The influence of nitric oxide and nitrite on coronary vascular resistance, platelet function and inflammation in patients undergoing revascularisation after NSTEMI and stable angina

Phillip Freeman

MBBS BSc MRCP

A DISSERTATION SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

To Cardiff University

June 2017

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed …… (candidate) Date ……

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD

Signed …… (candidate) Date ……

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated, and the thesis has not been edited by a third party beyond what is permitted by Cardiff University's Policy on the Use of Third Party Editors by Research Degree Students. Other sources are acknowledged by explicit references. The views expressed are my own.

Signed …… (candidate) Date ……

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available online in the University's Open Access repository and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed …… (candidate) Date ……

ACKNOWLEDGEMENTS

The PhD process is challenging, frustrating and on occasion rewarding. All of these features often came from directions that I never imagined when I started the process.

The simple truth is that this PhD would never have been completed without the considerable help, support and encouragement that I received from my partner in crime Malene Fischer Hansen and our two fantastic and smart children Leah Freeman and Zacharias Freeman. When I talk about stress and frustration I am well aware that my family experienced some of this translated through me. Their patients, understanding and strength has simply been unwavering.

It has also been a real pleasure to have the help and support of Colin Dayan from Cardiff University who's help, efficiency and considerably diplomatic skills enabled me to make the final (and most difficult) steps in getting this thesis past many barriers and submitted.

I am indebted to my "secret weapon", who was always willing to read protocols and papers and give me some great ideas from the outside, Jørgen Fischer Hansen.

Notable collaborators include both Valerie O'Donnell and Jerry McCann, from whom I gained invaluable advice on multiple areas at multiple times. I couldn't help but enjoy the thought provoking and intense academic interactions that both these fantastic scientists provided.

A critical component of this thesis was the data gained from complex protocols performed in the cardiac catheter lab, central to supporting this was the fantastic and highly professional team of cath lab nurses. I thank all of you for your help, support, flexibility and all round professionalism.

Lastly I thank both Philip James and Richard Anderson for providing a master class in how I both should and should not approach my academic career moving forward into the future.

ABSTRACT

Introduction

Coronary blood flow (CBF) is principally controlled by changes in coronary vascular resistance (CVR). Low CVR helps to maintain myocardial perfusion in the presence of epicardial stenosis, therefore factors that impair the reduction of CVR will have a direct effect on CBF leading to either a narrower "effective" perfusion pressure range or reduced ability to compensate for increased demand on myocardial contraction. There are several mechanisms which may be important in the control CVR in humans including both endothelial dependent production of NO and reduction of the simple anion inorganic nitrite (a metabolite of NO) back to NO (via several putative mechanisms). The synthesis of NO by both nitric oxide synthase (NOS) and the reduction of nitrite in the coronary circulation has been the subject of many animal and human clinical studies. Reduced systemic endothelial dependent production of NO has an association with worse cardiovascular outcomes in humans, the number of potential mechanisms are large and perhaps central is the effect on CVR. The reduction of nitrite to NO is seen, in some ways, to be the perfect compensatory mechanism, particularly when endothelial function is impaired. It is easy to hypothesise that this stoichiometric balance of NO production might be responsible for the perfect regulation of CVR and thus CBF.

Methods

This thesis investigates the influence and effect of both endothelial production of NO and the reduction of nitrite to NO on CVR in man.

First, an observational study assessing the impact of these mechanisms in patients undergoing percutaneous coronary intervention (PCI), in the treatment of both non-ST-elevation myocardial infarction and stable angina. Specifically, the metabolites of NO were measured from aortic root to coronary sinus together with the associated CVR both before and after PCI.

Second, using a systemic infusion of sodium nitrite ($NaNO_2$) in NSTEMI patients to assess the effect of nitrite reduction on CVR during PCI. The systemic nitrite concentration was increased 8-fold in the same experimental conditions as the observational study.

Third, beyond CVR control the influence of NO and nitrite was also assessed in terms of platelet reactivity and systemic inflammatory cytokines in the NSTEMI cohort both with and without $NaNO_2$ infusion.

Results

NSTEMI patients have a net increase in NO metabolites across the coronary circulation unlike healthy controls (historical data) and stable angina patients. This net increase is lost following PCI and is associated with a significant rise in CVR. Stable angina patients appear to compensate with increase collateral circulation and not NO synthesis. An 8-fold increase in nitrite concentration has no effect on CVR or platelet reactivity in NSTEMI patients.

Conclusions

In NSTEMI patients a net aorta to coronary sinus NO synthesis appears to be important to maintain a low CVR and thus CBF when haemodynamically significant epicardial disease is present. After the epicardial disease is treated this

net increase in NOx (Nitric Oxide metabolites), is lost and is associated with an acute increase in CVR. Stable angina patients have no net increase in NOx across the coronary circulation and after revascularisation have no change in CVR, this may reflect an alternate mechanism of compensation and microvascular perfusion maintained by collateral circulation as evident by the increase CFI. Despite the perfect environment for the reduction of nitrite to NO we saw no influence of an 8-fold increase in serum nitrite concentration on CVR in patients with NSTEMI either before or after PCI, suggesting that nitrite reduction to NO plays no role in CBF regulation in NSTEMI patients. Nitrite reduction depends on conditions that are found predominantly in the capillary bed or venules, thus any mechanism would need to rely on a feedback mechanism to signal back to the arterioles (where much of resistance change is created). Despite hypotheses by others that this may occur by the close arrangement of venules to arterioles, this appears not to be the case in NSTEMI patients. Other clinically relevant and important mechanisms also appear to be unaffected by this increase in serum nitrite, residual platelet function and cytokine concentrations at 24 hours.

FUNDING

British Heart Foundation

Project Grant (502370)

July 2011 £83,229

Cardiac microvascular function: Assessment and protection during coronary intervention. Dr P James, Dr P Freeman & Prof B Moser

St Jude Medical

Unrestricted Educational Grant,

Jan 2011

Dr P Freeman. £38,600

Cardiff University Startup grant

Clinical Lectureship:

Jan 2010

Dr Phillip Freeman £11,243

Publications and Abstracts

Freeman P, Moschonas K, Hinz C, O'Donnell V, Kinnaird T, James P, Anderson D. Changes in Platelet Function Independent of Pharmacotherapy following coronary intervention in non-ST-elevation myocardial infarction patients. Atherosclerosis 2015; 243: 320-327

Freeman P, Mochanos C, Kinnaird T, James P , Anderson D. Peri-Procedural Platelet Function Testing in Non-ST Elevation Myocardial Infarction Patients: The Importance of Timing. JACC 2014 63(12)

Freeman P, Anderson RA, James P. Fluxes in heart specific nitrite production before and after coronary intervention. Nitric Oxide 2012; 27; S28-S29

Freeman P, Kinnaird T, James P, Anderson D. Reliability and safety of measuring fractional flow reserve and index of myocardiam resistance with sodium nitroprusside bolus in ACS patients JACC 2014 63(12):A1784

TABLE OF CONTENTS

THE INFLUENCE OF NITRIC OXIDE AND NITRITE ON CORONARY VASCULAR RESISTANCE, PLATELET FUNCTION AND INFLAMMATION IN PATIENTS UNDERGOING REVASCULARISATION AFTER NSTEMI AND STABLE ANGINA

List of figures

List of tables

Abbreviations

1

12-Hydroxyeicosatetraenoic Acid (12-HETE)

8

8-Phenyltheophylline (8-PT)

A

Acidified Tri-Iodide (ATI)

Adenosine Diphosphate (ADP)

Adenosine Monophosphate (AMP)

Adenosine Triphosphate (ATP)

Allophycocyanin-(APC)

Aortic (Ao)

Arachidonic Acid (AA)

Area Under The Curve (AUC)

C

Cardiac Magnetic Resonance Imaging (CMR)

Cardiopulmonary Bypass (CPB)

Coronary Artery Bypass Surgery (CABG)

Coronary Blood Flow (CBF)

Coronary Sinus (CS)

Coronary Sinus Venous Pressure (Pv)

Coronary Vascular Resistance (CVR)

Coronary Wedge Pressure (Pw)

Creatinine Kinase (CK)

D

Distal Coronary Pressure (Pd)

E

Ejection Fraction (EF)

Electron Transport Train (ETC)

Endothelium Derived Hyperpolarising Factor (EDHF)

F

Fluoresceine-Isothiocyanate-(FITC)

Fractional Flow Reserve (FFR)

G

Glycoprotein IIb/IIIa Inhibitors (GPI)

H

Haemoglobin bound NO (HbNO)

High on Treatment Platelet Reactivity (HTPR)

Hydrogen Peroxide (H2O2)

Hyperaemic Transit Time (Tm_{hyp})

I

IMR at Rest (IMR_{rest})

IMR With Hyperaemia (IMR_{hyp})

Index of Microvascular Resistance (IMR)

Interferon-γ (IFN-γ)

Ischaemia Reperfusion Injury (IRI)

L

Late Gadolinium Enhancement (LGE)

Left Ventricular Hypertrophy (LVH)

Light Transmission Aggregometry (LTA)

M

Macrophage-1 Antigen (MAC-1)

Metabolites of Nitric Oxide (NOx)

Multiple Electrode Aggregometry (MEA)

N

Necrosis Factor-A (TNF-A)

N^G-Monomethyl-L-Arginine (L-NMMA)

Nitrate (NO_{3-}).

Nitric Oxide (NO)

Nitric Oxide Synthetase (NOS)

Nitrite (NO_{2-})

Non-ST Elevation Myocardial Infarction (NSTEMI)

NSTEMI Cohort Receiving Pre-PCI Loading With Sodium Nitrite ($NSTEMI_{inf}$)

Nω –Nitro-L-Arginine (L-NNA)

O

Ozone-Based Chemiluminescence (OBC)

P

Percutaneous Coronary Intervention (PCI)

Platelet Function Testing (PFT)

Potassium Hexacyanoferrate ($K3Fe_{3+}(CN)_6$)

Protein Kinase A (PKA)

Proximal Coronary Pressure (Pa)

P-Selectin Glycoprotein Ligand-1 (PSGL-1)

R

Reactive Oxygen Species (ROS),

Red Blood Cells (RBC)

S

S-Nitrosohaemoglobin (Hbsno)

S-Nitrosohaemoglobin (Snohb)

Sodium Hydroxide (NaOH

ST Elevation Myocardial Infarction (STEMI)

Stable Angina (SA)

Strain Gauge Plethysmography (SGP)

Superoxide Dismutase Group of Enzymes (SOD)

T

Thrombin Receptor Activating Peptide (TRAP)

Thrombin Receptor Activating Peptide 6 (TRAP-6)

Thromboxane A2 (TXA2)

Tumour Necrosis Factor (TNF)

Transforming Growth Factor B (TGF-B)

Transit Time At Rest (Tm Rest)

V

Vanadium III Chloride (VCl3).

Voltage Dependent Calcium Channels (VDCC)

Von Willibrand Factor (VwF)

Chapter 1: General introduction

Autoregulation of coronary blood flow in health and disease

The coronary circulation is unique in that it perfuses the organ that creates systemic perfusion pressure. Physiological, pathological and other interventions that alter the heart and/or systemic circulation effect coronary blood flow by altering the metabolic demands placed on the heart. [1] The mechanisms that control coronary blood flow are complex and multifaceted, another layer of complexity is achieved when we study these mechanisms in different physiological and pathological states. As conditions change, so does the emphasis or influence of any one mechanism of coronary blood flow control.

Even in the 21st century where we can influence coronary disease with a multitude of pharmacological and interventional treatments, relatively little is known about the basic mechanisms of coronary blood flow control in human pathological states.

This thesis focuses on one aspect of coronary blood flow and its influence in the context of two disease states (stable angina and acute coronary syndrome).

Oxygen extraction, perfusion pressure and coronary vascular resistance: influence on coronary blood flow

Oxygen extraction

The heart primarily depends on aerobic metabolism for production of adenosine triphosphate (ATP) and its ongoing heavy demands for continued function throughout life. Under resting conditions the heart can extract as much as 70 to 80% of the oxygen delivered to it by the coronary circulation, compared with 30 to 40% in skeletal muscle. Overall oxygen consumption per gram of tissue is 20 fold higher than skeletal muscle. [1,2]

This highly efficient oxygen extraction is made possible by a high capillary network density in comparison to skeletal muscle (3000 to 4000 capillaries/mm^2 vs. 600-1000 capillaries/mm^2 respectively). [3,4] Unsurprisingly because of the almost maximal oxygen extraction at rest there is almost no change in oxygen extraction during light exercise in humans [5] and a tendency towards only slightly increased extraction during moderate to heavy exercise. [6,7] This almost maximal oxygen extraction makes the heart almost completely dependent on increasing coronary blood flow (CBF) during exercise. This increase in CBF is seen in-vivo with four to five-fold and above increases in flow during heavy exercise (mean flow of 280 ml 280 $ml.min^{-1}.100g^{-1}$). [1,8,9]

Myocardial perfusion pressure

The gradient of pressure across the coronary bed (perfusion pressure), together with the total coronary vascular resistance, governs CBF. However, because the

coronary bed is embedded in the contracting myocardium the simple aortic to venous pressure drop does not equate to effective perfusion pressure. [1] Because of myocardial contraction in systole the majority of epicardial coronary flow occurs during diastole. The aortic pressure - and thus the radial pressure generated by myocardial contraction – govern diastolic pressure gradients across the myocardium. This diastolic pressure (or backflow pressure) is higher than right atrial pressure and is commonly referred to as the zero flow pressure (Pf=0). During exercise, healthy dogs have been shown to increase both their aortic inflow pressure and effective zero flow pressure (which opposes coronary flow) by 10-20mmHg. [10] This suggest that during exercise the effective perfusion pressure gradient does not change and thus any increase in CBF must be proportional to a reduction in coronary vascular resistance (CVR).

Canty et al [11] showed that in awake dogs CBF remains remarkably constant over a range of perfusion pressure (the auto-regulatory range), below the lower limit of this range (~ 40mmHg) CBF would drop rapidly with very small reductions in perfusion pressure down to no flow once the zero flow pressure was reached (Pf=0). The lower limit of this auto-regulatory perfusion pressure is raised by a number of factors that change oxygen extraction requirements. These factors would include increased heart rate (and decreased diastolic pressure time), Increase systolic blood pressure leading to increased contractility and radial wall stress; or reductions in oxygen supply such as anaemia and hypoxia. [12,13] These increases in oxygen demand or decreases in oxygen delivery increase resting flow and thus raise the lower limit of the auto-regulatory perfusion range; this

translates into ischaemia at higher perfusion pressures. (Figure 1: Autorgulation of coronary blood flow - relationship between coronary pressure and resistance)

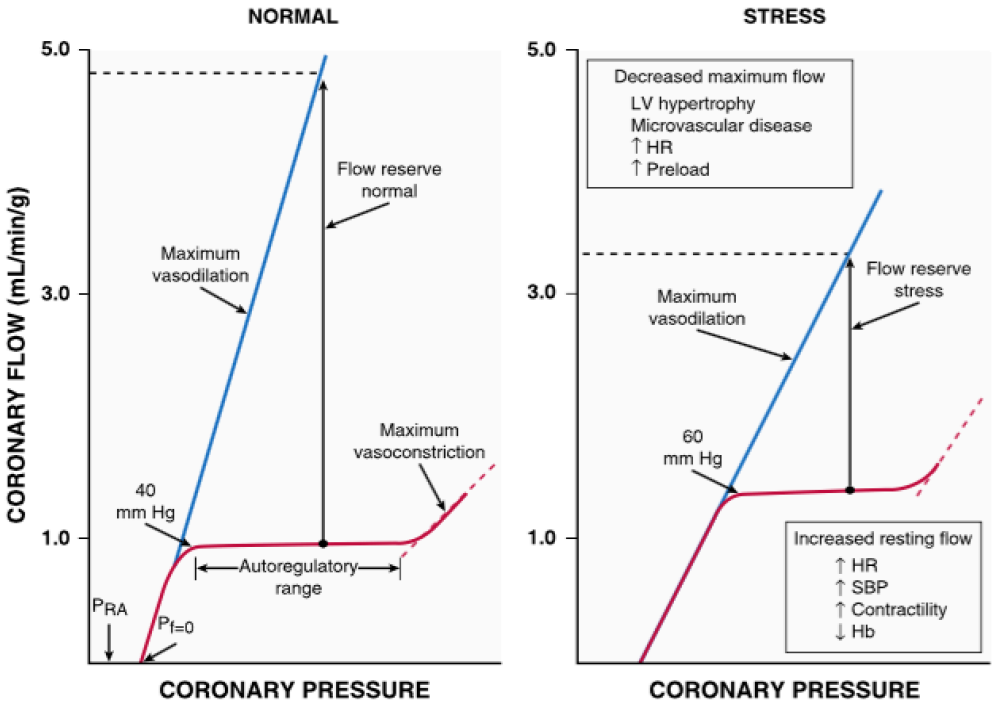

Figure 1: Autorgulation of coronary blood flow - relationship between coronary pressure and resistance

Autoregulation of CBF in terms of perfusion pressure and coronary vascular resistance at rest (left panel) and stress (right panel). Red lines correspond to CBF (y-axis) and describe its relationship to coronary pressure (x –axis) being constant when in the autoregulatory range. The blue line represents maximal vasodilation (or lowest CVR). At rest when the coronary pressure drops below its lower autoregulatory limit CVR is at its lowest and coronary flow drops rapidly, this would lead to ischaemia. During stress (increased HR, increased systolic BP, increased contractility, increased oxygen demand) the lower pressure limit of the autoregulatory range increases (here to 60mmHg as an example), thus CBF falls at high pressures. This may be further compounded by factors that reduce the ability of the microvasculature to maximally dilate (LVH, microvascular disease, increased preload / $P_{f=0}$). Reprinted from Canty and Duncker with permission, Coronary blood flow and myocardial ischaemia In: Bonow RO, Mann DL, Zipes DP, Libby P, eds. Braunwald's Heart Disease. 10th ed. Philadelphia: Elsevier 2014

Coronary Vascular Resistance (CVR)

We have now identified CVR as the principal determinant of auto-regulation of CBF. Whilst the principal component of CVR relates to vascular tone there are other determinants of CVR. These include the compressive forces of the cardiac cycle as well as the anatomy and distribution of subepicardial and subendocardial vessels. These compressive and anatomical features have been described extensively [14-17] For the purposes of this thesis I will focus on vascular tone and its control and effect on CVR and thus CBF regulation as this is the dominant mechanism, [18] in this context the thesis is focused on the control mechanisms of CVR with emphasis on nitric oxide (NO).

Along the course of the epicardial coronary artery there is very little pressure drop in the healthy heart, indicating negligible resistance in this conduit vessel. The dominant pressure drop in the coronary vessels occurs in those arteriolar vessels between 50 to 200μm in diameter, hence the term resistance vessels [19] There is very little contribution from capillaries and coronary venules to CVR, indeed resistance across these structures remains constant during changes in pressure, flow and metabolic demands. [20] (Figure 2: Distribution of coronary vascular resistance)

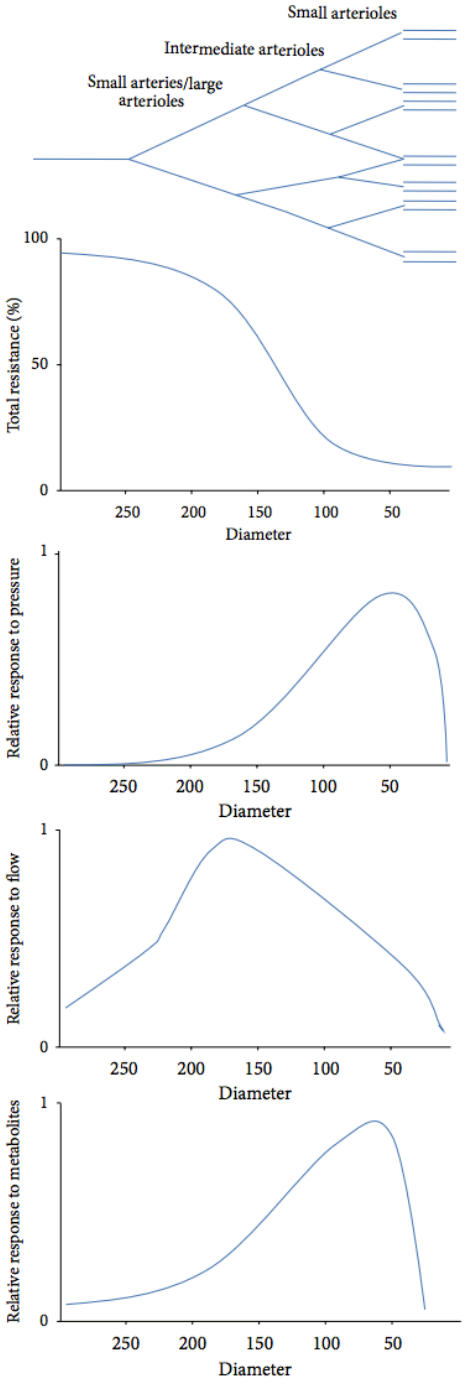

Figure 2: Distribution of coronary vascular resistance

Schematic representation of the distribution of coronary vascular resistance and a representation of the mechanisms that likely contribute to control of resistance in successive segments of the coronary vascular tree. Outlining the complex interaction of different factors controlling CVR. With permission[21]

The effect of epicardial coronary stenosis

Prior to an in-depth description of the mechanisms of control of CVR it is important to clarify and understand the effect of an epicardial stenosis on CVR and ultimately CBF. Unlike the subepicardial vessels the subendocardial vessels are exposed disproportionately to the extravascular tissue pressures caused by ventricular contraction. Minimal microvascular resistance is lower in the subendocardial vessels and this goes some way in compensating for the susceptibility of the subendocardial layer to ischaemia. [22] When the autoregulatory mechanisms of CBF are intact flow is uniform across the myocardium, however, with progressive narrowing of an epicardial coronary the distal coronary pressure (P_d) progressively reduces. In response to increasingly severe stenosis the resistance vessels will dilate and "auto-regulate" CBF through the myocardium. This compensation holds until P_d drops below the lower limit of the auto-regulatory range (equating to the exhaustion of the vasodilatory capacity of the resistance vessels and set by both mechanical and metabolic demands). As soon as P_d drops below this lower auto-regulatory threshold CBF drops sharply and delivery of oxygen is not adequate for the myocardial bed, [11,23,24] the extrinsic forces on the subendocardial layers of the heart will lead to ischaemia developing here first.[25]

Mechanisms of CVR control: feedback, feedforward, metabolic and non-metabolic

Feedback vs feedforward control of CVR

Many decades have passed in the search for a principal signal that regulates coronary blood flow. More than 40% of the total CVR, [20,26] is generated in arterioles, (diameter of 50-200 μm). If we assume the majority of oxygen delivered occurs at the systemic capillary bed, in any feedback mechanism the control of the resistance vessels must occur independent of direct oxygen tension unless a mechanism exists utilising the close proximity of post-capillary venules to pre-capillary arterioles. [27]

Indirect action of oxygen in feedback models has also been proposed the principal model being the adenine nucleotide model, Pradhan et al [28] suggest that haemoglobin derived ATP acts on purinergic receptors and causes upstream (feedback) signaling via endothelial gap-junctions to release NO. Nitrite is seen by many as the ultimate feedback molecule in the control of CVR and thus CBF. Produced by oxidation of NO nitrite can also be reduced back to NO under optimal conditions of low PO_2 and pH, [29] and again assuming low pH and PO_2 will occur principally at the point of oxygen delivery (capillaries) the requirement of close proximity of venous to resistance arteriole is required.

Subsequently, the notion of feed-forward mechanisms in the control of CBF has emerged. Whilst feedback mechanisms rely on an error signal based around oxygen consumption (not easily seen in the cardiac circulation), a feed-forward signal doesn't need a local oxygen error signal. Rather a metabolite produced

secondary to systemic oxygen utilisation would (See ROS and H_2O_2 section below page 37), control CVR and thus CBF direct. This means there is no requirement for oxygen mismatch to demand.

Feed-forward control may also be orchestrated by sensing key hemodynamic forces such as shear stress (NOS upregulation) and intramural pressure changes (myogenic control). [20] Oxygen should also not be excluded from a feed-forward model. Systemic and myocardial delivery of oxygen to tissues is a complex subject with some evidence that this may occur to a significant degree at the pre-capillary arterioles (resistance vessels). However, this principally occurs in skeletal muscle and there is no experimental evidence of this occurring in the mammalian heart microcirculation. [30]

Below each of the major potential mechanisms proposed to regulate CVR are described in more detail. These are the major examples of feedforward and feedback control systems and are categorised as metabolic or non-metabolic.

Metabolic control of CVR

Under physiological conditions CBF is adjusted to meet oxygen demand in an organ that is extremely efficient at oxygen extraction even at rest. The regulation of flow is so tightly coordinated that coronary venous pO_2 does not stray far away from 20mmHg regardless of the severity of exercise. [2]

Feed-forward control of CVR may occur by a multitude of mechanisms; from factors that reflect systemic oxidative metabolism to localised changes in coronary flow velocity (shear stress). Whilst it's tempting to consider only one signal for control of CVR it's clear that there are a number of control mechanisms that orchestrate this fine-tuned system.

Oxygen and carbon dioxide

The first obvious markers of systemic oxidative metabolism are oxygen and carbon dioxide, which have long been proposed as the principal determinants of CVR. Both arterial hypoxia and hypercapnia produce coronary vasodilation. [1,31] Broten et al [31] showed a synergistic effect of both hypoxia and hypercapnia occurring simultaneously with each molecule potentiating the other beyond an additive affect. However, in vivo changes in these gases in the arterial phase could only predict a small component of the change in CBF seen during pacing and an even smaller part during autoregulation, [32] suggesting that these molecules do not exert a dominant role as a feed-forward mechanism.

Both lung and tissue capillaries have an enormous surface area and it is this fact that leads many to the conclusion that these two networks are the "mirror images" of each other. It is intuitive to come to the conclusion that tissue (systemic) capillaries yield the oxygen absorbed by the lung in a final pathway of oxygen delivery to the tissues. [33] This process became a fundamental principal in physiology following the work of Krogh and Erlangen in developing the "Krogh cylinder model" in 1918. [34] The work describes a mathematical model of oxygen

delivery to skeletal muscle by an array of capillaries to a surrounding tissue cylinder. At the time of this work there was no method enabling the measurement of oxygen levels in microcirculatory vessels, therefore the model assumed that all oxygen exchange at the tissue level takes place in the capillary. By the 1970s this assumption was being questioned, Duling & Berne using new (for that time) techniques with micro-oxygen cathodes found that significant longitudinal oxygen gradients occurred in arterioles of 80 to 150 μm in diameter to the terminal arterioles (42 ± 3 to 21 ± 3 mmHg). [35] These observations were confirmed by a multitude of investigators using a large array of evolving techniques. [36-38]

The release of oxygen to - and its consumption by - the tissues is clearly highly complex, changing in different tissues with widely different characteristics. The reality is that these numerous studies have multiple methodological difficulties and their interpretation is often complex. [38] Suffice to say that the assumption that all oxygen delivery occurs at the systemic capillary network (after the resistance arterioles) is by no means without debate; thus we should not assume that oxygen tension has no role as a feed-forward mechanism in CVR control.

Adenine nucleotide / purinergic receptor control of CBF

In areas of increased oxygen demand (low PO_2 tension), adenosine triphosphate (ATP) is released from red blood cells (RBC). The adenine nucleotide model (Figure 3: Adenine nucleotide hypothesis of coronary vascular resistance control), postulates that ATP and its breakdown products adenosine diphosphate (ADP) and adenosine monophosphate (AMP), act on purinergic receptors on the

myocardial capillary endothelium. Via gap junctions this signal is conducted in a retrograde manner to the arteriolar endothelial cells where nitric oxide (NO) is generated via nitric oxide synthetase (NOS) and thus smooth muscle mediated dilation → reduced CVR→ increased coronary blood flow occurs. [39] Extensive evidence supports the release of ATP from erythrocytes in response to reduced oxygen tension. [40-42] Further research has suggested a defined second messenger signaling cascade controlling this mechanism via G-protein – cAMP – Protein kinase A (PKA) pathway that so far has stopped short of identifying a transmembrane protein that releases ATP. [39]. Beyond ATP release by RBC the interaction of ATP - and its breakdown products ADP and AMP – with capillary endothelial purinergic receptors has principally been studied in rodent cerebral microcirculation in which ATP (and to a lesser extent ADP) is a ligand to $P2Y_1$ receptor on the capillary endothelium causing dose dependent vasodilation. In a dose dependent response AMP is also a ligand to P_1 receptors resulting in vasodilation. [43,44] The steps involved in gap junction conduction (presumably hyperpolarizing factor), [45] and the link to arteriolar endothelial NOS stimulation and NO release are hypothesized steps only. [39]

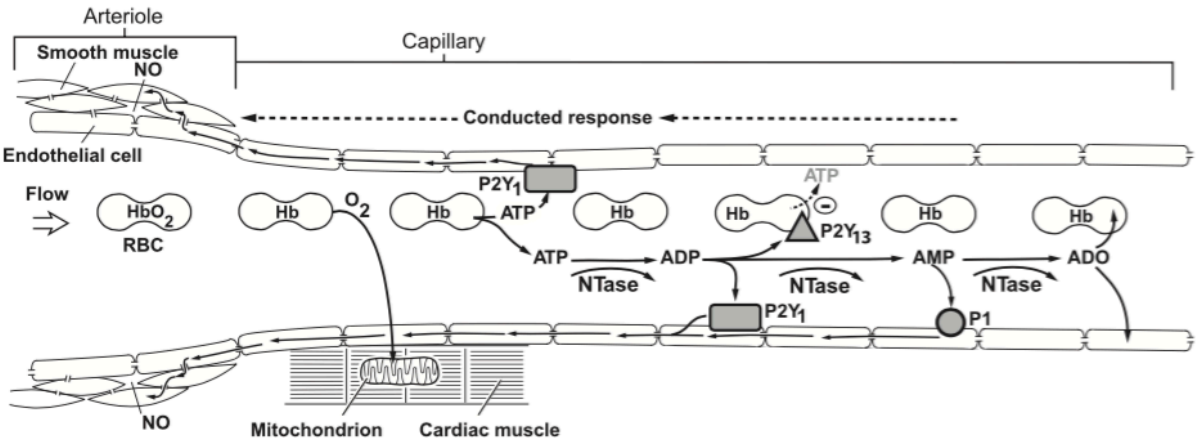

Figure 3: Adenine nucleotide hypothesis of coronary vascular resistance control

In myocardial capillaries ATP is released from RBC in areas of high oxygen demand (low PO_2 tension), this is facilitated by deoxy-haemoglobin. ATP (and its breakdown product ADP) stimulate endothelial $P2Y_1$ receptors and AMP also acts via P_1 receptor to generate a retrograde signal via gap junctions resulting in NO release from the arteriolar endothelium and smooth muscle vasodilation. Abbreviations ATP: Adenosine triphosphate, ADP: Adenosine monophosphate, AMP: adenosine monophosphate, HbO_2 oxy-haemoglobin, Hb: desoxy-haemoglobin, NO: nitric oxide. With permission [46]

Gorman et al tested the adenine / purinergic model in a canine closed chest coronary perfusion model. [46] Using $P2Y_1$, P_1, and NOS inhibitors they found a stepwise depression of the balance between oxygen delivery and myocardial oxygen consumption, lower oxygen tensions were required to create equivalent vasodilation as seen in baseline / control conditions.

The adenine nucleotide / purinergic model hypothesizes an alternate explanation for retrograde conduction of metabolic signals back to the resistance arterioles, and thus a mechanism by which CBF regulation can occur in a feedback system. In this model we also have a link between oxygen tension and the regulation of NOS as well as the standard shear stress model of NOS upregulation. [47] However, in terms of basic mechanistic science we are some way from joining the dots in this system, even the basics of ATP release from RBC, transduction cascade across the

capillary epithelium, and gap junction retrograde conduction are some way from being fully understood or proven. Whilst Gorman et al went some way in highlighting the role of ATP in a canine model again this is just an indication of mechanism and does not prove a causative link, particularly when trying to pick apart the interaction between purinergic receptor signaling and NOS stimulation. No experiment was performed with NOS inhibition blocking purinergic receptor stimulation.

Reactive oxygen species and hydrogen peroxide

A vasodilatory signal through reactive oxygen species (ROS), and - in particular - hydrogen peroxide (H_2O_2), has long been proposed as a feed-forward mechanism of CBF. Increased myocyte contraction would lead to greater mitochondrial electron transport train (ETC) activity and resultant production of superoxide radical ($\bullet O_2^-$) and conversion to H_2O_2 by superoxide dismutase group of enzymes (SOD). H_2O_2 itself was identified as an endothelium derived hyperpolarising factor in an ex-vivo model using human coronary arterioles. [48] H_2O_2 is capable of diffusion into smooth muscle in coronary resistance vessels leading to vasodilation and directly linking metabolic rate to oxygen delivery (CBF). Yada et al [49] demonstrated that H_2O_2 in cooperation with NO and adenosine played an important role in CBF autoregulation in the context of reducing coronary perfusion pressure. These elegant in-vivo canine experiments assessed the Acetylcholine induced vasodilation of coronary arterioles in controls, NOS inhibition with N^G-Monomethyl-L-Arginine (L-NMMA) and finally with NOS inhibition plus catalase (selectively dismutases H_2O_2) all receiving ibuprofen to

inhibit cyclooxygenase. NOS inhibition significantly attenuated vasodilation of large arterioles (≥100μm), with further attenuation once catalase was added. Medium and small arterioles (<100μm) were relatively resistant to NOS inhibition but markedly attenuated by catalase.

α and β mediated CVR control

Whilst sympathetically mediated α and β mediated coronary blood flow changes are not strictly metabolic the activation of the sympathetic system increases heart rate and contractility resulting in increased (metabolic) CBF. Whilst the direct dilating effect β adrenoreceptor activation can be seen on isolated microvessel [50,51] it is much more difficult to understand the role in vivo during exercise or ischaemia. This is because the large effect on local metabolic increases in CBF makes studying the direct affect β and certainly α adrenoreceptor activation on CBF challenging. Duncker et al [52] using a porcine treadmill model attempted to demonstrate the impact of β adrenoreceptor activation in a complex series of experiments that used the alternate blockade of α and β adrenoreceptors. Later Gorman et al [53] repeated the experiments in dogs with the addition of compensation for the enhanced α activity when the β receptors are blocked, finding that β-adrenoreceptor stimulation by norepinephrine can account for up to 25% of the reduction in CVR during exercise.

α-adrenoreceptor stimulation occurs simultaneously during sympathetic stimulation and vasoconstricts vessels >100 μm in size, [54] whilst these two actions seem at odds its clear that constriction of these up stream larger vessels together

with dilation by β-adrenoreceptor of the <100 μm vessels will result in an overall decrease in CVR and an increase in CBF.

The function of α and β adrenoreceptor during exercise of the healthy heart is well described. Much less data exists in humans with coronary atherosclerosis, Hansen et al [6] demonstrated that the myocardial upregulation of noradrenaline release was preserved (aortic to coronary sinus) during exercise in patients with significant stable coronary atherosclerosis.

Localised metabolic regulation of CVR, Adenosine

Proposed independently by Berne et al [55] and Gerlach et al [56] as a molecule that is released by cardiomyocytes during times of increased oxygen consumption and thus reduced myocardial oxygen tension. This mechanism would allow for the reduction in CVR and the increase in CBF allowing myocardial oxygen to remain within a physiological range. [57]Later experimentation led to a confusing set of studies supporting [58,59] and refuting [60,61] the hypothesis. One fundamental observation indicated that adenosine might not be central to the maintenance of myocardial oxygen tension; blockade of endogenous adenosine receptors did not decrease coronary flow during increased oxygen consumption. [62,63]This led Tune et al [60] to carefully measure coronary sinus adenosine and derive interstitial adenosine before and after adenosine receptor blockade, confirming that adenosine concentration changed little with exercise and remained under the threshold for causing localised metabolic vasodilation both with and without adenosine receptor blockade.

Adenosine, localised metabolic regulation of CVR during ischaemia

Whilst there is little contribution of adenosine in physiological regulation of CBF, during ischaemia the adenosine question resurfaces.[64] If changes in CVR and thus CBF are enough to compensate for epicardial coronary disease without ischaemia adenosine release is not augmented. However, when myocytes become ischaemic they will release adenosine in an attempt to augment CBF and oxygen delivery.

Adenosine has also been implicated in a number of other protective mechanisms including the down regulation of myocardial contractility,[65,66] myocardial pre and per-conditioning ,[67,68] inhibition of platelet aggregation [69,70] as well as a myriad of other proposed functions. However, the positive impact of adenosine within a clinical setting has been as unclear as its proposed ubiquitous function shortly after its discovery

Other potential localised metabolic regulators of CVR

Beyond the proposed headlining metabolic mechanisms of CVR regulation there are a long list of molecules and mechanisms all of which have their own body of publications behind them. Prostaglandins, [71,72] K_{ATP} channels,[73,74] endothelium derived hyperpolarising factor (EDHF), [59,75] to name but a few. All of these mechanisms have considerably less evidence (especially in-vivo) to support a significant role in CBF regulation. The principal theme around this thesis is NO and nitrite and for that reason I have chosen not to emphasise every possible mechanism of CBF regulation regardless of importance.

Non-metabolic mediators of CVR

Vessel wall stress or myogenic control

Myogenic vasoconstriction is an increase in vasomotor tone in response to an increase in arterial wall stress, which occurs independently of neural or humoral mechanisms. In healthy arterioles myogenic control plays an important role in regulation of a range of vascular beds including skeletal muscle, brain, renal and splanchnic. [76]

This response also plays its role in pathology such as hypertension were it reduces tissue perfusion[77] or in diabetes were the response is impaired resulting in vasculopathy at a microvascular level. [78]

Miller et al [79] demonstrated myogenic constriction of human coronary arterioles ranging from 44 to 227 μm with a graded response to pressure that constricted vessels up to 55 ± 4%. The confounding influence of shear stress was removed by setting up a constant pressure no flow system. Basal tone was lower in the presence of diltiazem, which inhibits the voltage dependent calcium channels (VDCC). Inhibition of Protein Kinase C also reduced basal tone and its activation increased basal tone.

Myogenic control was found to be independent of endothelial function by Kuo et al[80] in porcine arterioles around 100 μm in diameter. He again visualised arteriole tone both with and without endothelium at a range of perfusion pressures and

found no effect on myogenic smooth muscle tone when endothelium was removed.

Modulation of CVR by shear stress

The localised and upstream control of coronary resistance vessel tone by shear forces or "flow induced" dilation was first shown by Kuo et al. [81] His ex-vivo experiments using porcine arterioles demonstrated that this shear-induced dilation was endothelial dependent and mediated by NO, as well as being inhibited by NO synthase inhibitors. Given our previous description of adrenergic / purinergic receptor mediated control this section on shear stress and NO could equally sit in the metabolic section of this introduction.

Endothelial dependent production of nitric oxide is via the enzyme NOS using L-arginine as a substrate, there are a variety of analogues of L-arginine that inhibit NOS and are used extensively to understand the role of NO in a variety of systems.

In vitro work identifies NO as being central to a number of mechanisms controlling CVR. In dog coronary resistance vessels, reductions of CVR with administration of acetylcholine are blocked with the addition of the l-arginine analog (and inhibitor of NOS) LNMMA. [82] In isolated porcine coronary resistance vessels, blockade of NOS inhibited bradykinin induced relaxation. [80]

In vivo N^{ω} –nitro-L-arginine (L-NNA) another inhibitor of NOS was found to have no effect on basal CVR in conscious dogs but significantly attenuated the increased flow seen with the administration of both acetylcholine and adenosine. [83] Parent et al also demonstrated that inhibition of NOS also partially blocked β-adrenergic induced reductions in CVR using the same model. [84] Reactive hyperaemic flow after brief coronary occlusion (10 to 60 seconds) conceptually has a number of possible mechanisms including adenosine mediated, myogenic tone and potentially NO. Yamabe et al [85] in an anaesthetised dog model used both the NOS inhibitor L-NMMA and 8-phenyltheophylline (8-PT) a selective adenosine antagonist. Measurements were taken in terms of peak flow velocity and repayment of flow debt (%) following 10, 20 and 60 seconds of LCx occlusion. The group showed equivalent roles for inhibition of NOS and adenosine antagonism in terms of % change in flow repayment and an additive function when both L-NMMA and 8-PT were used. In part these finding were confirmed by Kostic et al [86] however they also found significant reduction in baseline (as well as hyperaemic) blood flow with NOS inhibition.

To add to the somewhat conflicting findings Smith et al [87] in a conscious dog model showed no effect of NOS inhibition on basal CBF or throughout the autoregulatory range, however, at pressures below the autoregulatory range NO did have an influence on trying to maintain CBF during what effectively amounts to ischaemia.

Others have studied in patients with stable coronary angina and those undergoing coronary artery bypass surgery (CABG), the predominant influence on flow-

induced dilation was found to be EDHF, rather than NO. [88] The mechanisms of flow-mediated response also seem to be a function of vessel size. For example Dube et al found that EDHF was more influential in porcine epicardial vessels and Kuo et al found NO being dominant in the smaller arteriole vessels. [89,90]

In a series of elegant experiments using a beating heart canine model Stepp et al [91] showed that NO limits coronary microvascular constriction. Stepp et al also demonstrated that this effect was more pronounced in smaller vessels >120 μm where shear stress was increased. In order to reveal the importance of flow (shear stress) in the NO-dependent dilation of coronary microvessels the experiments were repeated in the absence of flow, here inhibition of NO synthase activity by L-NMMA had no effect on the constriction induced by endothelin.

A multitude of both in vitro and in vivo experiments make up a substantial body of work relating to the role of NO in control of CVR and thus CBF. Despite this body of work a clear idea of the influence of NO in CVR and CBF has not been formulated. Partly this is secondary to the number of different experimental models in different disease states and using different techniques to assess the influence of blocking NO synthesis on CVR.

The role of NO in human regulation of CVR in health and disease

In vivo studies in both healthy humans subjects and subjects with risk factors for cardiac disease seem to indicate a role of NO in CBF regulation. Quyyumi et al [92]

managed to elucidate some of the importance of NO by showing that the reduction in coronary vascular resistance in response to rapid cardiac pacing was inhibited in healthy subjects administered NO synthase inhibitors L-NMMA. Quyyumi made in-vivo measurements of CVR using invasive doppler flow wire measures within the coronary artery. Further to this in patients with increased cardiac risk factors LNMMA had a negligible effect on increasing CVR during the same pacing protocol suggesting these patients had alternate mechanisms in the presence of likely endothelial dysfunction.

Quyyumi et al[93] went on to describe the role of NO in a cohort of patients with stable angina and compared them to a healthy population without epicardial coronary disease or risk factors for coronary disease. Quyyumi used the same measures of CVR and again found a differential effect of L-NMMA on these two populations. With L-NMMA healthy patients had a 38% ± 9 (mean ± SEM) increase in CVR and a 15 ± 2% reduction in epicardial lumen whereas patients with coronary risk factors had a 13 ± 4% increase in CVR and a 4 ± 1% reduction in lumen diameter. Inhibition of nitric oxide synthase also reduced the vasodilatory effect of acetylcholine in both groups but the effect in the atherosclerotic group was much smaller. The reduction in CVR and the increase in vessel diameter was similar in both groups following an infusion of sodium nitroprusside. These finding suggest that NO has a role in defining CVR in healthy humans and that this role is reduced in patients with cardiovascular risk factors and overt coronary atherosclerosis (chronic or stable coronary disease). This is presumed to be

secondary to endothelial dysfunction, which is expressed as dysfunctional or reduced function of NO synthase within the coronary vasculature.

Does nitrite have a role?

Whilst the inhibition of endothelial dependent NO synthesis has been shown to increase resistance vessel tone in these ischaemic models and also in healthy humans at rest and during pacing, [94-96] it has been more difficult to directly measure net increases in metabolites of NO (Serum Nitrite, Total Red Blood Cell (RBC) NOx) using sensitive methods in similar human models. [97] Part of the explanation for this absence of a net rise in direct measures of NO metabolites may be the role of serum Nitrite (NO_2-).

NO_2^- is the second most abundant metabolite of NO in blood after nitrate (NO_3^-). Using a human forearm model Gladwin et al [98] found a negative gradient of plasma $NO_2^-$from artery (540 ± 74nM) to vein (466 ± 79nM). The negative gradient of NO_2^- correlated with a positive gradient of HbNO observed by Jia et al (536 ± 99nM to 894 ± 126nM, artery to vein).[99] Gladwin also reported that the negative A to V gradient of plasma NO_2^- was enhanced by maximal exercise and L-NMMA; which both act to decrease O_2 levels. These observations clearly support NO_2^- acting as a potential store of NO that could be released and targeted to hypoxic tissues. Although no mechanism was investigated in this observational study, it was proposed that NO_2^- was being reduced to NO across the vascular bed and as a result contributing to arterial tone.

Doyle et al [100] described the kinetics of NO_2^- and deoxy-Hb in 1981, broadly speaking these describe the production of NO suggested by Gladwin.

$NO_2^- + H^+ + HbFe^{2+}(deoxy) \rightarrow HbFe^{3+} + OH^- + NO \rightarrow HbFe^{2+}\text{-}NO$

Cosby et al [101] demonstrated that isolated aortic rings were more sensitive to the vasodilator effect of NO_2^- - in the presence of erythrocytes - when O_2 levels were reduced. In the same paper it was reported that the reduction of NO_2^- began to occur at an O_2 tension of 50mmHg, which equates to a Hb saturation of approximately 50%.

Following these observations Gladwin proposed that the reduction of NO_2^- to NO occurs in hypoxic tissue and the reductase for this process is Deoxy-Hb occurring maximally at 50% O_2 saturations (Figure 4: Deoxy-haemoglobin nitrite reductase hypothesis). Within this hypothesis is a potential mechanism for maintenance of normal vessel tone and the phenomenon of hypoxic vasodilation. [102]

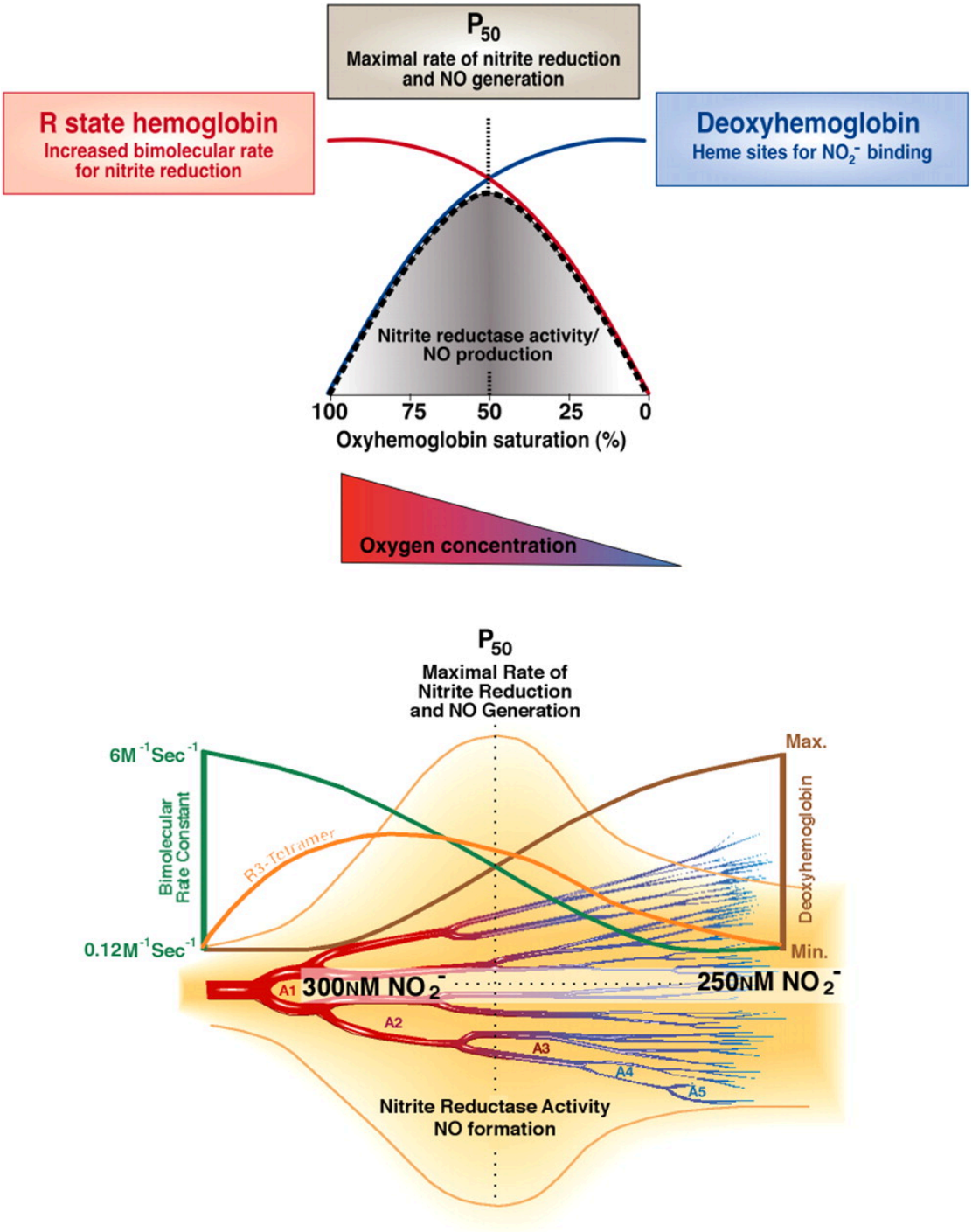

Figure 4: Deoxy-haemoglobin nitrite reductase hypothesis

Nitrite reductase equilibrium along the A1 to A5 arterioles. The majority of resistance is generated at A1, A2 and A3. Oxygen extraction may be higher at these sites as described by Tsai et al. [30]Gladwin et al suggest as heme moves from R state to deoxyhaem more haem sites become available for NO_{2^-} and maximal reductase activity with the formation of NO. The theoretical peak of this process occurs at 60-40% oxygen saturation, making the myocardial circulation the perfect environment to investigate this process. Reproduced with permission [102]

Whilst it's a very appealing mechanism, the deoxy-Hb hypothesis is not entirely without its limitations and potential pitfalls. The principal criticism is the lack of an explanation as to how the produced NO extracts itself from Hb (whose avidity will increase the lower the O_2), and further how it leaves the erythrocyte. [103] Rather than being a direct source of NO there is some evidence that the reaction of Nitrite with deoxy-Hb is the first step in the production of S-nitrosohaemoglobin (SNOHb), which is liberated once oxygen returns. [104] Alternatively there is the concept of an intermediate molecule that may bridge the liberation gap, however, substantial evidence of this is lacking. [105]

When total NO metabolites are measured from aorta to coronary sinus Rogers et al [97] found no net change of total NO metabolites indicating that there was neither production of NO by NOS or consumption of nitrite once all components were taken into account. It should be noted this was in healthy human subjects with no ischaemic heart disease.

There are also other potential mechanisms for the reduction of nitrite to NO. It has been noted that in an isolated vessel model, independent of haemoglobin, NO_2^- action on vasodilation is enhanced by hypoxia. [106] Xanthine oxidase is one of several enzymes present in vessel walls that have the capacity to reduce NO_2^- to NO more efficiently in the presence of hypoxia. [107]Beyond enzymatic reduction, simple acid disproportionation of NO_2^- to NO can occur; obviously this would require overt ischaemia rather than hypoxia. [108]

Thesis aims

Principally this thesis investigates the role of both endogenous NO and NO_2- in the regulation of CVR of patients with both acutely decompensated ischaemia, non-ST elevation myocardial infarction (NSTEMI) and chronic stable ischaemia (stable angina). There are a number of factors within this body of work that make it novel. NSTEMI and stable angina collectively represent a disease cohort that is responsible for a majority of the cardiovascular morbidity and mortality throughout the western world. This work aims to elucidate basic principal mechanisms of CVR regulation within these two disease cohorts (with emphasis on NSTEMI).

1. To examine the influence of NO and NO_2- within the compensatory mechanisms of CVR control in the ischaemic myocardium (NSTEMI and stable angina).
2. Following reperfusion and relief of myocardial ischaemia to understand how, and if, these mechanisms acutely adapt in response to the increase distal coronary pressure and increased flow in the absence of an epicardial stenosis.
3. In patients with acute NSTEMI does augmentation of NO_2- concentration influence other important factors in this cohort. Namely, platelet function and inflammatory/anti-inflammatory cytokines. Is there any correlation between platelet function and these cytokines.
4. In patients with acute NSTEMI does exogenous augmentation of serum NO_2- concentration have any influence on CVR either prior to or following revascularisation with coronary intervention.

Chapter 2: General methods

Methods structure

This general methods chapter is supplemented by chapter specific methods in each of the three results chapters. To reduce repetition the general methods section highlights those methods which are common to more than one of the following results chapters. Where a specific method is found in one of the three supplementary methods sections (and not here) there is a cross reference with a short description and page number to the location of the full method description.

Study populations

NSTEMI, NSTEMI$_{inf}$ and Stable angina

Two populations of patients were studied; NSTEMI and stable angina. The NSTEMI patients were principally recruited at University Hospital of Wales (UHW) whilst stable angina patients were recruited at both UHW and Papworth hospital (PH). The patients recruited at PH were done so under the auspices of another clinical project that was designed with identical cardiac catheter protocols (Principal Investigator Stephen Hoole). UHW patients were recruited and consented by the local team and cathlab protocols and percutaneous coronary intervention (PCI) also carried out at UHW. Sample preparation and analysis was completed at Cardiff University. For Papworth patients recruitment, consent, PCI and cathlab protocols were carried out by the team at Papworth Hospital, analysis of results was carried out in Cardiff University using identical techniques. Sample

preparation and storage conditions were identical between the two centres, storage times were similar as the samples were analysed in batches. The local research ethics committee approved the study in each centre and written informed consent was obtained from each patient.

Inclusion criteria

- Any patient over the age of 18 with the capacity to consent for study and who was receiving coronary angiography as a normal investigation and as part of their routine treatment for either stable angina or NSTEMI
- Normal left ventricular function ejection fraction (EF) (EF≥50%), assessed by echocardiography
- Lack of contraindication to the use of dual antiplatelet therapy (aspirin and clopidogrel).
- Patients that had one vessel PCI following coronary angiography with lesions that were suitable to allow fractional flow reserve and coronary flow reserve measurements.
- Patients with a successfully cannulated coronary sinus

Excluded criteria included:

- Patients with ongoing and unstable ischaemic symptoms
- Previous ST Elevation Myocardial Infarction (STEMI)
- Significant left main disease
- Patients with chronic total occlusions, index or otherwise
- Patients with earlier coronary artery bypass grafts

- Significant valvular disease
- Symptoms of heart failure
- Recent intravenous nitrate infusion
- Atrial fibrillation

Patients all had single vessel PCI of the culprit vessel only after a period of medical treatment. The culprit vessel was selected based on angiographic appearance, ECG changes and in the stable cohort in conjunction with non-invasive testing when indicated.

Platelet sub-study

We conducted a prospective observational study on high-risk NSTEMI patients who received clopidogrel at diagnosis and for a minimum period of 5 days before PCI was conducted.

Inclusion criteria

- Patients with an index admission following an NSTEMI (troponin positive and / or typical ECG changes with consistent clinical history), and a
- GRACE score >140 who subsequently went on to have significant disease treated by PCI and stent implantation.

Exclusion criteria

- Patient who received intravenous antiplatelet agents: glycoprotein IIb/IIIa inhibitors (GPI) or other) and
- Renal dysfunction (eGFR <40mls/min).

Local research ethics approval was granted and written informed consent was obtained from each patient.

Cardiac catheterisation laboratory protocol

Diagnostic angiography and coronary sinus cannulation

Patients received 2500 units heparin prior to a diagnostic angiogram. The default route for coronary angiography was via the radial artery with the femoral artery being used when a radial procedure was not possible or the operators preference. A standard diagnostic angiogram was performed and the index lesion was located.

If PCI was indicated and the patient met the inclusion criteria the venous access (for coronary sinus sampling) was prepared utilizing an earlier aseptically placed venflon (VENFLON™, Becton Dickinson, New Jersey, USA) to guide a radial introducer wire and exchanged with a six french radial sheath (Arrow® Teleflex medical), into either the basilica or cephalic vein of the antecubital fossa. Following a further dose of heparin was administered to a total of 70 to 100 IU / Kg.

An appropriate 6 French guiding catheter was then inserted into the relevant coronary ostium. A 5 French multipurpose MPA-2 (Cordis, Florida, USA) catheter was also placed into the coronary sinus (CS). Deep engagement into the CS was confirmed by injection of contrast. The MPA-2 catheter has side holes which are critical to allowing smooth low vacuum extraction of blood from the CS without this structure occluding the tip of the catheter.

Baseline (pre-PCI) blood sampling

Baseline blood sampling was taken from central venous, coronary sinus and proximal coronary artery together with a coronary artery and coronary sinus blood gas measurement. These samples were taken using a s 20ml syringe at low pressure. Samples were immediately transferred into vacuum released vacutainers (Becton Dickinson, New Jersey, USA). K2 EDTA for whole blood flow cytometry, Citrate for serum collection. For whole blood platelet aggregation samples were collected in Hirudin double walled tubes (Diapharma, Ohio, USA).

Baseline (pre-PCI) coronary physiology measurements

A Certus coronary temperature and pressure sensing wire (St Jude Medical, Minnesota, USA) was equalised to the guiding catheter proximal coronary pressure (P_a), the wire was then placed distal to the epicardial stenosis into the distal third of the vessel.

The index of microvascular resistance (IMR) was measured at rest (IMR_{rest}) and during hyperaemia (IMR_{hyp}). Microvascular resistance was measured using the techniques described by Fearon *et al*. [109] 3ml injections of room temperature saline were injected down the coronary artery via the guiding catheter to produce three consistent thermodilution curves. The average of these three curves was taken as a mean baseline transit time ($T_{m\ rest}$), shown to have a very close correlation with the inverse of absolute coronary flow. [110] The calculation of IMR_{rest} using the transit time at rest ($T_{m\ rest}$) reflects resting tone in the coronary microcirculation.[111] Hyperaemia was then induced with administration of intravenous adenosine (140 μg/kg/min) via the side arm of the 6 French venous sheath in the antecubital fossa. Hyperaemia was defined as the point after two minutes of intravenous adenosine infusion via the large bore sheath placed in the antecubital fosa. Clinical effect of adenosine was seen very quickly when delivered through this large bore venous route. The saline injections were then repeated and the hyperaemic transit time ($T_{m\ hyp}$) was derived from these thermodilution curves.

PCI was then performed as per the operator's preference with no limitations dictated by the study protocol. Prior to the first balloon or stent deployment hyperaemia was induced again and the distal coronary pressure measured once the vessel was occluded (Coronary wedge pressure, P_w).

Peri and Post PCI blood sampling

Immediately prior to deployment of the coronary stent a further coronary sinus blood sample was taken to confirm the concentrations of Nitric Oxide (NO) metabolites at the point just prior to revascularisation. Following successful PCI blood sampling was taken from central venous, coronary sinus and proximal coronary artery together with a coronary artery and coronary sinus blood gas measurement. These samples were taken using a 20ml syringe at low pressure. Samples were immediately transferred into vacuum released vacutainers (Becton Dickinson, New Jersey, USA). K2 EDTA for whole blood flow cytometry and nitric oxide metabolite analysis, Citrate for serum collection. For whole blood platelet aggregation samples were collected in Hirudin double walled tubes (Diapharma, Ohio, USA).

Post-PCI coronary physiology measurements

IMR at rest (IMR_{rest}) and IMR with hyperaemia (IMR_{hyp}) microvascular resistance was re-measured in an identical way to the baseline measurements, these post-PCI measurements were carried out at least 5 minutes after stent deployment.

Offline analysis of catheter lab haemodynamic data

IMR hyperaemic calculation

In the presence of significant epicardial stenosis IMR_{hyp} was calculated with the incorporation of both coronary sinus venous pressure (P_v) and coronary wedge pressure (P_w), using the equation below, described and validated here. [109,112,113] The IMR_{hyp} reflects how well preserved the microcirculation is and its maximal capacity of vasodilation. A number of factors can influence hyperaemic IMR including severe left ventricular hypertrophy (LVH) and myocardial infarction. [114-116]

$$IMR_{hyp} = (P_{a\ hyp}-P_v) \times T_{m\ hyp} (P_{d\ hyp} - P_w) / (P_{a\ hyp} - P_w)$$

P_a is the proximal aortic pressure; P_d is the distal coronary pressure both at hyperaemia.

IMR resting calculation

The calculation of IMR resting (IMR_{rest}) is identical to IMR $_{hyp}$ (minimal microvascular resistance), the only difference is the lack of an adenosine infusion to induce hyperaemia and uncouple autoregulation. This calculation reveals microvascular tone at rest (a surrogate of CVR), allowing us to relate CVR simultaneously to Ao and CS sampling and NO metabolite measures. Principally

IMR_{rest} gives the investigator an impression of perfusion at the time of blood sampling when any auto-regulatory mechanisms are in place. [111]

$$IMR_{rest} = (P_{a\ rest} - P_v) \times T_{m\ rest}(P_{d\ rest} - P_w) / (P_{a\ rest} - P_w)$$

Fractional Flow Reserve (FFR) method and calculation

FFR was measured simultaneously at the point of IMR_{hyp} measurement (pre and post PCI). The distal pressure sensor was placed distal to the epicardial stenosis. Pressure proximal to the stenosis was measured from the coronary guide catheter and distal to the epicardial stenosis via the distal pressure sensor of the coronary temperature and pressure sensing guide wire (St Jude Medical Certus wire 0.014). The measurement is taken at the hyperaemia phase of the protocol during measurement of IMR_{hyp}. FFR is a highly validated method of assessing if epicardial coronary lesions are flow limiting using comparisons to both surrogate and clinical end-points. [117,118]

Collateral Flow Index

Collateral Flow Index (CFI) was calculated via

$$CFI = (P_w - P_v)/(P_a - P_v)$$

Sodium nitrite infusion

In the NSTEMI cohort receiving pre-PCI loading with sodium nitrite ($NSTEMI_{inf}$), (page 102) the sodium nitrite infusion was started immediately after the first coronary sinus sample was taken pre-PCI. The sodium nitrite was supplied by

Tayside Pharmaceuticals, Ninewells Hospital and infusions prepared on the day of recruitment by St Mary's Pharaceutical Unit (SMPU), University Hospital of Wales. Final concentration of sodium nitrite was confirmed at SMPU using Griess Reagent Kit (Thermo Fischer Scientific). The infusion contained a total dose of 6.9mg (99 µmol) of sodium nitrite, infused over 30 minutes (3.3µmol/min). Following 5 minutes of this infusion an additional coronary sinus blood sample was taken to confirm the nitrite concentration at the point of the pre-PCI coronary physiology measures.

Measurement of Nitric Oxide metabolites

Blood preparation

Blood samples were collected in K2 EDTA vacutainers (Becton Dickinson, New Jersey, USA), and processed in two batches pre-PCI and post-PCI. Each batch was briefly stored on ice prior to centrifugation at 600 g for 10 min at 4°C. All reagents were purchased from Sigma (Poole, UK), unless stated otherwise. The red cell fraction and serum were immediately separated, aliquoted and flash frozen in liquid nitrogen. Samples were stored at -80°C for subsequent analysis. Summary of analysis (Figure 5: NO metabolite analysis overview)

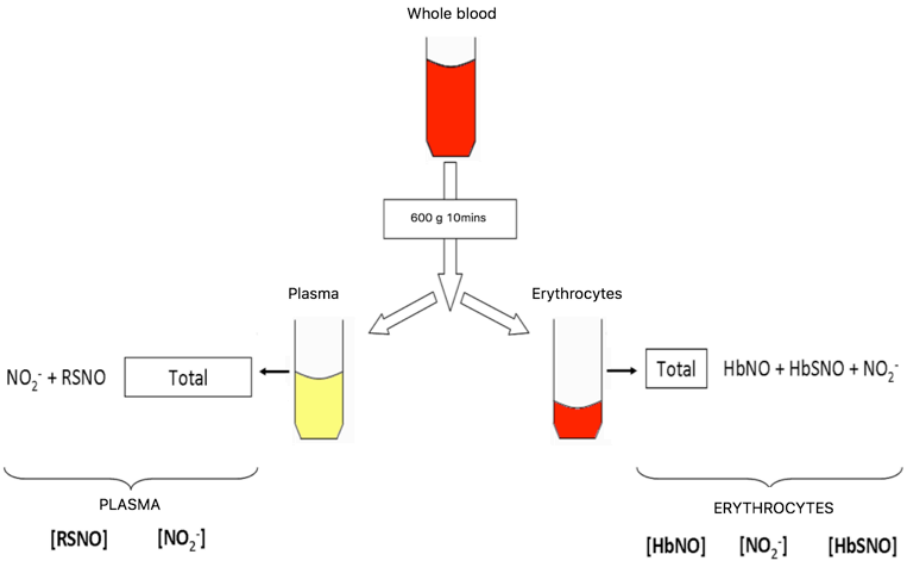

Figure 5: NO metabolite analysis overview

Overview of the analysis of blood samples and total NOx measured in both the plasma and erythrocyte fractions

Ozone-based chemiluminescence

The NO metabolites were assessed using ozone-based chemiluminescence (OBC), an established techniques within the group. [119] OBC detects NO liberated via chemical cleavage. [119] The chemical cleavage for non-nitrate quantification is split into two separate techniques described in more detail below (plasma and erythrocytes). For plasma nitrite analysis, 5ml of tri-iodide (I_3^-) was used, for erythrocytes the addition of potassium hexacyanoferrate ($K_3Fe^{3+}(CN)_6$) was made. Each cleavage reagent was placed in a glass purge vessel and heated at 50°C via a thermostatically controlled water bath. A constant flow of inert gas (O_2-free nitrogen) was bubbled through the reagent to collect the NO liberated. This gaseous mixture was then bubbled through a trap containing 25ml sodium hydroxide (NaOH 1mol/l). The gas was then analysed with an NO analyser (NOA280i, Sievers, Boulder, USA.). Ozone is mixed with the carrier gas and each

molecule of ozone (O_3), reacts with a molecule of NO, liberating a photon. The NOA controls this reaction and quantifies the liberation of photons by converting this signal via a photo-multiplier tube into a potential difference. This potential difference measured in mV is then quantified over time.

Plasma NOx quantification

As indicated above acidified tri-iodide (ATI) was used to quantify plasma metabolites of nitric oxide (NOx). ATI solution was mixed on the day of analysis. The reagent has been previously validated as an efficient reductive compound that provides a reproducible signal for the quantification of blood NO metabolites. [120] The solution was prepared using 70ml of glacial acetic acid mixed with 650mg iodine (I_2) crystals to this solution 1g of potassium iodide (KI), dissolved in 20ml HPLC (high performance liquid chromatography) grade water, was added.

The tri-iodide reagent was then added to the purge vessel (5ml) together with 30µl antifoam. Each sample was prepared using a fresh solution of tri-iodide and each sample was measured in duplicate. Each sample was thawed immediately prior to analysis and 200µl was injected into the side port of the purge vessel, to generate a plasma total signal (comprising NO_2^- and protein bound NO groups). This group is also known as plasma NOx.

Erythrocyte associated NO

The original tri-iodide solution was modified by the addition of potassium hexacyanoferrate ($K_3Fe^{3+}(CN)_6$) immediately prior to sample analysis, (1:10 to tri-iodide). This agent prevents auto-capture of liberated NO by Hb whilst in the assay.[121] 8ml modified tri-iodide reagent, together with 30µl antifoam, was placed into a purge vessel. 200µl of freshly thawed erythrocyte sample was injected into the purge vessel to generate an erythrocyte-total signal: Haemoglobin bound NO (HbNO), S-nitrosohaemoglobin (HbSNO) and erythrocyte-associated NO_2^-).

Plasma NO_3-

Total plasma inorganic nitrate (NO_3^-) was also quantified using OBC, the cleavage reagent used to liberate NO from NO_3^- was acidified vanadium III chloride (VCl_3).[122] When VCl_3 is used at higher temperatures (85°C) it is able to liberate NO from all plasma sources, including NO_3^-. A 50mM reagent solution was prepared by the addition of 0.785g VCl_3 (dissolved in 20ml HPLC grade water) to 80ml 1M HCl acid. 30ml of acidified VCl_3 was added to a larger-sized purge vessel. A Liebig condenser was connected between the purge vessel and the NaOH trap but otherwise the setup was unaltered from that previously stated. 20µl of freshly thawed plasma was injected into the purge vessel to generate a plasma NO_3^- & NOx signal. From this concentration the plasma [NOx] concentration was subtracted to give the plasma [NO_3^-] concentration.

Quantification of data produced by NOA

The real time continuous data produced by the NOA was analysed with O*rigin 7.0* (OriginLab Corps, Massachusetts, USA). This signal (in mV) was plotted vs time (in seconds) to give a raw trace and then a fifty-point adjacent averaging algorithm was used to smooth this trace. The *peak analysis package* for *Origin* was then used to calculate the area under curve (AUC) of each peak (mV.s). Using a standard curve (prepared every day to limit variability) the AUC was converted to a concentration of NO. (Figure 6: Example offline analsis of NO metabolite raw data)

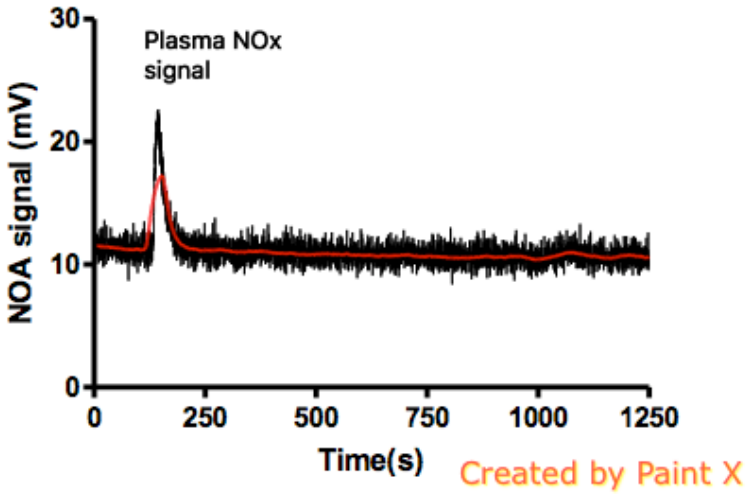

Figure 6: Example offline analsis of NO metabolite raw data

Example of raw data output (black) and smoothed data using Origin 7.0 (red). The first large peak is the signal generated with plasma total injection (plasma NOx), a much smaller second peak is seen (marked S), this is the signal after the addition of sulphanilamide to the sample and represents plasma protein bound NO (no attempt to quantify this signal was made in this thesis).

Calibration curve for quantification of OBC data

A fresh standard curve was produced daily using either $NaNO_2$ or $NaNO_3$ standards. For example, a range of 5 standards solutions of $NaNO_2$ was analysed on the OBC system and plotted (62.5 nM, 125 nM, 250 nM, 500 nM, 1000 nM). A

linear regression analysis of this data provides a multiplication factor by which NOA reading (mV.s) could be converted into a concentration (nM). (Figure 7: Example standard curve used in the quantification of NO metabolite data)

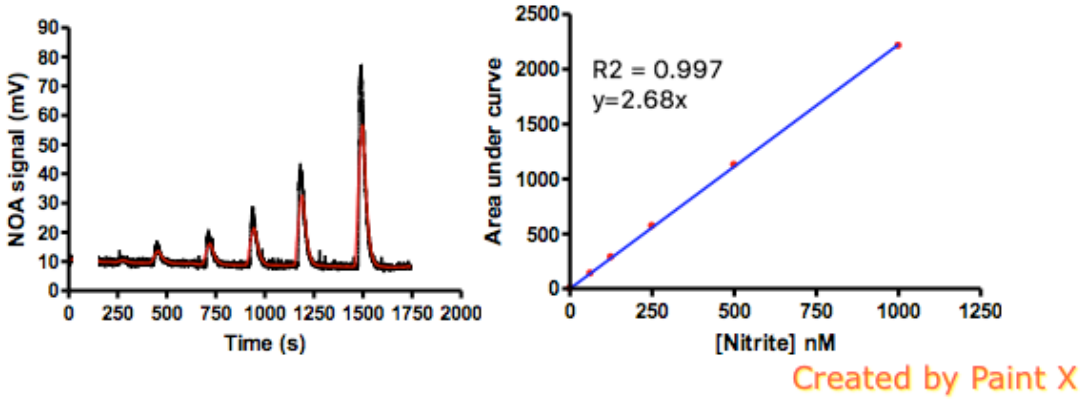

Figure 7: Example standard curve used in the quantification of NO metabolite data

Figure xx: A standard curve of known $NaNO_2$ concentrations (62.5 nM, 125 nM, 250 nM, 500 nM, 1000 nM), left panel, and the linear regression analysis, right panel. In this example the NOA output of experimental samples would be divided by 2.68 to convert into a concentration of NO_2^-.

Within the group NOA is an established and validated method with a limit of sensitivity for plasma nitrite of >10nmol/l and for plasma nitrate of >500nmol/l, the intra-assay coefficients of variation were <5 and <8%, respectively.[123]

Platelet analysis

Chapter 5: Results 3 contains multi-parametric analysis of platelet function and status as well as cytokine quantifcation

Impedance aggregometry

Whole blood impedance aggregometry (multiplate), full details of the chapter specific method can be seen on page 152

Flow cytometry assessment of platelet bound p-selectin

Whole blood flow cytometry with platelet markers and p-selectin markers. Full details of the chapter specific method can be seen on page 154

Soluble p-selectin quantification

Serum quantification of soluble p-selectin, electrochemiluminescence (Meso Scale Discovery). Full details of the chapter specific method can be seen on page 156

Assessment of 12-HETE by lipid extraction and reverse phase LC/MS/MS

Quantification of 12-hydroxyeicosatetraenoic acid (12-HETE) was made via reverse phase liquid chromatography and mass spectrometry analysis. Full details of the chapter specific method can be seen on page 157

Cytokine quantification

Serum quantification of TH1 and TH2 specific cytokines was made via a multispot electrochemiluminescence (Meso Scale Discovery). Full details of the chapter specific method can be seen on page 158

Statistical Analysis

Statistical analysis of data was performed using the software package GraphPad Prism 10.0 (La Jolla, California, USA). The individual tests performed are identified within the method section of each of the three results chapters.

Chapter 3: Results 1

The role of NO in coronary blood flow regulation in the presence of epicardial coronary disease

Abstract

Background

As the extraction of oxygen at rest is almost maximal the heart must increase CBF when metabolic demands on the heart increase (exercise). Perfusion pressure remains constant with equivalent rises in aortic and diastolic backpressure. The only remaining way to increase CBF is to reduce CVR. Nitric oxide - synthesised by both endothelial NOS and the (potential) reduction of its metabolite nitrite - is a potent vascular dilator. Its role in humans in the regulation of CVR is unclear, especially in the presence of epicardial coronary disease. This chapter outlines the influence of NO in humans with both acute epicardial coronary stenosis (NSTEMI) and stable epicardial stenosis.

Methods

Two groups of patients: stable angina (SA) n=35 and NSTEMI n=38. Both were scheduled to have an angiogram +/- PCI. Pre-PCI and post-PCI aortic and coronary sinus blood samples were taken together with measures of CVR (IMR_{rest} and IMR_{hyp}). Following rapid sample preparation both serum and red blood cell NOx were measured using ozone based chemiluminescence.

Results

A net increase in total NO metabolites (NOx) were found from aorta to coronary sinus (A-V) in the NSTEMI cohort pre-PCI (84.9 nM CI 9.5 to 160, p^{ANOVA} <0.05 *) and post-PCI this gradient disappeared. The formation of NO metabolites was principally driven by an increase in RBC NOx from A-V whilst serum nitrite did not change. Post-PCI RBC NOx was similar but nitrite concentration fell from A-V. This flux in NOx was accompanied by a change in IMR_{rest} (CVR) from 45.7 units (SD 21.7) to 57.6 units (SD 37.9), indicating an increase in CVR. Unlike the NSTEMI cohort the stable cohort had a fall in nitrite concentration from A-V both pre (-175nM p<0.05 ****) and post-PCI (-183.8nM p<0.05****). This was accompanied by a change in IMR_{rest} from 38.9 units (SD 18.4) to 38.99 (SD 3.96) indicating no change in CVR. Of note the collateral flow index (an indicator of coronary collateral formation) was significantly higher in the stable cohort.

Conclusions

NO appears to be important in the regulation of CVR in patients with acute epicardial stenosis (NSTEMI). We observe a net production of NO across the myocardial circulation (A-V) in the NSTEMI cohort. Immediately post-PCI we measure an acute drop of NO likely secondary to a the dilutional effect of increased flow and perhaps a reduction in shear force and down regulation of NOS. This drop is associated with an immediate increase in CVR. Net production of NO is not measured in the stable cohort pre+-PCI and thus a drop is not seen immediately post-PCI. We also see no change in CVR post-PCI. Coronary wedge pressure indicates significant collateral formation in the stable cohort perhaps indicating

an alternate chronic compensation mechanism that negates the need for NOS upregulation.

Introduction

Conceptually increased oxygen demand by the heart can be met in three ways: increased oxygen extraction, increased perfusion pressure or a reduction in total coronary vascular resistance. [1]

Even at rest the heart extracts 70 to 80% of the oxygen delivered to it by the coronary circulation, indeed the extraction per gram of tissue is 20 fold higher than skeletal muscle. [1,2] This almost maximal oxygen extraction at rest means there is almost no further increase in arterial-to-venous (A-V) oxygen extraction during light exercise [5] and a tendency towards only slightly increased A-V O_2 extraction during moderate to heavy exercise. [6,7].

The gradient of pressure across the coronary bed (perfusion pressure) can be measured from aortic pressure to diastolic pressure (or backflow pressure), the latter being generated by a combination of heart rate, diastolic and systolic left ventricular compressive forces which are in turn proportional to aortic pressure. During exercise the aortic inflow pressure rises and is matched with an equivalent rise in backflow pressure, therefore perfusion pressure remains constant. [10,11]

Changes in CVR appear to be the principal mechanism by which the heart can regulate CBF and fulfill the demands of increased myocardial oxygen requirements during exercise. [1] Control and adaptation of CVR means that CBF can be maintained for a number of different perfusion pressures or increased when the myocardium requires more oxygen. [11] The control of CVR has many proposed components including (but not limited to) the local concentration of adenosine, [58] NO, [95] and neural regulation. [124] This chapter focuses purely on the influence of endogenous NO production.

The role of both NO and its metabolite NO_2- have been studied in depth in the context of relatively healthy humans and those with overt coronary disease risk factors. Most of the clinical studies involving NO_2- have had an emphasis on augmenting its concentration for biological effect in terms of ischaemia-reperfusion injury and coronary blood flow regulation (covered in Chapter 4: Results 2). This chapter will touch on endogenous NO_2- concentration principally as a means of quantifying NO production but also touching on NO_2- as a bioactive metabolite itself as outlined in the general introduction. However, Chapter 4: Results 2 will cover this in much more detail.

Relevant human models to assess the role of NO in the regulation of CVR in health and disease

Quyyumi et al have described a series of experiments outlining the role of NO in both CVR resting tone as well as its role in decreasing CVR during increased demand (pacing), in healthy subjects. Quyyumi focused his investigation on the effect of blocking NO synthesis using L-NMMA to inhibit NOS and carrying out challenges with acetylcholine and pacing. [95] The same group went on to demonstrate diminishing endothelial function (in terms of NOS activity) in patients with risk factors for coronary heart disease. [92] This work then continued in patients with overt epicardial coronary disease (stable chronic lesions) using a similar model of assessing the effect of L-NMMA on both resting CVR and CVR following acetylcholine stimulation. [93] L-NMMA was found to have a smaller effect in patients with epicardial coronary stenosis at both rest and after stimulation with acetylcholine.

These studies made a valuable contribution to the field, however, there were some issues with patient characteristics that make the findings less clear. The principal issue lies with the lesion characteristics in the group with atherosclerosis; patients were selected if their lesions were less than 40% (to allow for the bulky doppler catheters to make accurate measurements). Significant differences were also present between the groups with and without atherosclerosis; control subjects were more often female (83% vs 25%) and significantly younger (40 ± 8 vs 56 ± 9). In terms of CVR and microvascular reactivity there are significant differences between men and women that make interpretation of these result difficult. [125]

Specifically, women investigated for potential ischaemia (which is true for these control patients), with normal coronary arteries have been shown to have less reactivity to adenosine; upregulation of NOS may occur to compensate for this generalized disruption of control of CVR. So, in reality, this work looked at the impact of gender on CVR control by NO with the added confounder of mild coronary lesions (that we wouldn't typically associate with overt ischaemia by current standards) and cardiovascular risk factors for atherosclerosis.

Minamino et al [126] measured plasma nitrate and nitrite concentrations in the coronary sinus - in patients with and without risk factors for coronary atherosclerosis, but all with normal coronary artery anatomy – at baseline and with an increase in myocardial oxygen consumption induced by rapid pacing. These experiments show that those with risk factors did not have a net increase in NO metabolites (serum nitrite and nitrate) in response to pacing (12.0±0.9 vs. 14.9±1.1 μmol/liter, mean ± SD $p<0.05$); the converse was true for those without risk factors for coronary atherosclerosis (11.1±1.1 vs. 12.2±1.1 μmol/liter). This work indicates a role of NO in the regulation of CVR in a healthy population and less so as cardiovascular risk factors increase. This goes some way in associating risk factors with endothelial dysfunction but does not tell us how this relates to overt epicardial coronary disease or if NO has a central role in compensating for overt flow limiting coronary disease. Again, this piece of work had its limitation, principally the method of NO metabolite quantification. No account is taken of the red cell metabolites of NO and we will read in the next paragraph how potentially in the hypoxic / ischaemic environment complete quantification of both

compartments is central to understanding NO synthesis. In terms of quantification of total serum nitrite + nitrate, the group used the Griess reaction a technique that dates from 1879. The Griess reaction is used in combination with absorbance at 540nm before and after passing the sample through a copper-plated cadmium column. It's μmol sensitivity is not sensitive enough to capture biologically significant nmol changes in NO_2- concentration, and there are also issues with specificity and the interference of other molecules in the plasma. [127]

Rogers et al [97] studied healthy patients - with normal epicardial coronary arteries - undergoing coronary angiogram as a routine investigation in the treatment of palpitations (electrophysiology study); these patients had no symptoms of ischaemia. The group measured both plasma and red blood cell NO metabolites (total NO metabolites) from aortic root to coronary sinus with and without L-NMMA. The group used ozone-based chemiluminescence, a highly sensitive method of quantification that allows ~ 99% recovery of NO added across a physiological and pharmacological range (nM sensitivity). [121] In terms of sensitivity, specificity and measuring both plasma and red cell components of NO metabolites, ozone-based chemiluminescence is far superior to methods based around the Griess reagent outlined above. [128] Rogers found that there was a net loss of NO_2- from aortic root to coronary sinus (366±39nM to 269±48nM mean±SEM $p<0.05$) as reported by Gladwin and others, [98] however this reduction in nitrite was mirrored by a doubling of total RBC HbNO from aorta to coronary sinus (107nM to 189 nM $p<0.05$ see paper for component figures and SEM) as the haem lost its oxygen across the myocardial bed. These observations were reversed

across the pulmonary bed as the NO was replaced by oxygen. Overall this "shuttling" of NO metabolites equated to no net change in total NO metabolites from aorta to coronary sinus (and pulmonary artery to aorta). The addition of L-NMMA increased systemic blood pressure and reduced epicardial coronary diameter but did not have any effect on the distribution or amount of any NO metabolite. This research demonstrated that in healthy patients endothelial NOS is unlikely to have a significant role in controlling CVR. The net component ofNO metabolites did not change from aorta to coronary sinus (just their distribution from serum to RBC as a function of O_2), suggesting that there is no consumption of nitrite that isn't explained by an increase in total RBC NO components that occurs as oxygen saturation falls.

Each of the studies described has its limitations in terms of understanding the role of both NO (and NO_2-) in terms of compensatory control of CVR in patients with myocardial ischaemia. However, each of the studies gives us an insight into how best to approach this question. It is clear that total NOx (in both serum and RBC) needs to be accurately quantified. Ideally this NOx quantification should measure the trans-myocardial gradients from aorta to coronary sinus. Patients selected should have overtly ischaemic coronary territories whose epicardial lesion can be quantified functionally. The addition of stable chronic ischaemia (stable angina) as well as acute decompensated ischaemia (non-ST elevation myocardial infarction, NSTEMI) should be included as separate groups as the mechanisms of compensation may differ. The assessment of CVR would be central to

understanding the role of NO and NO_2- together with the ability to reverse the ischaemic territory.

The core aim of this chapter is to understand the role of NO production in terms of regulation of CVR in patients with significant epicardial coronary disease and how this changes following treatment with PCI. Specifically, we will quantify NO concentration across the myocardial circulation both before and after PCI. Two defined populations will be studied; acute NSTEMI cohort and stable angina cohort both with quantified and ischaemic lesions.

Hypotheses

1/ In the NSTEMI cohort (pre-PCI) it was hypothesised patients will have reduced endothelial function (NOS activity) and be reliant on NO_2- reduction to NO to maintain low CVR. If this hypothesis were true we would expect a negative trans-myocardial (Ao to CS) NO_2- gradient and overall consumption of NO metabolites prior to revascularisation. Critical to this observation is the *total* NO metabolite pool in both plasma and red blood cell (RBC) compartments. In healthy a negative NO_2- gradient is seen but this is balanced by a positive gradient of NOx in the red cell compartment which equates to no net change from Ao to CS.

2/ Post-PCI following revascularisation we would expect a correction *towards* zero in the nitrite gradients reflecting reduced reliance on the NO_2- reduction to NO mechanism in the (now) non-ischaemic and less hypoxic myocardial bed.

3/ The stable angina cohort will have less reliance on nitrite reduction and rely on other chronically upregulated mechanisms of compensation such as endothelial (rather than nitrite) production of NO. This would be reflected in a total NOx positive gradient from aorta to coronary sinus (Serum and RBC, but excluding nitrate).

Methods

Study populations

Two populations of patients were studied; NSTEMI and stable angina. The NSTEMI patients were principally recruited at University Hospital of Wales (UHW) whilst stable angina patients were recruited at both UHW and Papworth hospital (PH). The patients recruited at PH were done so under the auspices of another clinical project that was designed with identical cardiac catheter protocols (Principal Investigator Stephen Hoole). UHW patients were recruited and consented by the local team and cathlab protocols and PCI also carried out at UHW. Sample preparation and analysis was completed at Cardiff University. For Papworth patients recruitment, consent, PCI and cathlab protocols were carried out by the team at Papworth Hospital, analysis of results was carried out in Cardiff University using identical techniques. Sample preparation and storage conditions were identical between the two centres, storage times were similar as the samples were analysed in batches. The local research ethics committee approved the study in each centre and written informed consent was obtained from each patient.

Inclusion criteria

- Any patient over the age of 18 with the capacity to consent for study and who was receiving coronary angiography as a normal investigation and as part of their routine treatment for either stable angina or NSTEMI
- Normal left ventricular function (EF≥50%), assessed by echocardiography
- Lack of contraindication to the use of dual antiplatelet therapy (aspirin and clopidogrel).
- Patients that had one vessel PCI following coronary angiography with lesions that were suitable to allow fractional flow reserve and coronary flow reserve measurements.
- Patients with a successfully cannulated coronary sinus

Excluded criteria included:

- Patients with ongoing and unstable ischaemic symptoms
- Previous STEMI
- Significant left main disease
- Patients with chronic total occlusions, index or otherwise
- Patients with earlier coronary artery bypass grafts
- Significant valvular disease
- Symptoms of heart failure
- Recent intravenous nitrate infusion
- Atrial fibrillation

In the NSTEMI group patients all had single vessel PCI of the culprit vessel only after a period of medical treatment as outlined in Table 1. The culprit vessel was selected on the basis of angiographic appearance, ECG changes and in the stable

cohort in conjunction with non-invasive testing when indicated. Index vessels and other baseline variables were well balanced between groups as per Table 1.

		NSTEMI N=30	Stable angina n=35
Age		63.8 ± 12	64.4 ± 9.6
Male (%)		32 (84)	29 (83)
Creatinine µmol/L		88.5 (71.3 – 99)	91 (81 – 98.3)
CRP		3 (2 – 6.25)	
Left ventricular function		55 (50-55)	52 (50-55)
Index vessel			
	LAD (%)	17 (57)	22 (63)
	LCx (%)	7 (23)	6 (17)
	RCA (%)	6 (20)	7 (20)
Hypertension (%)		24 (80)	20 (58)
Diabetes (%)		10 (33)	8 (23)
Hyperlipaedemia (%)		28 (93)	24 (70)
Smoking (%)		16 (53)	13 (37)
Previous PCI (%)		5 (17)	14 (40)
Clopidogrel > 5days (%)		30 (100)	21 (60)
Beta-blocker (%)		25 (83)	27 (77)
Calcium channel blocker		4 (13)	7 (20)
ACE Inhibitor (%)		22 (73)	21 (60)
Statin (%)		28 (93)	32 (91)

Table 1: Baseline clinical characteristics of NSTEMI patient population

Baseline clinical and laboratory characteristics of study population. Both pre-PCI and 24 hr post-PCI haemoglobin and total platelet count are included with no significant difference seen between either of these values. Abbreviations: PCI - percutaneous coronary intervention, CRP – C reactive protein, NSTEMI – non-ST-elevavation myocardial infarction, EF - ejection fraction, ACE - angiotensin converting enzyme inhibitor, LAD – left anterior descending, LCx – Left circumflex, RCA – right coronary artery

Cardiac catheterisation laboratory protocol

The cardiac catheterisation protocol can be seen in full in the general methods page 55

Measurement of Nitric Oxide metabolites

As described in general methods chapter: page 61

Statistical Analysis

Normally distributed data are expressed as mean ± SD unless otherwise stated. Non-gaussian data expressed as median (IQR). When the distribution of data was not clear the Shapiro-Wilk normality test was used. Multiple comparison between related groups of means were made with paired and unpaired ANOVA with Tukey's correction for multiple comparisons. Single comparisons were made with paired and unpaired student's t test as appropriate. Wilcoxon test was used for non-parametric single comparisons of paired data and Mann-Whitney test for unpaired data.

Results

Table 1 displays the baseline clinical characteristics of the two study groups together with key baseline physiological measures. Numerically baseline clinical characteristics were well matched between groups.

Trans-myocardial NO metabolites and oxygen

NSTEMI and stable cohorts

The trans-myocardial NO metabolite profiles for these two groups are outlined in Table 2 and Figure 8. With respect to the NSTEMI cohort (n=30) there was no

significant gradient of serum nitrite across the myocardium *pre PCI* comparing Ao to CS levels 204-5 nM (134.1 – 328.3) vs 203.4 nM (1245.3 – 293.9), respectively ($p^{ANOVA}>0.05$). Absolute measures of total red blood cell NO metabolites (RBC NOx) were 164.5 ± 99 nM in Ao and 247.2 ± 142.8 nM at the CS ($p^{ANOVA}<0.05$**). As expected we see increased total RBC NOx as the oxygen concentration reduces from Ao to CS. (Figure 2) The total NO species (excluding nitrate) were 403.9 ± 192.3 nM in Ao and 488.7 ± 265.9 nM in CS showing a positive gradient (net production) across the myocardium of 84.9 nM p^{ANOVA} <0.05 * (CI 9.5 to 160) in the presence of a significant epicardial stenosis with a mean FFR of 0.62 ± 0.03. (Table 4)

NSTEMI Pre PCI NOx (n=30)			
	Aorta	**Coronary Sinus**	P^{ANOVA} (CI)
HbO_2 (%)	96.6 ± 1.82	34.8 ± 8.7	-61.8
Serum NO_2^- (nM)	204.5 (134.1 to 328.3)	203.4	P^{ANOVA} >0.05
Total RBC (nM)	164.5 ± 99	247.2 ± 142.8	P^{ANOVA} <0.05* (13.5 to 152) mean diff 82.7nM
Total NOx (nM)	403.9 ± 192.3	488.7 ± 265.9	P^{ANOVA} <0.05 * (9.5 to 160) mean diff 84.9nM

NSTEMI Post PCI NOx (n=30)			
	Aorta	**Coronary Sinus**	P^{ANOVA} (CI)
HbO_2 (%)	96.7 ± 1.85	42.5 ± 12.4	
Serum NO_2^- (nM)	240.9	178.5	P^{ANOVA} >0.05
Total RBC	167.9 (±91.5)	228.1 (±120.9)	P^{ANOVA} >0.05 (-1.9 to 122.3)
Total NO	412.9 ± 170	429.1 ± 185.3	P^{ANOVA} >0.05 (-59 to 91)

Table 2: Trans-myocardial NO metabolites (NOx) in NSTEMI patients

Trans-myocardial nitric oxide metabolite concentration from aorta to coronary sinus Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, PCI - percutaneous coronary intervention, NOx – total nitric oxide metabolites, HbO_2 – haemoglobin oxygen saturation, NO_2 – nitrite, CI – confidence intervals, nM – nanomolar, NO – nitric oxide

Statistics: normally distributed data as mean ± SD, multiple comparisons with 1-way ANOVA. Not normally distributed median (IQR), multiple comparisons Friedman with Dunn's correction (marked ANOVA)

Following coronary intervention (post PCI) this total NO species gradient from Ao to CS reduced from its pre PCI value of 84.9 nM (CI 9.5 to 160) to 16.2 nM (CI -59.1 to 91.6), post PCI. Principally the loss of this Ao to CS gradient post PCI was secondary to the development of a negative gradient of serum nitrite from Ao to CS where pre PCI there had been no gradient i.e. Ao [nitrite] = CS [nitrite]. As expected the RBC NOx values continued to show their reciprocal relationship with oxygen saturation and the gradient from Ao to CS pre PCI was preserved post PCI. (figure 2) The sum of these two NO metabolites was 412.9 ± 170 nM in Ao and 429.1 ± 185.3 nM in CS (again excluding nitrate) giving a non-significant difference of +16.2nM cross heart once the epicardial stenosis had been treated by PCI. The post-PCI FFR was 0.92 ± 0.06 indicating that the epicardial stenosis in this group was adequately treated. (Table 4)

The stable cohort (n=35) underwent exactly the same Aortic and Coronary Sinus sampling pre and post PCI. From the perspective of measured serum nitrite concentrations these patients exhibited results that closely resemble a cohort of healthy patients with no coronary disease measured by our group previously.[97] (Table 1, Figure 8) The serum concentration of nitrite in the aorta of these stable patients pre and post PCI was very similar ($p^{ANOVA}>0.05$), 436nM (132.4) and 489.6 (173.5), both values significantly higher than the matched values in the NSTEMI group. Like the healthy controls measured previously, there was a significant negative gradient across the myocardium -175.5nM $P^{ANOVA}<0.05$ ****. This negative gradient was almost identical post PCI (-183.8nM).

With respect to CS saturations there was a significant increase in oxygen saturation post PCI. Pre to post PCI mean CS saturations were 38.7% (SD 8.4) and 50.0% (SD 12.1), respectively (p=0.0094). (Figure 9)

Stable Angina Pre-PCI NOx (n=35)			
	Aorta	**Coronary Sinus**	Delta P^{ANOVA}
HbO_2 (%)	97.1 ± 1.9	40.4 ± 7.2	-56.7
Serum NO_2^- (nM)	436.6 ± 132.4	261.1 ± 109.1	-175.5 P^{ANOVA} <0.05

Stable Angina Post-PCI NOx (n=35)			
	Aorta	**Coronary Sinus**	Delta P^{ANOVA}
HbO_2 (%)	97.3 ± 2.1	50.4 ± 9.6	-46.9
Serum NO_2^- (nM)	489.6 ± 173.6	305.8 ± 130.9	-183.8 P^{ANOVA} <0.05

Table 3: Trans-myocardial NO metabolites Stable Angina cohort

Trans-myocardial nitric oxide metabolite concentration from aorta to coronary sinus Abbreviations: PCI - percutaneous coronary intervention, NOx – nitric oxide metabolites, HbO_2 – haemoglobin oxygen saturation, NO_2 – nitrite, nM – nanomolar, NO – nitric oxide

Statistics: normally distributed data as mean ± SD

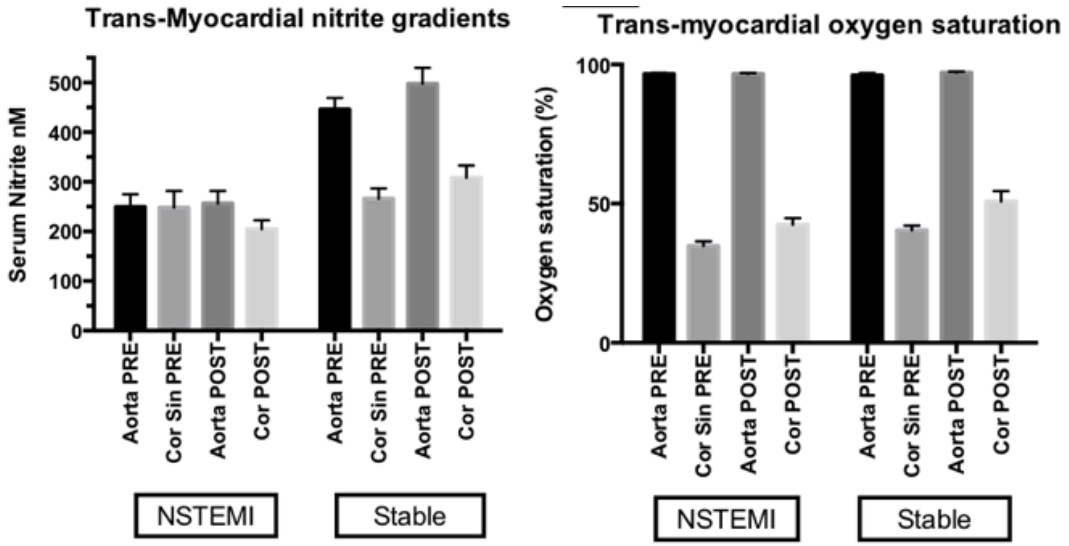

Figure 8: Trans-myocardial NO2- metabolites (NOx) in NSTEMI & Stable angina patients

Figure 9: Trans-myocardial oxygen saturation (%) in NSTEMI & Stable angina patients

Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, PCI - percutaneous coronary intervention, intervals, nM – nanomolar, NO – nitric oxide

Serum nitrate quantification

All three cohorts had nitrate concentration measured at the identical time points to nitrite and total RBC measures. Using a one-way ANOVA test there were no significant differences between time points within each group or across the different groups. Nitrate is present in μM concentrations as opposed to the nM concentration of serum nitrite and RBC NOx. Because nitrate does not change between sampling points - and there is no evidence that humans are able to reduce nitrate to nitrite across the coronary circulation – we exclude this from the calculations of total NO metabolites.

Coronary physiology measurements (CVR)

A summary of all coronary measures is reported in Table 4

In the NSTEMI cohort the mean FFR pre and post PCI was 0.62 (SD 0.18) and 0.91 (SD 0.06) confirming both significant ischaemia in the myocardial territory being studied and adequate restoration of epicardial vessel luminal area following PCI.[129] The stable cohort FFR measures were very similar Pre-PCI 0.69 (SD 0.16) and Post-PCI 0.93 (SD 0.05).

Prior to PCI the IMR_{rest} - a surrogate of CVR - in the NSTEMI cohort pre PCI was 45.7 units (SD 21.7). Immediately post- PCI this increased to 57.6 (SD 37.9), p=0.027 (Figure 3). These values represent a rise in resting microvascular tone immediately post PCI and at the point of aortic and coronary sinus sampling.

	NSTEMI (n=30)			Stable Angina (n=35)		
	Pre-PCI	**Post-PCI**		**Pre-PCI**	**Post-PCI**	
FFR	0.62 ± 0.03	0.92 ± 0.06	p<0.05****	0.73 ± 0.15	0.93 ± 0.06	p<0.05****
IMR_{rest}	46.2 ± 21.6	57.6 ± 37.9	p=0.038	38.9 ± 18.4	38.99 ± 22.1	p=0.97
IMR_{hyp}	22.3 ± 12.4	22.5 ± 19.8	p=0.95	16.1 ± 7.6	14.03 ± 7.1	p=0.16
Collateral Flow Index	0.09 ± 0.08			0.2 ± 0.13		p=0.0001

Table 4: Coronary physiology results summary data

Coronary physiology data pre-PCI and 5 minutes post-PCI

Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, PCI - percutaneous coronary intervention, FFR – Fractional flow reserve, IMR_{rest} – Index of microvascular resistance resting conditions, IMR_{hyp} – Index of microvascular resistance at hyperaemia, CFI – Collateral Flow Index.

Statistics: Normally distributed data presented as mean ± SD with significance calculated with one-way ANOVA with tukey's correction for multiple comparisons or paired t-test were ANOVA not possible. FFR is a ratio of P_d/P_a. IMR_{rest} and IMR_{hyp} are dimensionless.

In the stable cohort IMR_{rest} pre-PCI was 38.9 (SD 18.4) vs. post-PCI 38.99 (SD 3.96), p>0.05. Whilst the pre-PCI values were similar between the two cohorts unlike the NSTEMI cohort the stable cohort did not see a significant rise in IMR_{rest} post-PCI. (Table 4 and Figure 10)

Minimal microvascular tone (IMR_{hyp}) in the NSTEMI cohort pre and post-PCI did not change significantly; 22.3 (SD 12.4) vs 22.5 (SD 19.8) p>0.05. The same was true for the stable cohort; IMR_{hyp} did not change significantly pre to post-PCI; 16.14 (SD 7.55) vs 14.03 (SD 7.11), p=0.16. Overall IMR_{hyp} in the NSTEMI group

was significantly higher than the corresponding measures in the stable cohort as outlined in. (Table 4 and Figure 11)

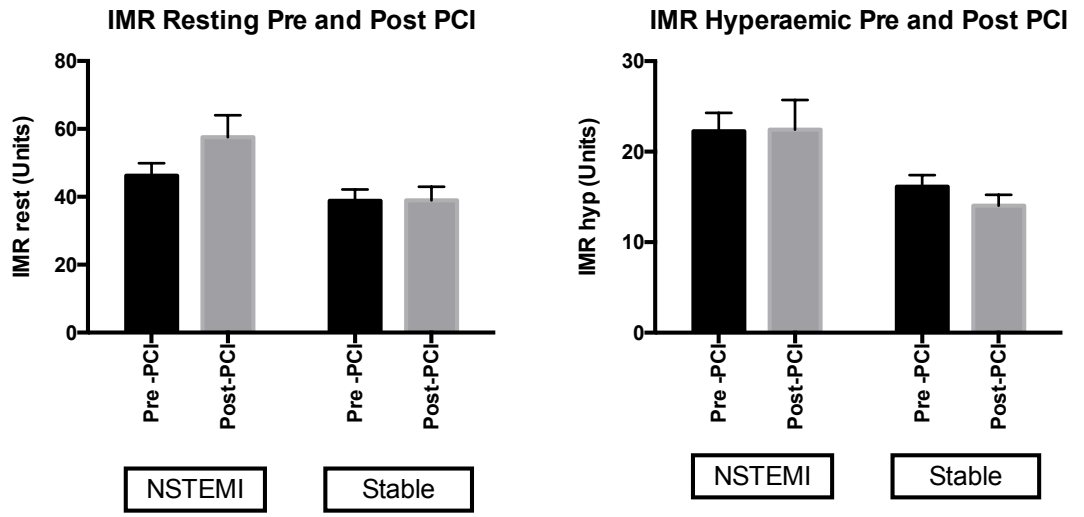

Figure 10: IMR_{rest} Pre and Post-PCI in both NSTEMI & Stable angina patients

Figure 11: IMR_{hyp} Pre and Post-PCI in both NSTEMI & Stable angina patients

Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, PCI - percutaneous coronary intervention, IMR_{rest} Index of microvascular resistance at rest, IMR_{hyp} index of microvascular resistance during hyperaemia (minimal IMR)

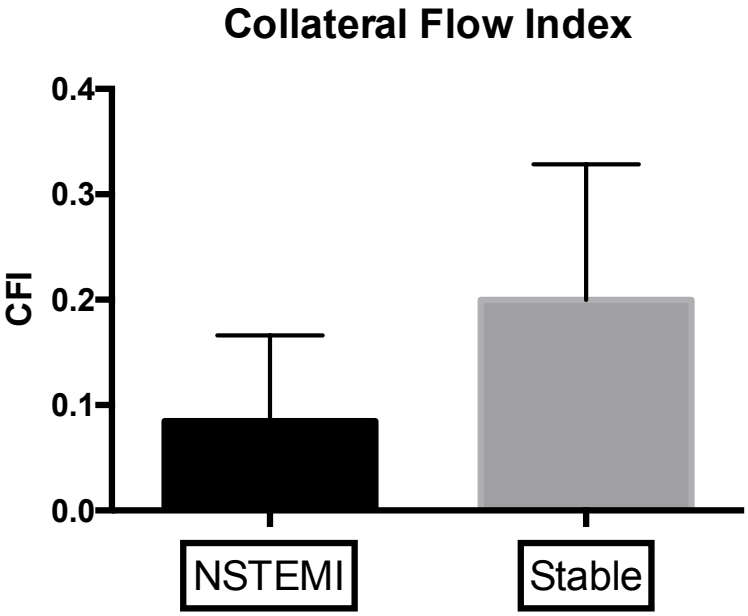

Figure 12: Collateral Flow Index (CFI) in both NSTEMI & Stable angina patients

Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, Stable – Stable angina pectoris

The coronary wedge pressure was measured in all groups pre-PCI in order to correct for collateral circulation in the calculation of microvascular tone. This wedge pressure was converted to the collateral flow index (CFI), the stable cohort had a significantly higher collateral flow index than the NSTEMI group; 0.2 (SD 0.13) vs. 0.08 (SD 0.08), $p < 0.0001$ (Figure 12).

Discussion

We have clearly indicated a significant net NO production across the myocardial bed in NSTEMI patients with significant epicardial stenosis. The net positive gradient in the NSTEMI cohort is 84.9 nM, principally the result of no apparent change in nitrite and an increase in RBC NOx across the coronary bed. This is in complete contrast to the stable angina cohort and data we have observed previously in healthy control patients. In the presence of a significant epicardial stenosis - and reduced CBF – the benefits of increased trans-myocardial NO production are clear. With a lower CVR via dilation of resistance vessels by locally produced NO this mechanism increases the effective trans-myocardial perfusion pressure, which in turn increases CBF.

Pre-PCI aortic root serum nitrite is significantly lower in the NSTEMI cohort (204.5 CI 134.1 to 328.3) in comparison to the stable angina cohort (436.6 ± 132.4 nM), this may reflect systemic rather than myocardial endothelial function. If this is the case the NSTEMI cohort have significant reduction in their systemic endothelial function / health in comparison to those patients with stable angina. It is not

understood how systemic NO production interacts with local trans-myocardial NO production. Improved systemic endothelial function in the stable angina cohort may go in some way to compensate for the epicardial stenosis by reducing CVR, it may also relate to the improved collateral formation indicated by the significantly higher CFI.

In contrast increasingly dysfunctional systemic endothelial function in patients with coronary disease has been associated with negative outcomes. [130] In the context of trans-myocardial NO production (in NSTEMI patients) perhaps we are seeing a localised up regulation of NOS in response to a highly dysfunctional systemic endothelium. Almost a "last-ditch" attempt to reduce CVR in an increasingly decompensated auto-regulatory system that is on the limit of being able to adjust and compensate for a severe epicardial stenosis. This data supports those observations and indicates a mechanism as to why these patients may have worsening cardiovascular outcomes as the endothelium loses its ability to maintain myocardial perfusion acutely. The resting IMR data in the stable cohort certainly supports the idea of a lower CVR prior to hyperaemia (Stable IMR_{rest} 38.9 ± 18.4 U vs NSTEMI IMR_{rest} 46.2 ± 21.6 U: both pre-PCI).

Supporting the trans-myocardial NOx observations in the NSTEMI group we see IMR_{rest} rise significantly from pre-PCI to post-PCI (46.2 Units ± 21.6 vs. 57.6 Units ± 37.9, p=0.038) following restoration of epicardial calibre. In the same cohort we see a sharp correction in trans-myocardial NO production from 85.1nM down to 24.5nM. This correction may partly reflect acute down regulation of endothelial

NOS secondary to the reduction in shear stress post-PCI, [81] and perhaps reduced RBC ATP release as oxygen saturation improve locally. [46] Conversely, this apparent reduction in NO production may just reflect the increased flow following PCI and be caused by a simple dilutional effect which leads to an acute rise in resting CVR (IMR_{rest}). The IMR_{hyp} reflects how well preserved the microcirculation is and its maximal capacity of vasodilation. A number of factors can influence hyperaemic IMR including severe left ventricular hypertrophy (LVH) and myocardial infarction. [114-116] In both the stable angina population and the NSTEMI population we see no significant difference in IMR_{hyp} pre to post PCI, we can assume therefore that these two patients groups have not suffered significant peri-procedural myocardial infarction (caused by the coronary intervention) which may have influenced our measurements of CVR (IMR_{rest}).[131]

If we assume that NOS activity remains constant and the reduction in NO_{2}- concentration is a dilutional effect secondary to increased flow, we can make a rudimentary calculation of how much flow would need to increase using the Fick principal (Figure 13). Using the measured NOx values in the NSTEMI population pre and post PCI we calculate that flow would need to increase 5.23 times in order to account for this reduction in NOx in terms of a pure dilutional effect from increased CBF following PCI.

A

$$VO_2 = (CO \times C_a) - (CO \times C_v)$$

$$VO_2 = CO \times (C_a - C_v)$$

$$CO = \frac{VO_2}{C_a - C_v}$$

B

Let the following

f_1 – flow before PCI
f_2 – flow after PCI
C_{Ao} - concentration in aorta
C_{CS1} - concentration in CS before PCI
C_{CS2} - concentration in CS after PCI
P - secretion rate of the compound

C

$$f_1 = \frac{P}{C_{CS1} - C_{Ao}}$$

$$P = f_1 \times (C_{CS1} - C_{Ao})$$

$$f_2 = \frac{P}{C_{CS2} - C_{Ao}}$$

D Replace P

$$f_2 = f_1 \times \frac{C_{CS1} - C_{Ao}}{C_{CS2} - C_{Ao}}$$

E Substitute measured values for NOx in the NSTEMI cohort (pre and post-PCI)

$$f_2 = 1\,x\,\frac{488.7 - 403.7}{429.1 - 412.9}$$

$$f_2 = 5.23$$

Figure 13: Fick calculation of change in coronary blood flow in NSTEMI cohort post-PCI

Panel A: Fick principal for calculating Cardiac output

Panel B&C: Substituting Fick equation for NOx concentration in Ao and CS, pre to post PCI

Panel D: Replacing P with pre-PCI Ao – CS concentration of NOx

Panel E: If pre-PCI flow = 1 then post-PCI flow = 5.23 times pre-PCI

Abbreviations: VO_2: Oxygen consumption, CO:Cardiac output, C_a & C_v: Arterial and venous oxygen saturation, C: concentration (nM), f= flow (dimensionless), NOx: total NO metabolite concentration (serum plus red cell excluding, nitrate).

Obviously, the calculation of increased flow is based on several assumptions which cannot be proven and are unlikely to be correct. These include the assumption that NO synthesis by NOS does not change following PCI. However, it is certainly possible that there is an acute regulation of NOS activity by either/both reduced shear force (reduced velocity across the epicardial lesion), and downregulation of NOS via the adenine / purinergic pathway (described in Chapter 1: General Introduction), it is also reliant on there being no reduction of nitrite to NO and loss to myocardial tissue.

Following PCI we only see a change in nitrite gradient (A-V), the RBC NOx content remains constant. The constant positive RBC NOx gradient can be explained by the reciprocal relationship between oxygen saturation and RBC NOx primarily caused by an increase in Hb-NO as the oxygen saturation drops. [97] Whilst this study would have benefited from the use of NOS inhibitors we see a direct relationship between reduced total NO concentration (post PCI) and an increase in IMR_{rest} which is consistent with the role of NO in the regulation of CVR. This CVR regulation compensates for severe epicardial stenosis and helps to maintain CBF.

It is important to note that minimal microvascular resistance IMR_{hyp} does not change from pre to post-PCI indicating that the rise in resting microvascular tone is not secondary to peri-procedural myocardial infarction or other factors.

In our NSTEMI cohort we have shown objective evidence of ischaemia and low oxygen saturations prior to PCI, yet despite this environment we see no evidence

of the nitrite consumption (reduction of nitrite to NO) that we would expect to see if this was the dominant mechanism of autoregulatory adaptation. Overall, we see a net increase in trans-myocardial NOx confirming net NO production across the human coronary bed in response to hypoxia and ischaemia. Of course, what this data cannot indicate is a dynamic situation in which both methods of NO production are occurring (NOS synthesis and Nitrite reduction), with competing influences on overall NO metabolite concentration. The addition of a NOS inhibitor would help delineate the influence of these competing systems however, this clearly would be unsafe in a group of patients with acute coronary syndromes and impaired coronary blood flow. Analysis of this dynamic situation is seen in the next chapter (Chapter 4: Results 2) with the recruitment of a further cohort of NSTEMI patients investigated in an identical manner but with augmentation of serum nitrite concentrations prior to, during and after revascularisation.

The findings in our stable cohort seem to be at odds with the observations made in the NSTEMI cohort. Whilst the stable angina cohort had similar baseline characteristics and equivalent levels of ischaemia (Table 4: Coronary physiology results summary data), there were some fundamental differences in both trans-myocardial NO gradients and microvascular tone. Pre-PCI we saw a similar resting tone (IMR_{rest}) in comparison to the NSTEMI patients (Stable 38.9±18.4 vs. NSTEMI 46.2±21.6, p=0.15), however, post-PCI instead of this rising (as it did in the NSTEMI cohort) there was no change in IMR_{rest} (pre-PCI 38.9±18.4 vs. post-PCI 38.99±22.06, p=0.98). The stable cohort also had a negative trans-myocardial gradient of serum nitrite, similar to typical healthy patients that can be ascribed

completely to the reciprocal relationship of oxygen with RBC-NOx distribution with no net rise in total NO metabolites. [97] A key observation was the mature coronary collateral circulation in the stable population as reflected by the significantly elevated CFI in comparison to the NSTEMI cohort Stable angina CFI 0.2 (SD 0.13) vs. NSTEMI CFI 0.08 (SD 0.08), p <0.0001 (Figure 12). The autoregulatory control of blood flow in these patients is more stable and this may be related to the non-acute nature of these patients and specifically their coronary lesions. We suggest the mechanism of compensation in stable patients may be related to the development of a mature coronary collateral circulation as indicated by the significantly elevated CFI in this group. This collateral circulation represents a form of chronic adaptation that may negate the need for upregulation of NOS and may explain the mixed findings of the role of NO in these patients described previously. [88,93]

Conclusion

The above study gives compelling evidence that in acute patients suffering NSTEMI, upregulation of endogenous production of NO - presumably by NOS - plays a role in autoregulatory adaptation of CVR to maintain myocardial perfusion. Patients with significant coronary atherosclerosis have consistently been shown to have reduced endothelial function, [92,93,130,132] it is clear from this data that the remaining endothelial function (in terms of localised myocardial NO production), still has a role to play. Overall the ability of individuals to compensate for

significant (and acute) epicardial stenosis may be related to their ability to upregulate NOS and reduce CVR.

Despite the perfect environment, we failed to find compelling evidence of increased dependence on nitrite consumption. However, without the use of NOS inhibitors it's difficult to get a clear understanding of the potential competing and dynamic influences of NO production by NOS and Nitrite reduction.

In stable patients, we do see a negative gradient of serum nitrite together with preserved resting microvascular tone post PCI. However, as coronary sinus oxygen saturations increase we do not see any reciprocal changes in this nitrite gradient. Another explanation for the preserved microvascular tone post PCI is the objective evidence of substantial collateral vessel formation with significantly elevated CFI. Collateral vessels formation may represent a chronic compensatory mechanism that negates the need for up-regulation of NOS.

LIMITATIONS

These experiments have several limitations that are inherent in this type of clinical research. The first set of limitations are those created by selecting compromises that allow this research to be carried out in humans, safely and in parallel to their clinical treatment following life threatening diagnosis. Amongst these limitations was the time constraint and sampling constraints in terms of how long and how often we could measure physiological and serum concentrations immediately

post-PCI. We can't exclude the possibility of later significant changes in CVR (IMR_{rest}) and NOx concentrations. For example, we may have seen a time dependent change in NOx formation indicating "recovery" of NO synthesis to compensate for increased flow post-PCI and a biphasic response in CVR (IMR_{rest})

Whilst we attribute the increase in CVR post-PCI (in the NSTEMI cohort), to the reduction in NOx metabolites (and thus NO synthesis by dilution or otherwise), we can't be sure the process is not a highly dynamic one between NO_{2-} reduction to NO and NOS synthesis of NO. Equally, there may be confounding influences on changes in CVR (IMR_{rest}), the likeliest being the intact myogenic mechanism (page 41). The use of a NOS inhibitor would have helped to elucidate these mechanisms, however, the use of these drugs in acute NSTEMI patients is not possible. However, in the following chapter we augment the serum concentration of NO_{2-} to further understand its role in autoregulation of myocardial blood flow.

Unfortunately, because of geographical constraints measurement of total red blood cell NO metabolites was not possible (stable cohort primarily recruited in Papworth, Cambridge). Without total RBC we can only assume that the inverse correlation of total RBC NOx to Oxygen saturation exists as per previous healthy controls and our groups here.

The split of recruitment between two centres potentially introduces experimental bias in terms of small differences in the way experimental protocols are performed and samples are handled.

Chapter 4: Results 2

Augmentation of serum nitrite in patients with non-ST elevation myocardial infarction: effect on coronary vascular resistance before and after coronary intervention.

ABSTRACT

Background

Nitrite, a metabolite of NO can itself be reduced to NO under favorable conditions. In the previous NSTEMI cohort resting CVR (IMR_{rest}) is significantly higher than in stable angina patients and rises significantly post-PCI in correlation with an acute drop in NO synthesis. This snapshot does not tell us if there is *both* NOS activity and nitrite reduction to NO competing with each other and shuttling between both NO and its metabolites. Augmented serum nitrite concentration would have the effect of "pushing" nitrite reduction to NO in favourable conditions.

Methods

15 patients were recruited following an NSTEMI and scheduled to have an angiogram +/- PCI ($NSTEMI_{inf}$). Pre-PCI aortic (Ao) and coronary sinus (CS) blood samples were taken prior to starting an intravenous infusion of sodium nitrite (3.3μmol/min for 30 minutes), a further CS sample was taken 5 minutes after the start of the infusion and just prior to measurement of CVR (IMR_{rest} and IMR_{hyp}). PCI was then carried out and a further Ao and CS blood sample was taken post-PCI. Both serum and red blood cell NO metabolites (NOx) were measured using

ozone based chemiluminescence. The control group (n=30) were the same patients recruited in the previous chapter and analysed with identical method.

Results

The $NSTEMI_{inf}$ cohort had a five fold increased concentration of serum nitrite in comparison to the NSTEMI cohort without nitrite infusion; pre-PCI NSTEMI 203.4 nM (125. to 293.9) vs. pre-PCI $NSTEMI_{inf}$ 1274nM (821.5 to 1475). No influence on IMR_{rest} ($NSTEMI_{inf}$ 47.1±30.9 vs. NSTEMI 46.2±21.6 p=0.91) was seen. During PCI median serum nitrite concentrations were 1422 nM (1032 to 1842). No influence was seen on post PCI microvascular tone (IMR_{rest}). Indeed, an almost identical rise in microvascular tone to that seen in NSTEMI without nitrite infusion ($NSTEMI_{inf}$ 59.63±40.1 vs. NSTEMI 57.6±37.9, p=0.87).

Discussion

The NSTEMI cohort had a net production of NO from A to V which correlated with changes in CVR. In a second cohort $NSTEMI_{inf}$, an infusion of intravenous sodium nitrite prior to and during PCI was administered with a 5 fold increase in serum NO_2- concentration. Despite this no effect was seen on CVR pre or post-PCI. Synthesis of NO in the coronary circulation seems to have a significant effect on CVR in NSTEMI patients, nitrite reduction to NO does not appear to have a significant role in CVR regulation of NSTEMI patients.

INTRODUCTION

The role of Nitrite in the ischaemic myocardium: the perfect model?

Whilst the inhibition of endothelial dependent NO synthesis has been shown to increase resistance vessel tone in ischaemic models and also in healthy humans at rest and during pacing, [94-96] it has been more difficult to directly measure net increases in metabolites of NO (Serum Nitrite, Total Red Blood Cell (RBC) NOx) using sensitive methods in similar human models. [97] Part of the explanation for this absence of a net rise in direct measures of NO metabolites may be the role of serum nitrite. Under favourable conditions (hypoxia, acidosis) nitrite (itself a metabolite of NO), may be re-cycled to NO acting as a bioavailable pool of NO made available to hypoxic and ischaemic tissue. One of the central tenets to this mechanism is thought to be the ability of deoxy-haemoglobin to reduce nitrite to NO and thus describes a plausible mechanism for hypoxia induced vasodilation.[101,102] Like other proposed mechanisms the "deoxy-haemoglobin" hypothesis has its limitations, the actual mechanism of nitrite reduction to NO is likely to be more complex and involve multiple pathways both enzymatic and non-enzymatic (see introduction page 46). Regardless of the mechanism multiple studies have reported the vasodilatory action of intravenous or oral doses of nitrite in various ischaemic models. [133-138]

Human studies of NO_2^- administration in health and disease

There are many small trials and cohort studies that have used intravenous administration of NO_2^- to study its role in control of vascular tone in humans. Here

we will look at three key studies and in addition appraise the two more recent clinical trials in patients with acute heart attacks (STEMI).

Cosby et al [101] infused sodium nitrite $NaNO_2$ into the brachial artery of healthy human subjects increasing serum nitrite from a baseline of 200nM to 221μM at peak in one group and 200nM to 2.56 μM in another. Forearm blood flow was measured with strain gauge plethysmography (SGP) and was found to increase by 175% and 25% respectively. Further, during exercise there was a decrease in venous NO_{2^-} and a further increase in forearm blood flow. Whilst the changes in forearm blood flow are interesting in terms of NO_{2^-} mechanism the reduction in serum NO_{2^-} during exercise seems predictable given the demonstrated reduction in venous oxygen content and how this creates a situation where the components of NO metabolism are redistributed between serum and RBC compartments as described by Rogers et al. [97]

Dejam et al[139] used a similar model to Cosby - with a brachial infusion of $NaNO_2$ - but this time looked at the influence of also inhibiting xanthine oxidase with allopurinol or adding ascorbic acid to promote acid disproportionation. Neither agent further enhanced forearm blood flow and a dose escalation protocol demonstrated the decay of both HbNO and plasma NO_{2^-} in the same direction. However, considering the avidity of deoxy-haem for NO it is difficult to understand how - in a hypoxic environment that is purportedly reducing NO – NO can be released from deoxy-haem to the surrounding endothelium / tissue and how both NO_{2^-} and HbNO can decay at the same rate.

Ingram et al [140] studied the effect of an intravenous $NaNO_2$ infusion on patients during dobutamine stress echocardiogram who had previously had a clinical diagnosis of angina confirmed with coronary angiography. This was a blinded randomised placebo controlled crossover study in which ten patients were recruited. He found that $NaNO_2$ infusion (1.5μmol/min for 20 mins) improved the functional response in ischaemic myocardium but had no effect in normally perfused areas of myocardium.

Lower dose of $NaNO_2$-: biologically active with minimal systemic arterial side-effects

High doses of $NaNO_2$ may not be safe in humans in the context of pre-existing cardiac disease. We know that these high doses may produce vascular collapse and syncope. [141] Ingram demonstrated in this study and another (using hypoxia and measuring pulmonary vascular dilation with an infusion of nitrite) that a lower total dose of $NaNO_2$ had a significant effect on pulmonary vasodilation. [135] These finding were consistent with other groups in particular Maher et al [133] who demonstrated a progressive dose related effect of brachial artery $NaNO_2$ infusion. He found biologically important effects on forearm blood flow (during hypoxia and normoxia) at doses from 314nmol/min (incremental steps of 40nmol/min) with maximal effect at 7.84μmol/min. Its important to note that the arterial flow was enhanced by the addition of hypoxia with the $NaNO_2$ infusion.

These studies confirm that lower doses than previously described [102] are both biologically active and importantly safe in healthy and non-healthy patients.

To date per-conditioning with sodium nitrite in patients with ST-elevation myocardial infarction has been carried out in two clinical trials. The first was "Intravenous sodium nitrite in acute ST-elevation myocardial infarction: a randomized controlled trial" (NIAMI). NIAMI was a trial carried out by Siddiqi et al, [142] a multi-centre, double-blind, placebo controlled trial in patients presenting with acute ST-elevation myocardial infarction (STEMI). An intravenous infusion of $NaNO_2$ was administered (totaling 70μmol) in the 5 minutes prior to reperfusion via primary-percutaneous coronary intervention (PCI). A total of 228 patients were studied (almost equal split) the outcomes were all surrogate and included infarct size assessed by cardiac enzyme release (Creatinine Kinase (CK) and Troponin I (TnI)), as well as infarct size measured by cardiac magnetic resonance imaging (CMR) and adjusted for area at risk (salvage index). The trial did not show any benefit of $NaNO_2$ infusion in terms of infarct size reduction as measured by cardiac enzyme or CMR salvage index.

Following on from NIAMI was a single centre, double-blind, randomised control trial comparing placebo to an intra-coronary bolus of sodium nitrite (1.8μmol over 30 seconds), or placebo (10mL of 0.9% NaCl). Carried out by Jones et al[143] surrogate infarct size end points were used with the primary specified as serum creatinine kinase (CK), area under the curve (AUC). Secondary endpoints were troponin T AUC, and infarct size assessed by late gadolinium enhancement (LGE) via CMR. Perhaps surprisingly salvage index was not used as a pre-specified

outcome which considering the trial is somewhat limiting. It should also be noted that no pre-specified subgroup analysis was identified at the outset of the trial.[144,145] The trial was also highly selective with 430 patients considered eligible and 82 going on to be randomised, the mean age of which was 57 years. Despite being labelled as an intention to treat analysis 2 patients were excluded after randomization from the placebo group. In a clear evolution from NIAMI this trial introduced a high dose bolus of sodium nitrite intracoronary rather than intravenous, further this bolus was delivered distal to the coronary occlusion via an over the wire balloon and distal port injection. No difference was seen in the primary outcome (CK AUC), or both secondary outcomes (troponin T AUC and LGE infarct size). A further post-hoc subgroup analysis using salvage index, ratio of LGE vs myocardia oedema / T2 STIR (area at risk); again this showed no significant difference between groups despite the group showing their techniques were of high quality with a positive association between LGE and CK AUC. A further post-hoc sub-group analysis looked at both the primary and secondary outcomes and found a significant difference between the groups (in favour of sodium nitrite) when patients with either profoundly restricted flow or complete absence of flow (TIMI ≤ 1) were analysed and the rest excluded. Here the authors found a significant difference in infarct size via LGE (%): 15.31 (12.36-18.27) vs 20.08 (16.72-23.43), p=0.03. This was also reflected in the salvage index calculations with a salvage index of 0.56 (0.50-0.62) vs 0.43 (0.37-0.49), p=0.002. CK AUC (but not troponin T) was also in favour of sodium nitrite; 44,608 AU (27,535-64,848) vs 55,666 AU (41,591-93,659), p=0.030. The CMR findings at baseline were not carried through to the six month CMR with no difference between groups regardless of sub-group analysis.

Somewhat surprisingly at 6 months the group receiving sodium nitrite with TIMI flow > 1 appeared to have a larger infarct size than the whole (unselected) cohort at 6 months; (TIMI ≤ 1 vs TIMI > 1 LGE %) 11.88 (9.52-14.24) vs 16.33 (-9.4-42.06). This difference was not seen in the placebo group; in fact, it was reversed (TIMI ≤ 1 vs TIMI > 1 LGE %) 13.15 (10.75-15.56) vs 10.59 (0.24-20.93).

Overall, this negative clinical trial generates some potentially interesting hypothesis with the post-hoc analysis but overall the trial suffers with its initial power calculation that has probably excluded the possibility of detecting smaller (clinically significant) infarct sizes. Part of the problem may be the low frequency of left anterior descending (LAD) artery occlusions 21 (26.25%) in the whole study population, this leads to a number of patients (non LAD) with relatively small infarct sizes, making it statistically more difficult to prove a difference between groups as the change in infarct size will be much smaller.

Also, noted by the authors is the absence of correction for multiple comparisons and/or the use of ANOVA in this analysis, important because small studies like this are at even greater risk of a type I error. Even looking beyond the limitations of unspecified post-hoc analysis, statistically we cannot really say with any certainty that there are differences between the two groups and this data should only be considered preliminary hypothesis generating in preparation for an adequately designed and controlled trial.[146]

Certainly the impressive protection of NO_{2^-} in ex-vivo animal has not been repeated thus far in terms of IR protection in the human heart. [147-150]

Coronary blood flow autoregulation and its relationship to ischaemic reperfusion injury

Principally this thesis investigates the role of NO and NO_{2^-} in the autoregulation of CBF in patient with severe epicardial stenosis (stable angina and NSTEMI). In the previous section on STEMI we have read about the potential role of NO_{2^-} in terms of reducing ischaemia reperfusion injury (IRI). Whilst IRI is not the topic of this thesis in the following section we clarify the potential mechanisms of NO_{2^-} in reducing IRI and how this relates to CBF autoregulation in the context of this thesis.

Beyond limited evidence that NO_{2^-} may have a direct effect on complex II of the mitochondrial respiratory chain (and thus protect against IRI), [151] the beneficial effects of NO_{2^-} are focused (but not limited to) its conversion to NO. As described in the general introduction (page 46) the mechanism of this conversion has many potential candidates: deoxy-haemoglobin reductase, [101,102] Xanthine oxidase, [107,152] acid disproportionation [108](as well as others). Fundamentally these mechanisms rely on the production of NO which in turn offers cardio protection via a multitude of well documented pathways. The presence of NO in the coronary circulation has been shown to contribute to the maintenance of regional myocardial blood flow in a canine model, [153] and in the research outlined in the previous chapter of this thesis, both models required a significant epicardial

stenosis. Beyond direct maintenance of CBF, NO inhibits platelet aggregation and adherence to the vascular endothelium which is also likely to have a positive effect on microvascular flow (see Chapter 5: Results 3, page 137). [133,154] During reperfusion NO augmentation in various ex-vivo and in-vivo models has been shown to have a beneficial effect on CBF and infarct size. [155-158] NO is also implicated in reducing mononuclear infiltration into the myocardium and reducing infarct size. [159] Beyond mononuclear infiltration NO also inhibits the release of TNFα (tumour necrosis factor α), itself associated with IRI. [160] NO interacts with mitochondrial respiration via the electron transfer chain. At low concentration NO donors like SNAP enhance mitochondrial membrane potentials and thus mitochondrial calcium uptake reducing IRI by reduced mitochondrial calcium overload. [161,162] Conversely, at high concentration NO can increase the production of peroxynitrite and shift from ATP production to oxygen free radical production with detrimental effects on IRI. [163]

It's possible that all of these mechanisms are at play when we study the effect of exogenous NO_{2^-} therapy in patients undergoing reperfusion therapy for STEMI. [142,143] Fundamentally, all the described mechanisms (beneficial or detrimental) are dependent on the reduction of NO_{2^-} to NO and in the context of mammalian in-vivo models one of the most predictable effects of NO is arterial and arteriolar (resistance arteriolar) vasodilation in multiple mammalian organs. [130,164-167] The previous chapter of this thesis outlines the importance of endogenous NO in terms of CVR in humans with NSTEMI. Whilst the current chapter does not investigate the effect of NO_{2^-} on markers of IRI, and certainly has no clinical (surrogate or

otherwise) endpoints, it does give insight into how relevant NO_2- reduction to NO is in terms of the possibility of enhancing local NO concentrations and reducing CVR within a human disease model that provides the perfect low oxygen and locally ischaemic environment. [168]

Nitrite a possible role in regulating coronary vascular resistance?

The oxygen gradient cross heart is one of the largest in the human circulation and locally this should be further exacerbated by critical epicardial stenosis, the perfect environment for hypoxic vasodilation mediated by nitrite reduction to NO and a reduction in CVR and increase in CBF.

Rogers et al [97] found no net change in NO metabolites in humans with no epicardial coronary disease. Certainly, in terms of serum nitrite concentration we found an almost identical pattern in those with stable coronary disease (Trans-myocardial NO metabolites and oxygen, page 82), and overt flow limiting stenosis. Unlike the NSTEMI cohort resting and minimal CVR (IMR_{rest}, IMR_{hyp}) showed no change pre and post PCI and both values were significantly lower than their equivalent values in the NSTEMI group (Table 4 page 89 and Figure 10). Despite these low values for CVR Ingram et al [140] found significant relief of ischaemia with a low dose infusion of sodium nitrite during stress echocardiography using pharmacological stress (dobutamine). The explanation for this is unclear, obviously, it is a different cohort of patients with a multitude of potential variances. Further, we have no data on the severity of epicardial lesions or a way to compare it to an invasive measure of epicardial stenosis (FFR), used in this study.

In Chapter 3: Results 1 we outlined evidence of a net NO synthesis in patients prior to PCI of significant epicardial stenosis who had suffered NSTEMI, we further outlined an increase in CVR post PCI in association with a reduction in net NO metabolites. These observations suggest upregulation of NOS is an important mechanism of compensation in these patients. The truth is unlikely to be so black and white; it is quite possible for the production of NO to be a balance of NOS synthesis and NO_2- reduction. This may help to explain the results of Ingram et al, however, Rogers et al inhibited NOS with LNMMA administration and found no influence of this on either total NOx or on the distribution of NOx between the serum and red blood cell compartments. From these studies, it is evident that the dynamics of NO synthesis can be quite different when using different human models (healthy vs. stable angina vs. NSTEMI), taking a snapshot of NO metabolite concentration will not be enough to indicate any shuttling of NO from both NOS and Nitrite reduction.

The observational study in Chapter 3: Results 1 falls into the same trap that all other clinical observational studies suffer in this and similar fields, we can outline an association between two measures but that doesn't equate to a causative link. The perfect answer to this would be the addition of a NOS inhibitor, however, in a cohort of acutely unstable patients this would not be in their best interests. It is impossible to pick apart the addition of NO via endothelial NOS versus the addition of NO via reduction of NO_2- and / or the likely shuttling of NO between these two states without changing the concentration of either one of these molecules.

Whilst using NOS inhibitors may be harmful in this patient group, enhancing [NO_2-] in patients following acute myocardial infarction has been shown to be well tolerated. [142] Ingram [140] showed the potential of even low dose nitrite to act as a potent anti-ischaemic agent. In the context of NSTEMI patients this effect may be even greater as we have shown previously this cohort has significantly higher resting CVR which further increases post PCI (Table 4 page 89).

The following is a description of the effect of augmenting the serum concentrations of NO_2- in a cohort of patients who have suffered an NSTEMI. The preparation of these patients and the cardiac catheter protocols are identical to those in the same group described in Chapter 3: Results 1. We chose to use a dose of sodium nitrite just over twice as high (3.3μmol/ml for 30 minutes, total of 99μmol) than that used by Ingram. The dose of sodium nitrite was based on the maximal dose used by Maher et al [133] without causing systemic hypotension but with maximal arterial vasodilation.

METHODS

As described in Chapter 2: General methods (Measurement of Nitric Oxide metabolites page 61) we used the same methods of NOx quantification to confirm the increased concentration of serum nitrite at the key points of measurements of

coronary physiology indices. We were careful to measure identical coronary physiology indices at identical time points of those described in Chapter 2: General methods (Cardiac catheterisation laboratory protocol page 55)

Unless stated methods are identical to those in Chapter 3: Results 1, major differences are highlighted (nitrite infusion and coronary sinus blood sampling). Methods are summarised here to allow quick reference within this chapter.

Study population (NSTEMI and NSTEMI$_{inf}$)

Patients were recruited from University Hospital of Wales following local research ethics committee approval of the study, written informed consent was obtained from each patient.

Inclusion criteria

- Any patient over the age of 18 with the capacity to consent for study and who was receiving coronary angiography as a normal investigation and as part of their routine treatment for either stable angina or NSTEMI
- Normal left ventricular function (EF≥50%), assessed by echocardiography
- Lack of contraindication to the use of dual antiplatelet therapy (aspirin and clopidogrel).
- Patients that had one vessel PCI following coronary angiography with lesions that were suitable to allow fractional flow reserve and coronary flow reserve measurements.
- Patients with a successfully cannulated coronary sinus

Excluded criteria included:

- Patients with ongoing and unstable ischaemic symptoms
- Previous ST Elevation Myocardial Infarction
- Significant left main disease
- Patients with chronic total occlusions, index or otherwise
- Patients with earlier coronary artery bypass grafts
- Significant valvular disease
- Symptoms of heart failure
- Recent intravenous nitrate infusion
- Atrial fibrillation

Patients all had single vessel PCI of the culprit vessel only after a period of medical treatment. The culprit vessel was selected based on angiographic appearance, ECG.

Non-ST elevation myocardial infarction (NSTEMI) patients were recruited prior to coronary angiography and continued in the study if they required PCI. Patients all had single vessel PCI of the culprit vessel, the culprit vessel was selected based on angiographic appearance, ECG changes. Index vessels and other baseline clinical variables were well balanced between the previous NSTEMI group in Chapter 3: Results 1 and this cohort as displayed in Table 5.

		NSTEMI *n=30*	NSTEMI$_{inf}$ *n=15*
Age		63.8 ± 12	64.7 ± 9.8
Male (%)		32 (84)	13 (87)
Creatinine µmol/L		88.5 (71.3 – 99)	87 (68 – 99)
CRP		3 (2 – 6.25)	3 (2-4)
Left ventricular function (EF)		55 (50-55)	55 (55-55)
Index vessel			
	LAD (%)	8 (53)	23 (61)
	LCx (%)	2 (13)	6 (15)
	RCA (%)	5 (33)	9 (24)
FFR pre-PCI (index vessel)		0.62 ± 0.17	0.58 ± 0.18
FFR post (index vessel)		0.92 ± 0.06	0.89 ± 0.08
Hypertension (%)		24 (63)	10 (67)
Diabetes (%)		10 (26)	6 (40)
Hyperlipaedemia (%)		30 (78)	9 (60)
Smoking (%)		16 (42)	5 (34)
Previous PCI (%)		7 (18)	2 (13)
Clopidogrel > 5days (%)		38 (100)	15 (100)
Beta-blocker (%)		32 (84)	12 (80)
Calcium channel blocker (%)		7 (18)	1 (7)
ACE Inhibitor (%)		29 (75)	11 (73)
Statin (%)		34 (89)	13 (87)

Table 5: Baseline clinical characteristics of study populations NSTEMI and NSTEMIinf .

Abbreviations: PCI - percutaneous coronary intervention, CRP – C reactive protein, NSTEMI – non-ST-elevation myocardial infarction, NSTEMI – with infusion of sodium nitrite, EF - ejection fraction, LAD – left anterior descending artery, LCx – left circumflex artery, RCA – right coronary artery, FFR – fractional flow ratio, ACE - angiotensin converting enzyme inhibitor

Cardiac Catheterisation laboratory Protocol

The cardiac catheterisation protocol was identical to that used in Chapter 3: Results 1 with the addition of sodium nitrite infusion in the NSTEMI$_{inf}$ cohort. A

full description of this method can be seen in general methods chapter: Cardiac catheterisation laboratory protocol page 55 and Sodium nitrite infusion page 60.

Sodium nitrite infusion

In the NSTEMI cohort receiving pre-PCI loading with sodium nitrite ($NSTEMI_{inf}$), the sodium nitrite infusion was started immediately after the first coronary sinus sample was taken pre-PCI. The sodium nitrite was supplied by Tayside Pharmaceuticals, Ninewells Hospital and infusions prepared on the day of recruitment by St Mary's Pharaceutical Unit (SMPU), University Hospital of Wales. Final concentration of sodium nitrite was confirmed at SMPU using Griess Reagent Kit (Thermo Fischer Scientific). The infusion contained a total dose of 6.9mg (99 µmol) of sodium nitrite, infused over 30 minutes (3.3µmol/min). Following 5 minutes of this infusion an additional coronary sinus blood sample was taken to confirm the nitrite concentration at the point of the pre-PCI coronary physiology measures.

Offline analysis of catheter lab haemodynamic data

Again, this method is identical to the previous chapter, full details of the method can be seen in the general methods (Offline analysis of catheter lab haemodynamic data page 59).

Measurement of Nitric Oxide metabolites

Again, this method is identical to the previous chapter, full details of the method can be seen in the general methods (Measurement of Nitric Oxide metabolites page 61)

Statistical Analysis

Normally distributed data are expressed as mean ± SD unless otherwise stated. Non-gaussian data expressed as median (IQR). When the distribution of data was not clear the Shapiro-Wilk normality test was used. Multiple comparison between related groups of means were made with paired and unpaired ANOVA with Tukey's correction for multiple comparisons. Single comparisons were made with paired and unpaired student's t test as appropriate. Wilcoxon test was used for non-parametric single comparisons of paired data and Mann-Whitney test for unpaired data.

RESULTS

Transmyocardial NO metabolites and oxygen

NSTEMI with Sodium Nitrite pre-loading (n=15)

15 NSTEMI patients had a pre-PCI infusion of intravenous sodium nitrite as outlined in the methods. A total of 6.9 mg (99 μmol) in a total volume of 30mls was infused over 30 minutes; this infusion was started following the first set of blood sampling ("baseline") and 5 minutes prior to coronary physiology measures (FFR, IMR_{rest}, IMR_{hyp}) and the simultaneous second "pre-PCI" coronary sinus sample. The large elevation of serum nitrite concentration makes detection of small variations of NO metabolites from aorta to coronary sinus both impossible and somewhat irrelevant. Serum nitrite measures here confirm the similarity at baseline to the NSTEMI group in Chapter 3: Results 1 (Table 2 page 84), and quantify the rise in serum nitrite at key points during the protocol (namely, coronary physiology measures at baseline and post-PCI). Figure 14

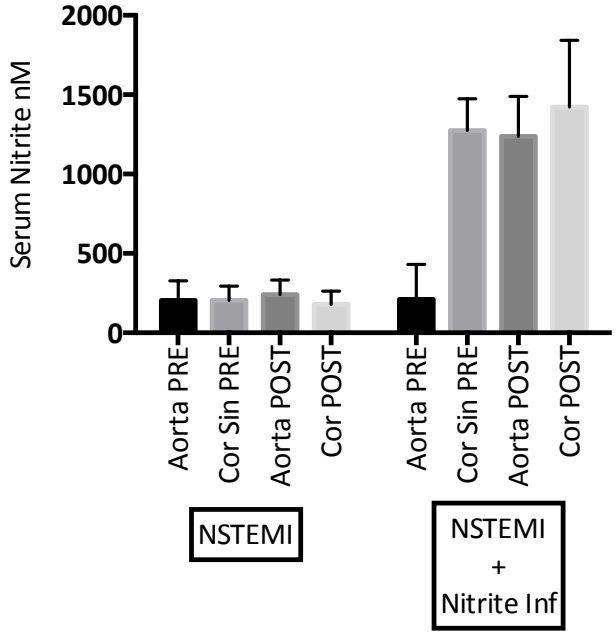

Figure 14: Trans-myocardial nitrite gradients (median with IQR)

Trans-myocardial NO_2^- nM in NSTEMI and NSTEMI with nitrite infusion ($NSTEMI_{inf}$). In the $NSTEMI_{inf}$ cohort aorta PRE – represents sample taken prior to nitrite infusion (baseline), Cor Sin PRE sample is taken at the point of pre PCI coronary physiology measures and 5 minutes after nitrite infusion was commenced. Cor POST is taken 5 minutes after stent deployment. Within groups there are no significant differences excluding any comparison with Aorta PRE. Statisitcs: not normally distributed data as median and IQR. Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, NSTEMI + Nitrite Inf – NSTEMI with nitrite infusion, PCI - percutaneous coronary intervention, Pre – pre-PCI, Post – post-PCI, Cor – coronary sinus, nM – nanomolar.

At baseline the median aortic (pre-PCI) nitrite concentration was 210.9 nM (150.3 – 431.3), median CS pre-PCI (and pre-infusion) was 192.1 nM (135.3 – 248.9). Following five minutes of sodium nitrite infusion the CS nitrite (pre-PCI) was 1274 nM (821.5 - 1475). Nitrite concentration in the CS 5 minutes post PCI was 1422nM (1032 - 1842). Post PCI aortic sampling was slightly delayed after the CS samples and the mean nitrite concentration was 1237nM (931 - 1489), this indicated a rise in nitrite concentration across myocardium that in retrospect we consider to be a sampling artifact (Figure 14, Table 6 and Table 7). The study was limited by ensuring the complexity and practice of PCI was not hindered, and as such the nitrite infusion had finished prior to the opportunity to take the post aortic

sample. Serum nitrite concentrations fall rapidly after the sodium nitrite infusion stops (as we and others have shown previously) with a serum half-life of ~22min.

NSTEMI Pre PCI NOx (n=30)			
	Aorta	**Coronary Sinus**	
HbO_2 (%)	96.6 ± 1.82	34.8 ± 8.7	-61.8
Plasma NO_2^- (nM)	204.5 (134.1 to 328.3)	203.4 (125.3 to 293.9)	P^{ANOVA} >0.05
Total RBC (nM)	164.5 ± 99	247.2 ± 142.8	P^{ANOVA} <0.05* (CI 13.5 to 152) mean diff 82.7nM
Total NOx (nM)	403.9 ± 192.3	488.7 ± 265.9	P^{ANOVA} <0.05 * (CI 9.5 to 160) mean diff 84.9nM
NSTEMI Post PCI NOx (n=30)			
	Aorta	**Coronary Sinus**	
HbO_2 (%)	96.7 ± 1.85	42.5 ± 12.4	
Plasma NO_2^- (nM)	240.9 (135.5 to 332.0)	178.5 (126.9 to 263.2)	P^{ANOVA} >0.05
Total RBC	167.9 (±91.5)	228.1 (±120.9)	P^{ANOVA} >0.05 (CI -1.9 to 122.3) mean diff 60.1nM
Total NO	412.9 ± 170	429.1 ± 185.3	P^{ANOVA} >0.05 (CI -59 to 91) mean diff 16.2 nM

Table 6: Trans-myocardial NO metabolites (NOx) in NSTEMI patients

Trans-myocardial nitric oxide metabolite concentration from aorta to coronary sinus Abbreviations: NSTEMI – Non-ST-Elevation MI cohort, Stable – stable angina cohort, PCI - percutaneous coronary intervention, NOx – nitric oxide metabolites, HbO_2 – haemoglobin oxygen saturation, NO_2 – nitrite, CI – confidence intervals, nM – nanomolar, NO – nitric oxide

Statistics: normally distributed data as mean ± SD, multiple comparisons with 1-way ANOVA. Not normally distributed median (IQR), multiple comparisons Friedman with Dunn's correction (marked ANOVA)

NSTEMI$_{inf}$ Pre PCI NOx (n=15)		
	Aorta	**Coronary Sinus**
HbO_2	98.2 ± 1.63	35.1 ± 4.8
Plasma NO_2^- (nM)	210.9 (150.3 to 431.3)	192.6 (135.3 to 248.9)
	PRE INFUSION	*PRE INFUSION*
NSTEMI$_{inf}$ Post PCI NOx (n=15)		
	Aorta	**Coronary Sinus**
HbO_2	97.7 ± 2.0	42.4 ± 9.5
Plasma NO_2^- (nM)	1237 (931 to 1489)	1422 (1032 to 1842)
	POST INFUSION	*POST INFUSION*

Table 7: Trans-myocardial NO metabolites NSTEMIinf cohort

Trans-myocardial nitric oxide metabolite concentration from aorta to coronary sinus Abbreviations: NSTEMI$_{inf}$ – Non-ST-Elevation MI cohort with nitrite infusion, PCI - percutaneous coronary intervention, NOx – nitric oxide metabolites, HbO_2 – haemoglobin oxygen saturation, NO_2 – nitrite, CI – confidence intervals, nM – nanomolar, NO – nitric oxide

Statistics: normally distributed data as mean ± SD. Not normally distributed median (IQR).

CS oxygen saturation followed a very similar profile to the other NSTEMI group with mean Pre-PCI saturation of 35.1% (SD 4.8) and Post-PCI saturation of 42.4% (SD 9.5), p=0.0078. Figure 15

Serum nitrate quantification

Nitrate concentration was measured at the identical time points to nitrite and total RBC measures. Using a one-way ANOVA test there were no significant differences between time points within this group or across the different groups (comparison

to Chapter 3: Results 1). Nitrate is present in μM concentrations as opposed to the nM concentration of serum nitrite and RBC NOx. Because nitrate does not change between sampling points - and there is no evidence that humans are able to reduce nitrate to nitrite across the coronary circulation – we have excluded this from the calculations of total NO metabolites.

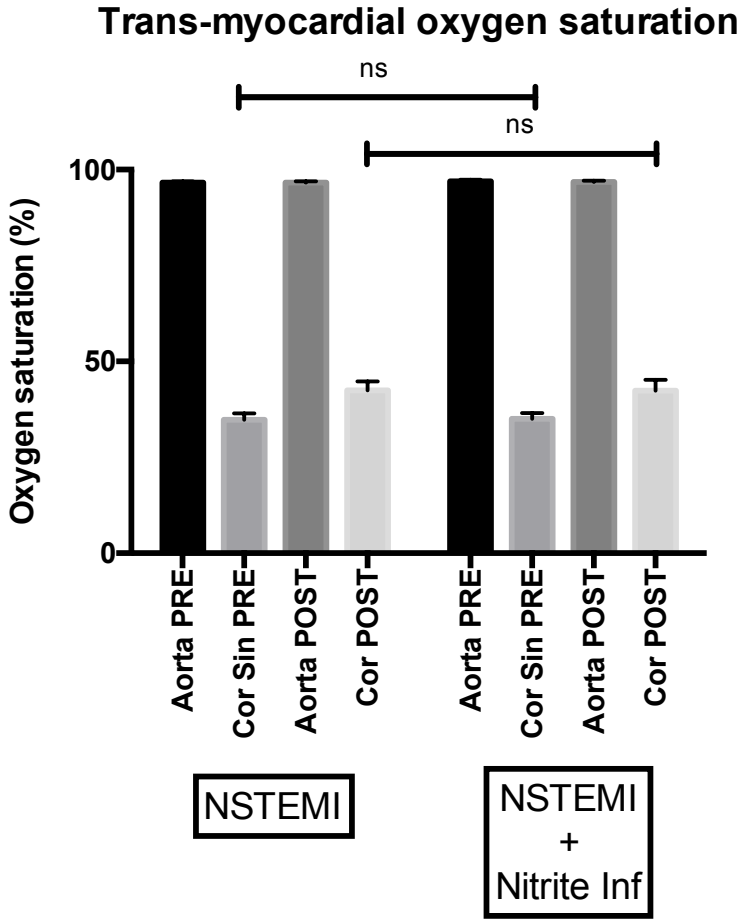

Figure 15:Aortic and coronary sinus haemoglobin oxygen saturation pre and post PCI

NSTEMI cohort left panel and $NSTEMI_{inf}$ cohort right panel.

** NSTEMI cohort Cor Sin PRE to Cor POST mean difference 7.7% (paired t, p=0.0034).*

***$NSTEMI_{inf}$ cohort Cor Sin PRE to POST mean difference 5.4% (paired t,p=0.0078).*

Abbreviations Aorta PRE: Aortic root sample pre-PCI, Cor Sin PRE: coronary sinus sample pre-PCI, Aorta POST: Aortic root sample post-PCI, Cor POST: coronary sinus sample post-PCI, $NSTEMI_{inf}$ is NSTEMI cohort with sodium nitrite infusion

Coronary physiology measures

NSTEMI with Sodium Nitrite pre-loading (n=15)

The cohort of NSTEMI patients receiving an infusion of sodium nitrite had no significant difference in baseline characteristics to the NSTEMI cohort in Chapter 3: Results 1. Blood sampling at baseline occurred prior to the start of the infusion. A further coronary sinus sample was taken 5 minutes following the start of the infusion and just before measuring the pre-PCI coronary physiology measures. FFR measures pre and post-PCI reveal a similar population to the NSTEMI cohort; 0.58 (SD 0.18) to 0.89 (SD 0.08). The measured coronary wedge pressure also revealed a similar low degree of collateral vessel formation to the NSTEMI group; CFI was 0.10 (SD 0.11) (SD 5.17) with no significant difference between the two groups. Table 8 and Figure 18

Resting microvascular tone was very similar to the NSTEMI (without nitrite infusion) both pre and post PCI; 47.13 (SD 30.9) vs. 59.6 (SD 40.1) with a significant rise immediately post PCI of 12.5 units (SD 15.5), p=0.017, matching almost identically the rise seen in the NSTEMI cohort without sodium nitrite infusion. Table 8 and Figure 16

Minimal (hyperaemic) microvascular tone was again almost identical to the NSTEMI group without nitrite infusion; 23.7 (SD 11.4) vs. 21.9 (SD 13.2) with no significant change from pre to post-PCI. Table 8 and Figure 17

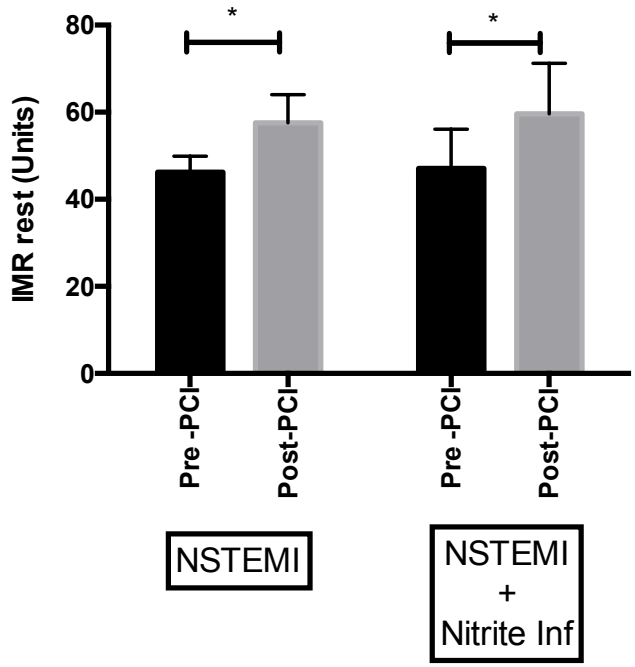

Figure 16: IMR resting pre and post PCI

Index of microvascular resistance during resting conditions, a surrogate of coronary vascular resistance in both cohorts: NSTEMI and NSTEMI$_{inf}$.

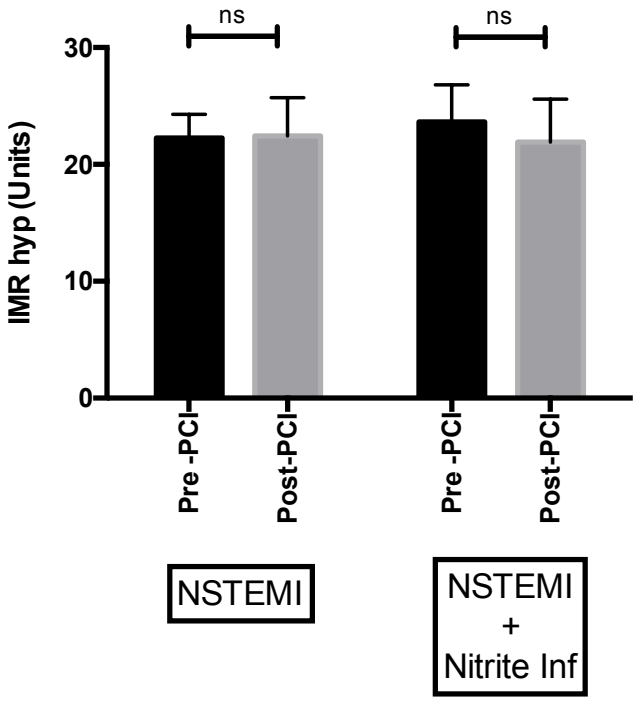

Figure 17: IMR hyperaemic pre and post PCI

Index of microvascular resistance during hyperaemic conditions (minimal microvascular resistance in all three experimental cohorts - NSTEMI, NSTEMI$_{inf}$, stable angina cohorts.

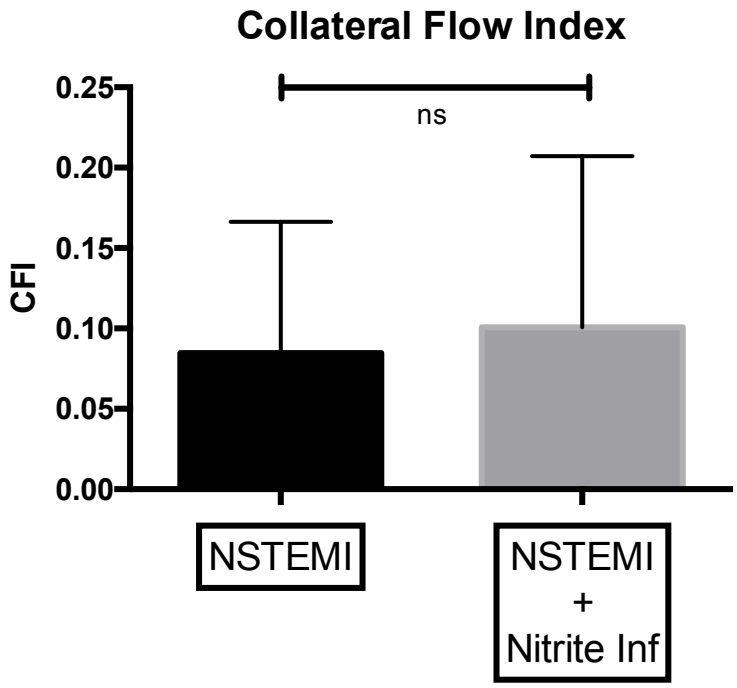

Figure 18: Collateral Flow Index

Coronary wedge pressure measured and converted to collateral flow index (CFI) at hyperaemia and prior to PCI in the NSTEMI and NSTEMI$_{inf}$ cohorts

*Fig 16, 17 & 18 Statistics: normally distributed data presented as mean with standard error of mean. Statistical significance where marked with * = < 0.05, Paired ANOVA and unpaired t-test (CFI)*

Abbreviations: IMR – index of microvascular resistance, hyp – hyperaemia, NSTEMI – Non-ST-Elevation MI cohort, NSTEMI$_{inf}$ – NSTEMI + nitrite infusion, Stable – stable angina cohort, PCI - percutaneous coronary intervention, Pre – pre-PCI, Post – post-PCI, Cor Sin – coronary sinus

	NSTEMI (n=30)		NSTEMI$_{inf}$ (n=15)	
	Pre-PCI	**Post-PCI**	**Pre-PCI**	**Post-PCI**
FFR	0.62 ± 0.03	0.92 ± 0.06	0.58 ± 0.05	0.89 ± 0.02
IMR$_{rest}$ units	46.2 ± 21.6	57.6 ± 37.9	47.1 ± 30.9	59.6 ± 40.1
IMR$_{hyp}$ units	22.3 ± 12.4	22.5 ± 19.8	23.7 ± 11.4	21.9 ± 13.2
CFI	0.08 ± 0.08		0.1 ± 0.1	*ns difference between cohorts for CFI p=0.59 (95% CI -0.04-to 0.08)*

Table 8: Coronary physiology summary data

Coronary physiology data pre-PCI and 5 minutes post-PCI

Abbreviations: NSTEMI$_{inf}$ – Non-ST-Elevation MI cohort with nitrite infusion, NSTEMI – Non-ST-Elevation MI cohort, PCI - percutaneous coronary intervention, FFR – Fractional flow reserve, IMR$_{rest}$ – Index of microvascular resistance resting conditions, IMR$_{hyp}$ – Index of microvascular resistance at hyperaemia, CFI – Collateral Flow Index (CFI).

Statistics: Normally distributed data presented as mean ± SD, for significance see graphical representations of data in figure 16, 17 & 18 above

DISCUSSION

In Chapter 3: Results 1 we demonstrated net NO metabolite (NOx) production from Aorta to coronary sinus (trans-myocardial) in the NSTEMI cohort. This net production disappeared post-PCI with an associated increase in CVR. The apparent net drop in NOx from pre to post PCI was driven primarily by a reduction in the trans-myocardial gradient of serum NO_{2-} (NO_{2-} gradient pre-PCI A to V -1.1nM, post-PCI -52.4nM). If this trans-myocardial drop in serum nitrite represents reduction to NO (which permeates into the endothelium and

myocardium) then we would expect to see a reduction in CVR. However, from pre to post-PCI CVR (IMR_{rest}) rose from 46.2±21.6 to 57.6±37.9, p=0.038 (mean ± SD).

By taking a snapshot of NO metabolites we have no idea of the potential (and likely) dynamic shuttling that may occur in NO and its metabolite nitrite. For instance, it is entirely plausible to imagine an acute down regulation in NOS (secondary to reduced CBF velocity and shear stress, [47] or adenine / purinergic receptor stimulation by ATP, [39] post-PCI), or a dilutional reduction in NO post-PCI (Figure 13: Fick calculation of change in coronary blood flow in NSTEMI cohort post-PCI page 95) partially compensated by reduction of nitrite to NO. To understand this relationship, we hoped to see a clear effect on CVR by augmenting serum nitrite concentrations by up to five-fold.

Despite a 5-fold augmentation of NO_{2-} concentration no effect is seen on resting CVR (IMR_{rest}) pre-PCI, pre-PCI the resting CVR is almost identical to the value observed in the NSTEMI cohort. The resting CVR post-PCI was almost identical to the NSTEMI cohort without nitrite infusion. Comparing the groups and as outlined in Table 5 there was no significant variance in baseline clinical characteristics and haemodynamic aspects of the epicardial lesions. Revascularisation outcomes measured objectively with FFR were also well matched between the groups. The collateral flow index was similar between groups indicating the same (poor) development of collateral vessels. Finally, from an oxygen gradient perspective pre-PCI coronary sinus oxygen saturations were almost identical between the two NSTEMI cohorts (NSTEMI: 34.8%±8.7, $NSTEMI_{inf}$:35.1%±4.8).

Gladwin et al [168] and colleagues have made a strong case for a role of nitrite reduction in hypoxic vasodilation. If one could select any organ system or circulation in mammals, then the coronary circulation would be the perfect environment to test this hypothesis, principally due to the extreme oxygen gradient from A to V (~99% to ~35% oxygen saturation). This takes us straight into the range at which it is proposed haemoglobin acts maximally as an allosterically regulated deoxy-haem based nitrite reductase producing NO. [102] Indeed from the perspective of a potential treatment in patients undergoing PCI for acute coronary syndromes this is a highly attractive targeted way to reduce CVR and potentially treat a common complication after PCI in these patients (coronary slow or no flow) following treatment of the stenosed epicardial vessel. [169]

This biological plausible, simple and effective hypothesis of the deoxy-haem model does have its shortcomings. The principal criticism is centered around a plausible mechanism by which the NO produced is able to extract itself from deoxy-haem, this in an hypoxic environment that will increase the avidity of deoxy-haem for NO. Once the NO has separated itself from deoxy-haem the next hurdle is to escape the erythrocyte (which is packed full of dexoy-haem with a high avidity for NO). To counter this argument some believe that rather than a direct source of NO nitrite reduction with deoxy-haem is the first step in the production of S-nitrosohaemoglobin (SNOHb), which is liberated once the RBC encounter normal oxygen conditions. [104]

Secondly, others have shown that whilst a negative A to V gradient of nitrite is present in the coronary circulation of healthy humans, this is balanced by an increase in RBC NO metabolite species (Hb-NO, Hb-SNO, nitrite) which increase inversely and proportionately to oxygen saturation. The net result of this results in no gradient of total NO metabolites and likely signifies shuttling of NO metabolites (which is reversed in the pulmonary vasculature). [97]

Thirdly, the vasodilator action of nitrite is seen in hypoxia in isolated vessel models both with haemoglobin, [170] and without. [106]. However, in both these models rather than physiological activity of nitrite at 300nM, [105] we only see activity at pharmacological concentration (both NO dependent and independent), around 10 μM and above.

Conclusion

Despite an optimal environment for nitrite reduction to NO we failed to see any evidence that nitrite had a role in hypoxic dilation or regulation of CVR. The human NSTEMI model we chose and the methods we developed have clear clinical importance with key questions being asked and answered in a relevant pathological situation.

Using sensitive NO assays and interrogating both serum and RBC NO metabolites we could show a net increase in NO metabolites that suggests NOS synthesis across the coronary circulation. This cohort have defined themselves as being different to healthy humans in this regard who don't seem to rely on NO synthesis to regulate CVR and thus coronary blood flow. [121] Further, it's important to recognise that NSTEMI patients do not display the negative gradient of nitrite seen in so many other animal and healthy human studies.

The 5-fold increase in serum nitrite concentrations observed in this study and the results obtained seem to suggest that any dynamic shuttling between nitrite production from NO and nitrite reduction to NO does not occur.

Limitations

As with any study - and in particular - clinical studies, any method will have limitations. The first and most obvious here is the dose of sodium nitrite used and the serum concentrations achieved. Others have previously shown much lower doses of nitrite have biological effect. [133,140] We chose this dose principally to ensure we had maximal vasodilation effect with minimal systemic blood pressure drop (potentially dangerous in patients with NSTEMI). Indeed, the selected dose highlights the practical limitations of using sodium nitrite in this clinical context, higher doses as well as being potentially dangerous also become progressively less relevant in the clinical context.

The method we chose to measure microvascular function is limited in a number of ways. IMR is a complex method in an already complicated situation where the emphasis is always the patient and their treatment and safety, limiting the number of times we can repeat the measures (for instance IMR pre and post nitrite infusion would have been very useful). IMR is also dimensionless and therefore only semi-quantitative, very good for making intra-patient or relative comparisons but less so across cohorts. We limited this by always placing the distal pressure and temperature sensor in the distal third of the artery. IMR also doesn't confirm the diagnosis of the patient, it is possible that some of our patients had suffered full thickness infarcts (ST elevation myocardial infarction) and this explains the lack of response to nitrite although there was no indication of this on echo.

In Chapter 5: Results 3 we further evaluate the potential action of nitrite in this cohort and its effect on platelet inhibition and inflammation.

Chapter 5: Results 3

Platelet function and inflammation in NSTEMI patients and the influence of augmented serum nitrite concentration

ABSTRACT

Background

Measuring platelet function around the time of an acute coronary syndrome is an unproven technique that does not correlate well with clinical outcomes. Despite this we continue to see these assays used (often in isolation), as surrogate markers of clinical effectiveness of compounds in the research setting. This chapter starts with a methodological assessment of platelet function assays in this setting and is followed by an analysis of the association between pro- and anti-inflammatory cytokine (TH1 / TH2) on platelet function and an analysis sodium nitrite augmentation and its effect on platelet function and inflammation.

Methods

Platelet function was measured in several ways for both peripheral venous and coronary sinus blood. Platelet aggregation was performed using both adenosine diphosphate (ADP) and thrombin receptor activating peptide (TRAP) as agonists using a multiplate analyser. Flow cytometry was used to identify platelets that expressed p-selectin on their surface. Soluble p-selectin and Th1/Th2 cytokines were quantified with a MSD multiplex assay. Finally, mass spectrometry was performed to quantify the eicosanoid 12-HETE.

Results

Platelet sub-study (PSS), n=42. Aggregation in response to ADP and TRAP significantly reduced peri-procedurally. Soluble p-selectin concentration remained constant and p-selectin surface expression also continued throughout.

Platelet function at both baseline and 24 hrs was almost identical between the NSTEMI and the $NSTEMI_{inf}$ cohorts.

Th1 / Th2 cytokines and the effect of nitrite. Nitrite infusion had no effect on any TH1 or Th2 cytokine at 24 hours, numerical differences could usually be explained by the differences seen at baseline concentration between the two groups.

Discussion

Platelet function assessment in acute coronary syndrome patients is complex with several competing and important factors including acute activation of platelets, pharmacological dynamics of powerful antiplatelet drug usage and the influence of invasive cardiovascular treatments that may also activate platelets. Outlined in these results we see the dangers of interpreting results based on just one assay (aggregation).

Nitrite had no effect on platelet function in terms of reducing this ongoing activation of platelets. These observations, interpretation and description of platelet function changes acutely are novel and may explain both the lack of clinical effect of nitrite in other studies and the poor performance of platelet function testing in randomised controlled trials aimed at tailoring antiplatelet pharmacology. The lack of an effect on platelet function was also reflected in the

lack of any effect of nitrite on the Th1 or Th2 cytokines, whilst we could quantify these cytokines in the pg/ml range.

INTRODUCTION

Nitric oxide and its role in platelet inhibition

Nitric oxide leads to vasodilation in mammals via its activation of guanylyl cyclase and production of cGMP. [171] As well as the endothelial production of NO there is evidence of nitrite reduction to NO by a number of mechanisms. [101] In this thesis we have discussed deoxy-haem reductase activity and a number of other mechanisms proposed such as acid disproportionation [150] and xanthine-oxidase reduction. [138]

NO has a well-documented role in inhibiting platelet reactivity via the same cGMP mediated mechanism central to many of its actions. [172,173] A significant limitation to NO and its direct role in serum is its very short half-life which can be measured in ms (1 to 2 ms), secondary to its rapid inactivation by free radicals (superoxide and lipid peroxyl radicals) and metalloproteins (in particular haemoglobin). [174]

NO complexes with thiol groups (to form RSNO) which protects NO from this rapid inactivation / decay in biological activity. RSNO can then release its NO and activate the guanylyl cyclase / cGMP dependent pathways and cGMP independent pathway giving rise to post-translational modification of proteins. [175-178] A convincing role for NO via *S*-nitrosothiols (RSNO) is seen in terms of platelet

function via direct inhibition of platelets, [179,180] and platelet-leukocyte binding to the vascular endothelium. [181-183]

Serum nitrite may have a role in platelet inhibition via its reduction to NO regardless of which mechanism this occurs through. Srihirun et al, [154] reported an effect of nitrite ex-vivo on inhibiting platelet reactivity using an LTA assay which was dependent on erythrocytes and deoxygenation; an effect abrogated with the addition of the NO scavenger C-PTIO.

Velmurugan et al, [184] also showed ex-vivo that nitrite itself has no direct effect on platelet reactivity. Whilst a multitude of in-vitro (NO and nitrite added to the platelet assay directly) studies have shown an effect of NO or nitrite (with intermediary metabolism), there is very little data on the in-vivo / ex-vivo action of NO or nitrite, *particularly* in pathological states. In the same paper Velmurugan et al used a dietary nitrate supplement in healthy volunteers to augment serum nitrite concentrations. A small rise in serum nitrite concentration was achieved (baseline ~200nM, post nitrate dose ~ 350nM), this was associated with a significant reduction in platelet reactivity measured using light transmission aggregometry (LTA) with a range of agonists. A selection of human nitrite infusion studies is outlined in Table 9.

Author / Year	Number patients	Intervention	Patient / Disease type	Platelet function analysis	Main result
Srihirun et al 2012 [185]	15	Ex-vivo Nitrite 0.01 to 10μM +/- deoxygenated erythrcytes +/- C-PTIO	Healthy	LTA and impedencd aggregometry (chrono-log), Flow cytometry. Using ADP as platelet agonist	Nitrite alone had no effect on platelet function, the addition of erythrocytes reduced platelet aggregation and reduced expression of platelet bound p-selectin. C-PTIO reversed both these actions
Kadan et al 2015 [186]	7	Ex-vivo Nitrite 10μM to 5mM	Healthy	LTA with ADP, collagen, epinephrine agonists	Significant platelet inhibition with nitrite alone (no erythrocytes), seen at doses of nitrite above 500μM
Akrawinthawong et al 2014 [187]	42	Ex-vivo Nitrite 0.01 to 10μM +/- deoxygenated and oxygenated erythrocytes +/- C-PTIO	Healthy	Flow cytometry with ADP, collagen and thrombin agonists	Nitrite alone had no effect on platelet p-selectin expression. The addition of deoxygenated erythrocytes significantly reduced the expression of p-selectin after stimulation with ADP, Collagen and thrombin.
Jones et al 2014[143]		Patients received 1.8μM sodium nitrite or placebo via intra-coronary injection at the time of P-PCI, ex-vivo platelet function	STEMI patients undergoing P-PCI	Whole blood aggregometry (Multiplate, Roche diagnostics), flow cytometry assessment of p-selectin expression	Reduced p-selectin platelet expression and whole blood aggregation in response to ADP (10μmol/L). At 30 mins, 4 hrs, 24 hrs and 6 months

Table 9: Summary table of contemporary studies focusing on the effect of augmented nitrite concentration and the effect on platelet function in humans

Summary table of contemporary studies focusing on the effect of augmented nitrite concentration and the effect on platelet function in humans. Abbreviations: LTA - light transmission aggregometry, C-PTIO – Carboxy-PTIO potassium salt (NO scavenger), ADP – Adenosine diphosphate, P-PCI – Primary percutaneous coronary intervention

There are a limited number of animal studies looking at nitrite supplementation and its effect on platelet reactivity. [188] Until recently the literature did not contain any trials looking at the effect of nitrite infusions on platelet function in patient populations with acute coronary syndromes (NSTEMI / STEMI), recently Jones et al, [189] published some platelet function data in a study that used intracoronary

sodium nitrite treatment in patients receiving P-PCI in STEMI (Table 9), This study did not reach its primary or secondary endpoints in terms of reperfusion injury protection but did show some effects on platelet function in the period after P-PCI. Principally the authors published a reduction in both ADP mediated aggregation and ADP mediated P-selectin expression on platelets. As will become evident in this chapter, the use of, and interpretation of these platelets assays has many pitfalls (Page 159: Platelet sub-study). Sensitive platelet assays should be interpreted with caution in a population undergoing super-acute coronary intervention during a disease process which has a strong influence on platelet activation / aggregation, whilst having acute and large doses of potent antiplatelet drugs. We will discuss the Jones et al paper in the discussion section of this chapter.

Platelet inhibition in NSTEMI patients

Platelet inhibition in NSTEMI patients is a fundamental aspect of the initial medical management. Up until very recently this has been achieved with a combination of aspirin and clopidogrel. Aspirin is an irreversible inhibitor of cyclo-oxygenase-dependent platelet aggregation, its efficacy within acute coronary syndrome patients was proven in one of the first large scale clinical trials within cardiology, ISIS-2. [190] Clopidogrel is an irreversible antagonist of the platelet P2Y12 receptor that is activated by ADP in a positive feedback loop following degranulation of platelets.

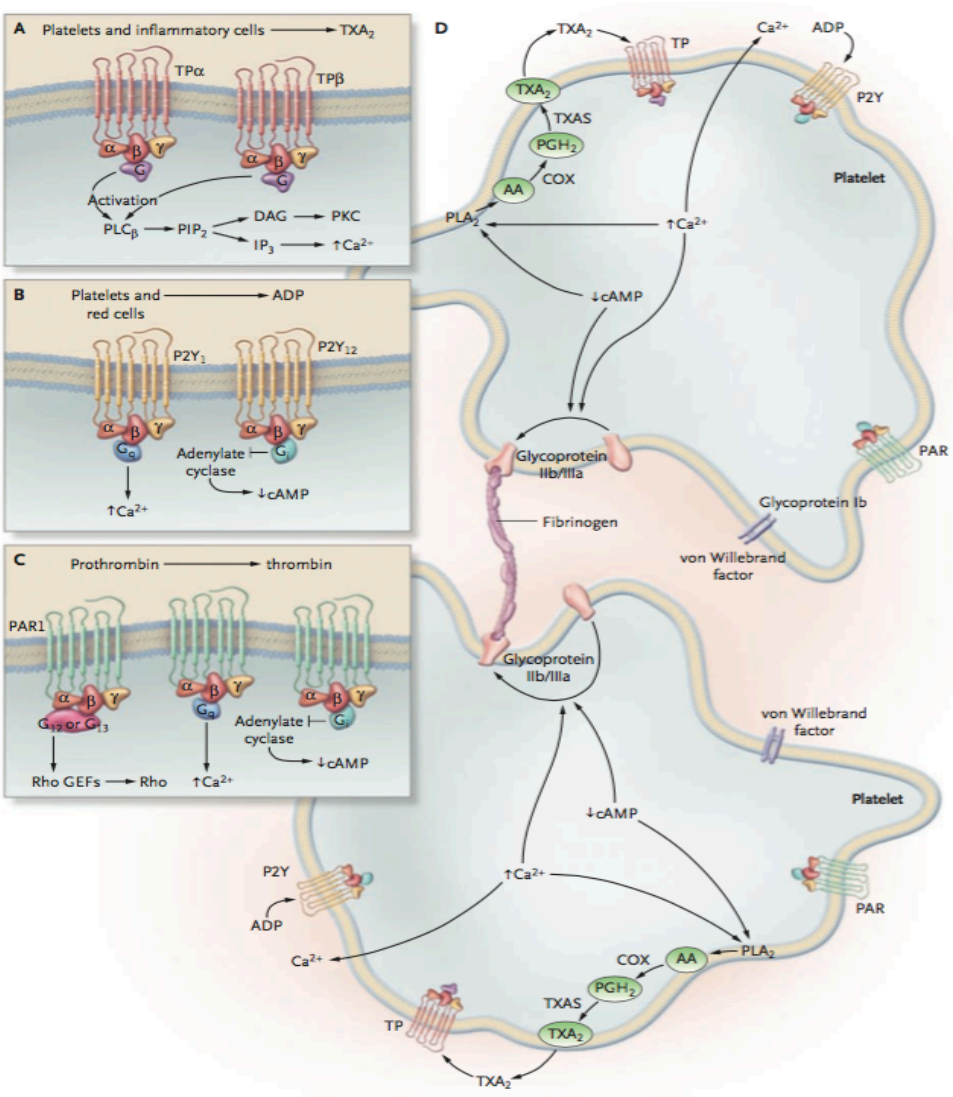

Figure 19: Platelet activation: A,B and C depict thromboxane A $_2$ (TXA_2), Adenosine diphosphate (ADP) and thrombin respectively. A/ Both TXA_2 and 12-HETE (Page 157) are synthesised from arachidonic acid (AA) via cyclooxygenase (COX). It causes a rise in intracellular Ca^{2+} via inositol triphosphate. TXA_2 can diffuse across the membrane to activate other platelets. B/ Activated platelets release ADP via α-granule release, it stimulates $P2Y_{12}$ and $P2Y_1$ receptors on platelets causing further intracellular calcium increase via cAMP. ADP and its key receptor $P2Y_{12}$ amplify and sustain platelet activation. C/ Thrombin release at sites of vascular injury mediates both fibrin generation and is the most potent platelet activator. It acts via the G-protein linked protease activated receptors (PAR1 to PAR4) which are activated following peptide cleavage off thrombin. Via 3 key G-proteins, thrombin initiates shape change (G_{12}/G_{13} – Rho), increase intracellular Ca^{2+} (G_q and G_i). D/ Depicts inside out platelet activation; specific emphasis on initial activation causing reduction in cAMP and increased Ca^{2+} as described in A,B and C. This then leads to a number of changes including further release of ADP and TXA_2 leading to a positive feedback loop and further activation. Glycoprotein IIb/IIIa also undergoes conformational changes allowing cross linkage within fibrin. With permission Davi et al [191]

Clopidogrel has been shown to confer a significant morbidity and/or mortality benefit in the prevention of ischaemic complications in patients with NSTEMI [192]

and/or after PCI. [193] Principally clopidogrel reduces clot formation (and occlusion of the vessel) following plaque rupture, this reduces ischaemic complications (recurrent ischaemia and / or STEMI). A significant proportion of NSTEMI patients have clinically important incomplete inhibition of $P2Y_{12}$ mediated platelet activation (up to 50%). High on treatment platelet reactivity (HTPR) in patients receiving clopidogrel has been identified as a risk factor for stent thrombosis and myocardial infarction and is inversely related to bleeding risk. [194,195] For this reason more potent and predictable platelet inhibition using newer antiplatelet agents (prasugrel and ticagrelor) in NSTEMI patients have shown clinical benefit at the expense of increased rate of bleeding. [196,197] Targeted release of NO from nitrite leading to further platelet inhibition is a very attractive target particularly in patients who may have a reduced response (secondary to a multitude of host factors) to clopidogrel.

Measuring platelet function

Platelet function testing (PFT) and in particular light transmission aggregometry (LTA) is notorious for producing a high degree of variability in results due largely to the need for conditions to be kept strictly constant in the context of multiple steps which all have the ability to "upset" platelets. [198] In a cohort of patients with variable clinical characteristics and receiving PCI at different times this is even more evident. [199] This variability of testing conditions and timing is no doubt part of the reason why multiple large scale clinical trials looking at tailored anti-platelet therapy have thus far failed to show any advantage of routine PFT in these patients in order to predict and treat partial response to clopidogrel. [200-203] All of the large-scale clinical trials are defined by their variability in method. Specifically

there is no consistency between the type of PFT assay used and the timing of PFT in relation to antiplatelet drug therapy and timing of testing around the time of PCI.

Based on a small pilot study in preparation for this thesis it was found that the greatest variability in PFT results was according to the timing of this test around the PCI itself, with large variability in platelet aggregation in response to ADP. This variability was similar to that seen and reported in patients undergoing CABG by Rinder et al. [204] Rinder found overt platelet activation confirmed with quantification of granule membrane protein 140 (gp140) by flow cytometry. The circulation of these "spent" platelets was associated with reduced aggregation in response to ADP (ex-vivo). Patients who have been on cardio-pulmonary bypass at the time of CABG appeared to have had a large activation of platelets secondary no doubt to the combination of no antiplatelet pharmacotherapy, and the mechanical insult secondary to cardio-pulmonary bypass itself. Pilot data collected during the initial preparation for this thesis indicated a significant shift in platelet function in NSTEMI patients, similar to that found by Rinder et al in CABG patients.

Inflammation and platelet function

In coronary disease, platelets are central to the relationship between inflammation, thrombosis and atherogenesis (Figure 20). The focus of this relationship is the way in which platelets interact with both endothelial cells and leukocytes (in particular monocytes). These interactions trigger a cascade of

autocrine and paracrine actions that promote development of coronary plaque and may have a role in plaque susceptibility to rupture. [205]

At points of endothelial damage and vascular lesions two key proteins are exposed to the blood; von Willibrand factor (vWF) and collagen. Platelets will then adhere to vWF via glycoprotein Ib/IX/V (GPIb/IX/V), [206] and collagen binding occurs via glycoprotein VI. [207] This is the first step in clot formation which is quickly followed by further recruitment of platelets and formation of fibrinogen bridges between pairs of platelet $\alpha_{IIb}\beta_3$ receptors.

Increasingly it is becoming evident that overt endothelial damage is not an absolute in terms of the ability of platelets to bind to the endothelium. The healthy endothelium has a number of anti-adhesive mechanisms principally these consist of the ecto-ADPase which metabolises local ADP (a platelet agonist released from α-granules), the arachidonic acid/prostacyclin pathway (PGI_2) and the NO pathway. [208] All three of these mechanisms have the potential to be disrupted; we have already described the association between endothelial dysfunction in terms of reduced NO production in patients with cardiovascular risk factors. [130] Further to this, inflammatory stimuli are capable of both down-regulating these anti-adhesive mechanisms and also upregulating the expression of adhesion molecules. [209] Inflammatory insults and expression of adhesive ligands are promoted by hyperlipidaemia and the accumulation of oxidised lipoprotein fractions.[210] The adhesive ligands expressed in this situation are p- and e-selectin as well as further expression of vWF. [211] Endothelial p-selectin-selectin binds to

the GPIbα receptor and the p-selectin glycoprotein-1 (PSGL-1), both of which enhance tethering and rolling of the platelet along the inflamed endothelium. The mechanisms of firm adhesion to the inflamed endothelium are not fully understood in-vivo, but may involve $\alpha_{IIB}\beta_3$ binding to fibrinogen, which is itself bound to the endothelium via ICAM-1 and other mechanisms. [211]

Platelets bound to endothelium are known to secrete IL-1β which appears to be the central mediator of platelet induced endothelial activation via increased IL-6 and IL-8 expression which in turn increases the expression of ICAM-1 and adhesion of monocytes to the endothelium. [209,212]

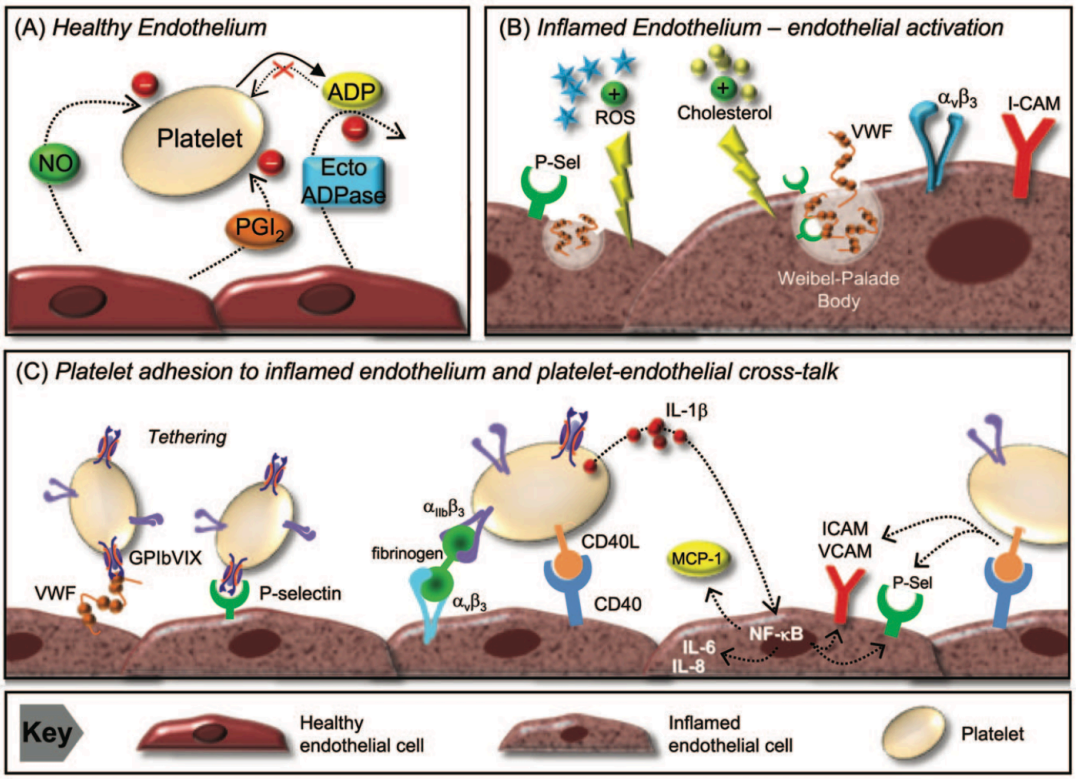

Figure 20: Interaction between the platelet and endothelium A/ Healthy endothelium and anti-adhesive mechanisms: ecto-ADPase reducing localised ADP concentrations, NO and PGI_2 which stimulate cGMP and cAMP production and thus reduce intracellular Ca^{2+} and reduce activation. B/ Hyperlipidaemia

induced endothelial activation via the action of lipoprotein particles and reactive oxygen species (ROS), leading to C/e, p-selectin and von-Willebrand factor (vWF) expression, which supports platelet tethering and rolling. Ultimately this allows platelet adhesion to endothelium via fibrinogen complexes. Once adherent platelets secrete a number of chemokines and cytokines including IL-1β which stimulates endothelial synthesis of IL-6, IL-8 and MCP-1 via NFκB. Ecto-ADPase: ecto-adenosine diphosphatase, NO: nitric oxide, MCP-1: monocyte chemoattractant protein-1. With permission[213]

Platelet leukocytes interaction

Platelets adherent to the endothelium have a central role in leukocyte recruitment and activation, this is particularly true for monocytes and T lymphocytes whose role in atherosclerosis is clear-cut. [205,214] A complex series of membrane receptor interactions are involved in monocyte and lymphocyte adhesion to the endothelium, a complex subject and one in which the reader can gain a brief and limited overview in this chapter but can gain a deeper insight by referring to the review of Hansson et al. [205]

Platelet, monocyte, and endothelial cell interaction

Once the platelet is adherent to the endothelium they also provide the initial steps of a complex multistep process to allow monocytes to be recruited to the vessel wall and ultimately differentiate into macrophages. The key steps involve selectin mediated rolling, monocyte activation, firm integrin-mediated adhesion and diapedesis. As seen in Figure 21 selectin mediated rolling occurs via the interaction of platelet bound p-selectin binding to the monocyte receptor PSGL-1 (p-selectin glycoprotein ligand-1). [215,216] This interaction secures the release of both IL-8 and TNFα by the monocyte and further firm adherence occurs via the MAC-1 (macrophage-1 antigen) to ICAM / GP interactions (intercellular adhesion molecules / Glycoprotein).

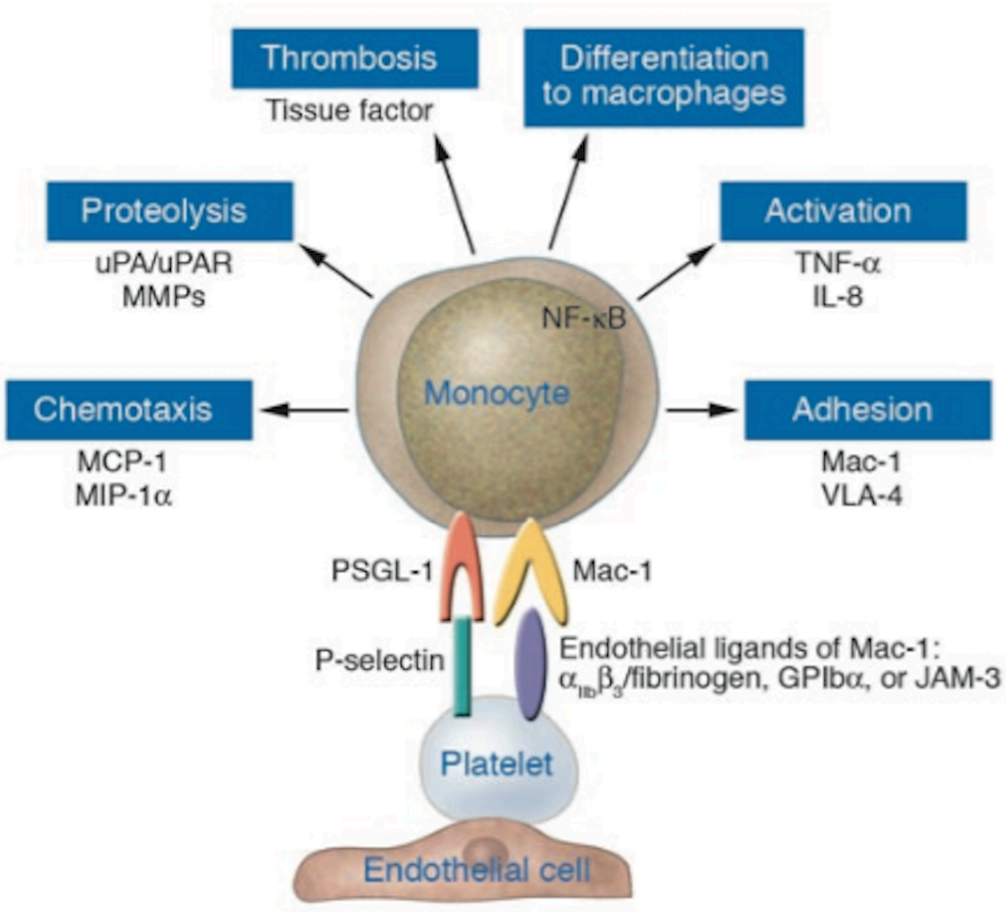

Figure 21: platelet monocyte interaction. Platelets adherent to the endothelium bind to monocytes via p-selectin glycoprotein ligand-1 (PSGL-1) and macrophage-1 antigen (MAC-1). This stimulates monocytes to release key chemokines, cytokines, tissue factor and further increased expression of adhesion molecules. This combination of activation is also accompanied by the monocyte differentiating into a macrophage. This outlines the central role of the platelet and monocyte in the atherogenic process and ultimately plaque formation. With permission from Gawaz et al [209]

Lymphocyte role in vascular inflammation

Lymphocytes and in particular T-cell lymphocytes are commonly found within atherosclerotic plaques, typically these are CD4+ (T helper) cells. On activation these CD4+ cells reveal themselves in two distinct populations; type 1 (Th1) and type 2 (Th2). Th1 cells activate macrophages and initiate a pro-inflammatory (and atherosclerotic) response whereas Th2 tend to promote a cytokine profile that is anti-inflammatory. [210] These populations are themselves controlled by a

regulatory T-cell population (Treg), via the secretion of IL-10 and transforming growth factor β (TGF-β), pro-inflammatory Th1 CD4+ cells are suppressed. See Table 10 for a list of TH1 and Th2 Interleukins and their actions.

Th1 Induced by IL-1β and IL-12 Suppressed by IL-6, IL-10 and TGF-β		**Th2** Suppressed by IFN-γ	
Cytokine	Action	Cytokine	Action
Interferon γ (IFN-γ)	Stimulate macrophages and vascular endothelial cells, inhibits Th2[210]	Interleukin-4 (IL-4)	Reduces atherosclerotic progression and plaque formation. Reduces tissue factor release. [217]
Interleukin-2 IL-2		Interleukin-5 (IL-5)	Effective marker of Th2 activity
Tumour Necrosis Factor α (TNF-α)	Stimulate macrophages and vascular endothelial cells[210]	Interleukin-6 (IL-6)	Secreted from monocytes, less when platelet activation occurs. Suppresses Th1 activated via Treg [218,219]
IL-8	Not a classic Th1 cytokine but a chemokine produced by epithelial cells which triggers firm adhesion of monocytes to the endothelium	Interleukin-10 (IL-10)	Released by activated platelets and associated with favourable outcome in ACS [218] Inhibits Th1 via downregulation of NF-κB [220]
		Interleukin-13 (IL-13)	Reduces tissue factor release. [217]

Table 10:Th1 and Th2 associated cytokines and their actions in the context of acute coronary syndromes. The Th1 / Th2 balance is itself regulated by Treg cells (CD28+ FoxP3+) principally through the release of IL-6,10 and TGF-β. Abbreviations: IL-interleukin, IFN-γ-interferon γ, TNF-α tumour necrosis factor β, Treg- T-cell regulatory.

Platelet metabolism of arachidonic acid

Metabolites of arachidonic acid play a key role in mediating thrombus formation and vessel tone in-vivo. [221] When the platelet is activated arachidonic acid concentration rises steeply as phospholipid hydrolysis occurs. [222] Arachidonic acid (AA) itself is metabolised via two distinct pathways; the first is cyclooxygenase which forms thromboxane A_2 (TXA_2) and the second is 12-lipoxygenase which produces 12-hydroperoxyeicosatetrenoic acid (12-HPETE) which then forms 12-hydroxyeicosatetraenoic acid (12-HETE). [223] Both TXA_2 [224,225] and 12-HETE [226,227] have proven roles in mediating thrombus formation via platelet activation. During a pilot run we were unable to detect and measure free TXA_2 in serum samples, however, we were able to consistently quantify small quantities of 12-HETE. We chose to measure serum 12-HETE in our cohorts as a further platelet activation marker.

Chapter aims

This chapter contains three main sections; the first is a methodological section (platelet sub-study) in a separate cohort of patients created to first establish the PFT assay in terms of limitations and optimisation in NSTEMI patients around the time of PCI.

The second section is a further study of the NSTEMI and $NSTEMI_{inf}$ cohorts specifically looking at baseline endogenous NO synthesis and the augmentation of nitrite and the influence on platelet function.

The third section analyses the cytokine profiles of the NSTEMI and $NSTEMI_{inf}$ cohorts and the association between pro-inflammatory status, endothelial function and platelet reactivity.

METHODS

Two principal studies on 3 different cohorts of similar patients are described in this chapter. Cohort 1 is the platelet sub-study of high risk NSTEMI patients. Cohorts 2 and 3 are the NSTEMI and $NSTEMI_{inf}$ population described in Chapter 3: Results 1 and Chapter 4: Results 2.

Platelet sub-study

Study patients

We conducted a prospective observational study on high-risk NSTEMI patients who received clopidogrel at diagnosis and for a minimum period of 5 days before PCI was conducted.

Inclusion criteria

- Patients with an index admission following an NSTEMI (troponin positive and / or typical ECG changes with consistent clinical history), and a
- GRACE score >140 who subsequently went on to have significant disease treated by PCI and stent implantation.

Exclusion criteria

- Patient who received intravenous antiplatelet agents (glycoprotein IIb/IIIa inhibitors (GPI) or other) and
- Renal dysfunction (eGFR <40mls/min).

Local research ethics approval was granted and written informed consent was obtained from each patient.

Platelet sub-study protocol

All patients received a loading dose of aspirin (300mg) and a maintenance dose of 75mg once daily initiated at the same time as the clopidogrel unless this was already taken prior to their hospital admission. To exclude any influence of variable clopidogrel loading only patients that had received a 600mg loading-dose followed by a minimum of 5 days of maintenance therapy (75mg OD) were recruited. [228] Timing of dose was also corrected for by ensuring the third sampling point was exactly (+/- 1hr) 24 hours after the baseline measurement and drugs received at the same time each day.

All patients received a weight-adjusted dose of intravenous unfractionated heparin 70 – 100 Units/kg bolus pre-procedurally. All blood samples were taken from the same large calibre venous sheath in the antecubital fossa and 3 samples collected in the 24-hour period (immediately pre- and post-PCI and 24hrs post-PCI). Samples were collected into a depressurised vacutainer (hirudin for MEA, citrate for flow cytometry, EDTA for mass spectrometry and ELISA). The 24 hour sample was taken at 24 hours (+/- 1 hour) and the morning dose of clopidogrel was administered at 8am on both days to ensure there a was no influence of dose timing on test results. The PCI itself was carried out with no restrictions and as

per the operator preference. Operators were blinded to the results of the platelet function testing.

NSTEMI and NSTEMI$_{inf}$ study protocol (platelet function and cytokine analysis)

The second part of the study describes platelet function and cytokine analysis of the NSTEMI and NSTEMI$_{inf}$ populations from Chapter 4: Results 2.

Study populations

Selection of patients, inclusion and exclusion criteria can be seen in general methods Page 52.

Cardiac catheterisation laboratory protocol

Full method details are seen in the general methods Page 55.

Measurement of Nitric Oxide metabolites

Full method details are seen in the general methods Page 61.

Impedance aggregometry

Whole blood aggregation was determined using Multiple Electrode Aggregometry (MEA), Multiplate Analyzer Figure 22 (Roche Diagnostics), which detects the change in electrical impedance due to the adhesion and aggregation of platelets on two independent electrode-set surfaces in the test cuvette. As per the manufacturer's recommendations, hirudin and Adenosine Diphosphate (ADP) were used as the sample anticoagulant and agonist respectively. A 1:2 dilution of whole blood and 0.9% NaCl was stirred at 37 °C for 3 min in the test cuvettes, ADP was added (final concentration 6.5 µM) and the increase in impedance was recorded continuously for 6 min. The process was repeated using Thrombin

Receptor Activating Peptide 6 (TRAP-6), (final concentration 32 μM), in order to assess platelet aggregation independently of clopidogrel. Each test cell generates two independent values, which are averaged and expressed as the area under the curve of the aggregation tracing (AUC). Consequently, manufacturer's software performs a Pearson's correlation coefficient on the two measurements and the result is rejected if the coefficient is <98% or the difference to the mean curve is >20%. AUC was reported as arbitrary units and a cutoff of 500 U was defined as the threshold above which a patient was characterised as having HTPR ("non-responder"), according to the mean of two previously proposed cut-offs. [195,229]Following a resting period of 20 minutes trained laboratory technicians processed the samples.

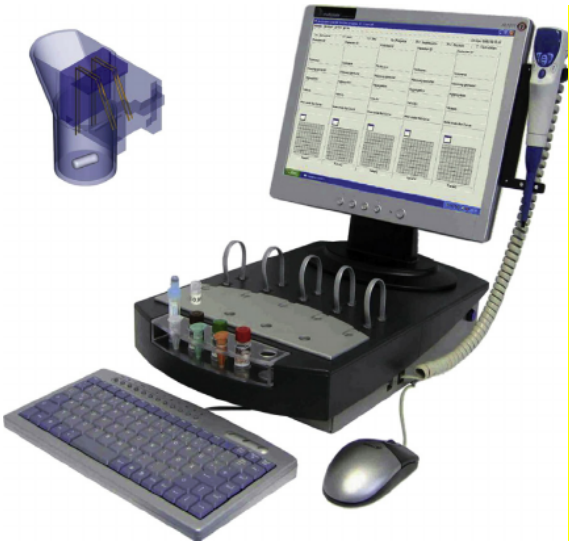

Figure 22: Multiplate platelet analyser. (Roche diagnostics). A five channel impedance analyzer, (inset) disposable cuvettes with electrodes and stir bar.

Flow cytometry assessment of Platelet bound p-selectin

Sample preparation:

20µl of whole blood was initially diluted in 50µl Tyrodes buffer (137mM NaCl, 2.68mM KCl, 1mM MgCl2, 1.8mM CaCl2, 0.2mM Na2HPO4, 12mM NaHCO3, pH7.4) and subsequently incubated in 10µl fluoresceine-isothiocyanate-(FITC)-mount-Anti-Human CD61, 20µl of allophycocyanin-(APC)-Mount-Anti-Human CD62P antibody or (APC)-Mount-IgG1 isotype control (BD Biosciences, Pharmigen™) and incubated at room temperature for 15 min. Incubation was terminated by fixation of 20µl of the reaction mixture to 1% paraformaldehyde. Samples were diluted four-fold in Tyrodes buffer prior to analysis. The Anti-CD61 antibody is targeted against the constitutively expressed IIIa domain of the GpIIb/IIIa receptor and identifies resting and activated platelets [230,231].

Sample analysis:

The samples were analysed by CyAn™ ADP-Analyzer (Beckman Coulter), Figure 23 on the same or the following day. Platelets were initially identified and gated at 50,000 events according to their side and forward scatter characteristics and consequently gated for CD61 positivity. Samples containing non-specific IgG1-APC antibodies were used as a negative control and to set an analysis marker so that 99.9% of the total population of platelets to be defined as negative. Platelets with fluorescence intensity higher that this value for anti-CD62P-APC antibody were defined as p-selectin positive and were expressed as a percentage of positive platelets compared to the total CD61 positive population. To correct for fluctuations of total platelet count at the different time-points, the ratio of platelet to total cells in the pre-PCI sample was used to correct the number of CD61 positive platelets in the subsequent time-point measurements.

Figure 23: CyAn flow cytometer. (Beckman Coulter), with Summit software. Capable of 11 simultaneous parameters (forward and side scatter with 7-9 colours and simultaneous width, peak, area and log on each).

Soluble p-selectin quantification

MSD Human Ultra-Sensitive p-selectin ECL assay (Electrochemiluminescence technique. MSD: Meso Scale Discovery, Gaithersburg, MD) was used as per manufacturers recommendations. A human anti-p-selectin coated MULTI-ARRAY 96-well small spot plates was initially blocked with bovine albumin followed by addition of serum samples and detection antibody. p-selectin calibration standards were run in duplicate on the same plate under the same conditions. Following incubation and washing the read buffer was added and the 96 well plate was analysed on an ImageSector 6000. Results were analysed using MSD workbench software. Samples were run as duplicates, and measurements with coefficient of variation less than 15% were considered acceptable.

Assessment of 12-HETE by lipid extraction and reverse phase LC/MS/MS

Lipid Extraction

For analysis of lipids in human plasma, 5 ng of 12-HETE-d8 were added to the samples before extraction, as an internal standard. Lipids were extracted by adding a solvent mixture (1 mol/L acetic acid, isopropyl alcohol, hexane (2:20:30, v/v/v)) to the sample at a ratio of 2.5 ml to 1 ml sample, vortexing, and then adding 2.5 ml of hexane. [232] After vortexing and centrifugation, lipids were recovered from the upper hexane layer. The samples were then re-extracted by addition of an equal volume of hexane. The combined hexane layers were dried and the aqueous layer was re-extracted by adding 3.75 ml of a chloroform and methanol mixture (1:2, v/v), vortexing, then adding 1.25 ml chloroform. After vortexing, 1.25 ml water was added and the mixture was vortexed again and centrifuged. Lipids were recovered in the chloroform layer, [233] and dried; the combined lipid extracts were consequently analysed for free 12-HETE using LC-MS/MS as described below.

Reversed phase LC/MS/MS of free HETE.

For 12-HETE quantitation lipids were separated on a C18 Spherisorb ODS2, 5 µm-1, 150 x 4.6 mm column (Waters, Hertfordshire, UK). The mobile phase was composed of water: acetonitrile: acetic acid (75:35:0.1, solvent A) and methanol: acetonitrile: acetic acid (60:40:0.1, solvent B), with flow rate 1 ml.min-1. Solvent B was increased from 50 % to 90 % over 10 min, held for 20 min, then returned to 50 %.[232] MS was performed using a Sciex 4000 Q-Trap, using DP -85V, CE -20V and a dynamic fill time monitoring the parent to daughter m/z (m/z 319.2 to 179.1, m/z 327.2 to 184.1 for 12-HETE or 12-HETE-d8, respectively). 12-HETE was

identified and quantified using 12-HETE and 12-HETE-d8 standards run in parallel under the same conditions.

Cytokine quantification

MSD Human Ultra-Sensitive TH1/TH2 10-plex assay kit (MSD: Meso Scale Discovery, Gaithersburg, MD) was used as per manufacturers recommendations. This 10-plex 96-well plate allowed the quantification of Interferon-γ (IFN-γ), Interleukin (IL)-1β, IL-4, IL-8, IL-10, IL-13 and tumor necrosis factor-α (TNF-α). 25µL of diluent 2 was dispensed into each well and incubated for 30minutes. 25µL of the serum sample or calibration standard was then pipetted into each well and incubated with shaking at 800rpm for 2 hours. The plate was then washed 3 times with PBS-T. 25µL of detection antibody was added to each well and incubated for 2 hours. The plate was then washed again 3 times with PBS-T. 150µL of 2 x Read buffer was then added to each well and the plate was analysed on an ImageSector 6000. Results were analysed using MSD workbench software. Samples were run as duplicates, and measurements with coefficient of variation less than 15% were considered acceptable.

RESULTS

Platelet sub-study

A total of 74 ACS patients were consented for the study; however 30 were excluded as they did not undergo PCI following coronary angiography. Two patients were further excluded due to peri-procedural glycoprotein IIb/IIIa inhibitor (GPI) use. The remaining 42 patients completed the study in full with no peri-procedural major complications.

The complete group of patients (42), showed a consistent and significant decrease in platelet reactivity in the first 24 hours after PCI as measured by MEA (Figure 24). Using ADP as an agonist the mean value of platelet aggregation pre-PCI was 499.1 AU (± 46.3), with 40.5% (17 patients) exhibiting HTPR. Immediately post PCI, this value decreased to 407.6 AU (±37.7) translating to 26.2% (11 patients) with HTPR ($p > 0.05$). At 24 hours post-procedure, platelet aggregation decreased to 269.1 AU (±24.6) further reducing the number of patients who appeared to have HTPR to 4.7% (2 patients). The mean change in platelet aggregation from Pre-PCI to 24 hrs Post-PCI was 230.0 AU (CI 104.5 to 355.6, $p=0.0002^{ANOVA}$). (Table 11)

When the population was split between those with and without HTPR, the group with HTPR had a mean reduction in ADP induced aggregation from Pre-PCI to 24 hrs Post-PCI of 379.1 AU (52%) (CI 255.9 to 502.2, $p=0.0001^{ANOVA}$). In comparison,

patients without HTPR had a proportionately smaller reduction of 98.6 AU (33.5%) over 24hours (CI 23.04 to 174.1, $p=0.0028^{ANOVA}$).

Baseline pre-PCI samples were collected before heparin administration in 32 out of the 42 patients. There was no significant difference between pre-PCI measurements collected before and after heparin administration; thereby demonstrating that heparin did not confound ADP-induced platelet aggregation as measured by MEA (Pre heparin mean 476.2 AU SEM 68.08, Post heparin mean 520.3 AU SEM 66.49. p=0.1795 CI -22.6 to 110.1).

The changes in ADP-induced platelet aggregation illustrated in Figure 24 were reflected to a lesser extent (and in proportion) with TRAP as the agonist for aggregation. For the total population the mean value of aggregation pre-PCI was 1000 AU (±51.6) reducing to 843.8 AU (±42.1) post-PCI ($p < 0.05^{ANOVA}$). At 24 hours post-PCI, aggregation further decreased to 792.0 (±41.0) equating to a mean reduction from Pre-PCI to 24 hrs Post-PCI of 208.4 AU (20.8%) (CI 114.1 to 302.6, $p=0.0002^{ANOVA}$), Figure 24.

TRAP induced platelet aggregation pre- and post-PCI changed to a greater extent in patients with HTPR compared to those without HTPR. Notably, in patients with HTPR, TRAP-induced aggregation had a larger mean numerical reduction in comparison to patients without HTPR (214.1 AU vs 203.9 AU respectively). Individually these reductions were both statistically significant. However,

between groups, comparisons did not differ significantly in relation to the initial absolute pre-PCI value.

n=42	Pre-PCI (SEM)	Post-PCI (SEM)	24hrs post PCI (SEM)	P ANOVA a/ Pre vs Post PCI **b/ Pre vs 24 hr PCI** c/ Post vs 24 hr PCI	95% CI of diff
ADP aggregation (AUC)	499.1 ± 46.3	407.6 ± 37.7	269.1 ± 24.6	*a/ p>0.05* *b/ p=0.0002** *c/ p<0.05**	*a/ -34.1 to 217* *b/ 104.5 to 355.6* *c/ 13 to 264.1*
TRAP aggregation (AUC)	1000 ± 51.6	869.2 ± 42.1	792.0 ± 41.0	*a/ p<0.05** *b/ p=0.0002** *c/ p>0.05*	*a/ 0.74 vs 261.4* *b/ 114.1 to 302.6* *c/ -29.5 to 184.1*
Soluble p-selectin (ng/ml)	32.1 ± 1.7	31.8 ± 1.9	36.1 ± 3.0	*a/ p>0.05* *b/ p=0.0225* *c/ p>0.05*	*a/ -3.7 to 3.7* *b/ 0.82 to 10.0* *c/ -0.9 to 12.0*
% p-selectin +ve plts ***n=16***	2.7 (1.4-5.0)	2.5 (2.1-3.6)	2.6 (2.0-5.5)	*a/ ns* *b/ ns* *c/ ns*	

Table 11: Summary table of key findings for aggregometry, soluble and platelet bound p-selectin (ALL patients).

Row 1 and 2 ADP and TRAP induced aggregation (AU), Row 3 soluble p-selectin (ng), row 4 percentage of platelets expressing p-selectin. Mean ± SD or median (IQR). P^{ANOVA} values with Tukey's multiple comparison (a= Pre-PCI vs Post-PCI, b=Pre-PCI vs Post-PCI). Except were not normally distributed or missing values prevent (Soluble p-selectin). Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention, ADP: adenosine diphosphate, TRAP: thrombin receptor agonist peptide, AUC: area under the curve

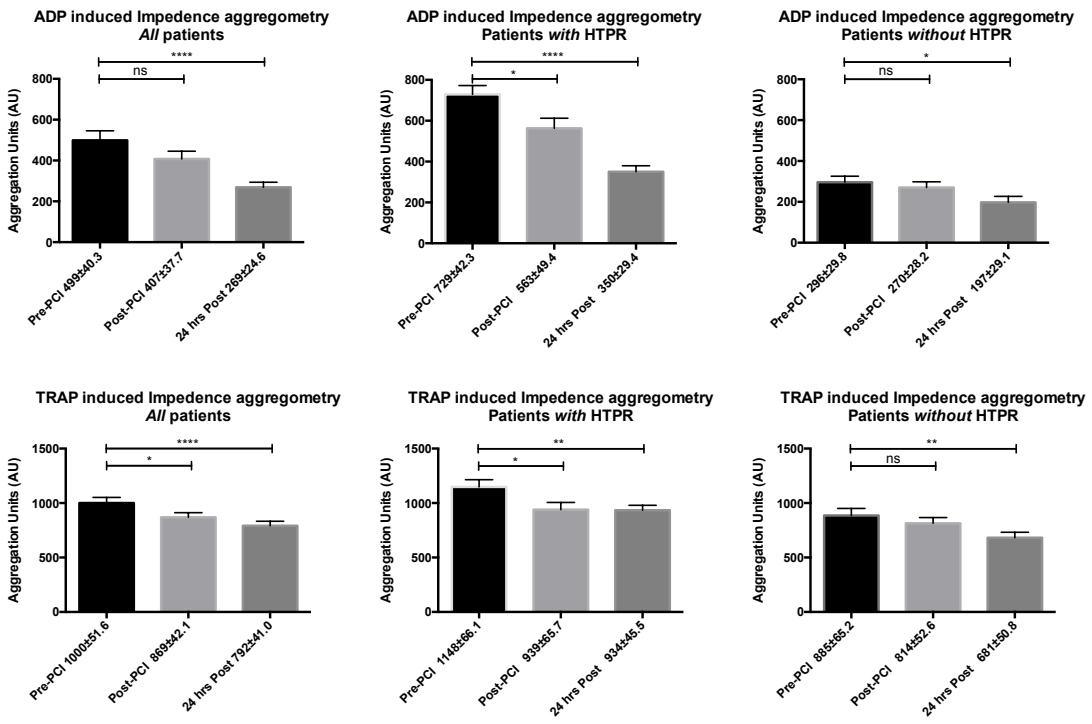

Figure 24: ADP (adenosine diphosphate) and TRAP (thrombin receptor agonist peptide) induced aggregation.

ADP (adenosine diphosphate) and TRAP (thrombin receptor agonist peptide) induced aggregation as measured by MEA (multi electrode aggregometry). Each agonist response in ALL patients those with HTPR (high on treatment platelet reactivity) and those without HTPR. Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention, ADP: adenosine diphosphate, TRAP: thrombin receptor agonist peptide

Despite a significant reduction in both ADP and TRAP induced aggregation we saw a consistent and significant rise in soluble p-selectin (4 ng/ml increase, CI 0.82 to 10.0, p=0.0225) between pre-PCI and 24hrs post-PCI (Table 11), mainly in the HTPR group (Figure 26). Overall the consistent levels of p-selectin found across all time-points suggest ongoing platelet activation in the face of a reduction in ADP and TRAP induced aggregation ex-vivo. In addition, ADP-induced platelet aggregation pre-PCI positively correlated with 24 hours post-PCI p-selectin levels (Pearson r 0.391, CI 0.071 to 0.64, p=0.0185), (Figure 25), suggesting that high baseline platelet reactivity pre-procedurally predicts activation after 24 hours.

There is no correlation when we compare 24hr post-PCI ADP induced aggregation to the same 24 hr post-PCI p-selectin concentration.

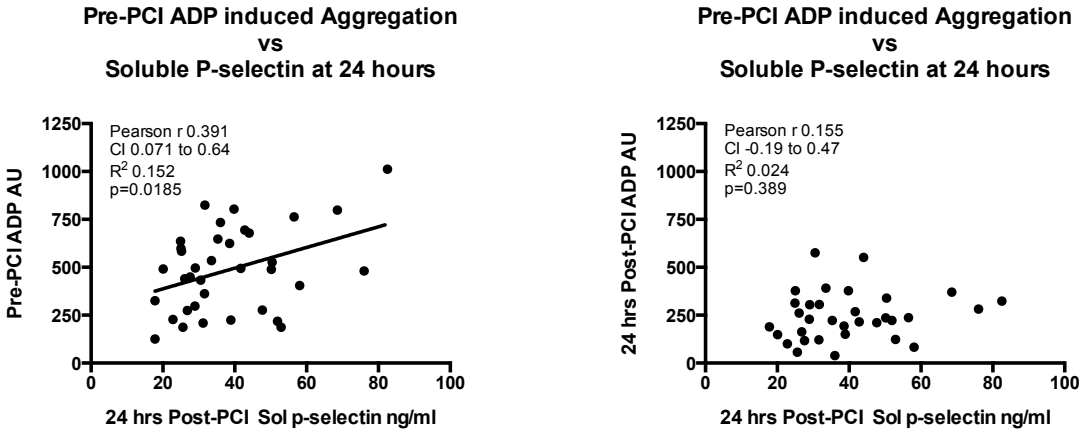

Figure 25: Correlation between pre-PCI ADP induced aggregation and soluble p-selectin.

Correlation between pre-PCI ADP induced aggregation and soluble p-selectin measured at 24hrs post-PCI (left panel), and 24hr post-PCI ADP induced aggregation and p-selectin measured at 24hrs (right panel). Pre-PCI ADP correlates with degree of platelet activation as indicated by soluble p-selectin concentration at 24hrs (Pearson r 0.391, R^2 0.152, p=0.0185). Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention

Since soluble p-selectin in plasma may be derived from endothelial cells as well as platelets, [234] we measured platelet bound p-selectin in a random population of the study's cohort (n=16). An overall increase in platelet bound p-selectin between pre-PCI and 24 hours post-PCI suggests that the soluble p-selectin measured may originate from platelets (Figure 28). Overall, median (IQR) percentage of platelets that were CD62P positive were pre-PCI, 2.7% (3.6) of platelets express p-selectin, 2.5% (1.5) immediately post-PCI and at 24 hours post PCI 2.6% (3.5) of platelets continue to express p-selectin. Whilst these numerical changes do not reach significance the importance of ongoing expression of platelet bound p-selectin in this context should be noted.

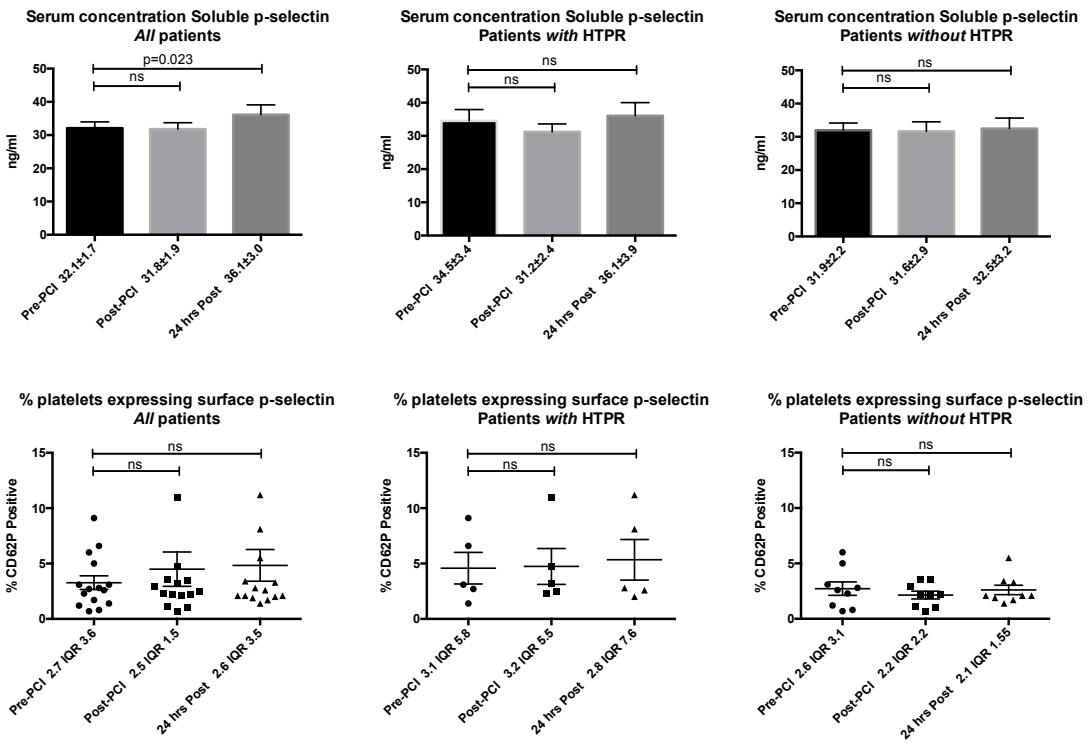

Figure 26: (top panel) Soluble p-selectin concentration pre, post and 24hrs post-PCI showing ongoing p-selectin secretion throughout the 24hr period, mean±SEM.

Figure 27: (bottom panel) Platelet bound p-selectin expressed as percentage of platelets that were CD62P positive median (IQR) (bottom row).

All patients (left column), those with HTPR (middle column) and those without HTPR.

Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention, CD62P: p-selectin protein present on platelet membrane

Specifically, in patients with HTPR the percentage of platelets CD62P positive remained constant throughout the 24-hour period, pre-PCI 3.1% (5.8), post-PCI 3.2%(5.45) and 24hrs post-PCI 2.8%(7.35). Again, suggesting both raised percentage at baseline and continued CD62P expression throughout the 24-hour period in a cohort of patients with significant and large reductions in ADP induced aggregation (Table 11 and Figure 27: (bottom panel) Platelet bound p-selectin expressed as percentage of platelets that were CD62P positive median (IQR) (bottom row).Figure 27).

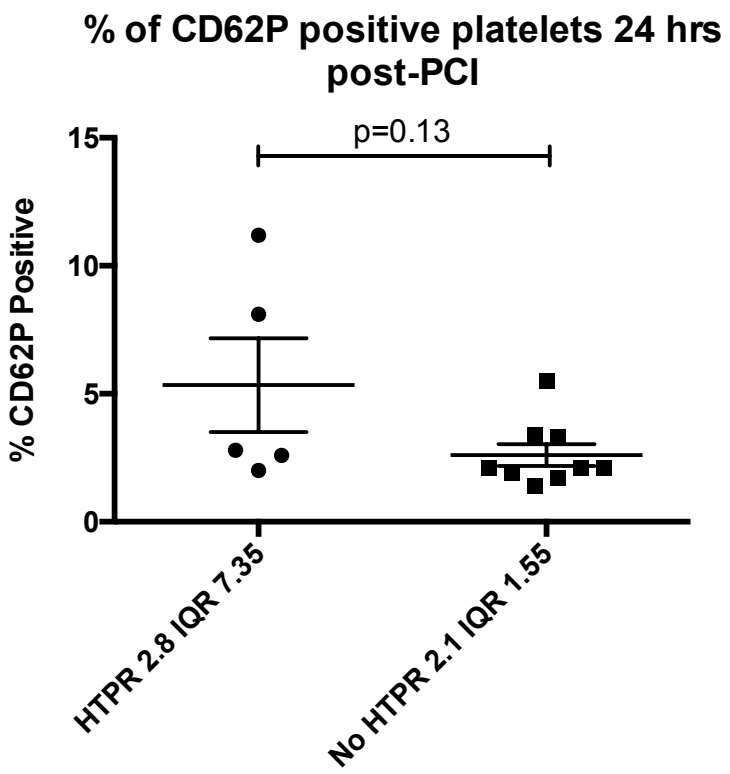

Figure 28: Percentage of platelets positive for CD62P at 24 hrs post-PCI

Patients with HTPR and without HTPR (high on treatment platelet reactivity). HTPR patients had a numerically higher percentage of CD62P positive platelets that did not reach significance. In the face of large decreases in ADP induced aggregation any ongoing expression of CD62P on platelets is significant. Expressed as median (IQR), Wilcoxin test for paired data. Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention

12-HETE is produced by platelet 12-lipoxygenase metabolism of arachidonic acid and appears to be both a marker and mediator of platelet aggregation. [227] In patients with HTPR, median levels of 12-HETE increased from 0.13 ng/ml (IQR 0.88) to 0.92 ng/ml (IQR 1.3) over the 24 hour period post-PCI (p=0.017),

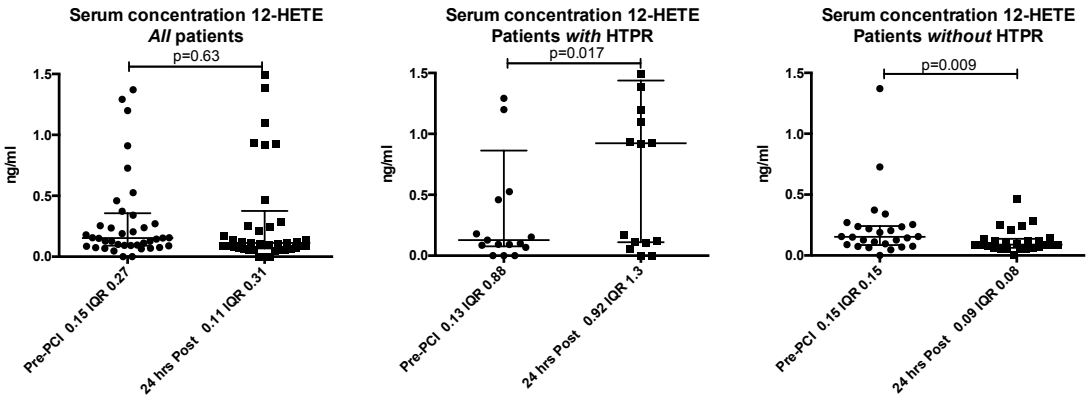

Figure 29. On the other hand, in patients without HTPR, median levels of 12-HETE decreased from 0.15 ng/ml (IQR 0.15) to 0.09 ng/ml (IQR 0.08) over the same period (p=0.009).

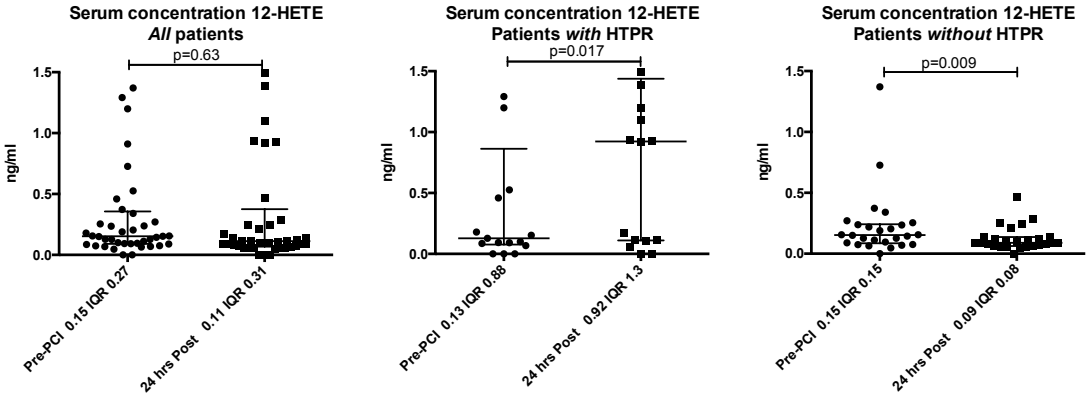

Figure 29: Serum 12-HETE concentration

All patients (left panel), HTPR patients (middle panel) and patients without HTPR (right panel). Data points are pre-PCI and 24 hrs post-PCI. Data expressed as median (IQR), ng/ml. Wilcoxin test used throughout. Abbreviations: 12-HETE: 12- Hydroxyeicosatetraenoic acid, HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention

NSTEMI and NSTEMI$_{inf}$: platelet function

Platelet aggregation: ADP

Overall when using ADP as an agonist there is no significant difference between the NSTEMI and the NSTEMI$_{inf}$ cohorts in terms of ex-vivo aggregation using MEA.

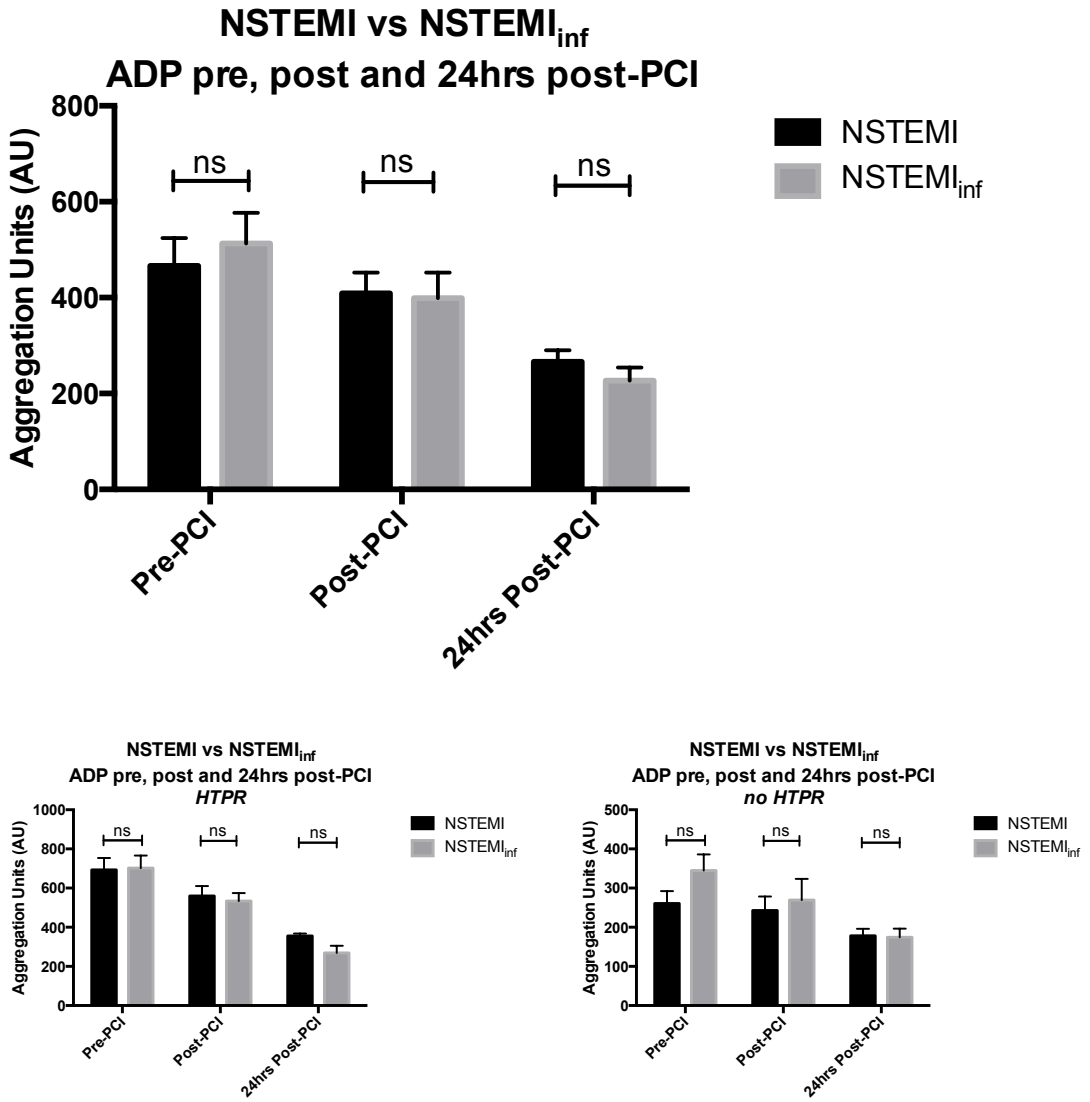

Figure 30: Comparison of NSTEMI vs NSTEMIinf – ADP induced aggregation pre, post and 24 hrs post-PCI.

Expressed as mean ± SEM, p^{ANOVA}. Panel A: All patients, panel B & C those with and without HTPR (high on treatment platelet reactivity). Nitrite augmentation does not appear to influence ADP aggregation. We see the same highly significant reduction throughout the 24 hour period as per the platelet sub-study. Abbreviations: HTPR: High on treatment platelet reactivity, PCI: percutaneous coronary intervention

When comparing NSTEMI with $NSTEMI_{inf}$ at each of the three time-points there was a very similar drop seen between the groups. The reduced ex-vivo response to ADP seen over this 24 hr period mimics that seen in the platelet sub-study. From pre to 24hrs post-PCI the platelet sub-study ADP response dropped from 499.1 ± 46.3 to 269.1 ±24.6 AU ($p^{anova}<0.005$), similar to both the NSTEMI cohort 466.4 ± 266 to 266.7 ± 101.6 ($p^{anova}<0.05$). and the $NSTEMI_{inf}$ cohort 512.9 ± 239.4 to 226.7 ± 96.5 AU ($p^{anova}<0.005$), Figure 30: Comparison of NSTEMI vs NSTEMIinf – ADP induced aggregation pre, post and 24 hrs post-PCI.Figure 30.

When both cohorts are split into those with HTPR and those with no HTPR, again we see no change in ADP induced aggregation. Visually the group with no HTPR (right panel Figure 30), appear to have increased platelet aggregation in response to ADP ($NSTEMI_{inf}$ cohort). It should be noted that the pre-PCI level (pre nitrite infusion) is raised in comparison to the NSTEMI cohort. Whilst it's easy to assume a pattern these variation are probably reflecting random variability given the low n number (The whole group was 15 but this sub-population is only 7).

Trans-coronary platelet activation

If nitrite is being reduced to NO the site we would expect this to happen is in the coronary circulation where haemoglobin saturation is the lowest and localised ischaemia is likely to be present. Below is a comparison of the two cohorts in terms of ADP induced platelet aggregation from aortic / coronary sinus (CS) samples taken pre and post-PCI.

Mean aortic aggregation was 502±261 vs 494.3±306 AU ($p^{ANOVA}>0.05$) for NSTEMI and $NSTEMI_{inf}$ respectively. Pre-PCI CS was also very similar 449.7±233 vs 424.8±205.4 AU ($p^{ANOVA}>0.05$), respectively. As an additional measure in the $NSTEMI_{inf}$ cohort a further pre-PCI sample was taken following 5 minutes of nitrite infusion (mean CS nitrite concentration: 1208±502.7nM), Figure 31. Here we see platelet aggregation in the CS go from 424.8±205.4nM pre-infusion to 456.4±231.1nM post infusion, $p^{ANOVA}>0.05$. Again, post-PCI there is very little difference between the two cohorts with a very comparable drop seen in both; 379.9±203 vs 343.6±181.2 nM respectively.

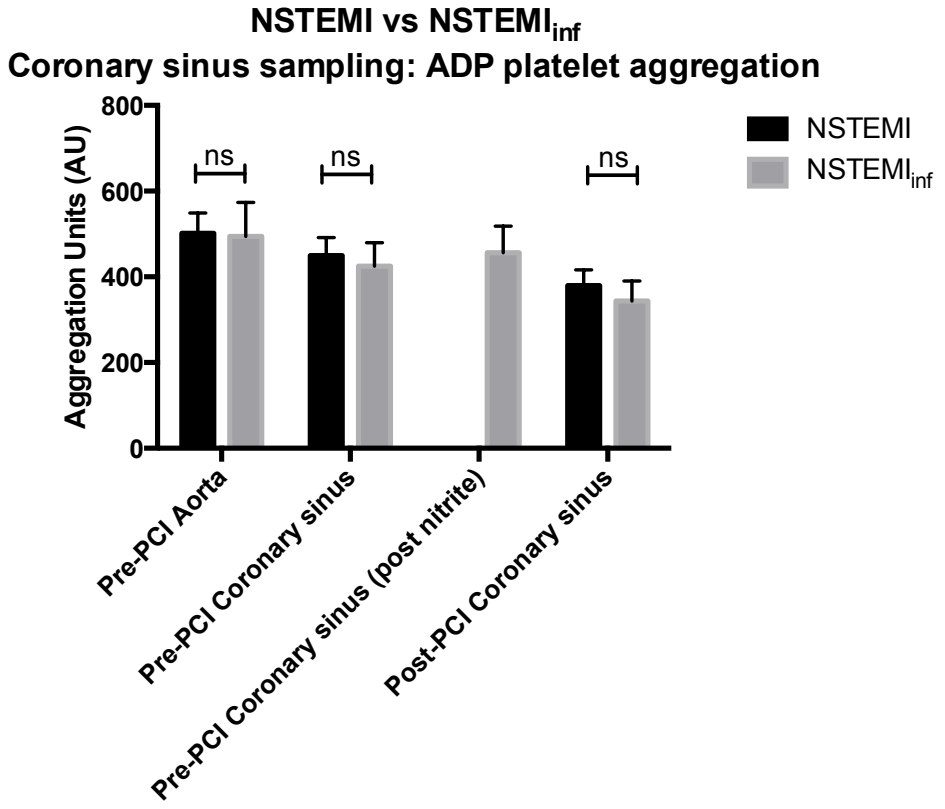

Figure 31: Comparison of central venous vs coronary sinus platelet aggregation.

(ADP) paired comparison (NSTEMI vs NSTEMI$_{inf}$), at anatomically and temporally identical points. No difference is seen between the two cohorts at all points. NSTEMI$_{inf}$ cohort has an additional coronary sinus (unpaired) sample pre-PCI and 5 minutes following sodium nitrite infusion.

Platelet aggregation: TRAP

Using TRAP as an agonist can help to indicate if the changes seen in aggregation to ADP are secondary to a platelet wide reduction (systemic) in aggregation or specific to ADP and $P2Y_{12}$ receptor. Platelet aggregation using TRAP as an agonist also reveals a similar pattern to the platelet sub-study cohort. Between the NSTEMI and NSTEMI$_{inf}$ cohorts there is no significant difference between each pair of results along the three time points. Pre-PCI mean±SD aggregation by TRAP was 998.3±318 vs 1026±313.5 AU for NSTEMI vs NSTEMI$_{inf}$ cohorts respectively. Post-PCI this reduced to 857.6±228.4 vs 912.9±297.1 AU, and 24 hrs post-PCI this further dropped in both groups to 741.2±202.3 vs 725.6±181.4 AU.

Again, in an identical manner to the platelet sub-study group we see the same significant reduction in TRAP aggregation from pre to 24 hrs post-PCI within the NSTEMI cohort 998.3 ± 318 vs 741.2 ± 202.3 ($p^{anova}<0.05$) and a similar drop in the $NSTEMI_{inf}$ cohort 1026 ± 313.5 vs 725.6 ± 181.4 (not reaching significance likely secondary to smaller cohort size), Figure 32. Comparing NSTEMI to $NSTEMI_{inf}$ even when we further split the group into those with and without HTPR there is still no effect of nitrite infusion seen on platelet function.

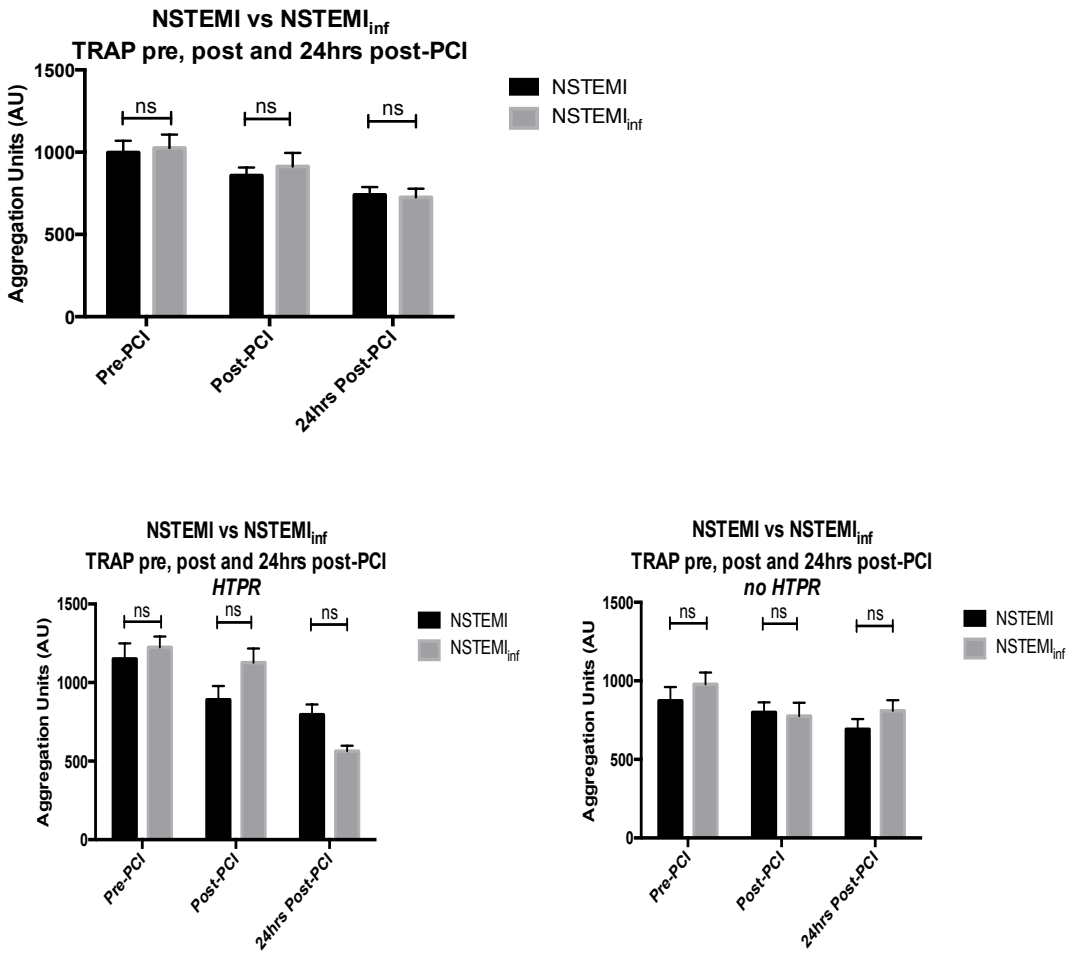

Figure 32: Comparison of NSTEMI vs NSTEMIinf – TRAP induced aggregation pre, post and 24 hrs post-PCI.

Expressed as mean ± SEM, p^{ANOVA}. Panel A: All patients, panel B & C those with and without HTPR (high on treatment platelet reactivity). Nitrite augmentation does not appear to influence ADP aggregation. We see the same highly significant reduction throughout the 24 hour period as per the platelet sub-study.

Soluble p-selectin

Between the two NSTEMI cohorts $NSTEMI_{inf}$ has significantly higher concentrations of soluble p-selectin at baseline (baseline sample taken prior to nitrite infusion). However, both cohorts show the same pattern of no net change in soluble p-selectin from pre to 24 hrs post-PCI, Figure 33. Again, this pattern does not change if the cohorts are split between those with and those without HTPR.

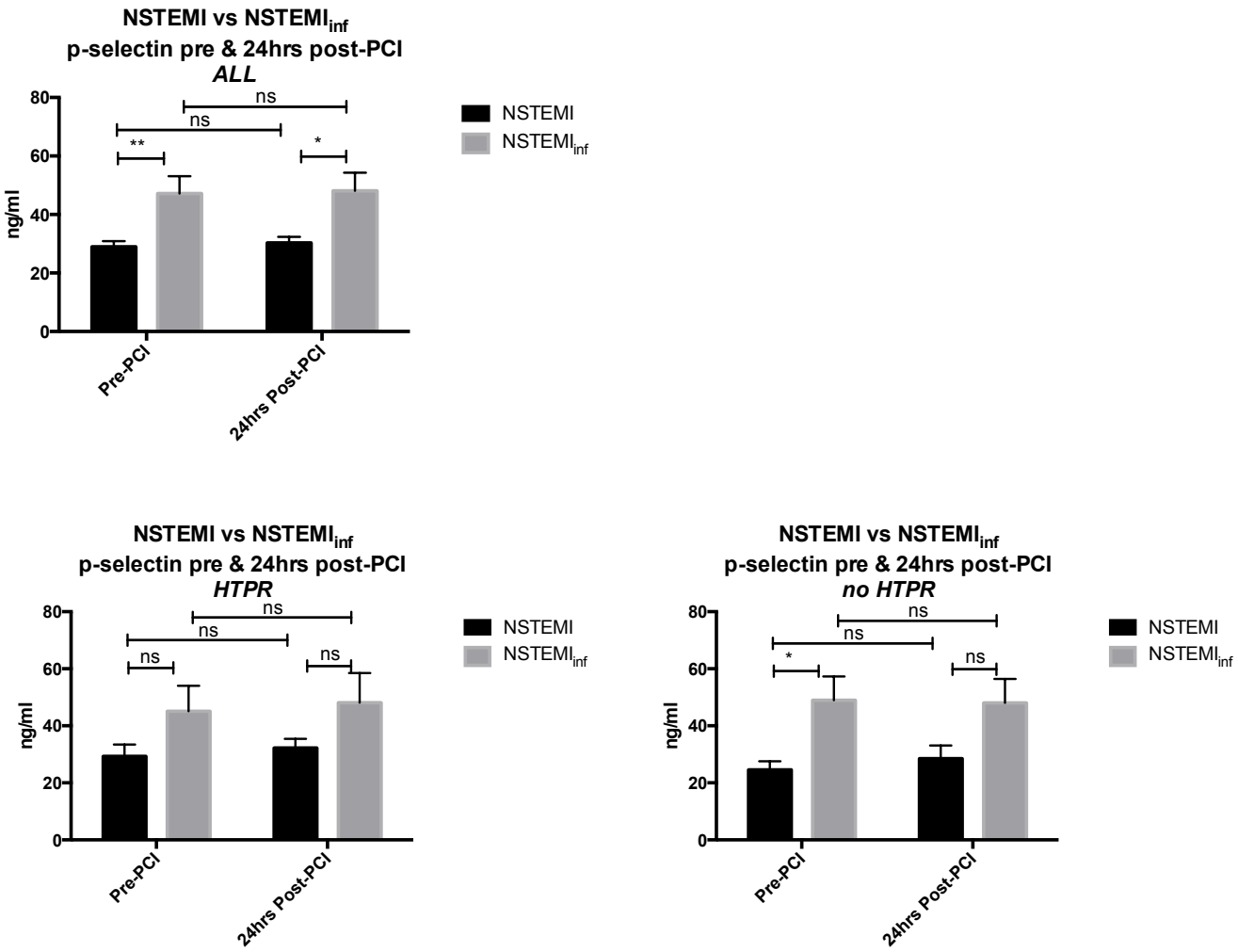

Figure 33: Comparison of NSTEMI vs NSTEMIinf – soluble p-selectin concentration pre, post and 24 hrs post-PCI.

Expressed as mean ± SEM, p^{ANOVA}. Panel A: All patients, panel B & C those with and without HTPR (high on treatment platelet reactivity). Nitrite augmentation does not appear to influence ongoing p-selectin secretion from pre to 24 hrs post-PCI. Baseline values (pre-infusion) are higher in the $NSTEMI_{inf}$ cohort, this likely represents assay variation or chance (low n number).

Inflammatory and anti-inflammatory cytokines

NSTEMI vs $NSTEMI_{inf}$

As outlined a range of both pro and anti-inflammatory cytokines were measured, specifically IL-1β, IL-6, IL-8 all have a role in platelet-endothelial interaction with the action of IL-1β and its NF-κB mediated upregulation of IL-6 and 8 synthesis. NO has an anti-inflammatory role principally by its anti-adhesive quality and thus

influence on inhibiting both leukocyte and platelet / endothelial binding and directly inhibiting platelets. [206]

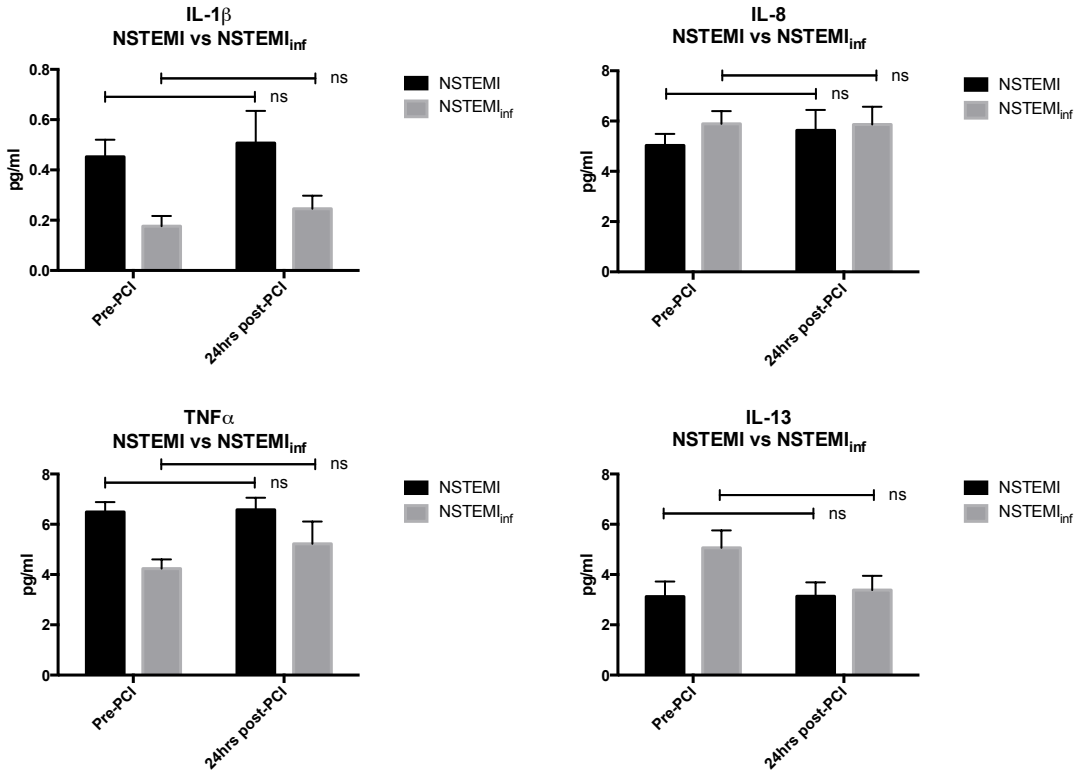

Figure 34: Quantification of IL-1β, IL-8, TNFα and IL-13. NSTEMI (30) vs NSTEMIinf (15) cohorts.

Expressed as mean ± SEM. There is a numerical difference between the two cohorts at baseline (pre sodium nitrite), this likely represents changing assay conditions or chance.

Experimentally it should be noted that the Pre-PCI samples were taken prior to the infusion of sodium nitrite and act as a baseline to ensure highlight any differences between the cohorts prior to the effect of nitrite.

IL-1β showed no significant change from Pre-PCI to 24hrs post-PCI in the NSTEMI cohort (Pre-PCI vs 24hrs Post-PCI: 0.45±0.36 vs 0.51±0.57 pg/ml) or the

$NSTEMI_{inf}$ cohort (Pre-PCI vs 24hrs Post-PCI: 0.18±0.13 vs 0.25±0.16 pg/ml). There was a significant difference at baseline pre-PCI when comparing $NSTEMI_{inf}$ cohort vs NSTEMI cohort: Pre-PCI ($NSTEMI_{inf}$ vs NSTEMI 0.18±0.13 vs 0.45±0.36 pg/ml, p=0.018), at 24 hrs Post-PCI a numerical but not significant difference was measured ($NSTEMI_{inf}$ vs NSTEMI 0.25±0.16 vs 0.51±0.57 pg/ml, p=0.19), Figure 34.

The chemokine IL-8 which plays a role in monocyte adhesion to the endothelium showed equivalent concentration both within and between groups. Pre-PCI to 24hrs post-PCI in the NSTEMI cohort (Pre-PCI vs 24hrs Post-PCI: 5.0±2.5 vs 5.6±3.7 pg/ml) or the $NSTEMI_{inf}$ cohort (Pre-PCI vs 24hrs Post-PCI: 5.9±1.7 vs 5.9±2.1 pg/ml). Concentrations of IL-8 were almost identical when we compared the two time points between $NSTEMI_{inf}$ cohort vs NSTEMI cohort: Pre-PCI ($NSTEMI_{inf}$ vs NSTEMI 5.9±1.7 vs 5.0±2.5 pg/ml) and 24 hrs Post-PCI ($NSTEMI_{inf}$ vs NSTEMI 5.9±2.1 vs 5.6±3.7 pg/ml), .Figure 34

TNFα is a pro-inflammatory cytokine belonging to the TH1 group. The release of TNFα is stimulated by IL-1β,. A similar concentration was seen within groups Pre-PCI to Post-PCI, NSTEMI cohort (Pre-PCI vs 24hrs Post-PCI: 6.5±2.1 vs 6.6±2.2 pg/ml) and the $NSTEMI_{inf}$ cohort (Pre-PCI vs 24hrs Post-PCI: 4.2±1.2 vs 5.2±2.7 pg/ml). Concentrations of TNFα were significantly different at baseline, pre-PCI between $NSTEMI_{inf}$ cohort vs NSTEMI cohort: Pre-PCI ($NSTEMI_{inf}$ vs NSTEMI 4.2±1.2 vs 6.5±2.1 pg/ml, p=0.0021), this significant difference was not sustained

24 hrs Post-PCI ($NSTEMI_{inf}$ vs NSTEMI 5.2±2.7 vs 6.6±2.2 pg/ml, p=0.16), Figure 34.

IL-13 is an anti-inflammatory TH2 cytokine which inhibits the release of tissue factor. Similar concentration between time points in the NSTEMI cohort were measured (Pre-PCI vs 24hrs Post-PCI: 3.1±2.9 vs 3.1±2.2 pg/ml). Whilst a numerical but not significant difference was seen in the $NSTEMI_{inf}$ cohort (Pre-PCI vs 24hrs Post-PCI: 5.1±2.7 vs 3.4±1.8 pg/ml). When comparing between cohorts we see a significant difference at baseline Pre-PCI ($NSTEMI_{inf}$ vs NSTEMI 5.1±2.7 vs 3.1±2.9 pg/ml, p=0.0443). This difference was not maintained to the 24 hrs Post-PCI ($NSTEMI_{inf}$ vs NSTEMI 3.4±1.8 vs 3.2±2.2 pg/ml, p=0.77), Figure 34.

There is a consistent numerical difference at baseline between the two cohorts that may be secondary to low n number and/or changing assay conditions (assays done on different plates on different days to reduce length of storage).

Platelet reactivity and inflammation

Within the NSTEMI cohort a further analysis was undertaken looking at associated changes in key cytokines between patient with and without HTPR. Following are 3 panels of matched cytokines (matched in their function), the measurements are made from the venous sample taken pre and 24 hrs post-PCI. The groups are further split into patients with HTPR and those without, using the cut-off of 469 AU for ADP (pre-PCI) as per Sibbing et al. [235]

On activation platelets release IL-1β to the endothelium with resultant up regulation of NFκB and formation of IL-6 and IL-8. The low concentrations of IL-1β are at the bottom end of the limits of detection. Whilst none of the changes reach statistical significance there is a clear numerical trend of increased IL-8 in the group with HTPR both pre and 24hrs post-PCI (pre-PCI: no HTPR 4.4±1.2 vs HTPR 6.0±3.5 pg/ml, 24hrs post-PCI: no HTPR 4.1±1.1 vs 5.4±2.2 pg/ml. Mean±SD), Figure 35. IL-8 is a chemokine produced by epithelial cells and triggers firm adhesion of monocytes to endothelium. [236]

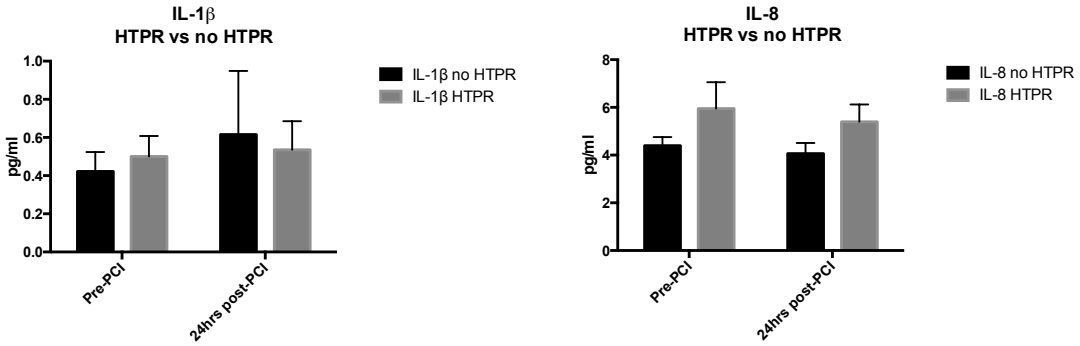

Figure 35: IL-1β and IL-8 quantification in patients with and without HTPR.

IL-1β (left panel) and IL-8 (right panel). Data split into pairs those with HTPR and those without, further expressed as pre and 24 hrs post-PCI. No significant changes between and within groups. Mean±SEM. IL: interleukin, pg/ml: picograms / milliliter, HTPR: high on treatment platelet reactivity. N=12 in each group from NSTEMI cohort

IL-10 is secreted by monocytes on binding to activated platelets via the p-selectin / PSGL-1 receptor interaction. [219] IL-10 is actually an anti-inflammatory cytokine whose principal role is as a negative feedback inhibitor via its inhibitory action of NFκB. Monocytes will also release TNFα in this interaction with activated platelets, causing further endothelial inflammation and leukocyte adhesion. [237] Again, we see a numerical trend that doesn't reach significance both in the pairs

and between groups for IL-10 and no overt trend for TNFα. IL-10 (pre-PCI: no HTPR 1.99±1.4 vs HTPR 3.70±3.3 pg/ml, 24hrs post-PCI: no HTPR 2.12±0.66 vs 3.7±4.4 pg/ml Mean±SD). TNFα (pre-PCI: no HTPR 6.0±2.8 vs HTPR 6.53±1.7 pg/ml, 24hrs post-PCI: no HTPR 5.2±2.7 vs 7.1±1.7 pg/ml Mean±SD), Figure 36.

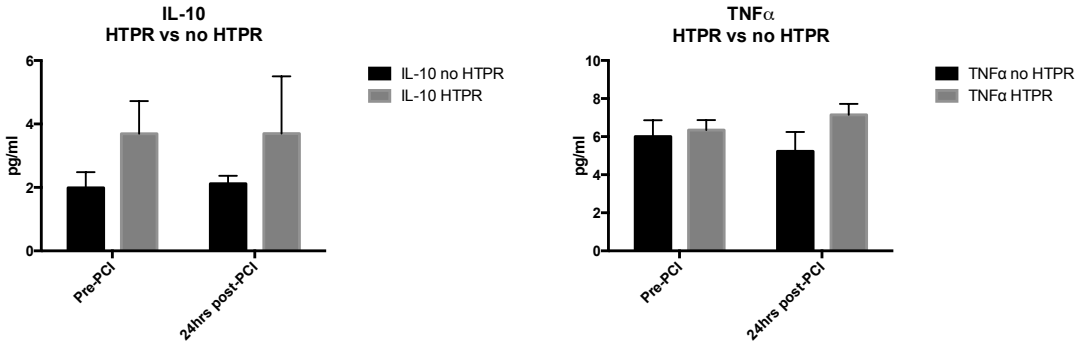

Figure 36: IL-10 and IL-TNFα concentration with and without HTPR

IL-10 (left panel) and IL-TNFα (right panel). Data split into pairs those with HTPR and those without, further expressed as pre and 24 hrs post-PCI. No significant changes between and within groups. Mean±SEM. IL: interleukin, pg/ml: picograms / milliliter, HTPR: high on treatment platelet reactivity, TNFα: tumor necrosis factor α. N=12 in each group from NSTEMI cohort

IL-4 and IL-13 are both anti-inflammatory TH2 cytokines, certainly IL-13 appears to be present in larger concentrations in patients without HTPR. IL-4 like IL-1β is at the bottom end of its sensitivity range. IL-4 (pre-PCI: no HTPR 0.7±0.7 vs HTPR 0.43±0.41 pg/ml, 24hrs post-PCI: no HTPR 0.3±0.25 vs 0.7±0.9 pg/ml Mean±SD). IL-13 (pre-PCI: no HTPR 3.9±4.4 vs HTPR 6.0±2.9 pg/ml, 24hrs post-PCI: no HTPR 1.85±1.5 vs 4.0±1.9 pg/ml Mean±SD). Figure 37.

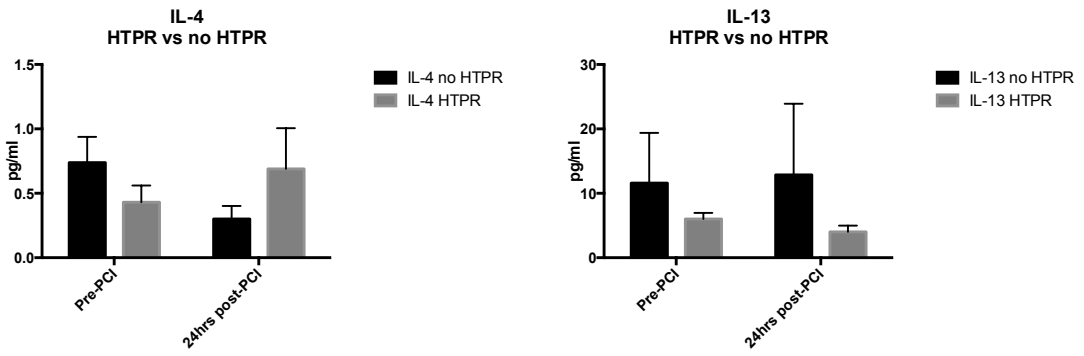

Figure 37: IL-4 and IL-13 concentration with and without HTPR.

IL-4 (left panel) and IL-13 (right panel). Data split into pairs those with HTPR and those without, further expressed as pre and 24 hrs post-PCI. No significant changes between and within groups. Mean±SEM. IL: interleukin, pg/ml: picograms / milliliter, HTPR: high on treatment platelet reactivity. N=12 in each group from NSTEMI cohort

Coronary vascular resistance: influence of platelets and inflammation

Conceptually patients with HTPR would appear to be at higher risk of microvascular disruption and thrombus formation. Certainly in the context of patients suffering with an acutely occluded coronary artery, STEMI, increased platelet reactivity appears to play a role. [238,239] The effect of residual platelet reactivity (HTPR) is assessed in relation to both resting and hyperaemic IMR.

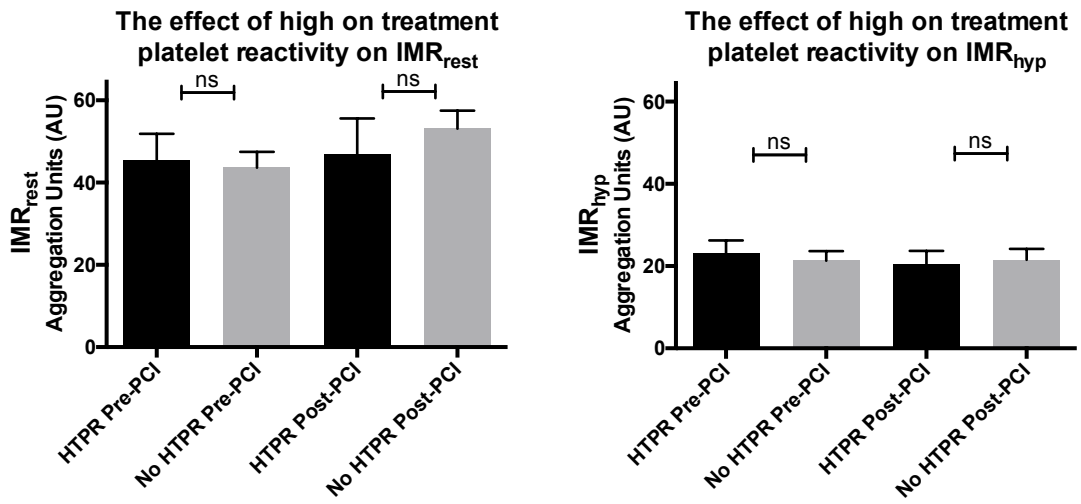

Figure 38: The effect of HTPR and no HTPR on both resting IMR and hyperaemic IMR.

HTPR (black column) No HTPR (grey columns). Resting IMR seen in the left panel and hyperaemic IMR on the right panel. Results for both pre-PCI and post-PCI. No difference is seen in respect of resting (left panel) and hyperaemic (right panel) IMR.

IMR$_{rest}$: Index microvascular resistance at rest, IMR$_{hyp}$: Index microvascular resistance at hyperaemia, HTPR: High on treatment platelet reactivity. Statistics: Mean±SEM, multiple comparisons analysed with one way ANOVA.

HTPR does not appear to influence resting microvascular tone suggesting that platelet function has little influence on CVR and CBF regulation in NSTEMI patients. IMR$_{rest}$ HTPR group pre to post-PCI, 45.3±24.6 units vs 46.9±31.5 units. IMR$_{rest}$ no HTPR group pre to post-PCI 43.6±15.4 units vs 53.1±16.9 units, Figure 38.

If HTPR were associated with peri-procedural myocardial infarction or microvascular disruption we should see this reflected in the minimal microvascular tone (IMR$_{hyp}$). [116] There was no significant change in IMR$_{hyp}$ in either the HTPR group or no HTPR group suggesting that despite the high proportion of clopidogrel resistance and HTPR, this has little influence on microvascular insult. IMR$_{hyp}$ HTPR group pre to post-PCI, 23.2±12.3 units vs

20.45±12.95 units. IMR_{hyp} no HTPR group pre to post-PCI 21.3±10.3 units vs 21.5±11.6 units.

DISCUSSION

Platelet sub-study

The purpose of the platelet sub-study was to develop and optimise a method to reproducibly measure platelet function that would allow the correct interpretation of platelet function around the time of PCI. The literature is littered with both small and large clinical and basic research that make assumptions about platelet status (often) based on one measure of platelet function / status and in wildly different settings. This study outlines significant changes in ex-vivo platelet aggregation that occur within 24 hours of PCI in high risk NSTEMI patients using bedside PFT. The platelet sub-study kept all conditions consistent between sampling points (pharmacotherapy, sampling and preparation technique) except for the PCI itself. We have outlined an important aspect of measuring platelet function / status at a clinically critical time. Whilst there were no changes in antiplatelet pharmacotherapy during the study period it is clear from the multiple measurements of platelet status that a large flux in platelet activation and clearance is occurring in this 24-hour period.

A consistent reduction in both ADP and TRAP induced aggregation is seen post-PCI and at 24 hrs. In contrast to this reduction in ex-vivo platelet aggregation we have demonstrated markers of ongoing platelet activation with increased soluble and platelet bound p-selectin. This p-selectin profile indicates α-granule secretion

with expression of p-selectin on the platelet membrane and subsequent cleavage and release in plasma as a free molecule. [240] Ongoing activation of platelets is also confirmed by the significant elevation of the eicosanoid 12-HETE, which is both secreted by and activates platelets per se. [227,241] These markers of platelet activation are seen to greatest extent in those patients defined as having HTPR at baseline (pre-PCI) using ADP induced aggregation.

Ongoing platelet activation and loss of ADP induced aggregation is well documented in patients during and following cardiopulmonary bypass (CPB), where recovery of platelet function has a more acute onset and offset. [204] This is the first study that raises the possibility of this phenomenon being present post coronary stenting as a potential stimulus for ongoing platelet activation, which may reflect their "near-maximal" activation in situ and consequent inability to contribute further to the ex vivo aggregation required by PFT assays.

The data presented supports the hypothesis that the observed flux in response to ADP – rather than being related to the type of bedside PFT device used – is the result of ongoing platelet activation occurring during the PCI and 24 hours following the procedure. Indeed, higher levels of pre-PCI ADP induced aggregation are a very good predictor of ongoing platelet activation at 24 hours as reflected by its positive association with soluble p-selectin levels at 24 hours post-PCI, a correlation that is lost when using 24hr Post-PCI ADP-induced aggregation. Figure 25

Put simply the data describes ongoing platelet activation in the 24-hour period after PCI. When we analyse whole blood samples ex-vivo in the MEA system we are effectively measuring how much residual platelet aggregation is possible with the addition of TRAP or ADP rather than increasing platelet inhibition over this 24-hour period.

Extensive attempts were made to exclude any confounding periprocedural drug effects in the methodology. In particular we have enrolled patients following loading and at least 5 days maintenance of clopidogrel in order to exclude cumulative clopidogrel response that occurs with acute periprocedural loading. The influence of heparin on platelet aggregation has been quantified and shown to be non-significant.

The 24-hour post-PCI ADP induced aggregation suggests that only 4.7% of patients had HTPR, if this is compared to other studies using the same bedside PFT apparent HTPR is much lower in our group. [195,242,243] However, it's clear that the first two studies use pre-PCI PFT and the degree of HTPR agrees with our data. The third by Aradi et al, does indeed use post-PCI PFT and the rates of HTPR are much higher post-PCI. However, it should be noted that the vast majority of patients (97%) received an acute loading dose of clopidogrel (600mg) just prior to the PCI and a further unspecified number also received the glycoprotein inhibitor tirofiban with further delay in PFT measurement. It is likely that these two important factors protect from the ongoing platelet activation we see at 24

hours as reflected in the low ADP signals with corresponding raised markers of platelet activation.

This platelet sub-study succeeded in providing both an insight into platelet status and also a way to interpret platelet status for the rest of the study. Also outlined is the importance of a consistent approach to the timing for future clinical tailored therapy trials where antiplatelet therapy is changed on the basis of peri-procedural aggregation tests. Heterogeneous clinical protocols for tailored therapy trials are no doubt part of the reason why these trials have failed to show any advantage to a conceptually attractive treatment strategy.

In the following discussion the effect of nitrite infusion on platelet function is considered where a smaller reduction (or complete absence of reduction) in ADP and TRAP induced aggregation is expected as this would indicate further inhibition of platelet function by nitrite and protection from the dramatic platelet activation that occurs in the 24 hr period post-PCI.

Nitrite and platelet inhibition

We have demonstrated in the previous chapter that nitrite concentration augmentation (~5 fold) has no influence on CVR and thus CBF regulation. Whilst unlikely, it is possible this may have been secondary to the method of CVR assessment (despite being able to measure subtle changes in CVR post-PCI). Therefore demonstrating a significant biological effect on platelet inhibition

would go some way in questioning the conclusions made in respect of nitrite and the lack of evidence of reduction to NO and effect on CVR.

The inhibitory action of NO on both platelet function and adhesion to the endothelium is well documented. [244,245] In terms of a role of nitrite and its action (via reduction to NO), the evidence is much less clear. Schafer et al, [246] demonstrated that healthy human platelets ex-vivo can clearly be inhibited in terms of aggregation but only at very high pharmacological concentrations (60μM), of nitrite. A clear issue here is not only the high concentration of nitrite but also that this was carried out with platelet rich plasma (red cell and haemoglobin free). In terms of effect on systemic blood pressure 60μM is far in excess of that which would cause significant hypotension even in healthy subjects. [133] In rat platelets ex-vivo Bryan et al failed to show a convincing anti-platelet effect in a whole blood ex-vivo system at nitrite doses from 0.1 up to 10 mg kg^{-1}. [247]

Other studies have indicated a role for nitrite in the inhibition of platelets either following a nitrite infusion or by augmenting nitrite serum concentration following oral nitrate supplements. [154,172,184] Jones et al [143] as previously discussed showed some effect of an intracoronary dose of sodium nitrite on patients undergoing P-PCI for STEMI. In both the main paper and supplementary material they suggest that nitrite also has a beneficial effect on platelet function. Central to this finding is the reduced aggregation to collagen seen in the patients receiving nitrite peri-procedurally, as previously discussed these types of changes in peri-

procedural platelet aggregation are difficult to interpret and *may* indicate platelet activation and less inhibition.[248] However, the authors also performed flow cytometry to quantify the amount of P-selectin expressed on platelets, unfortunately only data after stimulation with collagen is available with no reference to baseline platelet bound P-selectin (which would have helped us to differentiate between true inhibition versus true stimulation at baseline before the addition of platelet agonist).

High on treatment platelet reactivity (HTPR) is a common finding in this patient group (with clopidogrel) and the platelet sub-study in this chapter goes a long way in answering how clinically applicable and useful nitrite might be in this group. Indeed, clinically relevant HTPR in both cohorts of patients (NSTEMI and $NSTEMI_{inf}$) was 47.6% and 46.7% respectively. The coronary circulation is the perfect environment to reduce nitrite to NO in a focused and targeted fashion. Low oxy-haemoglobin saturations and localised ischaemia provide the optimal environment to deliver NO to the crux point in the pathological process of NSTEMI, namely ruptured plaque and thrombus formation.

Despite this we have seen no effect of nitrite in protecting the circulating platelets from the activation that occurs immediately post PCI and in the following 24-hour period. To further emphasise this we have also split the population into those with and without HTPR as defined by Siller-Matula et al. [235] Patients with overt HTPR with a pre-PCI ADP induced aggregation above 468 AU still fail to show any change in platelet reactivity following serum nitrite augmentation. To confirm this we also

see the same high concentrations of soluble p-selectin throughout this 24 hour period.

The lowest haemoglobin saturation in this experimental model is seen at the coronary sinus samples. If nitrite was to be reduced to NO this should be happening preferentially from aorta to coronary sinus (CS). Platelet aggregation was measured at the CS both pre and post-PCI in both cohorts. A further CS sample was taken from the CS 5 minutes into the sodium nitrite infusion (nitrite concentration 1208±502.7 nM). There was no difference between the cohorts in terms of ADP aggregation with both groups showing the same decline in ADP induced aggregation. The difference in ADP induced aggregation within the $NSTEMI_{inf}$ cohort pre nitrite infusion versus post infusion was non significant. This further suggests that significant nitrite reduction to NO is unlikely and is in agreement with the CVR findings in Chapter 4: Results 2.

Platelet reactivity and coronary vascular resistance

An important aspect of both resting and minimal CVR is platelet function. Minimal CVR measured during an infusion of adenosine (IMR_{hyp}) acts as a good indicator of the overall health of the microvasculature and its dilatory reserve. No difference was seen when comparing patients with and without HTPR in terms of IMR_{hyp}. This indicates that there was no significant peri-procedural infarction or disruption of the microvasculature during PCI. [116] When we look at the influence of HTPR on resting CVR (IMR_{rest}) we also see no difference between the groups indicating no role of platelet/inflammatory mediators on resting CVR.

Inflammation, platelet reactivity and nitrite

Coronary atherosclerosis is the principal cause of coronary thrombosis and myocardial infarction. Central to this process is the activation of platelets; on activation α-granules release inflammatory chemokines and cytokines that promote endothelial adhesion of both platelets and leukocytes. Endothelial inflammation is also promoted by platelet-released cytokines that may have a direct effect or induce expressions of further cytokines within the endothelium or by the attracted leukocytes. [210,249]

No influence of nitrite augmentation is detected on the concentration of the platelet-associated cytokines IL-1β, IL-8 or TNFα. Also, IL-10 and 13 – TH2 anti-inflammatory cytokines - do not change significantly in concentration at 24 hours. These results further underline a lack of any influence of nitrite augmentation on platelet inhibition and the secondary actions of platelets in terms of endothelial and leucocyte interaction. If nitrite had any inhibitory effect on platelets via NO we would expect down-regulation of IL-1β and a follow on reduction in IL-8 expression and TNFα. [250-252]

HTPR reflected in cytokine markers of endothelial/leukocyte interaction

When our NSTEMI population is split between those with and without high on treatment platelet reactivity (HTPR) we see some patterns emerging in terms of interleukins related to platelet aggregation. Patients with HTPR had a numerical trend to increased IL-8 and TNFα both upregulated via IL-1β and transcription activation via NFκB. This is indicated of active platelet endothelial and platelet-monocyte adhesion and binding. [219,231,236,237]

This gives support to the hypothesis in the platelet sub-study. The reduction in ADP and TRAP induced aggregation does not reflect increased inhibition of platelets but reflects ongoing platelet activation. Reduced ex-vivo aggregation at 24 hours reflects dysfunctional or "spent" platelets that have less ability to aggregate in response to ADP or TRAP.

CONCLUSIONS

The measurement of platelet aggregation in the 24-hour period starting just before PCI should be interpreted with caution. Certainly, no interpretation of platelet function and / or treatment decisions (in terms of pharmacotherapy) should be made using bedside tests immediately following and at 24 hours post-PCI. Reduced ADP and TRAP induced aggregation ex vivo reflects ongoing and high levels of platelet activation in vivo.

Augmentation of serum nitrite concentrations (~ 5 fold) has no influence on platelet function during the same 24 hour period. Even analysing coronary sinus samples failed to show any influence of nitrite augmentation.

No influence of nitrite augmentation is seen in terms of pro and anti-inflammatory cytokines (platelet associated or otherwise).

Chapter 6: General discussion

The role of NO and NO2- in patients with NSTEMI and stable angina

As we have read, the principal element of CBF regulation is a change in CVR. There is no single mediator of CVR and indeed the emphasis of different mediators appears to change according not just to the species but also to the specific situation (normal physiology, pacing, exercise, acute coronary syndromes and stable angina), as outlined in the general introduction (Page 44: The role of NO in human regulation of CVR in health and disease). Nitric oxide is implicated in many of these specific conditions with evidence for action in healthy humans and pathology alike. [80,93,95]

Specifically, in relation to healthy patients and those with stable angina Quyyumi et al[93] demonstrated that inhibition of nitric oxide synthetase with L-NMMA resulted in a much larger elevation of CVR in healthy patients when compared to those with stable angina. Halcox et al, [130] went on to show that increasing risk factors for ischaemic heart disease had a stepwise additive effect on endothelial function measured by flow mediated dilation.

In the first results (Page 69: The role of NO in coronary blood flow regulation in the presence of epicardial coronary disease) we have demonstrated that stable angina and NSTEMI patients appear to be fundamentally different in terms of the dynamics of NO production and CVR (IMR_{rest}). In the presence of an epicardial stenosis NSTEMI patients in comparison to stable angina patients had a

numerically higher CVR: IMR_{rest} NSTEMI 46.2 ± 21.6 vs Stable 38.9 ± 18.4 ($p>0.05$). Some of this difference in CVR at baseline may be secondary to the NSTEMI patients having suffered small subendocardial myocardial infarcts as reflected in the increased values of hyperaemic minimal microvascular resistance (Table 4: Coronary physiology results summary data). The NSTEMI patients had clear evidence of NO synthesis from coronary artery to coronary sinus with no net decrease in serum NOx and a clear increase in red cell NOx giving rise to a net increase from A to V of 84.9 nM. In the stable angina cohort, we see the typical pattern of serum NOx reduction from A to V (net change A to V serum NOx -183.8nM $p<0.05$), which is also seen in healthy patients. [97] Other studies have interpreted this reduction of serum NOx from A to V as evidence of serum NO_2-conversion (reduction) back NO, [101,168] however when the red cell NOx compartment is quantified this reduction of serum NOx is actually just secondary to the shuttling of these metabolites into red cells as haemoglobin becomes more desaturated and its avidity for NO increases. [97]

At first sight these observations of A-V NOx concentrations and CVR measurements seem at odds with each other. If NSTEMI patients are actively synthesising significant amounts of NO why would CVR be higher than patients with stable angina and equivalent epicardial lesions? Here our attention should turn towards the differences in coronary collateralisation seen between these patient groups. Our stable angina patients had a significantly higher CFI than the NSTEMI patients (0.2 ± 0.13 vs 0.08 ± 0.08 p 0.0001). Coronary collateralisation will have the effect of increasing the distal coronary pressure (Pd) particularly at

low perfusion pressure (Pa), and reducing the impact of an epicardial stenosis on resting microvascular resistance / IMR_{rest} (CVR) by maintaining the patency of the resistance vessels. [253] Both factors result in improved microvascular flow. So, it seems the mechanisms of regulation of microvascular patency are fundamentally different in these two pathologies. Whilst NSTEMI patients seem to compensate for an epicardial stenosis by upregulating NOS and thus NO synthesis to maintain microvascular perfusion, stable angina patients do not appear to rely on NOS but more on the (perhaps chronic) formation of collateral flow distal to the epicardial stenosis.

What happens to NOx and CVR post-PCI?

In terms of NOx and CVR in our two disease groups, we also observed an increase in CVR from pre to post PCI in the NSTEMI group (IMR_{rest} (units): 46.2 ± 21.6 vs 57.6 ± 37.9 p 0.038). In association with this rise in CVR we saw an acute drop in total NOx synthesis (from A to V), from +85nM to +16.2nM. The stable angina group had no significant (A to V) net changes in serum NOx from pre to post PCI (A to V net serum NO_2-: pre-PCI -175-5nM, post-PCI -183.8nM), in keeping with the theory that stable angina patients do not rely on local NO synthesis for microvascular perfusion. The stable angina group also had almost identical CVR from pre to post PCI (IMR_{rest} (units): 38.9 ± 18.4 vs 38.99 ± 22.1 p >0.05).

The sharp correction of NOx seen in the NSTEMI group together with the sharp increase in CVR may partly reflect acute down regulation of endothelial NOS secondary to the reduction in shear stress post-PCI, [81] and perhaps reduced RBC

ATP release as oxygen saturation improve locally. [46] However, increased coronary flow clearly has a role in this mechanism but is unlikely to be the whole explanation. If we assume that NOS activity remains constant and the reduction in NO_{2-} concentration is a dilutional effect secondary to increased flow, we would expect flow to increase by 5.23 tmes to account for this reduction in net NOx (pg xx). Whilst its entirely possible that flow could increase 5 fold post PCI there may also be an acute contribution to reduced NO synthesis by both reduced shear force (reduced velocity across the epicardial lesion), and downregulation of NOS via the adenine / purinergic pathway described in the general introduction Page 34: Adenine nucleotide / purinergic receptor control of CBF.

NSTEMI and local NO synthesis

In the presence of a relatively acute and significant epicardial stenosis - and reduced CBF – the benefits of increased trans-myocardial NO production in the NSTEMI group are clear. An increase (or maintenance) of CBF will be achieved with a relatively lower CVR via locally synthesized NO and dilation of resistance vessels. The mechanism of acute decompensation of patients leading to an NSTEMI event is by no means fully understood, however, NO has been implicated in many of the mechanisms from platelet aggregation, [254-256] to plaque stability[257,258] and now also here in maintaining microvascular perfusion. Of course, it is difficult to know if this local synthesis - in the coronary circulation – of NO is in acute response to the NSTEMI event or if there has been a gradual decline in this synthesis that has led to the acute event. Much of the data (referenced above) in relation to NO and NSTEMI suggests that chronic reduction

in NO synthesis may led up to these events. Although there are very few previous studies measuring cross heart A to V and none that do this in patients with NSTEMI.

This data supports the role of NO in maintaining microvascular perfusion in NSTEMI patients and indicates a mechanism as to why these patients may have worsening cardiovascular outcomes as the endothelium loses its ability to maintain myocardial perfusion acutely.

Nitrite (NO_2-) reduction to NO

In our NSTEMI cohort we have shown objective evidence of ischaemia as well as low oxygen saturations prior to PCI (Table 4: Coronary physiology results summary data, Page 89), according to many,[101,168,259] these conditions would appear to be ideal for the reduction of NO_2- to NO yet despite this environment we see no evidence of nitrite consumption. In fact, a net increase in trans-myocardial NOx was measured suggesting NOS synthesis of NO rather than reduction of NO_2-

.

Of course, it is possible we are observing a very dynamic situation in which both methods of NO production are occurring (NOS synthesis and nitrite reduction), with competing influences on overall NO metabolite concentration. The addition of a NOS inhibitor would help delineate the influence of these competing systems however, this clearly would be unsafe in a group of patients with acute coronary

syndromes and impaired coronary blood flow. Cosby et al [101] found that infusions of sodium nitrite infused into the brachial artery of healthy humans caused significant increases in flow during exercise down to concentrations of 900nM either with or without NOS inhibition using L-NMMA. Similar observations have been made by many others in various settings. [133,136-140]

We therefor recruited a virtually identical cohort (in terms of baseline characteristics), using identical methods to those in the observational NSTEMI group in Chapter 3: Results 1 (Page69). With the addition of a sodium nitrite infusion and augmentation of serum nitrite concentrations throughout the PCI procedure. In our group of NSTEMI patients (n=15), we increased the median coronary sinus NO_2- from 192.6nM (135.3 to 248.9) to 1422nM (1032 to 1842), well within the physiologically active range reported by both Maher et al[133] and Cosby et al. [101]

As reported in Chapter 4: Results 2 (Page 102) we saw no appreciable effect of this increased serum NO_2- on CVR (IMR_{rest}), minimal microvascular resistance (IMR_{hyp}), when compared to the NSTEMI patients without NO_2- augmentation. In fact, almost identical CVR values were seen both before PCI and the infusion group also experienced an almost identical rise of CVR after PCI with restoration of flow.

Why then do we fail to see any appreciable effect of NO_2- augmentation when others have observed good effect? As outlined earlier the influence of both NO and

NO_2- appears to be different according to the cohort studied. Healthy human volunteers have mixed reports of the influence of both NO and NO_2- on coronary and peripheral flow which is probably a reflection of both the interpretation of incomplete (no red cell component) of NOx and the way clinical outcomes are measured. [97,101] A subtle effect of NO_2- augmentation has been seen on the time to ischaemia of patients with stable angina undergoing stress echocardiography, [140] however, thus far all of the large RCT looking at the effect of NO_2- augmentation in cardiovascular disease (STEMI) have failed to show any meaningful effect on clinical endpoints.[142,143] Whilst STEMI and NSTEMI patients are fundamentally different the data in this thesis goes some way in explaining why there may have been no effect of NO_2- augmentation.

NO and NO_2- : platelet function and inflammation

The first part of the last results chapter (Platelet sub-study, Page 159) outlined the importance of careful measurement of platelet characteristics in this complex group of patients with an acute coronary syndrome and concomitant anti-platelet drug therapy and invasive coronary angiography. It is clear that a simple one dimensional approach to measuring platelet function can easily lead to misinterpretation of platelet function analysis.[248] The platelet sub-study clearly demonstrated that the acute reductions in platelet aggregation results during PCI actually reflected platelets that were fully activated with loss of function rather than acutely inhibited by some outside agent.

When comparing NSTEMI patients with and without augmentation of NO_2- no difference in platelet aggregation was observed when using both ADP and TRAP as agonists. Further when clopidogrel responders were separately analysed against clopidogrel non-responders (potential for more effect of NO_2-), no effect of NO_2- augmentation was evident. To test the hypothesis that this platelet inhibition may just be occurring locally across the coronary bed with a significant epicardial stenosis, measures of trans-coronary (aorta (A) to coronary sinus (V)) platelet function were taken and found to be almost identical in the two groups (Figure 31: Comparison of central venous vs coronary sinus platelet aggregation. Page 170). Soluble p-selectin was also identical between the two groups pre to 24hrs post PCI, with the same rises in p-selectin seen over this 24 hr period reflecting continuous platelet activation in this acute period.

In vitro and Ex-vivo studies have shown a clear role for NO and its role in platelet inhibition via the cGMP pathway, [173] and RSNO. [180] There is also in-vivo evidence of impaired NO (specifically platelet derived NO), production contributing to acute coronary syndromes (NSTEMI and STEMI). [260,261]

In terms of platelet inhibition in acute coronary syndromes NO_2- appears to have the potential to be a "magic bullet" in terms of its potential to be reduced to NO in areas of low oxygen saturation or pH. This was demonstrated by Srihirun et al [154] who demonstrated that nitrite had an effect ex-vivo and that this was indeed dependent on the presence of both erythrocytes and deoxygenation. Specifically in acute coronary syndrome Jones et al [143] demonstrated some effect of a nitrite

infusion on STEMI patients, however, the results were somewhat selective in terms of what was reported. There was also the suggestion of a long term (6 month) effect of a single nitrite infusion on platelet function, which seems biologically implausible and may reflect baseline differences in patient characteristics rather than biological activity.

However, the data outlined in this thesis suggests that when careful consideration is given to quantification of platelet function there is no overt effect of augmentation of serum NO_2- on platelet inhibition regardless of the propensity for those platelets to activate/aggregate.

Central to the pathophysiology of epicardial plaque rupture in NSTEMI is the activation of platelets; this activation leads to α-granules release of inflammatory chemokines and cytokines that promote endothelial adhesion of both platelets and leukocytes. Endothelial inflammation is also promoted by platelet-released cytokines that may have a direct effect or induce expressions of further cytokines within the endothelium or by the attracted leukocytes. [210,249]

Again, in this thesis, no influence of nitrite augmentation was detected on the concentration of the platelet-associated pro-inflammatory cytokines IL-1β, IL-8 or TNFα. Also, IL-10 and 13 - TH2 anti-inflammatory cytokines - did not change significantly in concentration at 24 hours. These results further underline a lack of any influence of nitrite augmentation on platelet inhibition and the secondary actions of platelets in terms of endothelial and leucocyte interaction. If nitrite had

any inhibitory effect on platelets via NO we would expect down-regulation of IL-1β and a follow on reduction in IL-8 expression and TNFα. [250-252]

Conclusions

The hypothesis to be investigated in this thesis are listed individually below together with a brief conclusion for each point

1. To examine the influence of NO and NO_2- within the compensatory mechanisms of CVR control in the ischaemic myocardium.

Nitric oxide and its influence on CVR depends on the specific pathology being studied. There is clear indication that NO has no role in CVR regulation in stable angina patients, chronic coronary collateral formation appears to have a role in maintaining microvascular perfusion. NSTEMI patients have a relatively poor formation of coronary collaterals but appear to rely on de-novo synthesis of NO to maintain a low CVR and CBF. There is no evidence of overt nitrite consumption in either group.

2. Following reperfusion and relief of myocardial ischaemia to understand how, and if, these mechanisms acutely adapt in response to the increase distal coronary pressure and increased flow in the absence of an epicardial stenosis.

Stable angina patients appear to have no flux in CVR once the epicardial stenosis is treated, unlike NSTEMI group in which a significant rise in CVR is demonstrated together with a sharp drop in net NO concentration from A to V (post-PCI). Assuming this drop in concentration is related to the increase in flow (secondary to the PCI), this equates to a 5 fold increase in coronary flow, this large increase in

flow may be possible but may also suggest an acute down regulation in NOS formation of NO.

3. In patients with acute NSTEMI does exogenous augmentation of serum NO_2- concentration have any influence on CVR either prior to or following revascularisation with coronary intervention.

No effect of NO_2- augmentation was found on CVR in patients with NSTEMI pre or post PCI. The same (NO dependent) rise in CVR was seen in both groups post-PCI with no action of NO_2- evident.

4. In patients with acute NSTEMI what effect does this augmentation of NO_2- concentration have on other important factors in this cohort. Namely, platelet function and inflammatory markers.

Once the complex nature of platelet function measures in acute NSTEMI patients was understood no influence of a significant augmentation of serum nitrite concentration was found on platelet function regardless of the residual (baseline) function of these patients. The same was true in terms of platelet derived inflammatory and ant-inflammatory cytokines when 24 hour concentrations were compared to baseline.

Limitations

As with any clinical study the practicalities of applying complex methods and protocols in a clinical situation will always lead to some element of compromise, particularly in unusual and acute environments like cardiac catheter labs.

The first compromise we chose to make was the dose of sodium nitrite used and the serum concentrations achieved. We chose this dose principally to ensure we had maximal vasodilation effect with minimal systemic blood pressure drop (potentially dangerous in patients with NSTEMI). Indeed, the actual median concentration achieved was 1422 nM (1032 to 1842), well above that reported by Cosby et al[101] (900nM) in which significant increases in blood flow were demonstrated in healthy human brachial arteries with exercise.

The assessment of CVR in patients undergoing invasive cardiac procedures is not easy and requires time, patients and consistency to produce a high standard of data quality. The method we used (IMR) has its limitations, the principal one being the fact that it is a dimensionless and thus semi-quantitative. To account for this we always made relative (paired) comparisons of the results within patient groups. Comparisons between different patient groups (NSTEMI, $NSTEMI_{inf}$ and stable angina), were always made following identical method in terms of delivering the pressure/temperature wire to the distal 3rd of the coronary artery, measuring after a standardized time after adenosine infusion etc.

REFERENCES

1. Feigl EO. Coronary physiology. Physiological Reviews 1983;63(1):1–205.

2. Duncker DJ, Merkus D. Acute adaptations of the coronary circulation to exercise. Cell Biochem Biophys 2005;43(1):17–35.

3. Laughlin MH, Tomanek RJ. Myocardial capillarity and maximal capillary diffusion capacity in exercise-trained dogs. Journal of Applied Physiology 1987;63(4):1481–6.

4. Plyley MJ, Groom AC. Geometrical distribution of capillaries in mammalian striated muscle. Am J Physiol 1975;228(5):1376–83.

5. GORLIN R, KRASNOW N, LEVINE HJ, MESSER JV. EFFECT OF EXERCISE ON CARDIAC PERFORMANCE IN HUMAN SUBJECTS WITH MINIMAL HEART DISEASE. AJC 1964;13:293–300.

6. Hansen JF, Christensen NJ, Hesse B. Determinants of coronary sinus noradrenaline in patients with ischaemic heart disease: coronary sinus catecholamine concentration in relation to arterial catecholamine concentration, pulmonary artery oxygen saturation and left ventricular end-diastolic pressure. Cardiovascular Research 1978;12(7):415–21.

7. Holmberg S, Serzysko W, Varnauskas E. Coronary circulation during heavy exercise in control subjects and patients with coronary heart disease. Acta Med Scand 1971;190(6):465–80.

8. Jorgensen CR, Kitamura K, Gobel FL. Long-term precision of the N 2 O method for coronary flow during heavy upright exercise. J Appl … 1971;

9. Nelson RR, Gobel FL, Jorgensen CR, Wang K, Wang Y, Taylor HL. Hemodynamic predictors of myocardial oxygen consumption during static and dynamic exercise. Circulation 1974;50(6):1179–89.

10. Duncker DJ, Zhang J, Pavek TJ, Crampton MJ, Bache RJ. Effect of exercise on coronary pressure-flow relationship in hypertrophied left ventricle. Am J Physiol 1995;269(1 Pt 2):H271–81.

11. Canty JM. Coronary pressure-function and steady-state pressure-flow relations during autoregulation in the unanesthetized dog. Circulation Research 1988;63(4):821–36.

12. Klocke FJ. Coronary blood flow in man. Progress in Cardiovascular Diseases 1976;19(2):117–66.

13. Duncker DJ, Bache RJ. Regulation of Coronary Blood Flow During Exercise. Physiological Reviews 2008;88(3):1009–86.

14. Kassab GS, Berkley J, Fung YC. Analysis of pig's coronary arterial blood flow with detailed anatomical data. Ann Biomed Eng 1997;25(1):204–17.

15. Algranati D, Kassab GS, Lanir Y. Mechanisms of myocardium-coronary vessel interaction. Am J Physiol Heart Circ Physiol 2010;298(3):H861–73.

16. Heineman FW, Grayson J. Transmural distribution of intramyocardial pressure measured by micropipette technique. Am J Physiol 1985;249(6 Pt 2):H1216–23.

17. Downey JM, Kirk ES. Inhibition of coronary blood flow by a vascular waterfall mechanism. Circulation Research 1975;36(6):753–60.

18. Tune JD. Matching coronary blood flow to myocardial oxygen consumption. Journal of Applied Physiology 2004;97(1):404–15.

19. Marcus ML, Chilian WM, Kanatsuka H, Dellsperger KC, Eastham CL, Lamping KG. Understanding the coronary circulation through studies at the microvascular level. Circulation 1990;82(1):1–7.

20. Chilian WM, Layne SM, Klausner EC, Eastham CL, Marcus ML. Redistribution of coronary microvascular resistance produced by dipyridamole. Am J Physiol 1989;256(2 Pt 2):H383–90.

21. Muller-Delp JM. The Coronary Microcirculation in Health and Disease. ISRN Physiology 2013;2013(2):1–24.

22. Spaan JA. Mechanical determinants of myocardial perfusion. Basic Res Cardiol 1995;90(2):89–102.

23. Canty JM, Suzuki G. Myocardial perfusion and contraction in acute ischemia and chronic ischemic heart disease. Journal of Molecular and Cellular Cardiology 2012;52(4):822–31.

24. Aversano T, Klocke FJ, Mates RE, Canty JM. Preload-induced alterations in capacitance-free diastolic pressure-flow relationship. Am J Physiol 1984;246(3 Pt 2):H410–7.

25. Bache RJ, Cobb FR. Effect of maximal coronary vasodilation on transmural myocardial perfusion during tachycardia in the awake dog. Circulation Research 1977;41(5):648–53.

26. Davis MJ, Hill MA, Kuo L. Local Regulation of Microvascular Perfusion. In: Microcirculation. Boston: Elsevier; 2008. p. 161–284.

27. Segal SS, DULING BR. Communication between feed arteries and microvessels in hamster striated muscle: segmental vascular responses are functionally coordinated. Circulation Research 1986;59(3):283–90.

28. Pradhan RK, Feigl EO, Gorman MW, Brengelmann GL, Beard DA. Open-loop (feed-forward) and feedback control of coronary blood flow during exercise, cardiac pacing, and pressure changes. Am J Physiol Heart Circ Physiol 2016;310(11):H1683–94.

29. Gladwin MT, Schechter AN, Kim-Shapiro DB, et al. The emerging biology of the nitrite anion. 2005. p. 308–14.

30. TSAI AG, JOHNSON PC, INTAGLIETTA M. Oxygen gradients in the microcirculation. Physiological Reviews 2003;

31. Broten TP, Romson JL, Fullerton DA, Van Winkle DM, Feigl EO. Synergistic action of myocardial oxygen and carbon dioxide in controlling coronary blood flow. Circulation Research 1991;68(2):531–42.

32. Broten TP, Feigl EO. Role of myocardial oxygen and carbon dioxide in coronary autoregulation. Am J Physiol 1992;262(4 Pt 2):H1231–7.

33. Weibel ER. Weibel: Delivering oxygen to the cells - Google Scholar. The Pathway for Oxygen; 1984.

34. Krogh A. Über die Versorgung der Gewebe mit Sauerstoff und über die Regulierung des Capillarkreislaufes. Journ. of physiol; 1918.

35. DULING BR, BERNE RM. Longitudinal Gradients in Periarteriolar Oxygen Tension A Possible Mechanism For the Participation of Oxygen in Local Regulation of Blood Flow. Circulation Research 1970;27(5):669–78.

36. Rubio R, BERNE RM. Regulation of coronary blood flow. Progress in Cardiovascular Diseases 1975;18(2):105–22.

37. Pittman RN, DULING BR. Measurement of percent oxyhemoglobin in the microvasculature. Journal of Applied Physiology 1975;38(2):321–7.

38. Kreuzer F. Oxygen supply to tissues: The Krogh model and its assumptions. Experientia 1982;38(12):1415–26.

39. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, Sprague RS. Erythrocytes: Oxygen Sensors and Modulators of Vascular Tone. Physiology 2009;24(2):107–16.

40. Bergfeld GR, Forrester T. Release of ATP from human erythrocytes in

response to a brief period of hypoxia and hypercapnia. Cardiovascular Research 1992;26(1):40–7.

41. Dietrich HH, Ellsworth ML, Sprague RS, Dacey RG. Red blood cell regulation of microvascular tone through adenosine triphosphate. AJP: Heart and Circulatory Physiology 2000;278(4):H1294–8.

42. Ellsworth ML. The red blood cell as an oxygen sensor: what is the evidence? Acta Physiologica 2000;168(4):551–9.

43. Dietrich HH, Kajita Y, Dacey RG. Local and conducted vasomotor responses in isolated rat cerebral arterioles. Am J Physiol 1996;271(3 Pt 2):H1109–16.

44. Yasukazu Kajita, Hans H Dietrich, Ralph G Dacey Jr. Effects of oxyhemoglobin on local and propagated vasodilatory responses induced by adenosine, adenosine diphosphate, and adenosine triphosphate in rat cerebral arterioles. http://dxdoiorg/103171/jns19968550908 2009;85(5):908–16.

45. Bagher P, Segal SS. Regulation of blood flow in the microcirculation: role of conducted vasodilation. Acta Physiologica 2011;202(3):271–84.

46. Gorman MW, Rooke GA, Savage MV, Jayasekara MPS, Jacobson KA, Feigl EO. Adenine nucleotide control of coronary blood flow during exercise. AJP: Heart and Circulatory Physiology 2010;299(6):H1981–9.

47. Boo YC, Jo H. Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. AJP: Cell Physiology 2003;285(3):C499–C508.

48. Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. Circulation Research 2003;92(2):e31–40.

49. Yada T, Shimokawa H, Hiramatsu O, et al. Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. Circulation 2003;107(7):1040–5.

50. Quillen J, Sellke F, Banitt P, Harrison D. The effect of norepinephrine on the coronary microcirculation. J Vasc Res 1992;29(1):2–7.

51. ZUBERBUHLER RC, BOHR DF. RESPONSES OF CORONARY SMOOTH MUSCLE TO CATECHOLAMINES. Circulation Research 1965;16:431–40.

52. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill

exercise: evidence for feed-forward beta-adrenergic control. Circulation Research 1998;82(12):1312–22.

53. Gorman MW, Tune JD, Richmond KN, Feigl EO. Quantitative analysis of feedforward sympathetic coronary vasodilation in exercising dogs. Journal of Applied Physiology 2000;89(5):1903–11.

54. Chilian WM, Layne SM, Eastham CL, Marcus ML. Heterogeneous microvascular coronary alpha-adrenergic vasoconstriction. Circulation Research 1989;64(2):376–88.

55. Berne RM. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am J Physiol 1963;204:317–22.

56. GERLACH E, DEUTICKE B, DREISBACH RH, ROSARIUS CW. [ON THE BEHAVIOR OF NUCLEOTIDES AND THEIR DEPHOSPHORYLATION DEGRADATION PRODUCTS IN THE KIDNEY IN ISCHEMIA AND SHORT-TERM POST-ISCHEMIC RE-ESTABLISHMENT OF BLOOD CIRCULATION]. Pflugers Arch Gesamte Physiol Menschen Tiere 1963;278:296–315.

57. Berne RM. The role of adenosine in the regulation of coronary blood flow. Circulation Research 1980;47(6):807–13.

58. Ely SW, Knabb RM, Bacchus AN, Rubio R, Berne RM. Measurements of coronary plasma and pericardial infusate adenosine concentrations during exercise in conscious dog: relationship to myocardial oxygen consumption and coronary blood flow. Journal of Molecular and Cellular Cardiology 1983;15(10):673–83.

59. Kauser K, Rubanyi GM. Bradykinin-induced, N omega-nitro-L-arginine-insensitive endothelium-dependent relaxation of porcine coronary arteries is not mediated by bioassayable relaxing substances. J Cardiovasc Pharmacol 1992;20 Suppl 12:S101–4.

60. Tune JD, Richmond KN, Gorman MW, Olsson RA, Feigl EO. Adenosine is not responsible for local metabolic control of coronary blood flow in dogs during exercise. AJP: Heart and Circulatory Physiology 2000;278(1):H74–84.

61. Yada T, Richmond KN, Van Bibber R, Kroll K, Feigl EO. Role of adenosine in local metabolic coronary vasodilation. Am J Physiol 1999;276(5 Pt 2):H1425–33.

62. Mallet RT, Lee SC, Downey HF. Endogenous adenosine increases O2 utilisation efficiency in isoprenaline-stimulated canine myocardium. Cardiovascular Research 1996;31(1):102–16.

63. Jones CE, Hurst TW, Randall JR. Effect of aminophylline on coronary functional hyperemia and myocardial adenosine. Am J Physiol

1982;243(3):H480–7.

64. Laxson DD, Homans DC, Bache RJ. Inhibition of adenosine-mediated coronary vasodilation exacerbates myocardial ischemia during exercise. Am J Physiol 1993;265(5 Pt 2):H1471–7.

65. DEGUBAREFF T, SLEATOR W. EFFECTS OF CAFFEINE ON MAMMALIAN ATRIAL MUSCLE, AND ITS INTERACTION WITH ADENOSINE AND CALCIUM. J Pharmacol Exp Ther 1965;148:202–14.

66. Dobson JG, Schrader J. Role of extracellular and intracellular adenosine in the attenuation of catecholamine evoked responses in guinea pig heart. Journal of Molecular and Cellular Cardiology 1984;16(9):813–22.

67. Uematsu M, Gaudette GR, Laurikka JO, Levitsky S, McCully JD. Adenosine-enhanced ischemic preconditioning decreases infarct in the regional ischemic sheep heart. The Annals of Thoracic Surgery 1998;66(2):382–7.

68. Thornton JD, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. Circulation 1992;85(2):659–65.

69. Anfossi G, Russo I, Massucco P, et al. Adenosine increases human platelet levels of cGMP through nitric oxide: possible role in its antiaggregating effect. Thrombosis Research 2002;105(1):71–8.

70. Edlund A, Sidén A, Sollevi A. Evidence for an anti-aggregatory effect of adenosine at physiological concentrations and for its role in the action of dipyridamole. Thrombosis Research 1987;45(2):183–90.

71. Friedman PL, Brown EJ, Gunther S, et al. Coronary vasoconstrictor effect of indomethacin in patients with coronary-artery disease. N Engl J Med 1981;305(20):1171–5.

72. Pacold I, Hwang MH, Lawless CE, Diamond P, Scanlon PJ, Loeb HS. Effects of indomethacin on coronary hemodynamics, myocardial metabolism and anginal threshold in coronary artery disease. AJC 1986;57(11):912–5.

73. Stepp DW, Kroll K, Feigl EO. K+ATP channels and adenosine are not necessary for coronary autoregulation. Am J Physiol 1997;273(3 Pt 2):H1299–308.

74. Richmond KN, Tune JD, Gorman MW, Feigl EO. Role of K+ATP channels in local metabolic coronary vasodilation. Am J Physiol 1999;277(6 Pt 2):H2115–23.

75. Fisslthaler B, Popp R, Kiss L, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 1999;401(6752):493–7.

76. Johnson PC. The Myogenic Response. In: The Resistance Vasculature. Humana Press; 1991. p. 159–68.

77. Hwa JJ, Bevan JA. A nimodipine-resistant Ca2+ pathway is involved in myogenic tone in a resistance artery. Am J Physiol 1986;251(1 Pt 2):H182–9.

78. Hill MA, Meininger GA. Impaired arteriolar myogenic reactivity in early experimental diabetes. Diabetes 1993;42(9):1226–32.

79. Miller FJ, Dellsperger KC, Gutterman DD. Myogenic constriction of human coronary arterioles. Am J Physiol 1997;273(1 Pt 2):H257–64.

80. Kuo L, Chilian WM, Davis MJ. Coronary arteriolar myogenic response is independent of endothelium. Circulation Research 1990;66(3):860–6.

81. Kuo L, Davis MJ, Chilian WM. Longitudinal gradients for endothelium-dependent and -independent vascular responses in the coronary microcirculation. Circulation 1995;92(3):518–25.

82. Myers PR, Banitt PF, Guerra R, Harrison DG. Characteristics of canine coronary resistance arteries: importance of endothelium. Am J Physiol 1989;257(2 Pt 2):H603–10.

83. Parent R, Paré R, Lavallée M. Contribution of nitric oxide to dilation of resistance coronary vessels in conscious dogs. Am J Physiol 1992;262(1 Pt 2):H10–6.

84. Parent R, al-Obaidi M, Lavallée M. Nitric oxide formation contributes to beta-adrenergic dilation of resistance coronary vessels in conscious dogs. Circulation Research 1993;73(2):241–51.

85. Yamabe H, Okumura K, Ishizaka H, Tsuchiya T, Yasue H. Role of endothelium-derived nitric oxide in myocardial reactive hyperemia. Am J Physiol 1992;263(1 Pt 2):H8–14.

86. Kostic MM, Schrader J. Role of nitric oxide in reactive hyperemia of the guinea pig heart. Circulation Research 1992;70(1):208–12.

87. Smith TP, Canty JM. Modulation of coronary autoregulatory responses by nitric oxide. Evidence for flow-dependent resistance adjustments in conscious dogs. Circulation Research 1993;73(2):232–40.

88. Liu Y, Gutterman DD. Vascular control in humans: focus on the coronary microcirculation. Basic Res Cardiol 2009;104(3):211–27.

89. Dube S, Canty JM. Shear stress-induced vasodilation in porcine coronary conduit arteries is independent of nitric oxide release. AJP: Heart and Circulatory Physiology 2001;280(6):H2581–90.

90. Kuo L, Davis MJ, Chilian WM. Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. Am J Physiol 1990;259(4 Pt 2):H1063–70.

91. Stepp DW, Merkus D, Nishikawa Y, Chilian WM. Nitric oxide limits coronary vasoconstriction by a shear stress-dependent mechanism. AJP: Heart and Circulatory Physiology 2001;281(2):H796–803.

92. Quyyumi AA, Dakak N, Andrews NP, et al. Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. J Clin Invest 1995;95(4):1747–55.

93. Quyyumi AA, Dakak N, Mulcahy D, et al. Nitric oxide activity in the atherosclerotic human coronary circulation. J Am Coll Cardiol 1997;29(2):308–17.

94. Duffy SJ, Castle SF, Harper RW, Meredith IT. Contribution of vasodilator prostanoids and nitric oxide to resting flow, metabolic vasodilation, and flow-mediated dilation in human coronary circulation. Circulation 1999;100(19):1951–7.

95. Quyyumi AA, Dakak N, Andrews NP, Gilligan DM, Panza JA, Cannon RO. Contribution of nitric oxide to metabolic coronary vasodilation in the human heart. Circulation 1995;92(3):320–6.

96. Tune JD, Richmond KN, Gorman MW, Feigl EO. Role of nitric oxide and adenosine in control of coronary blood flow in exercising dogs. Circulation 2000;101(25):2942–8.

97. Rogers SC, Khalatbari A, Datta BN, et al. NO metabolite flux across the human coronary circulation. Cardiovascular Research 2007;75(2):434–41.

98. Gladwin MT, Shelhamer JH, Schechter AN, et al. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc Natl Acad Sci USA 2000;97(21):11482–7.

99. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature 1996;380(6571):221–6.

100. Doyle MP, Pickering RA, DeWeert TM, Hoekstra JW, Pater D. Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. Journal of Biological Chemistry 1981;256(23):12393–8.

101. Cosby K, Partovi KS, Crawford JH, et al. Nitrite reduction to nitric

oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med 2003;9(12):1498–505.

102. Gladwin MT, Raat NJH, Shiva S, et al. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. AJP: Heart and Circulatory Physiology 2006;291(5):H2026–35.

103. Gladwin MT, Wang X, Reiter CD, et al. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O2/NO-linked allosteric function. Journal of Biological Chemistry 2002;277(31):27818–28.

104. Nagababu E, Ramasamy S, Rifkind JM. S-nitrosohemoglobin: a mechanism for its formation in conjunction with nitrite reduction by deoxyhemoglobin. Nitric Oxide 2006;15(1):20–9.

105. Dejam A, Hunter CJ, Pelletier MM, et al. Erythrocytes are the major intravascular storage sites of nitrite in human blood. Blood 2005;106(2):734–9.

106. Pinder AG, Pittaway E, Morris K, James PE. Nitrite directly vasodilates hypoxic vasculature via nitric oxide-dependent and -independent pathways. British Journal of Pharmacology 2009;157(8):1523–30.

107. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. Journal of Biological Chemistry 2001;276(27):24482–9.

108. Johnson G, Tsao PS, Mulloy D, Lefer AM. Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. J Pharmacol Exp Ther 1990;252(1):35–41.

109. Fearon WF. Novel Index for Invasively Assessing the Coronary Microcirculation. Circulation 2003;107(25):3129–32.

110. De Bruyne B, Pijls NHJ, Smith L, Wievegg M, Heyndrickx GR. Coronary Thermodilution to Assess Flow Reserve: Experimental Validation. Circulation 2001;104(17):2003–6.

111. Layland J, Carrick D, McEntegart M, et al. Vasodilatory Capacity of the Coronary Microcirculation is Preserved in Selected Patients With Non-ST-Segment-Elevation Myocardial Infarction. Circulation: Cardiovascular Interventions 2013;6(3):231–6.

112. Pijls NH, van Son JA, Kirkeeide RL, De Bruyne B, Gould KL. Experimental basis of determining maximum coronary, myocardial, and collateral blood flow by pressure measurements for assessing functional stenosis severity before and after percutaneous transluminal

coronary angioplasty. Circulation 1993;87(4):1354–67.

113. Aarnoudse W. Epicardial Stenosis Severity Does Not Affect Minimal Microcirculatory Resistance. Circulation 2004;110(15):2137–42.

114. Pijls NHJ, Sels J-WEM. Functional Measurement of Coronary Stenosis. JACC 2012;59(12):1045–57.

115. Fearon WF, Shah M, Ng M, et al. Predictive Value of the Index of Microcirculatory Resistance in Patients With ST-Segment Elevation Myocardial Infarction. Journal of the American College of Cardiology 2008;51(5):560–5.

116. McGeoch R, Watkins S, Berry C, et al. The Index of Microcirculatory Resistance Measured Acutely Predicts the Extent and Severity of Myocardial Infarction in Patients With ST-Segment Elevation Myocardial Infarction. JACC: Cardiovascular intervention 2010;3(7):715–22.

117. Tonino PAL, De Bruyne B, Pijls NHJ, et al. Fractional flow reserve versus angiography for guiding percutaneous coronary intervention. N Engl J Med 2009;360(3):213–24.

118. Barbato E. Validation of coronary flow reserve measurements by thermodilution in clinical practice. European Heart Journal 2004;25(3):219–23.

119. Pinder AG, Rogers SC, Khalatbari A, Ingram TE, James PE. The measurement of nitric oxide and its metabolites in biological samples by ozone-based chemiluminescence. Methods Mol Biol 2008;476:11–28.

120. Rassaf T. Circulating no pool: assessment of nitrite and nitroso species in blood and tissues. Free Radic Biol Med 2004;36(4):413–22.

121. Rogers SC, Khalatbari A, Gapper PW, Frenneaux MP, James PE. Detection of human red blood cell-bound nitric oxide. Journal of Biological Chemistry 2005;280(29):26720–8.

122. Braman RS, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. Anal Chem 1989;61(24):2715–8.

123. Willis GR, Udiawar M, Evans WD, Blundell HL, James PE, Rees DA. Detailed characterisation of circulatory nitric oxide and free radical indices-is there evidence for abnormal cardiovascular homeostasis in young women with polycystic ovary syndrome? BJOG: Int J Obstet Gy 2014;121(13):1596–603.

124. Deussen A, Ohanyan V, Jannasch A, Yin L, Chilian W. Mechanisms of

metabolic coronary flow regulation. Journal of Molecular and Cellular Cardiology 2012;52(4):794–801.

125. Pepine CJ, Anderson RD, Sharaf BL, et al. Coronary Microvascular Reactivity to Adenosine Predicts Adverse Outcome in Women Evaluated for Suspected Ischemia. JACC 2010;55(25):2825–32.

126. Minamino T, Kitakaze M, Matsumura Y, et al. Impact of coronary risk factors on contribution of nitric oxide and adenosine to metabolic coronary vasodilation in humans. JACC 1998;31(6):1274–9.

127. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the l-arginine/nitric oxide area of research. Journal of Chromatography B 2007;851(1-2):51–70.

128. MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. Journal of Chromatography B 2007;851(1-2):93–105.

129. Nijjer SS, Sen S, Petraco R, et al. Improvement in coronary haemodynamics after percutaneous coronary intervention: assessment using instantaneous wave-free ratio. Heart 2013;99(23):1740–8.

130. Halcox JPJ, Schenke WH, Zalos G, et al. Prognostic Value of Coronary Vascular Endothelial Dysfunction. Circulation 2002;106(6):653–8.

131. Layland JJ, Whitbourn RJ, Burns AT, et al. The index of microvascular resistance identifies patients with periprocedural myocardial infarction in elective percutaneous coronary intervention. Heart 2012;98(20):1492–7.

132. Prasad A, Husain S, Schenke W, Mincemoyer R, Epstein N, Quyyumi AA. Contribution of bradykinin receptor dysfunction to abnormal coronary vasomotion in humans. JACC 2000;36(5):1467–73.

133. Maher AR, Milsom AB, Gunaruwan P, et al. Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. Circulation 2008;117(5):670–7.

134. Allen JD, Stabler T, Kenjale A, et al. Plasma nitrite flux predicts exercise performance in peripheral arterial disease after 3months of exercise training. Free Radic Biol Med 2010;49(6):1138–44.

135. Ingram TE, Pinder AG, Bailey DM, Fraser AG, James PE. Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. AJP: Heart and Circulatory Physiology 2010;298(2):H331–9.

136. Hunter CJ, Dejam A, Blood AB, et al. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. Nat Med 2004;10(10):1122–7.

137. Tsuchiya K, Kanematsu Y, Yoshizumi M, et al. Nitrite is an alternative source of NO in vivo. AJP: Heart and Circulatory Physiology 2005;288(5):H2163–70.

138. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci USA 2004;101(37):13683–8.

139. Dejam A, Hunter CJ, Tremonti C, et al. Nitrite Infusion in Humans and Nonhuman Primates: Endocrine Effects, Pharmacokinetics, and Tolerance Formation. Circulation 2007;116(16):1821–31.

140. Ingram TE, Fraser AG, Bleasdale RA, et al. Low-dose sodium nitrite attenuates myocardial ischemia and vascular ischemia-reperfusion injury in human models. Journal of the American College of Cardiology 2013;61(25):2534–41.

141. Weiss S, Wilkins RW, Haynes FW. THE NATURE OF CIRCULATORY COLLAPSE INDUCED BY SODIUM NITRITE. J Clin Invest 1937;16(1):73–84.

142. Siddiqi N, Neil C, Bruce M, et al. Intravenous sodium nitrite in acute ST-elevation myocardial infarction: a randomized controlled trial (NIAMI). European Heart Journal 2014;35(19):1255–62.

143. Jones DA, Pellaton C, Velmurugan S, et al. Randomized phase 2 trial of intracoronary nitrite during acute myocardial infarction. Circulation Research 2015;116(3):437–47.

144. Isis I. Isis: Collaborative Group. Randomized trial of intravenou... - Google Scholar. Lancet; 1986.

145. Horton R. From star signs to trial guidelines. The Lancet; 2000.

146. Feise RJ. Do multiple outcome measures require p-value adjustment? BMC Medical Research Methodology 2002 2:1 2002;2(1):8.

147. Dezfulian C, Shiva S, Alekseyenko A, et al. Nitrite Therapy After Cardiac Arrest Reduces Reactive Oxygen Species Generation, Improves Cardiac and Neurological Function, and Enhances Survival via Reversible Inhibition of Mitochondrial Complex I. Circulation 2009;120(10):897–905.

148. Gonzalez FM, Shiva S, Vincent PS, et al. Nitrite Anion Provides Potent Cytoprotective and Antiapoptotic Effects as Adjunctive Therapy to

Reperfusion for Acute Myocardial Infarction. Circulation 2008;117(23):2986–94.

149. Hendgen-Cotta UB, Merx MW, Shiva S, et al. Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. Proc Natl Acad Sci USA 2008;105(29):10256–61.

150. Duranski MR, Greer JJM, Dejam A, et al. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. J Clin Invest 2005;115(5):1232–40.

151. Shiva S, Sack MN, Greer JJ, et al. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. Journal of Experimental Medicine 2007;204(9):2089–102.

152. Godber BL, Doel JJ, Sapkota GP, et al. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. Journal of Biological Chemistry 2000;275(11):7757–63.

153. Duncker DJ, Bache RJ. Inhibition of nitric oxide production aggravates myocardial hypoperfusion during exercise in the presence of a coronary artery stenosis. Circulation Research 1994;74(4):629–40.

154. Srihirun S, Sriwantana T, Unchern S, et al. Platelet inhibition by nitrite is dependent on erythrocytes and deoxygenation. PLoS ONE 2012;7(1):e30380.

155. Amrani M, Chester AH, Jayakumar J, Schyns CJ, Yacoub MH. l-Arginine reverses low coronary reflow and enhances postischaemic recovery of cardiac mechanical function. Cardiovascular Research 1995;30(2):200–4.

156. Sato H, Zhao Z-Q, McGee DS, Williams MW, Hammon JW Jr., Vinten-Johansen J. Supplemental l-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. The Journal of Thoracic and Cardiovascular Surgery 1995;110(2):302–14.

157. Lefer DJ, Nakanishi K, Johnston WE, Vinten-Johansen J. Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion of dogs. Circulation 1993;88(5):2337–50.

158. Nossuli TO, Hayward R, Scalia R, Lefer AM. Peroxynitrite Reduces Myocardial Infarct Size and Preserves Coronary Endothelium After Ischemia and Reperfusion in Cats. Circulation 1997;96(7):2317–24.

159. Wildhirt SM, Weismueller S, Schulze C, Conrad N, Kornberg A, Reichart B. Inducible nitric oxide synthase activation after

ischemia/reperfusion contributes to myocardial dysfunction and extent of infarct size in rabbits: evidence for a late phase of nitric oxide-mediated reperfusion injury. Cardiovascular Research 1999;43(3):698–711.

160. Thielmann M. Myocardial Dysfunction With Coronary Microembolization: Signal Transduction Through a Sequence of Nitric Oxide, Tumor Necrosis Factor-alpha, and Sphingosine. Circulation Research 2002;90(7):807–13.

161. Bell RM, Maddock HL, Yellon DM. The cardioprotective and mitochondrial depolarising properties of exogenous nitric oxide in mouse heart. Cardiovascular Research 2003;57(2):405–15.

162. Rakhit RD, Mojet MH, Marber MS, Duchen MR. Mitochondria as Targets for Nitric Oxide–Induced Protection During Simulated Ischemia and Reoxygenation in Isolated Neonatal Cardiomyocytes. Circulation 2001;103(21):2617–23.

163. Jekabsone A. Nitric oxide and calcium together inactivate mitochondrial complex I and induce cytochrome c release. Journal of Molecular and Cellular Cardiology 2003;35(7):803–9.

164. Joannides R, Haefeli WE, Linder L, et al. Nitric Oxide Is Responsible for Flow-Dependent Dilatation of Human Peripheral Conduit Arteries In Vivo. Circulation 1995;91(5):1314–9.

165. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest 1992;90(4):1248–53.

166. Taddei S, Virdis A, Mattei P, Salvetti A. Vasodilation to acetylcholine in primary and secondary forms of human hypertension. Hypertension 1993;21(6 Pt 2):929–33.

167. Egashira K, Inou T, Hirooka Y, et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation 1993;88(1):77–81.

168. Gladwin MT. Evidence Mounts That Nitrite Contributes to Hypoxic Vasodilation in the Human Circulation. Circulation 2008;117(5):594–7.

169. Galasso G, Schiekofer S, D'Anna C, et al. No-Reflow Phenomenon: Pathophysiology, Diagnosis, Prevention, and Treatment. A Review of the Current Literature and Future Perspectives. Angiology 2014;65(3):180–9.

170. Crawford JH, Isbell TS, Huang Z, et al. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. Blood 2006;107(2):566–74.

171. Garthwaite J. New insight into the functioning of nitric oxide-receptive guanylyl cyclase: physiological and pharmacological implications. Mol Cell Biochem 2010;334(1-2):221–32.

172. Webb AJ, Patel N, Loukogeorgakis S, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension 2008;51(3):784–90.

173. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 1990;87(13):5193–7.

174. Liu X, Miller M, Joshi MS. Diffusion-limited reaction of free nitric oxide with erythrocytes. Journal of biological … 1998;

175. Crane MS, Rossi AG, Megson IL. A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. British Journal of Pharmacology 2005;144(6):849–59.

176. Irwin C, Roberts W, Irwin C, et al. Nitric oxide inhibits platelet adhesion to collagen through cGMP-dependent and independent mechanisms: The potential role for S-nitrosylation. Platelets 2009;20(7):478–86.

177. Massberg S, Sausbier M, Klatt P, et al. Increased Adhesion and Aggregation of Platelets Lacking Cyclic Guanosine 3′,5′-Monophosphate Kinase I. Journal of Experimental Medicine 1999;189(8):1255–64.

178. Polanowska-Grabowska R, Gear AR. Role of cyclic nucleotides in rapid platelet adhesion to collagen. Blood 1994;83(9):2508–15.

179. Radomski MW, Rees DD, Dutra A, Moncada S. S-nitroso-glutathione inhibits platelet activation in vitro and in vivo. British Journal of Pharmacology 1992;107(3):745–9.

180. Simon DI, Stamler JS, Jaraki O, et al. Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium-derived relaxing factor. Arterioscler Thromb Vasc Biol 1993;13(6):791–9.

181. Prasad R, Giri S, Singh I, Singh AK. Gsno attenuates Eae disease by S-nitrosylation mediated modulation of endothelial-monocyte interactions. Journal of Neurochemistry 2006;96.

182. Gluckman TL, Grossman JE, Folts JD. Regulation of leukocyte function by nitric oxide donors: the effect of S-nitroso-thiol complexes. Journal of Toxicology … 2000;

183. Zampolli A, Basta G, Lazzerini G, Feelisch M, De Caterina R. Inhibition of Endothelial Cell Activation by Nitric Oxide Donors. J

Pharmacol Exp Ther 2000;295(2):818–23.

184. Velmurugan S, Kapil V, Ghosh SM, et al. Antiplatelet effects of dietary nitrate in healthy volunteers_ Involvement of cGMP and influence of sex. Free Radic Biol Med 2013;65(c):1521–32.

185. Srihirun S, Sriwantana T, Unchern S, et al. Platelet Inhibition by Nitrite Is Dependent on Erythrocytes and Deoxygenation. PLoS ONE 2012;7(1):e30380–9.

186. Kadan M, Doğanci S, Yildirim V, et al. In vitro effect of sodium nitrite on platelet aggregation in human platelet rich plasma--preliminary report. Eur Rev Med Pharmacol Sci 2015;19(20):3935–9.

187. Akrawinthawong K, Park JW, Piknova B, Sibmooh N, Fucharoen S, Schechter AN. A Flow Cytometric Analysis of the Inhibition of Platelet Reactivity Due to Nitrite Reduction by Deoxygenated Erythrocytes. PLoS ONE 2014;9(3):e92435–7.

188. Park JW, Piknova B, Huang PL, Noguchi CT, Schechter AN. Effect of Blood Nitrite and Nitrate Levels on Murine Platelet Function. PLoS ONE 2013;8(2):e55699–7.

189. Jones DA, Pellaton C, Velmurugan S. Randomized phase 2 trial of intracoronary nitrite during acute myocardial infarction. Circulation 2015;

190. Investigators I-2. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (…. Lancet; 1988.

191. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357(24):2482–94.

192. Yusuf S, Zhao F, Mehta SR, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001;345(7):494–502.

193. Fox KAA, Mehta SR, Peters R, et al. Benefits and risks of the combination of clopidogrel and aspirin in patients undergoing surgical revascularization for non-ST-elevation acute coronary syndrome: the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) Trial. Circulation 2004;110(10):1202–8.

194. Stone GW, Witzenbichler B, Weisz G, et al. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. Lancet 2013;382(9892):614–23.

195. Sibbing D, Braun S, Morath T, et al. Platelet reactivity after clopidogrel

treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. Journal of the American College of Cardiology 2009;53(10):849–56.

196. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007;357(20):2001–15.

197. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2009;361(11):1045–57.

198. M C. Light transmission aggregometry and ATP release for the diagnostic assessment of platelet function. Semin Thromb Hemost 2009;35(2):158–67.

199. Breet NJ, van Werkum JW, Bouman HJ, et al. Comparison of Platelet Function Tests in Predicting Clinical Outcome in Patients Undergoing Coronary Stent Implantation. JAMA 2010;303(8):754–62.

200. Price MJ, Berger PB, Teirstein PS, et al. Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA 2011;305(11):1097–105.

201. Trenk D, Stone GW, Gawaz M, et al. A randomized trial of prasugrel versus clopidogrel in patients with high platelet reactivity on clopidogrel after elective percutaneous coronary intervention with implantation of drug-eluting stents: results of the TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study. Journal of the American College of Cardiology 2012;59(24):2159–64.

202. Collet J-P, Cuisset T, Rangé G, et al. Bedside Monitoring to Adjust Antiplatelet Therapy for Coronary Stenting. N Engl J Med 2012;367(22):2100–9.

203. Montalescot G, Rangé G, Silvain J, et al. High on-treatment platelet reactivity as a risk factor for secondary prevention after coronary stent revascularization: A landmark analysis of the ARCTIC study. Circulation 2014;129(21):2136–43.

204. Rinder CS, Bohnert J, Rinder HM, Mitchell J, Ault K, Hillman R. Platelet activation and aggregation during cardiopulmonary bypass. Anesthesiology 1991;75(3):388–93.

205. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352(16):1685–95.

206. Ruggeri ZM. Platelets in atherothrombosis. Nat Med 2002;

207. Nieswandt B. Platelet-collagen interaction: is GPVI the central receptor? Blood 2003;102(2):449–61.

208. JIN RC, VOETSCH B, LOSCALZO J. Endogenous Mechanisms of Inhibition of Platelet Function. http://dxdoiorg/101080/10739680590925493 2009;12(3):247–58.

209. Gawaz M. Platelets in inflammation and atherogenesis. J Clin Invest 2005;115(12):3378–84.

210. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105(9):1135–43.

211. Wagner DD, Frenette PS. The vessel wall and its interactions. Blood 2008;111(11):5271–81.

212. Kaplanski G, Farnarier C, Kaplanski S, et al. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. Blood 1994;84(12):4242–8.

213. Kaplan ZS, Jackson SP. The role of platelets in atherothrombosis. Hematology Am Soc Hematol Educ Program 2011;2011(1):51–61.

214. Epstein FH, Ross R. Atherosclerosis — An Inflammatory Disease. N Engl J Med 1999;340(2):115–26.

215. Evangelista V, Manarini S, Rotondo S, Martelli N. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin …. Blood 1996;

216. Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. Thromb Haemost 1999;81(1):1–7.

217. Cardilo-Reis L, Gruber S, Schreier SM, et al. Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. EMBO Molecular Medicine 2012;4(10):1072–86.

218. Heeschen C, Dimmeler S, Hamm CW, et al. Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. Circulation 2003;107(16):2109–14.

219. Gudbrandsdottir S, Hasselbalch HC. Activated platelets enhance IL-10 secretion and reduce TNF-α secretion by monocytes. The Journal of … 2013;

220. Steppich BA, Moog P, Matissek C, et al. Cytokine profiles and T cell function in acute coronary syndromes. Atherosclerosis 2007;190(2):443–51.

221. Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. Annu Rev Biochem 1986;55:69–102.

222. Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. Blood 1989;74(4):1181–95.

223. Hamberg M, Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proc Natl Acad Sci USA 1974;71(9):3400–4.

224. Hirsh PD, Hillis LD, Campbell WB, Firth BG, Willerson JT. Release of prostaglandins and thromboxane into the coronary circulation in patients with ischemic heart disease. N Engl J Med 1981;304(12):685–91.

225. Fuster V, Dyken ML, Vokonas PS, Hennekens C. Aspirin as a therapeutic agent in cardiovascular disease. Special Writing Group. Circulation 1993;87(2):659–75.

226. Sekiya F, Takagi J, Usui T, et al. 12S-hydroxyeicosatetraenoic acid plays a central role in the regulation of platelet activation. Biochemical and Biophysical Research Communications 1991;179(1):345–51.

227. Katoh A, Ikeda H, Murohara T, Haramaki N, Ito H. Platelet-derived 12-hydroxyeicosatetraenoic acid plays an important role in mediating canine coronary thrombosis by regulating platelet glycoprotein IIb/IIIa activation. Circulation 1998;98:2891–8.

228. Quinn MJ, Fitzgerald DJ. Ticlopidine and clopidogrel. Circulation 1999;100:1667–72.

229. Siller-Matula JM, Christ G, Lang IM, Delle-Karth G, Huber K, Jilma B. Multiple electrode aggregometry predicts stent thrombosis better than the vasodilator-stimulated phosphoprotein phosphorylation assay. J Thromb Haemost 2010;8(2):351–9.

230. 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(5):885–96.

231. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating Monocyte-Platelet Aggregates Are a More Sensitive Marker of In Vivo Platelet Activation Than Platelet Surface P-Selectin: Studies in Baboons, Human Coronary Intervention, and Human Acute Myocardial Infarction. Circulation 2001;104(13):1533–7.

232. Thomas CP, Morgan LT, Maskrey BH, et al. Phospholipid-esterified Eicosanoids Are Generated in Agonist-activated Human Platelets and Enhance Tissue Factor-dependent Thrombin Generation. Journal of Biological Chemistry 2010;285(10):6891–903.

233. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37(8):911–7.

234. Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 1993;91(2):379–87.

235. Siller-Matula JM, Francesconi M, Dechant C, et al. Personalized antiplatelet treatment after percutaneous coronary intervention: the MADONNA study. International Journal of Cardiology 2013;167(5):2018–23.

236. Gerszten RE, Garcia-Zepeda EA, Lim Y-C, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999;398(6729):718–23.

237. Lou J, Donati YR, Juillard P, Giroud C. Platelets play an important role in TNF-induced microvascular endothelial cell pathology. The American journal … 1997;

238. Michaels AD, Gibson CM, Barron HV. Microvascular dysfunction in acute myocardial infarction: focus on the roles of platelet and inflammatory mediators in the no-reflow phenomenon. The American Journal of Cardiology 2000;85(5):50–60.

239. Jaffe R, Charron T, Puley G, Dick A, Strauss BH. Microvascular Obstruction and the No-Reflow Phenomenon After Percutaneous Coronary Intervention. Circulation 2008;117(24):3152–6.

240. Michelson AD, Barnard MR, Hechtman HB, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci USA 1996;93(21):11877–82.

241. Ozeki Y, Nagamura Y, Ito H, et al. An anti-platelet agent, OPC-29030, inhibits translocation of 12-lipoxygenase and 12-hydroxyeicosatetraenoic acid production in human platelets. British Journal of Pharmacology 1999;128(8):1699–704.

242. Sibbing D, Steinhubl SR, Schulz S, Schömig A, Kastrati A. Platelet Aggregation and Its Association With Stent Thrombosis and Bleeding in Clopidogrel-Treated Patients. JACC 2010;56(4):317–8.

243. Aradi D, Tornyos A, Pintér T, et al. Optimizing P2Y12 receptor inhibition in patients with acute coronary syndrome on the basis of platelet function testing: impact of prasugrel and high-dose clopidogrel.

Journal of the American College of Cardiology 2014;63(11):1061–70.

244. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. The Lancet 1987;2(8567):1057–8.

245. Salvemini D, de Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. Proc Natl Acad Sci USA 1989;86(16):6328–32.

246. Schafer A, Alexander R, Handin R. Inhibition of platelet function by organic nitrate vasodilators. Blood [Internet] 1980;Available from: http://bloodjournal.hematologylibrary.org/content/55/4/649.short

247. Bryan NS, Fernandez BO, Bauer SM, et al. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nat Chem Biol 2005;1(5):290–7.

248. Freeman PM, Moschonas KE, Hinz C, et al. Changes in platelet function independent of pharmacotherapy following coronary intervention in non-ST-elevation myocardial infarction patients. Atherosclerosis 2015;243(1):320–7.

249. Davì G, Patrono C. Platelet Activation and Atherothrombosis. N Engl J Med 2007;357(24):2482–94.

250. Avontuur JAM, Stam TC, Eggermont AMM, Braining HA, Jongen-Lavrencic M, van Amsterdam JGC. Effect of l-NAME, an inhibitor of nitric oxide synthesis, on plasma levels of IL-6, IL-8, TNFα and nitrite/nitrate in human septic shock. Intensive Care Med 1998;24(7):673–9.

251. Doganay S, Evereklioglu C, Er H, Türköz Y, Sevinc A. Comparison of serum NO, TNF-α, IL-1β, sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus. Eye 2002;16(2):163–70.

252. Apostolakis S, Vogiatzi K, Amanatidou V, Spandidos DA. Interleukin 8 and cardiovascular disease. Cardiovascular Research 2009;84(3):353–60.

253. Fearon WF. Microvascular Resistance Is Not Influenced by Epicardial Coronary Artery Stenosis Severity: Experimental Validation. Circulation 2004;109(19):2269–72.

254. Ichiki K, Ikeda H, Haramaki N, Ueno T, Imaizumi T. Long-term Smoking Impairs Platelet-Derived Nitric Oxide Release. Circulation 1996;94(12):3109–14.

255. Fuchs I, Frossard M, Spiel A, RIEDMÜLLER E, LAGGNER AN, Jilma B. Platelet function in patients with acute coronary syndrome (ACS)

predicts recurrent ACS. J Thromb Haemost 2006;4(12):2547–52.

256. Loscalzo J. Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis. Circulation Research 2001;88(8):756–62.

257. Boyle JJ. Macrophage Activation in Atherosclerosis: Pathogenesis and Pharmacology of Plaque Rupture. CVP 2005;3(1):63–8.

258. Tousoulis D, Charakida M, Stefanadis C. Endothelial function and inflammation in coronary artery disease. Heart 2006;92(4):441–4.

259. Webb AJ, Milsom AB, Rathod KS, et al. Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. Circulation Research 2008;103(9):957–64.

260. Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney JF, Vita JA. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. Circulation 1998;98(15):1481–6.

261. Chirkov YY, Holmes AS, Willoughby SR, et al. Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. JACC 2001;37(7):1851–7.