The Effect of Thienopyridines and Non-Thienopyridines on Nitric Oxide Metabolism in Patients with Stable Angina

by

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Submitted for the degree of

DOCTOR OF MEDICINE

Institute of Molecular and Experimental Medicine

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2016

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To my fiancée Vicky, thanks for your unwavering support in helping and encouraging me to complete this work.

ACKNOWLEDGEMENTS

First and foremost I would like to extend my sincere thanks to my supervisors Professor Phil James and Dr Richard Anderson. Without their continual advice, guidance, encouragement and patience this work would never have been completed.

I would also particularly like to thank all the staff on the cardiology day case unit at the University Hospital of Wales for their kind help with patient recruitment and Dr Keith Morris for his advice and support with this work.

Further thanks go to Dr Philip Freeman who helped greatly in the early stages and to Dr Shantu Bundhoo who not only provided the initial stepping stones for my work through his extensive research in this area but frequently provided guidance and advice throughout.

I would also like to specifically thank Dr Fairoz Abdul who provided support for the ticagrelor in vitro aspect of this study through his additional work with simulated stomach media.

I also wish to extend thanks to my friends and colleagues in the Wales Heart Research Institute Dr Gareth Willis, Dr Katie Connoly, Dr Ewelina Sagan, and Dr Jessica Tiplady.

More than five decades ago, Dr R. Altschul (1954) wrote: 'It has been said that one is as old as one's arteries. In view of the supreme importance of endothelium in arterial function, I should like to modify this statement by saying that one is as old as one's endothelium'

Contents

SUMMARY

Clopidogrel, prasugrel, and ticagrelor are antiplatelet agents used for the treatment of acute coronary syndromes. Clopidogrel is known to improve endothelial function in patients with coronary disease but little is known about either the more potent thienopyridine prasugrel, or the irreversible $P2Y_{12}$ inhibitor ticagrelor. The ability of clopidogrel to undergo S-nitrosation is recognised, and the thienopyridines' ability to form S-nitrosothiols (RSNO) has been confirmed in vitro, a finding of significant interest given the potent anti-aggregatory and vasomodulatory properties exhibited by S-nitrosothiols.

This study sought to investigate firstly, the effect of co-administration of oral nitrates and proton pump inhibitors on NO metabolites in patients treated with clopidogrel. Secondly, the effect of acute and chronic prasugrel treatment on NO metabolite formation was investigated, with particular emphasis on SNO bio-synthesis in-vivo. This lead to further interest in ticagrelor which, unlike the thienopyridines, lacks a free thiol group, to examine the effect of changing pH on its ability to dissolve, react and inhibit platelets, and ultimately establish whether ticagrelor could form RSNO.

Ozone-based chemiluminescence techniques were employed to measure the principal NO metabolites in blood samples, and platelet aggregation was measured using multiple electrode aggregometry.

The ability of clopidogrel and prasugrel to form RSNO is demonstrated both in vitro and in vivo. An acute rise in plasma RSNO levels occurs following a loading dose of prasugrel in patients with coronary disease.

Ticagrelor's platelet inhibitory response to ADP was found to decrease after lowering of the pH in vitro. However in the presence of nitrite and decreasing pH, it readily formed ticagrelor-induced RSNO which resulted in augmented platelet inhibition compared to ticagrelor alone.

These are exciting and novel findings with the potential to shape both our understanding of RSNO, and the pleiotropic effects of these commonly prescribed anti-platelet drugs.

ABBREVIATIONS

2Cs	Copper (I) chloride/cysteine
AA	Arachidonic Acid
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUC	Area under curve
Ca^{2+}	Calcium ion
CAD	Coronary artery disease
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanosine 3'-5' monophosphate
CHD	Coronary Heart Disease
CI	Confidence Interval
COX	Cyclo-oxygenase
csPDI	Cell surface protein disulphide isomerase
Cu^{+}	Cuprous ion
Cu^{2+}	Cupric ion
CVD	Cardiovascular disease
Cys	Cysteine
DAPT	Dual antiplatelet therapy
DES	Drug-eluting stent
DMSO	Dimethyl sulphoxide
EC	Endothelial cells
EC_{50}	Concentration required to achieve 50% effect
EDTA	Ethelene diamine tetra acetic
eNOS	Endothelial nitric oxide synthase
GPCR	G protein-coupled receptor

GSNO	S-nitrosoglutathione
H_2O	Water
H^+	Hydrogen ion
HCl	Hydrochloric acid
iNOS	Inducible nitric oxide synthase
K^+	Potassium ion
LTA	Light transmission aggregometry
MACE	Major adverse cardiovascular events
MI	Myocardial infarction
N_2	Nitrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
NOA	Nitric oxide analyser
NOS	Nitric oxide synthase
NO_x	Nitrate and nitrite anions
NSTEMI	Non ST-elevation myocardial infarction
$O_2^{-\bullet}$	Superoxide
OBC	Ozone based chemiluminescence
$ONOO^-$	Peroxynitrite
PAR	Protease-activated receptor
PBS	Phosphate buffer saline
PCI	Percutaneous coronary intervention
PPI	Proton pump inhibitor

PPP	Platelet poor plasma
PRP	Platelet rich plasma
ROS	Reactive oxygen species
RSH	Reduced thiols
RSNO	Protein S-nitrosothiol
SD	Standard deviation
sGC	Soluble guanylate cyclase
SMC	Smooth muscle cells
SNO	Nitrosothiol
STEMI	ST-elevation myocardial infarction
Th	Thienopyridine
Th-SNO	Thienopyridine-nitrosothiol
TIA	Transient ischaemic attack
TRAP	Thrombin receptor activating peptide
TXA_2	Thromboxane A2
VCl_3	Vanadium chloride

1 GENERAL INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is caused by disorders of the heart and circulatory system. The main forms of CVD are coronary heart disease (CHD) and cerebrovascular disease (stroke). The term also encompasses hypertension and peripheral artery disease as well as rheumatic heart disease, congenital heart disease and heart failure[1]. CVD causes over 4 million deaths in Europe and 1.9 million deaths in the European Union (EU) every year and is responsible for 47% of all deaths in Europe. It is the main cause of death in women in all European countries and the main cause of death in men in all but 6 countries[2]. By 2010, it was estimated that CVD was no longer responsible for the majority of deaths just in developed countries but had also become the leading cause of death in developing countries[1].

Furthermore, CHD is the United Kingdom's biggest single killer responsible for an average of 200 deaths per day, resulting in an estimated cost of £19 billion per year due to premature death, hospital treatment, prescriptions, and lost productivity[3]. These figures highlight the scope of the problem, and this is despite decreases in the CVD mortality rate across Europe over the last few decades following huge government expenditure and education programmes aimed to modify population behaviour. The prevalence of some of the medical risk factors for CVD, including type 2 diabetes mellitus and obesity, has increased, and there are already signs that some of the beneficial changes achieved in terms of risk factor modification such as dietary choice, smoking and physical activity, are no longer being maintained.

The burden of cardiovascular disease therefore poses a significant threat in view of its attendant death, disability, and social and economic costs on a worldwide scale. Research in

this field therefore plays an integral role allowing us to better understand the underlying mechanisms of CVD and establish strategies to aid its prevention and treatment.

1.2 Vascular endothelium

Fundamental to vascular health is an intact endothelium. The current view that the endothelium is a dynamic, heterogeneous organ with vital synthetic, secretory, metabolic, and immunologic functions evolved from work dating back to the 1950s[4]. Endothelial cells form the inner lining of all blood vessels, the structural and functional integrity of these cells being imperative for the maintenance of vascular homeostasis and inflammatory status. The total endothelial cell (EC) surface in an adult human comprises approximately 1 to 6×10^{13} cells and weighs approximately 1 kg with a surface area of approximately 1 to 7 m^2.[5]

The endothelium essentially serves as a physical barrier. However, endothelial cells also act as a semipermeable layer regulating the transfer of molecules and controlling important functions in vascular homeostasis, co-ordinated by the release of various hormones, neurotransmitters and vasoactive factors. Imbalance in the production of these mediators can lead to dysfunction of the endothelium and to compromised vessel responsiveness.

Furthermore, the endothelium is involved in haemostatic processes, platelet activation and aggregation, inflammation and immune modulation, vascular permeability, vascular smooth muscle cell proliferation and angiogenesis. Phenotypic variation exists between endothelial cells in different parts of the vascular tree, resulting in cells from different locations expressing different markers and also generating different responses to the same stimulus.

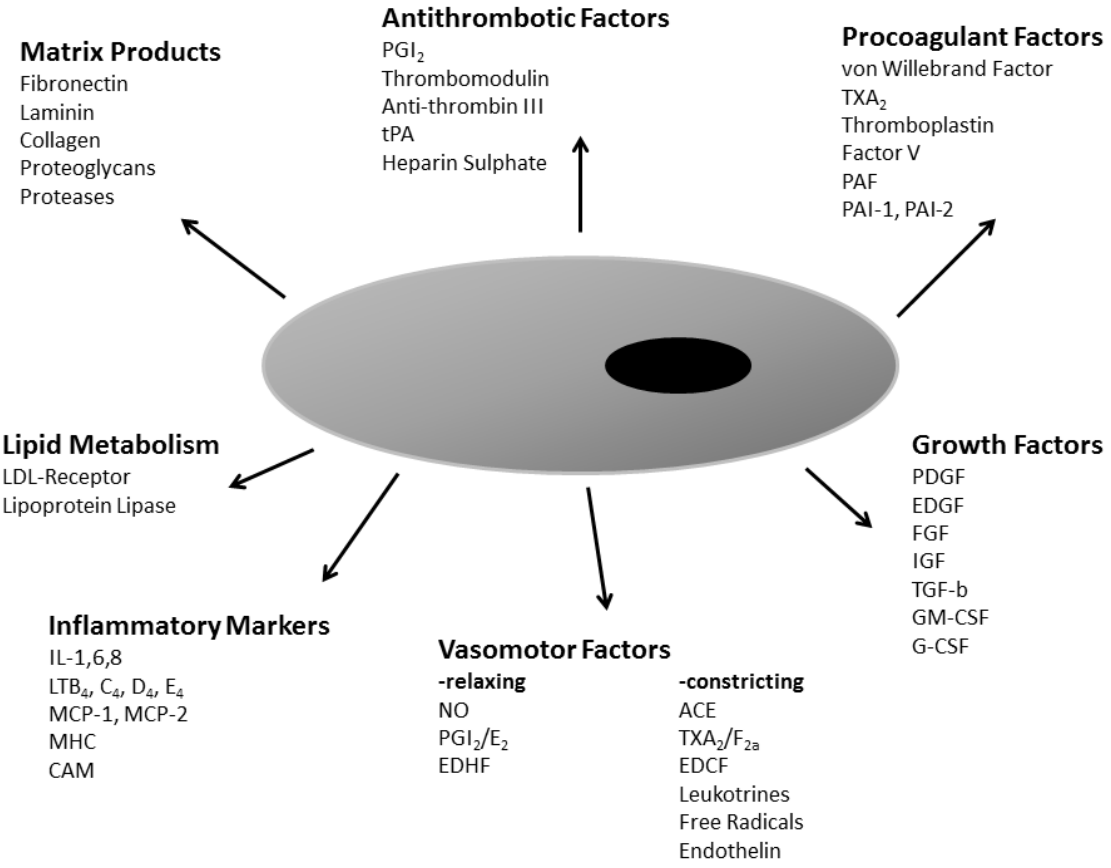


Figure 1: Schematic highlighting the important known secretory/expression products of endothelial cells relating to vessel physiology. (PGI_2 – Prostacyclin, tPA – Tissue Plasminogen Activator, TXA_2 – Thromboxane A2, PAF – Platelet Activating Factor, PAI – Plasminogen Activator Inhibitor, LDL – Low Density Lipoprotein, PDGF – Platelet Derived Growth Factor, EDGF – Epidermal Derived Growth Factor, FGF – Fibroblast Derived Growth Factor, IGF – Insulin Like Growth Factor, TGF-b – Transforming Growth Factor Beta, GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor, G-CSF – Granulocyte-Colony Stimulating Factor, IL – Interleukin, LT – Leukotriene, MCP – Monocyte Chemoattractant Protein, MHC – Major Histocompatibility Complex, CAM – Cell Adhesion Molecule, PGE_2 – Prostaglandin E2, EDHF – Endothelium Derived Hyperpolarising Factor, ACE – Angiotensin Converting Enzyme, TX – Thromboxane, EDCF – Endothelium Derived Contracting Factor)
Adapted from [6].

1.2.1 Anatomy of the vasculature

The vascular wall is made up of three layers; the tunica intima (inner layer) where endothelial cells lie, the tunica media (middle layer) and the tunica adventitia (outer layer). The structure and phenotype differs depending on the vessel[7], but essentially endothelial cells are thicker and more continuous in arteries and veins than they are in capillaries. Beyond this, the responses to stimuli differ not only between different vascular beds but even within the same bed[8].

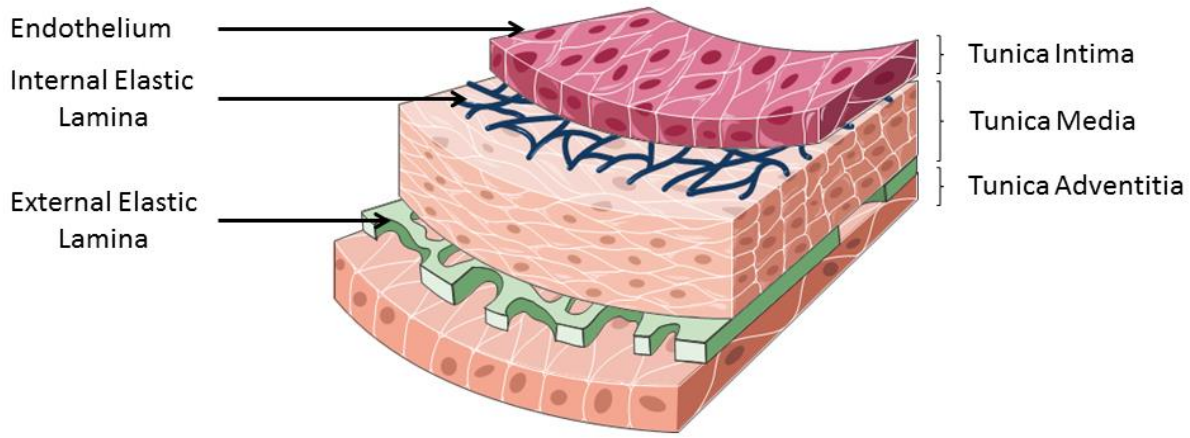


Figure 2: Structure of an artery. The artery wall consists of three layers as illustrated. The tunica adventitia is the strong outer layer consisting of connective tissue, collagen and elastic fibres. The middle layer is the tunica media which consists of smooth muscle and elastic fibres. The inner layer is formed by the tunica intima which lies in direct contact with the blood.

Arteries can be subdivided depending on where they lie within the arterial tree. The largest, called conducting arteries, have the most elastic tissue allowing them to cope with the blood pressure associated oscillatory changes induced by ventricular contractions, and include the aorta, pulmonary artery and carotid artery. Conducting arteries branch into conduit arteries such as the brachial, radial and femoral which direct blood to specific regions of the body before dividing further into resistance arteries. Resistance arteries form part of the microcirculation, and due to their predominance of smooth muscle cells and highly innervated sympathetic nerve supply, have the ability to finely regulate blood flow, and are therefore responsible for organ tissue perfusion.

Capillaries also form part of the microcirculation, and due to the presence of fenestrations and a single layer of endothelial cells, diffusion between tissue and blood is optimised allowing enhanced exchange of metabolites and gases. Gaseous exchange can continue as metabolite-rich blood flows into venules before feeding into peripheral veins and ultimately back to the heart[9].

1.2.2 Maintenance of vascular tone

The importance of the endothelium was initially recognized by its effect on vascular tone. This effect is achieved by production and release of several vasoactive molecules comprising vasodilatory factors such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarising factor (EDHF), and vasoconstrictive factors such as thromboxane (TXA_2) and endothelin-1 (ET-1), discussed in more detail below. The endothelium also responds to and modifies other circulating vasoactive mediators including bradykinin and thrombin. The maintenance of vascular tone is critical to the balance of tissue oxygen supply and metabolic demand, and is also involved in remodelling vascular structure and maintaining long-term organ perfusion[10].

Substance	Principle Effect	Other Effects	Secretion	Compound	Precursor Compound
NO	Vasodilation	Maintain vessel tone Inhibits leukocyte adhesion Inhibits platelet adhesion + activation Promotes platelet disaggregation Inhibits smooth muscle cell migration + proliferation	Paracrine/Constitutive Induced by • thrombin, • ADP • bradykinin, • substance P • muscarinic agonists • shear stress • strain • cytokines	Heterodiatomic free radical	L-arginine
PGI_2	Vasodilation	Retard platelet aggregation/ deposition	Paracrine/Induced at sites of vascular perturbation	Eicosanoid	Arachidonic acid
PAF TXA_2	Vasoconstriction	Promote leukocyte adhesion	Juxtacrine/Induced	Phospholipid	Arachidonic acid
EDHF	Vasodilation	Role in tissue perfusion and blood flow	Dependent on potassium channel activation	Various (see below)	Arachidonic acid and others
ET-1	Vasoconstriction	Mitogen for smooth muscle cells	Paracrine Induced by • hypoxia • shear stress • ischaemia	Amino acid Peptide	Preproendothelin-1

Table 1: Principal regulatory compounds synthesised by the endothelium, their effects on the vasculature and other processes, their mode of secretion, and the nature of their chemical composition and precursor compounds. Adapted from[11].

1.2.2.1 Nitric Oxide

Initially termed endothelium-derived relaxing factor, pioneering experiments first performed over 30 years ago identified this substance to be nitric oxide (NO)[12-14]. NO is a free gaseous signalling molecule with many different functions that is involved in the regulation of the cardiovascular, nervous and immune system. NO acts as a major endogenous vasodilator, countering the vasoconstrictor effects of the sympathetic nervous system and renin-angiotensin system. It is the smallest signalling molecule known and is produced by three isoforms of NO synthase (NOS), all of which utilise l-arginine and molecular oxygen as substrates. Neuronal NOS (nNOS), also known as NOS I, is a constitutively expressed low-output NOS, the prototypical enzyme being present in neurons[15]. Inducible NOS (iNOS), or NOS II, is a high-output NOS whose expression is induced by cytokines, and typically expressed by activated murine macrophages[16]. Endothelial NOS (eNOS) or NOS III, is also a low-output NOS that is constitutively expressed, and predominantly found in endothelial cells[17].

Additional cofactors are required to synthesise NO and these include reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4)[18].

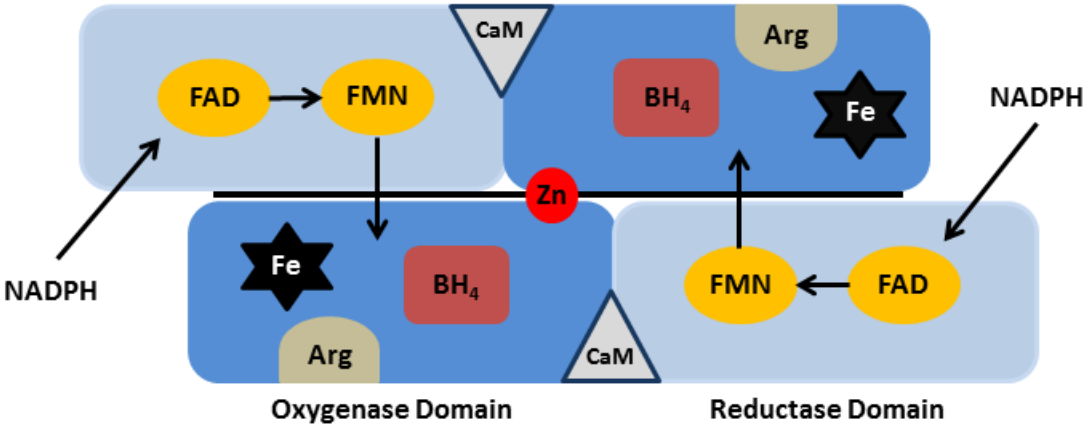


Figure 3: Diagram showing NOS monomer and associated NO cofactors. Each monomer is composed of an N-terminal oxygenase domain and a C-terminal reductase domain. A zinc (Zn) ion holds each subunit together, bound by two cysteines from each oxygenase domain. Calmodulin (CaM) is required for activation. The reductase domains, which supply electrons for the NOS reaction, contain two redox-active groups, Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN). Nicotinamide Adenine Dinucleotide Phosphate (NADPH) binds and passes an electron to FAD and then onto FMN. 5,6,7,8-Tetrahydrobiopterin (BH4) and Heme (Fe) are also involved as redox active groups.

All three NOS isoforms bind calmodulin, require haem for dimerisation[19] and have physiological and pathophysiological relevance in the cardiovascular system but eNOS is arguably the most important enzyme of the three in the vasculature. iNOS contains irreversibly bound calmodulin, making it largely independent of calcium, whereas activation of eNOS and nNOS is via elevation of intracellular calcium followed by the subsequent binding of Ca^{2+}/Calmodulin[20].

Intracellular calcium levels play a pivotal role in eNOS activation and this process of calcium regulation is referred to as store-operated Ca^{2+} entry or capacitative Ca^{2+} entry[21]. Inactive eNOS is bound to the protein caveolin, located in membrane invaginations called caveolae. Elevated levels of intracellular calcium lead to activation of eNOS by releasing it from caveolin whereas reduced intracellular calcium causes dissociation of the calcium-calmodulin complex from eNOS resulting in re-attachment to caveolin[22].

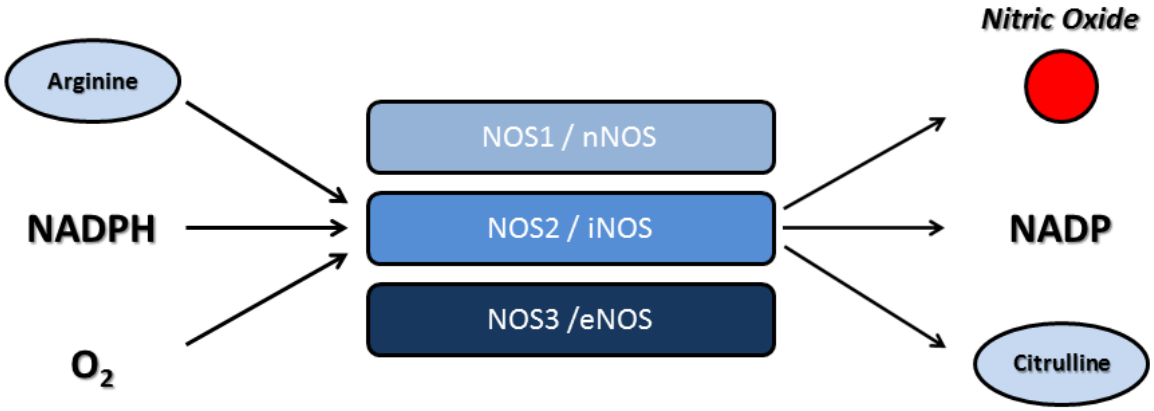


Figure 4: Simple schematic demonstrating NO generation. Nitric oxide synthases, of which there are three isoforms, convert arginine into citrulline using oxygen and NADPH as co-factors. This process leads to the production of nitric oxide. NADPH is the reduced form of NADP.

Whilst short term NO release is related to intracellular calcium levels, other mechanisms exist once calcium levels are depleted including eNOS phosphorylation via protein kinases[23], specifically protein kinase A and cGMP-dependent protein kinase II. Shear stress from blood flow through the vasculature can also induce eNOS phosphorylation via protein kinase A and protein kinase B (Akt)[24,25] but it can also lead directly to increased intracellular calcium by several mechanisms including the carriage of blood borne agonists allowing attachment to and stimulation of endothelial cell receptors, increased activation of G-proteins which participate in calcium signalling, and by increased permeability of the cell membrane to extracellular Ca^{2+} upon exposure to flow[26].

Even before EDRF had been identified as NO, Murad's group had shown that both NO and nitrovasodilators such as sodium nitroprusside activate soluble guanylyl cyclase (sGC) to effect smooth muscle relaxation and vasodilatation[27]. NO synthesis via eNOS is stimulated by various receptor agonists and by shear stress, but importantly is produced from the vascular endothelium under basal conditions. From the endothelium, NO diffuses into adjacent smooth muscle cells where it binds to sGC resulting in increased conversion of GTP to cyclic guanosine 3'-5' monophosphate (cGMP) resulting in decreasing smooth

muscle tension[28,29]. As mentioned above, NO which diffuses into the vascular lumen is key to the maintenance of vascular homeostasis. The NO agonists are typically calcium mobilising drugs and exert their effect by increasing calcium availability from the endoplasmic reticulum triggering the release of eNOS from caveolin; they include bradykinin, acetylcholine, adenosine di-phosphate, adenosine tri-phosphate and thrombin[30]. The release of NO by endothelial cells can be up-regulated by a variety of factors including oestrogens, insulin, adiponectin, exercise and dietary factors such as chronic intake of ω_3-unsaturated fatty acids. Down-regulation occurs secondary to oxidative stress, certain hormones including melatonin and long term exposure to aldosterone, smoking, ageing, obesity, sleep apnoea, and oxidised low-density lipoproteins amongst others[31].

Other mechanisms exist to enable vasodilation, but NO release is critical to maintaining vasodilator tone, and as such has an important role in blood pressure regulation, as demonstrated by administration of an NO antagonist such as L^G monomethyl-L-arginine (L-NMMA) which results in a dose-dependent increase in blood pressure[32].

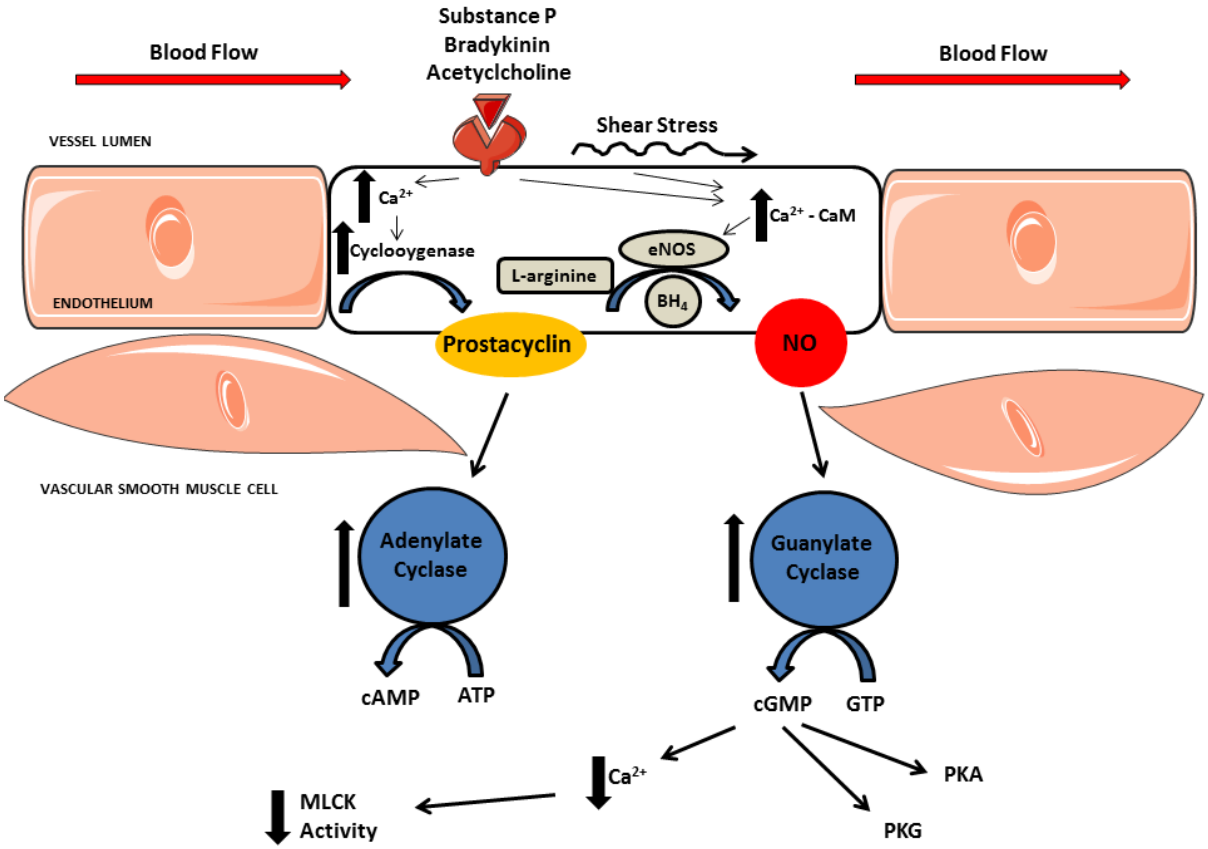


Figure 5: Vascular smooth cell relaxation. Nitric oxide formed via endothelial nitric oxide synthase (eNOS) acts downstream reducing platelet adhesion, decreasing leukocyte adhesion, inhibiting smooth muscle proliferation and migration, and inducing vasodilation. In addition, acetylcholine, adenosine triphosphate, adenosine, bradykinin, substance P and histamine all act on different receptors to generate downstream prostacyclin, which induces vasodilation and platelet inhibition. The release of Prostacyclin and NO generates Cyclic Adenosine Monophosphate (cAMP) and Cyclic Guanosine Monophosphate (cGMP) respectively which, through alteration of calcium/calmodulin binding, act on myosin light chain kinase (MLCK) resulting in smooth muscle cell relaxation. This is achieved through activation of Protein Kinase A (PKA) and Protein Kinase G (PKG), both cyclic nucleotide dependent, which result in vasodilatation by decreasing intracellular Ca^{2+}. (ATP – Adenosine Triphosphate, GTP – Guanosine Triphosphate) Adapted from [33].

1.2.2.2 Prostacyclin

Even before the discovery of nitric oxide, prostaglandins (PG) were first identified as endothelium-derived vasoactive paracrine substances in 1976[34]. We now know that the prostanoids, comprising prostaglandins and thromboxane A_2 form a group of bioactive substances which work together to modulate vascular tone and platelet activity under both physiological and pathophysiological conditions. Arachidonic acid (AA), the most common prostaglandin precursor, is released from extracellular membrane phospholipids and hydrolysed by the enzyme phospholipase A_2 (PLA_2) and then modified by cyclo-oxygenase enzymes to form an intermediate precursor prostaglandin G_2 (PGG_2) via the addition of two oxygen (O_2) molecules. Prostaglandin H_2 (PGH_2) is subsequently formed by the actions of peroxidase enzyme, releasing a single oxygen molecule. All prostanoids are then derived from this parent compound PGH_2.

Prostacyclin is the major metabolite of arachidonic acid produced in endothelial cells by the action of prostacyclin synthase on PGH_2, and is given the eicosanoid nomenclature PGI_2 (prostaglandin I_2). Besides PGI_2, the other principal bioactive prostanoids generated in vivo include prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thromboxane A_2 (TXA_2), all formed via their respective synthase enzymes[34,35].

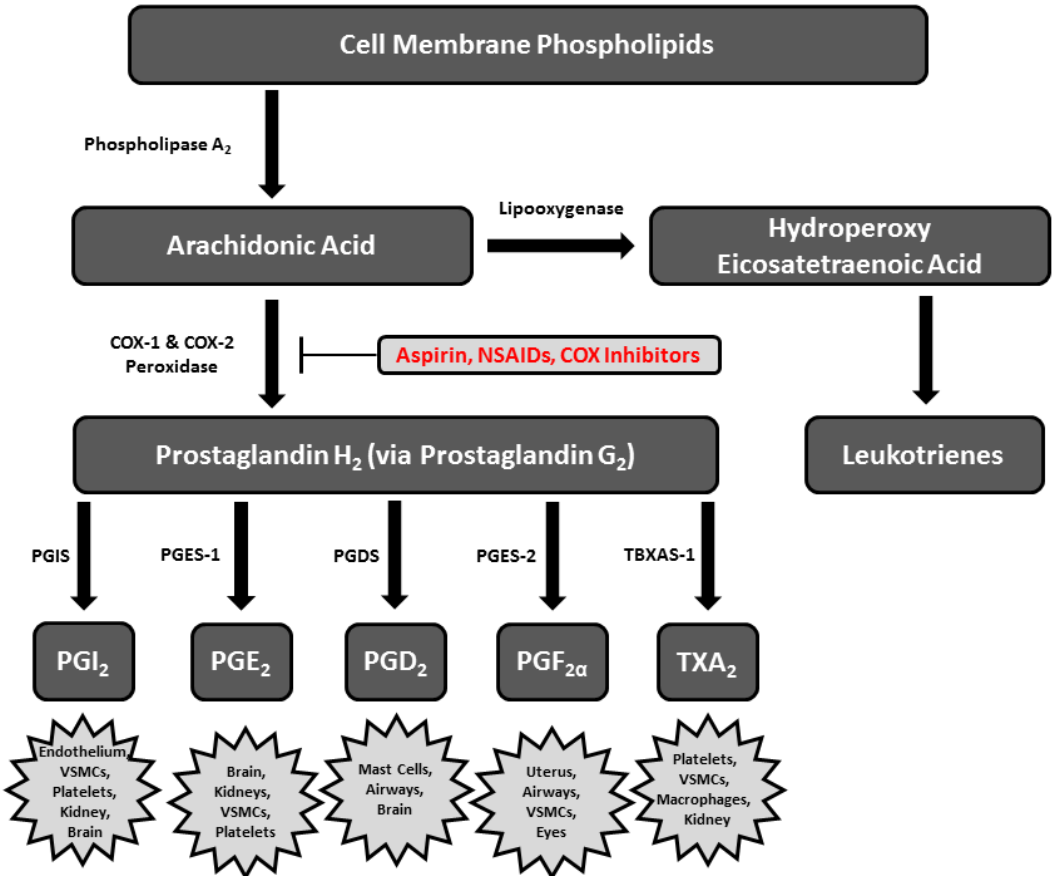


Figure 6: Prostaglandin pathway. Prostaglandins and thromboxane A_2 (TXA_2), collectively termed prostanoids, are formed when arachidonic acid, is released from the plasma membrane by phospholipases and metabolised by the sequential actions of PGG/H synthases or by cyclo-oxygenase (COX) and their respective synthases to form prostaglandin G_2 and prostaglandin H_2. There are then 4 principal bioactive prostaglandins in vivo: Prostacyclin (PGI_2), Prostaglandin E_2 (PGE_2), Prostaglandin D_2 (PGD_2) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). (VSMCs = vascular smooth muscle cells). Adapted from [36].

The conversion of AA into PGH_2 is catalysed by both cyclo-oxygenase enzymes; COX-1 is present in most cells including endothelial cells and its expression is therefore generally considered constitutive whereas expression of COX-2 is driven by damage to the endothelium or following exposure to inflammatory cytokines[37], and it is predominantly COX-2 that is responsible for the generation of PGI_2 within the systemic and pulmonary circulations in vivo[38]. Unstable at physiological pH it has a half-life in vivo of less than 2 minutes and as a result, rapidly breaks down into 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), an inactive hydration product[39].

Released partly in response to endothelial vasodilator agonists such as acetylcholine, and shear stress[40,41], PGI_2 exerts its effect through a seven-transmembrane-spanning G-protein coupled receptor (GPCR) known as the IP receptor (International Union of Pharmacology nomenclature). The human prostanoid IP receptor is functionally coupled to a signalling pathway in both smooth muscle cells and platelets that involves stimulation of intracellular cyclic adenine monophosphate (cAMP) via adenylyl cyclase[42]. Cyclic AMP activates protein kinase A in the same way as it does for NO leading to smooth muscle cell relaxation. In contrast to NO, it does not contribute to maintenance of basal tone in large conduit arteries[43], but it has been shown that PGI_2 can adopt a more compensatory role when NO bioavailability is reduced, typically in patients with endothelial dysfunction[44,45]. There is also some evidence that suggests PGI_2 effects on the vasculature might be mediated by the PPARδ pathway[46].

The primary function of PGI_2 is as an inhibitor of platelet aggregation but it is also a very effective vasodilator, vessel homeostasis being maintained by the balancing of its actions with thromboxane A_2. Amongst its additional physiological effects are inhibition of vascular smooth muscle cell proliferation[47], reduction of pulmonary blood pressure and bronchial hyper-responsiveness[48], and regulation of renal blood flow, glomerular filtration rate, and renin release[49].

1.2.2.3 Thromboxane A_2

The two prostanoids PGI_2 and thromboxane A_2 (TXA_2) act in synergy to maintain vascular function, so in contrast to PGI_2, TXA_2 causes platelet aggregation and vasoconstriction. The existence of TXA_2 was first demonstrated in platelets[50], and as illustrated above, it is synthesised by the action of thromboxane synthase on PGH_2, following COX-1 conversion of arachidonic acid[51]. It is an unstable AA metabolite with a half-life of about 30 seconds at

37°C and pH 7.4, resulting in non-enzymatic degradation into biologically inactive thromboxane B_2 (TXB_2).

TXA_2 activity, mediated through the thromboxane receptor (TP), couples with G proteins to regulate several effectors, including phospholipase C, small G protein Rho, and adenylyl cyclase[52]. Activation of TP mediates several physiological and pathophysiological responses, so besides platelet adhesion/aggregation and smooth muscle contraction and proliferation, TXA_2 plays a role in allergies, modulation of acquired immunity, atherogenesis, neovascularisation, metastasis of cancer cells, and activation of endothelial inflammatory responses[53].

1.2.2.4 EDHF

Endothelium dependent relaxation is not fully explained by NO synthase and cyclo-oxygenase pathways, implying the existence of an additional pathway[54]. This resulted in the discovery of the endothelium derived hyperpolarising factor (EDHF), a term first coined in 1987 to describe a hypothetical factor that caused endothelium-dependent myocyte relaxation that was not associated with either NO or prostacyclin[55]. However, the term is misleading as it has been found to represent a variety of different mediators, with even NO and prostacyclin having the ability to hyperpolarise vascular smooth muscle cells. EDHF seems to play a relatively minor part in the vasoactive responses of conduit vessels, its main responsibility lying in endothelium-dependent vasodilator response in resistance arteries[56].

There are two principle mechanisms by which EDHF-induced hyperpolarisation is mediated, either via the release of diffusible mediators in response to a number of stimuli including endothelial agonists such as acetylcholine, bradykinin and shear stress, or directly in response to the increase in cytoplasmic Ca^{2+} concentrations triggered by endothelium dependent vasodilators. In most arteries, the latter leads to activation of intermediate (IK_{Ca})

and small (SK_{Ca}) conductance calcium-activated potassium channels located in the endothelium which release potassium ions into the sub-endothelial space[57,58], resulting in endothelial cell hyperpolarisation.

Transmission of this hyperpolarisation to vascular smooth muscle cells occurs directly and is therefore more appropriately termed endothelium-dependent hyperpolarisation (EDH). Heterocellular coupling between endothelial and smooth muscle cells allows propagation of the hyperpolarisation current via myo-endothelial gap junctions[59]. In fact, expression of gap junctions and the occurrence of EDH-mediated responses is very closely correlated[60]. Alternatively, K^+ ions which have accumulated within the intracellular space following activation of the endothelial IK_{Ca} and/or SK_{Ca} potassium channels efflux towards the extracellular space. Depolarisation and contraction would be expected as predicted by the Nernst equation, but even a small increase in extracellular potassium concentration causes activation of both inward rectifying potassium (K_{IR}) channels and the Na^+/K^+ pump resulting in hyperpolarisation[57,61]. These two mechanisms of direct EDH-induced vasodilation can occur simultaneously or sequentially but may also act synergistically.

It is important to clarify again that even in the presence of inhibitors to NO and prostacyclin, NO can still itself lead to vascular smooth cell hyperpolarisation through the process of potassium channel activation[62], either due to residual production from endothelial cells or from NO stores including S-nitrosothiols[63], a concept that will be discussed later. Furthermore, hyperpolarisation secondary to the opening of potassium channels can also occur in response to prostacyclin. Large conductance calcium-activated potassium channels (BK_{Ca}), voltage-activated potassium channels (K_V) and/or inwardly rectifying potassium channels (K_{IR}) can all be associated with prostacyclin-induced relaxation[64].

Besides hyperpolarisation induced by myo-endothelial gap junctions and K^+ ion accumulation, EDHF is believed to comprise a selection of other endothelial factors including hydrogen peroxide, C-type natriuretic peptide (CNP), and arachidonic acid (AA) metabolites such as epoxyeicosatrienoic acids (EETs), 15-hydroxy-11,12-epoxyeicosatrienoic acids (15-H-11,12-EETA) and 11,12,15-trihdyroxyeicosatrienoic acid (THETA)[65].

- Hydrogen peroxide is constitutively expressed in some arteries but its effect varies with the vascular bed. It is generated from the hydration of superoxide by superoxide dismutase and accounts for a small part of the relaxation secondary to endothelial agonists[66].
- CNP, triggered by endothelial agonists is constitutively expressed in endothelial cells and relaxes smooth muscle cells by increasing cyclic GMP levels[67,68].
- EETs are cytochrome P450 (CYP) metabolites of AA. CYP2C and CYP2J are constitutively expressed in endothelial cells. They are synthesised by the endothelium and induce hyperpolarisation by activating large conductance calcium-activated potassium channels (BK_{ca})[69].
- 15-H-11,12-EETA and 11,12,15-THETA are endothelial 15-lipoxygenase (15-LO) metabolites of AA that mediate relaxation in response to acetylcholine in several arteries through activation of smooth muscle cell SK_{Ca} channels as well as by other less well characterised mechanisms. This 15-LO pathway represents an inducible EDHF[70].

The contribution of each of these mediators depends on the species and/or the vascular bed studied.

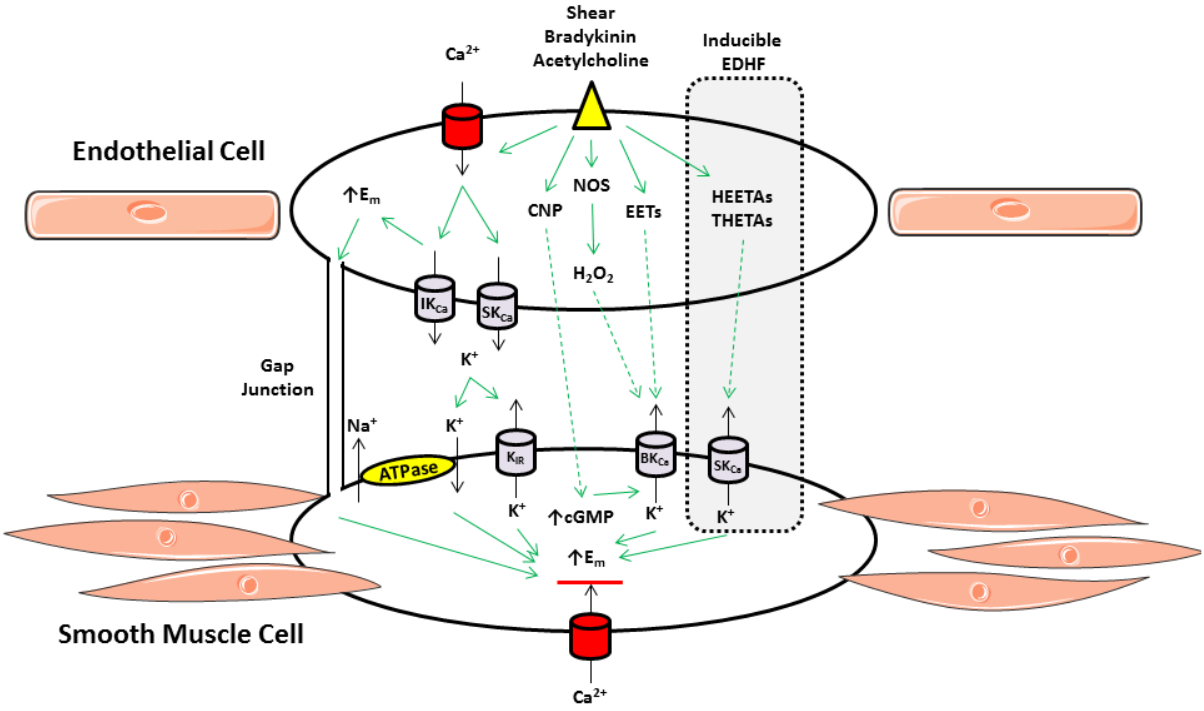


Figure 7: Endothelium Derived Hyperpolarising Factor (EDHF) Signalling Mechanisms. Shear stress or endothelium-dependent agonists including acetylcholine and bradykinin stimulate EDHF-dependent vascular relaxation. Mediators include: 1) electrical transmission through myoendothelial gap junctions, 2) Potassium (K) ions, 3) C-type natriuretic peptide (CNP), 4) hydrogen peroxide (H_2O_2), 5) epoxyeicosatrienoic acids (EETs) and 6) 15-lipoxygenase-1 (15-LO-1) metabolites, 15-H-11,12-EETA and 11,12,15-THETA. IK_{Ca} = intermediate conductance calcium activated potassium channels, SK_{Ca} = small conductance calcium activated potassium channels K_{ir} = inward rectifying potassium channels. BK_{Ca} = large conductance calcium activated potassium channels, E_m = membrane potential. Adapted from [70].

The relative involvement of EDHFs and EDH in the development of endothelial dysfunction remains relatively unknown and comprehensive investigations are still lacking. Nevertheless, alteration of EDHF-mediated responses has been reported with atherosclerosis, hypercholesterolaemia, heart failure, hypertension, eclampsia, diabetes, sepsis and ageing.

1.2.2.5 Endothelin-1

The endothelin (ET) family comprises four isoforms, ET-1, ET-2, ET-3[71], and ET-4 (vasoactive intestinal constrictor)[72] each made of up 21-amino acid peptides. Endothelin-1, the predominant isoform, is a potent vasoconstrictor synthesised and released from the

endothelium throughout the human vasculature. Prepro-endothelin mRNA is translated to form prepro-endothelin-1 which is cleaved to form Big ET-1 and this in turn is processed into endothelin predominantly by the action of endothelial converting enzyme[73]. ET-1 is unique amongst constrictor peptides in being released from human coronary artery endothelial cells via two distinct secretory pathways, a regulated pathway where release from storage in Weibel-Palade bodies occurs in response to external stimuli, and continuously released via a constitutive secretory pathway[74].

Once formed, vasoactive endothelin acts on G_i-protein–coupled receptors of which there are two in mammals, ET_A found on vascular smooth muscle cells and ET_B found predominantly on endothelial cells[75,76]. The binding of ET-1 to ET_A receptors activates phospholipase C, leading to accumulation of inositol triphosphate and intracellular calcium[77], resulting in vasoconstriction with an unusually long duration of action when compared to other endogenous vasoactive compounds[78]. Conversely, ET_B activation results in vasodilation secondary to release of NO and PGI_2[79,80], and is also responsible for mediating the clearance of circulating ET-1 via the lungs[81].

ET-1 effects include vasoconstriction, cell growth, cell adhesion, and thrombosis, predominantly via activation of ET_A receptors. As a result of these effects, ET-1 has been implicated in the pathogenesis of hypertension, coronary artery disease, pulmonary hypertension, and chronic heart failure.

Blockade of ET receptors has shown promise as a therapeutic target in both experimental and clinical studies involving a wide variety of diseases including hypertension, atherosclerosis, heart failure, and pulmonary hypertension. It has been demonstrated that selectively inhibiting ET_A receptors does result in vasodilation in patients with endothelial dysfunction but dual blockage of both ET_A and ET_B receptors may be superior [82]. A non-selective ET-1 receptor antagonist (bosentan) is currently used in the treatment of pulmonary hypertension.

1.2.3 Haemostasis

The intimal surface of healthy endothelium is both anticoagulant and antithrombotic. A careful balance must be maintained between pro-coagulant and anticoagulant factors to ensure haemostasis, so a normally functioning endothelium allows platelets to circulate without adhesion to the vascular wall. Fundamental to this process is the production of the major antiplatelet agents prostacyclin and NO by the endothelium[83].

Prostacyclin and NO release are constitutive, but molecules such as bradykinin and thrombin that are involved in the coagulation process, and secretion of ATP by aggregating platelets, lead to increased expression from endothelial cells. In addition to NO and prostacyclin, ectonucleotidases at the endothelial luminal surface act as important regulators of haemostasis by hydrolysing ATP and ADP, both potent platelet aggregating agents, into AMP and adenosine[84].

There are numerous anticoagulant pathways, the most important in the quiescent state being the protein C/protein S pathway. This is initiated when thrombin interacts with the endothelial cell receptor thrombomodulin. Protein C is activated but must form a complex with protein S (synthesised by endothelial cells) to be effective. It is then able to inactivate factors VIIIa and Va which are essential for blood coagulation[85]. Furthermore, thrombin and thrombomodulin form a complex which prevents the former from being able to clot fibrinogen or activate platelets[86].

Other important coagulation inhibitory proteins include antithrombin, which is bound to glycosaminoglycans on the endothelial cell surface, providing the main site for inactivation of active thrombin, and tissue factor pathway inhibitor (TFPI), a Kunitz-type protease inhibitor that inhibits the initial reactions of blood coagulation[87].

Cytokine release or vessel injury can upset the balance and tip the endothelium towards a pro-coagulant state. Furthermore, activated endothelial cells encourage platelet aggregation through the release of von Willebrand factor (vWF) and the lipid mediator platelet activating

factor (PAF). ECs are the main source of vWF, which is constitutively secreted into the plasma and the subendothelial matrix, and also stored in Weibel–Palade bodies.

Coagulation in vivo requires the availability of the transmembrane protein tissue factor[88]. It is present on tissue cells beyond the microcirculation and in the adventitia and plaques of conduit vessels. It is strongly induced in monocytes-macrophages in inflammation and sepsis[89], and initiates the extrinsic coagulation pathway by enhancing the proteolytic activity of factor VII. This in turn results ultimately in the formation of thrombin from prothrombin. Thrombin is an essential effector protease of the coagulation cascade which, in endothelial cells causes vWF release, the appearance of P-selectin at the plasma membrane, and production of PAF and chemokines. Thrombin signalling is mediated by binding to the G protein coupled protease-activated receptors (PAR), and also activates platelets by a similar mechanism which will be discussed later in section 1.8.

Endothelial cells also participate in fibrinolysis through production of tissue type plasminogen activator (t-PA), activated by the release of plasmin following fibrin binding. Plasminogen activator inhibitor type 1 (PAI-1) is the major plasma inhibitor of t-PA and is constitutively produced by endothelial cells, circulating in excess of t-PA.

These important pathways are highlighted in the table below.

Name	Pro/anti thrombotic	Mechanism
Proteoglycans	Anti	Negatively charged proteoglycans prevent platelet adhesion, some proteoglycans bind to antithrombin III which inhibit thrombin activity, dermatan sulfate in subendothelium promotes the antithrombotic activity of heparin cofactor II
Protein C	Anti	Inactivates factor Va and VIIIa
Thrombomodulin	Anti	Catalyses the activation of protein C by thrombin
Protein S	Anti	Cofactor for action of activated protein C
Tissue Factor Pathway Inhibitor	Anti	Binds to VIIa and Xa and inhibits activity of tissue factor and extrinsic coagulation pathway
Ectonucleotidase	Anti	Degrades platelet-stimulated ADP into AMP
Platelet Activating Factor	Pro	Promotes platelet adhesion to ECs
Annexin V	Anti	Binds to negatively charged phospholipids and inhibits the anchoring coagulation factor to the endothelium
Tissue Plasminogen Activator and Urokinase	Anti	Increase fibrinolytic activity – allows transformation of plasminogen into plasmin
vWF	Pro	Promote thrombosis – binds and stabilises coagulation factor VIII, and is a factor required for the binding of platelets to exposed extracellular matrix components
NO, PGI_2	Anti	Suppresses platelet adhesion and activation
Annexin II	Pro	Binds to t-PA and plasminogen and enhances plasmin generation

Table ii: Pro-thrombotic and Anti-thrombotic factors and their mechanisms. Adapted from [90].

1.2.4 Immunity & Inflammation

The endothelium also plays a key role in immune and inflammatory reactions through the regulation of lymphocyte and leukocyte movement into tissues and extravascular sites of inflammation. The main adhesion proteins involved in leucocyte transmigration are the selectins, the integrins, the immunoglobulin super-gene family and variants of the CD44 family.

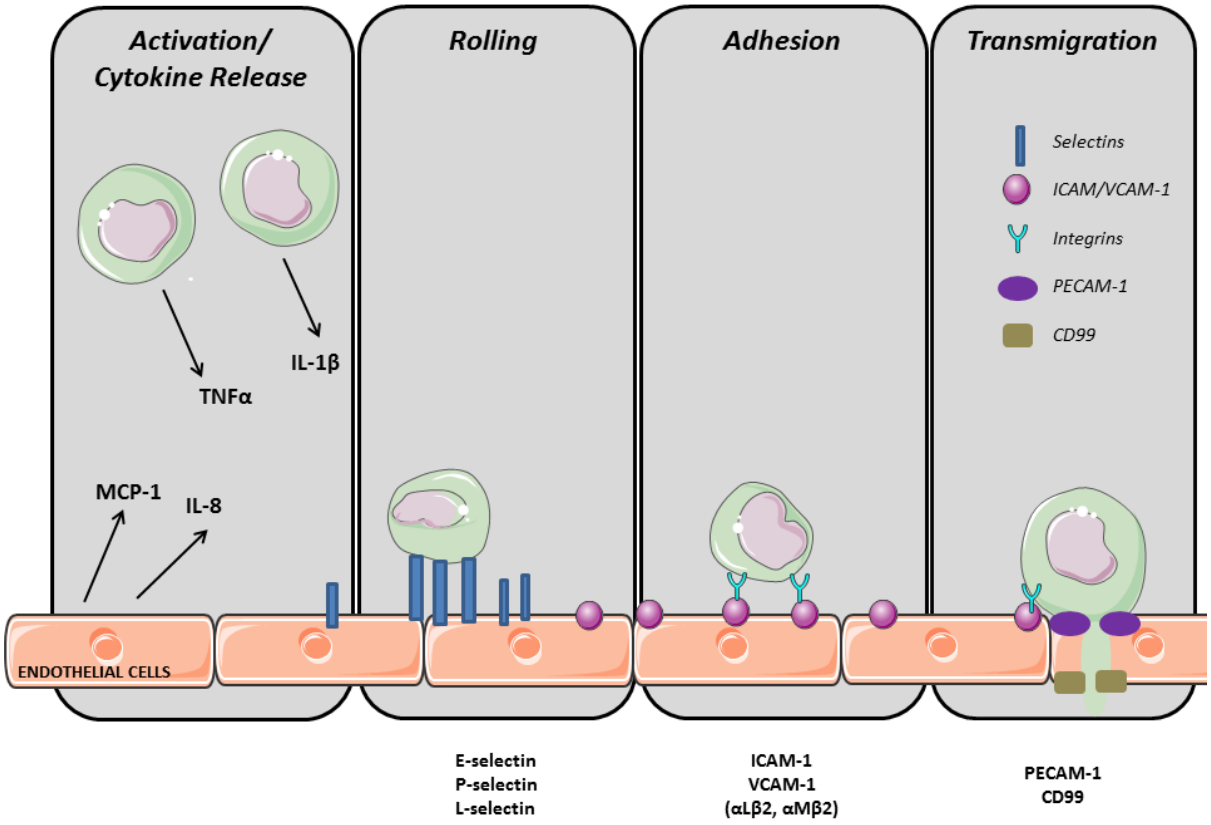


Figure 8: Schematic illustration of leukocyte extravasation. Following activation of endothelial cells and leukocytes pro-inflammatory factors are released. Exposure of cell surface adhesion molecules (selectins, ICAM-1/VCAM-1, integrins) leads to leukocyte rolling and leukocyte adhesion. PECAM-1 and CD99 are involved in subsequent leukocyte transmigration. (MCP-1 = monocyte chemotactic protein, IL = Interleukin, TNF-α = tumour necrosis factor, ICAM = intercellular adhesion molecule, VCAM = vascular cell adhesion molecule. PECAM = platelet/endothelial cell adhesion molecule). Adapted from [91] and [92].

The endothelium does not bind leucocytes in the quiescent state but once activated in response to cytokines including IL-1, TNFα and lipopolysaccharide[93], selectins initiate the first stage in leucocyte transmigration, allowing tethering and rolling of the leucocyte on the endothelial cell surface[94]. Specifically, L-selectin is found on most types of leukocytes[95], whereas E-selectin is specific for endothelial cells[96], and P-selectin is found on both endothelium and platelets[97].

Integrins are a group of heterodimeric transmembrane glycoproteins. They mediate cell–cell and cell–matrix interactions, and those relevant to leucocyte recruitment comprise β1 (VLA family) and the β2 integrins[98]. Their release leads to increased adhesion and subsequent promotion of leucocyte flattening and migration along the endothelium, known as diapedesis. Extravasation then occurs by migration through endothelial cell junctions resulting in attachment/migration on extracellular matrix components.

The immunoglobulin gene superfamily, comprising T cell receptors (CD4, CD8, CD3 and major histocompatibility complex class I and II) and adhesion molecules (ICAM-1, ICAM-2, ICAM-3 and VCAM-1), is particularly important in mediating the firm adhesion of neutrophils to endothelial cells by acting as ligands for leucocyte β2 integrins[99].

Finally, passage of leukocyte across the endothelium is also regulated by platelet/endothelial cell adhesion molecule-1 (PECAM-1), another member of the immunoglobulin superfamily, which is expressed on the surface of platelets, endothelial cells, monocytes, and neutrophils[100].

1.2.5 Vasculogenesis & Angiogenesis

Endothelial cells also play a major role in angiogenesis, and vasculogenesis or angiogenic sprouting. Vascular endothelial growth factor (VEGF)[101] and its endothelial cell-specific receptor, VEGFR2 is critical for vascular formation, initiating the formation of immature vessels by vasculogenesis[102], which occurs exclusively in the embryo. When the heart starts beating, morphogenesis leads to differentiation into arteries, post-capillary venules and veins. Angiopoietins then act in the next phase of embryological development, angiogenesis, the formation of new blood vessels from pre-existing endothelium, a process which is also affected by VEGF. The angiopoietin Ang1, and ephrin B2, ligand for the Eph receptor tyrosine kinase family of growth factor receptors, enable remodelling and maturation of this initially immature vasculature, at which point Ang1 is required to maintain the stability of the mature vasculature[103-105].

1.2.6 Neoangiogenesis

Proliferation of endothelial cells is low in adults, angiogenesis being predominantly limited to reproduction and wound healing. However, in the event of solid tumour development and metastasis, unregulated angiogenesis ensues resulting in neovascularisation and the construction of a new vascular network, activated by numerous angiogenic inducers including growth factors, chemokines, angiogenic enzymes, endothelial-specific receptors, and adhesion molecules[106].

1.3 Nitric oxide metabolism

Nitric oxide is a colourless gas with good water solubility and is an odd-electron species, the unpaired electron reducing the bond order to 2.5. NO has paramagnetic properties and its chemical structure prevents dimerisation and enhances reactivity with atoms and other free radicals. Despite being a structurally simple free radical it is involved in complex chemistry and wide and varied biological actions. It is fundamentally a signalling molecule that is synthesised by a number of cell types but because of its reactive nature and very short half-life in biological systems, has a small sphere of influence of only ~100 μm from its origin.

The steady-state concentration of NO is determined by its rate of formation and its rate of decomposition. Furthermore, the mode and rate of NO metabolism is dependent on its own concentration, diffusion, and the surrounding concentration of other bioreactants[107]. NO is able to diffuse long distances, and particularly at high concentrations can interact with molecular oxygen, thiols, and reduced haemoproteins.

Besides its reaction with O_2, several reactive oxygen derived species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are involved in the breakdown of NO, which principally reacts by gaining an electron to form the nitroxyl anion NO^-, or losing an electron to form NO^+, the nitrosonium ion.

1.3.1 NO Metabolism in blood and plasma

When exposed to molecular oxygen, NO becomes unstable generating various reactive nitrogen species. In aqueous solution, auto-oxidation of NO results in formation of nitrogen dioxide (NO_2) which itself can react with NO to form dinitrogen trioxide (N_2O_3) or dimerise to form dinitrogen tetroxide (N_2O_4). Hydrolysis of the former leads to the formation of nitrite, which in the absence of NO scavengers is the major breakdown product of NO in aqueous solutions, whilst the latter hydrolyses to equimolar amounts of nitrite and nitrate.

However, because NO breakdown also occurs in the presence of reactive oxygen species (discussed further in section 1.5) its half-life and the ratio of its corresponding metabolites in aqueous solutions depends on numerous factors including the type and amount of these oxygen-derived radicals, pO_2, pH, and concentration of transition metals and thiols.

In blood, NO undergoes many important interactions, amongst these are the reaction with oxyhaemoglobin to form methaemoglobin and nitrate, and also the reaction with thiols which results in the formation of S-nitrosothiols, such as S-nitrosocysteine and S-nitrosoglutathione (GSNO). The metabolic fate of NO involves metabolism by stepwise oxidation to nitrite and ultimately nitrate, but nitric oxide also circulates in plasma complexed in S-nitrosothiol species, the principal form being S-nitroso-serum albumin[108]. It is believed that NO can therefore be stabilised, S-nitroso-albumin acting as an intermediate reservoir before transfer, termed transnitrosation, to the more reactive and short-lived low molecular weight nitrosothiols. These then traverse cellular membranes permitting intracellular access to NO target cells[109].

Within the systemic circulation, haemoglobin represents a significant sink for NO[110]. In fact, the endothelium can produce up to 10- to 40-fold more NO than is needed to activate guanylate cyclase, but most will still be lost within the vascular compartment[111]. It is also important to note that the haemoglobin derivatives, haemoglobin (Hb), oxyhaemoglobin (HbO_2) and methaemoglobin (MetHb), all have a different affinity for NO. Without oxygen, NO has an affinity for haemoglobin about 1500 times higher than that of CO.

Parameter		Molecular Wt (g)	Concentration (µmol/L)		Half Life	
Nitric Oxide	NO	30	0.003-1.0	Plasma	0.05-1 s	Blood
Nitrite	NO_2^-	46	0.1-0.5	Plasma	110 s	Blood
Nitrate	NO_3^-	62	30-60	Plasma	5-8 h	Blood
Peroxynitrite	$ONOO^-$	62				
Haemoglobin	Hb	64 458	$2.2x10^3$	Blood		
Albumin	Alb	69 900	500	Plasma		
Glutathione	Glu	307	20	Plasma	25-45 min	
Cysteine	Cys	121	10-30	Plasma		
S-Nitrosoalbumin	SNO-Alb	69 029	0.25-7	Plasma	15-40 min	
S-Nitrosoglutathione	GSNO	361	0.02-0.2	Plasma	8 min	
S-Nitrosocysteine	CysNO	160	0.2-0.3	Plasma	<1 min	
Nitrosyl haemoglobin	NO-Hb	64 488	0.5(a)-0.9(v)	Blood	<1 min	
S-Nitrosohaemoglobin	SNO-Hb	64 487	0.3(a)-0.003(v)	Blood	<1 min	

Table iii: Table showing a summary of nitric oxide, N-oxides and their predominant bioreactants in circulating blood. (a) – arterial, (v) – venous, s = seconds, h = hours, min = minutes. Adapted from [112].

The dilator action of endothelium-derived NO, discussed in section 1.2.2.1, contributes to the control of basal and stimulated regional blood flow in man[113]. NO also has a powerful inhibitory effect on platelet aggregation and adhesion, via both cGMP-dependent and cGMP-independent mechanisms, and it also inhibits both inflammatory cell activation and monocyte activity[114].

Impairment of the production of NO accounts for many of the abnormalities in vascular reactivity that characterise a wide variety of disease states, but the primary role of NO arguably lies in its anti-atherothrombotic properties rather than its vasodilator effects, thus the impact of decreased NO availability is seen very evidently in conduit arteries as atherogenesis.

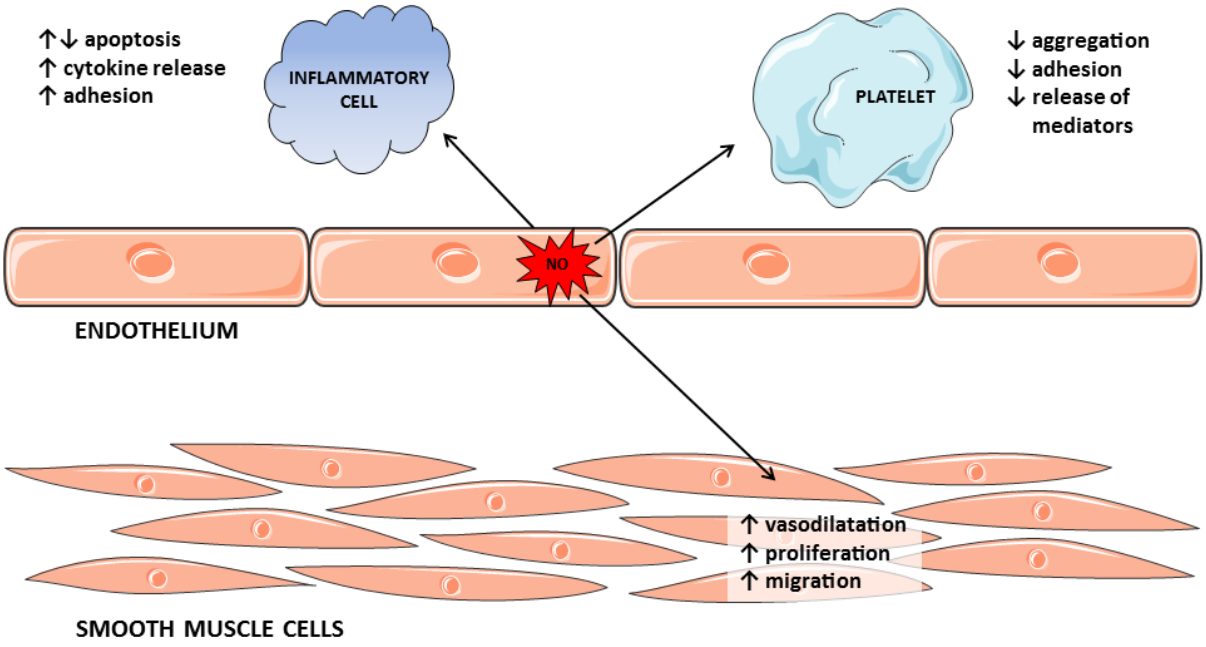


Figure 9: Figure highlighting the many actions of nitric oxide in the cardiovascular system. Adapted from [115].

In plasma, nitrite primarily derived from the conversion of l-arginine to NO by eNOS[116], remains stable for several hours, but in blood, depending on the redox conditions can be

rapidly oxidised to nitrate. In our laboratory, using similar detection methods to those I have utilised in the experiments herein, comprehensive studies were previously undertaken on the infusion of nitrite to humans[117], with an estimated half-life for nitrite in blood under normoxic conditions of 20-22 minutes, which is in good agreement with others[118]. An alternative source of nitrite includes peroxynitrate which can either form nitrosothiols first or decompose directly to nitrite and oxygen[119]. So, although NO detected at the luminal surface of the endothelium is unlikely to represent the total NO synthesised in the vascular endothelium, there is experimental data to support the measurement of NO oxidation products as a marker of total endothelial NO synthesis[120], and the James laboratory has significant experience utilising these methods in both healthy subjects and patient cohorts[121,122].

Nitrate levels are a marker for NO metabolism but they are unlikely to be sufficiently accurate alone to be used as a direct reflection of eNOS activity or indeed NO formation or endothelial dysfunction[123]. This is due to the fact that levels are influenced by a variety of factors, mainly dietary nitrate intake which probably accounts for around 70%[124], but also saliva formation, bacterial nitrate synthesis in the bowel, denitrifying liver enzymes, inhalation of atmospheric gaseous nitrogen compounds, and renal function.

1.3.2 NO metabolism in the gastro-intestinal tract

Salivary production is responsible for two thirds of the nitrite entering the stomach, the rest originating primarily form nutrients in food[125]. Bacteria in the oral cavity reduce nitrate to nitrite dependent on pH, oxygen, and type of bacterial flora with a resultant salivary nitrite concentration 30-210 µM and nitrate concentration of 200-600 µM[126].

Upon entering the gastric milieu, nitrite is acidified in the stomach resulting in dimerisation and dehydration to form N_2O_3 which can potentially form NO and NO_2. The salivary nitrite concentration is directly proportional to the amount of nitrate ingested and it has been shown that administration of oral potassium nitrate results in a significant increase in gastric NO

concentration[127]. The remaining nitrite continues into the small intestine where nitrate is formed through oxidation.

Food is not kept in the mouth but chewed and swallowed carrying nitrate through the gastro-intestinal tract. Nitrate is not absorbed in the stomach but uptake occurs primarily in the small intestine. This appears to be an active process with blood then transporting some nitrate back to salivary glands. Around 40-45% of the nitrate that passes into the intestines is metabolised, the rest filtered in the glomeruli and reabsorbed in the renal tubules prior to excretion in urine. The concentration of nitrate in urine is 250–2000 μM, whereas nitrite and NO are usually not detectable.

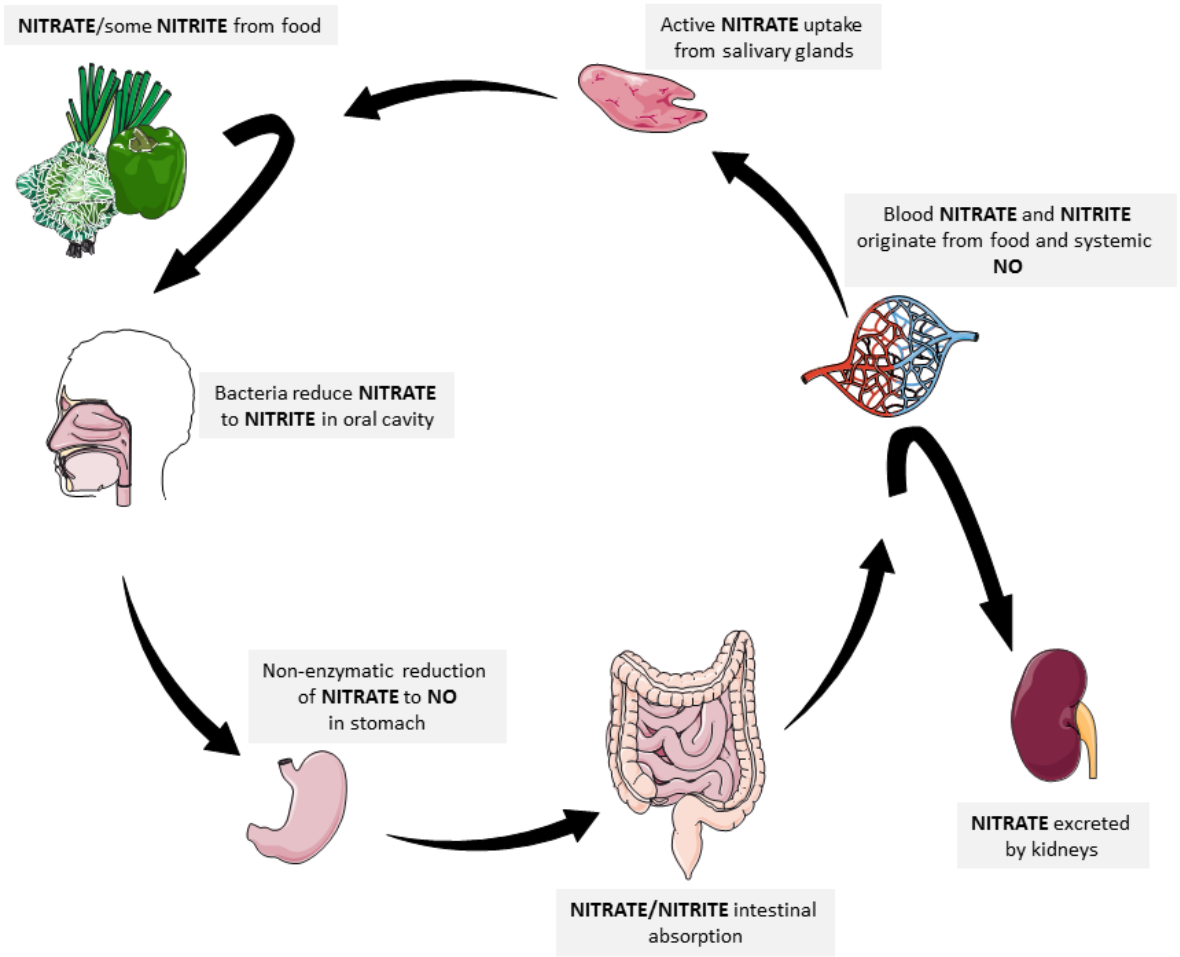


Figure 10: Nitrate-nitrite-nitric oxide pathway. Inorganic nitrates from the diet are absorbed in the small intestine, ~25% of which ends up in saliva. Commensal oral bacteria reduces nitrate to nitrite, which once swallowed is converted to NO and other nitrogen oxides in the acidic gastric juice. Nitrate and nitrite absorbed into blood and can serve as a source of NO in blood and tissue under hypoxic conditions. Adapted from [128].

1.4 S-Nitrosothiols

1.4.1 Background

There are a number of species that are capable of stabilising nitric oxide and thus minimise its oxidative inactivation. These agents therefore promote the biological actions of nitric oxide and one important class is the S-nitrosothiols. It has in fact been suggested that S-nitrosothiols may, in certain circumstances, be the direct proximate mediator of EDRF-like effects[108]. They are produced by the S-nitrosation of sulphydryl groups (usually cysteine thiols), ascribed the general formula RSNO. They occur naturally, having been demonstrated initially in human and rabbit plasma[108], as well as airway lining fluid where deficiency has been linked to severe asthma[129], and in neutrophils, where NO promotes the formation of intracellular S-nitrosothiol via activation of the hexose monophosphate shunt[130].

S-nitrosothiol derivatives of amino acids, peptides and proteins are natural products of NO metabolism, existing as S-nitroso derivatives of glutathione (GSH), cysteine, haemoglobin, BSA, and many other protein or non-protein thiols[131]. However, they are primarily attached to albumin with concentrations initially believed to be in the order of 7 µM in healthy humans. They are believed to act as stores of NO which can then be released when needed[108,132,133], and are involved in many physiological and pathophysiological processes, breaking down to release NO and the corresponding disulphide as follows:

$$2RSNO \rightarrow RSSR + 2NO$$

Debate has arisen regarding exact plasma levels due to chemical lability, interference of nitrite, and inherent errors attributable to the different analytical techniques used for measurement, with values ranging from 10 ηM to 10 µM[134-138].

1.4.2 Formation

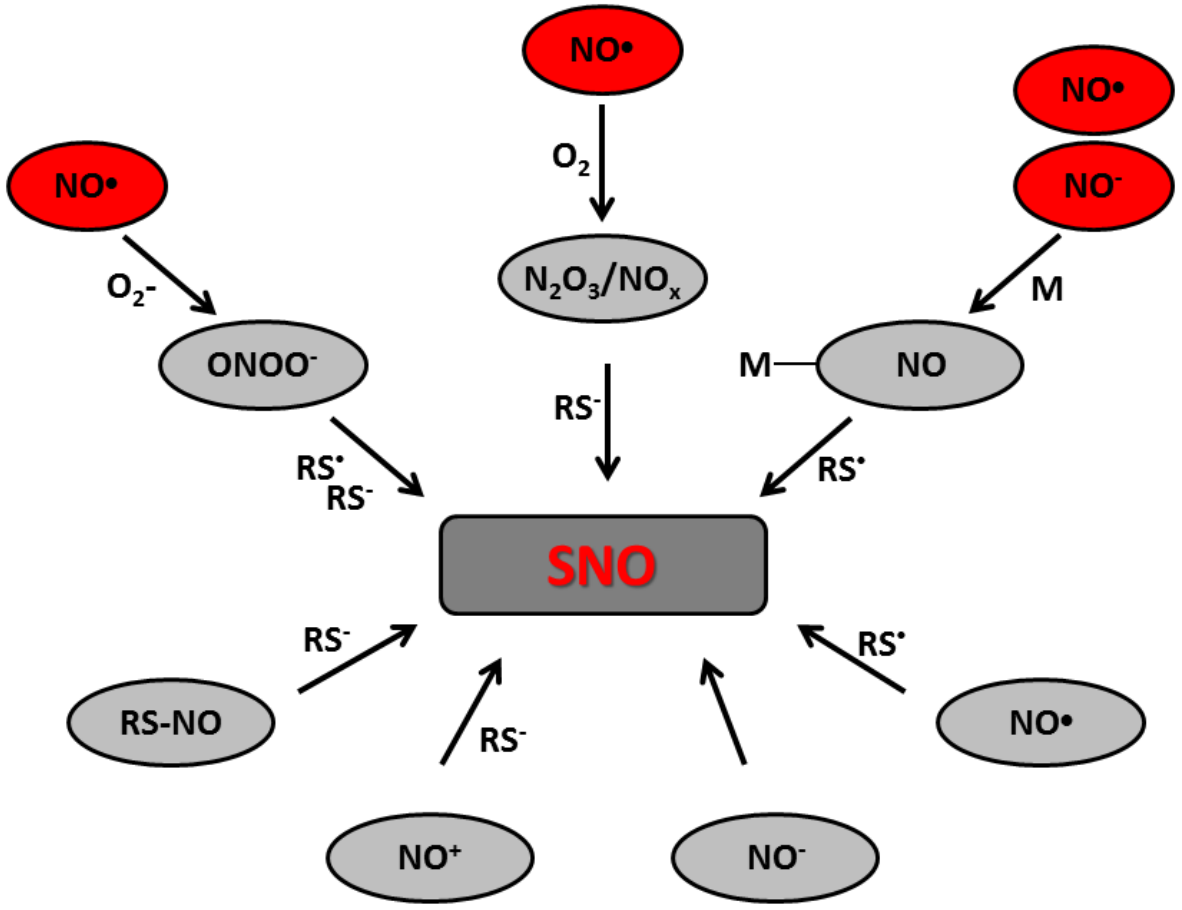


Figure 11: Illustration of the main mechanisms of S-nitrosothiols formation. (NO• = nitric oxide free radical, O_2 = oxygen, $O_2^{-\bullet}$ = superoxide, M = redox metals, $ONOO^-$ = peroxynitrite, N_2O_3/NO_x = nitric oxide oxides, M-NO = metal-nitric oxide complexes, RS• = thiyl radicals, RS^- = thiolate anion, NO^+ = oxidised nitrosonium cation, NO^- = reduced nitroxyl anion. Adapted from [139].

There are a number of ways S-nitrosothiols can be formed; the most important, as illustrated and described below.

NO, as a free radical (NO•), reacts primarily with superoxide ($O_2^{-\bullet}$), oxygen (O_2), and redox metals. These interactions result in the formation of S-nitrosylating agents including peroxynitrite (ONOO-), NO oxides (N_2O_3/NOx), and metal–NO complexes (M–NO) respectively. M-NO complexes also form as a result of the reaction between nitroxyl anions and redox metals. Furthermore, NO radicals can react directly with thiyl radicals (RS•) to form S-nitrosothiols.

Oxidised (nitrosonium cation (NO+)) and reduced (nitroxyl anion (NO-)) forms of NO can also act as S-nitrosylating agents. The direct reaction between thiols and NO- is dependent on the energy state of NO- and occurs only when NO- is present in the high energy singlet state.

1.4.3 Mechanisms of action

Shortly after the discovery that NO inhibited platelet function, it was identified that nitrovasodilator drugs including RSNO compounds could suppress platelet aggregation via sGC stimulation[140], and cGMP mediated inhibition of platelet adhesion, aggregation, granule secretion and fibrinogen binding[141-143].

This primary mode of action is secondary to the NO donor properties of these compounds, but other cGMP independent mechanisms also exist, including prevention of thromboxane synthesis[144], α-actinin nitration[145], platelet $P2Y_{12}$ ADP receptor inhibition[146], and S-nitrosylation[147] or altered phosphorylation[148] of platelet integrin αIIbβ3.

Although evidence exists for the role of NO, its direct influence on coagulation and fibrinolysis remains relatively unknown and under debate. For example nitrosative stress may promote a pro-thrombotic state[149] but an anti-thrombotic effect is seen with exposure of fibrinogen to GSNO by suppression of fibrin polymerisation[150]. Furthermore, another controversial hypothesis implicates the switching of tissue factor into a coagulation inactive form by RSNO compounds[151].

An alternative explanation as to how RSNO compounds exert their effect is cellular metabolism, specifically the transfer of NO from extracellular membrane-impermeant RSNOs across the plasma membrane of target cells, with cell surface protein disulphide isomerase (csPDI) probably representing the most promising mediator of RSNO signalling[152].

Interestingly, csPDI is known to exist on the external surface of the platelet plasma membrane[153].

Cellular uptake of an intact RSNO molecule via a membrane transporter has also been suggested. This has typically been shown with smaller RSNO species (cysNO and S-nitrosohomocysteine) transported via the amino acid transporter system-L (L-AT). The process involves extracellular cysteine transnitrosation to allow transporter uptake and subsequent intracellular signal transmission[154].

1.4.4 Decomposition

Decomposition of nitrosothiols occurs via several methods and at different rates depending on conditions. Firstly, they can be catalysed by Cu^{+} ions, which can themselves also be formed by reduction of Cu^{2+} ions by thiols[155]. This results in release of NO, with additional production of RS^{-} and Cu^{+} as depicted below.

$$Cu^{2+} + RS^{-} \longleftrightarrow \text{Intermediate substance (? } RSCu^{-}) \longrightarrow Cu^{+} \quad RS^{\bullet}$$

$$Cu^{+} + RSNO \longleftrightarrow \text{Intermediate substance} \longrightarrow Cu^{2+} + RS^{-} + NO$$

$$2RS^{-} \longrightarrow RSSR$$

Indeed, this is the basis of the “2Cs” assay used for nitrosothiols quantification in this thesis and described in detail in methods section 2.2.4.

The type of S-nitrosothiols present determines which of the above pathways is rate-limiting, and the quantity of thiol is also critical as it determines whether catalysis of S-nitrosothiols is sped up, or slowed down perhaps through formation of Cu^{2+} complexes[155].

Alternatively, S-nitrosothiols can decompose to release NO via transnitrosation which involves the transfer of NO to other thiols[156].

Enzymatic decomposition has also been reported, specifically g-glutamyl transpeptidase can cause breakdown of GSNO to NO in the presence of Cu^{+} once converted to another less stable S-nitrosothiol compound[157].

Importantly, homolytic cleavage of the *S*-N bond also occurs secondary to decomposition by photosensitisers; photolysis of GSNO has been demonstrated at 340 nm and 545 nm with release of nitric oxide[158,159].

Other methods comprise homolytic cleavage of the S–N bond to give NO and an alkyl thiyl radical secondary to thermal decomposition, and also the presence of ascorbate which leads to S-nitrosothiols reduction either through reduction of Cu^{2+} to Cu^{+}, or directly when, at high concentrations, it acts as a nucleophile[160,161].

Although S-nitrosothiols have established NO donor properties and as such potential therapeutic applications, it has been shown that their biological effects do not correlate with the rate of nitric oxide release in solution, and therefore biological activity cannot necessarily be inferred from their rate of decomposition in an experimental buffer[162,163]. Nevertheless, the prospect of their use therapeutically remains appealing, enhanced by the fact they are naturally occurring, which would suggest low toxicity.

1.4.5 Clinical Effects

S-nitrosothiols are not used therapeutically at present, but there is a vast growing library of evidence supporting their potential clinical use. This relates to their ability to transport and transfer NO, and will therefore be discussed in more detail in section 1.6.4 Endothelial Dysfunction – therapeutic targets.

1.5 Oxidant stress

Reactive oxygen species and oxidant stress play a pivotal role in cardiovascular disease and endothelial dysfunction.

Reactive oxygen species (ROS) comprise a family of oxygen-derived molecules produced by all aerobic cells[164]. Small rises in ROS are important in the maintenance of physiological functions, as they act as signalling molecules in redox biology, but when produced excessively they result in oxidant stress, a harmful process in which biological macromolecules including DNA, protein, carbohydrates, and lipids become oxidised.

Combustion of organic compounds with oxygen by aerobic metabolism allows organisms to obtain much more energy than organisms utilising anaerobic processes. However, the resultant generation of ROS can damage and destroy cell structures. The most important reactive oxygen species include the superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). Cellular levels of ROS should remain essentially stable so long as the balance between constant mitochondrial production of ROS and their continuous neutralisation by antioxidant enzymes, such as superoxide dismutases (SOD), catalase, glutathione peroxidase, thioredoxins and peroxiredoxins, remains equal.

Endothelial dysfunction as a result of decreased NO bioavailability may be caused by several factors including decreased eNOS expression, reduced substrate and cofactor availability for eNOS, alterations in cellular signalling and importantly accelerated NO degradation by ROS[165].

Repeated injury to the vascular endothelium as seen in association with many of the traditional risk factors for cardiovascular disease including hyperlipidaemia, hypertension, diabetes, and smoking, are associated with overproduction of ROS or increased oxidative stress[166].

There are numerous sources of ROS in vascular cells, but the most important are probably xanthine oxidase[167], NADH/NADPH oxidase[168], lipoxygenases, cytochrome p450 and NO synthase. The latter is an important source of $O_2^{-\bullet}$ and H_2O_2, formed when eNOS lacks either L-arginine or its cofactor (BH_4) in a process referred to as NOS uncoupling[169]. Furthermore, this uncoupling phenomenon has been implicated as a cause of nitrate tolerance[170], although other ROS-derived mechanisms have also been implicated in this process as described in section 1.6.5 Endothelial dysfunction – NO donors (Nitrates).

Evidence suggests that xanthine oxidase, which is present in the vascular endothelium, catalyses the oxidation of hypoxanthine to $O_2^{-\bullet}$ leading predominantly to vascular dysfunction and end organ damage secondary to hypertension[171].

The NAD(P)H oxidases (nicotinamide adenine dinucleotide phosphate-oxidase) represent a family of seven multi-subunit enzymes that catalyse $O_2^{-\bullet}$ production by the 1-electron reduction of O_2 using NADPH or NADH. This family comprises the superoxide-producing enzymes Nox1, Nox2 (formerly referred to as gp91phox [*ph*agocyte *ox*idase] when first identified due to its expression in phagocytic cells involved in host defence), Nox3, Nox4, Nox5, and the dual oxidases Duox1 and Duox2 that release hydrogen peroxide but not superoxide. There are multiple signalling pathways responsible for vascular NAD(P)H oxidase activation including cytokines, mechanical forces, several growth factors, and metabolic factors such as G protein–coupled receptor agonists[172,173].

ROS generated as a result of NAD(P)H oxidase activation have been implicated in the regulation of vascular tone both directly, and indirectly by decreasing NO bioavailability, these processes contributing to vascular damage and remodelling in hypertension and other cardiovascular diseases.

The role of oxidant stress is therefore integral to the development of endothelial dysfunction, but this can in part be ameliorated with the use of traditional antihypertensive agents such as β-adrenergic blockers, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers, AT1 receptor antagonists, and calcium channel blockers. These beneficial effects have been attributed to both NAD(P)H oxidase activity inhibition and to intrinsic antioxidant properties of the drugs.

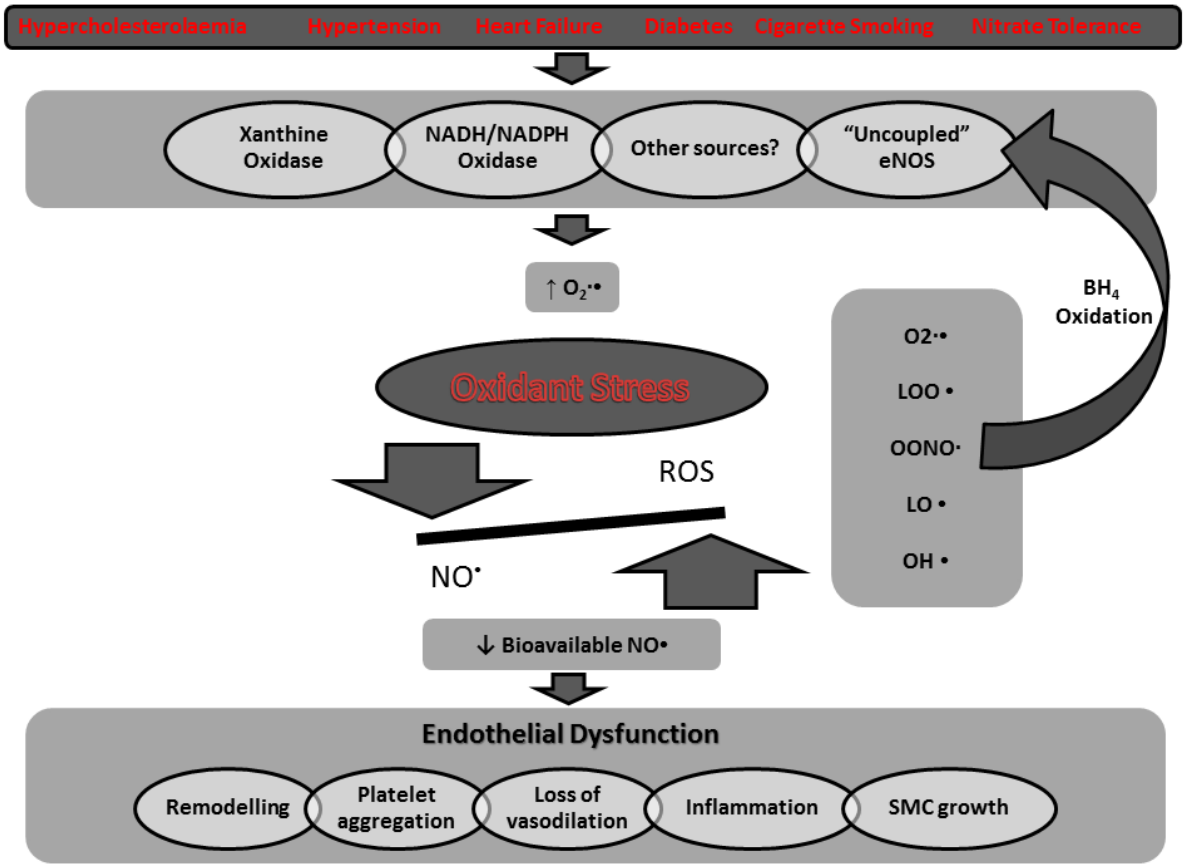


Figure 12: Overview of the mechanisms of oxidant stress induced endothelial dysfunction in cardiovascular disease. Many reactive oxygen species (ROS) possess unpaired electrons and thus are free radicals, including superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$) and lipid radicals (LO• and LOO•). Other ROS, which are not free radicals but having oxidising effects, include peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl). (BH_4 = tetrahydrobiopterin). Adapted from [166].

1.6 Endothelial activation and endothelial dysfunction

Endothelial dysfunction and endothelial cell activation are important converging concepts that represent an alteration of normal endothelial physiology with potentially serious deleterious effects that culminate in vascular disease.

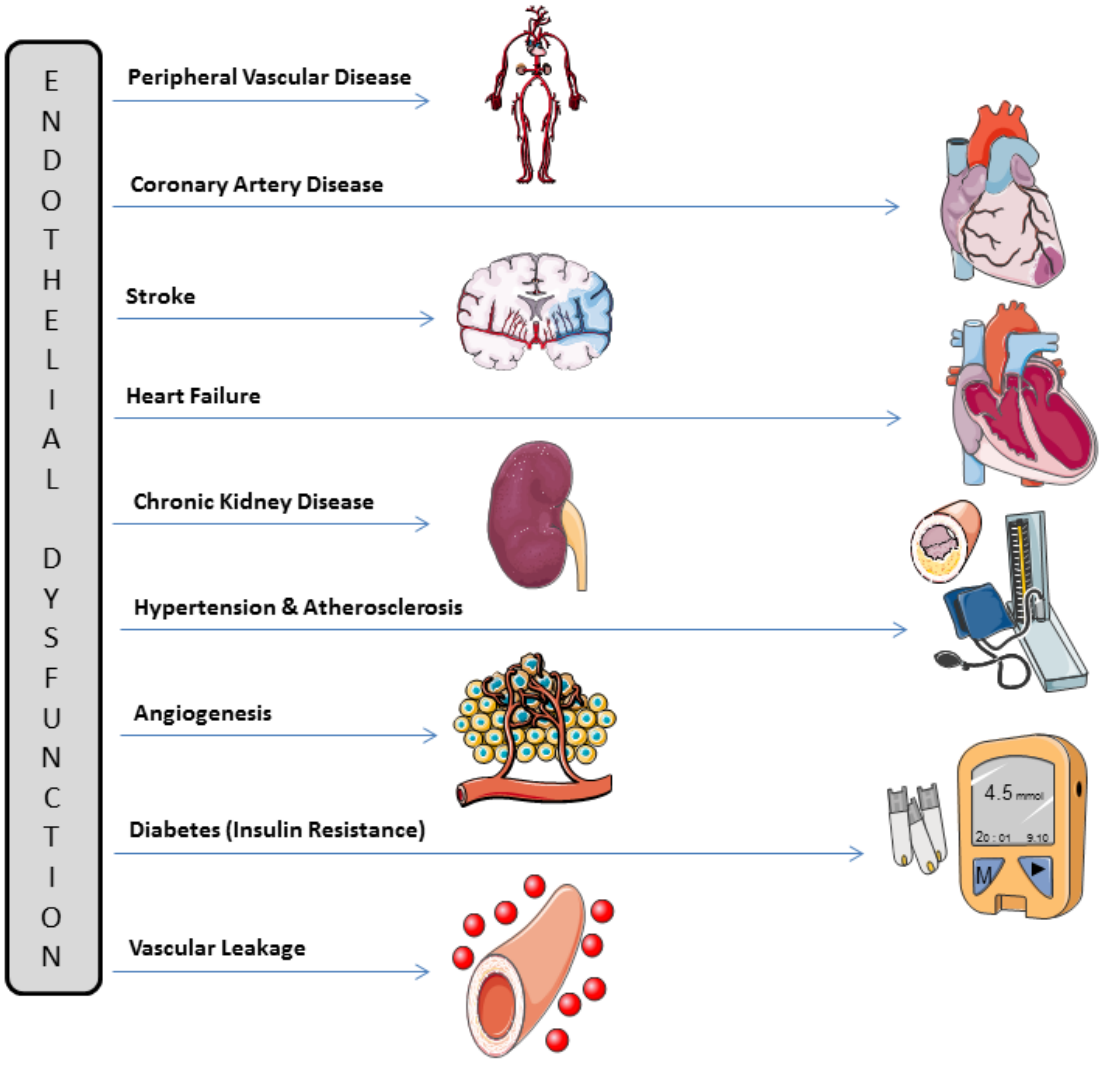


Figure 13: Schematic showing the disease states that are commonly associated with endothelial dysfunction.

1.6.1 Endothelial dysfunction

Dysfunction of the endothelium is characterised by reduced vasodilation, a pro-inflammatory state, and increased pro-thrombotic properties. Traditional risk factors including diabetes mellitus[174], hypertension[175], cigarette smoking[176], and hypercholesterolaemia[177] are all implicated in the alteration of endothelial function which results in a chronic inflammatory process and abnormal vasoreactivity. Other more recent associations include obesity[178], elevated C-reactive protein[179] and chronic systemic infection[180].

It is characterised by decreased synthesis, release, and bioavailability of vasodilators, particularly endothelium-derived NO, and/or an increase in endothelium-derived contracting factors, resulting in the impairment of endothelium-dependent vasodilation. Decreased expression of endothelial cell NO synthase (eNOS)[181], a lack of substrate or cofactors for eNOS[182], alteration of cellular signalling such that eNOS is not appropriately activated[183], and accelerated $NO^{\cdot}$ degradation by ROS[165] can all result in decreased NO bioavailability.

There is a close association between endothelial cell dysfunction and cardiac events[184], with a multivariate analysis of almost 2500 patients demonstrating a strong and independent association with cardiovascular events such as cardiac death, myocardial infarction, and need for revascularisation[185]. There is a lack of correlation between endothelial dysfunction and the presence of traditional cardiovascular risk factors, but nevertheless it can be considered reflective of an integrated index of all atherogenic and atheroprotective factors present in an individual. Endothelial dysfunction thus reflects a vascular phenotype prone to atherogenesis[186].

1.6.2 Endothelial cell activation

Under normal homeostatic conditions there is little or no expression of pro-inflammatory factors, but the endothelium can undergo changes which allow it to participate in the inflammatory response. This is known as endothelial cell activation, a term coined by Willms-Kretschmer in the 1960s[187] and re-introduced in the 1980s by Pober[188]. There are five core changes implicated in endothelial cell activation comprising loss of vascular integrity, expression of leukocyte adhesion molecules, change in phenotype from antithrombotic to prothrombotic, cytokine production, and up-regulation of HLA molecules. The cell-surface adhesion molecules include VCAM-1, ICAM-1, and endothelial leukocyte adhesion molecule (ELAM, also known as E-selectin). Release of pro-inflammatory cytokines such as TNF-α and IL-6 typically induce endothelial cell activation and also facilitate recruitment and attachment of circulating leukocytes to the vessel wall.

The processes involved in endothelial cell activation are diverse but they appear to share a common intracellular control mechanism through transcription factor activation. One regulatory factor in particular, nuclear factor κB, (NF-κB), once activated, is transported into the nucleus where it binds to promoter areas of genes which are upregulated in endothelial cell activation[189]. The precursor to this is a switching from the quiescent endothelial phenotype, where NO-mediated silencing predominates, towards one where the host defence response is activated and ROS signalling dominates. ROS molecules target NF-κB and phosphatases, leading to endothelial cell activation. This can occur physiologically in the context of host defence, or pathophysiologically in the presence of cardiovascular risk factors[190].

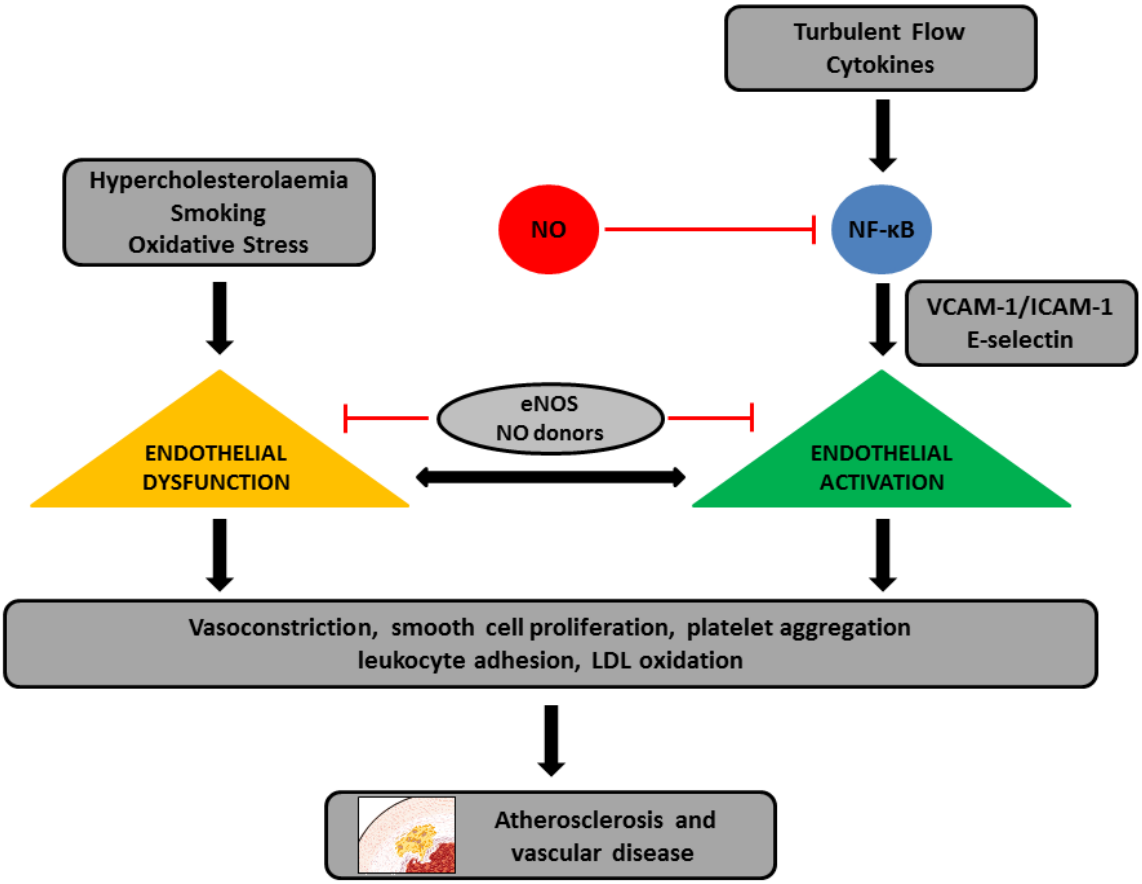


Figure 14: Endothelial dysfunction and endothelial cell activation in vascular disease. Cardiovascular risk factors are important mediators of endothelial dysfunction. However, turbulent flow and pro-inflammatory cytokines promote endothelial cell activation via activation of the transcription factor, nuclear factor-kappaB (NF-κB). NO reduces endothelial activation by inhibiting NF-κB whereas loss of NO increases endothelial activation. Both endothelial dysfunction and endothelial activation lead to atherosclerosis. Adapted from [191].

1.6.3 Clinical assessment of endothelial function

With better understanding of the vascular biology of the endothelium has followed the development of clinical tests to evaluate the functional properties of normal and activated endothelium[192]. Importantly, the assessment of endothelial function serves as a good predictor of future cardiac events both in individuals at risk of CVD, and those with established CVD[193].

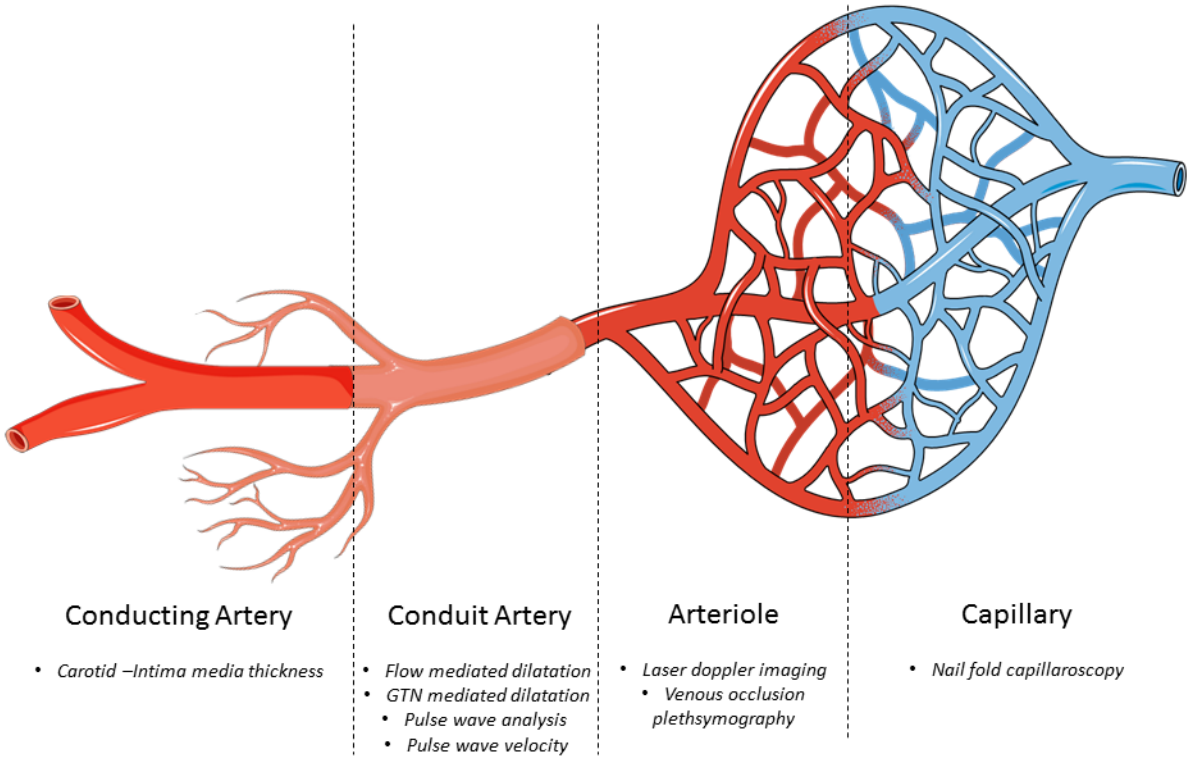


Figure 15: Overview of the different types of techniques available for the assessment of endothelial function and vascular structure performed in different vascular beds. Adapted from[194].

Initial clinical studies of endothelial function used coronary angiography to evaluate change in coronary diameter following local infusion of acetylcholine[195]. The responses to other endothelial agonists including substance P, adenosine, and bradykinin have also been measured[196], as has the response to specific NO antagonists such as L-NMMA[197]

A less invasive approach involves measurement of forearm resistance vessel tone by venous occlusion plethysmography following pharmacological infusion[198].

This measures microvascular pathophysiology, so for an even less invasive test with the potential for improved repeatability and standardisation, brachial artery flow mediated dilatation (FMD) represents the gold standard for clinical research on conduit artery endothelial biology as long as the technique is correctly applied[199]. Alternative non-invasive approaches are available for the study of vascular biology in the peripheral circulation predominantly through measurement of pulse wave analysis[200].

Finally, circulating markers can be measured as surrogate indicators of endothelial function, including measures of NO biology, inflammatory cytokines, adhesion molecules, and regulators of thrombosis but many are difficult and expensive to measure with the inherent risk of confounding factors leading to erroneous interpretation.

1.6.4 Endothelial dysfunction – therapeutic targets

Endothelial dysfunction is potentially reversible, and the mainstay of current treatment is aimed at strategies that reduce cardiovascular risk factors. Cholesterol lowering, antihypertensive therapy, smoking cessation, ACE inhibitor therapy, oestrogen replacement therapy in postmenopausal women, supplementation with folic acid, and physical exercise, translate into improved endothelial health.

Medications that control CVD risk factors such as antihypertensive agents and statins may also have beneficial effects on endothelial function through decreasing oxidative stress and lipid accumulation. Additionally, some of these established strategies target direct vasoactive substances including endothelin, and NO activation which is enhanced for example by statins and L-arginine.

Beta-blockers are considered to have minimal effect on endothelium-dependent vasodilation, although nebivolol does induce vasodilation by a direct effect on NO synthase and an antioxidant effect[201] whilst carvedilol suppresses ROS generation[202]. Calcium-channel blockers are also able to reverse impaired endothelium-dependent vasodilation, mainly in the microcirculation[201], and ACE inhibitors and angiotensin receptor blockers (ARBs) exert several pleiotropic effects, which reduce oxidative stress and stimulate bradykinin to help increase NO bioavailability[203]. Current therapeutic strategies are summarised below;

Treatment Associated With Improvement of Endothelial Dysfunction in Humans	
Acute	**Chronic**
LDL lowering with apheresis	LDL lowering with statins
ACE inhibition/ARBs	Antioxidants (probucol with lovastatin)
Vitamins C + E	ACE inhibition /ARBs
Oestrogen	Oestrogen
L-arginine, D-arginine	Oestrogen and progesterone
Tetrahydrobiopterin, methyltetrahydrofolate	Exercise
Deferoxamine	L-arginine
Glutathione	Metformin/Thiazolidine to improve insulin resistance
Calcium Channel Blockers	β-blockers

Table iv: Established treatment strategies for endothelial dysfunction.

Other potential pharmacological therapy comprises drugs which enhance EPC, drugs which reduce ADMA, tetrahydrobiopterin, and inhibitors of PKC and TNF-α. Drugs which enhance NO release warrant particular mention and will be discussed next.

1.6.5 Endothelial dysfunction – NO donors

Given that loss of endogenous NO production is integral to endothelial dysfunction which manifests as vasoconstriction, increased smooth muscle cell proliferation, and activation of platelets and inflammatory cells with resultant adherence at sites of endothelial damage, there has been extensive research in an attempt to try and establish a role for exogenous NO as a therapeutic target. Unfortunately, relatively little progress has been made in recent years, but drugs capable of releasing NO including those listed below remain a focus for

potential therapeutic application. Except for its use in pulmonary hypertension and in neonates where it can be used in gaseous form, NO generally requires a 'carrier' (NO donor drugs) allowing its transport and stabilisation until its release is required.

Organic Nitrate:

Organic nitrates have been a favoured treatment for angina pectoris for many years. Nitroglycerin, isosorbide dinitrate and mononitrate are the agents of choice, and although they alleviate symptoms of angina through vasodilation, they do not appear to alter the progression of platelet and inflammatory cell activation associated with atherosclerosis. The mechanism of NO release also remains unclear, although is likely to be mediated by specific enzymes in vivo[204,205]. Furthermore, drug tolerance, although ameliorated with a nitrate free period, remains a hindrance to its use with some reports of a paradoxical increase in cardiac events with long term use[206]. Nitrate tolerance, thought to be mediated by increased vascular production of superoxide ion ($O_2^{-\bullet}$) probably occurs via several mechanisms, which account for the associated endothelial dysfunction seen with continuous organic nitrate use; increased peroxynitrite formation reduces NO availability, vascular oxidative stress inhibits sGC and PKG, and mitochondrial ROS inactivate mtALDH which is necessary for nitrate bio-activation[207-209].

Some confirmation that ROS are central to nitrate tolerance and endothelial dysfunction is suggested in the finding that co-administration of antioxidants including vitamin C, vitamin E, folic acid, and hydralazine can ameliorate and even negate the effect.

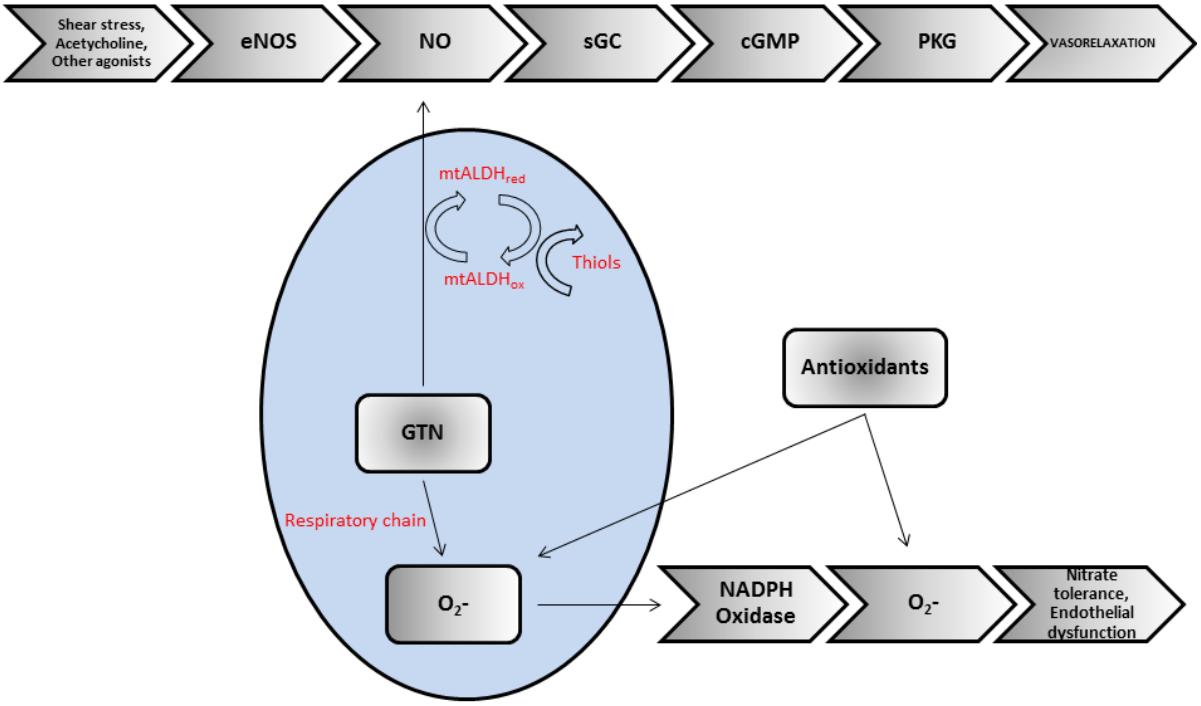


Figure 16: Biotransformation of nitroglycerine. GTN is biotransformed by mitochondrial aldehyde dehydrogenase (mtALDH) to release nitric oxide (NO) which results in vasodilatation via the well-described sGC-cGMP-PKG activation pathway . The same transduction mechanism is used by factors which lead to eNOS activation. Simultaneously, GTN uncouples the mitochondrial respiratory chain to increase superoxide anion production. This results in results in activation of membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase Adapted from [205].

Sodium Nitroprusside (SNP):

SNP is used clinically for rapid lowering of blood pressure in hypertensive crises and in clinical studies as the gold standard NO-dependent, but endothelium-independent vasodilator. Its use is limited by the need for intravenous administration, potency and sensitivity to photolysis.

Diazeniumdiolates (NONOates):

These agents decompose spontaneously in solution at physiological pH and temperature, to generate up to 2 molar equivalents of NO, but as yet, pending further studies on long-term

safety have no therapeutic role in clinical practice despite a wealth of early promising studies[115].

S-Nitrosothiols:

The S-Nitrosothiols have been discussed in detail in section 1.4. They generally have less stringent metabolic requirements, with some evidence of tissue selectivity and an apparent absence of tolerance following long term use, giving them clear advantages over other NO donors[210]. Furthermore, their ability to transfer NO^+ species between different thiol groups (transnitrosation) enables protection of NO from oxidative stress prior to release. They have been investigated extensively, showing promise in human and animal studies predominantly in the cardiovascular system.

S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) have proven NO donor properties, with evidence of diverse and remarkable biological effects; SNAP is a potent vasodilator[211], and a 3-minute intracoronary infusion before ischaemia in anaesthetised pigs has been shown to decrease infarct size and improve coronary endothelial function[212]. GSNO has exhibited significant protection to the ischaemic myocardium in an isolated rat heart model[213] and the ability to reduce lesion size in transgenic mice with early atherosclerosis[214].

Early studies confirmed that S-nitrosoalbumin could reduce platelet adhesion and neointimal thickening in angioplasty-damaged blood vessels both as an infusion in a rabbit model[215] and as a stent-coating in a model using canine blood platelet adhesion in a plate glass chamber[216].

This use as an anti-platelet agent represents a particular area of interest but was initially limited due to concerns over profound co-existent vaso-dilatory and hypotensive effects.

However, with appropriate dosing, GSNO infused via the brachial artery was shown to be highly effective as a platelet inhibitor without associated vasodilation, suggesting that it is possible to achieve selective antiplatelet and potentially antithrombotic effects with these NO donors[217], and this was also confirmed following intravenous GSNO administration[218]. This, together with other supportive data, demonstrates the useful downstream effects of RSNO, its relative stability allowing carriage of NO and delivery not just locally but to more distal sites. Furthermore, it is well established that platelets are activated following percutaneous transluminal coronary angioplasty (PTCA) and percutaneous coronary intervention (PCI) with potentially harmful effects, but GSNO administered before coronary angioplasty was shown to significantly inhibit platelet surface expression of P-selectin and glycoprotein IIb/IIIa without altering blood pressure[219], and has been shown to reduce platelet adhesion in bypass grafts[220].

Other benefits in the cardiovascular system have been demonstrated, with GSNO leading to a decrease in both occurrence of cerebral embolism following carotid endarterectomy in patients already receiving aspirin and heparin[221], and emboli that dissociate from carotid plaques[222].

Further large scale studies are required before S-nitrosothiols can establish a therapeutic role as antiplatelet agents and also in other conditions where they have shown some promise, particularly immune and inflammatory processes, pre-eclampsia[223], neuroprotection specifically delaying progression of neurodegenerative disorders[224], improved wound healing[225], and as an adjunct for nitrate tolerance[226], amongst others.

It is important to state that NO exerts its cellular influence predominantly in a cGMP-independent manner and NO-mediated modification of protein cysteine residues through S-nitrosylation and generation of S-nitrosothiols plays a major role. In particular the redox reaction between NO and protein cysteine thiol side chains affects structure and function, thus S-nitrosylation is responsible for widespread cellular effects within the cardiovascular

system, and conversely protein denitrosylation has been shown to have an important role controlling cellular S-nitrosylation. Precisely regulated equilibrium between these two pathways, in addition to transnitrosylation reactions (the transfer of a NO from one thiol to another) between a variety of peptides and proteins is critical to SNO-based signal transduction.[227]

Accumulating evidence highlights the role of S-nitrosylation both in normal physiology and in a broad spectrum of human diseases. Given this influence of protein S-nitrosylation on the ubiquitous action of nitric oxide on cellular signal transduction, the use of exogenously-derived SNO represents an exciting therapeutic opportunity but its administration must be carefully controlled and regulated to avoid interference with normal physiological S-nitrosylation pathways.

Sydnonimines:

These are heterocyclic compounds derived from morpholine, the liberation of NO requiring an alkaline pH and the presence of oxygen[228].

C-nitroso compounds:

These compounds have been studied for over 120 years, but only recently have they been explored with regards to their potential use as nitric oxide donors. Once exposed to light, C-nitroso compounds may undergo homolytic cleavage of the C-NO bond to generate NO[229].

Inorganic nitrate:

Although organic nitrates have been the mainstay of angina treatment for over a century[230] the Chinese used inorganic nitrate as far back as 700AD for the treatment of coronary artery disease before the West followed between the 14th and 17th centuries [231]. This practice has not been commonplace since concerns over potential carcinogenicity were raised in the early 20th century.

Both organic and inorganic nitrates exert their effects via nitric oxide, but their different pharmacokinetic properties result in very different bioavailability and metabolic profiles. Inorganic nitrates are found in abundance in green leafy vegetables and beetroot, with many linking this to the recent association between diets rich in fruit and vegetables and reduced cardiovascular morbidity and mortality. There is certainly a correlation between vegetarian diets and lower blood pressure. The bioactivation of orally ingested nitrate to nitrite and resultant NO formation as part of the entero-salivary circulation is discussed in section 1.3.2 [232].

NO-mediated vasodilatation and decreases in blood pressure have been demonstrated following oral nitrate administration but the exact mechanisms have been unclear. A recent study however has demonstrated that exogenous administration of inorganic nitrate in the form of beetroot juice or as a potassium nitrate capsule supplement to healthy volunteers resulted in attenuation of ex vivo platelet aggregation responses to ADP and collagen, predominantly in males. This was associated with a reduction in platelet P-selectin expression and elevated levels of platelet-derived cGMP [233].

Preventing the development of endothelial dysfunction and progression to atherothrombotic complications remains a fundamental target, and as opposed to the other NO donors, this simple dietary modification could represent one of the easiest and safest strategies.

1.7 Atherosclerosis

Atherosclerosis is a process that begins in childhood, progressing silently through a long preclinical stage that culminates in clinical disease usually from middle age. The link between endothelial dysfunction and this process was first identified over 35 years ago[234], and ever since, the study of human endothelium has remained at the forefront of cardiovascular research, its exact role in influencing the development and progression of vascular disease being the focus. This important lining of cells has emerged as the key regulator of vascular homeostasis, acting as a signal transducer with the ability to adopt a phenotype that facilitates inflammation, thrombosis, vasoconstriction, and ultimately atherosclerotic lesion formation in the presence of coronary artery disease risk factors[235].

Notably, levels of endothelial-derived vasoactive molecules are altered in atherosclerosis. Elevated concentrations of the ET peptide have been demonstrated within atherosclerotic vessels[75], and also in the plasma of patients with both advanced atherosclerosis[236] and acute myocardial infarction[237]. Large epicardial coronary arteries are particularly prone to atherosclerosis whereas small resistance coronary arteries are less susceptible to plaque formation. However, due to the latter's downstream location and consequent exposure to ischaemic conditions, they tend to exhibit increased levels of ET-1.

Furthermore, in some situations, induction of NOS has been shown to result in a focal increase of NO levels within atherosclerotic plaques[238], a reflection of the underlying inflammatory process. However, in atherosclerotic coronary artery disease, the overall basal release of NO from the endothelium is decreased[239].

The steps that culminate in atherosclerosis and ultimately clinical events include initiation of endothelial activation and inflammation followed by promotion of intimal lipoprotein deposition, release of cytokines, growth factors and chemokines, and build-up of cholesterol-engorged macrophages termed foam cells. In the early stages of atherosclerosis, lesions are labelled "fatty streaks" which although not clinically significant, are the precursor to more

complex plaque formation as lipid-rich necrotic debris and smooth muscle cells accumulate. With progression, occlusive plaques can form, but clinical events such as myocardial infarction and stroke are typically initiated when a plaque ruptures exposing the inner contents to vasoactive substances within blood which trigger thrombus formation. Smooth muscle cells (SMC) and an extracellular matrix form a “fibrous cap” overlying the lipid rich necrotic debris and this can be particularly prone to ulceration and rupture. This process of plaque formation has been established as a human disease for thousands of years[240], but acceleration of atherosclerosis through effects on low-density lipoprotein (LDL) particles and inflammation is attributable to risk factors including hypertension, cigarette smoking, obesity, diabetes mellitus, and genetic predisposition.

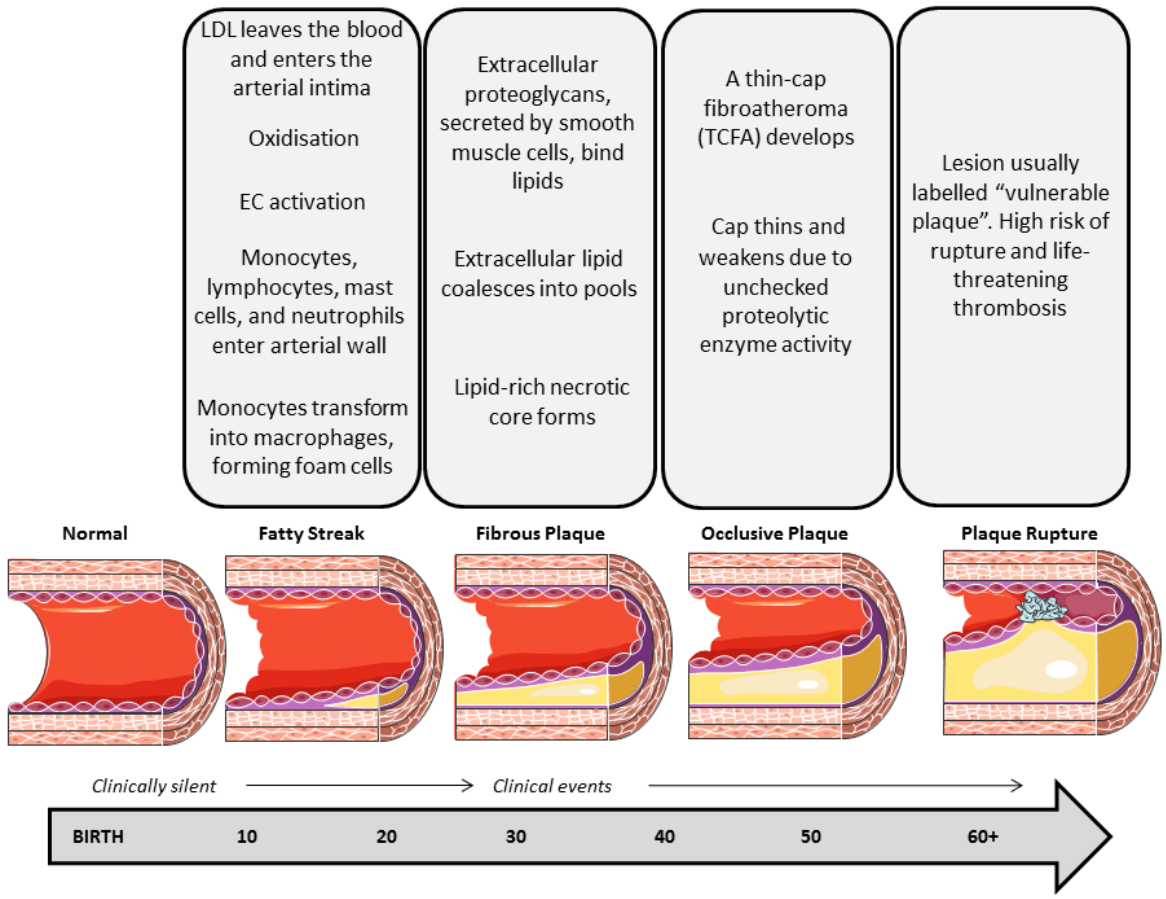


Figure 17: Atherosclerotic plaque formation and progression. The illustration depicts the pathological progression of atherosclerosis, from no visible disease at birth to the development of complex plaques in mid to late adulthood with the potential for rupture and thrombosis.

Given that the accumulation of different plasma lipoproteins appears to be of primary importance in the aetiology of atherosclerosis, the mainstay of treatment currently comprises lipid lowering therapy. The clinical manifestations of atherosclerosis can be significantly reduced, proportional to the reduction in LDL cholesterol, by the use of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins). These have been shown to reduce the risk of clinical events, reduce plaque size, alter cellular composition and plaque chemical composition, and improve plaque biological activity primarily through mechanisms effecting inflammation and lipid metabolism[241,242], with regression of atherosclerosis reported[243,244]. Whilst lowering LDL levels is therefore important, elevated levels of high-density lipoprotein (HDL) have been considered to be atheroprotective[245].

1.8 The role of platelets

Platelets are anucleated cells of 1–2 μm in length, originating from multinuclear megakaryocytes in the bone marrow. They usually circulate in a quiescent state, but increased platelet activation is seen in patients with established cardiovascular disease.

In atherosclerosis, platelets facilitate the recruitment of inflammatory cells towards lesion sites and release a variety of inflammatory mediators. They are central to the pathogenesis of atherosclerosis and thrombosis with platelet activation being pivotal in ischaemic syndromes affecting both the cerebral[246] and coronary circulations[247]. They participate in both primary and secondary haemostasis, involving platelet adhesion, aggregation and pro-coagulant activity. Once activated, platelets produce cytokines and chemokines, released from α-granules[248], leading to increased levels of circulating platelet-leukocyte aggregates, which are fundamental to local atherothrombosis and inflammatory immune reactions at the vessel wall[249]. Activated platelets also release serotonin, soluble p-selectin and thrombocidins. Platelet cell surface receptors such as p-selectin and CD40 ligand are important in platelet-leukocyte interaction and promotion of atherosclerosis[250,251].

Thrombus formation occurs following initial platelet tethering mediated by glycoprotein GPIbα. This is followed by activation and firm adhesion, aggregation with recruitment of more platelets, and then thrombus stabilisation. Thrombin generation at the site of vascular injury is the most potent platelet activator and causes platelets to change shape, enabling integrin activation and granule secretion, with adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2) release promoting further activation. Thrombin mediates platelet activation via protease-activated receptors (PARs) located on platelets, endothelial cells, smooth muscle cells, mononuclear cells and fibroblasts, and receptor antagonists have attracted recent attention as potential novel antiplatelet agents.

1.9 Antiplatelet therapy

Aspirin remains the reference antiplatelet drug. In addition, ADP is fundamental to platelet activation and aggregation, so the $P2Y_{12}$ receptor has become the main target of current antiplatelet agents. The ADP $P2Y_1$ receptor also represents a potential novel target[252].

The αIIbβ3 (glycoprotein IIb/IIIa) antagonists (abciximab, eptifibatide and tirofiban) also form part of the current antiplatelet armament through their mechanism of inhibition of the final common pathway of platelet aggregation, usually in the setting of percutaneous coronary intervention.

Finally, vorapaxar has been introduced as a first-in-class PAR-1 antagonist, effective in the secondary prevention of atherothrombosis currently licensed for use in the USA[253,254].

1.9.1 Aspirin

Aspirin's effects on blood clotting (as an antiplatelet agent) were first noticed in 1950 by Lawrence Craven. However, it wasn't until the early 1970s when the first clinical trials

confirmed mortality and morbidity benefit that aspirin developed an established role in the treatment of cardiovascular disease.

Aspirin permanently acetylates platelet cyclo-oxygenase 1 (COX-1), blocking the synthesis of TXA_2, which promotes activation of other platelets and is a potent vasoconstrictor. Cyclo-oxygenase 2 (COX-2) expressed by cytokines, secondary to inflammatory stimuli and some growth factors, is also inhibited but much higher doses of the drug are required. Selective COX-2 inhibitors (including celecoxib, rofecoxib and valdecoxib) have been shown to improve endothelial function and reduce markers of inflammation[255]. However, serious safety concerns were raised following an observed increase in the incidence of cardiovascular events, attributable to their effect of blocking PGI_2 without inhibiting TXA_2 resulting in platelet activation, adhesion and aggregation[256].

It has been suggested that aspirin can induce the formation of NO[257], and also modulate signalling through NF-κB[258].

Aspirin has no effect on other platelet activators, such as adenosine diphosphate (ADP), thrombin or serotonin.

1.9.2 Thienopyridines

Ticlopidine, clopidogrel and prasugrel are members of the thienopyridine family of platelet anti-aggregants. Ticlodipine was the first FDA licensed thienopyridine for clinical use in 1991 and clopidogrel followed soon after in 1997. Unfortunately, ticlopidine causes severe neutropenia in more than 1% of patients and thrombotic thrombocytopenic purpura (TTP) in about 0.2% of patients, in which up to 25-50% can be fatal[259]. Clopidogrel is synthesised by the addition of a substituted ester linkage to the base molecule ticlopidine. Both compounds are converted from the inactive parent compound to labile active metabolites in the liver. The active metabolites irreversibly inhibit platelet aggregation by selectively decreasing binding

of adenosine diphosphate (ADP) to its platelet receptor, $P2Y_{12}$, via the single active thiol group which is revealed in the active molecule, thereby interfering with subsequent ADP-mediated activation and glycoprotein IIb/IIIa signalling.

These agents are widely used for the secondary prevention of cardiovascular disease, and until expiry of its US patent in 2012, clopidogrel had been ranked as the second most prescribed drug worldwide[260]. Use of these agents is well established for the prevention of ischaemic complications in patients undergoing percutaneous coronary intervention[261-263] and for the treatment of acute coronary syndromes.

	Clopidogrel	Prasugrel	Ticagrelor	Cangrelor
Chemical class	Thienopyridine	Thienopyridine	Cyclopentyl-triazolo-pyrimidine	ATP analogue
Route	Oral	Oral	Oral	Intravenous
Pro-drug	Yes (requires hepatic cytochrome P450 activation)	Yes (requires hepatic cytochrome P450 activation)	No	No
Standard dose	300 mg/ 600 mg loading	60 mg loading	180 mg loading	30 µg/kg bolus
	75 mg OD	10 mg OD	90 mg BD	4 µg/kg/min
Reversibility of binding	Irreversible	Irreversible	Reversible	Reversible
Excretion	50% renal, 46% biliary	68% renal, 27% faeces	Biliary	Not dependent on hepatic or renal clearance

Table v: Characteristics of $P2Y_{12}$ receptor antagonists.

However, clopidogrel has limitations. It has a modest antiplatelet effect, a delayed onset of action and considerable inter-patient variability in drug response. 15–40% of patients are poor responders to treatment[264]. The distribution of responses to clopidogrel is wide, and a large fraction of the population carries a gene that may account for some of the inter-patient variation.

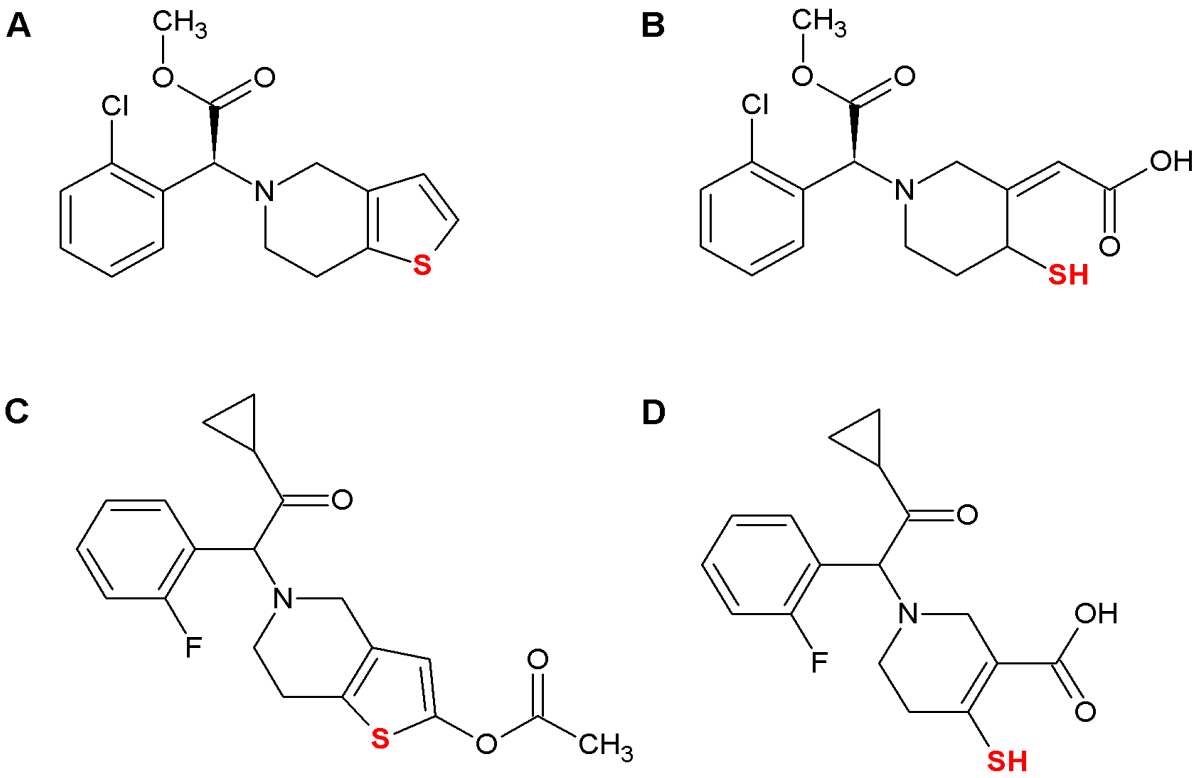


Figure 18: Clopidogrel and Prasugrel chemical structure. A) Native Clopidogrel, B) Active Clopidogrel, C) Native Prasugrel, D) Active Prasugrel. Thiol group is shown in red.

These disadvantages motivated the development of prasugrel, approved by the FDA in 2009. It has a greater antiplatelet effect than clopidogrel because it is metabolised more efficiently. Prasugrel is about 10 times more potent than clopidogrel and 100 times more potent than ticlopidine. All three thienopyridines act via the single thiol group revealed in the active molecule. Treatment with 5mg of prasugrel results in inhibition of platelet activity (distributed in a Gaussian curve) very similar to that produced by 75mg of clopidogrel. Even a maintenance dose of 150mg of clopidogrel inhibits platelet activity to a lesser degree than 10mg of prasugrel (46% vs 61%) so clopidogrel appears to reach a plateau of platelet inhibition that prasugrel can overcome[265]. Some of the differences in metabolism between clopidogrel and prasugrel may be explained by genetic polymorphisms affecting the cytochrome P-450 system.

TRIAL NAME	TRIAL DETAILS	PATIENTS	RESULTS
Percutaneous Coronary Intervention			
REAL LATE, ZEST LATE, 2010 *(n=2701)*	Clopidogrel + Aspirin vs Aspirin after 12 months of DAPT	Patients who had received DES, free of major adverse cardiac/cerebrovascular events and major bleeding for >12 months	After a median 19.2 months, the cumulative risk of primary end point (MI or death from cardiac causes) was not significantly different: DAPT group 1.8% vs 1.2% in aspirin group.
GRAVITAS, 2011 *(n=5429)*	High dose Clopidogrel vs regular Clopidogrel	Patients receiving DES with high residual platelet activity (PRU>230, tested with VerifyNow) on regular clopidogrel (platelet-function tested12-24 hours after PCI)	High dose compared to standard dose clopidogrel did not reduce incidence of death from cardiovascular (CV) causes, non-fatal MI, or stent thrombosis
Müller, 2000 *(n=700)*	Clopidogrel 75mg vs Ticlopidine 325mg	A randomised comparison of clopidogrel and aspirin versus ticlopidine and aspirin after the placement of coronary-artery stents.	Non cardiac events significantly reduced with clopidogrel. Aspirin and clopidogrel is comparably safe and effective as ticlopidine and aspirin.
CLASSICS, 2000 *(n=1020)*	Clopidogrel 300mg vs Ticlopidine 250mg	Double-blind study of clopidogrel safety with and without a loading dose (plus aspirin) compared with ticlopidine (plus aspirin) after coronary stenting	The safety/tolerability of clopidogrel (plus aspirin) is superior to that of ticlopidine (plus aspirin)
TOPPS, 2000 *(n=1016)*	Clopidogrel 300mg vs Ticlopidine 500mg	Patients underwent coronary stent placement and were randomised to clopidogrel vs ticlopidine to assess effectiveness at preventing stent thrombosis	Clopidogrel better tolerated than ticlopidine, and offered similar protection against sub-acute stent thrombosis and MACE. Stent thrombosis rate was 1.53% in ticlopidine compared to 2.02% in clopidogrel patients.
Piamsomboon, 2001 *(n=68)*	Clopidogrel 300mg vs Ticlopidine 250mg	Patients undergoing coronary stenting were randomised to assess stent thrombosis prevention with clopidogrel plus aspirin compared with ticlopidine plus aspirin	Clopidogrel and aspirin is an effective coronary stenting regime comparable to ticlopidine and aspirin.
Acute Coronary Syndrome			
CURE PCI substudy, 2001 *(n=2658)*	Clopidogrel + Aspirin vs Placebo + Aspirin	Patients with NSTEMI undergoing PCI	Clopidogrel + aspirin demonstrated a 31% relative risk reduction from randomisation to end of follow up, and a 25% relative risk reduction in composite end point of MI or CV death with long term use.
COMMITT, 2005 (n=45852)	Clopidogrel vs placebo	Patients admitted to hospital within 24 hours of suspected acute myocardial infarction onset	1st primary endpoint was death from any cause, 2nd endpoint was first occurrence of re-infarction, stroke or death. Clopidogrel significantly reduced relative risk of 1st endpoint by 7%, and 2nd endpoint by 9%, an absolute reduction of 0.5% and 0.9%, respectively.
CLARITY-TIMI 28, 2005 *(n=3491)*	Clopidogrel 300mg then 75mg vs placebo	Patients, 18 to 75 years of age, within 12 hours after the onset of a STEMI	15% of patients in clopidogrel group and 21.7% in placebo group reached the primary endpoint (occluded infarct-related artery on the pre-discharge angiogram, or death or recurrent MI before coronary angiography.
CURE, 2001 *(n=12562)*	Clopidogrel 300mg then 75mg + aspirin vs aspirin + placebo	NSTEMI within 24 hours after the onset of symptoms	CV death, MI, or stroke occurred in 9.3% in the clopidogrel-treated group and 11.4% in the placebo-treated group, a 20% relative risk reduction.

CURRENT OASIS 7, 2010 *(n=25087)*	Double vs standard dose Clopidogrel	ACS patients referred for invasive strategy (scheduled for PCI <72 hours after randomisation)	Double dose clopidogrel reduced stent thrombosis and MACE in the PCI group, but showed no difference in the non-PCI group.
Cardiovascular Protection			
CAPRIE, 1996 *(n=19185)*	Clopidogrel 75mg vs aspirin 325mg	Patients with atherosclerotic vascular disease (recent ischaemic stroke or MI, or symptomatic peripheral arterial disease)	Clopidogrel significantly reduced the incidence of new ischaemic events (combined end point of myocardial infarction, ischaemic stroke and vascular death) when compared to aspirin
CHARISMA, 2006 *(n=15603)*	Clopidogrel 75mg + aspirin vs placebo + aspirin	Patients with either clinically evident CVD or multiple risk factors	No significant difference in the primary efficacy endpoint, but a composite of the primary endpoint plus hospitalisation for unstable angina, TIA, or a revascularisation procedure, was significantly lowered by 7.7% in the clopidogrel plus aspirin group.
ASCET, 2012 *(n=1001)*	Clopidogrel 75mg vs aspirin 160mg	Patients with documented CAD and treated with aspirin	No difference in the composite endpoint between the groups
Atrial Fibrillation			
ACTIVE W, 2006 *(n=3335)*	Clopidogrel 75mg + aspirin vs oral anticoagulant (OAC)	Patients with atrial fibrillation (AF) plus one or more risk factor for stroke	Demonstrated that OAC treatment with vitamin K antagonists was more effective than with clopidogrel and ASA
ACTIVE A, 2009 *(n=7554)*	Clopidogrel 75mg + aspirin vs aspirin alone	Patients with AF and at least one risk factor for stroke, who are not candidates for warfarin therapy	Strokes occurred in 7.8% patients receiving clopidogrel + aspirin and 10.8% of patients receiving placebo + aspirin
CLAAF, 2004 *(n=30)*	Clopidogrel 75mg + aspirin vs OAC	Non high-risk patients with permanent AF or with persistent AF awaiting cardioversion	Aspirin plus clopidogrel and warfarin were equally safe and effective in preventing thromboembolism.
Stable Angina/CAD			
CAPRIE, 1996 *(n=19185)*	Clopidogrel 75mg vs aspirin 325mg	Patients with atherosclerotic vascular disease (recent ischaemic stroke or MI, or symptomatic peripheral arterial disease)	Clopidogrel significantly reduced the incidence of new ischaemic events (combined end point of MI, stroke and vascular death) when compared to aspirin.
CABG			
CASCADE, 2009 *(n=113)*	Aspirin 162mg + Clopidogrel vs aspirin + placebo	Patients after CABG involving at least two saphenous vein grafts (SVG)	Aspirin plus clopidogrel did not significantly reduce SVG intimal hyperplasia following CABG when compared to aspirin alone.

Table vi: Table summarising the main clopidogrel trials.

1.9.3 Non-thienopyridines

Whereas the active metabolites of the thienopyridine prodrugs (ticlopidine, clopidogrel, and prasugrel) covalently bind to the $P2Y_{12}$ receptor and are irreversible, indirect platelet inhibitors, the newer, direct-acting $P2Y_{12}$ inhibitors (cangrelor, ticagrelor and elinogrel) are not based on thienopyridines and act by changing the conformation of the $P2Y_{12}$ receptor, resulting in reversible, concentration dependent inhibition of the receptor. More importantly,

these do not require prior metabolism and act directly without involving the binding of an integral thiol group.

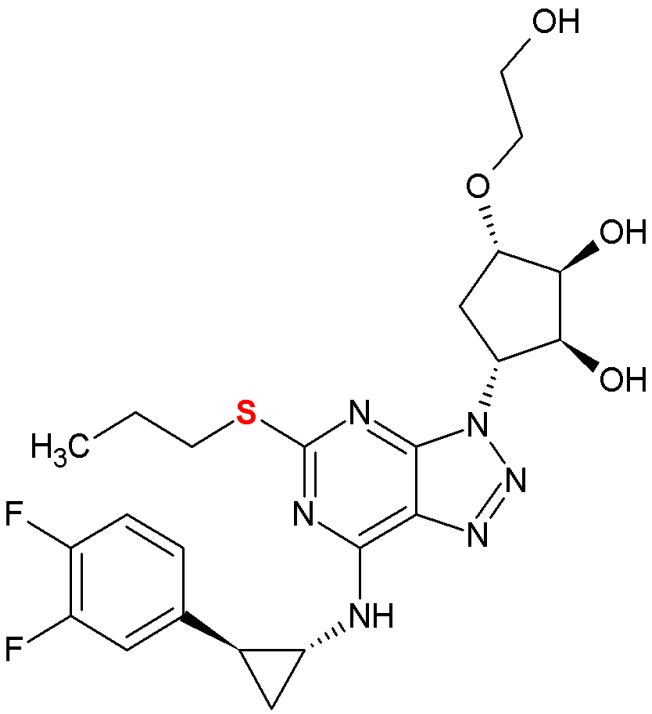


Figure 19: Ticagrelor chemical structure. Thiol group is shown in red.

Ticagrelor is quickly absorbed and has a rapid antiplatelet effect and onset of action. Early trials in patients with acute coronary syndromes suggest it may be superior to clopidogrel in reducing death from vascular causes, MI or stroke[266]. Furthermore, recent evidence has shown that long-term dual antiplatelet therapy in the form of ticagrelor and aspirin instead of placebo and aspirin leads to a reduction in major adverse cardiac events when given to patients who have suffered a myocardial infarction within the previous 2 years[267]. The mechanism behind this benefit, so distant form the acute coronary event, remains to be explained.

1.10 Pleiotropic effects

Attention has turned to potential properties of thienopyridines beyond their inhibitory action on platelets. Substantial evidence now exists to support these extra-platelet manifestations, presumed to be explained by the fact that besides the platelet, the $P2Y_{12}$ receptor can be found in several different regions and tissues of the body including the brain, vascular smooth muscle cells[268], leukocytes[269], macrophages[270], microglial[271] and dendritic cells[272]. Alternatively, off-target mechanisms with no relation to the $P2Y_{12}$ receptor have also been hypothesised. To date, the main areas of interests with regard pleiotropic effects have been endothelial function, vascular tone, inflammatory processes, plasma adenosine levels and cardio-protection. A brief summary of these discoveries follows.

1.10.1 Clopidogrel Pleiotropic effects

Clopidogrel's beneficial effect on endothelial function was the first implication that off-target effects existed, with both clopidogrel and ticlopidine exhibiting vasomodulatory activity in murine, rabbit and canine models[262,263]. Jakubowski et al have also shown that clopidogrel (and its inactive metabolites) can stimulate guinea pig coronary endothelial cells to release nitric oxide[273]. Simon et al[274] have identified the $P2Y_{12}$ receptor on rat brain capillary endothelial cells, whilst Shanker et al[275] have demonstrated the same in human coronary artery endothelial cells, HUVEC, and on human aortic smooth muscle cells. Oral administration of a single loading dose of clopidogrel to patients with stable coronary artery disease leads to a dose-dependent improvement of endothelial dysfunction which is independent of platelet activation[276], although the same group also believe the beneficial effects of this short term treatment on the endothelium is abolished following a longer duration of clopidogrel, 28 days in their study[277]. Heitzer et al have also reported improvement of endothelial nitric oxide bioavailability with clopidogrel in a similar patient group[278], and Schafer et al have shown improved endothelial function and nitric oxide

bioavailability in association with clopidogrel administered to rats with congestive heart failure[279]. Froldi et al studied the direct activity of thienopyridines in rat caudal arterial rings and aortic smooth muscle cells in culture, revealing in vitro arterial relaxation is endothelium-independent[280].

The role of inflammation in atherosclerosis is well established, and evidence now exists linking clopidogrel treatment with reduced levels of CD40 ligand, CRP, P-selectin and platelet-leukocyte aggregate formation, all inflammatory markers associated with activated platelets[281]. Patients recruited to the PLATO trial taking clopidogrel were also shown to have reduced leukocyte counts confined to the treatment period[282], and reduced inflammatory markers secondary to clopidogrel treatment have been demonstrated both in patients on long term treatment before undergoing PCI[283], and in patients with superficial femoral artery stenosis[284].

1.10.2 Prasugrel Pleiotropic effects

Similarly, prasugrel is also capable of modulating inflammation as shown by Liverani et al, who demonstrated inhibition of neutrophil transmigration, CD16 surface expression, and neutrophil-platelet aggregation by a prasugrel metabolite mixture[285], through direct neutrophil targeting. Platelet-leucocyte interaction was also inhibited by prasugrel active metabolites in mice pre-treated with endotoxin to induce an acute inflammatory reaction. Specifically, prasugrel treatment reduced TXB_2 and tumour necrosis factor-α synthesis whilst increasing nitric oxide metabolites in vivo[286]. Furthermore, using a mouse model to measure therapeutic index and bleeding relating to the thienopyridines, prasugrel exhibited dose- and time-dependent off target $P2Y_{12}$-independent effects of a reversible nature at the vessel wall secondary to inhibition of vascular tone[287].

1.10.3 Ticagrelor Pleiotropic effects

Questions regarding probable ticagrelor pleiotropic effects have arisen due to the discovery of a disproportionate mortality benefit despite only a moderate reduction in myocardial infarction in the ticagrelor arm of the PLATO trial[266]. This has been attributed to a significant reduction in deaths related to sepsis and pulmonary adverse events as compared to the clopidogrel arm of the study[282]. Dyspnoea, ventricular pauses and transient rises in uric acid and creatinine levels, observed in the PLATO trial, are known adverse effects of ticagrelor. It is tempting to speculate a link between ticagrelor and increased adenosine release as these effects are also typical for adenosine.

Augmented plasma adenosine levels in ticagrelor treated ACS patients have been shown[288], as has adenosine-induced enhanced coronary vasodilatation secondary to ticagrelor[289]. Furthermore, ticagrelor can induce ATP release from red blood cells with subsequent enzymatic degradation to adenosine[290]. An alternative explanation for the increased sensation of breathlessness observed in these patients has been attributed to the reversible inhibition of $P2Y_{12}$ receptors on sensory neurons[291].

Whilst adenosine does have a wide range of physiological effects including vasodilatation, release of endothelial factors, cardio-protective and anti-inflammatory effects, whether any of these explanations or indeed an altogether different mechanism is responsible for the observations in the PLATO trial remains to be confirmed. The argument for adenosine may seem compelling but it has failed to show positive clinical outcomes in ACS patients[292], suggesting an alternative explanation may exist.

Consistent with the thienopyridines' effects on the vascular endothelium, substantial improvement in peripheral arterial function has also been observed in a cohort of ACS patients treated with ticagrelor[293], with additional evidence in a rat model suggesting that orally administered ticagrelor prevents ADP-induced vascular smooth muscle cell contraction[294]. Findings of increased carcinogenicity with prasugrel[295] and decreased with

ticagrelor[266] have attracted further hypothesis generation, but more evidence is required to corroborate these findings.

1.10.4 Pleiotropic Effects Conclusion

Taken together, all of these findings imply mechanisms of action beyond the inhibition of platelet aggregation and may point to direct effects on vascular endothelium/smooth muscle that may, or may not be specific to the active metabolite and could be ascribed to the parent drug.

In vivo, the active metabolite of clopidogrel exhibits a critical thiol group that governs its interaction with platelets. This prompted investigation at the Wales Heart Research Institute (WHRI) into whether active clopidogrel might form nitrosothiols derivatives. Indeed, the James group have recently shown direct nitrosothiols formation from clopidogrel, prasugrel, and ticlopidine formulations[296,297]. Thus a circulating SNO store can be formed which has demonstrated additive anti-platelet properties and acts directly to vasodilate isolated blood vessels, thereby acting as an NO donor.

Novel work at the Wales Heart Research Institute has recently confirmed that following administration of clopidogrel to patients, there is an increase in circulating NO metabolites measured in blood samples[298]. It has also become apparent that in vivo conditions promote the formation of SNO – acidic thienopyridine enters the stomach where further acidity and copious levels of nitrite provide ideal reaction conditions.

In patients at the WHRI, chronic clopidogrel treatment was noted to significantly alter the profile of nitric oxide metabolites in blood, predominantly via increased nitrite. Both $P2Y_{12}$ dependent and $P2Y_{12}$ independent components were implied following platelet aggregability.

1.11 Thesis Aims

Ample data exists relating to the antiplatelet effect of the thienopyridine group of drugs predominantly via their action on the $P2Y_{12}$ receptor. Relatively little information is available on the direct vascular effects, and given the varied patient response to these agents, further clarity is needed regarding the relevance and importance of the pleiotropic effects, and in particular whether responses are affected by concurrent use of proton pump inhibitors and oral nitrates, commonly co-prescribed with antiplatelet drugs.

Furthermore, given the improved efficacy of novel $P2Y_{12}$ receptor antagonists, such as prasugrel and ticagrelor, that have started to replace clopidogrel use in clinical practice, it would be interesting to investigate whether the pleiotropic effects of clopidogrel also relate to these novel compounds and whether they are characterised by large inter-individual differences.

Endothelial nitric oxide bioavailability has been shown to increase following an oral loading dose of clopidogrel but the mechanisms behind this are not fully understood. In their active forms, both clopidogrel and prasugrel contain a readily available thiol group (see figure 18), so it can be speculated that in an appropriate environment, RSNO could be produced to serve as an NO reservoir independent of NOS. This would imply an additional mechanism of action of these drugs with anti-aggregatory and vasomodulatory properties in addition to the established $P2Y_{12}$ inhibition.

On this basis and given the observations outlined above, the objectives of this study are to determine whether, in addition to $P2Y_{12}$ inhibition, the antiplatelet drugs clopidogrel, prasugrel and ticagrelor can also act as NO donors through the formation of RSNO compounds, and specifically whether;

1) Co-administration of oral nitrate drugs to patients treated with clopidogrel leads to enhanced levels of circulating NO metabolites by improving the yield of RSNO.

2) Co-administration of proton pump inhibitors to patients treated with clopidogrel leads to a reduction in the levels of circulating NO metabolites by impairing the environment necessary for RSNO production.

3) An increase in circulating NO metabolites is also measured following oral administration of prasugrel, or indeed whether the NO profile is more greatly enhanced due to prasugrel's increased potency as compared to clopidogrel.

4) Ticagrelor administration has the same effects on circulating NO metabolites as the thienopyridines, given that it does not contain a readily available thiol group.

1.11.1 Hypothesis

- *Elevated nitrite and nitrosothiols levels secondary to clopidogrel loading will be augmented by concurrent oral nitrate treatment, and reduced by concurrent proton pump inhibitor therapy.*

- *Prasugrel contains a thiol group so will therefore be able to form a vasoactive nitrosothiols derivative under physiological conditions and will enhance NO bioavailability following administration of a loading dose* in vivo*.*

- *Ticagrelor will not form a vasoactive nitrosothiols derivative due to the lack of a critical thiol group at its active centre (or because its mode of action does not involve a critical thiol group).*

2 GENERAL METHODS

2.1 Blood Collection

Blood was required from both healthy volunteers and patients with coronary artery disease for the purposes of the study, and taken in accordance with guidance set out by Cardiff and Vale Health Board. Approval was gained from the Local Research Ethics Committee for Wales and the study was conducted according to the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 (and later revisions).

For patient studies, an 18G cannula was inserted into the ante-cubital fossa vein and blood drawn directly into vacutainers (Vacuette Greiner Bio-One^{TM}). For NO metabolites analysis, 2x 4 ml K_3EDTA per patient sample were collected, and for platelet analysis, 1x 3 ml Hirudin vacutainer (Roche Hirudin Vacutainer). Repeat samples were required dependent on patient group, and these were taken from the same cannula. Cannulas were flushed with 0.9% normal saline after each use, so prior to repeat sampling, a tourniquet was re-applied above the cannula and the first 10 ml of whole blood discarded prior to sampling to prevent saline contamination. NO metabolites degradation is rapid so the K_3EDTA tubes were placed on ice immediately and for the duration of transportation.

Platelet studies were performed on the Hirudin vacutainer samples. These were transported at room temperature taking care not to shake or agitate the tubes in order to minimise platelet activation. They were analysed by multiple electrode aggregometry (Multiplate$^{®}$) between 30 and 45 minutes after acquisition.

2.1.1 Platelet Poor Plasma (PPP) Preparation

PPP was prepared for NO metabolites measurements. Immediately following return to the laboratory, typically within 15 minutes of acquisition, the K_3EDTA tubes were centrifuged at 3000rpm for 10 minutes at 4°C. Samples were then carefully separated and aliquoted into plasma and erythrocyte fractions prior to immediate snap freezing in liquid nitrogen and placed in storage at -80°C. When ready for NO metabolite analysis, aliquots were thawed for 3 minutes in a water bath kept at 37°C and analysed immediately.

2.1.2 Platelet Rich Plasma (PRP) Preparation

PRP from healthy volunteers was used for all *in-vitro* experiments including platelet and nitrosothiols studies. Blood was drawn from the ante-cubital fossa vein and carefully aliquoted into vacutainers containing hirudin anticoagulant (Roche Hirudin Vacutainer 3mL). Following centrifugation (800rpm at 20°Celcius) for 10 minutes, the formed PRP was immediately drawn off taking care not to agitate the sample, and left to stand for 10 minutes at room temperature. In order to ensure adequate PRP preparation and consistency of technique, the platelet count was confirmed on several samples using a standardised flow cytometer (Horiba Medical ABX Pentra ML) and comparison was also made with paired whole blood at 30 and 60 minutes post venepuncture. In view of degradation in platelet quality, analysis of hirudin-collected plasma was performed within 30 minutes. PRP was used in lieu of whole blood for all Multiplate® analysis to test platelet response to both ADP and thrombin-receptor agonist peptide (TRAP) agonists in a variety of settings as described in the relevant results chapters.

2.2 Measurement of NO derivatives by Ozone Based Chemiluminescence

Ozone based chemiluminescense (OBC) was utilised to quantify the nitric oxide metabolites nitrite, nitrosothiols and nitrate.

Other techniques are available for the measurement of NO derivatives and some undoubtedly have distinct advantages over OBC, such as ease of operation, minimal specialist equipment, and the ability to process multiple samples simultaneously, but OBC provides a sensitivity of better than 1 pico mole NO and can specifically measure different NO metabolites following appropriate sample pre-treatment.

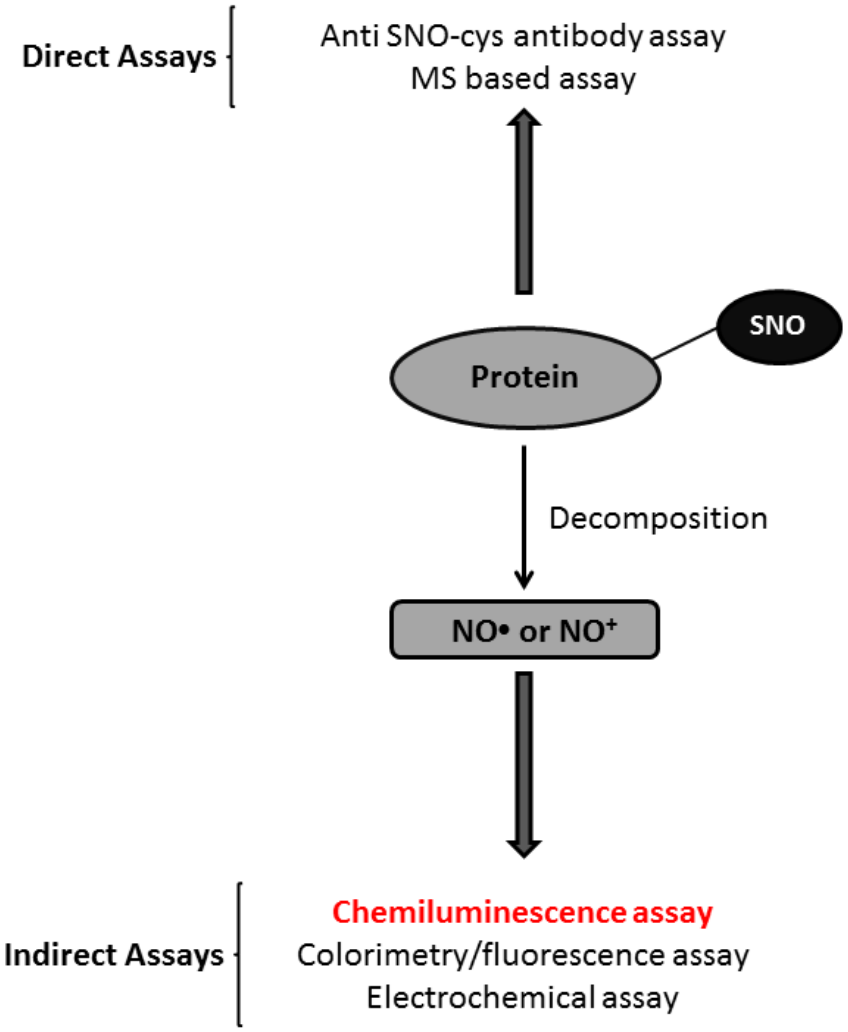


Figure 20: Techniques for measuring S-nitrosothiols. There are a number of methods for the "direct" detection of the S–NO bond which rely on measurement of its characteristic absorbance band at 340 nm, but sensitivity tends to be low. "Indirect" chemical methods require cleavage of the S–NO bond and detection of the species formed

OBC measurement first requires specific cleavage reagents to be added to a reaction vessel in order to liberate $NO^{\bullet}$ from the species of interest within the specified samples, typically solutions or human plasma. This is then carried by inert N_2 gas at a flow rate of $200cm^3$ per minute through a trap containing 40mls of 1N sodium hydroxide which serves two main purposes, firstly it prevents contamination from any other $NO^{\bullet}$ generated by acids, and secondly it protects the analyser form hot acidic vapour. From here, the generated $NO^{\bullet}$ is carried onto the NO analyser (Sievers NOA 280i, Analytix, UK). Here it reacts with ozone (O_3) as per the equation below,

$$\mathbf{NO^{\bullet} + O_3 \rightarrow NO_2^{\bullet} + O_2 \rightarrow NO_2 + O_2 + \mathit{hv}}$$

Energy released from this reaction in a form of a photon (*hv*) is amplified in a photo-multiplier tube (PMT) and recorded as a potential difference (mV).

This mV signal provides a graphical data series seen as peaks by the NO Analysis software (Liquid). These peaks are then analysed by measuring the area under curve (AUC) corresponding to each sample using the Liquid software or, if needed in the case of nitrosothiols for increased accuracy, Origin software. This AUC correlates with the quantity of NO metabolites as determined from relevant standard curves performed daily.

OBC analysis has been investigated thoroughly and extensively through previous work at the WHRI[299]. The NOA requires minimal maintenance; as per the manufacturer's guidelines, consumables require replacement approximately every six months (which equates to around 900 hours of operation). The firmware tracks operating time and alerts the user as necessary, but to ensure robust performance routine manufacturer maintenance is performed every 6 months.

2.2.1 Special Considerations

The half-life of NO and the ratio of the final NO metabolites in aqueous solutions, namely nitrite and nitrate, will depend on surrounding conditions including pH and temperature, and the concentration of transition metals and thiols. In whole blood, nitrite is rapidly converted to nitrate with an approximate half-life of 110 seconds.

Furthermore, serum nitrosothiols can destabilise in part during sampling, and also with the passage of time to form nitrite. Therefore, in order to ascertain reliable nitrite and nitrosothiols levels which accurately reflect NO release, prompt sample preparation is paramount. Assuming samples are immediately snap frozen in liquid nitrogen, storage in the freezer for up to 6 months tends not to alter the total NO metabolite levels when compared with analysis of freshly measured samples. However, the apportionment of NO between metabolite species does begin to alter; RSNO starts declining significantly after 7 days and nitrite within weeks. Ultimately, plasma RSNO and nitrite are gradually oxidised to nitrate[299].

Analysis of S-nitrosothiols levels is also subject to error. Decomposition is affected by numerous factors including light, temperature, pH, and contaminating transition metal ions[300]. Elimination of these confounding factors when performing OBC analysis is thus pivotal to the accurate measurement of NO metabolites. It has been shown that biologically relevant S-nitrosothiols are stable in the dark in the presence of transition metal ion chelators. Furthermore, metal-ion catalysed decomposition of S-nitrosothiols in the dark generates nitric oxide and disulfides without the intermediate production of thiyl radicals[301] which can often have additional and unknown effects of their own. Careful attention is therefore paid to performing analysis in the dark where possible and maintaining consistency of these additional confounding factors.

2.2.2 Measurement of plasma nitrite and nitrosothiols

The cleaving agent used to quantify plasma nitrite and nitrosothiols by OBC is tri-iodide (I_3), consisting of 0.78 M glacial acetic acid, 66.9 mM potassium iodide and 28.5 mM iodine.

The assay reagent (5ml) is placed in a glass purge vessel with a rubber septum covered injection inlet. Oxygen free nitrogen gas is bubbled through the reagent mix, which is heated to 50ºC (± 1ºC) in a water bath on a thermostatically controlled hotplate. A stock solution of acidic/tri-iodide cleavage reagent (70mls) is prepared fresh each day and 200 microlitre freshly thawed plasma samples are injected directly into the 5ml reagent, and analysed in duplicate as demonstrated in figure 24. 20 µL of Antifoam™ is added to the reagent chamber to prevent foaming caused by bubbling of nitrogen gases and plasma proteins. This assay is sensitive to <10nM nitrite with an accuracy better than ±10%.

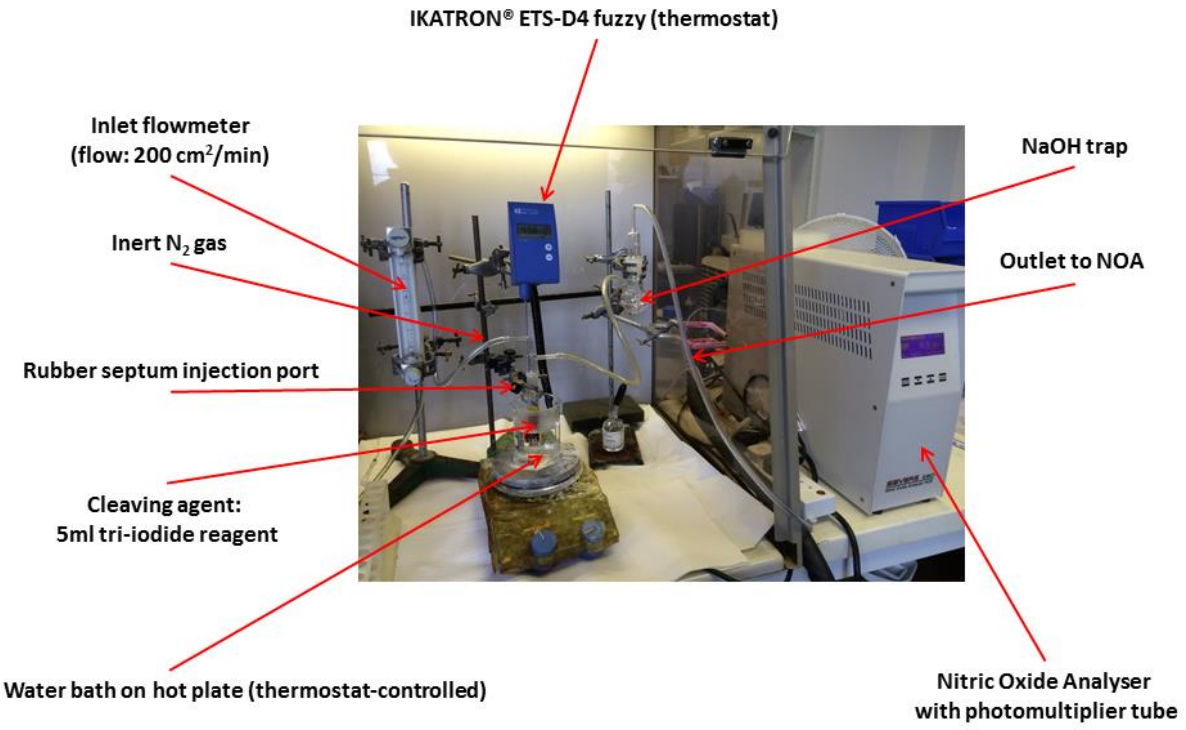


Figure 21: NOA setup for measurement of plasma nitrite. Sample and Antifoam™ is injected through the rubber septum and into a vessel containing tri-iodide reagent, the temperature of which is maintained at 50°C by a beaker of water kept on a hot plate linked to a thermostat. Released NO is carried in a nitrogen (N_2) gas stream controlled by a flowmeter into a round-bottomed flask containing 1 M Sodium Hydroxide (NaOH). Neutralised NO vapour is directed to the NO analyser

Use of the tri-iodide reagent allows cleavage of both plasma nitrite and nitrosothiols as illustrated in the following equations. Firstly, nitrite conversion to nitrous acid occurs due to excess acid and is followed by reduction to NO and water;

$$H^+ + NO_2^- \leftrightarrow HNO_2$$

$$HNO_2 + 2I^- + 2H^+ \rightarrow 2NO + I_2 + 2H_2O$$

Nitrosothiols are reduced to $NO^\bullet$ and water due to the excess iodine;

$$I_2 + I^- \rightarrow I^-_3$$

$$I^-_3 + 2RSNO \rightarrow RS\text{-}SR + 2NO^\bullet$$

$$2NO^\bullet + 2I^- + 2H^+ \rightarrow 2NO + I_2 + H_2O$$

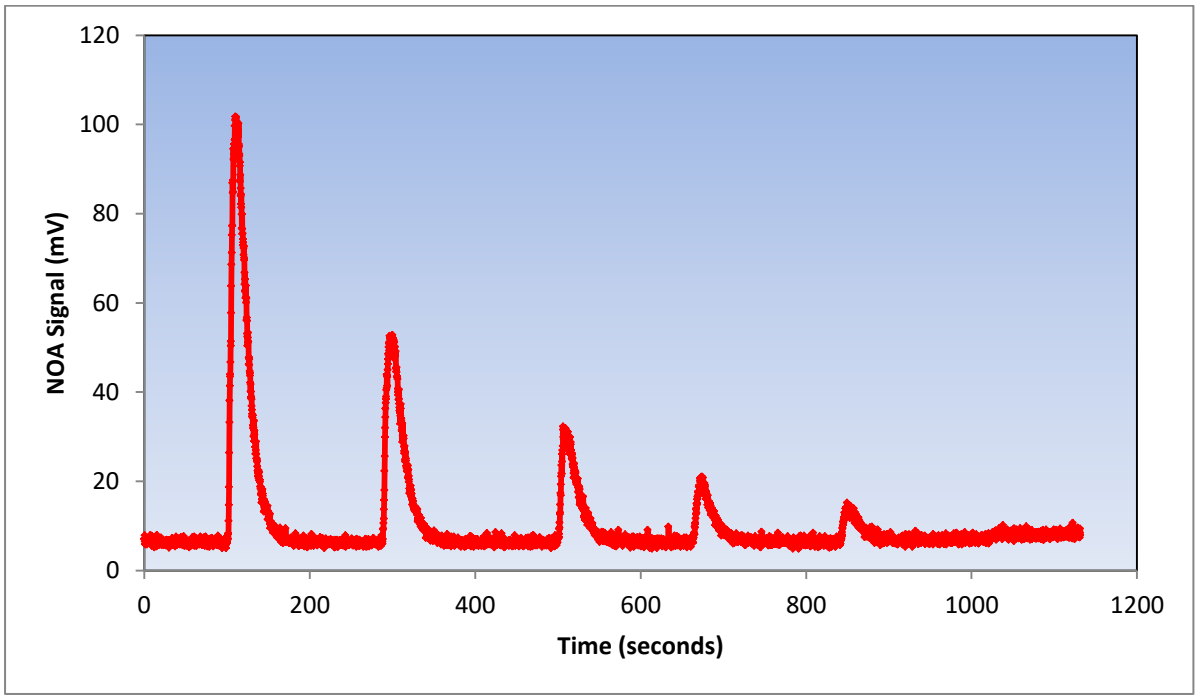


Figure 22: Analysis of standard curve for plasma nitrite and RSNO measurement. Peaks, in order, correspond to sodium nitrite concentrations of 1000 nM, 500 nM, 250 nM, 125 nM, 62.5 nM, and finally a blank HPLC-grade water signal.

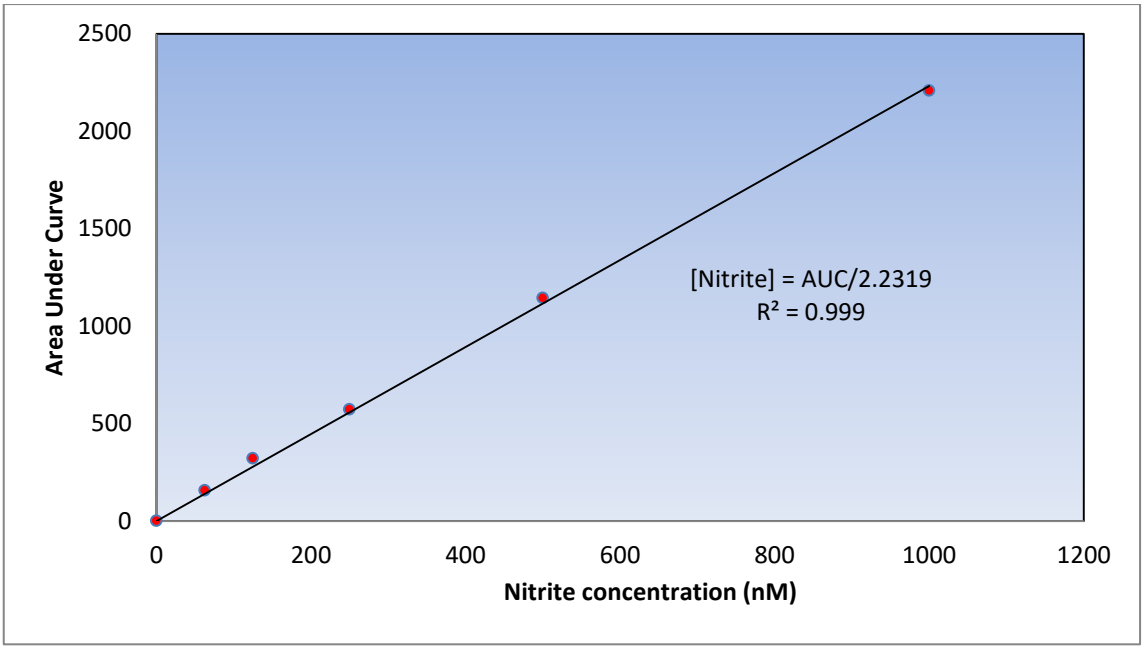


Figure 23: Nitrite standard curve - corresponding areas under the curve of the detected peaks shown in figure 22 (corrected by AUC of water) were used to calculate the slope coefficient of the standard curve.

A standard curve was constructed using HPLC-grade water and different concentrations of sodium nitrite 62.5 nM, 125 nM, 250 nM, 500 nM and 1000 nM. The corresponding AUC of detected peaks were used to calculate the slope coefficient of the standard curve. Correction was made for area under curve for water to eliminate the risk of nitrite contamination.

To distinguish between nitrite and RSNO, the same sample was run before and after a pre-treatment with 5 % acidified sulphanilamide (290 mM). This binds nitrite ions rendering plasma nitrite undetectable and allows selective measurement of the residual signal attributable to plasma RSNO. Acidified sulphanilamide is typically mixed with the plasma sample and left in the dark for 15 minutes to reduce all the nitrite.

RSNO signals are small with a typical noise to signal ratio for plasma nitrite/RSNO of <10. Origin version 8.0 software is better suited to calculating the AUC in this situation as the signal is smoothed prior to analysis, the standard curve being analysed the same way.

The intra-assay coefficient of variation (CV) has been determined previously in our lab as 7%[302], and the inter-assay CV is 8%. For each patient sample, duplicate measurements are

made for plasma nitrite before treatment with sulphanilamide and measurement of RSNO as shown in figure 24. Additional CV measurements have been performed on a random selection of these duplicate samples as illustrated in table vii to highlight the consistency seen with OBC analysis.

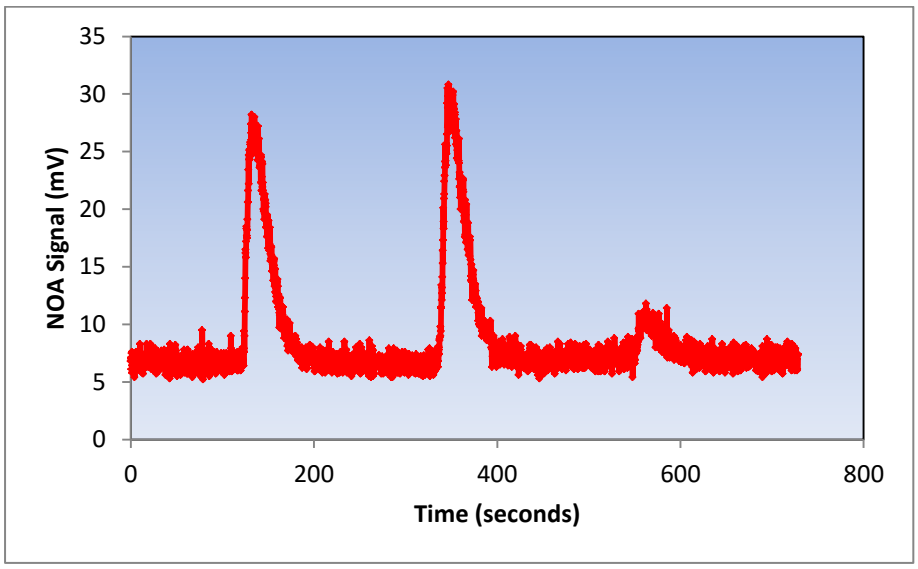


Figure 24: Typical duplicate nitrite signal and RSNO signal from the same plasma sample.

PLASMA NITRITE	Sample number 1 (ηM)	Sample Number 2 (ηM)	Mean (ηM)	Coefficient of Variation
1	248.4	242.4	245.4	0.017
2	247.7	260.9	254.3	0.037
3	279.2	275.1	277.2	0.010
4	114.1	120.2	117.1	0.037
5	223.6	228.4	226.0	0.015
6	172.5	195.1	183.8	0.087
7	262.9	256.0	259.4	0.019
8	177.3	176.5	176.9	0.003

Table vii: Random patient selection chosen for analysis. Table demonstrates paired plasma nitrite sample results and the calculated coefficient of variation for each. Mean CV is 0.028.

2.2.3 Measurement of plasma nitrate

The agent of choice for cleavage of plasma nitrate is vanadium chloride (VCl_3). The reagent contains 49.9 mM VCl_3 and 0.8 M HCl and is a more potent reductive agent than tri-iodide reducing nitrate to $NO^{\bullet}$ as shown in the following equation;

$$2VCl_3 + 4HCl + NO_3^- \rightarrow 2VCl_5 + 2H_20 + NO$$

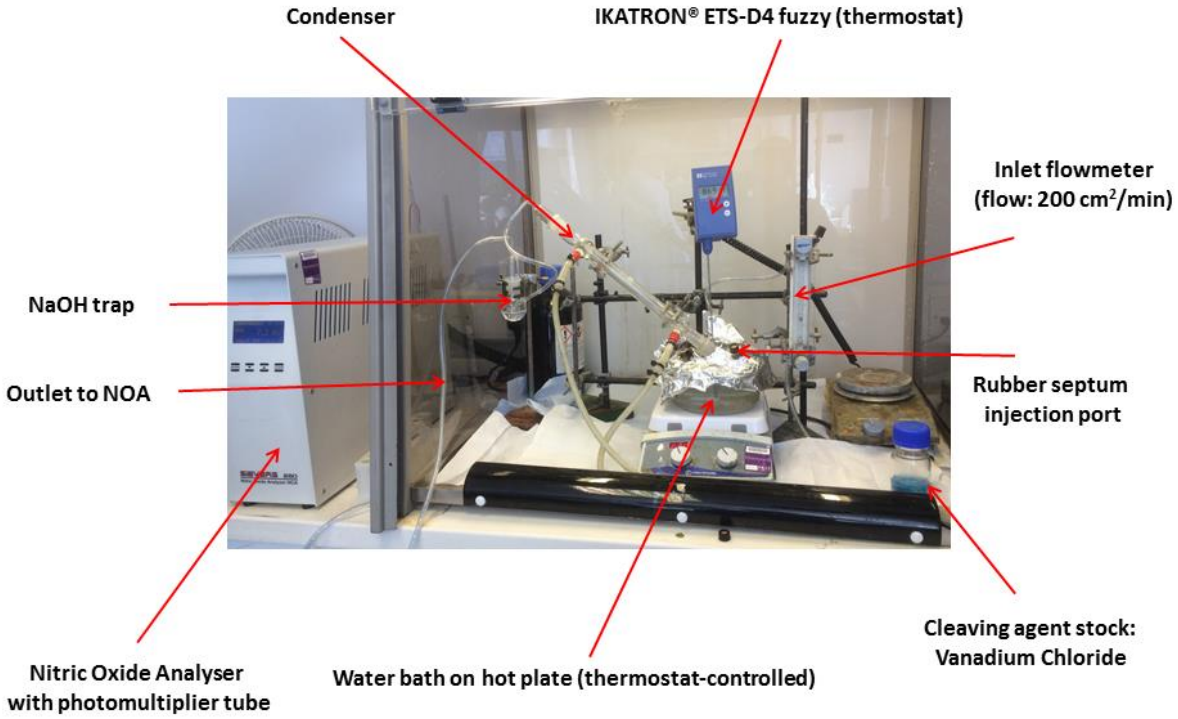


Figure 25: NOA setup for measurement of plasma nitrate. The sample is injected through the rubber septum into a 2-neck round-bottomed flask containing vanadium chloride reagent, the temperature of which is maintained at 85°C by a beaker of water kept on a hot plate linked to a thermostat. Released NO is carried in a nitrogen (N_2) gas stream controlled by a flowmeter and directed through a condenser then to a 1 M NaOH trap. Neutralised NO vapour is directed to the NO analyser.

Vanadium chloride is mixed for 15 minutes and then filtered through a 0.22 µm filter to remove undissolved salt prior to addition to the reagent chamber which is kept at a steady 85°Celsius for effective cleavage. A large purge vessel is used to allow any vaporised reagent to condense back into the chamber. 30ml of vanadium chloride is used and this typically allows 20 plasma samples of 20 µL each to be analysed before deterioration in the quality and reliability of the OBC signal is seen[302]. Again, each patient plasma sample is analysed in duplicate. 20 µL of Antifoam™ is added to the reagent chamber to prevent foaming caused by bubbling of nitrogen gases and plasma proteins.

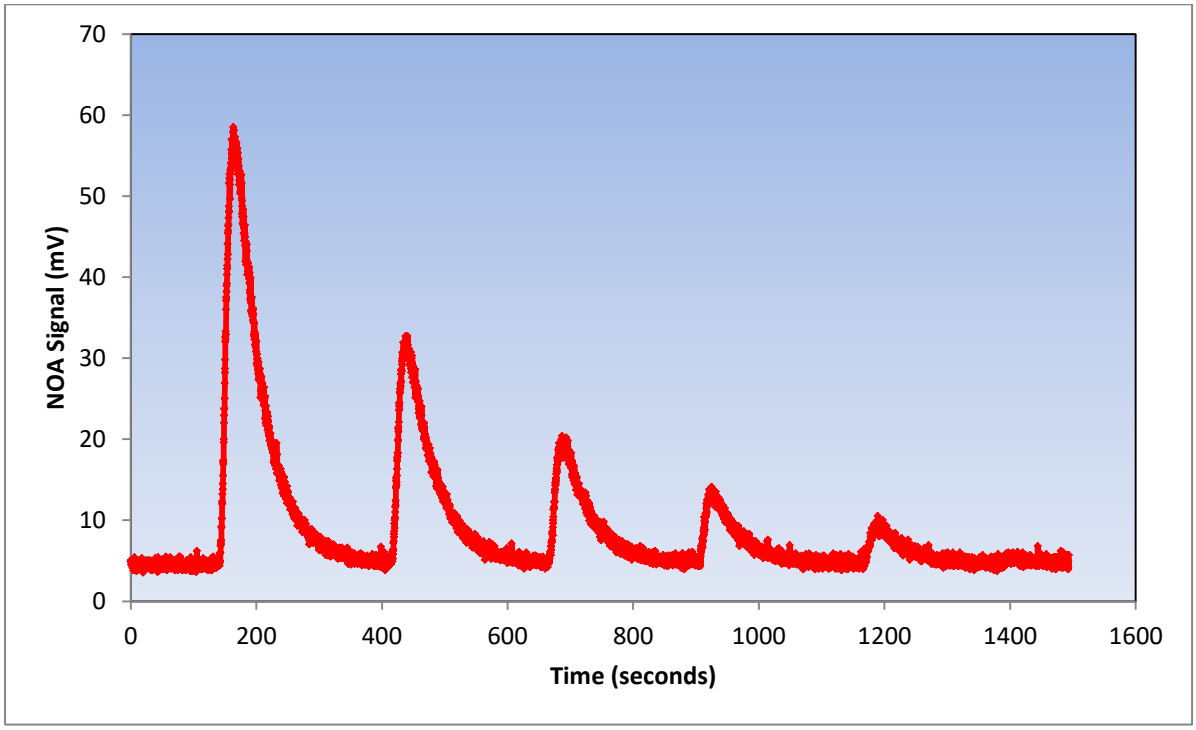


Figure 26: Analysis of standard curve for plasma nitrate measurement. Peaks, in order from left to right, correspond to sodium nitrate concentrations of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, and finally a blank HPLC-grade water signal.

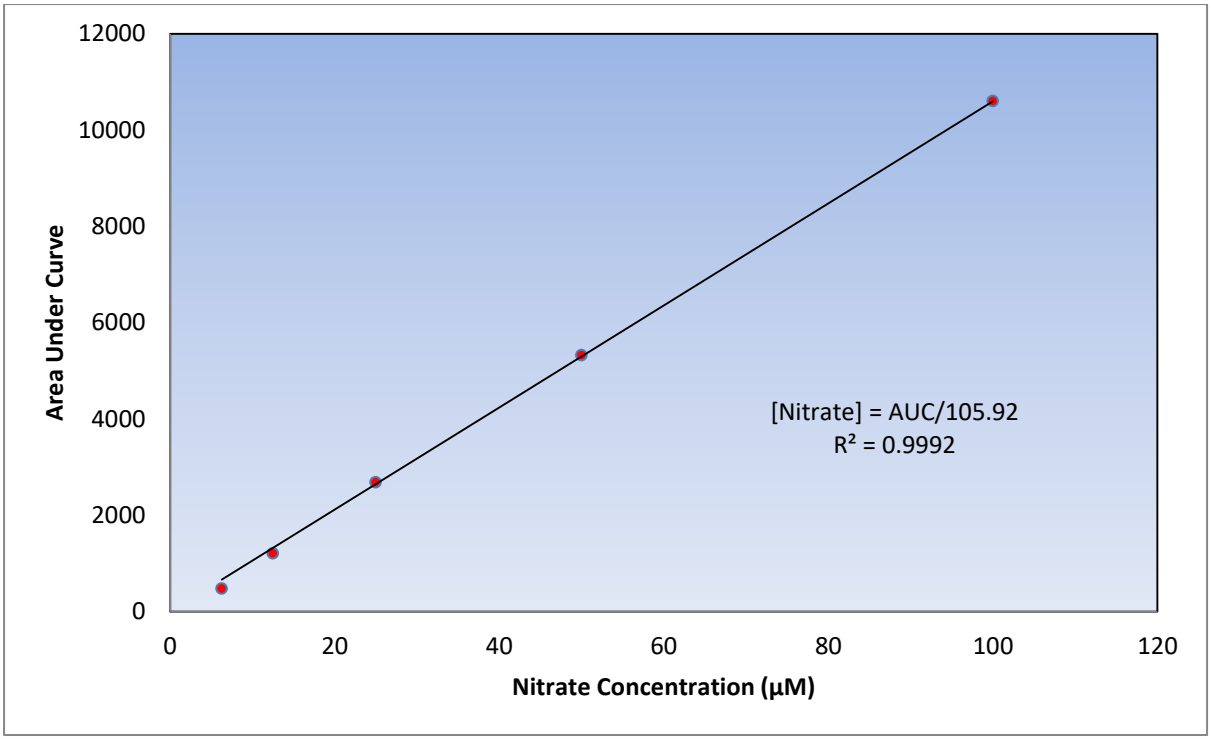


Figure 27: Nitrate standard curve - corresponding areas under the curve of the detected peaks shown in figure 26 (corrected by AUC of water) were used to calculate the slope coefficient of the standard curve.

A standard curve was constructed using HPLC-grade water and different concentrations of sodium nitrate; 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM. Due to its reductive power, vanadium chloride reduces not only nitrate, but also nitrite and RSNO to NO, so to calculate an accurate nitrate level the total nitrite and RSNO measured by tri-iodide analysis must first be subtracted from that observed with the VCl_3 reagent.

The intra-assay CV has been determined previously in our lab as 4%[302], and the inter-assay CV is 4%. As with plasma nitrite analysis, each patient sample is run in duplicate before averaging to establish a mean plasma nitrate result. Additional CV measurements have been performed on a random selection of these duplicate samples as illustrated in table viii.

PLASMA NITRATE	Sample number 1 (μM)	Sample Number 2 (μM)	Mean (μM)	Coefficient of Variation
1	67.0	64.7	65.9	0.025
2	57.7	55.5	56.6	0.027
3	34.0	32.4	33.2	0.034
4	43.4	42.0	42.7	0.023
5	83.2	83.1	83.1	0.0009
6	19.9	17.0	18.4	0.111
7	23.7	27.3	25.5	0.099
8	73.4	73.7	73.6	0.003

Table viii: Random patient selection chosen for analysis. Table demonstrates paired plasma nitrate sample results and the calculated coefficient of variation for each. Mean CV is 0.040.

2.2.4 Measurement of Nitrosothiols in Thienopyridine-SNO preparations

Artificially synthesised nitrosothiols are measured using OBC, with cysteine and copper (1) chloride as the cleavage agent. This "2Cs" reagent comprises 0.1 mM CuCl and 0.97 mM cysteine, with the following equation illustrating the cleavage mechanism;

$$\mathbf{RS\text{-}NO + Cu^{+} + H^{+} \rightarrow RSH + Cu^{2+} + NO}$$

$$\mathbf{2RSH + 2Cu^{2+} \rightarrow RS\text{-}SR + 2Cu^{+} + 2H^{+}}$$

Assay reagents (5ml) are placed in a glass purge vessel with a rubber septum covered injection inlet, into which is injected 200 μL samples. As described above for nitrite measurement using tri-iodide, the set-up is the same bubbling oxygen free nitrogen gas through the reagent mix, which is heated to 50ºC (± 1ºC) in a water bath, and linked to a trap containing 20mls of 1M sodium hydroxide, and then connected to the NO analyser. PBS is

used rather than water to maintain neutrality of the 2Cs reagent and avoid detection of nitrite and nitrate contaminants.

N-acetyl cysteine-SNO (NAC-SNO) is prepared fresh using 1 M N-acetyl cysteine (NAC) and 1.1 M sodium nitrite and used in increasing concentrations of 250 nM, 500 nM, 1000 nM, 2000 nM and 4000 nM to create a standard curve. To maintain stability and prevent decomposition, the NAC-SNO is kept in the dark and on ice. The intra-assay CV has been determined previously in our lab as 7%[302], and the inter-assay CV is 18%.

UV spectrophotometry is used to confirm the precise NAC-SNO concentration prior to NOA analysis based on the following direct relationship with maximal light absorbance occurring at 334 nM. The absorption coefficient is ε 727.

[NACSNO] = Light absorbance / Absorption coefficient

The advantage of the 2Cs reagent lies in its neutrality which ensures that other metabolites such as nitrites and nitrates remain undetected in biological samples, thus providing specificity[303]. This makes it ideal for measurement of RSNO species in aqueous samples. However, the sensitivity and practicality of undertaking measures of plasma RSNO using 2Cs is poor; it cannot accurately determine levels within the range necessary for analysing human plasma samples under normal conditions (<50 nM). Furthermore, it is not suitable for samples with high protein content due to the formation of foam in the reaction vessel at neutral pH. Therefore, use of the 2Cs method is reserved for analysis of laboratory based samples throughout this work, and tri-iodide for plasma samples[299]. Both methods have been extensively characterised and are well proven[304].

2.3 Platelet Function Testing Using Multiple Electrode Impedance Aggregometry (MEA)

The role of dual antiplatelet therapy (DAPT) is well established in the treatment of acute coronary syndromes and following percutaneous coronary intervention to prevent major adverse cardiovascular events (MACE). Interest has arisen in measuring platelet function to assess the risk of arterial thrombosis in these clinical contexts because individuals with platelet hyper-reactivity are known to be at greater risk[305]. Furthermore, those with high residual platelet reactivity (HRPR) despite antiplatelet therapy are also at increased thrombotic risk[306,307]. Unfortunately, the definitions of resistance vary widely dependent on the platelet function test, to the point that patients classified as non-responders by one test were considered responders by another. HRPR is frequent, particularly amongst clopidogrel users (30% to 40%), but the low frequency of MACE following PCI (0.5% to 2.5%) makes establishing a causal relationship between platelet function result and event difficult. It was hoped that bedside platelet testing would facilitate antiplatelet prescribing, tailoring appropriate medication and dosing to the individual with the aim of reducing MACE, but this has not been borne out in practice, trials suggesting no role for this approach[308,309].

Numerous tests exist to analyse platelet function.

Light transmission aggregometry (LTA) was invented in the early 1960s and despite being regarded as the gold standard for platelet function testing, its use is poorly standardised with wide variations in laboratory practice[310], and is certainly impractical as a bedside test.

Additional point-of-care tests have been developed since and most commonly used are the PFA-100 (Siemens Medical, Munich, Germany)[311] and VerifyNow (Accumetrics, San Diego, California)[312] but others include multiple electrode platelet aggregometry (MEA), vasodilator-stimulated phosphoprotein (VASP) phosphorylation, Cone-and Plate(let) assay (IMPACT-R, DANED SA, Beersel, Belgium), Plateletworks (Helena Laboratories, Beaumont, Texas) and others as shown in table ix below.

Method	Pros	Cons
Bleeding time	Quick No WB processing	Invasive Poorly standardised Multiple variables
LTA	Gold standard Flexible Diagnostic Different platelet pathways Sensitive to therapy	Manual sample processing Pre and analytical variables High sample volume Time consuming
Impedance aggregometry	No sample processing Diagnostic Flexible Different platelet pathways Sensitive to therapy POC	Limited haematocrit Limited platelet count range
VerifyNow	POC No WB processing Easy and Quick	Inflexible Expensive Monitoring anti-platelet therapy Limited haematocrit Limited platelet count range
Plateletworks	POC Minimal sample prep Easy and Quick	Indirect Requires platelet count Scarce data
PFA-100, Innovance PFA-200	In vitro standardised BT Easy and Quick POC	Closed system Platelet count and haematocrit dependent
Impact Cone and Plate(let) Analyser	WB assay Small sample volume Global platelet method	Expensive Experience required Limited evidence Limited availability

Global thrombosis test	POC Global haemostasis test Small sample volume	Insufficient evidence Limited availability
TEG (thrombo-elastography) platelet mapping	POC Global haemostasis test Reduces blood transfusion	Insufficient evidence
ROTEM (rotational thrombo-elastometry)	POC Predicts bleeding Reduces blood transfusion Global haemostasis test WB platelet aggregometry	Limited haematocrit Limited platelet count range Insufficient evidence

Table ix: Platelet Function Tests available and their pros and cons (Adapted from Platelet function tests: a comparative review[313]). POC = point-of-care. WB = whole blood. BT = bleeding time.

There is relatively poor correlation between the different platelet function tests available so the decision to use MEA was based on the need for an easy to use, reliable and repeatable test which provided multiple channels for simultaneous recording.

Impedance aggregometry involves stirring whole blood or PRP at 37°C whilst measuring electrical impedance as platelets adhere to the surface of two fine, precious metal, wire electrodes[314]. Impedance aggregation measurements in whole blood may be influenced by parameters such as haematocrit, platelet count, and elevated white cell count[315,316]. Technical problems associated with whole blood impedance aggregation have been obviated by the development of newer machines including the Multiplate® Analyser (Dynabyte, Munich, Germany) which employs disposable electrodes, standardised reagents and 5 channels for simultaneous recording. Use of this technique has been shown to be easy, reproducible and sensitive for assessing stimulated platelet aggregation, and evaluating antiplatelet drugs in diluted whole blood. The use of hirudin rather than citrate as an anticoagulant is recommended[317]. Trisodium citrate dihydrate has been the preferred anticoagulant agent for platelet function testing, but it chelates calcium making its use for in

vivo assessment sub-optimal. Resultant inhibitory effects on coagulation are likely to lead to under- or overestimation of the true inhibitory effects of $P2Y_{12}$ receptor antagonists[318].

Additional common sources of error comprise both pre-analytical, including variable venepuncture technique, collection, blood/anticoagulant ratio, transportation, and storage, and analytical, such as calibration, reagent issues, methodology and instrument problems. Limitation of pre-analytical error has been discussed. Analytical error was minimised by daily calibration of the Multiplate® equipment, use of fresh, standardised reagents and agonists, plus the inherent benefits of single use disposable test cells and internal controls.

2.3.1 Multiplate® setup

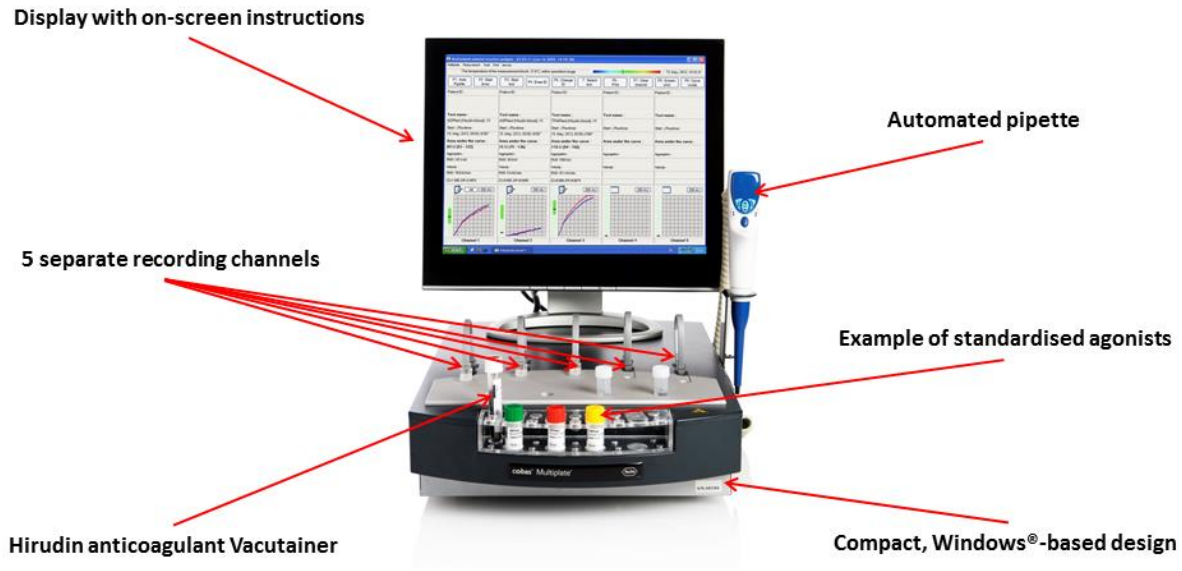


Figure 28: Standard Multiplate® Analyser set-up. The figure shows the bench top device and different components of the electrical impedance aggregometer. It is an easy to use instrument that allows standardised measurement of platelet function using small quantities of whole blood. The five channel analyser has a wide menu of CE marked tests.

Briefly, a test cell is attached to an electrical sensor cable. Into this cell 300 µL of 0.9% normal saline is added to 300 µL of blood collected in Hirudin anticoagulant and incubated for 3 minutes prior to the addition of an agonist, namely ADP and TRAP for the purposes of this study. One Multiplate® test cell incorporates two independent sensor units. The increase in impedance, proportional to the adherence of platelets to the electrodes is converted by each sensor unit into arbitrary aggregation units (AU) that are plotted against time. The duplicate sensors serve as an internal control.

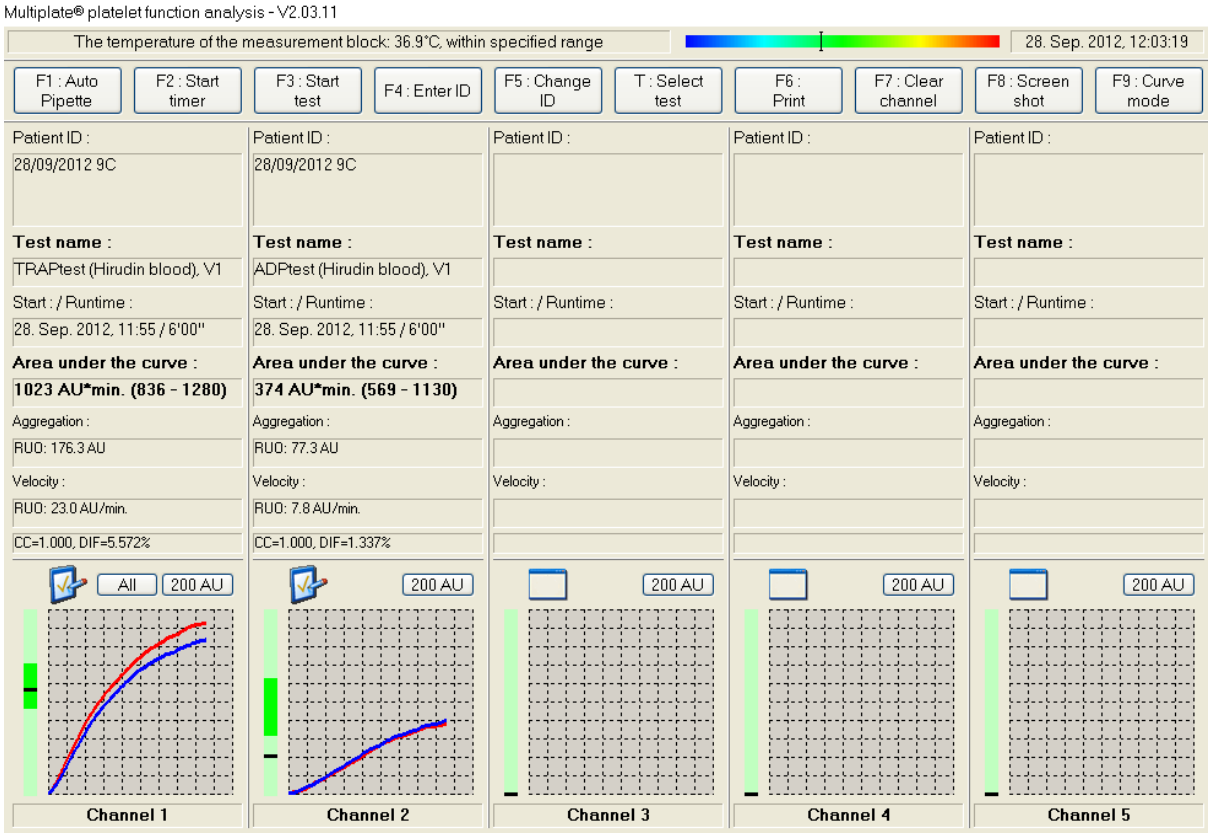


Figure 29: Typical example of a Multiplate® signal trace using 2 of the 5 channels simultaneously. The aggregometer uses a windows®-based design providing live viewing of the impedance aggregometry, plotting platelet aggregation measurements against time (in AU*min). The red and blue lines represent the measurements taken from each of the pair of independent sensors within each test cell. Each sensor comprises two pairs of electrodes.

Whole blood is used in the patient studies. Platelet rich plasma has been substituted in the *in-vitro* studies due to the need to measure a direct effect of NO-induced platelet inhibition produced by ticagrelor-derived SNO. This cannot be measured in whole blood due to the scavenging effect of RBCs on nitric oxide.

To ensure optimal performance of the Multiplate® analyser, the manufacturer guidelines were followed as per the operator's manual. Prior to each daily use, an electronic quality control measurement was performed, allowing at least 20 minutes for the analyser to reach its operating temperature of 37°C. Reagent vials were marked with the date of reconstitution, not refrozen and used within 24 hours. Pre-heated sterile saline solution (0.9% NaCl) was used as stated. Daily inspections of the electronic pipette and sensor cables were performed, with changing of the pipette filter as required. Routine analyser servicing was performed annually.

2.4 cGMP ELISA

The *'R & D Systems Parameter™ cGMP Assay Kit'* was used for cyclic guanosine 3',5'-monophosphate (cGMP) quantification and was chosen due to the wide detection range (0-500 pmol/ml). As previously described, cGMP is a second messenger converted from guanosine triphosphate (GTP) via the action of guanylyl cyclase. Cyclic GMP generation can occur either through a soluble pathway by the action of cytoplasmic nitric oxide-activated GC or via a particulate pathway through the action of transmembrane proteins typically involving atrial (ANP) and B-type natriuretic peptides (BNP)[319]. Cyclic GMP acts through four different pathways; cGMP-dependent protein kinases (PKG/GK), cyclic nucleotide-gated (CNG) channels, cAMP-dependent protein kinase (PKA), and phosphodiesterases.

2.4.1 Assay principle

This ELISA kit is based on competitive binding of the substrate using a rabbit polyclonal antibody. Cyclic GMP present in plasma samples compete with a fixed amount of horseradish peroxidase-labelled cGMP on the antibody, which, during incubation, then binds to goat anti-rabbit antibody embedded on the plate. Conjugate and unbound sample is then washed off prior to the addition of the substrate to determine bound enzyme activity. Colour development is stopped before reading the absorbance, and quantifying cGMP levels which are inversely proportional to colour intensity. This ELISA method is concentration dependant, and the signal output is inversely correlated to the amount of cGMP in the sample.

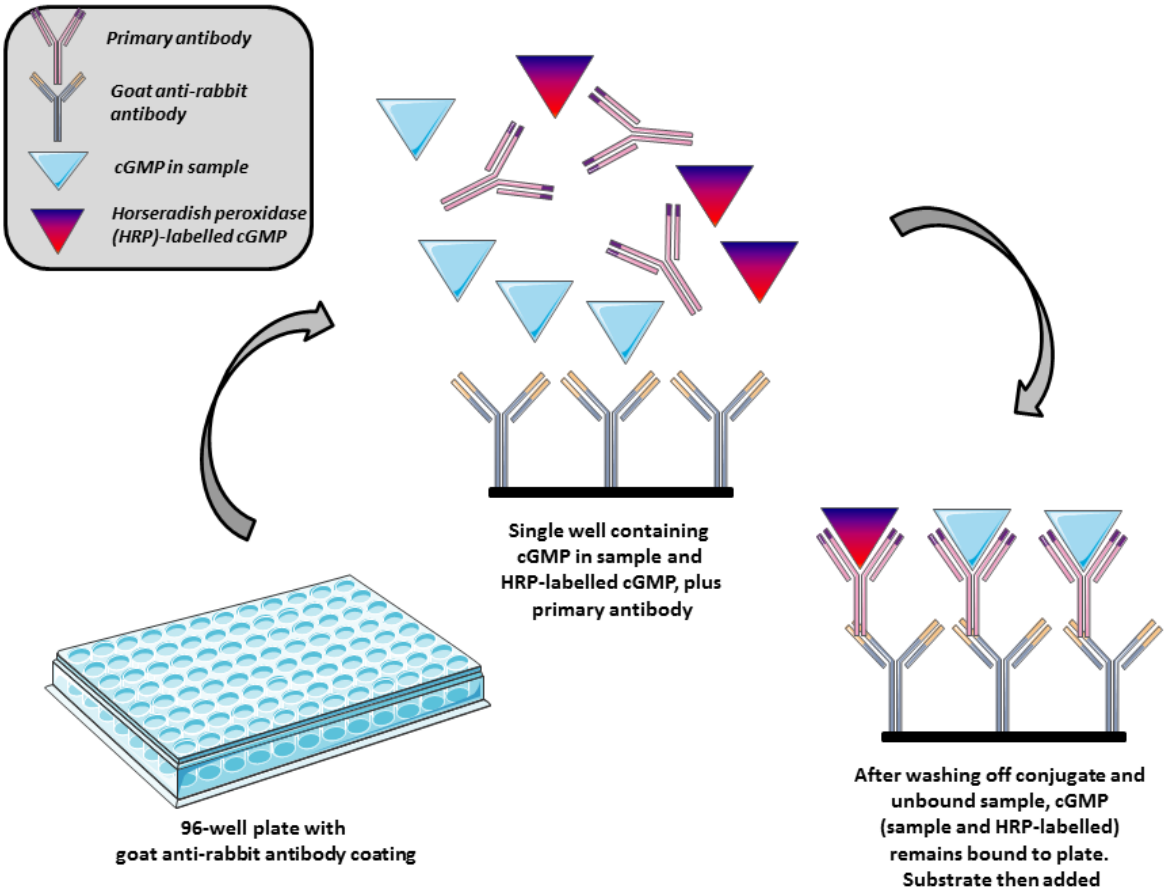


Figure 30: Competitive cGMP ELISA. Based on the principle that the antigen in the sample competes for limited antibody binding sites. cGMP present in the sample competes with a fixed amount of horseradish peroxidase-labelled antibody for sites on a rabbit polyclonal antibody. The antibody binds to anti-rabbit antibody coated on the microplate during incubation. Washing then removes unbound sample and conjugate following which a substrate solution is added to determine bound enzyme activity.

2.4.2 cGMP Assay Preparation:

The kit comprises the following:

- Goat anti-rabbit microplate
- cGMP Conjugate
- cGMP Standard
- Primary Antibody Solution
- Calibrator Diluent RD5-5
- Wash Buffer Concentrate
- Colour Reagent A and Colour Reagent B
- Stop Solution
- Adhesive Plate Sealers

With regards reagent preparation, 20 mL of Wash Buffer Concentrate was diluted in de-ionised water to prepare 500 mL Wash Buffer. The cGMP standard was reconstituted in 1 mL of de-ionised water to produce a stock solution of 5000 pmol/mL which was diluted further to prepare diluted standards at concentrations of 500 pmol/mL, 167 pmol/mL, 56 pmol/mL, 18.5 pmol/mL, 6.2 pmol/mL and 2.1 pmol/mL.

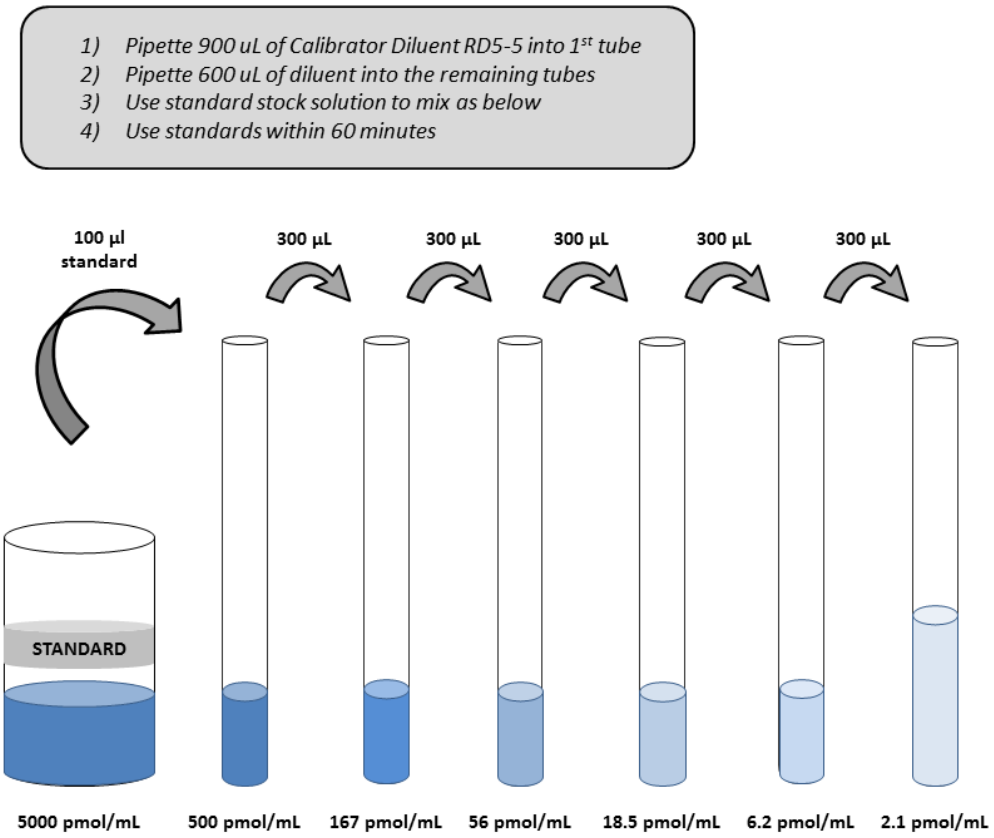


Figure 31: Diagram showing preparation of diluted standards. cGMP standard is reconstituted in 1 mL of de-ionised water to produce the stock solution from which the diluted standards are made. Each tube is thoroughly mixed and pipette tips are changed between each transfer.

Stored plasma samples, collected using EDTA as the anticoagulant, were thawed at 37°C for 3 minutes prior to diluting 20 fold with Calibrator Diluent RD5-5. Each sample was mixed thoroughly prior to adding to the 96-well plate.

2.4.3 Assay Procedure

All reagents and samples are brought to room temperature before use, and every sample, control and standard is assayed in duplicate to minimise error.

- The plate layout comprises non-specific binding (NSB) wells which serve as the control, zero standard wells and remaining standard and sample wells.
- 150 µL of Calibrator Diluent is first added to the NSB wells and 100 µL to the zero standard wells, followed by 100 µL of standard or sample to the remaining wells.
- 50 µL of cGMP conjugate is added to each well producing a slight red colour.
- 50 µL of Primary Antibody Solution is then added to each well, excluding the NSB wells. This results in a slight violet appearance to all except the NSB wells.
- The plate is covered with an adhesive strip.
- Then follows a 3 hour incubation phase, gently stirring the plate on a microplate shaker at 500 ± 50 rpm at room temperature.
- Plate washing is then performed; well contents are aspirated and replaced with 400 µL of diluted wash buffer, repeated 4 times, and then blotted dry.
- Colour reagent A (stabilised hydrogen peroxide) and colour reagent B (stabilised chromogen (tetramethylbenzidine)) are mixed in equal quantities to produce the Substrate solution.
- 200 µL of this Substrate Solution is added to each well within 15 minutes of its preparation, covered with foil and protected from light, then incubated for a further 30 minutes at room temperature.
- 50 µL of Stop Solution (containing 2 N sulphuric acid) is added to each well, which should lead to a colour change from blue to yellow, but often requires tapping of the plate for thorough mixing.
- Optical density of each well is then determined within 30 minutes, using a microplate reader set to 450 nm. Density is also measured at 540 nm and subtracted from the 450 nm value. This corrects for optical imperfections in the plate.

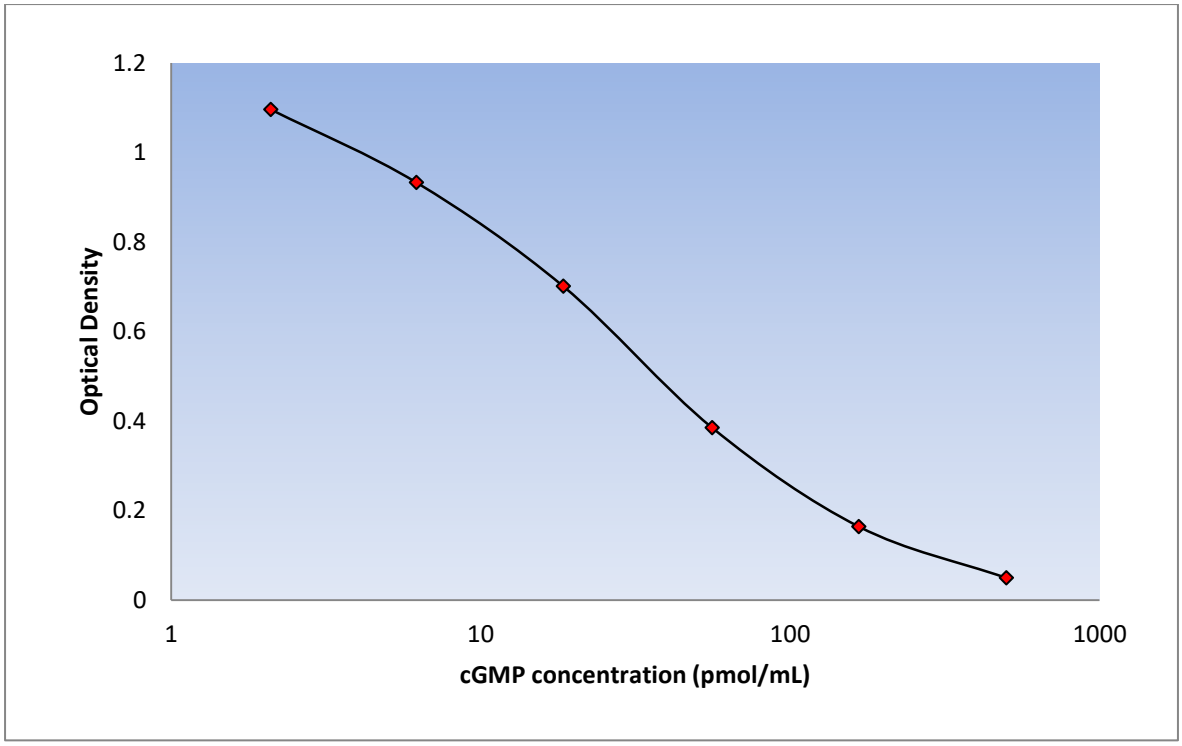


Figure 32: Typical R&D parameter assay standard curve. A standard curve is generated for each set of samples assayed. For each standard, control and sample duplicate readings are made and then averaged. The standard curve is then created by plotting mean absorbance on a linear y-axis against the concentration on a logarithmic x-axis. A best fit curve is then drawn through the points on the graph.

2.5 Artificial Stomach Medium Preparation

2.5.1 Gastric Physiology

Absorption of orally administered antiplatelet agents is an important determinant to drug effectiveness and speed of onset. Gastric physiology is therefore integral to a full understanding of how oral anti-platelet agents work. The stomach normally produces about 2 litres of gastric juice a day, in response to eating, but during fasting there is little or no secretion. The main constituents are hydrochloric acid, intrinsic factor, pepsinogens, mucus, water and electrolytes, particularly K^+ and Cl^-, with a resultant pH of 1–1.5. The stomach empties when pressure generated by the antral pump exceeds pyloric sphincter resistance. Emptying occurs at an exponential rate proportional to the volume of the stomach, mediated by vagal excitatory reflexes. In a normal stomach, 95% of ingested clear liquid reaches the duodenum within 1 hour and 50% of a meal will pass the pylorus in 2 hours.

2.5.2 Stomach Medium Set-up

In order to replicate physiological conditions as closely as possible, simulated human gastric fluid (SGF) was prepared according to a formula reported by Beumer et al[320]. This comprises the following; Peptone 4.15g, D-Glucose 1.75g, NaCl 1.025g, KH_2PO_4 0.3g, $CaCl_2$ 0.055g, KCl 0.37g, Porcine Bile 0.025g, Lysozyme 0.05g, Pepsin 0.00665g, Resazurin 0.001g. This was made up to 500 mL with de-ionised water and acidified using 1 M HCl.

It is known that the pH of a fasted stomach ranges from 1 to 3 and fasted volumes average 25 mL but are frequently higher. Furthermore, when a tablet is ingested, fluid is typically co-administered, and in pharmacokinetic studies, this is usually in the range of 200-250 mL. Once prepared, SGF was gently stirred, and filtered through a 0.22µM pore. 100 mL was added to a conical flask and kept at a steady temperature of 37°C on a hotplate.

3 Clopidogrel: The effect of Proton Pump Inhibitors and organic nitrates on NO Metabolites

3.1 Introduction

Clopidogrel, a second-generation thienopyridine, cemented its status as an essential treatment in acute coronary syndromes as a result of the CURE study in 2001[321], and subsequently as a treatment in patients undergoing percutaneous coronary intervention.

Clopidogrel is an inactive prodrug that undergoes a two-step activation process. It is absorbed in the intestine[322], mediated by the intestinal efflux transporter P-glycoprotein (Pgp), encoded by ABCB1, and must then undergo a two-step oxidative bio-activation process in the liver.

Several cytochrome P450 (CYP) enzymes are implicated including CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4/5, but the relative contribution of each remains unclear. However, it was suggested by Kazui et al that CYP2C19 contributes substantially to both oxidative steps and CYP3A4 predominantly the second oxidative step[323]. (see figure 33)

In view of this reliance on CYP-dependent metabolism, concern therefore exists about potential drug-drug interactions with clopidogrel and the impact this may have on resultant platelet inhibition, amplified by the known increased risk of stent thrombosis seen in clopidogrel “non-responders”[325]. Interactions resulting in low efficacy of clopidogrel have been reported with morphine[326], fluoxetine[327], ketoconazole[328], the oral hypoglycaemic sulfonylureas[329], the calcium-channel blockers (CCB) but primarily the non-Pgp-inhibiting CCB amlodipine[330,331], and the proton-pump inhibitors omeprazole and esomeprazole[332,333]. Pantoprazole and rabeprazole have been shown not to affect the pharmacokinetics and antiplatelet efficacy of clopidogrel[332].

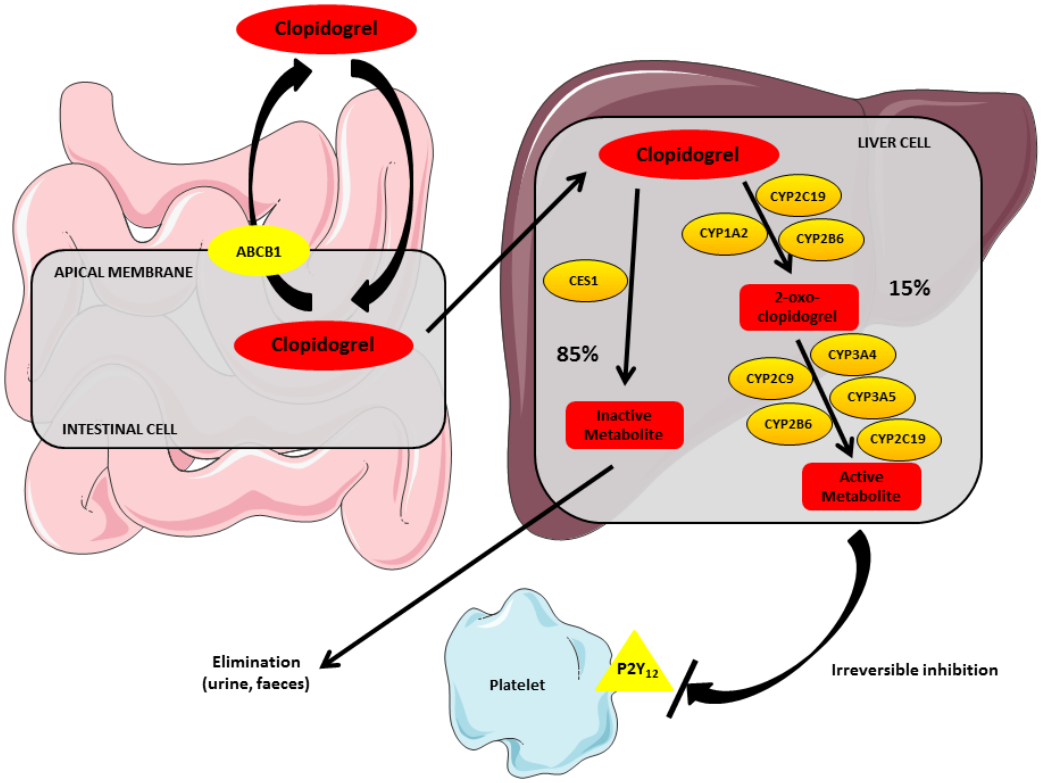


Figure 33: Clopidogrel bio-activation. Diagram highlighting the main candidate genes involved in clopidogrel metabolism and its primary mechanism of action. Adapted from [324].

Presumed to be related to liver metabolism, several reports have implicated cytochrome P450 enzymes, in particular, showing a higher rate of cardiovascular events in patients carrying CYP2C19 loss-of-function alleles[334], with the genetic variant CYP2C19*2 (loss of function allele “star 2”) thought to account for most of the associated diminished platelet response to clopidogrel[335].

However, there are many factors that may account for this decreased response to clopidogrel besides genetic polymorphisms, including age, presence of diabetes, renal failure, and reduced left ventricular function[336]. Furthermore, the implication that genetic polymorphisms alone can be used to determine clopidogrel responsiveness and likelihood of clinical sequelae has been contested[337]. Hochholzer et al identified the major independent predictors of insufficient antiplatelet response to clopidogrel as CYP2C19*2 carrier status, age, diabetes mellitus, and body mass index. However, analysis using a model that only

comprises CYP2C19*2 carrier status could explain only 4.6% of the variability in on-clopidogrel residual platelet aggregation. Furthermore, when allowing for all factors including genetic variance together with all demographic and clinical predictors, CYP2C19*2 carrier status could only explain 11.5% of residual platelet aggregation.

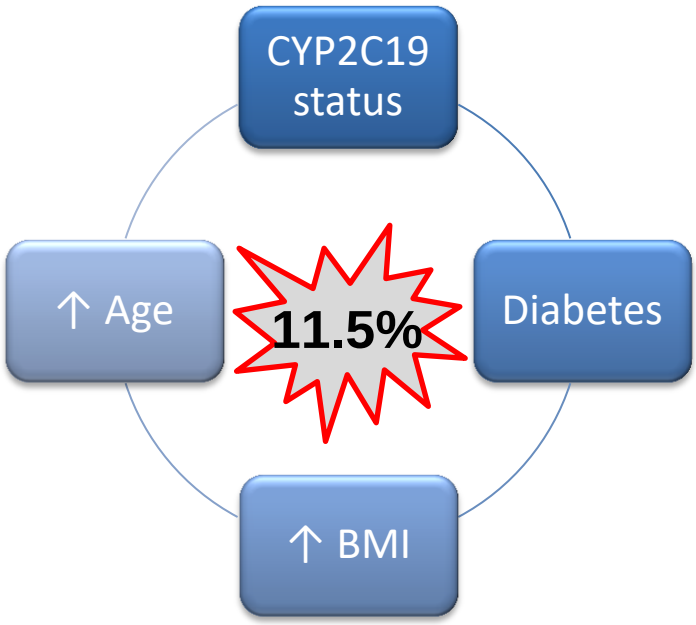


Figure 34: Schematic highlighting the main parameters responsible for decreased responsiveness to clopidogrel.

So despite these variables showing a statistically significant association with clopidogrel response, they are insufficient to fully predict high on-clopidogrel residual platelet aggregation and should not be used for clinical decision-making.

By implication, there must be other, as yet undiscovered factors accounting for clopidogrel non- and reduced- responsiveness. This may be attributable to other genetic polymorphisms including for genes encoding for drug metabolism, enzymes, transporter proteins such as P-glycoprotein, and drug target proteins, although there is little evidence to support a significant clinical effect to date[338-340]. Another focus of interest involves paraoxonase-1

(PON1), a high-density-lipoprotein (HDL) associated enzyme with anti-oxidative and anti-atherogenic properties. Synthesised in the liver, it is a genetically controlled enzyme involved in the formation of the thiol active metabolite from clopidogrel with esterase and more specifically paraoxonase activity. It is a crucial enzyme responsible for clopidogrel bioactivation[341].

So, various hypotheses exist to explain the reduced effectiveness of clopidogrel in certain individuals, but the biological mechanisms underlying this remain largely unclear, with genetic variants only accounting for a minority of the response variability identified thus far.

Previous work undertaken at the Wales Heart Research Institute has revealed that all clopidogrel salts, when in an acidic milieu akin to that in the human stomach, form S-nitrosothiols (RSNO) derivatives with anti-aggregatory and vasomodulatory properties. This effect is reliant on the availability of the free thiol within the clopidogrel molecule[297,342].

Importantly, this work also showed clopidogrel induces significant increases in circulatory nitrite in both the acute (compared to clopidogrel naïve) and chronic treatment of patients. Given that plasma nitrite not only reflects vascular endothelial NO production but is also a breakdown product of RSNO, this result is indicative of increased circulating NO metabolites following clopidogrel administration[298].

Given that the formation of RSNO is dependent on low pH, and the presence of nitrite, it must be established whether co-administered drugs which affect these parameters have an effect on the RSNO yield. Proton pump inhibitors (PPIs) block the gastric acid pump, H(+)/K(+)-adenosine triphosphatase (ATPase), inhibiting gastric acid secretion thus raising pH. Oral administration of organic nitrates for the treatment of angina leads to increased gastric nitrite availability as explained by the entero-salivary nitrate-nitrite-nitric oxide pathway described in section 1.3.2

Therefore, patient studies were devised to establish whether;

- Patients taking regular clopidogrel who were also on concomitant PPIs had lower RSNO plasma levels than those not taking PPIs.
- Patients taking regular clopidogrel who were also on concomitant organic nitrate medication had higher RSNO plasma levels than those not taking organic nitrates.

3.2 Methods

All biochemical methods including analysis of plasma NO metabolites and platelet function testing are as described in the Methods section.

3.2.1 Patient Recruitment

Patients for all the clinical studies were recruited from the cardiac day-case unit at the University Hospital of Wales. For this study, it was decided that the most effective and simplest population to evaluate in order to assess response to antiplatelet agents are elective patients attending routinely for coronary angiography +/- percutaneous coronary intervention. They are easily identifiable, clinically stable, and fasted. Furthermore, these patients all attend at least 3 days prior to their procedure for pre-assessment allowing an ideal opportunity to meet and discuss the study with potential participants before recruitment.

With clopidogrel, maximum inhibition of platelet aggregation occurs 3 to 5 days after starting therapy with 75mg daily without a loading dose[343], but within 4 to 6 hours if a loading dose of 300 to 600mg is given.

Therefore, all patients attending the cardiac day case unit who were receiving long term clopidogrel therapy (>1 month) were studied. All patients have an 18G intravenous cannula inserted by an experienced doctor as part of their routine care on the morning of their

admission. These ‘chronic’ patients, identified at the pre-assessment clinic, require a single 15 mL blood sample to be taken from their intravenous cannula before their coronary artery intervention.

These samples are then individually analysed for platelet aggregation and nitrite/nitrate/NO metabolites in the Wales Heart Research Institute.

Participation was voluntary. Full ethical approval for the study was sought from and approved by the Local Research Ethics Committee for Wales, and then conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 (and later revisions).

3.2.2 Inclusion Criteria

Clopidogrel treatment is the focus of this chapter, but work highlighted in later chapters involves treatment with prasugrel and ticagrelor. All patient studies are intended to be a study of the real world use of the antiplatelet drugs clopidogrel, prasugrel and ticagrelor. The decision to prescribe one of these agents is made by the responsible consultant cardiologist, conforming with both nationally and internationally approved criteria, and is in no way linked to any research team decisions or opinions. Therefore, inclusion criteria are as follows;

1) Any adult over the age of 18 years with stable coronary artery disease (confirmed on coronary angiography) admitted routinely to the University Hospital of Wales for coronary angiography +/- percutaneous coronary intervention.

2) All patients must be taking aspirin.

3) Patient must be due to start or already be taking a thienopyridine/non-thienopyridine drug for a clinically-approved condition. For the purposes of this study, this

encompasses patients due to have a coronary stent implanted or who have already had a stent implanted, and patients who have previously had a myocardial infarction. (The duration of treatment in each situation varies and is ultimately down to the discretion of the responsible cardiologist).

4) Patients must have fasted for at least 6 hours

3.2.3 Exclusion Criteria

Patients whose baseline characteristics are likely to interfere with NO metabolite measurement and platelet function evaluation are excluded, so the exclusion criteria are therefore as follows;

1) Patients with contra-indication to, or are unable to take Clopidogrel, Prasugrel or Ticagrelor.

2) Patients who are unable to provide informed consent.

3) Patients presenting with acute coronary syndromes (STEMI, NSTEMI, unstable angina).

4) Patients using non-steroidal anti-inflammatory drugs (NSAIDs).

5) Patients using long term anti-coagulant therapy (for example warfarin or novel oral anticoagulants such as dabigatran, rivaroxaban and apixaban).

6) Patients receiving intravenous or subcutaneous anti-thrombin therapy.

7) Co-existent pro-inflammatory condition (for example malignancy)

INCLUSION CRITERIA	EXCLUSION CRITERIA
Adult >18 years of age	Patient unable to provide informed consent
Concurrent Aspirin use of at least 75mg daily	Patients presenting with ACS (i.e. STEMI/NSTEMI/unstable angina)
Due to start or already taking one of; - Clopidogrel - Prasugrel - Ticagrelor	Contraindication to or inability to take Clopidogrel or Prasugrel or Ticagrelor
Fasted for ≥6 hours	Concurrent use of NSAIDs
Know coronary artery disease	Concurrent use of Warfarin/long term anticoagulant therapy
	Concurrent IV or SC anti-thrombin therapy
	Pro-inflammatory condition

Table x: Summary of inclusion and exclusion criteria.

3.2.4 Statistical Analysis

Based on previous plasma analysis performed in the laboratory at the WHRI, typical nitrite and nitrate levels measured by ozone based chemiluminescence (OBC) are 160 nM and 30 µM respectively, with a coefficient of variation of 4% for plasma nitrates and 7% for plasma nitrites/nitrosothiols. OBC has been shown to be highly sensitive for the determination of nanomolar quantities of NO and NO-related species in biological fluids. Nagababu measured fasting plasma nitrite levels in the range 56-210 nM (mean 110+/- 36 nM) with high sensitivity and an accuracy of 97%[344]. Baseline plasma nitrate levels are normally around 25 µM. D'Agostino & Pearson omnibus normality testing was used to facilitate determination of normally distributed data. For 2 groups comparisons, either the unpaired Students t-test or Mann-Whitney test was applied depending on distribution of the data. For comparison of more than 2 groups, one way ANOVA or Kruskal Wallis was applied as explained in the text. The presented data show means with error bars representing the standard deviation. A *p* value of <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism™ version 5 software.

3.3 Results

Patient demographics are shown in table xi. All patients were questioned to confirm both medication compliance and a fasting duration in excess of 6 hours.

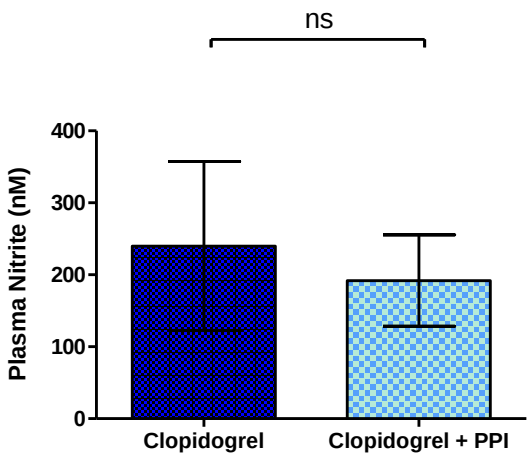
	Clopidogrel only	Clopidogrel + PPI	Clopidogrel + oral nitrate	Clopidogrel + PPI + oral nitrate
Number of patients	15	14	22	8
Mean age (years)	62	60	63	71
Male (%)	81	86	68	89
β-blockers (%)	69	79	68	67
ACEi (%)	75	36	59	67
CCB (%)	38	43	50	56
Statin (%)	75	79	100	89
Diabetes (%)	0	0	0	0
LV impairment	6	7	5	11

Table xi: Patient demographics for all clopidogrel patient groups, comprising number of patients, mean age, sex, concurrent medication use, diabetes mellitus and significant impairment of the left ventricle (LV)

3.3.1 Effect of Co-administration of PPIs with Clopidogrel on plasma NO metabolites

15 patients were recruited into the clopidogrel only arm, and 14 patients on a combination of clopidogrel and a PPI. The PPIs used comprised omeprazole (n=7), lansoprazole (n=6) and esomeprazole (n=1).

A



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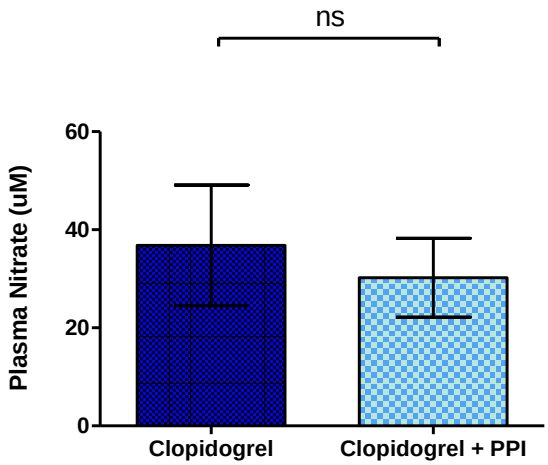

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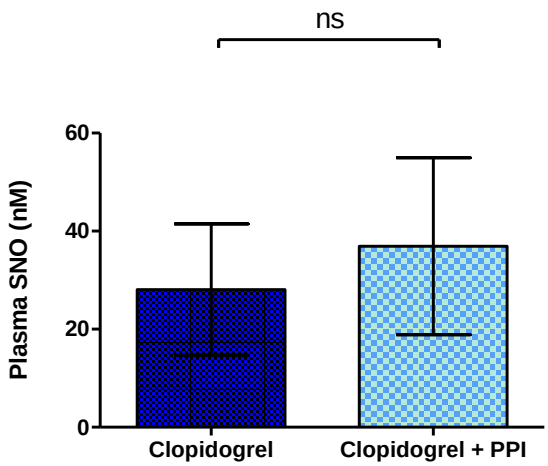


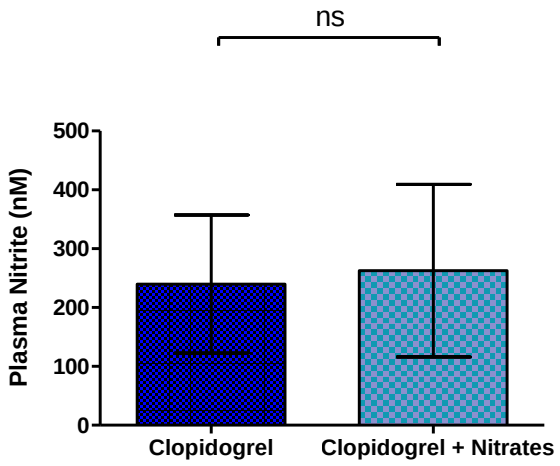
Figure 35: Plasma NO metabolites in fasted patients with CAD taking clopidogrel and a PPI compared to patients on clopidogrel alone.

D’Agostino & Pearson omnibus normality testing confirmed Gaussian distribution of the data. There was no statistical difference between measured plasma NO metabolites in patients taking a PPI compared to those taking clopidogrel alone with p values of 0.186, 0.098 and 0.144 for plasma nitrite, nitrate and SNO respectively. For plasma nitrite the difference between means was 48.0 +/- 35.4, with a 95% confidence interval (CI) of -24.7 to 120.7. For plasma nitrate, the difference between means was 6.6 +/- 3.9, with a 95% CI of -1.3 to 14.5, and for plasma SNO the difference between means was -8.8 +/- 5.9, with a 95% CI of -20.9 to 3.2.

3.3.2 Effect of Co-administration of organic nitrates with Clopidogrel on plasma NO metabolites

The 15 patients recruited into the clopidogrel only arm were compared with 22 patients taking a combination of clopidogrel and an oral nitrate. Nitrates included all brands of organic nitrate approved for use in angina, including both short and long acting forms. (Imdur, Monomil, ISMN, Ismo, and Monomax).

A



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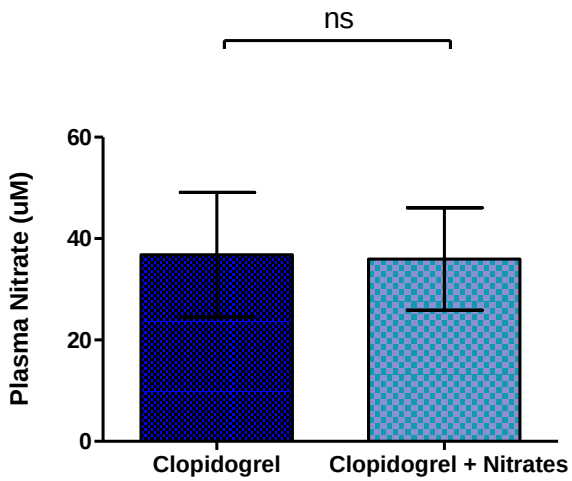

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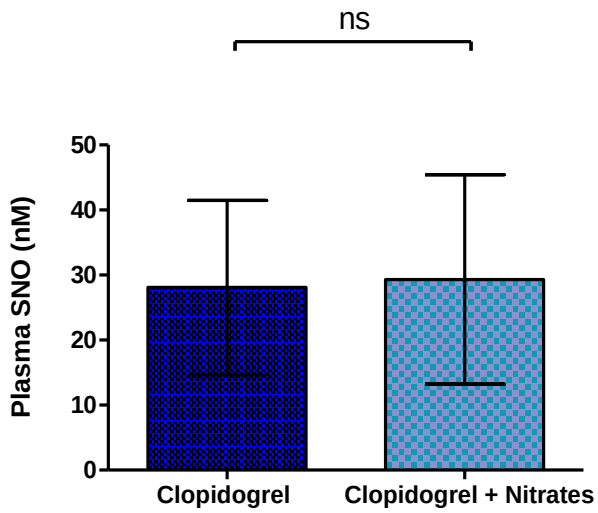


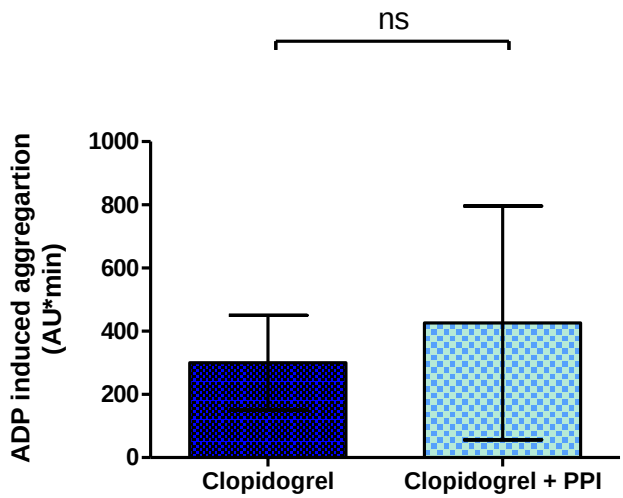
Figure 36: Plasma NO metabolites in fasted patients with CAD taking clopidogrel and an oral nitrate compared to patients on clopidogrel alone.

There was no statistical difference between measured plasma NO metabolites in patients taking an oral nitrate compared to those taking clopidogrel alone. P values for plasma nitrite, nitrate and SNO were 0.622, 0.821 and 0.809 respectively. For plasma nitrite the difference between means was -22.6 +/- 45.5, with a 95% CI of -115.0 to 69.7. For plasma nitrate, the difference between means was 0.8 +/- 3.6, with a 95% CI of -6.5 to 8.2, and for plasma SNO the difference between means was -1.2 +/- 5.0, with a 95% CI of -11.5 to 9.0.

3.3.3 Effect of Co-administration of PPIs with Clopidogrel on platelet function

Platelet function was measured using multiple electrode aggregometry with Multiplate®, as described in section 2.3.1, in response to both ADP and TRAP agonists.

A



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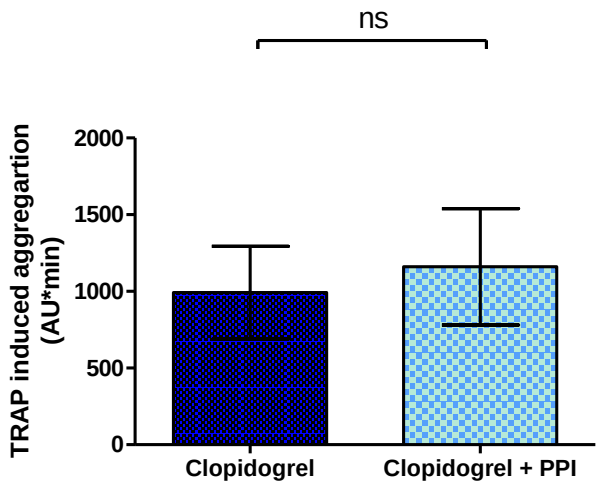


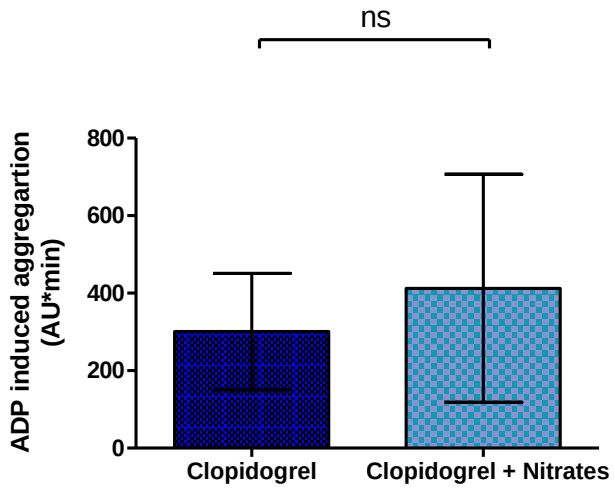
Figure 37: Platelet function tested with Multiplate® in fasted patients with CAD taking clopidogrel and a PPI compared to patients on clopidogrel alone, using A) ADP agonist and B) TRAP agonist.

Data for ADP-induced aggregation was not distributed normally so the Mann-Whitney test was used, revealing no statistical difference in platelet response to ADP (p=0.378). Using the unpaired t-test, there was also no statistically significant change in response to TRAP-

induced aggregation between the two groups (p=0.211). The difference between means was -168.2 +/- 131.2, with a 95% CI of -438.5 to 102.

3.3.4 Effect of Co-administration of organic nitrates with Clopidogrel on platelet function

A



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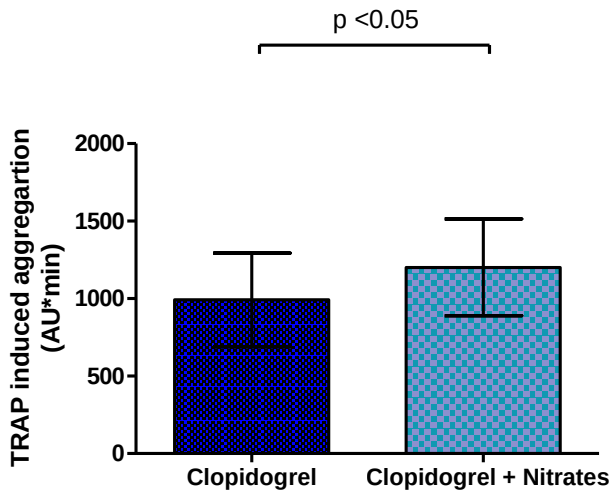


Figure 38: Platelet function tested with Multiplate® in fasted patients with CAD taking clopidogrel and an oral nitrate compared to patients on clopidogrel alone, using A) ADP agonist and B) TRAP agonist.

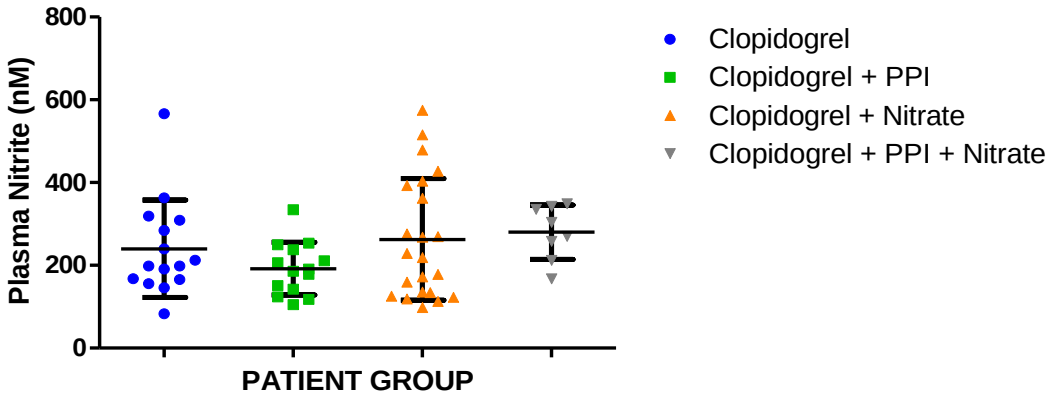
There was no statistically significant difference in platelet response to ADP (p=0.484) using the Mann-Whitney test, but TRAP-induced platelet aggregation was significantly higher (p=0.050) in those taking concomitant oral nitrates. The difference between means was -209.6 +/- 103.2, with a 95% CI of -419.4 to 0.2.

3.3.5 Comparison of all chronic Clopidogrel patient groups

Besides patients taking either clopidogrel and a PPI, or clopidogrel and an organic nitrate, a cohort of patients on all three agents were also recruited. 8 patients were taking clopidogrel with an oral nitrate and a PPI.

There was no statistical difference between the groups. Using one way ANOVA, p values for plasma nitrite, plasma nitrate, plasma SNO and TRAP-induced aggregation were 0.249, 0.231, 0.333, and 0.209 respectively. For ADP-induced aggregation p = 0.814, using non-parametric testing with the Kruskal-Wallis test.

A



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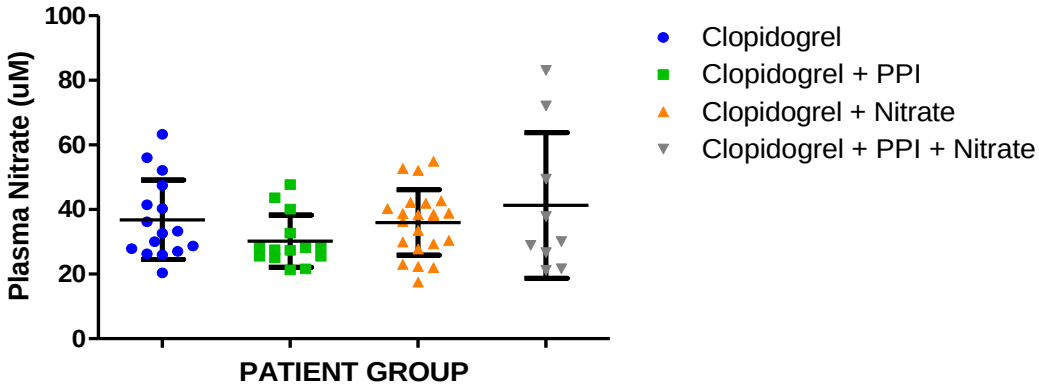
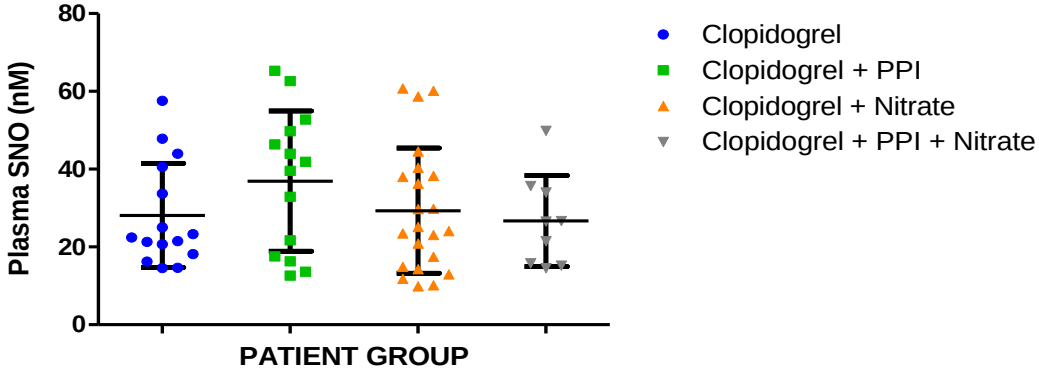
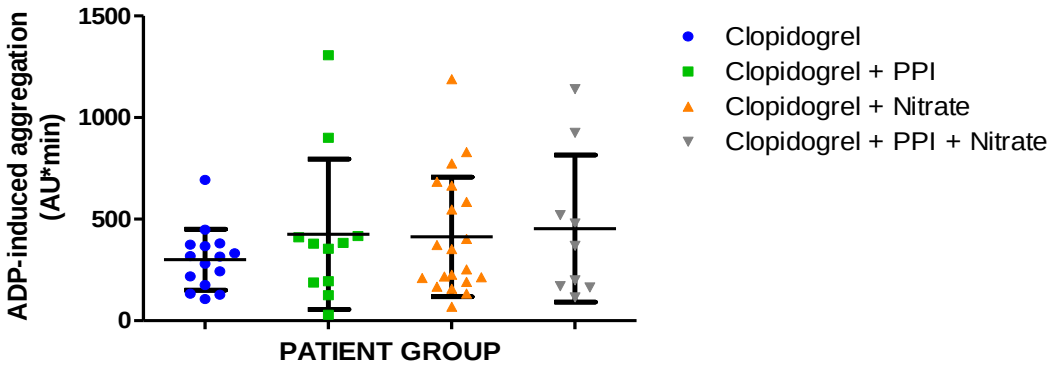

Plasma Nitrate (uM)
100
80
60
40
20
0
PATIENT GROUP
Clopidogrel
Clopidogrel + PPI
Clopidogrel + Nitrate
Clopidogrel + PPI + Nitrate

C


Plasma SNO (nM)
80
60
40
20
0
PATIENT GROUP
Clopidogrel
Clopidogrel + PPI
Clopidogrel + Nitrate
Clopidogrel + PPI + Nitrate

D


ADP-induced aggregation (AU*min)
1500
1000
500
0
PATIENT GROUP
Clopidogrel
Clopidogrel + PPI
Clopidogrel + Nitrate
Clopidogrel + PPI + Nitrate

E

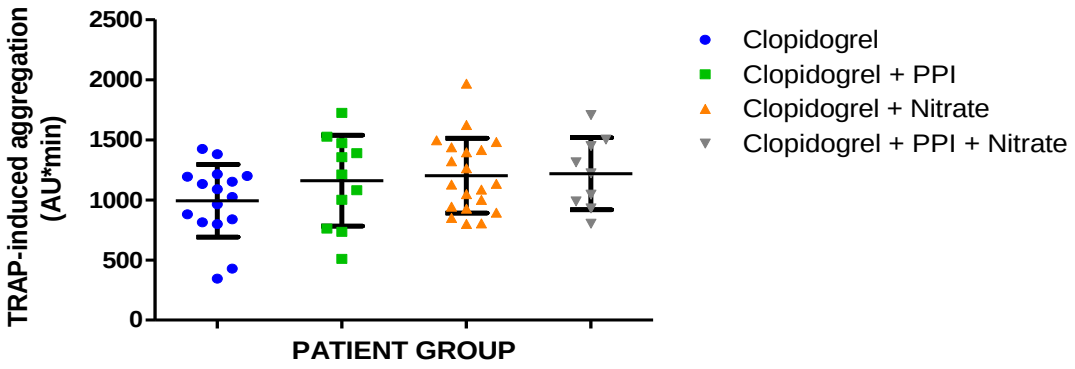


Figure 39: Summary of results per patient group. A) Plasma nitrite. B) Plasma nitrate. C) Plasma SNO. D) Response to ADP. E) Response to TRAP.

3.4 Discussion

Clopidogrel is safer than aspirin in terms of gastro-intestinal (GI) bleed risk, but the risk is not negligible, and it is often prescribed together with aspirin as recommended in treatment guidelines for ACS and post PCI[345,346], thereby further increasing the risk. In addition, elderly patients, and those also taking steroids and/or non-steroidal anti-inflammatory drugs are at even greater risk of bleeding. Proton pump inhibitors are therefore commonly co-prescribed to patients treated with dual anti-platelet therapy in order to minimise the chance of bleeding from the GI tract[347]. To confound the difficulty with identifying the true risks associated with taking this combination of drugs, many patients either take non-prescribed over-the-counter PPIs, or use the medication only intermittently. It is an important consideration however, because there is evidence to suggest PPIs are associated with increased cardiovascular risk irrespective of clopidogrel use following myocardial infarction[348]. Furthermore, Shih et al conducted observational studies which revealed that PPI use even in patients without prior cardiovascular history may be independently associated with an increased risk of MI[349].

Clopidogrel-treated patients taking regular proton pump inhibitors would be expected to have increased gastric pH, and as such a less favourable environment for the yield of nitrosothiols (RSNO) derivatives. However, this study has shown no statistical difference in measured plasma metabolites between those taking PPIs and those taking clopidogrel alone. The results also confirm no significant change in platelet reactivity between the two groups as measured with multiple electrode aggregometry in response to both ADP and TRAP agonists.

There are several explanations for these findings. Firstly, the nitrosothiols yield from clopidogrel, the weakest of the currently used non-aspirin antiplatelet drugs, is small even in optimum conditions, so identifying a significant reduction secondary to reduced acid availability is unsurprisingly difficult. Furthermore, it was not within the remit of this study to measure gastric pH prior to measurement of plasma nitrosothiols levels, so given the known compliance issues and evidence that PPIs must be taken regularly to maintain elevated gastric pH levels[350], doubt could exist as to whether these results are reflective of a true population with suppressed stomach acidity. Indeed, variance in both stomach pH and nitrosothiols levels is likely to exist, so a solitary plasma measurement may not be representative, and serial measurements would be preferential. Furthermore, it is known that use of PPIs simply raises stomach pH but does not reach neutrality. As such, there remains an acidic environment (although reduced) in which to form RSNO. It should also be noted that 48% of all clopidogrel-treated patients were also prescribed a calcium channel blocker, which is known to interact with clopidogrel activation, and may therefore influence the witnessed response to additional PPI use[351].

Of course, PPI users who are poor CYP metabolisers are likely to have higher intra-gastric pH (median pH 6) as well as being poor responders to clopidogrel. Extensive metabolisers, although responsive to clopidogrel, would be expected to have a median intra-gastric pH of 3-4[352], a level where according to previous work at the WHRI[302] clopidogrel may still be able to form nitrosothiols. So it would be interesting to evaluate plasma nitrosothiols levels

specifically in these groups of patients, i.e. according to genetic phenotype. An explanation is still needed to understand the relationship between response to clopidogrel, PPI use and adverse cardiovascular events.

Nevertheless, these results are consistent with those found by other groups who also suggest that concomitant PPI use does not attenuate the anti-platelet effect of clopidogrel[353].

Notwithstanding these observations, as previously discussed, patients with coronary artery disease who are treated with clopidogrel have poorer clinical outcomes if they have CYP2C19*2 carrier status[354]. So current recommendations are that co-prescribing of PPIs with clopidogrel should be avoided unless deemed necessary based on an individual risk-benefit assessment taking into account both cardiovascular and gastrointestinal bleed risks, and avoidance of omeprazole and esomeprazole, the most potent CYP2C19 inhibitors[355], is advocated[356].

It was also anticipated that clopidogrel-treated patients taking anti-anginal medication in the form of oral nitrates would exhibit higher levels of plasma nitrosothiols, primarily due to the greater availability of gastric nitrite. However, there was no statistical difference between these two groups of patients. The most likely explanation for this is simply, insufficient augmentation of intra-gastric nitrite concentration. The bio-availability of inorganic nitrate is very high following oral ingestion, but this is not true for organic nitrates which undergo first pass metabolism. Metabolism of inorganic nitrates is via the nitrate-nitrite-NO pathway (see chapter 1, section 1.3.2 NO metabolism in the gastro-intestinal tract), whereas most organic nitrate undergoes rapid activation via the cytochrome P450 system, and some higher potency nitrates also undergo metabolism via various enzymes including mitochondrial aldehyde dehydrogenase[357], xanthine oxidase, glutathione-S-transferase and glutathione dependent reductases[205,358]. Some organic nitrates such as ISMN and ISDN have better bioavailability profiles[359,360], but it appears that in-sufficient nitrate is available for conversion to nitrite and subsequent generation of intra-gastric S-nitrosothiols.

However, it is believed that bio-activation and metabolism of organic nitrates results in the generation of glycerol-1,2-dinitrite, inorganic nitrite, and both NO and S-nitrosothiols, so even in the absence of an increase in gastric S-nitrosothiols, it may be expected that increased plasma nitrosothiols levels would be measurable. Corollary to this, development of nitrate tolerance (and therefore presumed increased ROS generation), leads to a decrease in bio-activation, and, as with PPI co-prescribing nitrate compliance is difficult to confirm, both of these factors likely to be contributory to the negative results seen. Furthermore, from the baseline measurements in patients receiving both clopidogrel and organic nitrate, there is no increase in NO, nitrite or nitrate as a result of the latter. This is not entirely expected as increases in NO bioavailability are evident (flow, MABP, etc).

Interestingly, there appeared to be a trend towards increased ADP-induced platelet aggregation and a significant rise in TRAP-induced aggregation in patients taking clopidogrel in conjunction with oral nitrates. Speculatively, this could be explained by the concept of nitrate tolerance and increased ROS generation leading to greater platelet aggregation.

Previous studies have shown that in the presence of acid, increasing nitrite concentrations do correlate with greater nitrosothiols formation, but this effect is small with clopidogrel, and greater with prasugrel. Furthermore, pH must be <5, and optimally 2 or less. Of interest, if clopidogrel-treated patients were supplemented with inorganic nitrates in lieu of the organic compounds investigated above, increased S-nitrosothiols production might be anticipated. In addition, it would of course be intriguing to re-evaluate the effect of using inorganic nitrates on the platelet aggregation studies given the effects seen on TRAP-induced aggregation in particular.

3.4.1 Study limitations

There is significant intra- and inter-individual variability in terms of circulating plasma nitrate levels and urinary nitrate excretion; this is strongly influenced by variance in daily nitrate intake which ranges from 75 to 150 mmol, so ideally plasma levels should only be measured after a low nitrate/nitrite diet. This has been previously recommended to be after at least 4 days[361], a time scale and goal that is difficult to achieve in this selected cohort of patients.

Although levels drop dramatically after 24 hours, patients were only fasted overnight so dietary influences are possible and the lack of individual food diaries is a limitation here, and certainly something that should be considered in any future patient studies.

Furthermore, serial NO metabolites and platelet function measurements would likely be more helpful in identifying the existence of any link between these and PPI and nitrate use in clopidogrel-treated patients.

It has to be acknowledged that medication compliance is a key determinant to achieving accurate results, and unfortunately it is known that adherence to long-term therapy for chronic illnesses in developed countries averages only 50%[362]. All patients were questioned with regards their adherence to taking prescribed medication to minimise this.

3.5 Conclusions

PPI co-administration to patients taking chronic clopidogrel therapy had no effect on measured plasma NO metabolites or platelet function testing. Furthermore, treatment with organic nitrates did not augment the NO metabolites profile as would be expected.

With regards PPI use, to prove the validity of these findings, it would be preferable to confirm intra-gastric pH prior to plasma NO metabolites measurements and interrogate findings dependent on both cytochrome P450 phenotype and brand of PPI.

The role of organic nitrates is well established, but with increased interest in their inorganic cousins, questions remain unanswered as to whether these agents could augment intra-gastric nitrite sufficiently to result in therapeutic plasma levels of clopidogrel-derived nitrosothiols.

CHAPTER SUMMARY

It has previously been shown that clopidogrel induces a significant increase in circulatory nitrite in patients on both chronic treatment and acutely loaded, but this study has shown that;

- *Co-administration of proton pump inhibitors with clopidogrel had no effect on plasma NO metabolites (plasma nitrite, plasma nitrate and plasma SNO)*

- *Co-administration of organic nitrates with clopidogrel had no effect on plasma NO metabolites (plasma nitrite, plasma nitrate and plasma SNO)*

- *Co-administration of proton pump inhibitors with clopidogrel had no effect on platelet function tests (using ADP and TRAP agonists)*

- *Co-administration of organic nitrates with clopidogrel had no effect on platelet function tests (using ADP and TRAP agonists)*

4 Prasugrel: The effect of drug loading on plasma NO metabolites and in vivo formation of nitrosothiols

4.1 Introduction

The use of dual anti-platelet therapy has played an integral role in the reduction of thrombotic complications following percutaneous coronary intervention (PCI). Furthermore, clopidogrel used in conjunction with aspirin, has proven morbidity and mortality benefit when given to patients presenting with acute coronary syndromes (ACS).

Due to the large inter-patient variability, delayed onset of action and modest anti-platelet effect associated with clopidogrel[363,364], the newer thienopyridine prasugrel[365] has been used widely as an alternative agent in patients with ACS undergoing PCI. When compared with clopidogrel use in patients with coronary artery disease (CAD), prasugrel exhibits more potent and less variable platelet inhibition with a quicker onset of action. It has also been shown to significantly reduce rates of ischemic events compared to clopidogrel in patients with ACS undergoing PCI[295,366]. Prasugrel is a pro-drug requiring bio-activation, and once in its active state also possesses a free thiol as previously shown with clopidogrel.

Widespread use of the thienopyridines has led to increased interest in their pleiotropic effects. This particularly applies to clopidogrel in which improvement in endothelial function[276], anti-inflammatory effects[278], and reduced endothelial injury after PCI have all been reported, although the exact mechanisms remain unclear.

The interaction between platelets and endothelial cells is critical to the development and progression of atherosclerosis, and NO pathways play an integral role in maintaining both vascular endothelial and platelet homeostasis. NO acts by directly activating platelet guanylyl cyclase, causing an increase in intraplatelet cGMP (Cyclic Guanosine Monophosphate). This cGMP pathway is distinct from the cAMP pathway which is modulated by PGI_2 and $P2Y_{12}$, but studies have shown that activation of both of these

inhibitory pathways produces a synergistic antiplatelet effect. Furthermore, it has been shown that a NO donor drug can boost the effectiveness of $P2Y_{12}$ inhibitors against thrombus formation ex vivo[367,368].

S-nitrosothiols are a class of compound produced by S-nitrosation of reduced sulphydryl groups that act as NO donors. They are capable of cGMP generation, and have been shown to induce vessel relaxation and exhibit potent platelet anti-aggregatory effects[296]. They exist naturally in blood and tissues predominantly as S-nitrosoalbumin and S-nitrosohaemoglobin but also as low molecular weight forms such as S-nitrosoglutathione, typically in the low nanomolar range. Usually ascribed RSNO, they have been considered an attractive source in the hunt for more potent yet safer anti-thrombotic agents[369,370]. Stimulation of sGC is considered perhaps the most important mechanism by which S-nitrosothiols exert their effect[140]. However, there has been recent progress in this field and numerous pathways have been postulated, including denitrosation at the cell surface, transport via the amino acid transporter system-L, regulation of key cell surface targets[371], and transnitrosation of secondary intracellular targets to modulate effect.

Importantly, it has been demonstrated by our group previously that by virtue of their crucial thiol group (-SH), clopidogrel and prasugrel both readily form RSNO compounds under laboratory conditions. Critically, formation of thienopyridine induced RSNO compounds was found to be dependent on the availability of inorganic nitrite, an acidic environment, and the resulting stability of the SNO produced[297] (see also section 3.1). Prasugrel exhibits the greatest capacity for RSNO production of all thienopyridines tested, largely attributable to possession of more free thiol and the stability of the resulting RSNO.

In vivo, this perfect milieu for RSNO formation exists in the stomach where nitrite concentrations in the saliva and stomach are typically 20-210 µmol/L and 0.6-20 µmol/L, respectively, and pH is usually between 1 and 4[372,373]. Despite this, evidence for in vivo RSNO formation in blood by thienopyridines is absent to date.

In patients with CAD undergoing planned PCI, experiments conducted at the WHRI have recently demonstrated that after a loading dose of 600mg of clopidogrel there is a time-proportional increase in NO bioavailability as reflected by elevated levels of plasma nitrite and cGMP[298].

Given the increased potency of prasugrel over clopidogrel in terms of platelet inhibition[374] and the increased capacity for prasugrel to undergo S-nitrosation in vitro, it was investigated firstly whether prasugrel could form detectable RSNO in patients with CAD and if so its potential relationship with platelet inhibition in the acute and chronic setting. Secondarily, confirmation was sought regarding the importance of in vivo RSNO formation by examining whether proton pump inhibition, which reportedly does not affect total prasugrel active metabolite formation[375] but elevates stomach pH, had an impact on these parameters.

4.2 Materials and Methods

4.2.1 Patient Recruitment and Collection of blood samples

A prospective, single centre study was undertaken. Approval was granted by the Local Research Ethics Committee, and the study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964.

A total of 60 patients with coronary artery disease undergoing elective PCI were enrolled and informed consent was obtained from all patients prior to venepuncture. All patients were fasted for at least 6 hours and on a maintenance dose of Aspirin 75mg daily. Patients taking concurrent anticoagulants, anti-thrombin agents or anti-inflammatory drugs in the previous 7 days were excluded. They were divided into 2 separate groups.

The first group comprised 34 thienopyridine-naïve patients. They each received a standard oral loading dose of prasugrel (Effient® Daiichi Sankyo UK, tablets containing 60 mg prasugrel hydrochloride) prior to their procedure. Blood samples were collected from an 18G intravenous cannula pre and 2 hours post prasugrel loading into vacutainers containing K_3EDTA (Vacuette Greiner Bio-One™) or Hirudin. Immediate centrifugation of the EDTA samples was performed (1500g for 10 minutes at 4°C) to prevent NO metabolite degradation. Platelet poor plasma was isolated, snap frozen with liquid nitrogen and then stored at -80°C in preparation for batch analysis. Platelet function testing was performed on the Hirudin samples within 45 minutes as described below.

The second group comprised 26 patients already on a maintenance dose of prasugrel 10mg daily. One patient had been taking the treatment for 7 days, all others for over 28 days. A single blood sample was taken and analysed as described for the first group above.

4.2.2 Platelet Function Testing Using Multiplate® Multiple Electrode Impedance Aggregometry

Multiplate® is one of the most widely used bedside kits for testing platelet function, with evidence to support its use in clinical practice[376,377]. Analysis using whole blood is based on impedance aggregometry and is described in the methods chapter, Chapter 2: section 2.3.

4.2.3 Measurement of plasma NO metabolites

Plasma nitrite, nitrate and RSNO were measured using well established ozone-based chemiluminescense techniques, as described previously[299]. This is described in the methods chapter, Chapter 2: section 2.2.

4.2.4 cGMP ELISA

cGMP was quantified using a cGMP Enzyme Immunoassay kit (R&D systems KGE003). Patient platelet poor plasma was diluted 20 times with assay calibrator diluent and the assay procedure was then performed according to the manufacturer's instructions as described in the methods chapter, Chapter 2: section 2.4

4.2.5 Statistical Analysis

The results of plasma NO metabolites levels and of platelet function tests are expressed as the mean with standard deviation (in brackets) and presented data also show means with error bars representing the standard deviation. D'Agostino & Pearson omnibus normality testing was used to facilitate determination of normally distributed data. Proportions in Tables were analysed using Fisher's exact test. Two-tailed paired *t*-test were used to compare the means for normally distributed data, and the 2-tailed Mann-Whitney test to compare the medians of non-parametric distributions. The unpaired *t*-test was also used when analysing differences between patients on chronic prasugrel treatment (>28 days) and those 2 hours after acute loading. A p value of <0.05 was considered statistically significant. Pearson's method was used to determine correlation coefficients.

Analysis was performed using GraphPad Prism^{TM} version 5 software.

4.3 Results

4.3.1 Patient groups and characteristics

This prospective, single centre study was undertaken in 60 patients with coronary artery disease undergoing elective PCI. The first group comprised 34 thienopyridine-naïve patients who were sampled pre and 2hrs post administration of prasugrel. The second group comprised 26 patients already on a maintenance dose of prasugrel for >28 days. The patients in both groups demonstrated no significant difference in the incidence of clinical characteristics including hypertension, hyperlipidaemia, cigarette smoking, family history of ischaemic heart disease and drug treatment, except for the use of calcium channel blockers which was higher in the thienopyridine-naïve group, and angiotensin converting enzyme inhibitors (ACEIs) which was higher in the chronically-treated patients (Table x). Whilst some of the differences between groups appear large it is only these latter two that differ significantly. It is acknowledged that these differences could be important and any subsequent analysis could try to take account of these covariates.

The increased use of ACE inhibitors, and also of beta-blockers and statins in the chronic group is unsurprising following the diagnosis and treatment of coronary artery disease. Furthermore, treatment of coronary disease with percutaneous revascularisation frequently enables the discontinuation of anti-anginal medication such as nitrates and calcium channel blockers. Dual anti-platelet therapy is also likely to increase the incidence of gastro-oesophageal related side-effects and is reflected in the increased prescription of PPIs.

Characteristic	Chronic (n=26)	Acute Loading (n=34)	*P*
Age (years)	62 +/- 8.7	65 +/- 8.9	ns
Male %	88	78	ns
Hypertension %	54	56	ns
Hyperlipidaemia %	67	56	ns
Cigarette Smoker %	43	19	ns
Family History of ischaemic heart disease %	19	32	ns
Beta-blocker %	84	76	ns
Statin %	91	82	ns
Calcium Channel Blocker %	8	41	0.01
ACE inhibitor %	92	35	0.0007
Nitrate %	8	29	ns
Proton Pump Inhibitor %	46	34	ns

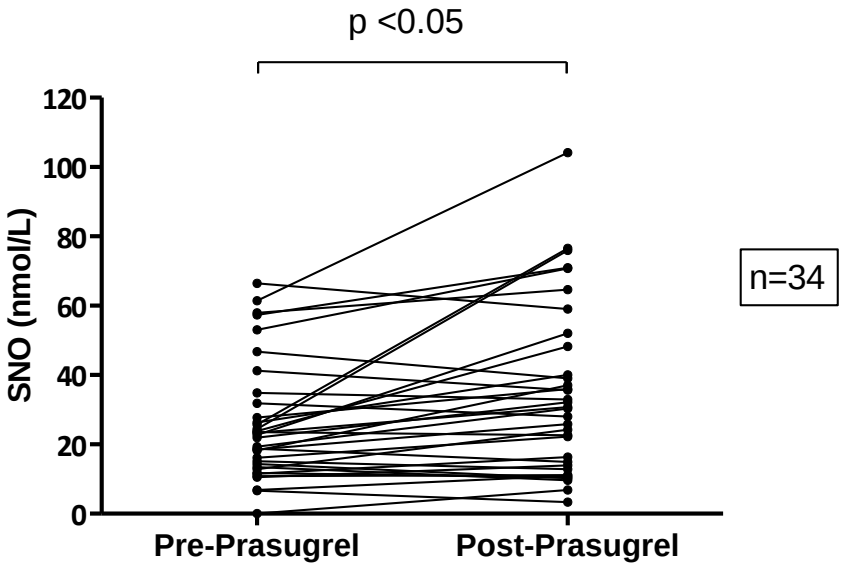
Table xii: Clinical characteristics of patients in the chronic treatment and acute loading groups. Mean age (as shown +/- standard deviation) was compared using the two-sample *t*-test and all other proportions were compared using the Fisher's exact test.

4.3.2 Influence of acute prasugrel loading on NO metabolites

Concentration of mean plasma RSNO rose significantly (p=0.0018) from a baseline of 25.8 (17.1) nmol/L to 34.7 (24.5) nmol/L 2hrs following prasugrel loading (Figure 40A). The mean of differences was -9.0 with a 95% CI of -14.3 to -3.6. Plasma RSNO was shown to be predominantly associated with plasma protein, as reflected by the finding that protein removal from the sample reduced RSNO by 58.1+/-3.5%. Two hours after a loading dose of prasugrel plasma nitrite concentration also rose significantly (p=0.0044) from 239.9 (128.8) to 268.2 (125) nmol/L (Figure 40B). Mean of the differences was -28.3 and 95% CI was -47.1 to -9.4. Plasma nitrate concentration fell significantly (p=0.0053) from 40.6 (24.5)

µmol/L before prasugrel administration to 38.2 (24.2) µmol/L 2 hours post dose (Figure 40C). 95% CI was 0.7 to 3.9 with a mean of differences of 2.3. There was no significant difference (p=0.3847) between circulating cGMP levels measured in the plasma samples taken before and after prasugrel loading. Mean before was 214.4 (36.4) pmol/mL, and after was 209.4 (35.5) pmol/mL (Figure 40D), with a mean of differences of 5.0 and 95% CI from -6.6 to 16.7.

A



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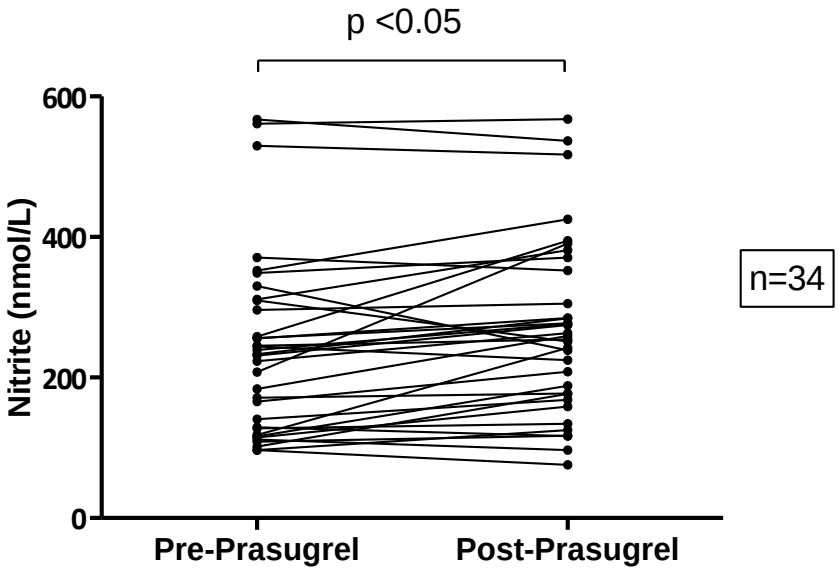

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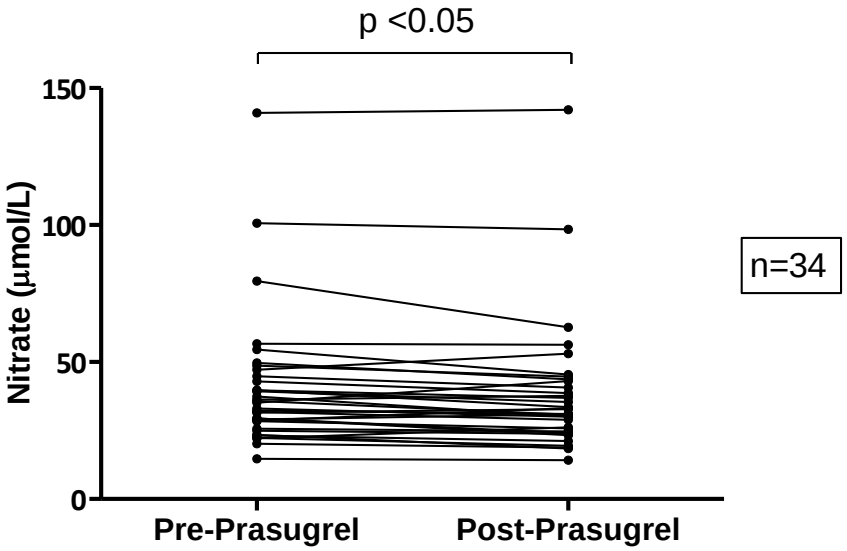

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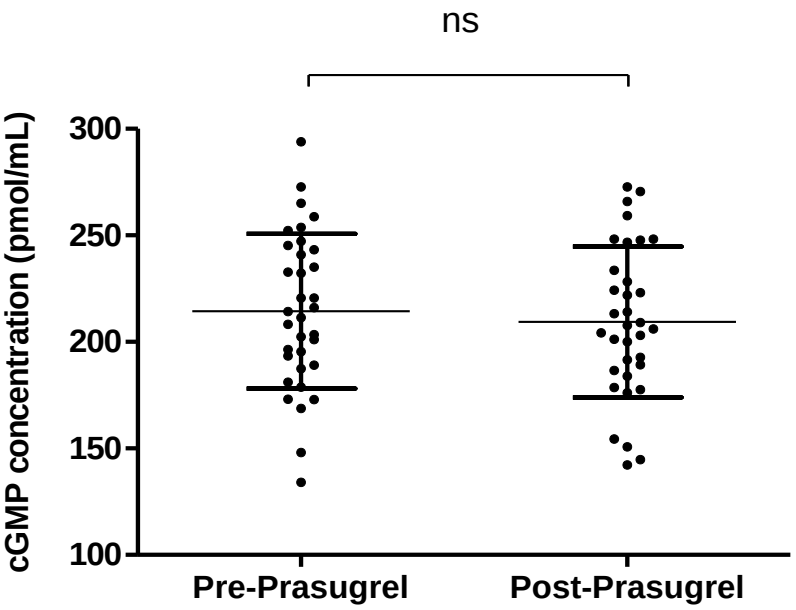
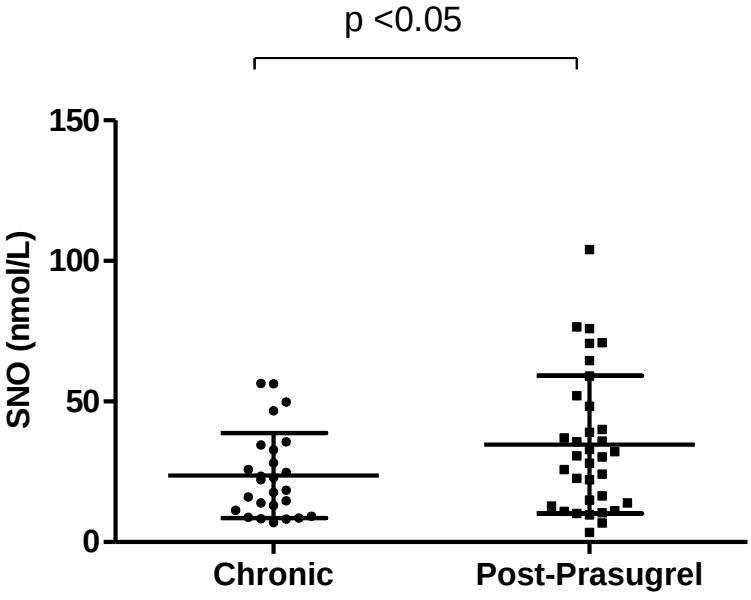


Figure 40: Charts showing the effect of acute prasugrel loading on plasma NO metabolites and cGMP.
A) Plasma SNO
B) Plasma nitrite
C) Plasma nitrate
D) Plasma cGMP

4.3.3 Effect of chronic treatment vs acute loading of prasugrel on NO metabolites

The data is normally distributed taking into account experimental design, and using D'Agostino & Pearson omnibus normality testing. Comparison between patients after acute prasugrel loading and those on chronic therapy was therefore performed using an unpaired *t*-test. RSNO means were 23.7 (15.1) nmol/L and 34.7 (24.5) nmol/L in the chronic group and acute prasugrel loading group respectively. This represented a statistically significant drop in plasma RSNO in the chronic group (p=0.0478), with a difference between means of -11.0 +/- 5.5 and 95% CI from -22.0 to -0.1. Mean plasma nitrite was significantly lower (p=0.0034) in the chronic group at 180.8 (80.3) mol/L vs the post-prasugrel group, 268.2 (125) nmol/L. The difference between means in this group was -87.4 +/- 28.6 and 95% CI was -144.6 to -30.1. Mean plasma nitrate remained virtually unchanged (p=0.479). Mean plasma nitrate in the chronic group was 34.4 (13.6) µmol/L and in the post-prasugrel group 38.2 (24.2) µmol/L (see Figure 41). Difference between means was -3.8 +/- 5.4, and 95% was -14.6 to 6.9.

A



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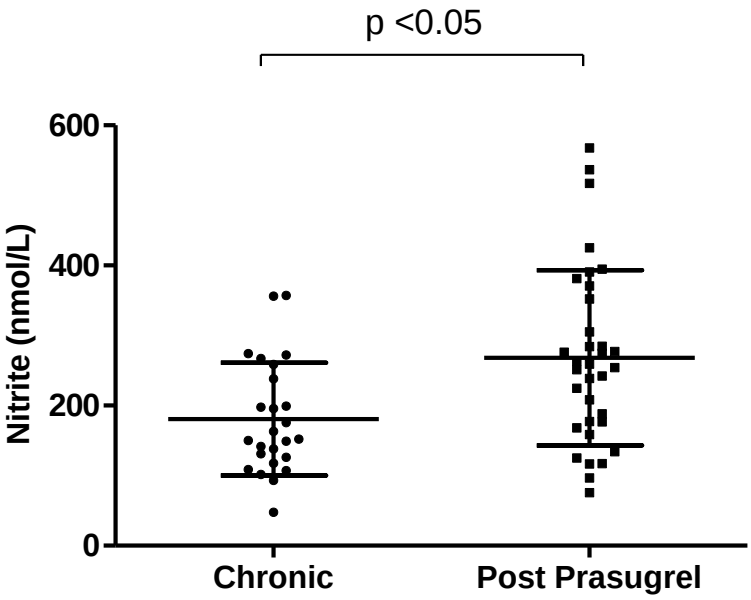

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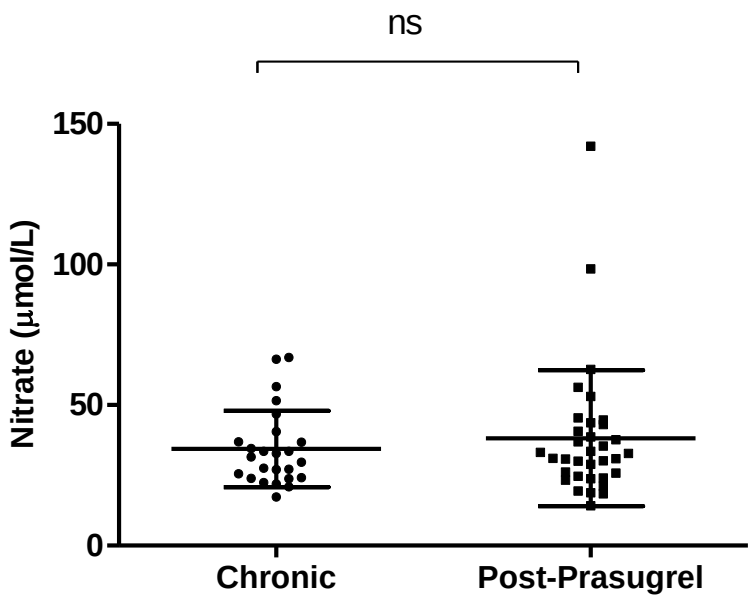


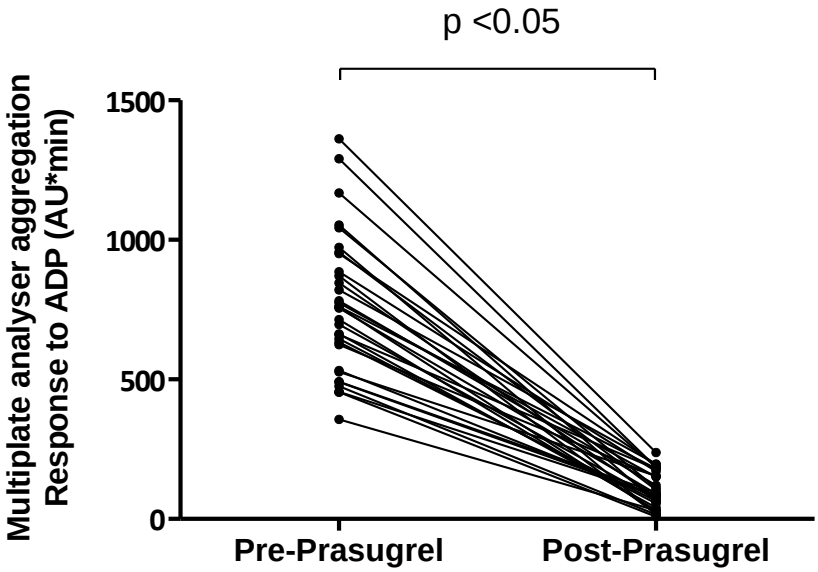
Figure 41: Charts showing the effect of chronic prasugrel therapy on NO metabolites.
A) Plasma SNO
B) Plasma nitrite
C) Plasma nitrate

4.3.4 Effect of prasugrel loading on platelet aggregation

Multiple electrode aggregometry using Multiplate® confirms a mean platelet response to ADP of 755.2 (248.9) AU*min before loading with prasugrel, and a mean of 102.3 (65.6) AU*min 2 hours following administration. This represents a significant drop with a p value <0.0001, mean of differences of 662.4, and 95% CI from 582.4 to 742.4. Platelet response to TRAP was 1312 (282.4) AU*min at baseline and 994.7 (256.7) AU*min following prasugrel loading. This also represents a significant drop with a p value <0.0001, mean of differences of 322.0, and 95% CI from 257.8 to 386.2. Taken together this confirms efficient inhibition of platelet aggregation following prasugrel loading. All patients were on long term aspirin treatment of 75mg daily (see Figure 42).

Patients receiving chronic therapy exhibit greater residual platelet aggregation in response to ADP measured at a mean of 174.7 (121.0) AU*min compared with a mean of 102.3 (65.6) AU*min two hours following a prasugrel loading dose. This is statistically significant with p=0.0071 using the Mann-Whitney test. No significant difference (p=0.4341) in platelet responses to TRAP is observed when comparing the acute versus chronic group. Mean response in the acute group is 994.7 (256.7) AU*min versus 935.8 (295.0) AU*min in the chronic group (see Figure 43). Difference between the means is -58.9 +/- 74.7 with 95% CI from -208.8 to 91.1.

A



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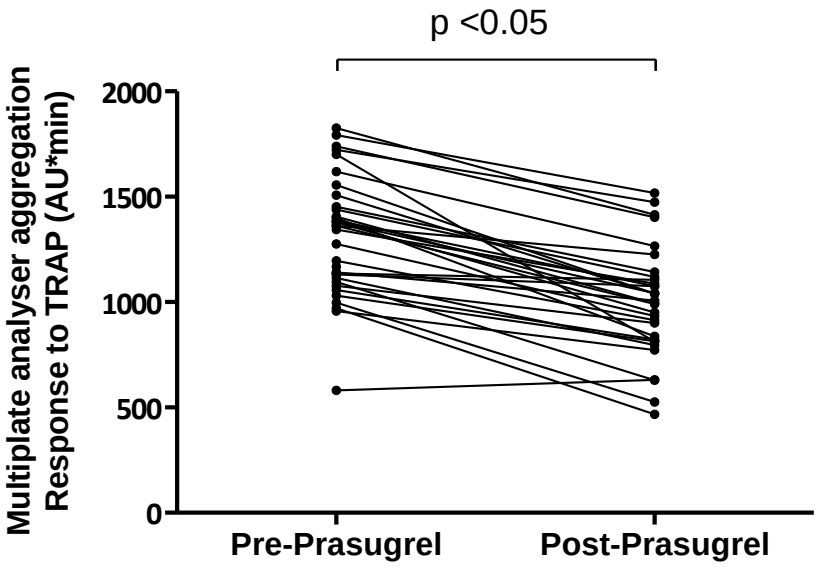
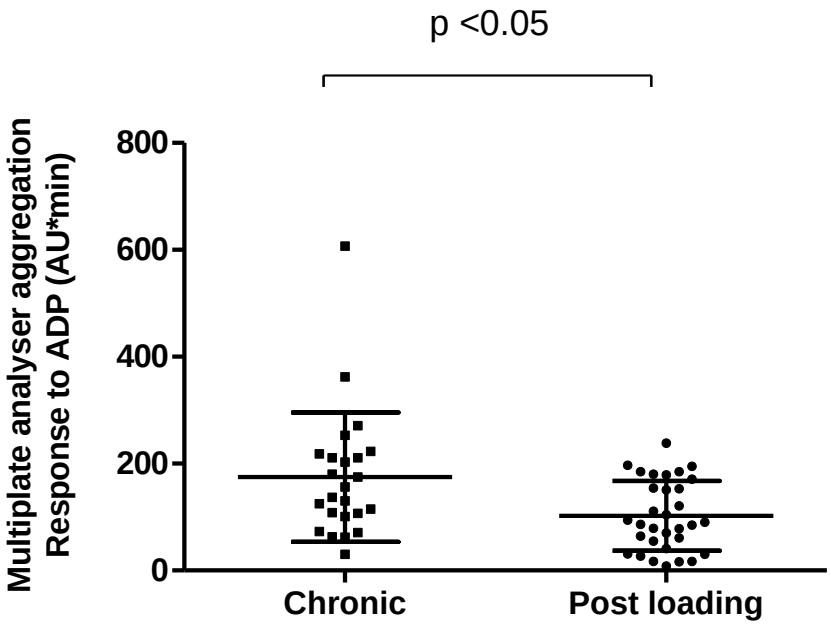


Figure 42: Charts showing the effect of acute prasugrel loading on platelet aggregation in response to the agonists
A) ADP and B) TRAP.

A



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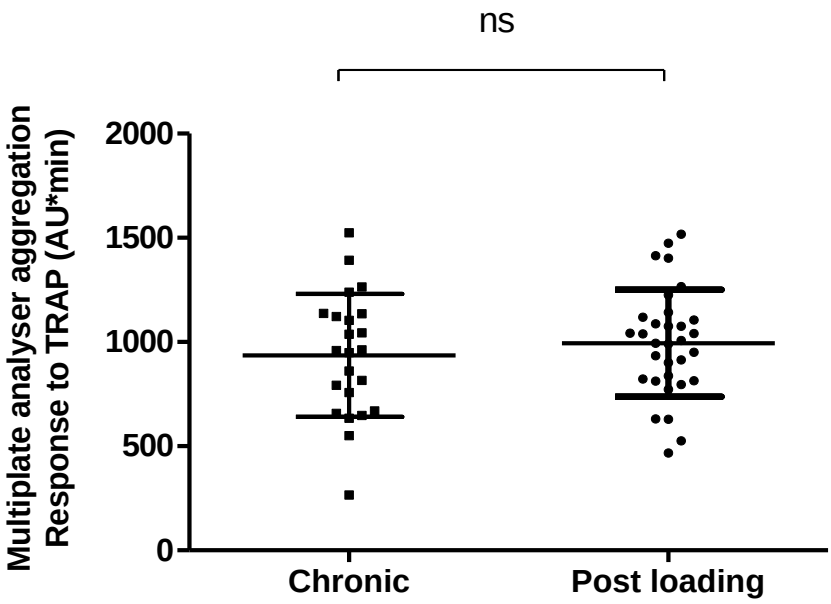
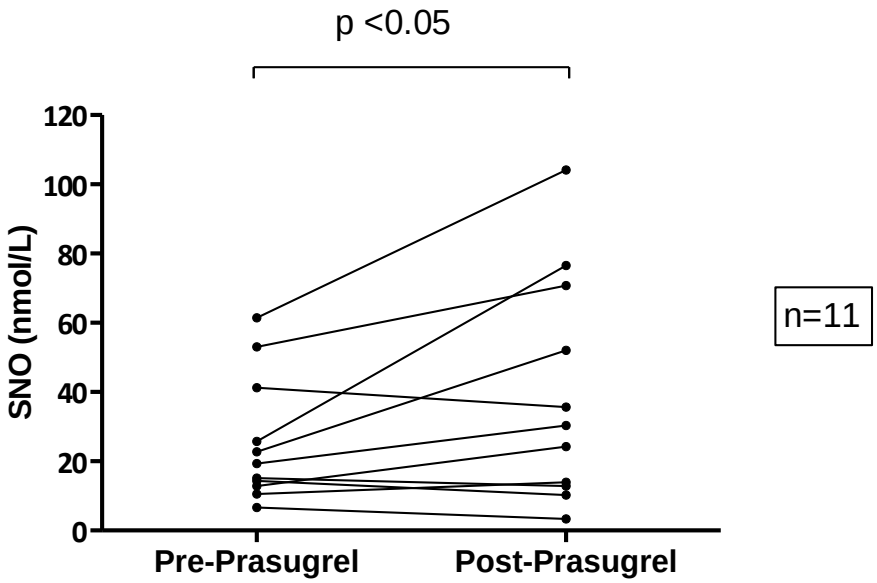


Figure 43: Charts showing the effect of chronic prasugrel therapy on platelet aggregation in the response to the agonists
A) ADP and B) TRAP.

4.3.5 Effect of PPI on indices of NO and platelet aggregation following prasugrel loading

Normal distribution was confirmed. Using the paired t-test, a significant rise in mean plasma RSNO was detected whether patients were using PPIs (p=0.0422) or not (p=0.0197) as shown in Figures 44A and 44B respectively. Mean plasma SNO was almost identical at baseline, rising from 25.7 (18.2) nmol/L to 39.5 (32.5) nmol/L in the on PPI group, and 25.8 (17.0) nmol/L to 32.4 (20.1) nmol/L in those not taking a PPI group. Mean of differences was -13.7 in the PPI group with a 95% CI from -26.9 to -0.6, and mean of differences in the non-PPI group was -6.7 with a 95% CI from -12.2 to -1.2.

A



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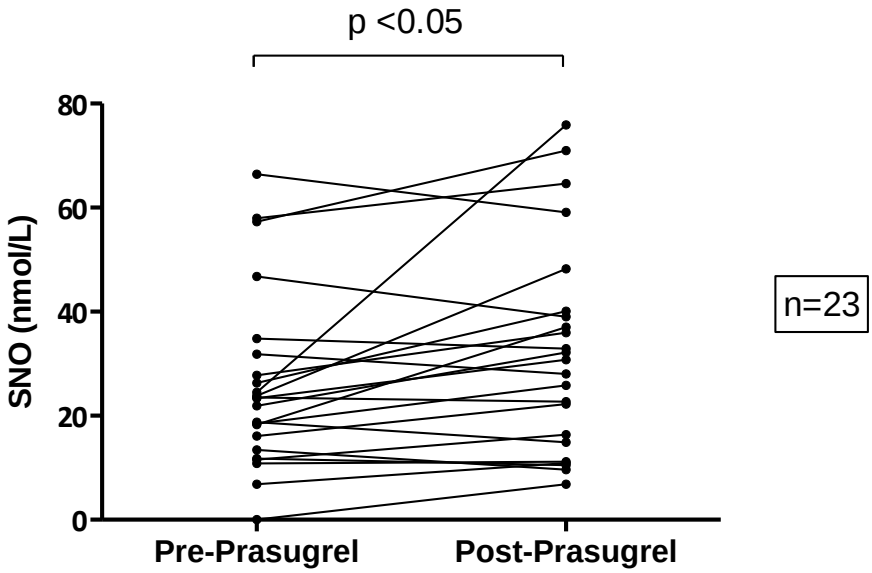
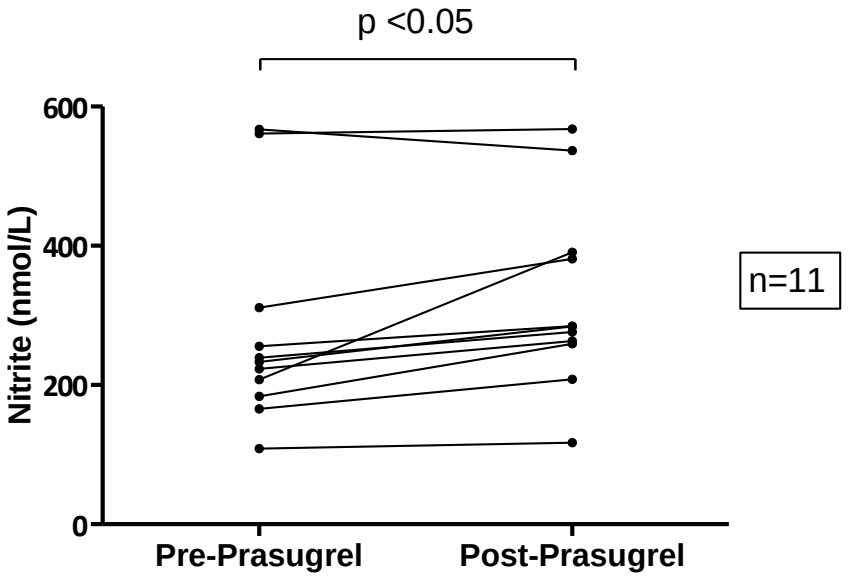


Figure 44: Charts showing the effect of concurrent PPI therapy on plasma SNO in patients loaded with prasugrel.
A) Patients on concurrent PPI treatment.
B) Patients not taking PPI.

A significant increase in nitrite was exhibited when comparing pre and post prasugrel in patients receiving concurrent PPI medication (p=0.0173), whereas patients not receiving a PPI showed no change in plasma nitrite when compared to their baseline values (p=0.0895), as shown in Figure 45A and Figure 45B, respectively. The mean of differences in the group receiving a PPI was -46.5 (with a mean of 278.0 (150.7) nmol/L before and mean of 324.5 (135.0) nmol/L after loading) with a 95% CI from -82.9 to -10.1. The mean of differences in the group not taking concurrent PPI treatment was -19.6 (mean of 221.7 (116.1) nmol/L before and 241.2 (113.1) nmol/L after loading) with a 95% CI from -42.4 to 3.3.

A



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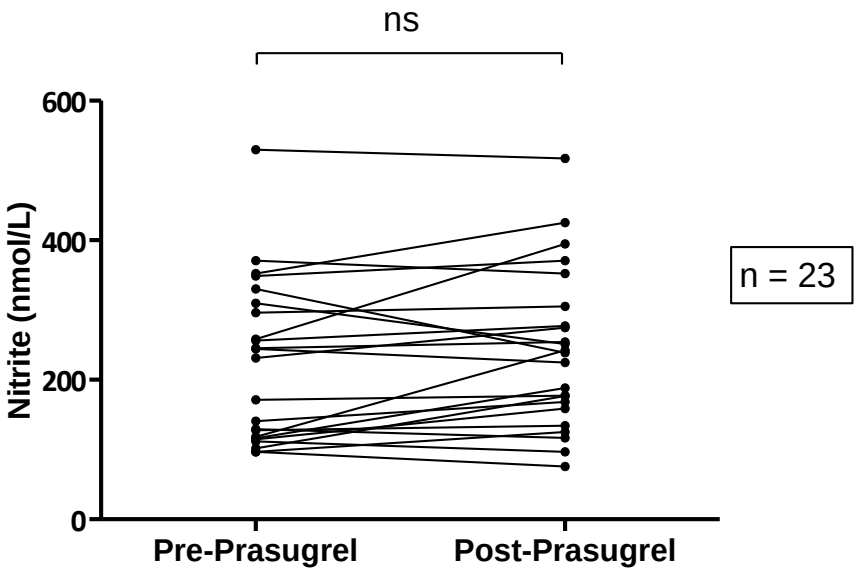
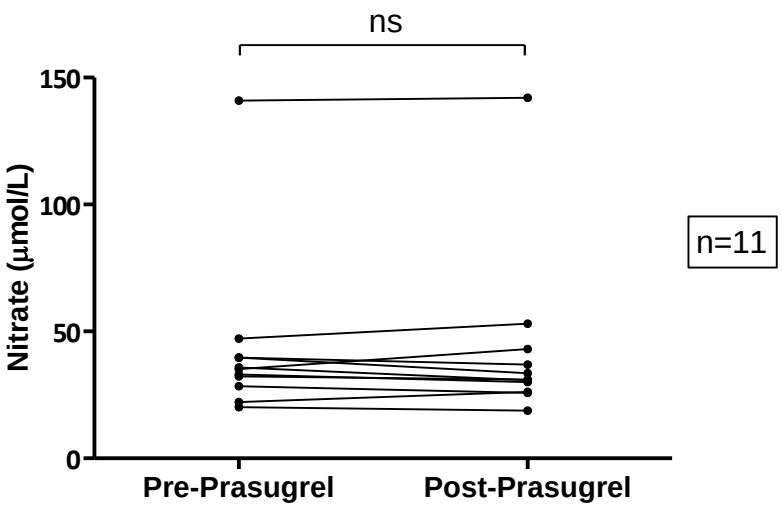


Figure 45: Effect of concurrent PPI therapy on plasma nitrite in patients loaded with prasugrel.
A) Patients on concurrent PPI treatment.
B) Patients not taking PPI.

No significant difference in plasma nitrate concentration was seen before and after prasugrel loading in patients taking concurrent PPI therapy (p=0.8359). Mean before loading was 43.1 (33.3) µmol/L and after loading was 42.9 (34.2) µmol/L. Mean of differences was 0.3 with 95% CI from -2.7 to 3.3. However, a significant drop in nitrate was detected in patients not

using PPIs (p=0.0014) with a mean before prasugrel loading of 39.4 (19.7) µmol/L and after loading of 36.0 (18.2) µmol/L. Mean plasma nitrate differences in those not taking a PPI was 3.3 with a 95% CI from 1.4 to 5.2. These results are shown in Figures 46A and 46B

A



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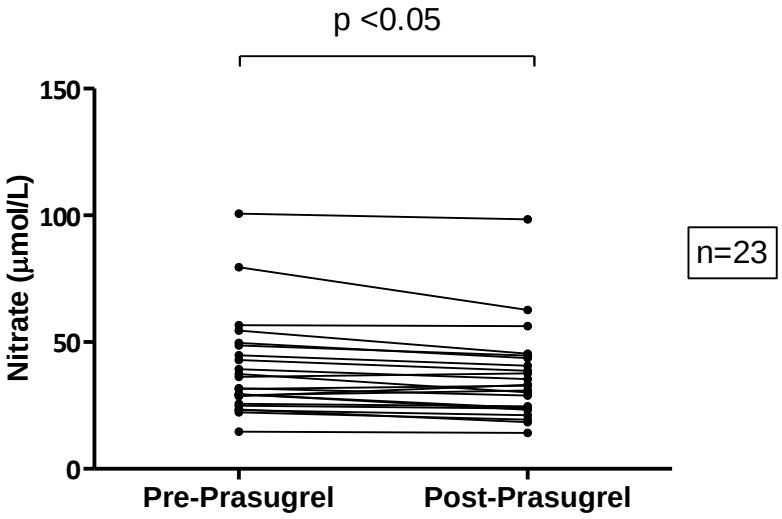


Figure 46: Charts showing the effect of concurrent PPI therapy on plasma nitrate in patients loaded with prasugrel.
A) Patients on concurrent PPI treatment.
B) Patients not taking PPI.

ADP induced aggregation, measured pre and post prasugrel administration to naïve patients, was considered separately in patients receiving PPI versus those who did not. The degree of inhibition afforded by prasugrel was greater in patients not receiving PPI (p=0.0253), shown in Figure 47, confirming a direct link between stomach acidity and anti-platelet activity of prasugrel in vivo.

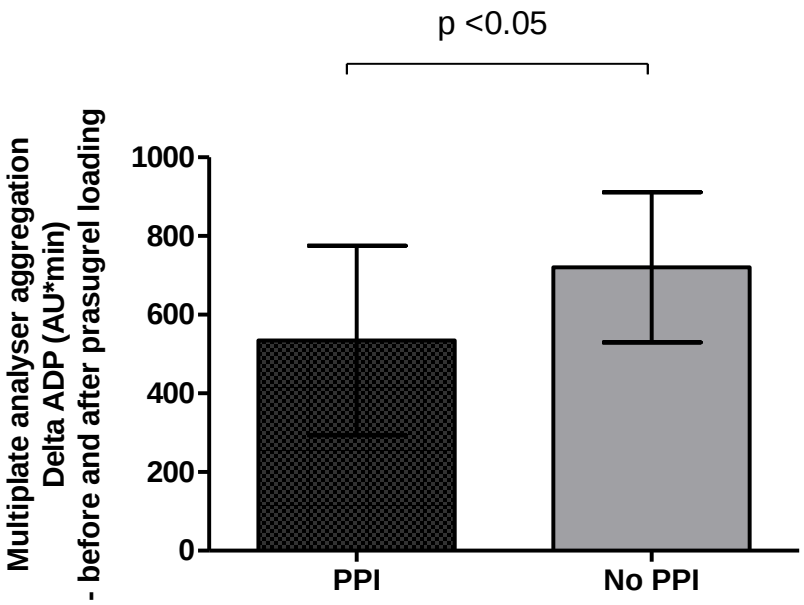


Figure 47: Degree of platelet inhibition in response to ADP, as measured by Multiplate®. Bars represent change in ADP reading before and after loading with prasugrel in patients on a PPI as compared to those off a PPI.

4.3.6 Effect of PPI on indices of NO and platelet aggregation following chronic prasugrel treatment

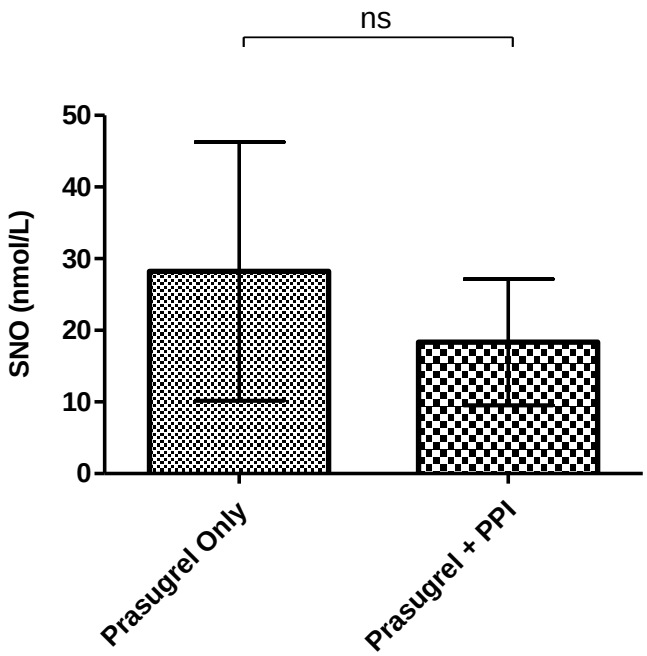
Mean plasma SNO, nitrite and nitrate was measured in all patients taking chronic prasugrel therapy. Of the 26 patients in this group, 12 were receiving concurrent PPI therapy and 14 were not taking a PPI. Normal distribution was confirmed. Mean plasma SNO was higher in those taking prasugrel only, 28.2 (18.0) nmol/L, compared to those taking prasugrel with a PPI, 18.3 (8.8) nmol/L but did not reach statistical significance (p=0.0972). Difference between means was 9.9 +/- 5.7 with a 95% CI from -1.9 to 21.7.

Mean plasma nitrite was 191.2 (83.1) µmol/L in those taking prasugrel alone and 167.6 (78.5) µmol/L in those taking the combination of prasugrel and a PPI, with no statistical significance between the groups (p=0.4773). Difference between means was 23.6 +/- 32.7 with a 95% CI from -44.0 to 91.3.

There was also no statistical difference (p=0.1698) in mean plasma nitrate between the 2 groups, with means of 30.2 (9.1) nmol/L in those on a PPI versus 37.8 (15.8) nmol/L in those not taking a PPI. The difference between means was 7.6 +/- 5.4 with a 95% CI from -3.5 to 18.7.

These results are shown in Figure 48.

A



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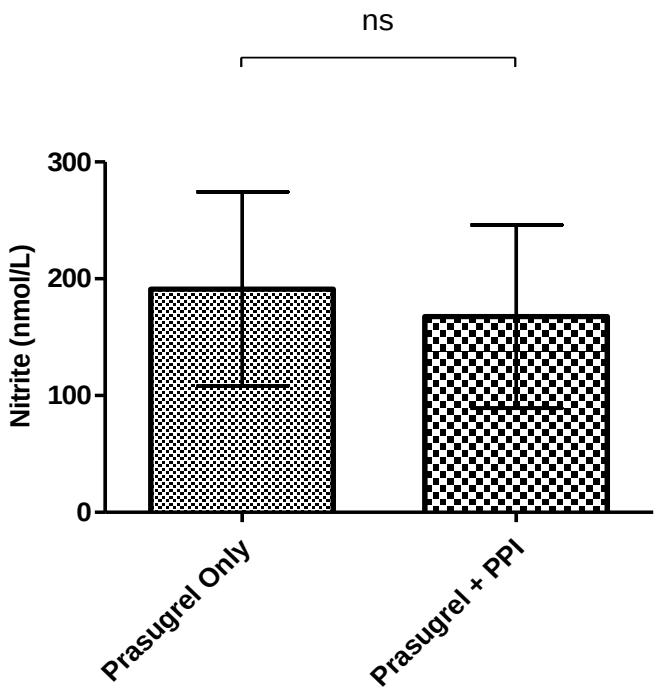

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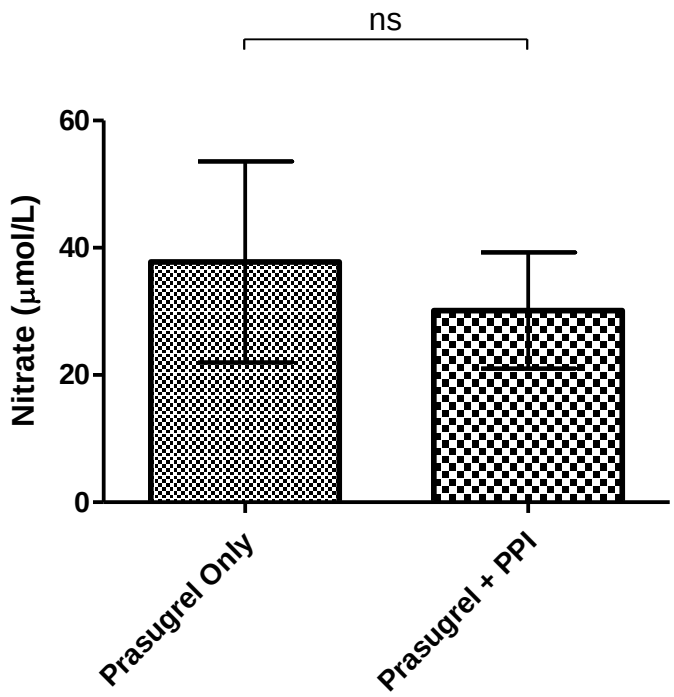


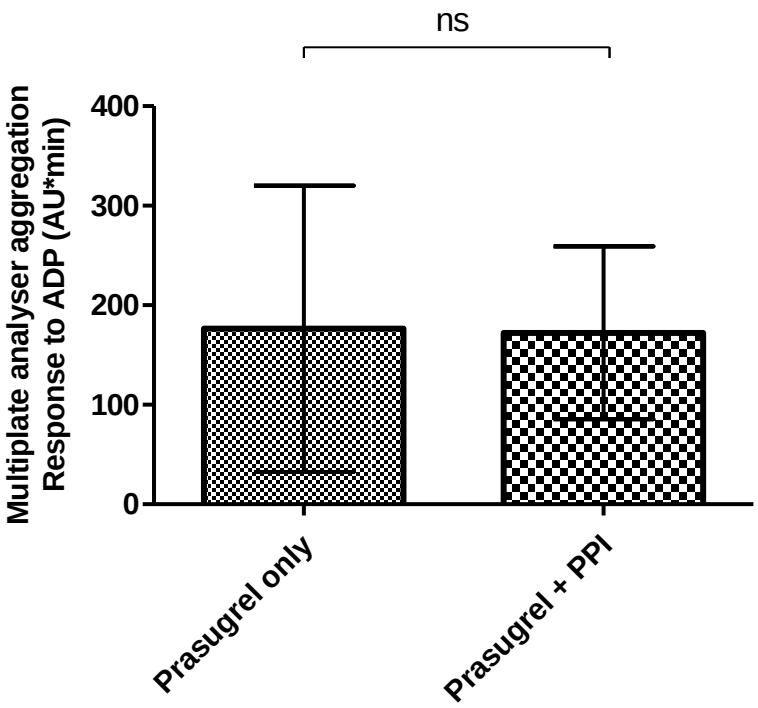
Figure 48: Mean NO metabolites in patients treated with chronic prasugrel alone compared to those receiving chronic prasugrel and PPI treatment.
A) Mean plasma SNO
B) Mean plasma nitrite
C) Mean plasma nitrate

Patients receiving chronic prasugrel treatment also had platelet function testing performed in response to ADP and TRAP specifically to identify any difference in those taking a PPI compared to those taking prasugrel alone. No statistical difference was found in response to ADP (p=0.9376) or TRAP (p=0.2155). Mean aggregation response to ADP was 176.4 (143.7) AU/min in the prasugrel only group and 172.3 (86.8) AU/min in the prasugrel plus PPI group. Difference between means was 4.1 with a 95% CI from -102.1 to 110.2.

Mean aggregation response to TRAP was 867.8 (285.3) AU/min in the prasugrel only group and 1024 (298.2) AU/min in the prasugrel and PPI group. Difference between means was -156.3 +/- 122.4 with a 95% CI from -410.7 to 98.2.

These results are shown in Figure 49.

A



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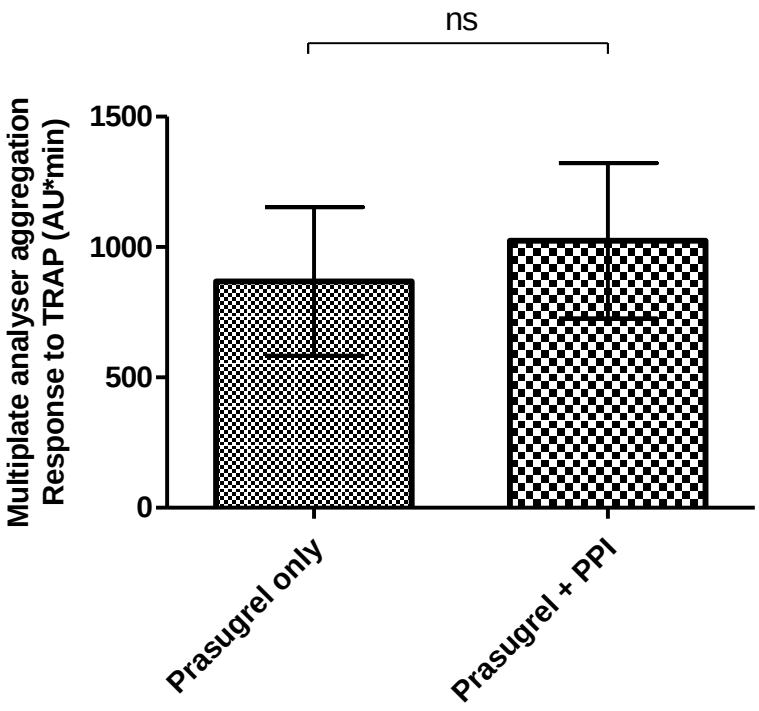

Figure 49: Charts showing the mean platelet response to A) ADP and B) TRAP in patients receiving prasugrel and a PPI vs those receiving prasugrel alone.

4.3.7 Results Summary Table

The following table summarises all the results for patients receiving prasugrel. Plasma measures of SNO, nitrite, nitrate, cGMP and platelet response to ADP and TRAP agonists are shown for the various patient groups.

GROUP	N	SNO (nmol)	Nitrite (nmol)	Nitrate (µmol)	cGMP (pmol)	ADP (AU*min)	TRAP (AU*min)
Pre-Prasugrel	34	25.8 +/-17.1	239.9 +/-128.8	40.6 +/-24.5	214.4 +/-36.4	755.2 +/-248.9	1312 +/-282.4
+ PPI	11	25.7 +/-18.2	278.0 +/-150.7	43.1 +/-33.3		606.5 +/-265.0	1182 +/-210.7
no PPI	23	25.8 +/-17.0	221.7 +/-116.1	39.4 +/-19.7		829.6 +/-208.7	1377 +/-295.0
Post-Prasugrel	34	34.7 +/-24.5	268.2 +/-125.0	38.2 +/-24.2	209.4 +/-35.5	102.3 +/-65.6	994.7 +/-256.7
+PPI	11	39.5 +/-32.5	324.5 +/-135.0	42.9 +/-34.2		87.3 +/-60.9	773.6 +/-176.7
no PPI	23	32.4 +/-20.1	241.2 +/-113.1	36.0 +/-18.2		109.1 +/-67.9	1095 +/-223.7
Chronic Prasugrel	26	23.7 +/-15.1	180.8 +/-80.3	34.4 +/-13.6		174.7 +/-121.0	935.8 +/-295.0
+PPI	12	18.3 +/-8.8	167.6 +/-78.5	30.2 +/-9.1		172.3 +/-86.8	1024 +/-298.2
no PPI	14	28.2 +/-18.0	191.2 +/-83.1	37.8 +/-15.8		176.4 +/-143.7	867.8 +/-285.3

Table xiii: Prasugrel results summary. Values shown represent mean +/- standard deviation

4.4 Discussion

This study shows that administration of a single loading dose of prasugrel to CAD patients previously naïve to thienopyridine treatment results in a significant increase in circulating RSNO and nitrite acutely at 2hrs, although this is not evident in CAD patients receiving prasugrel for >28 days. Platelet aggregability was significantly inhibited by prasugrel, but effectiveness was markedly reduced in patients receiving PPI who also exhibited reduced RSNO.

The discovery that significant rises can be detected in not only plasma nitrite concentration but more importantly plasma RSNO concentration following a standard loading dose of prasugrel is both novel and noteworthy and may have important implications in terms of different mechanisms of action of thienopyridine agents. Time-proportional increases in NO metabolites following clopidogrel loading have been demonstrated previously by colleagues at the WHRI[298], reflected predominantly by elevated nitrite, and corresponding with increased plasma cGMP. Given that plasma nitrite itself is considered a marker of vascular NO production/bioavailability, and circulating cGMP is derived primarily from endothelium (and to a lesser extent platelets), these results confirm clopidogrel exhibits beneficial off-target effects on the vasculature. The fact that demonstrable increases in RSNO following prasugrel that were not evident in studies previously conducted with patients receiving clopidogrel could be considered consistent with the earlier laboratory findings[298]. In vitro, prasugrel consistently produced more RSNO/mole drug (x10) across a very broad range of nitrite concentrations without the need for bioconversion to the active drug metabolite, which if translated to the in vivo setting may result in significant increases in RSNO.

The fact that plasma cGMP concentration did not change whereas increases in circulating RSNO resulted following prasugrel administration is key. This implies that the significant increase in RSNO did not result in vasodilation, a finding consistent with the fact prasugrel dosing is not associated with acute changes in blood pressure per se. RSNO are NO donors

with established antiplatelet[140] and probable antithrombotic[378] effects, and there is evidence to suggest they also reduce stent thrombosis post PCI[219]. Evidence from previous in vivo studies using the endogenous RSNO, nitroso-glutathione (GSNO) suggests a degree of platelet selectivity, with platelet inhibition at doses that failed to produce significant vasodilatation[379]. In fact, this is one of the characteristics of RSNOs that makes them appealing as possible therapeutic agents. In addition, some examples show tissue selectivity, they do not have the disadvantage of tolerance that is associated with organic nitrates and given that they tend to be naturally occurring are unlikely to induce cytotoxicity. Thus, detection of elevated nitrite and augmented RSNO concentrations as demonstrated in this study in patients undergoing PCI therefore has clear clinical relevance, and may account for firstly some of the putative early benefit of prasugrel use in patients undergoing STEMI PCI and secondly decreased stent thrombosis[366].

There does appear to be a trend towards decreased plasma NO metabolites, including RSNO, following chronic prasugrel therapy as compared to the early post-dosing levels. Furthermore, there is a statistically significant increase in platelet aggregation seen in response to ADP, but not TRAP, following prolonged prasugrel treatment. This is likely to be due to the reduced dose of prasugrel used for regular prescribing as compared to the loading dose resulting in less $P2Y_{12}$ inhibition, but the associated change seen in plasma NO metabolites could also be indicative of a reduced bio-availability of nitrosothiols and thereby resulting in reduced platelet inhibition. Certainly, a larger dose of thienopyridine and hence thiol availability together with plentiful stomach acid and nitrite would have a much greater potential to yield more RSNO.

The measures of platelet responsiveness to prasugrel loading in patients with stable coronary disease in this study are consistent with previous studies using other modalities of platelet function testing, and do correlate closely with those reported by other groups[379,380]. However, the effect of co-treatment with PPIs and thienopyridines has been and continues to be the source of much debate. Pharmacological interaction between clopidogrel and some

PPIs has been proposed based on mutual CYP450-dependent metabolism, but available evidence of subsequent clinical sequelae has ultimately been inconsistent[381]. This has been discussed in chapter 3. Conversely, prasugrel metabolism to its active metabolite(s) is reportedly unaffected by PPI use[375], allowing utilisation of the PPI effect on neutralising stomach pH to directly test the influence of RSNO formation on platelet inhibition by prasugrel. This provided an in vivo model that enabled separation of the classic $P2Y_{12}$ inhibition pathway and alternative RSNO/NO pathway of inhibition.

Co-treatment of CAD patients with prasugrel and a PPI significantly reduced the effectiveness of platelet inhibition in this study. Taken together with a tendency to reduced RSNO formation in this same patient group may suggest a link between neutralising stomach pH, reducing plasma RSNO and reduced effectiveness of prasugrel in patients. However, in order to prove this association, accurate information on stomach pH through the use of nasogastric aspirates would be required.

Although prasugrel-induced RSNO formation was reduced with PPI, significant increases remained compared to pre-prasugrel levels. This may relate to the fact stomach pH is unlikely to reach neutrality in patients on chronic PPI therapy[372,373,382-384] and RSNO formation from nitrite occurs across the range <pH7, with marked reduction only when approximating neutrality. The human stomach typically contains ~6-20 µmol/L inorganic nitrite which provides plentiful substrate for the ingested 60 mg prasugrel (equivalent to 16 mmol/L assuming a stomach volume of 100mL) – concentrations which were shown in vitro to generate prasugrel-SNO in a pH dependent manner.

Of note, measuring RSNO is notoriously difficult and dependent on the technique used. Nevertheless, OBC is considered perhaps the most robust method for accurate analysis of NO metabolites and the set-up used in the WHRI is tried and tested, and well validated[299]. There has been much debate about the relevance of RSNO and the exact circulating concentration at baseline in plasma. Results of this study reveal baseline RSNO

concentrations consistent with those measured previously both by colleagues at the WHRI and other groups[138] (25+/-17 nmol/L), which is towards the lower end of what is measurable with OBC in plasma samples (~5-10 nmol/L). By employing a paired study design, it is shown for the first time that an acute and significant rise in plasma RSNO can be measured in vivo in patients.

It is acknowledged that the measurement is a composite of all RSNO species present in plasma at any one time as opposed to specific quantification of prasugrel-SNO. The latter was beyond the scope of this study and may be beyond the detection limits of complex analytical mass spectrometry. In a sub-cohort of plasma samples, the removal of protein components was found to result in a decrease in plasma RSNO in the aqueous compartment by >58%. This implies very strongly that RSNO elevation following prasugrel is primarily protein associated (whereas prasugrel and prasugrel-SNO is completely soluble in aqueous media). This is in keeping with previous work in vitro in which it has been demonstrated by our group that clopidogrel-SNO readily undergoes rapid and efficient trans-nitrosation with transfer of the NO moiety to albumin-SNO.

The fall in nitrate concentrations detected in patients following prasugrel treatment is surprising given that more than 70% of circulating plasma nitrate is dietary in origin. Patients were fasted and remained so following loading with prasugrel. The drop is potentially explained by the theory that RSNO and resulting nitrite could be formed preferentially over nitrate.

It is intriguing to postulate that this difference in thienopyridine-induced RSNO formation could possibly further explain differences in platelet inhibition and the clinical results seen with these agents. In particular, patients with endothelial dysfunction, a characteristic of conditions such as diabetes mellitus, are at increased cardiovascular risk largely due to the decrease in NO bio-availability may accrue particular clinical benefit. Given the novel discovery that prasugrel loading increases RSNO concentrations and plasma nitrite levels, it

would be tempting to speculate that its ability to undergo S-nitrosation could account for its potency in this patient subgroup. This needs further proof, but the case for investing further interest and resources into S-nitrosothiols as potential therapeutic agents for patients with cardiovascular disease is becoming more compelling. Dietary manipulation of stomach nitrite content via ingestion of beetroot concentrate or other products high in inorganic nitrate is also now well established and may also provide a new and complimentary route by which the action of current anti-platelet drugs could be modified.

4.5 Conclusion

Prasugrel induces an acute rise in plasma RSNO and nitrite following a loading dose in patients with coronary artery disease which is not maintained with chronic prasugrel treatment. Patients receiving PPI exhibited reduced RSNO formation and reduced platelet inhibition, confirming the potent antiplatelet effects of prasugrel-induced RSNO in vivo. This offers a new and alternative mechanism of action that contributes significantly to the potency of prasugrel.

The fact that prasugrel forms RSNO in vivo may not preclude it from exhibiting a dual effect on platelets – inhibition of $P2Y_{12}$ in parallel with delivery of SNO. Confirmation of a potential role of thienopyridine derived SNO in future therapeutics needs further evaluation and study to establish more direct correlation with platelet inhibition in clinical trials, but these results may go some way into demonstrating beneficial pleiotropic effects in patients.

CHAPTER SUMMARY

Given the previously demonstrated effects of clopidogrel treatment on plasma nitrite, the effect of treatment with prasugrel was assessed;

- *A statistically significant acute rise in plasma RSNO is seen in patients following acute prasugrel loading*

- *A statistically significant acute rise in plasma nitrite is seen in patients following acute prasugrel loading*

- *This rise in plasma RSNO and nitrite does not persist in patients on chronic prasugrel treatment*

- *The acute rise in plasma RSNO seen following acute prasugrel loading is seen irrespective of whether or not a concurrent PPI was being used*

- *The degree of platelet inhibition afforded by prasugrel (in response to ADP) was greater in patients not receiving PPI*

- *Patients receiving a PPI who were on chronic prasugrel treatment exhibited reduced RSNO formation, although this was not statistically significant*

5 Ticagrelor: In vitro nitrosothiols formation and modification of drug effect by acidification

5.1 Introduction

The use of dual anti-platelet therapy has played an integral role in the reduction of thrombotic complications following percutaneous coronary intervention (PCI)[385] and clopidogrel has proven morbidity and mortality benefit when given to patients presenting with acute coronary syndromes (ACS)[321]. Newer, more potent antiplatelet agents have been developed to overcome some of the perceived weaknesses of clopidogrel, already discussed, such as the large inter-patient variability, delayed onset of action and modest anti-platelet effect[363,364]. Ticagrelor has been tested in various ACS populations in the PLATO trial, exhibiting more potent anti-platelet effects, with more consequent bleeding side effects, but showing overall clinical superiority to clopidogrel in reducing death from vascular causes, myocardial infarction or stroke[266].

It has been shown in previous chapters and previously by our group that the thienopyridines, clopidogrel and prasugrel, expose a free thiol group (Figure 50) in their chemical structure and in the presence of nitrite (from saliva and the stomach) form nitrosothiol derivatives (Thienopyridine-SNO-(RSNO))[297,342]. These RSNO compounds exhibit typical nitrosothiol biochemistry; they can undergo transnitrosation allowing potential transportation via plasma proteins to their effector destination and potential localised delivery of nitric oxide (NO) within the circulation. RSNO compounds can inhibit platelets directly[217,386] and exert a vasomodulatory effect on the vascular tree[276,278], thereby offering a potentially synergistic antiplatelet effect in addition to the intended $P2Y_{12}$ receptor inhibition exhibited by thienopyridines.

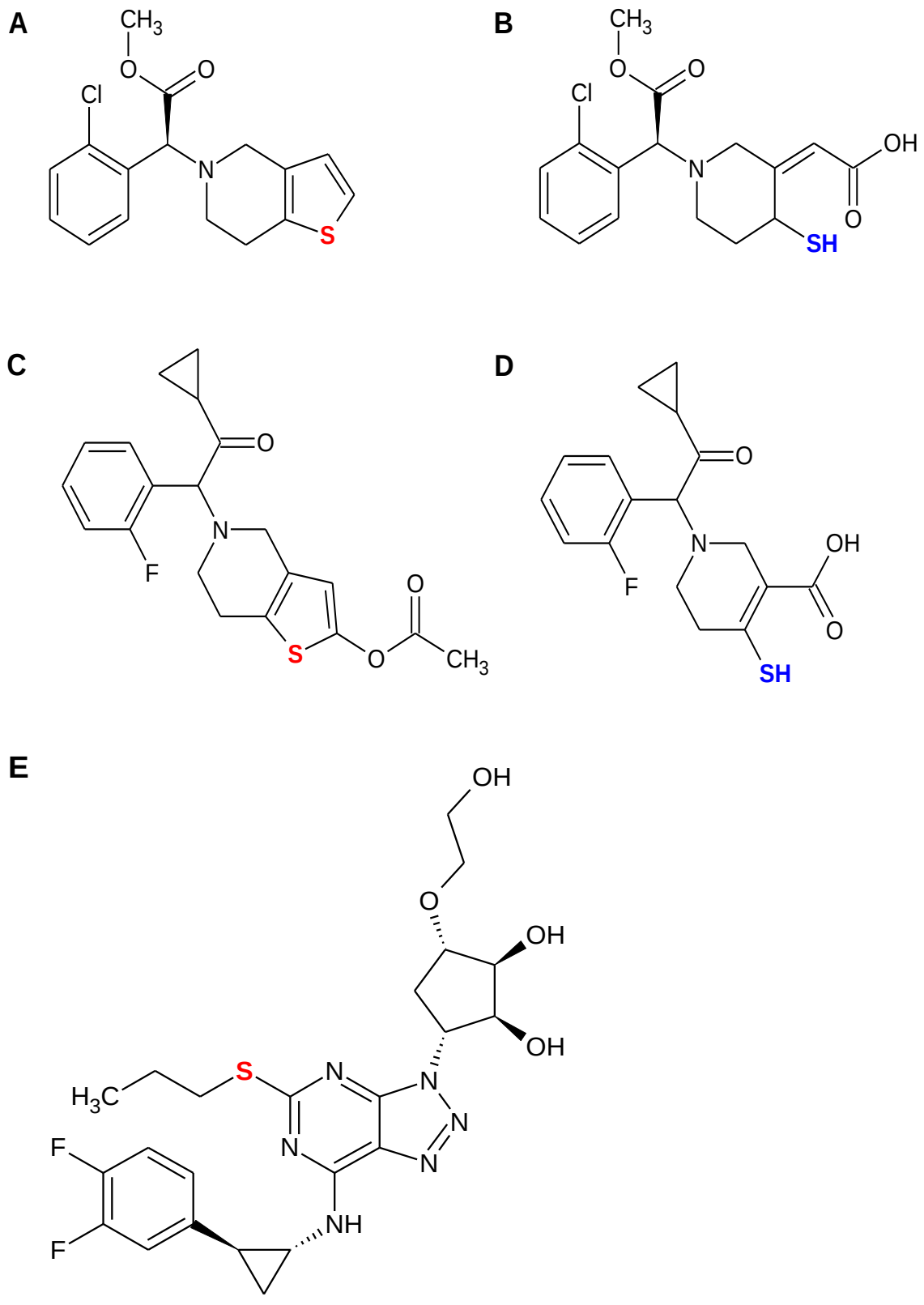

Figure 50: Chemical structures of;

A) Native clopidogrel. B) Clopidogrel active metabolite. C) Native prasugrel. D) Prasugrel active metabolite (*S group (red) shown in native thienopyridines, with exposed thiol group after bio-activation (blue highlighted)).*

E) Ticagrelor with S-group shown (No thiol group is exposed).

Given that ticagrelor lacks a free thiol group with the sulphydryl group contained within the ring structure of the molecule (as shown in Figure 50E) it is hypothesised that it will not have the ability to form an S-nitrosothiol compound. Unlike the thienopyridines, which irreversibly inhibit platelet aggregation by selectively decreasing binding of adenosine diphosphate (ADP) to its platelet receptor, it acts directly by changing the conformation of the $P2Y_{12}$ receptor[387]. This results in reversible, concentration dependent inhibition of the receptor[388]. More importantly, ticagrelor is quickly absorbed although its exact mechanism of absorption is unknown and does not require in vivo metabolism so has a rapid antiplatelet effect and onset of action within 30 minutes[389]. Median time to T_{MAX} is 3 hours (range 1 to 4 hours) for ticagrelor and 4 hours for the major (active) circulating metabolite AR-C124910XX, with a C_{MAX} of 931 ng/mL following a standard 180mg loading dose[390].

Interestingly, in the PLATO trial there was a significant attenuating effect of proton pump inhibitors (PPIs, which typically raise the gastric pH levels in patients to >5) on the mortality benefit seen with both clopidogrel and ticagrelor[391]. Given the previous experimental data on the effect of pH on thienopyridine induced RSNO formation[392], the following merited investigation;

Firstly, the potential effect of changing pH on native ticagrelor's ability to inhibit platelets, and to dissolve in gastric media. Secondly, whether ticagrelor, as previously demonstrated with the thienopyridines, could form ticagrelor induced RSNO when in an acidic milieu containing physiological levels of nitrite. Thirdly, whether ticagrelor could form ticagrelor induced RSNO in an artificial gastric environment using a simulated stomach media to allow exploration of the interplay between the stomach constituents, different nitrite concentrations, and ticagrelor on any resultant RSNO formation. Finally, and importantly from a clinical perspective, to examine whether any formed ticagrelor induced RSNO could exhibit or account for any of the antiplatelet effect of ticagrelor.

5.2 Methods

5.2.1 Preparation of Ticagrelor solution

Ticagrelor (Brilique™, Astra-Zeneca, London, UK) film-coated tablets containing 90mg ticagrelor were crushed individually and mixed with 17.2mls 0.9% sodium chloride to create a stock 10 mmol/L milky solution.

Pure ticagrelor was unavailable so crushed tablets were used which also contain the following excipients; mannitol (E421), calcium hydrogen phosphate dihydrate, magnesium stearate (E470b), sodium starch glycolate type A and hydroxypropyl-cellulose (E463) in the tablet core, and talc, titanium dioxide (E171), iron oxide yellow (E172), macrogol 400 and hypromellose (E464) in the tablet coating.

Whilst the effects of Brilique™ excipients on platelet function were not specifically assessed, the whole tablet was used for the purposes of this study in an attempt to more closely replicate in vivo use.

Furthermore, in initial experimentation, the waxy coating was scraped off prior to forming the solution but this inevitably led to loss of some of the drug and a tendency to inconsistent results. It was later appreciated that aggressive mixing meant that removal of the coating was unnecessary, allowing more realistic interpretation of real-world ticagrelor use.

5.2.2 The Effect of Lowering pH on the Activity of Ticagrelor

In order to specifically test the effect of lowering pH on the effectiveness of ticagrelor, 1mL aliquots of prepared ticagrelor suspension were adjusted to different pH (2–7) by adding 1M HCl and incubating for 10 minutes at 37°C. The mixture was then neutralised prior to adding to PRP and performing platelet inhibition testing with Multiplate®. No sodium nitrite was

added to these samples because the intention was to assess ticagrelor activity once acidified, without interference from concomitant production of ticagrelor induced-SNO.

5.2.3 Ticagrelor acidification and addition of nitrite

In vivo, ticagrelor would be exposed to intra-gastric nitrite, so using the 10 mmol/L stock solution, 1mL of ticagrelor was added to 1mL of sodium nitrite ($NaNO_2$) in a small brown bottle to protect from light and incubated in a water bath for 10 minutes at 37°C. The solution was then neutralised and immediate quantification of SNO content confirmed using ozone based chemiluminescense and the 2Cs analysis method as described in section 2.2.4. This experiment was repeated by adding 1M HCl to the ticagrelor/sodium nitrite mixture to create increasingly acidic (pH 2-7) solutions prior to incubation and subsequent neutralisation. 0.5 µL HCl increments were added to the ticagrelor solution and tested both with litmus paper and with formal pH testing to establish volume required to achieve desired acidity.

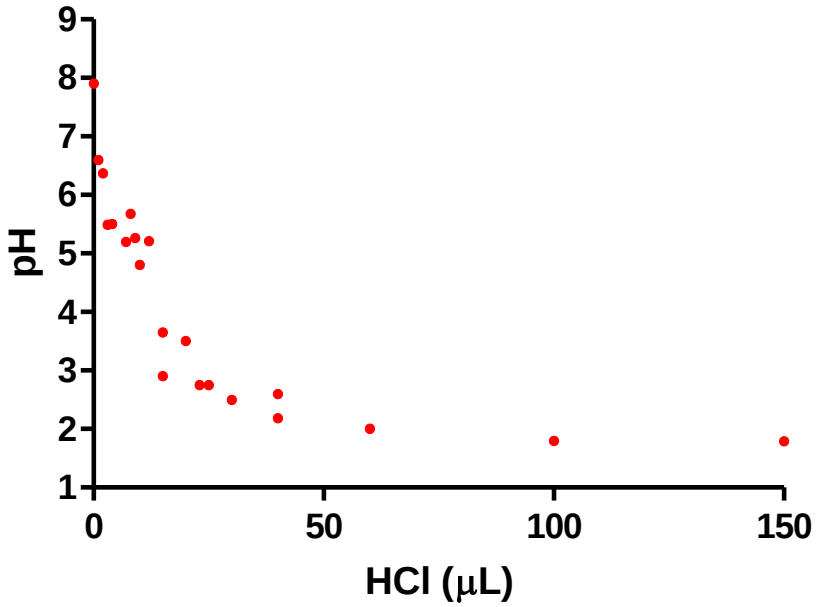

Figure 51: Increments of hydrochloric acid were added to the ticagrelor solution. Graph shows the volume of HCl required to achieve the desired pH of ticagrelor solution. Summarised below.

Desired pH	2	3	4	5	6
HCl volume added to Ticagrelor Solution	60 µL	22 µL	14 µL	10 µL	2.5 µL

5.2.4 Precautions regarding application of 2Cs for measurement of RSNO

The 2Cs method is well established as a means of specifically cleaving NO form SNO and is able to measure nanomolar quantities with a high degree of accuracy by OBC. However, when using very high concentrations of nitrite, it has been shown by our group at the WHRI[392] that a background NO signal can also be detected, attributable to nitrite itself. Therefore, to ensure that only RSNO derived NO signals were reported, appropriate nitrite controls were performed on a daily basis and respective areas under curve (AUC) were subtracted from the total AUC generated by drug-SNO.

5.2.5 Ticagrelor dose inhibition analysis

Reference ticagrelor dose inhibition was assessed by simply adding ticagrelor solution (after 10 minutes incubation at 37°C and neutralisation) to PRP or whole blood in a Multiplate® test cell. Ticagrelor induced RSNO was prepared by adding 1mL ticagrelor solution to 1mL nitrite of varying concentrations, acidifying to pH2, and then adding to PRP in a Multiplate® test cell, again after incubation and neutralisation. The ticagrelor solutions are used in lieu of the normal saline used in the standard test protocol.

5.2.6 Statistical Analysis

Comparison of ticagrelor effectiveness before and after acidification was performed using PRP from healthy volunteers. Multiple channels in the Multiplate® aggregometer allow simultaneous recording of platelet function tests, so the paired 2-tailed students *t*-test was applied. Concentration-responses to inhibitors of platelet aggregation were fitted by nonlinear regression curves and the respective doses producing 50 % platelet inhibition (IC_{50}).

The presented data show means with error bars representing standard error of the mean. A p value of <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism™ version 5 software. A normal distribution is assumed.

5.3 Results

5.3.1 Effect of acidifying ticagrelor

Crushed ticagrelor tablets were mixed with 0.9% normal saline in order to create a solution, the pH of which could then be adjusted. It was observed that the process of acidifying the solution with 1 M HCl, incubating at 37°C, and then neutralising with 1 M NaOH had a dramatic effect on the parent drug's ability to inhibit platelet aggregation when activated by the agonist ADP. Platelet function testing was performed on the prepared solutions both before and after the acidification process. Importantly, samples were only added to PRP in the Multiplate® aggregometer once neutral pH had been confirmed.

At neutral pH (and without exposure to acid conditions), addition of a 2.5 mmol/L preparation of ticagrelor in normal saline solution to PRP from healthy volunteers effectively inhibits platelet aggregation in response to ADP as would be expected. Preliminary experiments revealed that lowering the pH of the ticagrelor solution significantly reduced

effectiveness of inhibition, and at pH 3 or below, the ability of the parent drug to inhibit platelet aggregation is lost altogether as demonstrated in Figure 52.

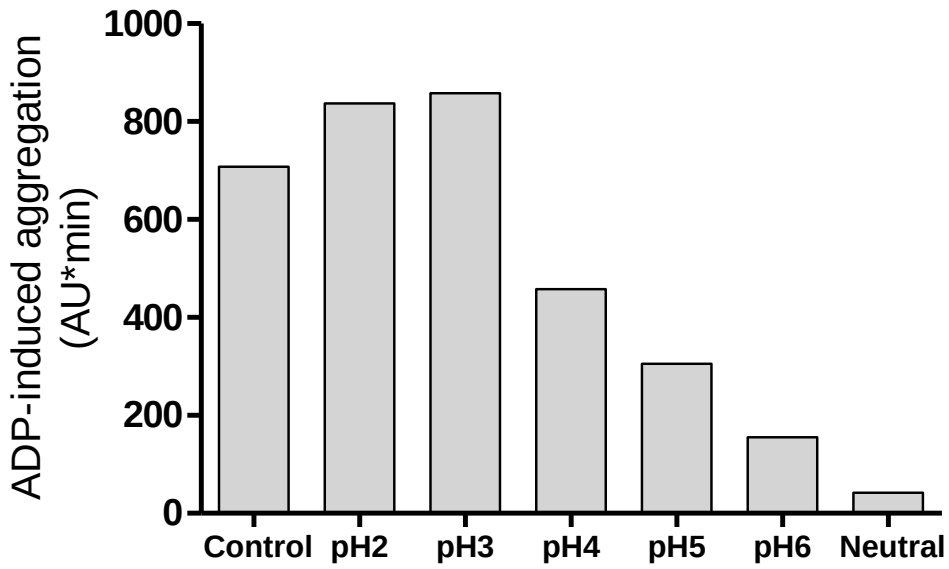

Figure 52: Graph showing the effect of increasing ticagrelor acidity on its ability to inhibit platelet aggregation as measured with Multiplate® multiple electrode aggregometer using a standard ADP agonist.

PRP from healthy volunteers was added separately to ticagrelor at neutral pH and ticagrelor that had been acidified for 10 minutes at pH 2 then returned to neutrality prior to use. Simultaneous test cells were then run using Multiplate® to assess response to ADP in nine paired experiments. This is shown in Figure 53 confirming that ticagrelor exposed to an acidic environment is unable to inhibit platelet aggregation via $P2Y_{12}$ receptor blockade in vitro.

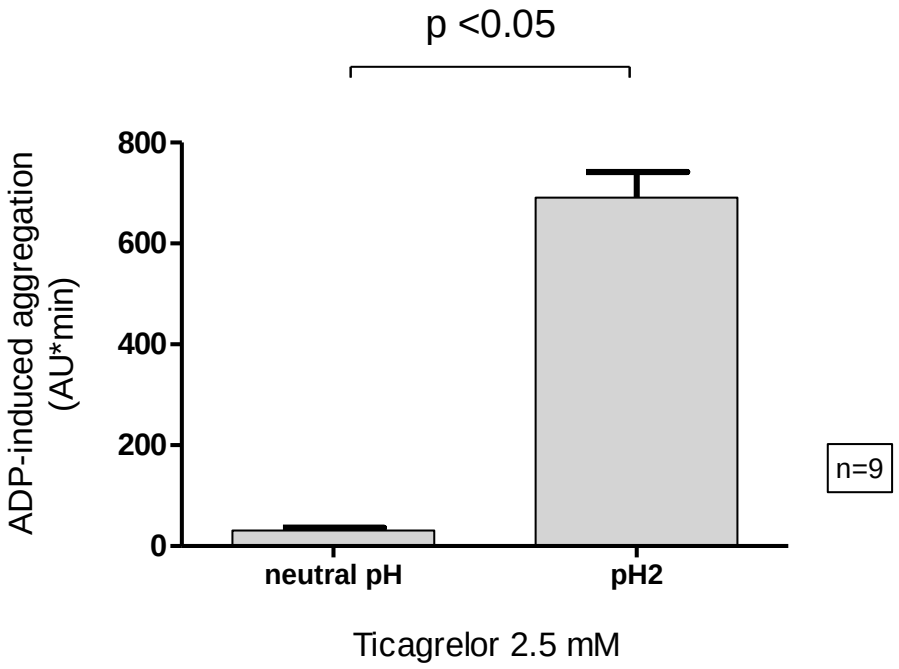

Figure 53: Graph showing the comparison of acidified ticagrelor vs ticagrelor at neutral pH on ADP response as measured with Multiplate®. (n=9, *** p<0.0001).

The exact mechanism of ticagrelor absorption in vivo is unknown. The marketed drug has a waxy coating but given the findings that the action of ticagrelor is impaired in an acidic environment, the effect of adding a standard 90mg tablet of ticagrelor to simulated gastric fluid was investigated.

The time required for firstly the coating and then the whole tablet to dissolve, were recorded at various induced pH as shown in the table xiv . Phosphate buffered solution was used as a control.

Simulated Gastric Fluid (pH)	Time for coating to dissolve (min:sec)	Time for whole tablet to dissolve (min:sec)
1.6	1:30	**2:35**
1.95	2:00	**3:50**
2.9	1:45	**3:00**
5.15	1:40	**3:25**
6.8	1:10	**3:00**
PBS control 7.6	**1:20**	**2:15**

Table xiv: Time, shown in minutes and seconds, taken for 90mg ticagrelor (Brilique™) tablet coating and then the whole tablet to dissolve at various induced pH when added to simulated gastric fluid.

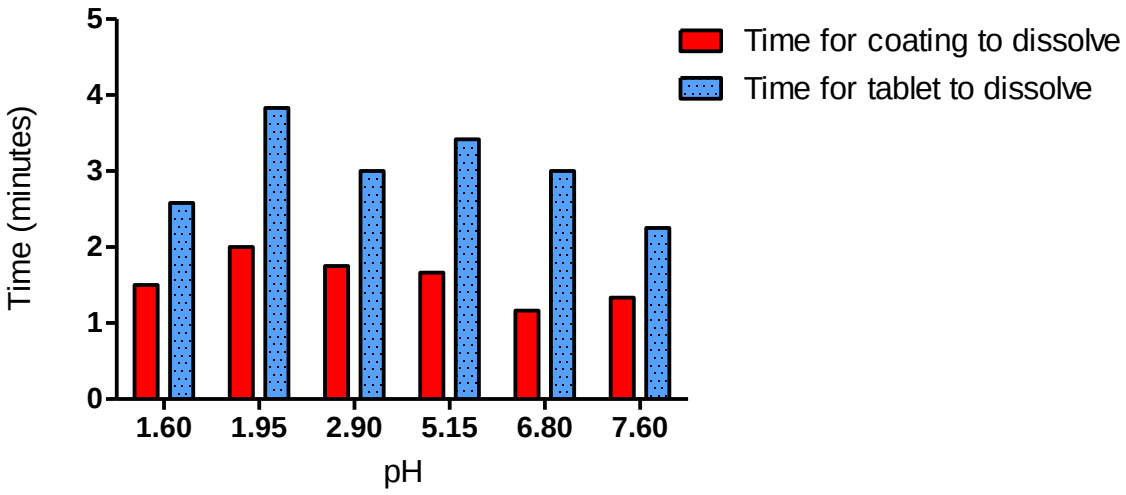

Figure 54: Time taken for 90mg ticagrelor tablet to dissolve at various pH when added to simulated gastric fluid.

No measurable difference is noted in time taken to dissolve and irrespective of pH, ticagrelor appears to dissolve completely within 4 minutes. Extrapolated to the in vivo patient scenario, this data implies that ticagrelor would be fully exposed to the acidic human stomach environment within the first 4 minutes of ingestion.

5.3.2 Ticagrelor transformation after acidification (in the presence of nitrite)

In order to assess whether ticagrelor could form nitrosothiols derivatives, 1mL of the stock 10 mmol/L ticagrelor solution was added to 1ml sodium nitrite and incubated at 37 °C for 10 minutes prior to neutralisation. Immediate analysis by OBC using the Cu^{+}/Cys (2Cs) method was then performed. This was also concurrently performed for control samples containing sodium nitrite only, and these results subtracted from the ticagrelor/nitrite mixture to ensure that displayed SNO quantities reflect production from ticagrelor alone. Following initial analysis at the drug's native pH of 7.4, experiments were repeated in an increasingly acidic environment to determine the effect of pH on nitrosothiols yield.

This confirmed that ticagrelor has the ability to form RSNO compounds in the presence of nitrite in vitro. Experiments were repeated four times with mean ticagrelor-SNO production and SEM shown in Figure 55 below where the ratio of drug to nitrite is 10 mmol/L to 1.

Ticagrelor forms RSNO efficiently as the pH drops below 3, and to a greater degree than RSNO formation found with the thienopyridines, ticlopidine, clopidogrel and prasugrel[297].

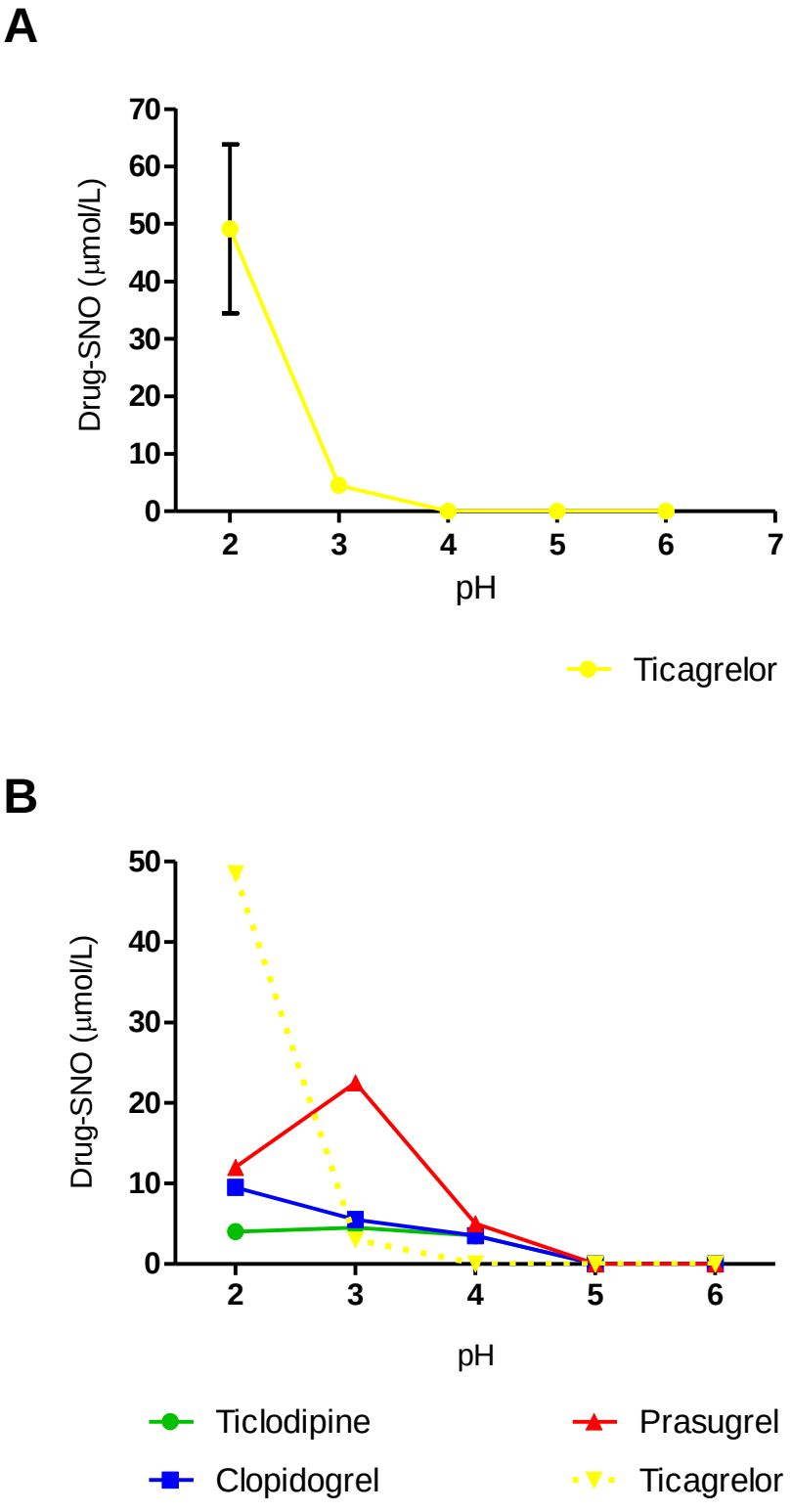

Figure 55: Drug-SNO is shown for ticagrelor relative to the thienopyridines at different forced pH (Figure 55B adapted with permission). 1mL of stock 10 mmol/L ticagrelor solution is added to 1ml sodium nitrite, and immediate analysis performed by OBC using the 2Cs method, with experiments repeated at different forced pH.

5.3.3 Ticagrelor induced RSNO formation in simulated gastric media

RSNO generation in a simulated gastric media both with and without ticagrelor was measured. This experiment was conducted by a colleague at the WHRI (Dr Fairoz Abdul, MBBS, MRCP) using the same gastric media as described above.

Adding nitrite, at varying concentrations, to gastric media resulted in the formation of RSNO from the endogenous proteins within the media (Figure 56). Furthermore, when ticagrelor was added to gastric media in addition to nitrite, RSNO production was augmented. Although this effect was only modest at physiological levels of nitrite, it was measurable at the low doses (Figure 57A), but occurred particularly at higher nitrite levels (Figure 57B shown in grey).

Increased RSNO formation in the ticagrelor group was significant at both 5000 µmol/L and 1000 µmol/L concentrations of nitrite. P values are shown below, applying the 2 way ANOVA and Bonferroni post-test analysis to assess significance between the ticagrelor and gastric media group and the gastric media group alone.

Nitrite (µmol/L)	P value
5000	**< 0.001**
1000	**< 0.05**
500	> 0.05 (ns)
50	> 0.05 (ns)
25	> 0.05 (ns)
12.5	> 0.05 (ns)

Figure 56: Comparison between the ticagrelor and gastric media group, and the gastric media group alone at decreasing nitrite concentrations. Grouped analysis was performed using 2way ANOVA, and Bonferroni post-test analysis to compare replicate means. N=5 for each concentration.

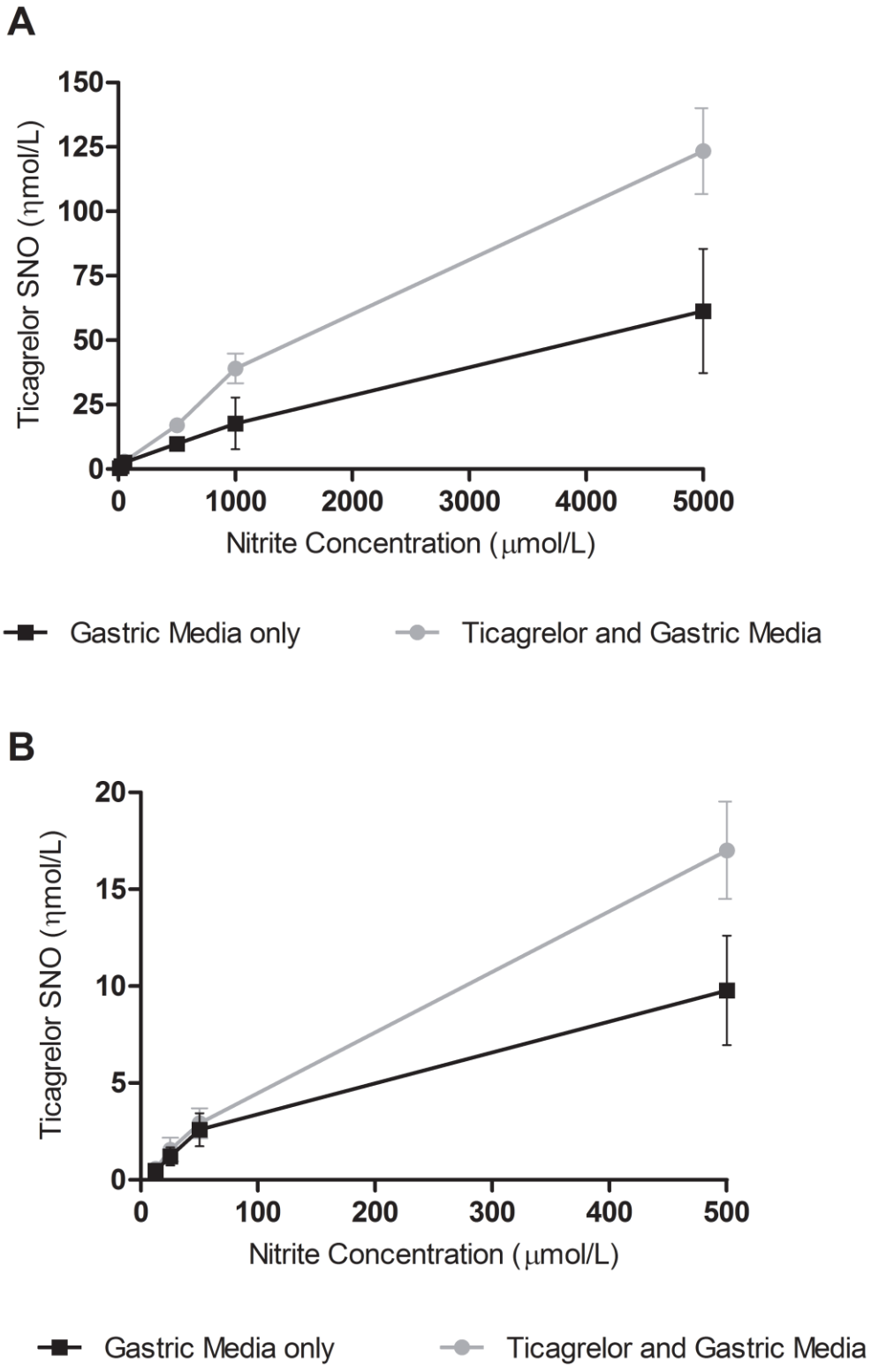

Figure 57: Graphs showing the quantity of drug-SNO generated in a simulated gastric media both with and without ticagrelor. SNO formation is plotted against high (57A) and low (57B) dose nitrite showing augmented RSNO production with drug in gastric media. (90mg ticagrelor dissolved in 30mls of gastric media).

5.3.4 Ticagrelor dose inhibition curves

A ticagrelor dose inhibition curve was created at neutral pH as a reference. Responses to ADP (6.5 µmol/L) for both PRP and whole blood were analysed as shown in Figure 58.

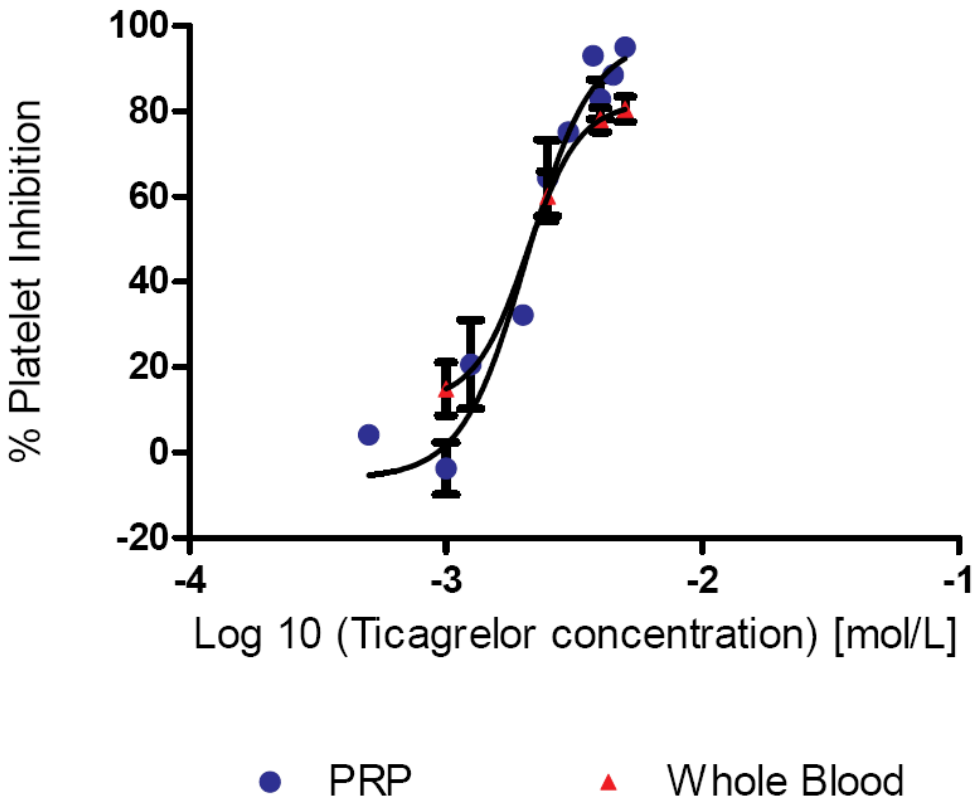

Figure 58: Ticagrelor dose-inhibition curve at neutral pH. Response to ADP agonist for both PRP and whole blood is plotted against log 10 ticagrelor concentration. Multiple whole blood and platelet rich plasma samples were taken from healthy volunteers. (n=15). A non-linear best fit log(inhibition) vs response curve is applied.

Platelet inhibition in response to ticagrelor-SNO (produced at pH 2) was also measured using PRP from healthy volunteers. Analysis was performed at neutral pH in response to both ADP and TRAP agonists using different SNO concentrations, as confirmed with the 2Cs measurement, to create dose inhibition curves as follows.

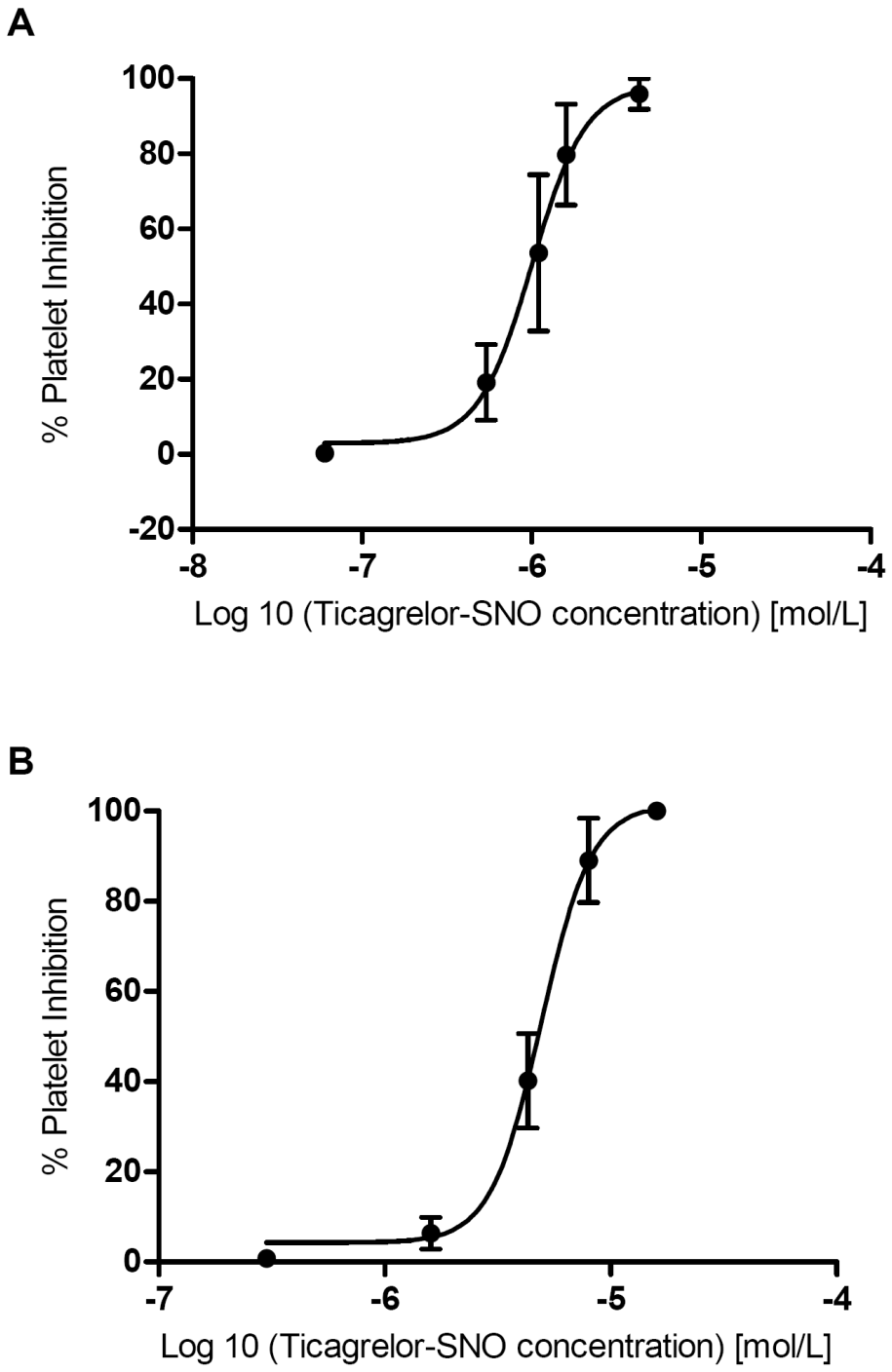

Figure 59: Graphs showing ticagrelor-SNO dose-inhibition curve (ticagrelor-SNO produced at pH 2) in response to A) ADP agonist, and B) TRAP agonist using platelet rich plasma from healthy volunteers. (n=15)

In an attempt to match physiological conditions as closely as possible, a final concentration of 5 mmol/L ticagrelor was added to a range of nitrite concentrations from 50 µmol/L to 1.25 mmol/L to produce ticagrelor-SNO. The ability to measure the effect of ticagrelor induced

RSNO on platelet reactivity in relative isolation is only possible due to the earlier discovery that the parent drug is no longer able to inhibit platelets once exposed to a highly acidic environment. The above Figures (59A and 59B) are therefore an accurate reflection of specific drug induced RSNO action on platelet rich plasma. They demonstrate the concentration of drug-SNO needed to fully inhibit platelets from healthy volunteers in vitro, using the standard manufacturer-recommended concentrations of ADP and TRAP.

This makes it possible to derive an IC_{50} for ticagrelor induced RSNO on platelet inhibition in response to ADP and TRAP of 1.0 µmol/L and 4.7 µmol/L, respectively. This difference in IC_{50} for the two agonists is not unexpected as they act via independent pathways and receptor. Various other determinants including patient factors will also influence the final result.

Importantly, the IC_{50} for ticagrelor induced RSNO is a magnitude lower than that required for native ticagrelor implying that relatively smaller amounts of ticagrelor-SNO are required to inhibit platelets compared to native ticagrelor.

5.4 Discussion

The most striking finding is that, in vitro, acidifying ticagrelor reduces its ability to inhibit platelet aggregation in response to either ADP or TRAP agonists, in platelet rich plasma. This poses a conundrum given that the pH of a fasting stomach is typically between 1 and 3 at which, in vitro, the inhibitory effect of native ticagrelor appears to be completely lost. Furthermore, with a gastric emptying half-life of 20-40 minutes in patients, ticagrelor will not be protected from this harsh environment by its waxy coating due to its rapid dissolution at any pH within 4 minutes, as shown in the studies using an artificial stomach medium.

Furthermore, in the in vitro experiments ticagrelor does readily form RSNO molecules (presumed ticagrelor-SNO) in a biochemical milieu that is analogous to the human stomach

i.e. following acidification (pH<3) in the presence of biologically relevant levels of nitrite. In addition, this RSNO compound is shown to be a potent inhibitor of platelet aggregation when compared with the parent drug ticagrelor. Extrapolating to the in vivo scenario this raises the intriguing possibility that whereas the anticipated mode of action of ticagrelor (via $P2Y_{12}$ inhibition) is lost following metabolism at pH<3, the drugs antiplatelet action is not only maintained, but potentially enhanced by the formed ticagrelor induced RSNO, which is known to be extremely potent. This suggests a completely novel mechanism for platelet inhibition which is dependent on low stomach pH. These findings may go some way to explaining the observation that proton pump inhibitors (via raising stomach pH) blunt the clinical effectiveness of this drug in the PLATO trial[391]. This is of course speculative as with higher pH, RSNO formation would be lost but the expected $P2Y_{12}$ inhibitory effect predominates.

It should be stated that the PLATO trial authors do suggest that the PPI effect is related to possible confounding, but there is conflicting evidence in the literature. The effect of PPI treatment on cardiovascular outcomes using a variety of different antiplatelet agents remains unclear and a causal link is difficult to establish.

The pharmacokinetics, metabolism and excretion of [^{14}C]ticagrelor have been investigated by other groups, and it has been revealed that ticagrelor has one active metabolite, AR-C124910XX which is at least as potent at the $P2Y_{12}$ receptor as the parent drug. In the proposed metabolic pathway for the formation and elimination of ticagrelor metabolites, the authors state that total recovery of the radiolabelled drug reached only 84.3%, the lower than expected result likely due to limitations of the procedure[393]. Notably, the radiolabel used by the research team was attached between the thiol and free methyl group, so the formation of an RSNO group as implied by our results could conceivably account for at least a proportion of the ‘lost’ radiolabel. This could potentially be investigated by expansion of their LC-MS/MS analysis technique.

This demonstrated ability to generate nitrosothiols is nevertheless an unexpected discovery because ticagrelor lacks a free thiol moiety. By implication, the formation of ticagrelor-derived RSNO is dependent on liberation of the thiol group from within its structure, and although the mechanism of this remains unclear, it is likely that it becomes available following the breakdown of ticagrelor in the acidic stomach environment. The in vitro model used utilises a very clean system with only ticagrelor in normal saline, thereby implying that any derived nitrosothiols formed on addition of nitrite must come from here.

It is important to highlight that physiological conditions ideally match those in the laboratory, and favour ticagrelor-induced RSNO production. Nitrite concentrations in the saliva and stomach are typically 20-210 µmol/L and 0.6-20 µmol/L respectively[127]. The in vitro experiments show that RSNO signals were generated when ticagrelor solutions were mixed with physiological nitrite concentrations (well below 50 µmol/L). However, elevated pH, very low stomach nitrite concentrations and dilution of ticagrelor will all result in an insufficient SNO yield to inhibit platelet aggregation. In the clinical setting stomach nitrite levels could be augmented with exogenous supplementation. In addition, taking ticagrelor on an empty stomach and avoidance of agents such as PPIs or H2 antagonists which will lead to elevation of the fasting stomach pH could reinforce these findings further. Furthermore, evidence already exists in humans demonstrating RSNO formation in stomach fluid aspirates, associated with anti-aggregatory and vascular modulatory effects after an oral nitrate load[394].

Once formed, the fate of ticagrelor-SNO in vivo is unclear. The tissue effects of all RSNOs are largely determined by their ability to release NO, although correlation between rate of NO release and potency of RSNOs is known to be poor[395]. Assuming a fasting stomach volume of 30mls, ingestion of a typical dose of 90mg ticagrelor would result in a gastric concentration of 6.9 mmol/L of ticagrelor, which in turn could yield RSNO as demonstrated in the studies. The data suggest an IC_{50} of approximately 1 µmol/L in platelet rich plasma, but it remains to be proven whether standard dosing of ticagrelor is sufficient to produce

physiologically relevant plasma levels of RSNO in vivo. Measuring RSNO is notoriously difficult and dependent on the technique used, and if the nitrosothiols group remained attached to the drug, quantifying accurate plasma concentrations would require complex analytical mass spectrometry. However, this seems unlikely as it has been shown previously that clopidogrel derived nitrosothiols can participate in transnitrosation reactions with bovine and human albumin with the potential to shuttle around the human circulation[297]. It has also been demonstrated that in patients following an oral dose of prasugrel, the rise in plasma RSNO measured is largely the result of protein based-SNO (albumin-SNO) which implies in-vivo transfer and circulatory stability. (also see section 4.3.2). The process of transnitrosation is well described and is typically a reversible second-order reaction between a nitrosothiol and a thiol, with high molecular weight SNO such as albumin-SNO representing a much more stable pool of NO than low molecular weight SNOs.

Nitrosothiols, and therefore potentially ticagrelor-SNO, can exhibit platelet anti-aggregatory properties similar to biologically occurring nitrosothiols like glutathione-SNO[297]. There are 3 potential antiplatelet activity targets. Firstly, downstream dampening of the $P2Y_{12}$ mediated activation pathway by activating soluble guanylate cyclase (sGC) causing inhibition of intracellular calcium flux[371]. Secondly, by acting as a source of NO, thrombin-induced platelet activation is decreased via direct inhibition of PI3K pathway activation by TRAP[396]. The third target involves nitrosation reactions in platelets, specifically protein tyrosine residues of the COX1 enzyme which inhibit the conversion of arachidonic acid to thromboxane-A_2[397]. Activation of platelet sGC to produce cyclic guanosine monophosphate (cGMP) causes a fall in intracytoplasmic calcium levels, which inhibits platelet shape change and glycoprotein IIb/IIIa expression, but how much platelet inhibition mediated by NO donor compounds is cyclic GMP-dependent and how much is via cyclic GMP-independent pathways remains unclear[398]. Nitrosovasodilation can also occur with nitrosothiols via NO (or more correctly, NO^+) donation which can induce relaxation of vascular smooth muscle, mediated via classic sGC signal transduction, a finding predominantly seen in vitro, but likely to occur in vivo also.

Vasodilation has been observed in patients as soon as 2 hrs after a loading dose of clopidogrel with an increase in NO bioavailability and effective vasodilation, as reflected by higher levels of plasma nitrite and cGMP[298,371].

5.4.1 Limitations

There are limitations to this work. It is well established that each of the major constituents of whole blood, plasma, platelets and red blood cells contribute towards clot formation, and will thus affect Multiplate® results. Only platelet rich plasma has been used to demonstrate effects on platelet aggregation, so although these results can be extrapolated to real world populations they need to be interpreted with appropriate caution. Furthermore, definitive proof of in vivo ticagrelor induced RSNO production remains to be established.

5.5 Conclusion

Ticagrelor loses capacity to inhibit platelet aggregation in response to the agonists ADP and thrombin after relatively brief exposure to acidic conditions in vitro. However, in the presence of even trace amounts of inorganic nitrite, ticagrelor readily formed RSNO resulting in potent platelet inhibition via a possible alternative mechanism to that of native ticagrelor. Furthermore, ticagrelor dissolves readily in gastric media within 4 minutes to form RSNO in vitro. This is an exciting and novel finding for this drug that may explain some of the putative pleiotropic effects of ticagrelor and its rapid onset of action. This could have implications in the search for potent anti-platelet agents without the inherent bleeding associated with $P2Y_{12}$ receptor inhibition, and potentially with the benefits associated with a drug capable of acting as an NO donor.

CHAPTER SUMMARY

Ticagrelor does not contain a free thiol group so would not be expected to be able to form nitrosothiols derivatives. Ticagrelor acidification and addition of nitrite was investigated.

- *Ticagrelor is unable to inhibit platelet aggregation in response to ADP and thrombin following exposure to acidic conditions in vitro*

- *Ticagrelor does readily form RSNO when exposed to nitrite and an acidic environment*

- *Ticagrelor dissolves readily in simulated gastric media to form RSNO*

- *Ticagrelor induced RSNO leads to profound platelet inhibition in vitro*

6 GENERAL DISCUSSION

Cardiovascular disease is the leading cause of death globally, and accounted for 31.5% of all deaths in 2013[399]. The progression of atherosclerosis, the major precursor to cardiovascular disease, can be delayed by aggressive control of modifiable risk factors, but antiplatelet drugs currently form the basis of treatment for atherosclerosis and prevention of atherothrombosis. Over recent years, the hunt for the 'perfect' antiplatelet drug which protects against ischaemic events without increasing bleeding risk has led to rapid expansion of the use of thienopyridines, and more recently, non-thienopyridines including ticagrelor and cangrelor for short and long term use[400,401].

Varying approaches exist when it comes to prescribing these ubiquitous drugs because of their relative novelty and a new and expanding evidence base, so given what is at stake a complete understanding of the action of these drugs is paramount. It is clear that all three of the most commonly prescribed agents ticagrelor, prasugrel and clopidogrel exert effects beyond those predicted at the level of the platelet receptor. A variety of pleiotropic effects have been demonstrated, and specific work carried out at the Wales Heart Research Institute has revealed both the ability of parent thienopyridines to form vasoactive nitrosothiols without prior metabolism, and also to enhance plasma NO species in patients following loading with clopidogrel.

These exciting initial findings prompted the work outlined in this thesis, and further novel discoveries have followed, specifically related to the ability of clopidogrel, prasugrel and ticagrelor to form vasoactive nitrosothiol derivatives both in vitro and in vivo. Additional work has focussed on how the co-administration of other commonly prescribed drugs effects the nitrosothiols yield from these anti-platelet drugs.

These findings are summarised as follows;

1) The co-administration of proton pump inhibitors with chronic clopidogrel therapy in patients with established coronary artery disease had no adverse effect on measured plasma NO metabolites or platelet function testing.

2) Treatment with organic nitrates did not augment the NO metabolites profile of patients taking regular clopidogrel.

3) Prasugrel induces an acute rise in plasma RSNO and nitrite following a loading dose when administered to patients with coronary artery disease.

4) Patients treated with prasugrel in the acute setting (large loading dose) who were also receiving a proton pump inhibitor exhibited reduced plasma RSNO formation and reduced platelet inhibition.

5) Exposure of ticagrelor to acidic conditions in vitro causes it to lose its capacity to inhibit platelet aggregation in response to the agonists ADP and thrombin.

6) In the presence of even trace amounts of inorganic nitrite, ticagrelor readily formed RSNO resulting in potent platelet inhibition via a possible alternative mechanism to that of native ticagrelor.

The novel finding that the critical thiol group within thienopyridine drugs, that by design is more usually associated with binding to and inhibiting the platelet $P2Y_{12}$ receptor, can in fact be a source of nitrosothiols formation under physiological conditions even without prior metabolism in vitro was discovered by Bundhoo et al[297] at the Wales Heart Research Institute. This early discovery has now been expanded in this work, and although plasma nitrosothiol levels did not increase in patients loaded with clopidogrel, a statistically significant rise was seen in patients loaded with prasugrel. Plasma nitrite levels were previously shown to rise following long term clopidogrel use in patients with coronary artery disease[298] and the current study confirms elevation of plasma nitrite levels following acute loading with prasugrel, a finding that is considered reflective of enhanced systemic $NO^{\cdot}$ availability either through enhanced production or increased RSNO breakdown.

Furthermore, ticagrelor, which does not require bio-activation in patients was also shown to form nitrosothiols derivatives in the presence of acid and trace amounts of nitrite in vitro although confirmation of this effect in vivo is still awaited.

The excitement surrounding the discovery of an agent that can generate nitrosothiols exists because of its potential to act as a nitric oxide donor. Loss of endogenous NO production is integral to endothelial dysfunction and the search for carrier agents able to transport and release NO as required throughout the circulation still continues.

Nitric oxide itself plays a vital role in maintaining normal vascular function, and since the early work carried out over three decades ago by Furchgott et al into establishing the identity of endothelium derived relaxing factor, our knowledge of the complex mechanisms of action and important biological properties of this signalling molecule have developed vastly.

NO formed by vascular endothelium diffuses rapidly into the blood where it binds to haemoglobin, and into vascular smooth muscle cells where it binds to and activates guanylyl cyclase which catalyses dephosphorylation of GTP to cGMP. The resultant vascular effects of NO are extensive, most importantly;

- direct vasodilation
- indirect vasodilation by inhibition of vasoconstrictor responses
- anti-thrombotic effects through inhibition of platelet adhesion to the vascular endothelium
- anti-inflammatory effect by inhibiting leukocyte adhesion,
- anti-proliferative effect through inhibition of smooth muscle hyperplasia

Reduced bioavailability of NO therefore results in vasoconstriction, thrombosis, inflammation, and vascular hypertrophy, so a drug capable of delivering exogenous nitric oxide has clear therapeutic benefit. Furthermore, amongst the various NO donors discovered to date, nitrosothiols have shown perhaps the greatest potential[210].

Nitrosothiols are naturally occurring and produced by the S-nitrosation of sulphydryl groups, typically cysteine thiols, with the ability to transfer NO^+ species between different thiol groups, a trait which protects NO from oxidative stress prior to release.

The formation of nitrosothiols highlighted in this study through the use of the critical thiol group within the thienopyridines and non-thienopyridines is reliant on the acidic gastric environment, and therefore interplay in this environment between the antiplatelet drugs and proteins, other stomach constituents and co-administered drugs is likely to be important. The reliance on low pH and availability of nitrite mean that PPI use and exogenous nitrate/nitrite use need to be considered as both are likely to affect nitrosothiols yield.

In fact, the use of proton pump inhibitors in general, and particularly their administration to patients with cardiovascular disease continues to generate a lot of interest both amongst clinicians and the lay press. Although CYP2C19*2 carrier status is probably the most notable independent predictor of insufficient antiplatelet response to clopidogrel, it is certainly not the sole predictor and there are likely to be numerous, some as yet undiscovered, factors contributing to this reduced response. Furthermore, it is likely that these factors are additive

so likelihood of drug non-responsiveness will increase depending on the total number and combination of these, the antiplatelet drug dose and the choice of PPI agent.

The case against PPIs, and their link with adverse outcomes in patients with cardiovascular disease has predominantly revolved around the association with cytochrome P450 genetic polymorphisms as discussed, but amongst the numerous postulated causes, recent groups have suggested a direct link between PPI use and endothelial dysfunction through elevation of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, in a murine model[402]. However, it should be noted that the same group have more recently gone on to perform a human prospective cross-over pilot study[403], and although not completely dispelling their earlier theory, there was no statistically significant difference in plasma ADMA levels.

The argument that nitrosothiols yield is reduced by concurrent PPI use is a compelling one, particularly given the sound physiological explanation, and certainly the results in this study have shown reduced platelet aggregation in patients loaded with prasugrel who are taking a concomitant PPI as compared to those not on PPI therapy. No changes were noticed in the clopidogrel-treated patients, but this can be explained by the reduced potency of clopidogrel and therefore reduced available thiol for nitrosothiols formation.

In fact, a recent study conducted in two-kidney, one-clip hypertensive rats treated with omeprazole showed that orally administered nitrite lowered blood pressure and increased plasma S-nitrosothiol concentrations independently of circulating nitrite levels. Furthermore, increasing gastric pH secondary to omeprazole treatment did not affect plasma nitrite or nitrate concentrations but was noted to severely attenuate the increases in plasma S-nitrosothiol. This completely blunted the antihypertensive effects of nitrite, and further reinforces this credible concern that concomitant PPI use can also limit the effectiveness of antiplatelet therapy assuming the role of thienopyridine-induced nitrosothiols. The authors go on to state that co-administration of buthionine sulfoximine, which induces partial thiol

depletion, attenuated the increases in S-nitrosothiol concentrations and antihypertensive effects of oral nitrite. They conclude that gastric S-nitrosothiols formation drives the antihypertensive effects of oral nitrite and nitrates[404].

This emphasises the need for further studies to tease out the exact risks of prescribing PPIs to patients with cardiovascular disease, and given all the permutations with regards choice of antiplatelet drug, genetic variance, concomitant drug related interactions and other potential predictors of non-responsiveness, these will need to be large scale and well conducted.

Whilst the regular use of PPIs makes the gastric environment less conducive to formation of nitrosothiols derivatives, increased nitrite availability should enable increased production. The other facet of this study was to further assess this interaction, but to date, this project has shown no conclusive proof that organic nitrate taken regularly by patients with coronary artery disease has any significant impact on NO metabolites.

The role of organic nitrates is well established in cardiovascular disease, but the use of inorganic nitrates lost favour due to concerns over potential carcinogenic effects. However, there is already ample evidence to suggest that conversion of inorganic nitrate into nitrite in the gastro-intestinal tract can acutely elevate vascular nitric oxide levels with the potential to result in clear therapeutic benefit. Differences in chemistry between the two classes mean their pharmacokinetics and pharmacodynamics differ, and this has re-ignited interest in inorganic nitrates due to their vascular benefits and potential antiplatelet properties. However, questions remain unanswered as to whether these agents could augment intra-gastric nitrite sufficiently to result in therapeutic plasma levels of clopidogrel-derived nitrosothiols.

Although many effects of nitrite and nitrate are attributed to increased circulating concentrations of nitrite and its ultimate conversion to NO, nitrite is known to generate nitrosating species at low pH promoting S-nitrosothiols formation in the stomach and there is no doubt that further studies are required to investigate this intriguing interaction within the

gastric milieu to establish the perfect “recipe” for nitrosothiols generation. Diets rich in fruit and vegetables reduce blood pressure and the risk of ischaemic heart disease and ischaemic stroke, thought to be attributable at least in part to the presence of inorganic nitrate. This beneficial effect is thought to result from the formation of nitric oxide and has led to various manufacturers producing and promoting “sports” sachets which contain relatively large quantities of inorganic nitrate with the promise that they will “provide an innovative way to enhance endurance performance”. Indeed, multiple studies support nitrate supplementation as an effective method to improve exercise performance[405].

More interestingly, it would be prudent to establish whether these gels could enhance the NO profile of patients with cardiovascular disease, and in particular whether they could sufficiently increase intra-gastric nitrite levels to enhance thienopyridine and non-thienopyridine derived nitrosothiols.

6.1 Future Directions

The nitrosylation properties of the thienopyridines have been explored but the effects of ticagrelor and cangrelor in vivo remain to be investigated. If the in-vitro effects of ticagrelor demonstrated in this work regarding augmentation of plasma SNO and nitrite through generation of nitrosothiols derivatives were effected in vivo this could explain some of the interesting off-target findings in the PLATO trial and potentially open the door to a new understanding about the effects of this widely prescribed anti-platelet agent.

Expansion of its therapeutic application through the dual mechanism of both $P2Y_{12}$ inhibition and NO donation through nitrosothiols formation could lead the way to creation of new and very powerful drugs for the treatment of coronary artery disease and all forms of cardiovascular disease. Therefore, measurement of NO metabolites in patients loaded with, and taking chronic ticagrelor would be the obvious target for future investigation.

Furthermore, further clarity is required regarding the thienopyridines' and non-thienopyridines' exact effect on NO profile and this would require multiple sampling points following drug loading to include not only post-loading and chronic treatment, but plasma nitrite/nitrate/SNO following drug discontinuation as well to establish the longevity of any beneficial effects of enhancement of plasma NO metabolites.

The findings in the pilot study highlight the need for further investigation into the effects of inorganic nitrate, and provided there is favourable ethical approval, the observed response to these gels in a population of patients with coronary artery disease would yield potentially exciting results. In addition, the interactions between anti-platelet drugs and, not only nitrates and PPIs, but other commonly prescribed drugs including calcium channel blockers and statins amongst others should also be the focus of future work.

The vasomodulatory effects of increased plasma nitrosothiols formation should be quantified and even simple measures such as forearm plethysmography and arterial waveform recording using pulsed wave analysis could be employed. The vasomodulatory effects of antiplatelet agents on coronary flow are more difficult to assess but this would remain the gold standard when trying to evaluate the benefits of these drugs in patients with coronary artery disease if safe and reliable techniques for assessing this could be established.

Further studies should be appropriately tailored to answer the pertinent questions that remain about the three main antiplatelet agents currently in clinical use.

Regarding clopidogrel use, we know that a large proportion of patients are non-responders. It has been shown that all clopidogrel salts can form RSNO derivatives with anti-aggregatory and vasomodulatory properties when in an acidic milieu in vitro, and in vivo increases in circulatory nitrite are seen with acute and chronic clopidogrel treatment. Although this effect is weak, could it be enhanced by the co-administration of inorganic nitrates, particularly in the cohort of patients who are poor responders? A simple crossover study using SIS® sports

supplement gels to increase inorganic nitrate availability in patients on clopidogrel treatment could satisfactorily answer this.

In view of the known problems with clopidogrel use, and the general trend for using the newer antiplatelet agents, focus should perhaps turn to prasugrel and in particular, ticagrelor. Prasugrel is a much more potent anti-platelet agent, so augmenting its anti-platelet effect further may not be beneficial. However, the elevated plasma RSNO and nitrite levels seen in these patients may be relevant. Recent analysis of the TRILOGY ACS trial, specifically focusing on the ACS cohort who were managed without revascularisation, and randomised to clopidogrel or prasugrel and also receiving a PPI, has shown a decreased MI rate in the prasugrel arm [406]. Whether or not this difference can be attributable to differing plasma RSNO production correlating to altered stomach pH is unknown. However, it would be intriguing to analyse the gastric contents of patients loaded with clopidogrel and prasugrel to quantify RSNO levels. This would require passage of a nasogastric tube to allow aspiration of stomach contents, and although difficult, a pilot study could be considered in those patients already intubated requiring administration of antiplatelet drugs.

Ticagrelor is still a relatively new drug compared to the thienopyridines and the pleiotropic effects of its use in patients with coronary artery disease remain unknown so warrant further evaluation. Interestingly, the recent PEGASUS study suggested that treatment with ticagrelor significantly reduced the risk of cardiovascular death, myocardial infarction, and stroke when used in patients who had suffered a myocardial infarction more than 1 year previously[267]. It remains unclear why a $P2Y_{12}$ inhibitor would confer such long term benefits, and further justifies investigation into whether there are additional actions that perhaps enhance endothelial function. Proof or otherwise about the ability of ticagrelor to form plasma nitrosothiols derivatives in vivo could be established with an extension of this study.

It remains an exciting time for antiplatelet agents as interest in finding the perfect drug shows little sign of waning.

6.2 Conclusion

The pleiotropic effects of antiplatelet drugs have been a focus of interest for several years now. However, a safe drug which inhibits unwanted platelet aggregation whilst also acting as a nitric oxide donor and at the same time minimising bleeding risk could be considered the Holy Grail for the therapeutic management of patients with cardiovascular disease and particularly those with coronary artery disease and acute coronary syndromes.

The findings in this study add to our knowledge about the current generation of anti-platelet drugs clopidogrel, prasugrel and ticagrelor. Specifically, the exciting novel discovery that prasugrel augments plasma nitrosothiols levels in vivo, and ticagrelor readily forms RSNO that results in potent platelet inhibition in vitro complements our recent discovery that clopidogrel also enhances NO species in patients and effects blood vessels with enhancement of the effectiveness of NO donors.

This novel mechanism of action could lead to the production of a newer generation of antiplatelet drugs resulting in improved outcomes for a huge number of patients suffering from coronary artery disease.

Appendix I – Ethical Approval Letter

Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
Yn rhan o seilwaith ymchwil Cymru a ariannir gan y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd, Llywodraeth Cymru

NISCHR
Gwasanaeth Moeseg Ymchwil | RES | Research Ethics Service

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09 October 2012

Dr Laurence V Thornhill
Clinical Research Fellow
Cardiff and Vale University Health Board
9 Park Avenue
Bath BA2 4QD

Dear Dr Thornhill

Study title:	**Effect of Thienopyridines and Non-thienopyridines on Endothelial Dysfunction and NO metabolites in patients with stable angina undergoing percutaneous coronary intervention.**
REC reference:	**12/WA/0290**
Protocol number:	**3.0**

Thank you for your letter of 25 September 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman, Dr. Gordon Taylor.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites
The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion
The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter	signed Dr Thornhill	
Investigator CV	Philip James; dated August 2012	
Investigator CV	Dr Laurence Thornhill	29 August 2012
Letter from Sponsor	signed Professor Jonathan I Bisson, Cardiff and Vale Research Review Service	17 August 2012
Other: Patient flow charts : groups	Group 1 - acute loading group - 105 subjects; version 2	11 July 2012
Other: Patient flow charts : groups	Group 2 - chronic use group - 320 subjects; version 2	11 July 2012
Other: Patient Flow Charts : Overview	2	11 July 2012
Participant Consent Form	2	24 September 2012
Participant Information Sheet	2	24 September 2012
Protocol	4.0	24 September 2012
REC application	signed electronically by Dr. Thornhill; electronically by Mrs. Lee Hathaway, sponsor's representative; and electronically by Dr. Philip James, academic supervisor	30 August 2012
Response to Request for Further Information	Email from Dr. Thornhill	25 September 2012

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements
The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

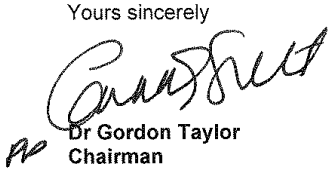
Feedback
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/WA/0290	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



pp **Dr Gordon Taylor**
Chairman

Email: corinne.scott@wales.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Prof Julian Halcox, Cardiff and Vale University Health Board*

Appendix II – Patient Information Sheet

PATIENT INFORMATION LEAFLET

1. Study Title

Effects of Anti-platelet Drugs on Endothelial Dysfunction

2. What is the purpose of the study?

The overall purpose of the study is to see if the anti-platelet drugs clopidogrel, prasugrel and ticagrelor, drugs which are used to thin the blood of patients who are undergoing coronary stenting or those who are at risk of having coronary disease and heart attacks, have an additional benefit on the blood vessel wall.

3. Why have I been chosen?

You have been chose because you will be undergoing a procedure called coronary stenting, where you will be given one of the drugs clopidogrel, prasugrel or ticagrelor before the procedure. In order to carry out the procedure safely, you need to have your blood thinned by taking one of the drugs clopidogrel, prasugrel or ticagrelor about 2 hours before the procedure. This is a standard form of treatment given to all patients who undergo coronary stenting.

Patients undergoing coronary stenting who are already taking one of these three drugs may also be invited to take part in the study.

4. Do I have to take part?

Your participation in this study is entirely voluntary. You can decline to take part or withdraw at any time without explanation.

5. What will happen to me if I take part?

We will fully explain the procedure and ask you to sign a consent form. The study will take place at the Cardiac Day Case Unit, University Hospital of Wales, Cardiff. You will be given one of the drugs, clopidogrel, prasugrel or ticagrelor in the form of tablets to swallow about 2 hours before you have your stenting procedure. Before giving you the drug, we will take a blood sample from a vein. After 2 hours, we shall take another blood sample from your vein through the same drip needle.

If you are already taking one of the drugs clopidogrel, prasugrel, or ticagrelor, we only require one blood sample from your vein before your stenting procedure.

You may also be invited to re-attend the hospital once you have finished the full course of drug treatment. This will usually be between 1 and 12 months after your coronary stenting procedure. All that will be required at this final visit is one further blood sample from a vein. If needed, travel expenses can be provided for you for this follow-up visit.

6. What do I have to do?

Once you have read this form and had time to think about the study, you will be contacted by Dr James's research team. If you agree to participate then you will be asked to sign a consent form. The study involves taking blood samples, before and after you have taken the drug, from a **single** drip needle (a tiny piece of plastic that sits in the vein) that will have already been placed into the vein of your arm for the purpose of your procedure. It avoids the need to puncture the vein multiple times.

7. What are the drugs that are being tested?

Patients who have coronary disease or diabetes are prone to have poor function of the endothelium. The endothelium is a lining of special cells that cover all the inner layer of all the arteries (blood vessels carrying oxygen). Their function is to keep the arteries healthy and allow blood to flow to all of the organs. Clopidogrel, prasugrel and ticagrelor are similar drugs that keep the blood thin, make the blood less sticky and prevent the formation of blood clots. They are widely used in patients who have had heart attacks or diseased coronary arteries as well as in patients who undergo coronary stenting. We are however testing whether the drugs have additional beneficial effects on the endothelium apart from their known function to keep the blood thin.

8. What are the side effects of taking part?

Before your doctor decides to perform the coronary stenting procedure, (s)he will check whether you would be suitable to take clopidogrel, prasugrel, or ticagrelor. It is a vital requirement of your procedure that you take these drugs regularly; side effects from the drugs are rare. It is possible you may have some bruising to your forearm after the drip needle has been removed at the end of the study, or after a simple blood sample is taken when you re-attend after stopping the drug. There are no direct side effects or consequences related to your taking part in this study.

9. How much blood would be taken for the study?

The total amount of blood required for each sample will be about a quarter of an eggcup full (15mls). Most patients will require two blood samples on the day of the procedure, unless you are already taking one of the drugs clopidogrel, prasugrel or ticagrelor, in which case only one blood sample is necessary. If you are invited to re-attend on another day once you have stopped your drug, one further blood sample will be needed.

10. What are the possible benefits of taking part?

There is no benefit to you, but by measuring any biologically active chemicals, we may be better able to understand people with diseased arteries. This study does not affect your treatment in any way.

11. What happens when the research study stops?

You may be asked to re-attend for one further blood sample once you have stopped taking the drug clopidogrel, prasugrel or ticagrelor. You will not be asked to attend any additional follow up visits for the purpose of the study.

12. What if something goes wrong?

This study is being sponsored by the University Hospital of Wales. Therefore if you suffer negligent harm as a result of participation in the study you will be covered by the NHS indemnity scheme.

13. Will my taking part in this study be kept confidential?

Dr James, Dr Anderson, Dr Thornhill and their study personnel will collect information about you. This will remain confidential. This data will be kept in a secure office at the Wales Heart Research Institute. Anonymity will be maintained throughout the trial.

14. What will happen to the results of the research study?

The data from this study may be used in publications. However, your name will not appear in the publications.

15. Who is organising and funding the research?

The study has been funded by the Cardiff and Vale University Health Board. It has been organised jointly with the Wales Heart Research Institute, Cardiff University.

16. Who has reviewed the study?

The study has been reviewed by the Research and Development Office at Cardiff and Vale University Health Board, and the Research Ethics Committee for Wales.

17. Where can I obtain independent information about being involved in a research study?

You can contact Dr Tim Kinnaird (Consultant Cardiologist) who is a colleague at the University Hospital of Wales but is not involved with this study. He is extremely experienced in patient participation in research and clinical trials.

Dr Tim Kinnaird, Department of Cardiology,

Wales Heart Research Institute, Cardiff,

CF14 4XN

029 2074 7747

18. Contact for further information.

If you or your relatives have any questions about the study, please call Dr Laurence Thornhill 029 2074 4192, email laurencethornhill@yahoo.com or write to:

Dr. Laurence Thornhill

Clinical Research Fellow in Cardiology

Wales Heart Research Institute, Heath Park, Cardiff, CF14 4XN

Appendix III – Patient Consent Form

PATIENT CONSENT FORM

Patient Identification Number for this trial:

Effects of Anti-platelet Drugs on Endothelial Dysfunction

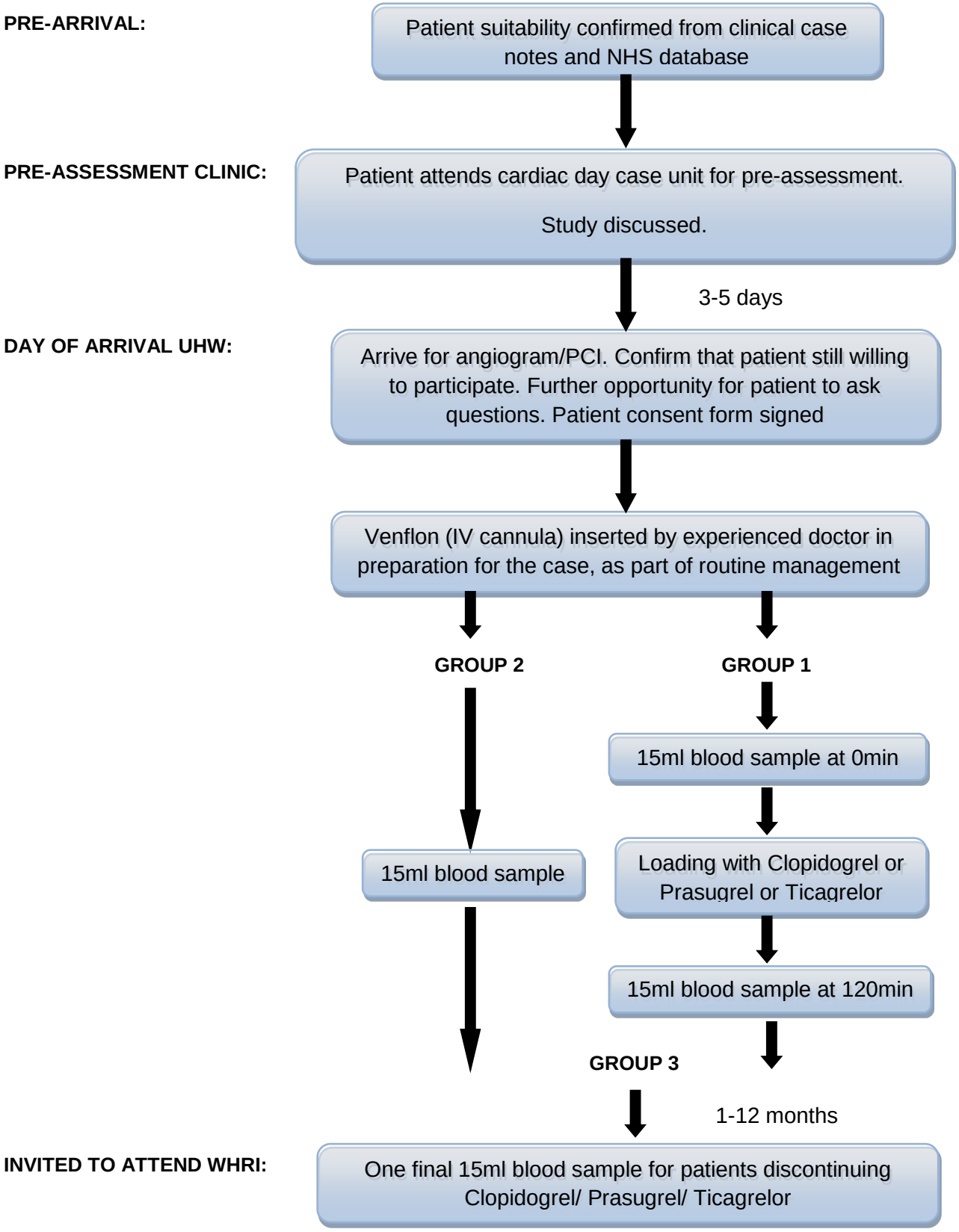
Name of researchers: Dr Philip James, Dr Richard Anderson, Dr Laurence Thornhill

Please initial each box

1.	I confirm that I have read and understood the information sheet dated 24/09/2012 for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason.	☐
3.	I agree to take part in the study.	☐
4.	I agree to being contacted by the research team once the drug treatment course has been completed (between 1 and 12 months after procedure).	☐

_____	_____	_____
Name of Volunteer	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix IV – Patient Flow Chart: Overview



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Publications and Presentations

Publications:

1. Muggeridge DJ, Sculthorpe N, Grace FM, Willis G, Thornhill L, Weller RB, James PE, Easton C

   Acute whole body UVA irradiation combined with nitrate ingestion enhances time trial performance in trained cyclists

   Nitric Oxide. 2015;48:3-9

2. Thornhill L, Morris K, Anderson RA, James PE

   Prasugrel loading leads to an increase in circulating plasma nitrosothiols levels in patients with coronary artery disease undergoing PCI

   Submitted, awaiting review

3. Thornhill L, Abdul F, James PE, Anderson RA

   Ticagrelor's antiplatelet effect is modified by acidification and nitrites, and readily forms nitrosothiol compounds in vitro: An alternative mechanism for the potent antiplatelet effect of Ticagrelor.

   Submitted, awaiting review

Abstracts:

1. Thornhill L, Morris K, Anderson RA, James PE

   Prasugrel-induced nitrosothiols in patients with coronary artery disease

   8th International Nitric Oxide Conference August 2014, Cleveland, Ohio – oral presentation

2. Thornhill L, James PE, Anderson RA

   Ticagrelor's Antiplatelet Effect is Modified by Acidification and Nitrites, and Readily Forms Nitrosothiol Compounds to Augment its Antiplatelet Effect in Vitro

   ACC March 14th–16th 2015, San Diego, California – poster presentation

3. Thornhill L, Anderson RA, James PE

   Circulating plasma Nitrosothiols levels rise in response Prasugrel loading in patients with coronary artery disease undergoing elective percutaneous coronary intervention

   BCS June 8th-10th 2015, Manchester, UK – poster presentation