result of

exposure to collagen and thrombin in atheromatous lesions, occurs in patients with CAD. The present study demonstrates that RIPC ameliorates this. The effect of RIPC in reducing procoagulant platelets under unstimulated circulating conditions was apparent in the subgroup of patients with significant CAD. There was no correlation between the effect of RIPC on the levels of procoagulant platelets and on GPIIb-IIIa activation, implying that RIPC modulates these processes independently.

Depolarisation of the platelet mitochondrial membrane by formation of the MPTP is thought to be a pivotal step during the formation of a procoagulant phenotype (178). Stabilisation of the membrane may be detected by retention of TMRE within the platelet mitochondria and this was defined as a TMRE+ platelet event in this study. There was a significant increase in the proportion of platelets which were TMRE+ after RIPC. Additionally, RIPC significantly increased platelet mitochondrial membrane stabilisation after stimulation with low dose thrombin, which would be expected to be more likely to occur *in vivo* in patients with stable coronary disease. This effect was also seen at higher concentrations of thrombin, with and without collagen. There was a significant correlation between the decrease in the proportion of circulating pro-coagulant platelets after RIPC and the change in the number of platelets with stable mitochondrial membrane potential, suggesting this pathway is a relevant mechanism during RIPC.

The MPTP is a non-specific, high conductance channel that forms in the inner mitochondrial membrane upon myocardial reperfusion. It mediates cell death by uncoupling oxidative phosphorylation leading to depletion of ATP (190). Ischaemic preconditioning and postconditioning have been shown to inhibit the opening of the MPTP in cardiac myocytes by causing resistance to calcium induced MPTP formation in a cyclophilin D-dependent process (191,192). There are also suggestions that RIPC

may also affect MPTP opening during IR injury, leading to cardiac protection(193). Thus it is possible that RIPC would inhibit the opening of the MPTP in circulating cells such as platelets. RIPC could influence mitochondrial membrane potential stability by affecting formation of the MPTP. Hence the effect of RIPC on procoagulant platelet formation may be a manifestation of an effect on the MPTP.

This study demonstrated a novel effect of RIPC on platelet activity. Procoagulant platelets are increasingly recognised contributors to the pathogenesis of thrombotic diseases and the reduction in procoagulant platelet activity may help explain the beneficial effects of RIPC in clinical settings including acute myocardial infarction. The apparent greater effect of RIPC in patients of aspirin monotherapy will require further exploration as will the effect of antiplatelet therapy on mitochondrial membrane potential and the changes induced by conditioning.

5.5 Conclusions

Remote ischaemic preconditioning reduces the proportion of circulating procoagulant platelets and their formation in response to thrombin based stimulation in patients with coronary artery disease. This appears to occur by stabilising the platelet mitochondrial membrane potential, preventing depolarisation. This effect of RIPC may be important in diseases associated with extensive thrombus formation such as STEMI and may help explain the benefit of preconditioning in this clinical context.

Chapter 6: The effect of remote ischaemic preconditioning on circulating microRNAs

6.1 Introduction

MicroRNAs are short (approximately 22 nucleotides in length), non-coding segments of RNA that have gene regulatory functions in human biology (194). They play a role in post-transcriptional regulation of protein expression, by direct regulation of messenger RNA (mRNA) or by inhibition of translation of the target mRNA (195,196). MicroRNAs are synthesised as a primary transcript, pri-miRNA, in the nucleus by RNA polymerase II (197). This primary transcript is cleaved to form pre-miRNA. Once transported out of the nucleus the pre-miRNA is cleaved in the cytoplasm by the RNAse III enzyme DICER to form a duplex which is between 20-22 nucleotides long (198,199). The two miRNA strands are indicated by the suffixes -5p and -3p, respectively. Although both strands can perform the miRNA function, usually only one strand is incorporated into the RNA induced silencing complex (RISC), where the miRNA and target mRNA interact (200).

There is evidence supporting the role for miRNAs in the development of atherosclerosis and coronary artery disease (201). Lipid homeostasis can be influenced by miRNAs, with inhibition of miRNA-122 leading to reduction in plasma cholesterol in animal models (202,203). Further, other miRNA species promote atherosclerotic plaque development and the formation of high risk plaque features while others have atheroprotective properties (204-207). Specific miRNA species have been shown to promote endothelial cell dysfunction and promote leukocyte invasion into the vessel wall leading to vascular inflammation (208,209). Activation of human platelets can lead

to changes in miRNA expression, which consequently affects protein expression, integrin activity and inflammatory pathways (199,210). Further, specific miRNA profiles may influence the cardiac response to IR injury and hence the degree of myocardial injury and the potential for repair (211,212). Finally, endothelial regeneration and smooth muscle proliferation are influenced by miRNA expression (213), which has implications for revascularisation by PCI and CABG.

6.1.1 MicroRNA and remote ischaemic preconditioning

There is precedent that RIPC may affect miRNA expression in some experimental models. MicroRNA-144 is upregulated by RIPC in both mouse myocardium and plasma, and in this species is associated with promotion of cellular survival pathways and down-regulation of mammalian target of rapamycin (45). In this same study, miRNA-144 was also upregulated in the plasma of healthy human volunteers subjected to RIPC. While local ischaemic preconditioning is associated with a rise in myocardial miRNA-1 and miRNA-21 in the rat heart, RIPC and ischaemic postconditioning reduced miRNA-1 in the myocardium and had no effect on miRNA-21 (50). Changes in miRNA-1 and miRNA-21 have also been suggested by other studies. The reports describing these animal studies did not comment on the effect of RIPC on other miRNA species.

In patients undergoing CABG, RIPC altered the atrial myocardial expression of specific miRNAs, attenuating expression of miRNA-1 in response to surgery and promoting miRNA-338-3p expression (51). A rat model demonstrated a more complex relationship between RIPC and expression within the myocardium. MicroRNA-1 was initially decreased by RIPC after IR injury, but after 6 h RIPC had increased miRNA-1 levels in the myocardium (214). Changes in plasma miRNA profiles with RIPC were not

reported in these studies. In a separate study, children undergoing cardiopulmonary bypass for congenital heart disease surgery were treated with RIPC and this was associated with an increase in levels of miRNA-21 in urine and blood and a reduction in markers of acute kidney injury compared to patients in the control arm (215).

RIPC is thought to have protective effects beyond the heart and animal studies have demonstrated the contribution of miRNA to the protection of other organs against IR injury and against the effects of sepsis (216-218). As discussed in chapter 1, the mediators of RIPC are most likely transported to distant target organs in the circulation. Hence the aim of this study was to study the effect of RIPC on the plasma miRNA profile in patients undergoing coronary angiography for the investigation of CAD.

6.2 Methods

6.2.1 Patient population

Patients referred for coronary angiography were recruited for participation in this study prior to their planned procedure. The patients were randomised to receive RIPC or sham treatment and had blood collected before and after the allocated treatment. This patient population was described in chapters 4 and 5.

6.2.2 Blood collection and plasma storage

The blood collection and storage of plasma was performed as described in sections 2.4 and 2.5. In brief, a 20G cannula was inserted into a large cubital fossa vein in the contralateral arm to the limb where RIPC/sham treatment was delivered. After the first 3 mL of blood was discarded, blood was slowly drawn through the cannula, without a tourniquet or haemostatic valve, at a rate of 0.5 mL/s into a syringe and gently

transferred into tubes containing 3.2 % buffered sodium citrate. The blood collection procedure was repeated in an identical manner after the patient's allocated treatment. Blood was transported carefully at room temperature for processing within 20 min of collection. The blood was centrifuged twice at 2500 g for 15 min at room temperature and aliquoted for storage. Plasma was stored at -80 °C for miRNA extraction at a later time, without previously being thawed.

6.2.3 Remote ischaemic preconditioning

As described in section 2.1, RIPC was delivered to the left upper limb by inflation of a sphygmomanometer to 200 mmHg or 50 mmHg greater than systolic blood pressure (whichever was greater) for 5 min, followed by deflation for 5 min. This cycle was performed a total of 3 times. Sham treatment was delivered in an identical manner except the sphygmomanometer was inflated to 10 mmHg. Patients were randomised to RIPC or sham treatment by way of a closed envelope system.

6.2.4 Assessing for haemolysis in stored plasma

The degree of haemolysis in the stored plasma was determined by spectrophotometry in duplicate on an EnSpire Multimode Plate Reader as described in section 2.7.1, using A414nm as this is the absorbance peak of free haemoglobin (127). Haemolysis has been shown to alter the miRNA profile of plasma (127,128).

6.2.5 MicroRNA extraction from plasma

MicroRNA was extracted as described in section 2.7.2.5 using the NucleoSpin miRNA Plasma kit as per the manufacturer's recommendations. The extraction was performed in duplicate from 100 µL of each patient sample with mussel glycogen added during the

extraction process to increase the extraction efficiency. After extraction, the RNA sample was eluted in 30 μL of water.

The RNA extracted from the duplicate plasma samples was pooled and underwent a precipitation step as described in section 2.7.2.5. In brief, the miRNA was mixed with 0.1 volumes of 3 M sodium acetate, 2.5 ng mussel glycogen and 2.5 volumes ice cold 100% ethanol before incubation overnight at -80 °C. Once thawed, the sample was centrifuged and the pellet was carefully washed with 1 mL 80% (v/v) of ice cold ethanol. After further centrifugation, for 10 min at 4 °C, the supernatant was carefully removed and the pellet air dried at room temperature for 10 min before being resuspended in 10 μL of RNase free water. The miRNA was stored at -80 °C and used within 10 days.

6.2.6 MicroRNA array

A total of 4 patients who were treated with RIPC were selected from the larger cohort of 60 randomised patients for the purpose of miRNA profiling by TaqMan qPCR array cards. Patients with the greatest attenuation of PAC-1 binding in response to thrombin and collagen and with insignificant amounts of haemolysis in both pre and post RIPC stored plasma samples were chosen. After miRNA extraction, miRNAs were measured using the TaqMan Array MicroRNA Cards (A and B cards) together with the Megaplex^{TM} primers, human Pool Set v3.0 (Thermofisher). Each card (A or B) allowed assessment of up to 381 specific miRNA species. Megaplex RT reaction and card arrays were performed as instructed by the manufacturer with reactions performed with the Viia 7 system and analysed with the QuantStudio software.

Prior to the RT reaction, miRNA-16 specific RT and qPCR reactions were performed to assure successful isolation of miRNA. Additionally, after the megaplex RT reaction,

specific qPCR reactions for miRNA-16 (card A samples) and miRNA-1274B, another common species in plasma (card B samples), were performed to ensure the multiplex RT reaction was successful. The RT and qPCR reactions were performed as described in section 2.7.2.5.

After the miRNA array, microRNA species with at least a 2-fold increase with RIPC, in at least 3 of the 4 patients, were deemed to be potentially upregulated by RIPC. These candidate species were validated by specific RT and qPCR reactions in the larger study cohort. A threshold of 2-fold increase was chosen to improve the chances of validation (219).

6.2.7 Validation of array findings with reverse transcription, pre-amplification and quantitative polymerase chain reactions

To validate the results of the array, RNA was extracted from the 60 patients randomised to RIPC or sham described in chapters 4 and 5. In order to increase consistency, a multiplex RT reaction, with all 8 miRNA-specific RT primers placed in the same reaction was performed, after confirming that combining the primers did not interfere with the RT reaction. The multiplex RT reactions were performed using specific primers for the 2 reference species (miRNA-16 and RNU6-1) and the 6 candidate miRNA species that appeared to be upregulated by RIPC according to the array analysis. The multiplex RT reaction was performed in 10 μL with 1.67 μL of RNA, 1 nM dNTPs, 33 U MultiScribe reverse transcriptase, 1 μL 10x RT buffer, 2.4 U RNAse inhibitor, 4 μL of an equal mix of each of the 8 primers (5x) and 2.45 μL water, using the same RT cycle settings: 16 °C for 30 min, then 42 °C for 30 min then 85 °C for 5 min. Each primer concentration in the multiplex RT reaction was 4-fold lower than

individual RT reactions preformed. After the multiplex RT reaction, 57.8 µL of water was added to dilute each cDNA sample for subsequent qPCR analysis.

To ensure that the 8 primers were compatible, a multiplex reaction was compared to separate individual RT reactions for the same 8 miRNA species using miRNA extracted from 2 separate patients with significant CAD. The multiplex RT reaction was performed as described above. The individual RT reactions were performed as described in section 2.7.2.5 except 28.9 µL of water was added to each sample after the reaction was complete.

Specific miRNA-16 qPCR reactions were performed on the cDNA from 12 random samples from the entire cohort of 60 randomised patients. This was performed to ensure the multiplex RT reaction had worked prior to processing all samples for the 6 candidate and 2 reference miRNAs.

As average Ct values for some miRNA species were > 35 cycles in the array, a pre-amplification step prior to the specific qPCR reactions in the validation cohort was introduced to more accurately assess any changes in miRNAs. Each pre-amplification was performed in 20 µL containing 1x KAPA Probe Fast Master Mix, 1x Rox Low, 5 µL of 1:100 dilution of the 8 miRNA probes (final concentration 0.05x) and 0.1 µL water. Pre-amplification conditions were 95 °C for 10 min followed by 10 cycles of 95 °C for 15 sec and 60 °C for 4 min and were performed on a Viia 7 system. After pre-amplification, samples were diluted with 20 µL of water. To ensure this step was effective in increasing the amount of cDNA, the cDNA of the two patients whose samples were used to test the multiplex RT reaction underwent pre-amplification as above. These results were compared with the results of qPCR reactions for the 6 candidate and 2 reference miRNA species with non-pre-amplified cDNA.

RNA from all patients was reverse transcribed using the multiplex RT reaction, then pre-amplified as described, above after which specific qPCR reactions for each of the 6 miRNA candidate species and the 2 reference species were performed in duplicate. The qPCR reactions were performed in a 96 well plate in 10 μL as described in section 2.7.2.5. The patient treatment (RIPC/sham) allocations were concealed until after the experiments and data analysis was completed.

There were several samples which showed high Ct values for each of the miRNA species investigated, including the reference miRNAs. This suggested reduced quality or quantity of miRNA in these samples. The samples resulting in the highest Ct values in at least 6 of the 8 miRNA species were identified. These outlying samples were excluded from the analysis together with their corresponding pre or post sample. This resulted in exclusion of 14 subjects from the cohort.

6.2.8 Statistical analysis

The mean of the Ct values for duplicate reactions was used in each analysis. With respect to the array, the reference species used was RNU6-1 in all samples as this species was present on both card A and card B. In addition, for card A samples, a separate analysis was performed with miRNA-16 as the reference species. For the validation PCR reactions, both RNU6-1 and miRNA-16 were included as reference miRNA species. The fold change in miRNA species with RIPC or sham treatment was calculated using the double delta method. In short, for each sample, the Ct value of the reference miRNA species was subtracted from the Ct of the miRNA species in question. This was performed in pre and post RIPC/sham samples (ΔCt-pre and ΔCt-post respectively). To determine the double delta Ct value (ΔΔCt), the difference between

ΔCt-post and ΔCt-pre was calculated. The fold change in miRNA species expression with RIPC or sham was calculated by $2^{-\Delta\Delta Ct}$.

The fold change in miRNA species expression with the allocated treatment was expressed as median (IQR) and were compared with the Mann-Whitney U tests for non-parametric data. Correlations between variables were assessed by Pearson r correlation or Spearman rank correlation for parametrically and non-parametrically distributed data respectively. Calculations and statistical analyses were performed with Excel (Microsoft, Redmond, Washington, USA) and GraphPad Prism.

6.3 Results

6.3.1 Baseline clinical characteristics

The baseline characteristics of the 60 patients recruited to study the effects of RIPC were described in section 4.3.1 and Table 4.1. Amongst this cohort, 4 patients treated with RIPC were selected for the array. These 4 patients were all male, presented with stable angiographically significant CAD, showed an average attenuation of PAC-1 binding by 8.2 ± 10.8% by RIPC and had insignificant levels of haemolysis in both pre and post plasma samples (data not shown).

6.3.2 Changes in plasma microRNA based on card array analysis

6.3.2.1 Verification of microRNA-16 levels after extraction

Following extraction and precipitation of small RNA the presence of miRNA-16 was assessed to ascertain successful isolation of miRNA. MiRNA-16 specific RT and qPCR reactions demonstrated similar Ct values (Figure 6.1). The consistency of the miRNA-

16 Ct values implied similar miRNA-16 levels across the samples and suggested successful miRNA isolation.

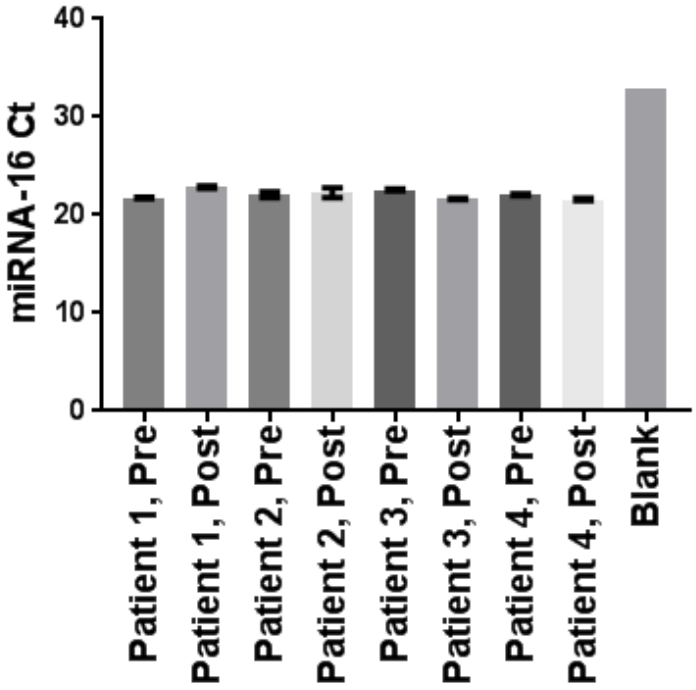


Figure 6.1 MicroRNA-16 content of plasma microRNA samples for array analysis

MicroRNA was extracted from the stored plasma of 4 patients with CAD who received RIPC. Specific miRNA-16 RT and qPCR reactions were performed. Values represent mean Ct value of duplicate reactions for each sample pre and post RIPC, with lower values indicating a greater concentration of miRNA-16 in the sample. The blank sample was a control reaction with water added rather than miRNA. Bars represent range.

CAD, coronary artery disease, Ct, cycle threshold; miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

6.3.2.2 Verification of Megaplex Pool A and B reverse transcription reactions

After establishing successful miRNA extraction, RT reactions using the Megaplex primer pools A and B were performed. Prior to array card analysis, miRNA-16 (pool A)

and miRNA-1274B (pool B) levels were checked. Specific qPCR reactions identified consistent levels of miRNA-16 and miRNA-1274B in the cDNA produced after the Megaplex RT reactions (Figure 6.2). This indicated that the RT reaction was successful and the cDNA could be used for analysis with the array cards.

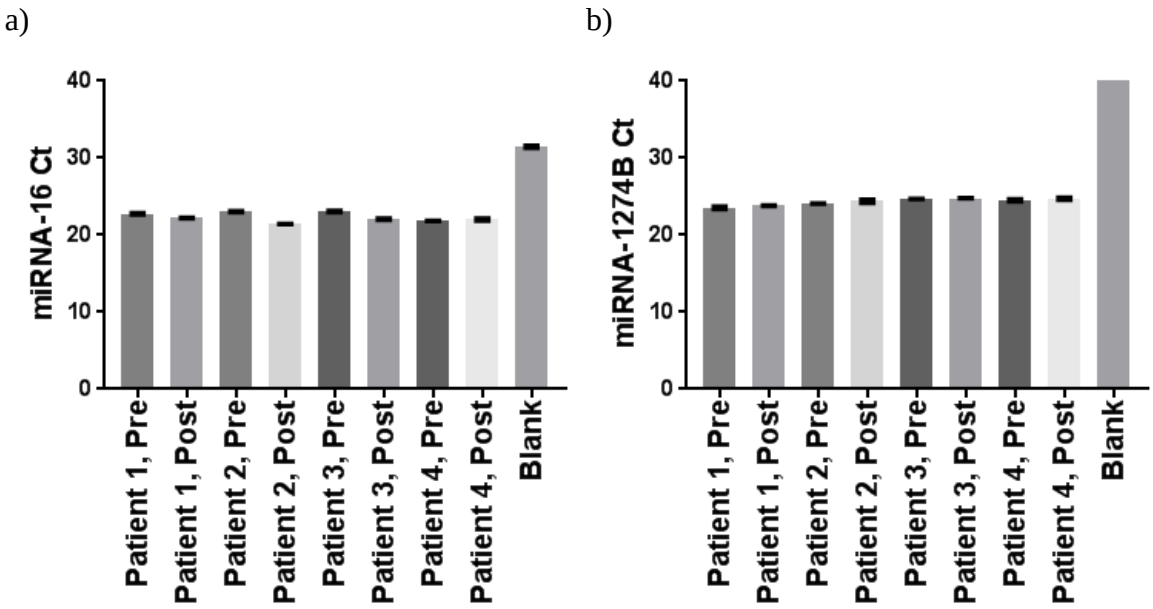


Figure 6.2 MicroRNA-16 and microRNA-1274B levels in complementary deoxyribonucleic acid produced by the Megaplex reverse transcription reactions

After a Megaplex RT reaction, specific qPCR reactions were performed on the cDNA. Values represent mean Ct value of duplicate reactions for each sample pre and post RIPC, with lower values indicating a greater concentration of miRNA-16 (a) or miRNA-1274B (b) in the sample. The blank sample was a control reaction with water added rather than cDNA. Bars represent range.

cDNA, complementary deoxyribonucleic acid; Ct, cycle threshold; miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning; RT, reverse transcription;

6.3.2.3 Array results

In total, 8 A cards and 8 B cards were processed. The reference species RNU6-1 was present in similar levels across the samples for both A and B cards, indicating similar levels of miRNA and successful running of the card PCR reactions (Figure 6.3).

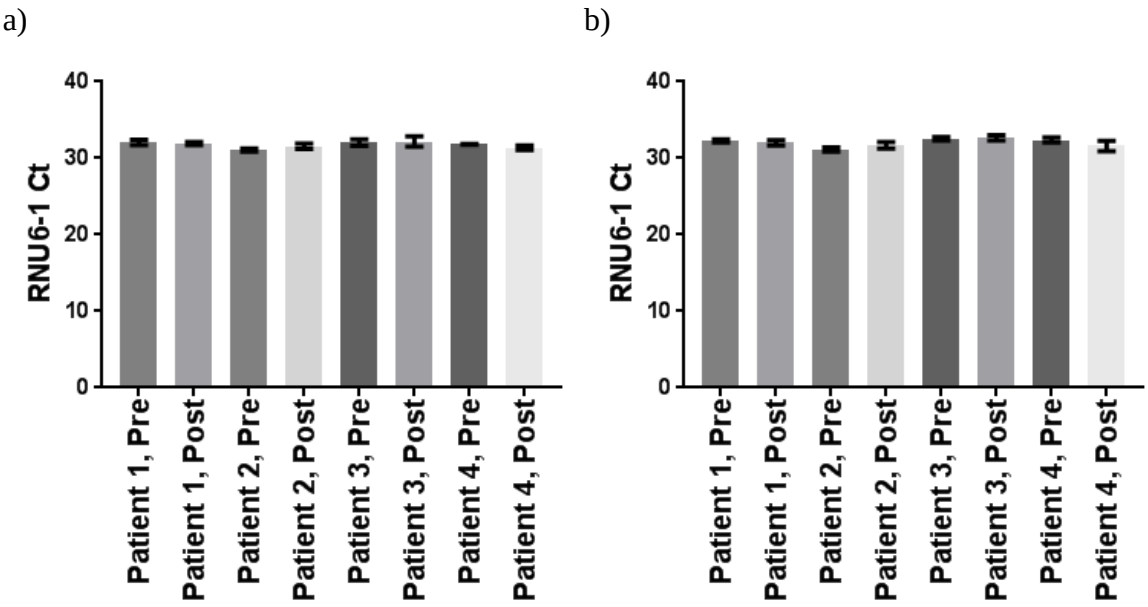


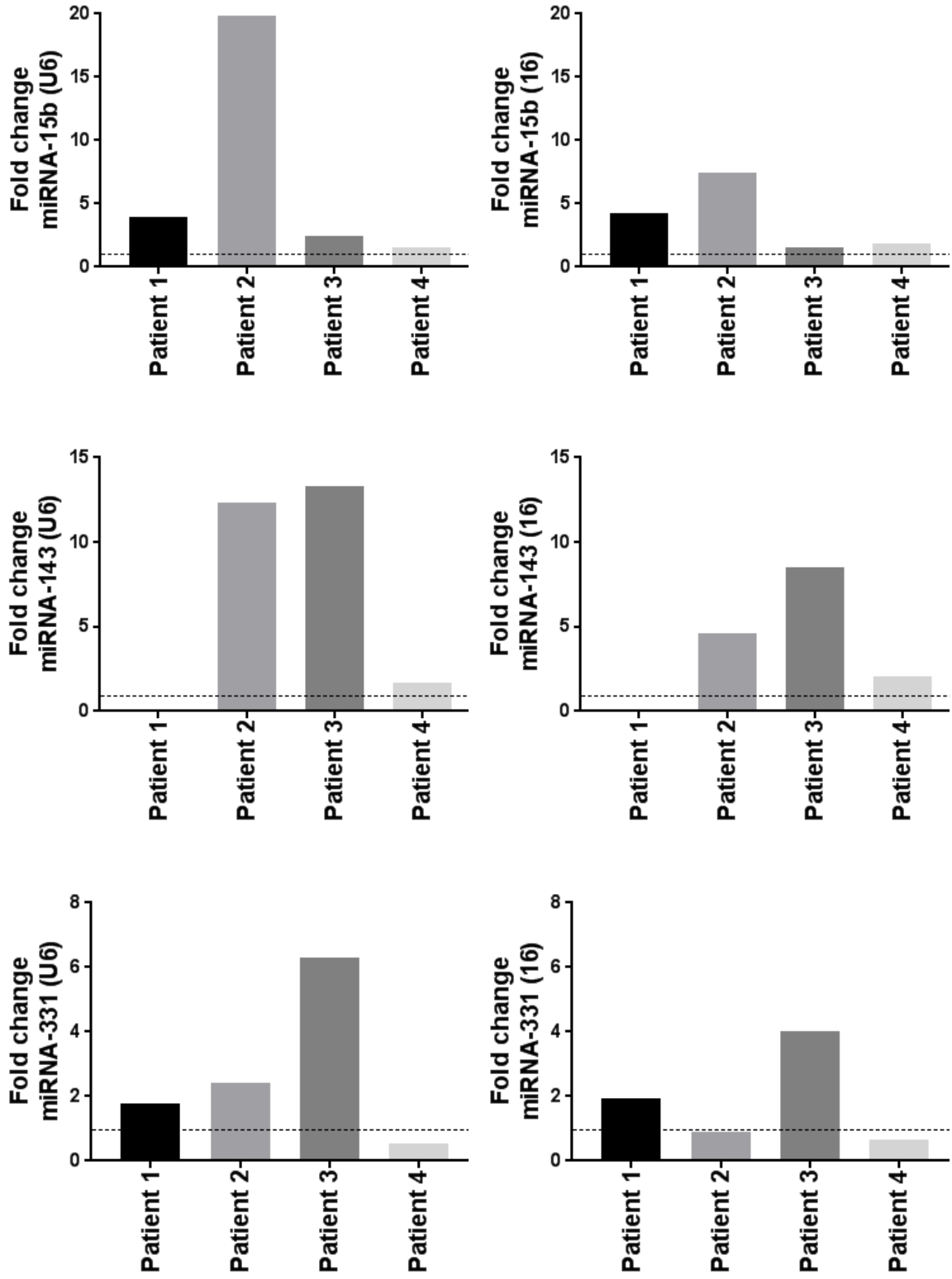
Figure 6.3 RNU6-1 content in TaqMan array card samples

Results of TaqMan array card based qPCR reactions for RNU6-1. Values represent mean Ct valve of 4 qPCR reactions for each sample, pre and post RIPC, with lower values indicating a greater concentration of RNU6-1 in card A samples (a) and card B samples (b). Bars represent ± SD.

Ct, cycle threshold; RIPC, remote ischaemic preconditioning; qPCR, quantitative polymerase chain reaction;

After the fold change with RIPC was calculated for each miRNA species in each patient, 6 miRNA species emerged as potentially being upregulated by conditioning. The species from card A were miRNA-15b, miRNA-143, miRNA-331 and miRNA-425-5p (Figure 6.4), while the species from card B were miRNA-30e-3p and miRNA-151-3p (Figure 6.5). The average fold change in each of these species with RIPC is

demonstrated in Table 6.1 demonstrating large differences in fold change between different patients.



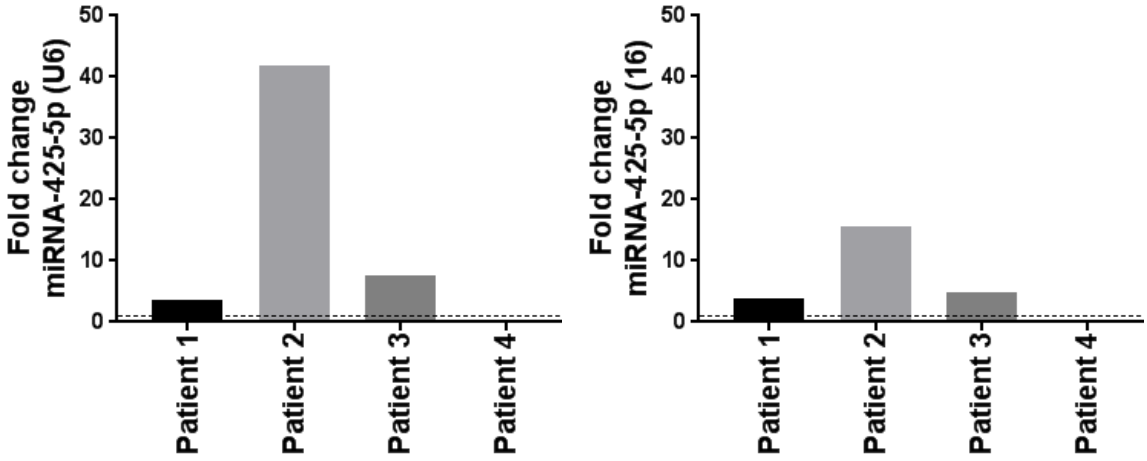


Figure 6.4 MicroRNA species which appeared to be upregulated by remote ischaemic preconditioning amongst TaqMan array card A samples

Card A species with at least a 2-fold increase in expression with RIPC in at least 3 of 4 patients. Fold change calculated with RNU6-1 (left panels) or miRNA-16 (right panels) as the reference species. A value >1 implies an increase in expression with the treatment, whereas a value <1 implies a decrease. Where no fold change reported, amplification curve of either pre RIPC or post RIPC sample did not reach threshold. The dotted line denotes a fold change of 1.

miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

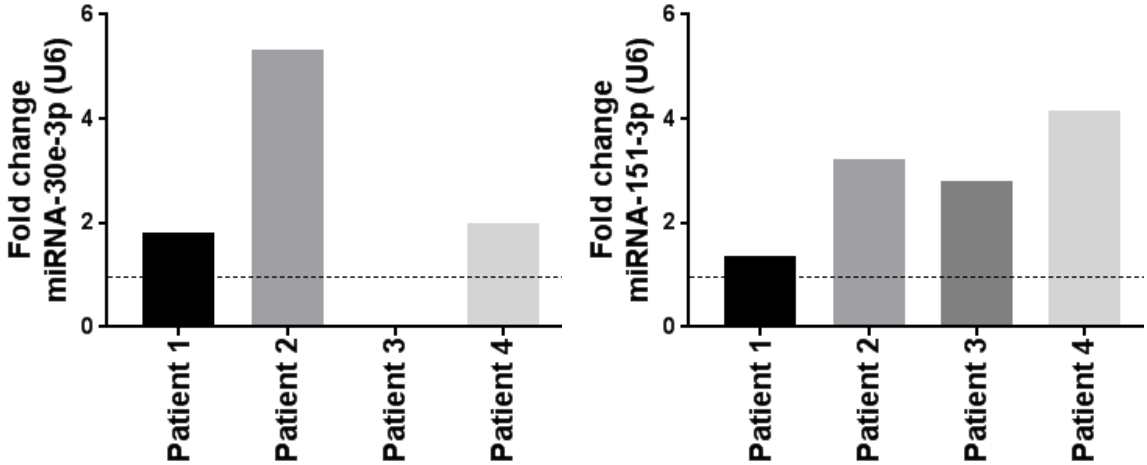


Figure 6.5 MicroRNA species which appeared to be upregulated by remote ischaemic preconditioning amongst TaqMan array card B samples

Card B species with at least a 2-fold increase in expression with RIPC in at least 3 of 4 patients. Fold change calculated with RNU6-1 as the reference species. A value >1 implies an increase in expression with the treatment, whereas a value <1 implies a decrease. Where no fold change reported, amplification curve of either pre RIPC or post RIPC sample did not reach threshold. The dotted line denotes a fold change of 1.

miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

Table 6.1 Fold change in candidate microRNA species with remote ischaemic preconditioning

Species	RNU6-1	miRNA-16
miRNA-15b	6.9 ± 8.7	3.8 ± 2.7
miRNA-30e-3p*	3.0 ± 2.0	
miRNA-143	6.8 ± 7.0	5.0 ± 3.2
miRNA-151-3p*	2.9 ± 1.2	
miRNA-331	2.8 ± 2.5	1.9 ± 1.5
miRNA-425-5p	13.4 ± 19.3	6.2 ± 6.6

Values represent mean ± SD fold change in each miRNA species with RIPC corrected for RNU6-1 or miRNA16. A value >1 or <1 implies an increase or decrease in expression with the treatment, respectively. n=4.

* miRNA-16 reaction not present on card B.

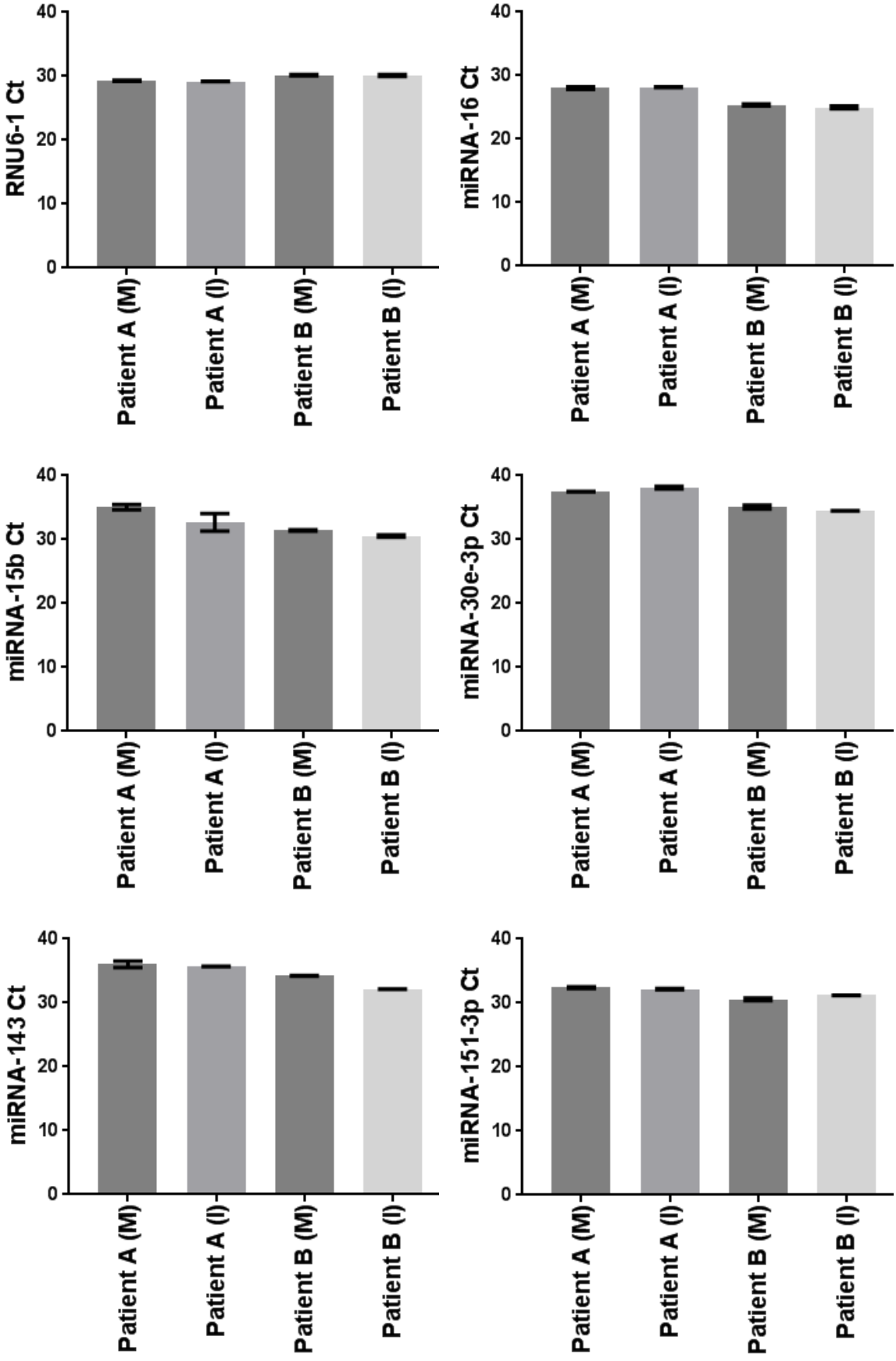
miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

Prior studies have demonstrated RIPC-induced increases in miRNA-144 and miRNA-21 in the circulation of healthy volunteers and children undergoing cardiopulmonary bypass respectively. MicroRNA-1 has been shown to be reduced while miRNA-338-3p increased in atrial tissue of patients undergoing CABG after treatment with RIPC. The array data demonstrated that miRNA-144 was increased in 2 of the 4 patients while miRNA-21 was increased in 1 of the 4 patients after RIPC (data not shown). All of the qPCRs for miRNA-1 and miRNA-338-3p did not reach the threshold, indicating inadequate levels of these species to reliably assess changes in any of these miRNA species after RIPC in this study cohort.

6.3.3 Validation of array findings

6.3.3.1 Comparison of multiplex and individual reverse transcription reactions

A comparison of the Ct values derived after specific qPCR reactions showed similar miRNA levels between the multiplexed RT sample and the individual RT samples for the 6 candidate and the 2 reference species (RNU6-1 and miRNA-16), suggesting the multiplex RT reaction was successful and that combining the primers did not lead to interference in the subsequent qPCR reaction (Figure 6.9). These results indicated that the primers for the 8 miRNA species were compatible in the multiplex RT reaction.


RNU6-1 Ct
miRNA-16 Ct
miRNA-15b Ct
miRNA-30e-3p Ct
miRNA-143 Ct
miRNA-151-3p Ct
0
10
20
30
40
Patient A (M)
Patient A (I)
Patient B (M)
Patient B (I)

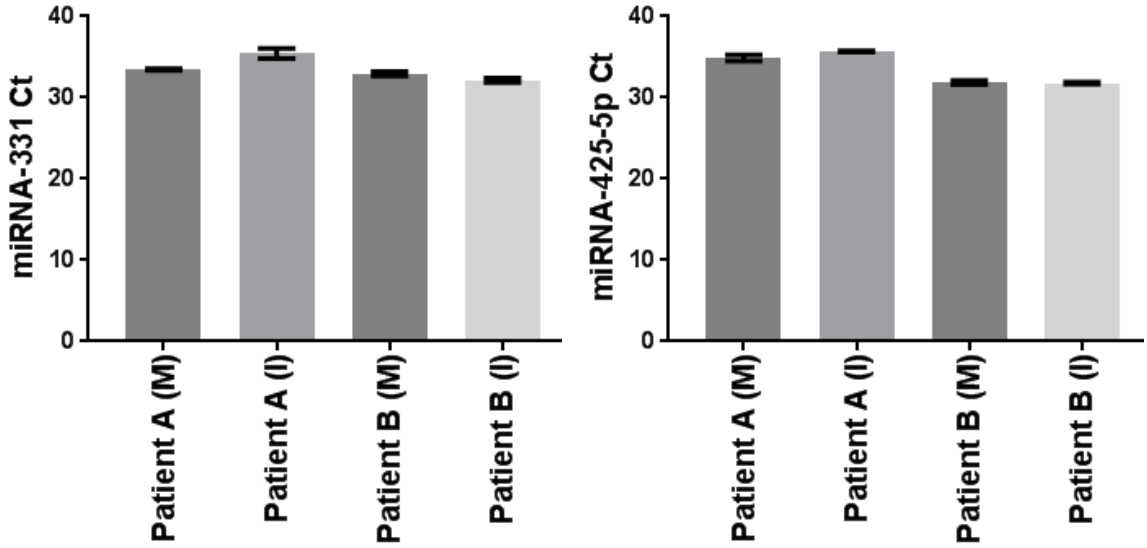


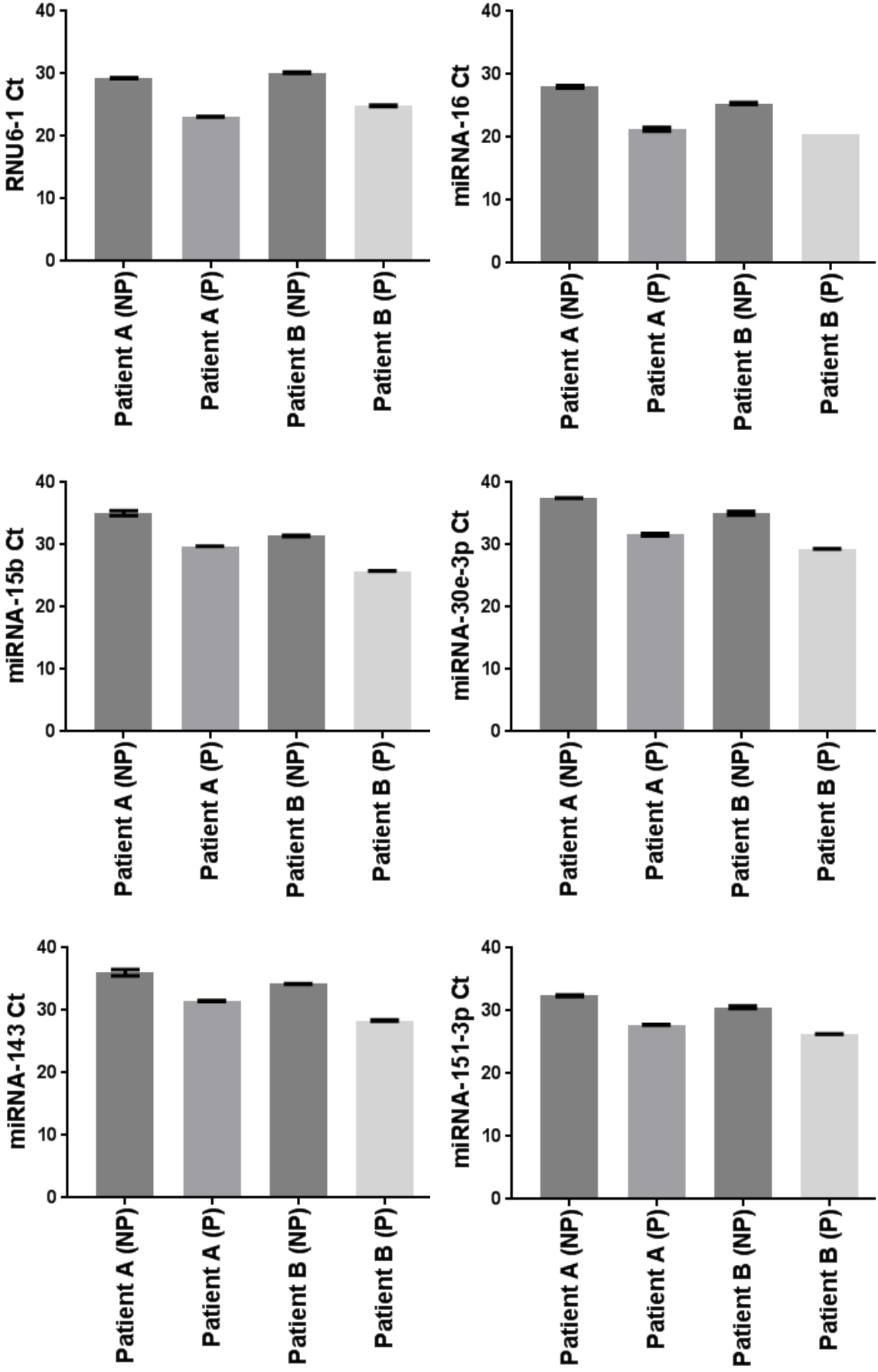
Figure 6.6 Comparison of multiplex and individual reverse transcription reactions

Values represent mean Ct value of duplicate reactions for each sample that underwent either a multiplex RT (M) or individual RT (I) reactions. Lower values indicate a greater concentration of the miRNA species. Bars represent range.

Ct, cycle threshold; miRNA, miRNA, micro ribonucleic acid;

6.3.3.2 Comparison of pre-amplified and non-pre-amplified samples

The effect of pre-amplification on the Ct values resulting from qPCR reactions for the 6 candidate and 2 reference miRNA species is displayed in Figure 6.7. Pre-amplification was effective in reducing the Ct values by 5-7 cycles compared to qPCR of non-pre-amplified cDNA in all miRNA species and thus, was used as part of the validation study in the larger patient cohort.


RNU6-1 Ct
miRNA-16 Ct
miRNA-15b Ct
miRNA-30e-3p Ct
miRNA-143 Ct
miRNA-151-3p Ct
0
10
20
30
40
Patient A (NP)
Patient A (P)
Patient B (NP)
Patient B (P)

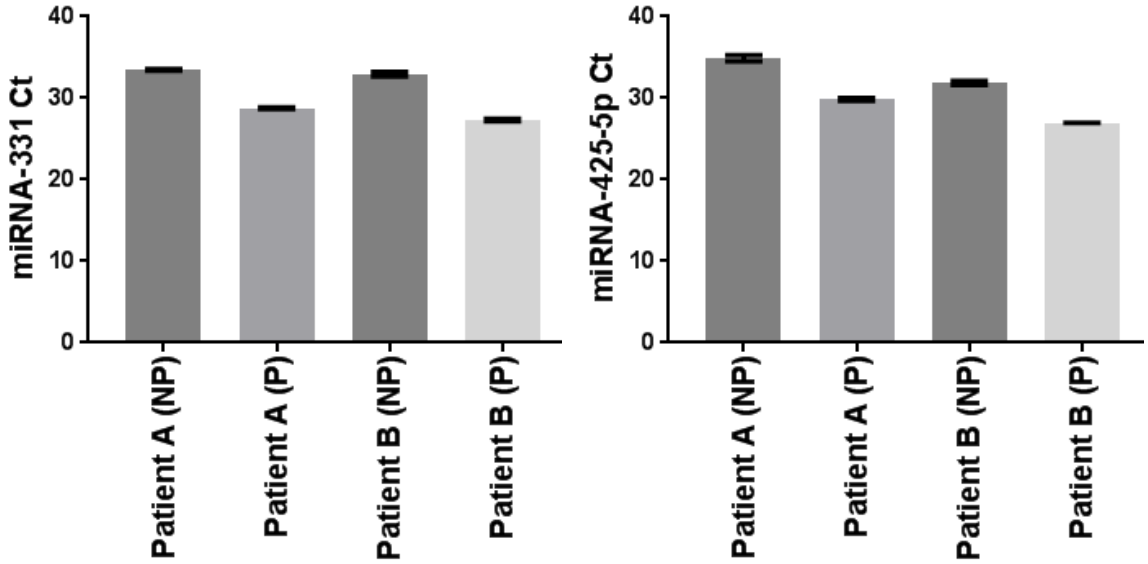


Figure 6.7 Comparison of non-pre-amplified and pre-amplified samples

Values represent mean Ct value of duplicate reactions for the product of the multiplex RT reaction with no pre-amplification (NP) or with pre-amplification (P). Lower values indicate a greater concentration of the miRNA species. MicroRNA-16 could not be quantified after pre-amplification in one reaction for patient B due to an error during setup. Bars represent range.

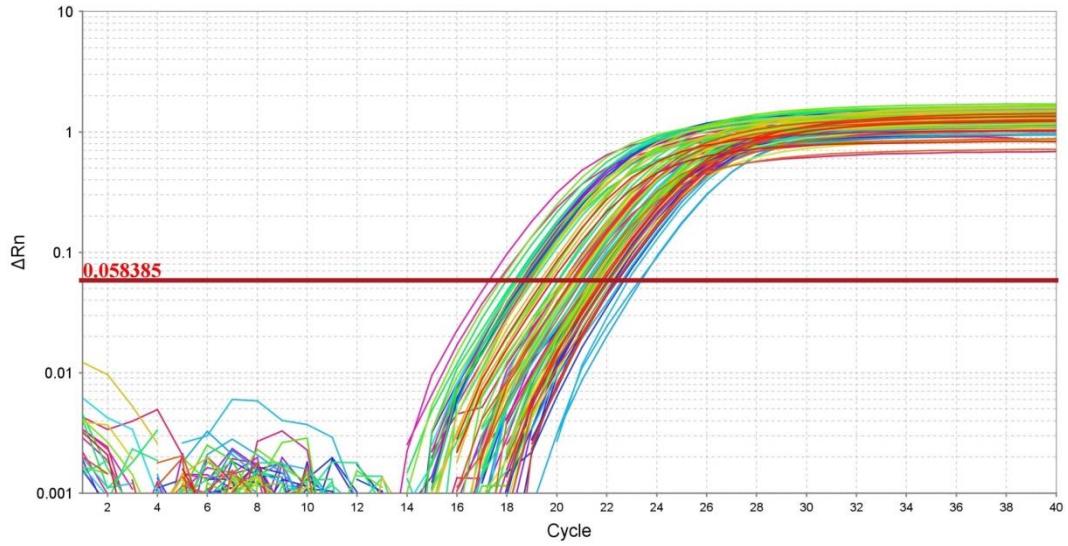
Ct, cycle threshold; miRNA, miRNA, micro ribonucleic acid; RT, reverse transcription;

6.3.3.3 Results of specific quantitative polymerase chain reactions to validate array findings

In order to validate the findings of the array, specific RT and qPCR reactions were performed for the 6 candidate miRNA species and the 2 reference miRNA species in RNA extracted from plasma stored before and after either RIPC or sham treatment in 60 patients. After a multiplex RT reaction, a random sample from each batch of miRNA extractions was assessed for miRNA-16 with qPCR. This demonstrated the presence and comparable levels of miRNA-16 in each of the tested samples (data not shown). Subsequently all samples underwent pre-amplification and specific qPCR reaction for the 6 candidate miRNA species and the 2 reference species. A representative example

of the amplification curves for RNU6-1 and miRNA-16 for a subset of samples from the entire cohort is shown in Figure 6.8.

a)



b)

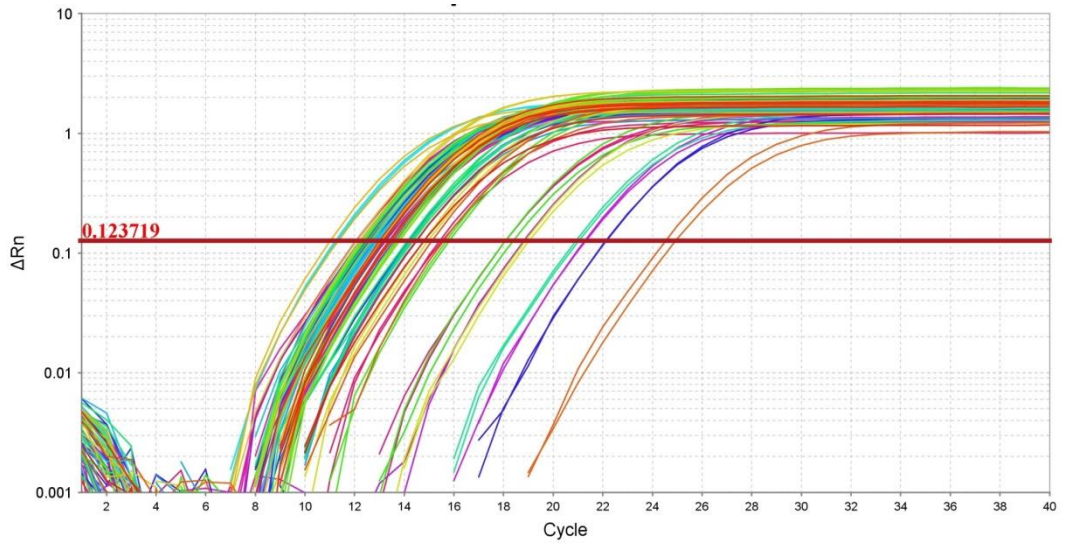


Figure 6.8 Amplification curves from quantitative polymerase chain reactions for RNU6-1 and microRNA-16

Representative amplification curves from qPCR reactions for RNU6-1 (a) and miRNA-16 (b) for 24 patients, with samples pre and post RIPC/sham treatment. Duplicate reactions represented by the same colour in each figure. The threshold for each curve is indicated by the horizontal red line. The magnitude of the fluorescent signal generated by the qPCR reaction is denoted by ΔRn.

miRNA, micro ribonucleic acid; qPCR, quantitative polymerase chain reaction; RIPC, remote ischaemic preconditioning;

There were samples which resulted in late amplifying curves during the qPCR reaction (Figure 6.9) including reactions for miRNA-16 (Figure 6.8b). The reactions with the highest Ct values, across the 8 miRNA species, were predominantly from the plasma of 14 patients (6 RIPC, 8 sham), who were excluded from the analysis, leaving 92 samples and 46 patients with paired plasma miRNA samples for analysis. There was a moderately strong correlation between the Ct values for the two reference species, RNU6-1 and miRNA-16 (Figure 6.10).

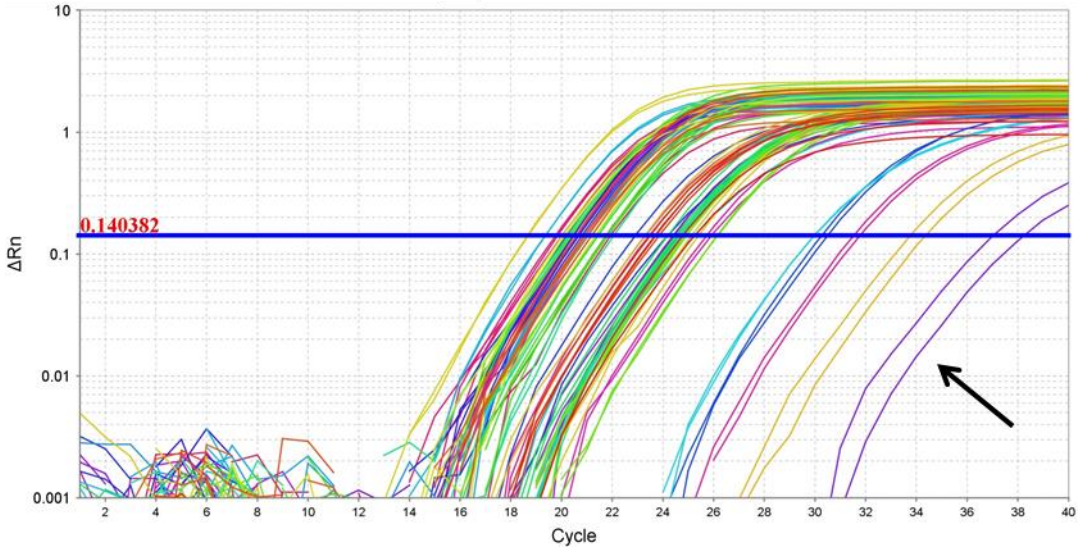


Figure 6.9 Amplification curves with samples amplifying at late cycles

Representative amplification curves from qPCR reactions for miRNA candidate species for 24 patients, with samples pre and post RIPC/sham treatment. Duplicate reactions represented by the same colour. The threshold for each curve is indicated by the horizontal blue line. The magnitude of the fluorescent signal generated by the qPCR reaction is denoted by ΔRn. Late amplifying samples indicated by arrow. miRNA, micro ribonucleic acid; qPCR, quantitative polymerase chain reaction;

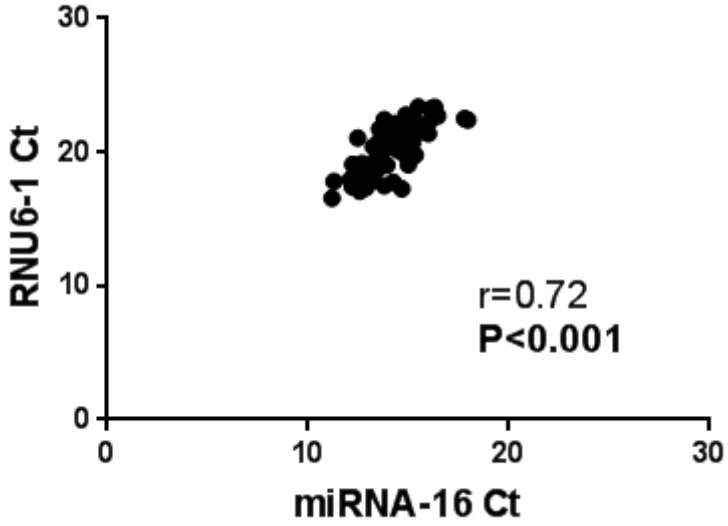


Figure 6.10 Correlation between cycle threshold values for RNU6-1 and microRNA-16

Correlation between Ct values for RNU6-1 and miRNA-16 from qPCR reaction of multiplex reverse transcribed and pre-amplified cDNA. Each value represents the mean Ct value of the duplicate reactions for each species. n=92.

cDNA, complementary deoxyribonucleic acid; Ct, cycle threshold; miRNA, micro ribonucleic acid;

Amongst the 4 RIPC patients that were initially studied with the TaqMan card array system, the specific qPCR data demonstrated similar patterns of enhanced miRNA expression in the validation study but only with RNU6-1 as the reference species. There was a fold change greater than 1 in the majority of RIPC treated patients and species after RNU6-1 adjustment. However, the finding of increased expression with RIPC was not supported by the miRNA-16 adjusted data with only a minority displaying a fold change greater than 1 (Figure 6.11). This suggested more variability in the miRNA-16 data. A comparison of the fold change with RIPC in these 4 patients in the array and the validation data, corrected for RNU6-1, demonstrated larger magnitudes of change suggested by the array data (Table 6.2).

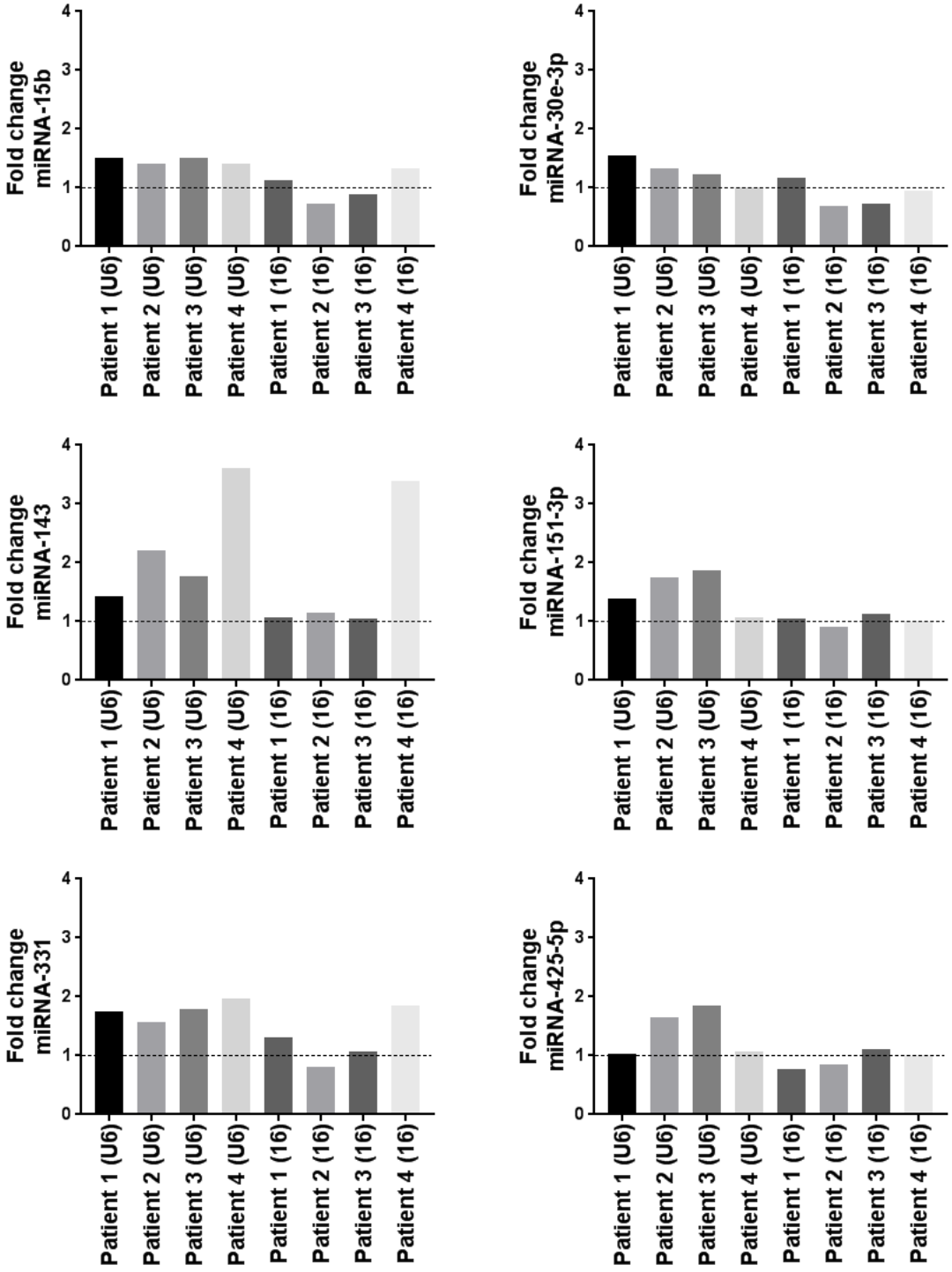


Figure 6.11 Changes in microRNA species with remote ischaemic preconditioning in patients assessed with array - Validation data

Fold change with RIPC in the 4 patients who were analysed with the array card system. RNU6-1 (U6) or miRNA-16 (16) used as the reference species. A value >1 implies an increase in expression with RIPC, whereas a value <1 implies a decrease. The dotted line denotes a fold change of 1.

miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

Table 6.2 Comparison of fold change in candidate microRNA species demonstrated by array and specific quantitative polymerase chain reactions

Species	Array card	qPCR validation
miRNA-15b	6.9 ± 8.7	1.5 ± 0.1
miRNA-30e-3p	3.0 ± 2.0	1.3 ± 0.2
miRNA-143	6.8 ± 7.0	2.3 ± 1.0
miRNA-151-3p	2.9 ± 1.2	1.5 ± 0.4
miRNA-331	2.8 ± 2.5	1.8 ± 0.2
miRNA-425-5p	13.4 ± 19.3	1.4 ± 0.4

Values represent the mean ± SD fold change in each candidate miRNA species, corrected for RNU6-1, as demonstrated by the TaqMan card array and specific qPCR reactions in the same 4 patients treated with RIPC. n=4.

miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

Considering the larger study cohort of 46 patients, 25 treated with RIPC and 21 with sham, there was no significant increase in expression of any of the 6 candidate miRNA species with conditioning (Table 6.3). This was the case after adjustment with either RNU6-1 or miRNA-16. There was a significantly greater increase in expression of miRNA-151-3p and miRNA-425-5p with sham treatment over RIPC, when correction with RNU6-1 was performed. This difference was not significant when the data was corrected for miRNA-16.

Table 6.3 Fold change in microRNA species with remote ischaemic preconditioning

Species*	RIPC (n=25)	Sham (n=21)	P
RNU6-1			
miRNA-15b	1.0 (0.4-1.5)	1.2 (0.8-2.7)	0.24
miRNA-30e-3p	0.8 (0.5-1.5)	1.1 (0.6-2.6)	0.25
miRNA-143	0.7 (0.4-1.6)	1.1 (0.6-3.3)	0.20
miRNA-151-3p	0.7 (0.4-1.4)	1.4 (0.7-2.3)	**0.04**
miRNA-331	0.7 (0.4-1.6)	1.5 (0.6-2.6)	0.08
miRNA-425-5p	0.7 (0.4-1.1)	1.4 (0.9-3.1)	**0.006**
miRNA-16			
miRNA-15b	1.1 (0.9-1.7)	1.0 (0.7-1.3)	0.13
miRNA-30e-3p	1.2 (0.7-1.9)	0.9 (0.5-1.2)	0.15
miRNA-143	1.0 (0.7-2.1)	0.8 (0.4-1.6)	0.18
miRNA-151-3p	1.0 (0.6-1.3)	0.9 (0.7-1.3)	0.90
miRNA-331	1.1 (0.7-1.5)	1.0 (0.7-1.7)	0.56
miRNA-425-5p	0.8 (0.5-1.5)	1.2 (0.7-1.5)	0.28

Values represent median fold change in Ct value in each miRNA species with RNU6-1 or miRNA-16 as the reference. A value >1 implies an increase in expression with the treatment, whereas a value <1 implies a decrease. P values compare fold changes in miRNA species in patients treated with RIPC and sham treatment using the Mann-Whitney U test for non-parametrically distributed data.

Ct, cycle threshold; miRNA, micro ribonucleic acid; RIPC, remote ischaemic preconditioning;

6.3.3.4 Haemolysis in plasma samples

Despite careful plasma preparation, there was a significant degree of haemolysis in 85 (70.8%) of the 120 samples processed for the validation study based on spectrophotometry. Figure 6.12a demonstrates the absorbance at 414 nm for each of the 120 samples. Although plasma miRNA-16 has been shown in prior studies to be increased by haemolysis, there was no significant correlation between the absorbance at 414 nm on spectrophotometry and the Ct values for miRNA-16 (Figure 6.12b).

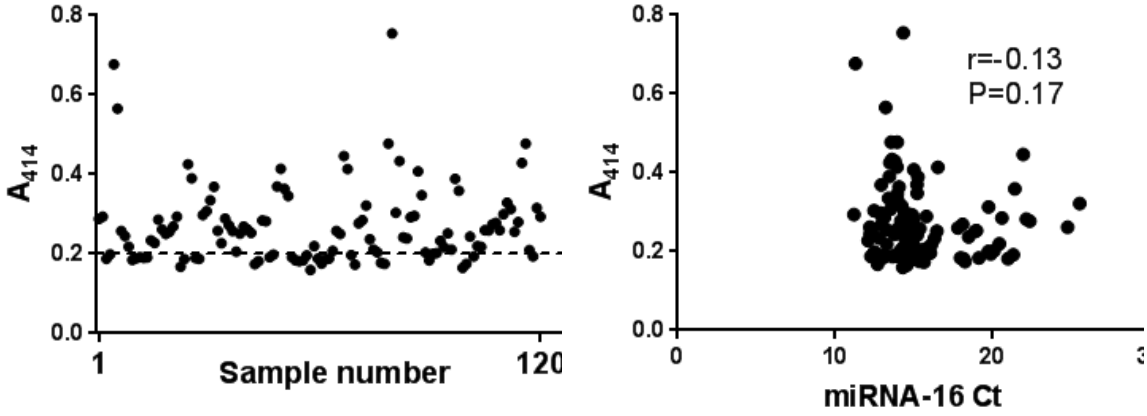


Figure 6.12 Haemolysis in plasma samples and correlation with miRNA-16

Absorbance at 414 nm (A_{414}) was used as a measure of haemolysis in each plasma sample. Each symbol represents the mean of the duplicate measurements for each sample. The threshold of 0.2 is marked by a dotted line, with an A_{414}>0.2 indicative of a significant degree of haemolysis in the sample (a). Correlation between A_{414} and the Ct value of qPCR reactions for miRNA-16 (b). n=120.
Ct, cycle threshold; miRNA, micro ribonucleic acid;

6.4 Discussion

In recent years there has been increased interest in studying miRNA as regulators of many biological and pathological processes including cardiovascular disease (201,204,205,208,213). These small, non-coding RNAs have been implicated in the pathogenesis and development of atherosclerosis and have been studied as biomarkers of disease. MicroRNA species have also been associated with cardiac repair and regeneration in animal models, raising further interest as potential therapeutic targets in the treatment of CAD (212). Given the benefits conferred by RIPC in clinical studies (83,138,220-222), miRNA have been considered as potential mediators of RIPC-mediated cardiac protection. Prior studies have demonstrated an effect of RIPC on miRNA expression, both in cardiac tissues and in the circulation (45,50,51). For

example, miRNA-144 has been shown to be upregulated in the plasma of healthy volunteers by RIPC and to be associated with cellular protection in animal models (45). However, changes in miRNAs have not been investigated in CAD patients after RIPC. One of the predominant theories behind the protective effects of RIPC is the humoral hypothesis. This theory proposes that a factor is released into the circulation by the tissue subject to RIPC, which circulates to the organ of interest to promote protective pathways (26). Given the likely role of circulating mediator and the emerging contribution of miRNA to both CAD pathogenesis and cardiac repair and protection, and prior literature indicating long-term benefits of RIPC in patients with CAD, a study of the effect of RIPC on plasma miRNA profile was conducted.

A commercially available qPCR-based card array system was used to screen for RIPC-medicated changes in 762 miRNA species in stored plasma. The results of the array suggested 6 candidate miRNA species that were potentially upregulated by RIPC. A validation study was subsequently conducted to confirm these findings in a larger cohort of patients. Specific RT and qPCR reactions were used to quantify the levels of these 6 species in miRNA extracted from stored plasma collected before and after either RIPC or sham treatment. The array findings were validated in the same 4 patients by the specific qPCRs. However, the magnitude of fold change in miRNA species differed between the array and validation qPCRs in the same 4 patients, which may be explained by slight differences in the miniaturised array conditions in the card configuration compared to the standard qPCR setup. Differences in magnitude of change have been reported between hybridisation-based arrays and qPCR (219). However, in the entire cohort there was no increased expression of the 6 candidate miRNA species with RIPC, suggesting that the effects of RIPC on platelet activation, as described in chapters 4 and 5, are most likely not mediated by changes in circulating miRNAs.

Evaluation of miRNA levels in plasma is traditionally challenging for several reasons. The RNA yields are generally low with multiple methods for extraction described but no definite gold standard technique (137). Additionally, the quantity and composition of the miRNA component of RNA extracted from plasma can be altered by factors such as the degree of haemolysis during the blood collection process, centrifugation and storage (127,128) and cannot be easily assessed by standard assays such as using a Nanodrop (Thermofisher) or Bioanalyzer. After testing multiple commercially available extraction kits and techniques to optimise quality and yield of miRNA (chapter 2), a published method was adapted and employed to investigate the effect of RIPC on plasma miRNA (127). Despite checking the miRNA-16 levels at multiple time points and these levels appearing relatively constant, the results of the miRNA array could not be validated in a larger cohort. This suggests variability in the patient data and the need for a larger discovery cohort of patients to study circulating miRNA.

Analysis of circulating miRNA includes multiple steps, introducing various sources of extra variability and error. The entire process involves blood collection, preparation of plasma, plasma storage, miRNA extraction, miRNA precipitation, RT reaction and qPCR with or without pre-amplification. A small degree of variability during any of these steps could lead to significant discrepancies between samples, which may not have been accounted for by the modest cohort size. As fold-change is an exponential calculation, any mild variation in the level of the reference species in the pre and post treatment samples, may also lead to significant differences in apparent expression of the candidate species. Therefore, stringent and critical assessment of protocols and data and a large patient cohort is necessary.

Specific qPCRs demonstrated a similar pattern of enhanced expression of the candidate miRNA species in the 4 patients assessed by array. This was only the case however,

after correction for RNU6-1 with discrepant results after correction with miRNA-16. There was also incongruence between RNU6-1 and miRNA-16 corrected data in the larger study cohort. There was a moderate correlation between the Ct values of the two reference species (Figure 6.10), indicating some variability between the two species which may explain some of the discrepant results. The fold change in each of the miRNA species is corrected with either of the reference species, hence variability in the reference will lead to unreliable calculation of fold changes in expression. The use of reference genes is common in RNA research, with miRNA-16 and U6 previously used as reference species for the study of circulating miRNA (223,224). However, debate exists about which species is most suitable in investigating circulating miRNAs and as this study shows, different results may be obtained using different reference miRNA species.

Certain miRNA species can be affected by significant levels of haemolysis in plasma. These include miRNA-16 and miRNA-425-5p which are increased by haemolysis (127). Haemolysis, identified by spectrophotometric absorbance at 414 nm, was observed in 70.8% of the samples, despite careful plasma preparation. However, there was no significant correlation between absorbance at 414 nm and the level of miRNA-16 in the samples and only weak correlation with miRNA-425-5p levels (data not shown). Due to the significant levels of haemolysis in the majority of samples, a sensitivity analysis in patients without haemolysis could not be performed. Hence, it was not possible to exclude that haemolysis was confounding the miRNA-16 corrected data or the analysis of miRNA-425-5p. The other candidate miRNA species studied have been reported not to be significantly affected by haemolysis. Hence, it is unlikely that haemolysis affected the RNU6-1 corrected results or the analysis of the other miRNA species.

Samples with high Ct values in reference miRNAs, indicative of lower quantities or lower quality miRNA, were excluded from the analysis. Despite the apparent changes in miRNA expression by RIPC based on the 4 patients studied with the array, in the larger study cohort, there was no evidence to support an increased expression of any of the candidate miRNA species by RIPC over sham treatment. There was a numerically greater increase in miRNA expression with sham treatment, reaching statistical significance for miRNA-151-3p and miRNA-425-5p when corrected for RNU6-1. There was a discrepancy with the miRNA-16 corrected results, with the differences in expression of these two miRNA species with sham treatment no longer reaching statistical significance. It is unclear why these two miRNA species might be increased by sham treatment. Furthermore, for both miRNA species the increase was 1.4-fold with sham. Previously studies have demonstrated fold changes in miRNA species of at least 2.5 in response to RIPC (45,215). Hence the clinical and physiological significance of these changes with sham treatment are unclear.

Prior studies have demonstrated upregulation of miRNA-144 and miRNA-21 after RIPC in healthy volunteers and children undergoing cardiopulmonary bypass. In the 4 patients assessed with the array, 2 had an increased expression of miRNA-144 and 1 had an increased expression of miRNA-21. These initial results in 4 patients did not suggest an effect of RIPC on these miRNA species and was therefore not investigated further.

The small size of the derivation cohort may be seen as a limitation of this study. A cohort of 4 patients (8 samples) was chosen as a balance between increasing the accuracy of the results and managing the significant costs of the card array system.

Given the method optimisation studies performed prior to conducting this study, it is likely that these results are valid and accurate. However, a larger derivation cohort may have led to array results which were more likely to be validated.

6.5 Conclusions

MicroRNAs are increasingly being recognised as important regulators of a vast range of biological and pathological processes. RIPC had been shown to alter miRNA expression in animal models and in the plasma of healthy volunteers. The results of this methodologically rigorous study did not identify miRNAs that may mediate the beneficial effects of RIPC in CAD patients. As there are however several limitations in methodology in this area, future developments are necessary to adequately assess circulating plasma miRNA as biomarkers of disease or efficacy of theraputic interventions.

Chapter 7: Conclusion and future directions

7.1 General conclusions

Remote ischaemic preconditioning is supported by extensive animal data demonstrating protection against myocardial injury. The clinical trials of RIPC prior to elective and primary PCI have demonstrated a reduction in myocardial injury and a suggestion of reduced ischaemic events during long term follow up. This thesis has explored novel mechanisms which may contribute to improved outcomes at the time of PCI including: 1) the effect of RIPC on coronary physiology assessed by invasively measured indices of microcirculatory function; 2) the effect of RIPC on specific pathways of platelet activation and procoagulant platelet formation; and, 3) the effect of RIPC on the plasma miRNA profile of patients with suspected CAD.

In patients who had clinical indications for FFR measurement, RIPC was associated with a significant reduction in microcirculatory resistance and a significant increase in coronary flow reserve. This was mediated by an increase in hyperaemic coronary flow. The mechanism behind these observed changes is unclear with no clear evidence of a responsible hormonal pathway based on interrogation of stored plasma samples. RIPC was also associated with an attenuation of platelet GPIIb-IIIa activation in response to SFLLRN and a combination of thrombin and collagen. RIPC was also associated with a reduction in basal levels of procoagulant platelets in the circulation and inhibited their induction by soluble agonist stimulation. The reduced GPIIb-IIIa activation was not associated with detectible changes in platelet intracellular signalling pathways. Unexpectedly, there was a reduction in plasma nitrite levels, which correlated with the change in GPIIb-IIIa activation, suggesting that RIPC influences nitric oxide/ nitrite

metabolism. The influence of RIPC on procoagulant platelet formation appeared to be due to stabilisation of the mitochondrial membrane which reduced depolarisation in response to agonists. There did not appear to be any definite effect of RIPC on the plasma miRNA profile when blood was collected immediately after conditioning.

This thesis has demonstrated novel effects of RIPC which are particularly relevant to patients with CAD. The findings suggest that in these patients, the benefit of RIPC is contributed to by an improvement in microcirculatory function and an attenuation of platelet activation.

7.2 Future directions

7.2.1 Remote ischaemic preconditioning and coronary physiology

The coronary microcirculation is recognised as an important contributor to the prognosis of patients with CAD. Microcirculatory function, however, is not easily amenable to pharmacological manipulation. Reduced microcirculatory resistance and improved flow reserve is associated with improved outcomes in patients with CAD, including those undergoing PCI. An acute improvement in microcirculatory function was demonstrated in this study but the duration and timing of maximal improvement could not be determined. Given that the clinical studies in PCI suggest that RIPC is ideally delivered 1-2 hours prior to PCI, defining the time course of the microcirculatory response to RIPC would be of clinical utility as this may help define the optimal timing of PCI after delivery of conditioning.

The mechanism by which changes in microcirculatory resistance occurs has not been defined. The use of specific inhibitors of potential pathways is a commonly used strategy in animal studies. This however is more difficult in studies involving patients.

A potential agent with an acceptable safety profile is glibenclamide, which is an inhibitor of potassium ATP channels, which have previously been implicated in RIPC mediated protection and are also known to be involved in regulation of the microcirculation. Amelioration of changes in coronary microcirculatory function in patients taking or randomised to take glibenclamide would suggest that potassium ATP channels are involved in this RIPC-mediated phenomenon. This is an area for future exploration.

Due to the mechanistic nature of the studies conducted, correlation between changes in coronary physiology with clinical outcomes could not be performed. Given the important role of the microcirculation in determining the prognosis of patients undergoing PCI, a large clinical trial powered for outcomes could ideally be conducted to confirm the contribution of changes in microcirculatory function to RIPC-mediated cardiac protection. A possible role in the setting of poor reflow after myocardial infarction may be clinically important.

The observation that RIPC augments coronary microcirculatory function not only provides an insight into a potential mechanism of cardiac protection, but also raises the possibility of other areas of RIPC related research and clinical application. RIPC may be considered for application in disease processes where microcirculatory dysfunction is important such as microvascular angina and heart failure. Additionally, areas of extra-cardiac pathology such as wound healing and microvascular complications of diabetes may also be potential areas for clinical application of RIPC.

7.2.2 Remote ischaemic preconditioning and platelet activation

A major component of this thesis was the exploration of the effect of RIPC on platelet activation. This was investigated by measuring specific pathways of platelet activation

through measurement of a wide range of markers in response to *ex* vivo platelet stimulation. The current study was performed in a cohort of patients with predominantly stable CAD and demonstrated that RIPC attenuates specific pathways of platelet activation. Importantly, these findings were observed in patients taking DAPT, indicating that RIPC provides a novel antiplatelet strategy additional to current best therapy. It is unclear whether patients with unstable presentations of CAD will exhibit the same effect with RIPC, although analysis of this subgroup of patients (data not shown) showed similar effects to those seen in stable patients, suggesting this is likely. ACS is associated with a pro-thrombotic, inflammatory state, which differs from stable presentations of CAD and studying platelet effects in this particular population would require independent confirmation in a suitably powered study. Given that RIPC has been shown to be beneficial in the setting of STEMI, the effects on platelet activation during ACS would be an interesting area to explore.

Clear effects on the levels of circulating procoagulant platelets and inhibition of procoagulant platelet induction by agonist stimulation were also identified. There was a clear effect of RIPC on mitochondrial depolarisation, suggesting a candidate mechanism for inhibition of procoagulant platelet formation. Cyclophilin D-mediated regulation of the mitochondrial permeability transition pore (225) may represent a key target of RIPC and an avenue for further investigation. While the effect of RIPC in attenuating GPIIb-IIIa activation appeared to be additive to the effects of DAPT, its inhibition of procoagulant platelet formation was less evident in patients receiving both aspirin and a P_2Y12 inhibitor, clearly dissociating the pathways involved in RIPC attenuation of GPIIb-IIIa activation and formation of procoagulant platelets. Further investigation into the effect of RIPC on procoagulant platelet pathways might best be performed in patients on aspirin monotherapy. Exploring the interaction between disease state, such

as acute coronary syndrome, antiplatelet therapy, GPIIb-IIIa activation and procoagulant platelet formation are areas for future research.

This thesis did examine some potential pathways through which RIPC may attenuate platelet activation. Although a change in VASP phosphorylation was not detectable with western blotting, due to the important role of VASP in modulating GPIIb-IIIa activity, this may be further explored with an alternative method to assess the phosphorylation state of VASP. A flow cytometry based technique has been described to measure VASP phosphorylation (226) and this could be considered to elucidate the mechanism behind the changes in PAC-1 binding that were observed. We also found no significant effect of RIPC on cAMP, cGMP or prostacyclin. Inhibitory pathways are less well defined than activatory pathways in platelets and it is possible that new pathways, identified by array-based approaches may reveal new avenues for research. Importantly, the duration and sustainability of platelet inhibition was not defined by this study. Exploring the duration of platelet inhibition, through serial assessments of platelet activation, may help guide the optimal timing of RIPC delivery prior to a known platelet activating insult such as PCI. In a similar manner to the coronary physiology arm of this project, the mechanistic focus of the platelet studies did not allow correlation between platelet inhibition and clinical outcomes. Given the minimally invasive nature of the platelet activation assays, platelet assessments are an ideal adjunct to clinical studies of RIPC, allowing correlation between platelet inhibition and clinical outcomes.

7.3 Final remarks

This thesis has examined potential mechanism behind the beneficial effects of RIPC in the setting of PCI. Novel effects on microcirculatory function and attenuation of platelet

activation have been demonstrated. Although the adoption of RIPC into clinical management guidelines will depend on ongoing large clinical trials powered for clinical outcomes, these results warrant future studies to understand the biological effects of RIPC. These will in turn help guide clinical application, guide selection of populations that are likely to benefit and potentially identify novel therapeutic targets for the treatment of coronary artery disease.

References

1. Global, regional, and national disability-adjusted life-years (dalys) for 315 diseases and injuries and healthy life expectancy (hale), 1990-2015: A systematic analysis for the global burden of disease study 2015. Lancet 2016;388:1603-1658.
2. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: A systematic analysis for the global burden of disease study 2015. Lancet 2016;388:1459-1544.
3. Montalescot G, Sechtem U, Achenbach S et al. 2013 ESC guidelines on the management of stable coronary artery disease: The task force on the management of stable coronary artery disease of the european society of cardiology. Eur Heart J 2013;34:2949-3003.
4. Roffi M, Patrono C, Collet JP et al. 2015 ESC guidelines for the management of acute coronary syndromes in patients presenting without persistent st-segment elevation: Task force for the management of acute coronary syndromes in patients presenting without persistent st-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2016;37:267-315.
5. Windecker S, Kolh P, Alfonso F et al. 2014 ESC/EACTS guidelines on myocardial revascularization: The task force on myocardial revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) developed with the special contribution of the european association of percutaneous cardiovascular interventions (eapci). Eur Heart J 2014;35:2541-619.

6. Serruys PW, Morice MC, Kappetein AP et al. Percutaneous coronary intervention versus coronary-artery bypass grafting for severe coronary artery disease. N Engl J Med 2009;360:961-72.
7. Selvanayagam JB, Porto I, Channon K et al. Troponin elevation after percutaneous coronary intervention directly represents the extent of irreversible myocardial injury: Insights from cardiovascular magnetic resonance imaging. Circulation 2005;111:1027-32.
8. Mohammed AA, Agnihotri AK, van Kimmenade RR et al. Prospective, comprehensive assessment of cardiac troponin t testing after coronary artery bypass graft surgery. Circulation 2009;120:843-50.
9. Kizer JR, Muttrej MR, Matthai WH et al. Role of cardiac troponin t in the long-term risk stratification of patients undergoing percutaneous coronary intervention. Eur Heart J 2003;24:1314-22.
10. Nageh T, Sherwood RA, Harris BM, Thomas MR. Prognostic role of cardiac troponin i after percutaneous coronary intervention in stable coronary disease. Heart 2005;91:1181-5.
11. Kathiresan S, Servoss SJ, Newell JB et al. Cardiac troponin t elevation after coronary artery bypass grafting is associated with increased one-year mortality. Am J Cardiol 2004;94:879-81.
12. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 1993;87:893-9.
13. Davies WR, Brown AJ, Watson W et al. Remote ischemic preconditioning improves outcome at 6 years after elective percutaneous coronary intervention: The crisp stent trial long-term follow-up. Circ Cardiovasc Interv 2013;6:246-51.

14. Hoole SP, Heck PM, Sharples L et al. Cardiac remote ischemic preconditioning in coronary stenting (crisp stent) study: A prospective, randomized control trial. Circulation 2009;119:820-7.

15. Hoole SP, Heck PM, White PA et al. Remote ischemic preconditioning stimulus does not reduce microvascular resistance or improve myocardial blood flow in patients undergoing elective percutaneous coronary intervention. Angiology 2009;60:403-11.

16. Iliodromitis EK, Kyrzopoulos S, Paraskevaidis IA et al. Increased c reactive protein and cardiac enzyme levels after coronary stent implantation. Is there protection by remote ischaemic preconditioning? Heart 2006;92:1821-6.

17. Luo SJ, Zhou YJ, Shi DM, Ge HL, Wang JL, Liu RF. Remote ischemic preconditioning reduces myocardial injury in patients undergoing coronary stent implantation. Can J Cardiol 2013;29:1084-9.

18. Pedersen CM, Cruden NL, Schmidt MR et al. Remote ischemic preconditioning prevents systemic platelet activation associated with ischemia-reperfusion injury in humans. J Thromb Haemost 2011;9:404-7.

19. Thielmann M, Kottenberg E, Kleinbongard P et al. Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: A single-centre randomised, double-blind, controlled trial. The Lancet 2013;382:597-604.

20. Zografos TA, Katritsis GD, Katritsis DG. Remote ischemic preconditioning reduces peri-procedural myocardial injury in elective percutaneous coronary intervention: A meta-analysis. Int J Cardiol 2014;173:530-2.

21. Ahmed RM, Mohamed el HA, Ashraf M et al. Effect of remote ischemic preconditioning on serum troponin t level following elective percutaneous coronary intervention. Catheter Cardiovasc Interv 2013;82:E647-53.

22. Meybohm P, Bein B, Brosteanu O et al. A multicenter trial of remote ischemic preconditioning for heart surgery. N Engl J Med 2015;373:1397-407.

23. Hausenloy DJ, Candilio L, Evans R et al. Remote ischemic preconditioning and outcomes of cardiac surgery. N Engl J Med 2015;373:1408-17.

24. Loukogeorgakis SP, Williams R, Panagiotidou AT et al. Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a k(atp)-channel dependent mechanism. Circulation 2007;116:1386-95.

25. Prasad A, Gossl M, Hoyt J et al. Remote ischemic preconditioning immediately before percutaneous coronary intervention does not impact myocardial necrosis, inflammatory response, and circulating endothelial progenitor cell counts: A single center randomized sham controlled trial. Catheter Cardiovasc Interv 2013;81:930-6.

26. Hausenloy DJ, Yellon DM. Ischaemic conditioning and reperfusion injury. Nat Rev Cardiol 2016;13:193-209.

27. Heusch G, Botker HE, Przyklenk K, Redington A, Yellon D. Remote ischemic conditioning. J Am Coll Cardiol 2015;65:177-95.

28. Steensrud T, Li J, Dai X et al. Pretreatment with the nitric oxide donor snap or nerve transection blocks humoral preconditioning by remote limb ischemia or intra-arterial adenosine. Am J Physiol Heart Circ Physiol 2010;299:H1598-603.

29. Wong GT, Lu Y, Mei B, Xia Z, Irwin MG. Cardioprotection from remote preconditioning involves spinal opioid receptor activation. Life Sci 2012;91:860-5.

30. Shimizu M, Tropak M, Diaz RJ et al. Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: Evidence suggesting cross-species protection. Clin Sci (Lond) 2009;117:191-200.

31. Surendra H, Diaz RJ, Harvey K et al. Interaction of delta and kappa opioid receptors with adenosine a1 receptors mediates cardioprotection by remote ischemic preconditioning. J Mol Cell Cardiol 2013;60:142-50.

32. Rentoukas I, Giannopoulos G, Kaoukis A et al. Cardioprotective role of remote ischemic periconditioning in primary percutaneous coronary intervention: Enhancement by opioid action. JACC Cardiovasc Interv 2010;3:49-55.

33. Skyschally A, Gent S, Amanakis G, Schulte C, Kleinbongard P, Heusch G. Across-species transfer of protection by remote ischemic preconditioning with species-specific myocardial signal transduction by reperfusion injury salvage kinase and survival activating factor enhancement pathways. Circ Res 2015;117:279-88.

34. Leung CH, Wang L, Nielsen JM et al. Remote cardioprotection by transfer of coronary effluent from ischemic preconditioned rabbit heart preserves mitochondrial integrity and function via adenosine receptor activation. Cardiovasc Drugs Ther 2014;28:7-17.

35. Zhao Y, Zheng ZN, Liu X, Dai G, Jin SQ. Effects of preconditioned plasma collected during the late phase of remote ischaemic preconditioning on ventricular arrhythmias caused by myocardial ischaemia reperfusion in rats. J Int Med Res 2018;46:1370-1379.

36. Breivik L, Helgeland E, Aarnes EK, Mrdalj J, Jonassen AK. Remote postconditioning by humoral factors in effluent from ischemic preconditioned

rat hearts is mediated via pi3k/akt-dependent cell-survival signaling at reperfusion. Basic Res Cardiol 2011;106:135-45.

37. Hepponstall M, Ignjatovic V, Binos S et al. Remote ischemic preconditioning (ripc) modifies plasma proteome in humans. PLoS One 2012;7:e48284.

38. Hepponstall M, Ignjatovic V, Binos S et al. Remote ischemic preconditioning (ripc) modifies the plasma proteome in children undergoing repair of tetralogy of fallot: A randomized controlled trial. PLoS One 2015;10:e0122778.

39. Wider J, Undyala VVR, Whittaker P, Woods J, Chen X, Przyklenk K. Remote ischemic preconditioning fails to reduce infarct size in the zucker fatty rat model of type-2 diabetes: Role of defective humoral communication. Basic Res Cardiol 2018;113:16.

40. Contractor H, Stottrup NB, Cunnington C et al. Aldehyde dehydrogenase-2 inhibition blocks remote preconditioning in experimental and human models. Basic Res Cardiol 2013;108:343.

41. Hibert P, Prunier-Mirebeau D, Beseme O et al. Apolipoprotein a-i is a potential mediator of remote ischemic preconditioning. PLoS One 2013;8:e77211.

42. Rassaf T, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M. Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. Circ Res 2014;114:1601-10.

43. Davidson SM, Selvaraj P, He D et al. Remote ischaemic preconditioning involves signalling through the sdf-1alpha/cxcr4 signalling axis. Basic Res Cardiol 2013;108:377.

44. Vicencio JM, Yellon DM, Sivaraman V et al. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. J Am Coll Cardiol 2015;65:1525-36.

45. Li J, Rohailla S, Gelber N et al. Microrna-144 is a circulating effector of remote ischemic preconditioning. Basic Res Cardiol 2014;109:423.

46. Minghua W, Zhijian G, Chahua H et al. Plasma exosomes induced by remote ischaemic preconditioning attenuate myocardial ischaemia/reperfusion injury by transferring mir-24. Cell Death Dis 2018;9:320.

47. Li H, Borinskaya S, Yoshimura K et al. Refined geographic distribution of the oriental aldh2*504lys (nee 487lys) variant. Ann Hum Genet 2009;73:335-45.

48. Diehl P, Fricke A, Sander L et al. Microparticles: Major transport vehicles for distinct micrornas in circulation. Cardiovasc Res 2012;93:633-44.

49. McManus DD, Freedman JE. Micrornas in platelet function and cardiovascular disease. Nat Rev Cardiol 2015;12:711-7.

50. Duan X, Ji B, Wang X et al. Expression of microrna-1 and microrna-21 in different protocols of ischemic conditioning in an isolated rat heart model. Cardiology 2012;122:36-43.

51. Slagsvold KH, Rognmo O, Hoydal M, Wisloff U, Wahba A. Remote ischemic preconditioning preserves mitochondrial function and influences myocardial microrna expression in atrial myocardium during coronary bypass surgery. Circ Res 2014;114:851-9.

52. Shan LY, Li JZ, Zu LY et al. Platelet-derived microparticles are implicated in remote ischemia conditioning in a rat model of cerebral infarction. CNS Neurosci Ther 2013;19:917-25.

53. Oberkofler CE, Limani P, Jang JH et al. Systemic protection through remote ischemic preconditioning is spread by platelet-dependent signaling in mice. Hepatology 2014;60:1409-17.

54. Ropcke DM, Hjortdal VE, Toft GE, Jensen MO, Kristensen SD. Remote ischemic preconditioning reduces thrombus formation in the rat. J Thromb Haemost 2012;10:2405-6.

55. Stazi A, Scalone G, Laurito M et al. Effect of remote ischemic preconditioning on platelet activation and reactivity induced by ablation for atrial fibrillation. Circulation 2014;129:11-7.

56. Battipaglia I, Scalone G, Milo M, Di Franco A, Lanza GA, Crea F. Upper arm intermittent ischaemia reduces exercise-related increase of platelet reactivity in patients with obstructive coronary artery disease. Heart 2011;97:1298-303.

57. Lanza GA, Stazi A, Villano A et al. Effect of remote ischemic preconditioning on platelet activation induced by coronary procedures. Am J Cardiol 2016;117:359-65.

58. Albrecht M, Zitta K, Bein B et al. Remote ischemic preconditioning regulates hif-1alpha levels, apoptosis and inflammation in heart tissue of cardiosurgical patients: A pilot experimental study. Basic Res Cardiol 2013;108:314.

59. Gedik N, Kottenberg E, Thielmann M et al. Potential humoral mediators of remote ischemic preconditioning in patients undergoing surgical coronary revascularization. Sci Rep 2017;7:12660.

60. Heinen NM, Putz VE, Gorgens JI et al. Cardioprotection by remote ischemic preconditioning exhibits a signaling pattern different from local ischemic preconditioning. Shock 2011;36:45-53.

61. Heidbreder M, Naumann A, Tempel K, Dominiak P, Dendorfer A. Remote vs. Ischaemic preconditioning: The differential role of mitogen-activated protein kinase pathways. Cardiovasc Res 2008;78:108-15.

62. Simkhovich BZ, Przyklenk K, Kloner RA. Role of protein kinase c in ischemic "conditioning": From first evidence to current perspectives. J Cardiovasc Pharmacol Ther 2013;18:525-32.

63. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. Stat5 activation and cardioprotection by remote ischemic preconditioning in humans: Short communication. Circ Res 2012;110:111-5.

64. Kottenberg E, Musiolik J, Thielmann M, Jakob H, Peters J, Heusch G. Interference of propofol with signal transducer and activator of transcription 5 activation and cardioprotection by remote ischemic preconditioning during coronary artery bypass grafting. J Thorac Cardiovasc Surg 2013.

65. Jang YH, Kim JH, Lee YC. Mitochondrial atp-sensitive potassium channels play a role in reducing both myocardial infarction and reperfusion arrhythmia in remote ischemic preconditioned hearts. Anesthesiology and pain medicine 2017;7:e42505.

66. Frankenreiter S, Bednarczyk P, Kniess A et al. Cgmp-elevating compounds and ischemic conditioning provide cardioprotection against ischemia and reperfusion injury via cardiomyocyte-specific bk channels. Circulation 2017;136:2337-2355.

67. Camici PG, Crea F. Coronary microvascular dysfunction. N Engl J Med 2007;356:830-40.

68. Santillo E, Migale M, Balestrini F et al. Coronary flow response to remote ischemic preconditioning is preserved in old cardiac patients. Aging Clin Exp Res 2017.

69. Kono Y, Fukuda S, Hanatani A et al. Remote ischemic conditioning improves coronary microcirculation in healthy subjects and patients with heart failure. Drug Des Devel Ther 2014;8:1175-81.

70. Corcoran D, Young R, Cialdella P et al. The effects of remote ischaemic preconditioning on coronary artery function in patients with stable coronary artery disease. Int J Cardiol 2018;252:24-30.

71. Camici PG, d'Amati G, Rimoldi O. Coronary microvascular dysfunction: Mechanisms and functional assessment. Nat Rev Cardiol 2015;12:48-62.

72. Ricciardi MJ, Davidson CJ, Gubernikoff G et al. Troponin i elevation and cardiac events after percutaneous coronary intervention. Am Heart J 2003;145:522-8.

73. Blusztein DI, Brooks MJ, Andrews DT. A systematic review and meta-analysis evaluating ischemic conditioning during percutaneous coronary intervention. Future Cardiol 2017;13:579-592.

74. Botker HE, Kharbanda R, Schmidt MR et al. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: A randomised trial. Lancet 2010;375:727-34.

75. Eitel I, Stiermaier T, Rommel KP et al. Cardioprotection by combined intrahospital remote ischaemic perconditioning and postconditioning in st-elevation myocardial infarction: The randomized lipsia conditioning trial. Eur Heart J 2015;36:3049-57.

76. White SK, Frohlich GM, Sado DM et al. Remote ischemic conditioning reduces myocardial infarct size and edema in patients with st-segment elevation myocardial infarction. JACC Cardiovasc Interv 2015;8:178-88.

77. Prunier F, Angoulvant D, Saint Etienne C et al. The ripost-mi study, assessing remote ischemic perconditioning alone or in combination with local ischemic

postconditioning in st-segment elevation myocardial infarction. Basic Res Cardiol 2014;109:400.

78. Sloth AD, Schmidt MR, Munk K et al. Improved long-term clinical outcomes in patients with st-elevation myocardial infarction undergoing remote ischaemic conditioning as an adjunct to primary percutaneous coronary intervention. Eur Heart J 2014;35:168-75.

79. Yellon DM, Ackbarkhan AK, Balgobin V et al. Remote ischemic conditioning reduces myocardial infarct size in stemi patients treated by thrombolysis. J Am Coll Cardiol 2015;65:2764-5.

80. Crimi G, Pica S, Raineri C et al. Remote ischemic post-conditioning of the lower limb during primary percutaneous coronary intervention safely reduces enzymatic infarct size in anterior myocardial infarction: A randomized controlled trial. JACC Cardiovasc Interv 2013;6:1055-63.

81. Hausenloy DJ, Kharbanda R, Rahbek Schmidt M et al. Effect of remote ischaemic conditioning on clinical outcomes in patients presenting with an st-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention. Eur Heart J 2015;36:1846-8.

82. Zografos TA, Katritsis GD, Tsiafoutis I, Bourboulis N, Katsivas A, Katritsis DG. Effect of one-cycle remote ischemic preconditioning to reduce myocardial injury during percutaneous coronary intervention. Am J Cardiol 2014;113:2013-7.

83. Healy DA, Carroll PJ, Clarke Moloney M et al. Systematic review and meta-analysis of remote ischaemic preconditioning in percutaneous coronary intervention. IJC Metabolic & Endocrine 2013;1:13-19.

84. Yetgin T, Manintveld OC, Boersma E et al. Remote ischemic conditioning in percutaneous coronary intervention and coronary artery bypass grafting. Circ J 2012;76:2392-2404.

85. Xu X, Zhou Y, Luo S et al. Effect of remote ischemic preconditioning in the elderly patients with coronary artery disease with diabetes mellitus undergoing elective drug-eluting stent implantation. Angiology 2014;65:660-6.

86. Munk K, Andersen NH, Schmidt MR et al. Remote ischemic conditioning in patients with myocardial infarction treated with primary angioplasty: Impact on left ventricular function assessed by comprehensive echocardiography and gated single-photon emission ct. Circ Cardiovasc Imaging 2010;3:656-62.

87. Miyoshi T, Ejiri K, Kohno K et al. Effect of remote ischemia or nicorandil on myocardial injury following percutaneous coronary intervention in patients with stable coronary artery disease: A randomized controlled trial. Int J Cardiol 2017;236:36-42.

88. Ejiri K, Miyoshi T, Kohno K et al. Protective effect of remote ischemic preconditioning on myocardial damage after percutaneous coronary intervention in stable angina patients with complex coronary lesions- subanalysis of a randomized controlled trial. Circ J 2018;82:1788-1796.

89. Liu Z, Wang YL, Hua Q, Chu YY, Ji XM. Late remote ischemic preconditioning provides benefit to patients undergoing elective percutaneous coronary intervention. Cell Biochem Biophys 2014;70:437-42.

90. Zhou FZ, Song W, Yin LH et al. Effects of remote ischemic preconditioning on myocardial injury and endothelial function and prognosis after percutaneous coronary intervention in patients with acute coronary syndrome. Eur Rev Med Pharmacol Sci 2017;21:4642-4648.

91. Ghaemian A, Nouraei SM, Abdollahian F, Naghshvar F, Giussani DA, Nouraei SA. Remote ischemic preconditioning in percutaneous coronary revascularization: A double-blind randomized controlled clinical trial. Asian Cardiovasc Thorac Ann 2012;20:548-54.

92. Soraas CL, Friis C, Engebretsen KV, Sandvik L, Kjeldsen SE, Tonnessen T. Troponin t is a better predictor than creatine kinase-mb of long-term mortality after coronary artery bypass graft surgery. Am Heart J 2012;164:779-85.

93. Payne RE, Aldwinckle J, Storrow J, Kong RS, Lewis ME. Ripc remains a promising technique for protection of the myocardium during open cardiac surgery: A meta-analysis and systematic review. Heart Surg Forum 2015;18:E074-80.

94. Candilio L, Malik A, Ariti C et al. Effect of remote ischaemic preconditioning on clinical outcomes in patients undergoing cardiac bypass surgery: A randomised controlled clinical trial. Heart 2015;101:185-92.

95. Hong DM, Lee EH, Kim HJ et al. Does remote ischaemic preconditioning with postconditioning improve clinical outcomes of patients undergoing cardiac surgery? Remote ischaemic preconditioning with postconditioning outcome trial. Eur Heart J 2013;35:176-83.

96. Murphy PG, Myers DS, Davies MJ, Webster NR, Jones JG. The antioxidant potential of propofol (2,6-diisopropylphenol). Br J Anaesth 1992;68:613-8.

97. Ney J, Hoffmann K, Meybohm P et al. Remote ischemic preconditioning does not affect the release of humoral factors in propofol-anesthetized cardiac surgery patients: A secondary analysis of the ripheart study. International journal of molecular sciences 2018;19.

98. Rahman IA, Mascaro JG, Steeds RP et al. Remote ischemic preconditioning in human coronary artery bypass surgery: From promise to disappointment? Circulation 2010;122:S53-9.

99. Ali N, Rizwi F, Iqbal A, Rashid A. Induced remote ischemic pre-conditioning on ischemia-reperfusion injury in patients undergoing coronary artery bypass. J Coll Physicians Surg Pak 2010;20:427-31.

100. Meybohm P, Kohlhaas M, Stoppe C et al. Ripheart (remote ischemic preconditioning for heart surgery) study: Myocardial dysfunction, postoperative neurocognitive dysfunction, and 1 year follow-up. Journal of the American Heart Association 2018;7.

101. Pijls NH, Van Gelder B, Van der Voort P et al. Fractional flow reserve. A useful index to evaluate the influence of an epicardial coronary stenosis on myocardial blood flow. Circulation 1995;92:3183-93.

102. Pijls NH, De Bruyne B, Peels K et al. Measurement of fractional flow reserve to assess the functional severity of coronary-artery stenoses. N Engl J Med 1996;334:1703-8.

103. Pijls NH, De Bruyne B, Smith L et al. Coronary thermodilution to assess flow reserve: Validation in humans. Circulation 2002;105:2482-6.

104. De Bruyne B, Pijls NH, Smith L, Wievegg M, Heyndrickx GR. Coronary thermodilution to assess flow reserve: Experimental validation. Circulation 2001;104:2003-6.

105. Fearon WF, Balsam LB, Farouque HM et al. Novel index for invasively assessing the coronary microcirculation. Circulation 2003;107:3129-32.

106. Yong AS, Layland J, Fearon WF et al. Calculation of the index of microcirculatory resistance without coronary wedge pressure measurement in the presence of epicardial stenosis. JACC Cardiovasc Interv 2013;6:53-8.

107. Tonino PA, De Bruyne B, Pijls NH et al. Fractional flow reserve versus angiography for guiding percutaneous coronary intervention. N Engl J Med 2009;360:213-24.

108. De Bruyne B, Pijls NH, Kalesan B et al. Fractional flow reserve-guided pci versus medical therapy in stable coronary disease. N Engl J Med 2012;367:991-1001.

109. Ng MK, Yeung AC, Fearon WF. Invasive assessment of the coronary microcirculation: Superior reproducibility and less hemodynamic dependence of index of microcirculatory resistance compared with coronary flow reserve. Circulation 2006;113:2054-61.

110. Kern MJ, Lerman A, Bech JW et al. Physiological assessment of coronary artery disease in the cardiac catheterization laboratory: A scientific statement from the american heart association committee on diagnostic and interventional cardiac catheterization, council on clinical cardiology. Circulation 2006;114:1321-41.

111. Yong AS, Ho M, Shah MG, Ng MK, Fearon WF. Coronary microcirculatory resistance is independent of epicardial stenosis. Circ Cardiovasc Interv 2012;5:103-8, S1-2.

112. Yong AS, Ng AC, Brieger D, Lowe HC, Ng MK, Kritharides L. Three-dimensional and two-dimensional quantitative coronary angiography, and their prediction of reduced fractional flow reserve. Eur Heart J 2011;32:345-53.

113. Yong ASC, Javadzadegan A, Fearon WF et al. The relationship between coronary artery distensibility and fractional flow reserve. PLoS One 2017;12:e0181824.

114. Kelle S, Hays AG, Hirsch GA et al. Coronary artery distensibility assessed by 3.0 tesla coronary magnetic resonance imaging in subjects with and without coronary artery disease. Am J Cardiol 2011;108:491-7.

115. Jeremias A, Spies C, Herity NA et al. Coronary artery compliance and adaptive vessel remodelling in patients with stable and unstable coronary artery disease. Heart 2000;84:314-9.

116. Yong AS, Pennings GJ, Chang M et al. Intracoronary shear-related up-regulation of platelet p-selectin and platelet-monocyte aggregation despite the use of aspirin and clopidogrel. Blood 2011;117:11-20.

117. Schmitz G, Rothe G, Ruf A et al. European working group on clinical cell analysis: Consensus protocol for the flow cytometric characterisation of platelet function. Thromb Haemost 1998;79:885-96.

118. Bye AP, Unsworth AJ, Gibbins JM. Platelet signaling: A complex interplay between inhibitory and activatory networks. J Thromb Haemost 2016;14:918-30.

119. Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987;70:307-15.

120. Hua VM, Abeynaike L, Glaros E et al. Necrotic platelets provide a procoagulant surface during thrombosis. Blood 2015;126:2852-62.

121. Agbani EO, van den Bosch MT, Brown E et al. Coordinated membrane ballooning and procoagulant spreading in human platelets. Circulation 2015;132:1414-24.

122. McFadyen JD, Jackson SP. Differentiating haemostasis from thrombosis for therapeutic benefit. Thromb Haemost 2013;110:859-67.

123. Pasalic L, Wing-Lun E, Lau JK et al. Novel assay demonstrates that coronary artery disease patients have heightened procoagulant platelet response. J Thromb Haemost 2018;16:1198-1210.

124. Li N, Goodall AH, Hjemdahl P. Efficient flow cytometric assay for platelet-leukocyte aggregates in whole blood using fluorescence signal triggering. Cytometry 1999;35:154-61.

125. Rendu F, Brohard-Bohn B. The platelet release reaction: Granules' constituents, secretion and functions. Platelets 2001;12:261-73.

126. Pennings GJ, Yong AS, Kritharides L. Expression of emmprin (cd147) on circulating platelets in vivo. J Thromb Haemost 2010;8:472-81.

127. Kirschner MB, Edelman JJ, Kao SC, Vallely MP, van Zandwijk N, Reid G. The impact of hemolysis on cell-free microrna biomarkers. Frontiers in genetics 2013;4:94.

128. Kirschner MB, Kao SC, Edelman JJ et al. Haemolysis during sample preparation alters microrna content of plasma. PLoS One 2011;6:e24145.

129. El-Khoury V, Pierson S, Kaoma T, Bernardin F, Berchem G. Assessing cellular and circulating mirna recovery: The impact of the rna isolation method and the quantity of input material. Sci Rep 2016;6:19529.

130. Moret I, Sanchez-Izquierdo D, Iborra M et al. Assessing an improved protocol for plasma microrna extraction. PLoS One 2013;8:e82753.

131. Berckmans RJ, Sturk A, van Tienen LM, Schaap MC, Nieuwland R. Cell-derived vesicles exposing coagulant tissue factor in saliva. Blood 2011;117:3172-80.

132. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microrna. Nucleic Acids Res 2011;39:7223-33.

133. Arroyo JD, Chevillet JR, Kroh EM et al. Argonaute2 complexes carry a population of circulating micrornas independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108:5003-8.

134. Canfran-Duque A, Lin CS, Goedeke L, Suarez Y, Fernandez-Hernando C. Micro-rnas and high-density lipoprotein metabolism. Arterioscler Thromb Vasc Biol 2016;36:1076-84.

135. Benson EA, Skaar TC. Incubation of whole blood at room temperature does not alter the plasma concentrations of microrna-16 and -223. Drug Metab Dispos 2013;41:1778-81.

136. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microrna biomarkers in plasma and serum using quantitative reverse transcription-pcr (qrt-pcr). Methods 2010;50:298-301.

137. McAlexander MA, Phillips MJ, Witwer KW. Comparison of methods for mirna extraction from plasma and quantitative recovery of rna from cerebrospinal fluid. Frontiers in genetics 2013;4:83.

138. Lau JK, Pennings GJ, Yong A, Kritharides L. Cardiac remote ischaemic preconditioning: Mechanistic and clinical considerations. Heart Lung Circ 2017;26:545-553.

139. Taqueti VR, Everett BM, Murthy VL et al. Interaction of impaired coronary flow reserve and cardiomyocyte injury on adverse cardiovascular outcomes in patients without overt coronary artery disease. Circulation 2015;131:528-35.

140. Lee JM, Jung JH, Hwang D et al. Coronary flow reserve and microcirculatory resistance in patients with intermediate coronary stenosis. J Am Coll Cardiol 2016;67:1158-69.

141. Ahn SG, Hung OY, Lee JW et al. Combination of the thermodilution-derived index of microcirculatory resistance and coronary flow reserve is highly predictive of microvascular obstruction on cardiac magnetic resonance imaging after st-segment elevation myocardial infarction. JACC Cardiovasc Interv 2016;9:793-801.

142. Taqueti VR, Hachamovitch R, Murthy VL et al. Global coronary flow reserve is associated with adverse cardiovascular events independently of luminal angiographic severity and modifies the effect of early revascularization. Circulation 2015;131:19-27.

143. Ng MK, Yong AS, Ho M et al. The index of microcirculatory resistance predicts myocardial infarction related to percutaneous coronary intervention. Circ Cardiovasc Interv 2012;5:515-22.

144. Fearon WF, Low AF, Yong AS et al. Prognostic value of the index of microcirculatory resistance measured after primary percutaneous coronary intervention. Circulation 2013;127:2436-41.

145. Gutterman DD, Chabowski DS, Kadlec AO et al. The human microcirculation: Regulation of flow and beyond. Circ Res 2016;118:157-72.

146. Tsuruda T, Kato J, Kuwasako K, Kitamura K. Adrenomedullin: Continuing to explore cardioprotection. Peptides 2019;111:47-54.

147. Dong W, Yu P, Zhang T, Zhu C, Qi J, Liang J. Adrenomedullin serves a role in the humoral pathway of delayed remote ischemic preconditioning via a hypoxia-inducible factor-1alpha-associated mechanism. Mol Med Rep 2018;17:4547-4553.

148. Kleinbongard P, Skyschally A, Heusch G. Cardioprotection by remote ischemic conditioning and its signal transduction. Pflugers Arch 2017;469:159-181.

149. Taqueti VR, Shaw LJ, Cook NR et al. Excess cardiovascular risk in women relative to men referred for coronary angiography is associated with severely impaired coronary flow reserve, not obstructive disease. Circulation 2017;135:566-577.

150. Majmudar MD, Murthy VL, Shah RV et al. Quantification of coronary flow reserve in patients with ischaemic and non-ischaemic cardiomyopathy and its association with clinical outcomes. Eur Heart J Cardiovasc Imaging 2015;16:900-9.

151. Naya M, Tamaki N, Tsutsui H. Coronary flow reserve estimated by positron emission tomography to diagnose significant coronary artery disease and predict cardiac events. Circ J 2014;79:15-23.

152. Lee JF, Barrett-O'Keefe Z, Garten RS et al. Evidence of microvascular dysfunction in heart failure with preserved ejection fraction. Heart 2016;102:278-84.

153. Ahn JH, Kim SM, Park SJ et al. Coronary microvascular dysfunction as a mechanism of angina in severe as: Prospective adenosine-stress cmr study. J Am Coll Cardiol 2016;67:1412-1422.

154. Heusch G. The coronary circulation as a target of cardioprotection. Circ Res 2016;118:1643-58.

155. Kuntscher MV, Kastell T, Altmann J, Menke H, Gebhard MM, Germann G. Acute remote ischemic preconditioning ii: The role of nitric oxide. Microsurgery 2002;22:227-31.

156. Hoffman JI, Spaan JA. Pressure-flow relations in coronary circulation. Physiol Rev 1990;70:331-90.

157. Whittle BJ, Moncada S, Vane JR. Some actions of prostacyclin (pgi2) on the cardiovascular system and the gastric microcirculation. Acta Biol Med Ger 1978;37:725-8.

158. Hirayama N, Kitamura K, Imamura T, Kato J, Koiwaya Y, Eto T. Secretion and clearance of the mature form of adrenomedullin in humans. Life Sci 1999;64:2505-9.

159. Yuan M, Wang Q, Li C et al. Adrenomedullin in vascular endothelial injury and combination therapy: Time for a new paradigm. Curr Vasc Pharmacol 2015;13:459-66.

160. Schoemaker RG, van Heijningen CL. Bradykinin mediates cardiac preconditioning at a distance. Am J Physiol Heart Circ Physiol 2000;278:H1571-6.

161. Oosterlinck W, Dresselaers T, Geldhof V et al. Diabetes mellitus and the metabolic syndrome do not abolish, but might reduce, the cardioprotective effect of ischemic postconditioning. J Thorac Cardiovasc Surg 2013;145:1595-602.

162. Amsterdam EA, Wenger NK, Brindis RG et al. 2014 AHA/ACC guideline for the management of patients with non-st-elevation acute coronary syndromes: A report of the american college of cardiology/american heart association task force on practice guidelines. J Am Coll Cardiol 2014;64:e139-228.

163. Harbeck B, Huttelmaier S, Schluter K, Jockusch BM, Illenberger S. Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. J Biol Chem 2000;275:30817-25.

164. Horstrup K, Jablonka B, Honig-Liedl P, Just M, Kochsiek K, Walter U. Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur J Biochem 1994;225:21-7.

165. Zhao W, Meng R, Ma C et al. Safety and efficacy of remote ischemic preconditioning in patients with severe carotid artery stenosis before carotid artery stenting: A proof-of-concept, randomized controlled trial. Circulation 2017;135:1325-1335.

166. Pryds K, Kristiansen J, Neergaard-Petersen S et al. Effect of long-term remote ischaemic conditioning on platelet function and fibrinolysis in patients with chronic ischaemic heart failure. Thromb Res 2017;153:40-46.

167. Stalker TJ, Newman DK, Ma P, Wannemacher KM, Brass LF. Platelet signaling. Handb exp pharmacol, 2012:59-85.

168. Joo SJ. Mechanisms of platelet activation and integrin alphaiibeta3. Korean circulation journal 2012;42:295-301.

169. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol 2010;30:2341-9.

170. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nature reviews Drug discovery 2008;7:156-67.

171. Chan MV, Knowles RB, Lundberg MH et al. P2y12 receptor blockade synergizes strongly with nitric oxide and prostacyclin to inhibit platelet activation. Br J Clin Pharmacol 2016;81:621-33.

172. Apostoli GL, Solomon A, Smallwood MJ, Winyard PG, Emerson M. Role of inorganic nitrate and nitrite in driving nitric oxide-cgmp-mediated inhibition of platelet aggregation in vitro and in vivo. J Thromb Haemost 2014;12:1880-9.

173. Moncada S, Whittle BJ. Biological actions of prostacyclin and its pharmacological use in platelet studies. Adv Exp Med Biol 1985;192:337-58.

174. Tsai AL, Wu KK. Structure-activity relationship between prostacyclin and its platelet receptor. Correlation of structure change and the platelet activity. Eicosanoids 1989;2:131-43.

175. Whittle BJ, Moncada S. Platelet actions of stable carbocyclic analogues of prostacyclin. Circulation 1985;72:1219-25.

176. Moncada S, Vane JR. Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. N Engl J Med 1979;300:1142-7.

177. Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B. Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. Am J Physiol 1997;273:H1707-12.

178. Jobe SM, Wilson KM, Leo L et al. Critical role for the mitochondrial permeability transition pore and cyclophilin d in platelet activation and thrombosis. Blood 2008;111:1257-65.

179. Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: Different populations, different functions. J Thromb Haemost 2013;11:2-16.

180. Hua VM, Chen VM. Procoagulant platelets and the pathways leading to cell death. Semin Thromb Hemost 2015;41:405-12.

181. Munnix IC, Kuijpers MJ, Auger J et al. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: Regulation by transient integrin activation. Arterioscler Thromb Vasc Biol 2007;27:2484-90.

182. Choo HJ, Kholmukhamedov A, Zhou C, Jobe S. Inner mitochondrial membrane disruption links apoptotic and agonist-initiated phosphatidylserine externalization in platelets. Arterioscler Thromb Vasc Biol 2017;37:1503-1512.

183. Arachiche A, Kerbiriou-Nabias D, Garcin I, Letellier T, Dachary-Prigent J. Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization. Arterioscler Thromb Vasc Biol 2009;29:1883-9.

184. Topalov NN, Yakimenko AO, Canault M et al. Two types of procoagulant platelets are formed upon physiological activation and are controlled by integrin alpha(iib)beta(3). Arterioscler Thromb Vasc Biol 2012;32:2475-83.

185. Park D, Don AS, Massamiri T et al. Noninvasive imaging of cell death using an hsp90 ligand. J Am Chem Soc 2011;133:2832-5.

186. Pasalic L, Wang SS, Chen VM. Platelets as biomarkers of coronary artery disease. Semin Thromb Hemost 2016;42:223-33.

187. Gilio K, van Kruchten R, Braun A et al. Roles of platelet stim1 and orai1 in glycoprotein vi- and thrombin-dependent procoagulant activity and thrombus formation. J Biol Chem 2010;285:23629-38.

188. Schoenwaelder SM, Darbousset R, Cranmer SL et al. 14-3-3zeta regulates the mitochondrial respiratory reserve linked to platelet phosphatidylserine exposure and procoagulant function. Nature communications 2016;7:12862.

189. Agbani EO, Poole AW. Procoagulant platelets: Generation, function, and therapeutic targeting in thrombosis. Blood 2017;130:2171-2179.

190. Hausenloy DJ, Yellon DM. The mitochondrial permeability transition pore: Its fundamental role in mediating cell death during ischaemia and reperfusion. J Mol Cell Cardiol 2003;35:339-41.

191. Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J Physiol 2003;549:513-24.

192. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: United at reperfusion. Pharmacol Ther 2007;116:173-91.

193. Zhang SZ, Wang NF, Xu J et al. Kappa-opioid receptors mediate cardioprotection by remote preconditioning. Anesthesiology 2006;105:550-6.

194. Ambros V. Micrornas: Tiny regulators with great potential. Cell 2001;107:823-6.

195. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and micrornas. Nature reviews Genetics 2007;8:93-103.

196. Ambros V. The functions of animal micrornas. Nature 2004;431:350-5.

197. Tetreault N, De Guire V. Mirnas: Their discovery, biogenesis and mechanism of action. Clin Biochem 2013;46:842-5.

198. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-micrornas and short hairpin rnas. Genes Dev 2003;17:3011-6.

199. Gatsiou A, Boeckel JN, Randriamboavonjy V, Stellos K. Micrornas in platelet biogenesis and function: Implications in vascular homeostasis and inflammation. Curr Vasc Pharmacol 2012;10:524-31.

200. Rana TM. Illuminating the silence: Understanding the structure and function of small rnas. Nat Rev Mol Cell Biol 2007;8:23-36.

201. Economou EK, Oikonomou E, Siasos G et al. The role of micrornas in coronary artery disease: From pathophysiology to diagnosis and treatment. Atherosclerosis 2015;241:624-33.

202. Esau C, Davis S, Murray SF et al. Mir-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell metabolism 2006;3:87-98.

203. Elmen J, Lindow M, Schutz S et al. Lna-mediated microrna silencing in non-human primates. Nature 2008;452:896-9.

204. Wei Y, Nazari-Jahantigh M, Neth P, Weber C, Schober A. Microrna-126, -145, and -155: A therapeutic triad in atherosclerosis? Arterioscler Thromb Vasc Biol 2013;33:449-54.

205. Wei Y, Schober A, Weber C. Pathogenic arterial remodeling: The good and bad of micrornas. Am J Physiol Heart Circ Physiol 2013;304:H1050-9.

206. Rader DJ, Parmacek MS. Secreted mirnas suppress atherogenesis. Nat Cell Biol 2012;14:233-5.

207. Haver VG, Slart RH, Zeebregts CJ, Peppelenbosch MP, Tio RA. Rupture of vulnerable atherosclerotic plaques: Micrornas conducting the orchestra? Trends Cardiovasc Med 2010;20:65-71.

208. Urbich C, Kuehbacher A, Dimmeler S. Role of micrornas in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res 2008;79:581-8.

209. Nazari-Jahantigh M, Wei Y, Noels H et al. Microrna-155 promotes atherosclerosis by repressing bcl6 in macrophages. J Clin Invest 2012;122:4190-202.

210. Cimmino G, Tarallo R, Nassa G et al. Activating stimuli induce platelet microrna modulation and proteome reorganisation. Thromb Haemost 2015;114.

211. Choi E, Cha MJ, Hwang KC. Roles of calcium regulating micrornas in cardiac ischemia-reperfusion injury. Cells 2014;3:899-913.

212. Yang Y, Cheng H, Qiu Y et al. Microrna-34a plays a key role in cardiac repair and regeneration following myocardial infarction. Circ Res 2015;117:450-9.

213. Gareri C, De Rosa S, Indolfi C. Micrornas for restenosis and thrombosis after vascular injury. Circ Res 2016;118:1170-84.

214. Brandenburger T, Grievink H, Heinen N et al. Effects of remote ischemic preconditioning and myocardial ischemia on microrna-1 expression in the rat heart in vivo. Shock 2014;42:234-8.

215. Kang Z, Li Z, Huang P et al. Remote ischemic preconditioning upregulates microrna-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass. Pediatr Nephrol 2018;33:911-919.

216. Duan YF, An Y, Zhu F, Jiang Y. Remote ischemic preconditioning protects liver ischemia-reperfusion injury by regulating enos-no pathway and liver microrna expressions in fatty liver rats. Hepatobiliary Pancreat Dis Int 2017;16:387-394.

217. Duan YF, Sun DL, Chen J, Zhu F, An Y. Microrna-29a/b/c targets inos and is involved in protective remote ischemic preconditioning in an ischemia-reperfusion rat model of non-alcoholic fatty liver disease. Oncol Lett 2017;13:1775-1782.

218. Jia P, Wu X, Dai Y et al. Microrna-21 is required for local and remote ischemic preconditioning in multiple organ protection against sepsis. Crit Care Med 2017;45:e703-e710.

219. Morey JS, Ryan JC, Van Dolah FM. Microarray validation: Factors influencing correlation between oligonucleotide microarrays and real-time pcr. Biol Proced Online 2006;8:175-93.

220. Heusch G, Rassaf T. Time to give up on cardioprotection? A critical appraisal of clinical studies on ischemic pre-, post-, and remote conditioning. Circ Res 2016;119:676-95.

221. Healy DA, Khan WA, Wong CS et al. Remote preconditioning and major clinical complications following adult cardiovascular surgery: Systematic review and meta-analysis. Int J Cardiol 2014;176:20-31.

222. Sukkar L, Hong D, Wong MG et al. Effects of ischaemic conditioning on major clinical outcomes in people undergoing invasive procedures: Systematic review and meta-analysis. BMJ 2016;355:i5599.

223. Li Y, Xiang GM, Liu LL et al. Assessment of endogenous reference gene suitability for serum exosomal microrna expression analysis in liver carcinoma resection studies. Mol Med Rep 2015;12:4683-91.

224. Wang X, Gardiner EJ, Cairns MJ. Optimal consistency in microrna expression analysis using reference-gene-based normalization. Molecular bioSystems 2015;11:1235-40.

225. Obydennyy SI, Sveshnikova AN, Ataullakhanov FI, Panteleev MA. Dynamics of calcium spiking, mitochondrial collapse and phosphatidylserine exposure in platelet subpopulations during activation. J Thromb Haemost 2016;14:1867-81.

226. Pampuch A, Cerletti C, de Gaetano G. Comparison of vasp-phosphorylation assay to light-transmission aggregometry in assessing inhibition of the platelet adp p2y12 receptor. Thromb Haemost 2006;96:767-73.