
# The Effect of Inorganic Nitrate and Antiplatelet Drugs on NO Metabolites and Platelet Reactivity in Patients with Stable Coronary Artery Disease

By

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*This thesis is dedicated to my parents Dilshad Begaum and late Khader Basha, to my wife Rumana and our two children Sarah and Rayan, for their continuous support, their patience and encouragement that has immensely helped me in completing this work.*

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# Contents

# Abbreviation

| | |
|---|---|
| 2Cs | Copper (I) chloride/cysteine |
| AA | Arachidonic acid |
| ACE | Angiotensin-converting enzyme |
| ACS | Acute coronary syndrome |
| ADP | Adenosine diphosphate |
| ALDH-2 | Aldehyde dehydrogenase-2 |
| AMP | Adenosine monophosphate |
| ATP | Adenosine triphosphate |
| AUC | Area under curve |
| $BH_4$ | Tetrahydrobiopterin |
| BMI | Body mass index |
| BR juice | Beetroot juice |
| CABG | Coronary artery bypass graft |
| CAD | Coronary artery disease |
| CAM | Cell adhesion molecule |
| cAMP | Cyclic adenosine monophosphate |
| CCB | Calcium channel blockers |
| cGMP | Cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CNS | Central nervous system |
| COPD | Chronic obstructive pulmonary disease |

| | |
|---|---|
| COX | Cyclooxygenase |
| COX-2 | Cyclooxygenase-2 |
| CuCl | Cuprous (I) chloride |
| CVA | Cerebrovascular disease |
| CVD | Cardiovascular disease |
| DAPT | Dual antiplatelet Drug |
| EC | Endothelial cells |
| EDHF | Endothelium-derived hyperpolarising factor |
| EDTA | Ethylenediaminetetraacetic acid |
| eNOS | Endothelial nitric oxide synthase |
| FAD | Flavin adenine dinucleotide |
| FGF | Fibroblast derived growth factor |
| FH | Familial hypercholesterolemia |
| FMD | Flow mediated dilation |
| FMN | Flavin mononucleotide |
| GSNO | S-Nitrosoglutathione |
| GM-CSF | Granulocyte-colony stimulating factor |
| GTN | Glyceryl trinitrate |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| HbNO | Nitrosylated haemoglobin |
| HbSNO | S-nytrosylated haemoglobin |
| HCl | Hydrochloric acid |
| HDL | High density lipoprotein |

| | |
|---|---|
| HFpEF | Heart failure with preserved ejection fraction |
| IGF | Insulin like growth factor |
| IHD | Ischemic heart disease |
| IL-1 | Interleukin-1 |
| iNOS | Inducible nitric oxide synthase |
| $IP_3$ | Inositol triphosphate |
| $IP_3R$ | Inositol triphosphate receptor |
| LDL | Low-density lipoprotein |
| LT | Leukotriene |
| LTA | Light transmission aggregometry |
| MACE | Major adverse cardiovascular events |
| MCP | Monocyte chemoattractant protein |
| MHC | Major histocompatibility complex |
| mtNOS | Mitochondrial NOS |
| NAC | N-acetyl-cysteine |
| NACSNO | Acetyl-cysteine-SNO |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| $NaNO_2$ | Sodium nitrite |
| NaOH | Sodium hydroxide |
| NCD | Non-Communicable disease |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| $NO^+$ | Nitrosonium |
| $NO_2^-$ | Nitrite |

| | |
|---|---|
| $NO_3^-$ | Nitrate |
| NOA | Nitric oxide analyser |
| NOS | Nitric oxide synthase |
| NR | Nitrate reductase |
| $O_3$ | Ozone |
| OBC | Ozone based chemiluminescence |
| OH. | Hydroxyl radical |
| $ONOO^-$ | Peroxynitrite |
| PAD | Peripheral arterial disease |
| PAF | Platelet activating factor |
| PAI-1 | plasminogen activator inhibitor-1 |
| PAMPS | Pathogen associated molecular patterns |
| PAR | Protease-activated receptor |
| PBS | Phosphate buffered saline |
| PC | Phosphatidylcholine |
| PCI | Percutaneous coronary intervention |
| PDGF | Platelet derived growth factor |
| PG | Prostaglandin |
| PGE2 | Prostaglandin E2 |
| $PGH_2$ | Prostaglandin $H_2$ |
| $PGI_2$ | Prostacyclin |
| PPI | Proton Pump inhibitors |
| PVD | Peripheral vascular disease |
| REC | Research Ethics Committee |

| | |
|---|---|
| ROS | Reactive oxygen species |
| RSNO | S-Nitrosothiol |
| SEM | Standard error of the mean |
| SIS® $G0^+$ | Sports in science Go Plus nitrate supplements |
| TIA | Transient Ischaemic attack |
| TNF-α | Tumour necrosis factor-α |
| tPA | Tissue plasminogen activator |
| TRAP | Thrombin receptor activating peptide |
| $TxA_2$ | Thromboxane $A_2$ |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| $VCl_3$ | Vanadium III Chloride |
| vWF | von Willebrand factor |
| XOR | Xanthine oxidoreductase |

# Summary

$P2Y_{12}$ antagonists are commonly prescribed in coronary artery disease (CAD) patients undergoing coronary intervention, however non-P2Y12 mediated effects have also been observed. The thienopyridine-Clopidogrel, for example, forms S-nitrosothiols (RSNO) at low pH, in the presence of nitrite in-vitro but it is unclear whether this occurs substantially in-vivo. It is unknown whether the newer class of non-thienopyridine antiplatelets(ticagrelor) has similar properties. Dietary sources of inorganic nitrate ($NO_3^-$) are also known to provide alternative pathways for NO production.

This thesis investigates the effect of dietary nitrate supplement with or without clopidogrel therapy on NO metabolites and platelet inhibition in CAD patients and explores the potential role of ticagrelor in RSNO biosynthesis.

In vitro studies demonstrate dietary $NO_3^-$ (in the form of *SIS®-Go+* and *Beet-It®)* gets converted to nitrite via bacterial nitrate reductase and thereby readily formed S-nitrosothiols in the simulated acidic gastric medium with and without clopidogrel.

In CAD patients, dietary $NO_3^-$ (SIS® $Go^+$) along with or without clopidogrel therapy results in a significant rise in plasma nitrate, nitrite and RSNO levels. $NO_3^-$ + clopidogrel therapy caused significantly more inhibition of TRAP mediated platelet activation with patients receiving clopidogrel with little change in the ADP mediated platelet inhibition, suggesting a non-ADP mediated effect due to RSNO. Concomitant PPI therapy has no effect.

Importantly, Ticagrelor has the ability to form RSNO in vitro and in-vivo. RSNO increased significantly in patients following a loading dose of Ticagrelor. However, elevated RSNO are not sustained in patients receiving a maintenance dose of Ticagrelor.

In conclusion, dietary $NO_3^-$ therapy significantly augments RSNO level with or without clopidogrel in CAD. Ticagrelor exhibits RSNO formation in-vitro and in CAD patients. Augmented RSNO may explain the $P2Y_{12}$ independent effects seen with these agents and represents a novel therapeutic approach in future management of CAD.

# Publications

**FB Abdul**, R Anderson, L Thornhill, K Morris, Phillip E. James
A Ticagrelor loading results in increased plasma S-nitrosothiols formation acutely in patients with stable angina undergoing PCI but not on chronic therapy: Possible evidence of an acute pleiotropic effect ***Journal of the American College of Cardiology****, Volume 69, Issue 11, Supplement, 21 March 2017, Page 41*

Burnley-Hall N, **Abdul F**, Androshchuk V, Morris K, Ossei-Gerning N, Anderson R, Rees DA, James PE (2017) Dietary nitrate supplementation reduces circulating platelet-derived extracellular vesicles vascular disease patients on clopidogrel therapy: a randomised, double-blind, placebo-controlled study. ***Thrombosis & Haemostasis****.* doi: 10.1160/TH17-06-0394

Witczak JK, Burnley-Hall N, **Abdul F**, Androshchuk V, Ossei-Gerning N, Anderson R, Rees DA, James PE (2016). The 2nd United Kingdom Extracellular Vesicle Forum Meeting Abstracts: Differences in circulating extracellular vesicles between healthy volunteers and patients with established erectile dysfunction – do endothelial microvesicles play an ambivalent role? ***Journal of Extracellular Vesicles****.* 5: 10.3402/jev.v5.30924. doi: 10.3402/jev.v5.30924

# Poster Presentations

In vitro model of conversion of physiological in-organic nitrate from dietary source to nitrite and nitrosothiols. Welsh Cardiovascular Society Annual Meeting (Park Plaza Hotel, Cardiff, UK, April 2017)

# 1 General Background

## 1.1 Cardiovascular disease

Cardiovascular disease (CVD) is a leading non-communicable disease (NCD) and a major cause of death across the globe. CVD includes coronary artery disease (CAD), cerebrovascular accidents (CVA), peripheral vascular disease (PVD), congenital heart disease, cardiomyopathies, heart failure, and aortic disease.

In 2013, approximately 54 million deaths occurred worldwide, of which 17.3 million (32%) deaths were attributable to CVD (1). By 2030 CVD deaths are expected to rise to more than 23.6 million (2). In the United Kingdom, CVD was the biggest killer in 2010 responsible for approximately one in three of all deaths in that year. Nearly half of these arise from coronary artery disease, making it the most common singular cause of death. The British Heart Foundation have estimated the annual cost of CVD to the United Kingdom's economy is approximately £19 billion annually (3).

CAD and CVA are a leading cause of premature deaths across the globe. Approximately 30-50% of all CVD cases are due to coronary artery disease. Approximately 70,000 deaths in the UK each year are due to CAD and most deaths are due to acute myocardial infarction. The lifetime risk of developing CAD at the age of 55 is 67.1% in men and 66.4% in women (4). In recent years, there has been an overall decline in incidence across Europe including the UK, but in contrast the incidence and prevalence of CAD and CVA deaths have doubled in developing countries and approximately 80% of CVD deaths occur at younger ages (<60yr) (2). The burden of CAD is rising with an ageing population and increasing prevalence of type 2 diabetes and obesity.

Stable CAD is characterised by episodes of reversible myocardial demand/supply mismatch, leading to ischaemia or hypoxia. These episodes are often reproducible and are inducible by exercise, stress or sometimes spontaneously. Stable CAD is a stable subtype in the continuum of CAD. It is associated with an estimated annual mortality rates of 1.2-2.4% per annum (5) and an annual incidence of 0.6-1.4% cardiac death and 0.6 to 2.7% of non-fatal myocardial infarction (6) (7).

Angina is often the most common clinical manifestation in patients with ischemic heart disease. Stable angina pectoris or stable angina is defined as discomfort in the chest, jaw, shoulder, back or arm, occurring at certain levels of exertion and is relieved with rest or nitro-glycerine (itself an organic or pharmacological “NO” donor, typically called “Nitrates”). A diagnosis of stable angina is made based on a classical history of angina pectoris, with associated one or more risk factors for atherosclerotic CAD.

Lifestyle modification such as smoking cessation, increase in physical activity, healthy diet, dietary sodium reduction, avoidance of excess alcohol, and weight management, along with therapeutic management of diabetes and hypertension, have significant clinical beneficial effects on cardiovascular morbidity and mortality(8-11).

There has been a significant decline in the rate of deaths due to CVD in UK over the last four decades, but this remains relatively high compared to other European countries (12). This decline is attributed to advances in medical and surgical treatments with modification of cardiovascular risk factors. An estimated 58% mortality decline was attributed to reduction of major cardiovascular risk factors and the remaining 42% of mortality decline was due to individualised patients care where secondary prevention plays a significant role (12).

Antiplatelet therapy forms a core component of medical therapy in patients with CAD especially in acute coronary syndromes and in CAD patients undergoing percutaneous coronary interventional (PCI) procedures. PCI procedures are now more commonly undertaken for patients with CAD compared to a decade earlier. Despite this, adverse cardiac events can still occur in this group of patients.

Continuing research in this field is vital, to facilitate a better understanding of the pathophysiology involved in CVD. This would pave a way for the development of more effective treatment and prevention strategies.

## 1.2 Vascular endothelium and its functions

The cardiovascular system comprises the heart and the blood vessels forming the coronary, cerebral and peripheral circulation. Atherosclerotic plaque formation within the lumen of the

blood vessels is the principal pathological process of development of CVD. These plaques cause a disruption to the flow of blood to the vital organs leading to their dysfunction or death. A review of the vascular structure and their function is essential in the understanding of CVD.

The blood vessels are grossly divided into arteries and veins. Arteries carry oxygenated blood to all organs of the body. Anatomically, the vascular wall comprises three layers- the tunica intima, tunica media and the tunica adventitia.

The tunica adventitia is the outermost layer of the vessel, which is comprised of connective tissue and contains a network of smaller vessels (vasa vasorum) and nerves.

The tunica media is the mid-layer of the artery, which is the thickest and has concentric layers of smooth muscle in the fibrous matrix.

The tunica intima is the inner most layer made up of a single layer of simple squamous ECs. A thin layer of sub endothelial connective tissue called the internal elastic lamina supports this layer.

An intact endothelial layer is vital for cardiovascular health. It is a dynamic organ which responds to various physical and humoral conditions. The endothelium is less than 0.2μm thick, comprising of 1 to $6x10^{13}$ endothelial cells with a total surface area of 4000-7000 $m^2$ and approximate weight of 1 kg in an average-sized human (13). It acts as a physical barrier with a feature to act as a semipermeable layer regulating the movement of molecules. In addition to this it has a major role in the regulation of vascular tone, haemostasis, smooth muscle proliferation, cellular adhesion, and inflammation. These functions are co-ordinated by the synthesis and release of various neurotransmitters, vasoactive factors and hormones, which influences cellular function throughout the body (Figure 1.1) (14, 15).

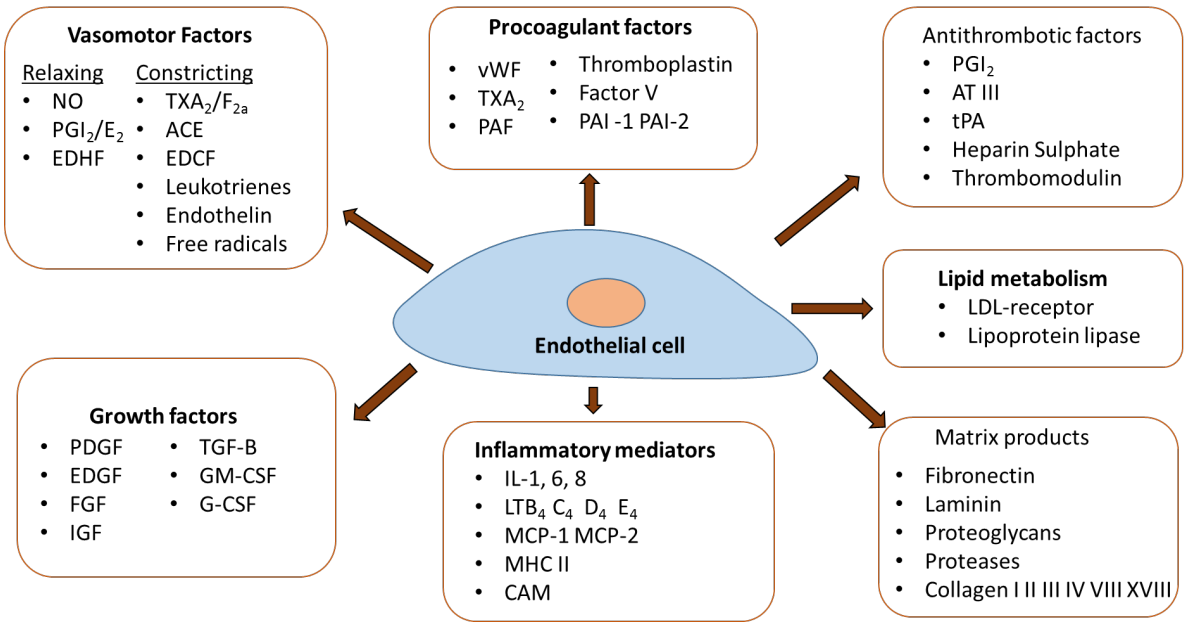



*Figure 1.1: Vascular endothelium is a dynamic, multifunction organ, which secretes a spectrum of mediators influencing cardiovascular physiology. This diagram summarises the mediators with their effects. Adapted from Sumpio et al(16).* ***(NO: Nitric Oxide, PGI2 : Prostacyclin, tPA : Tissue Plasminogen Activator, PGE2 : Prostaglandin E2, EDHF :Endothelium Derived Hyperpolarising Factor TXA2 : Thromboxane A2, ACE : Angiotensin Converting Enzyme, EDCF : Endothelium Derived Contracting Factor, vWF: Von Willebrand Factor, 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, TX : Thromboxane)***

### 1.2.1 Haemostasis

The intimal surface of healthy endothelial cells has both procoagulant and anticoagulant features. Under normal physiological conditions there is a critical balance between thrombotic and antithrombotic properties. It is the major site for anticoagulant reactions involving thrombin (17). The predominant antithrombotic factors are protein C, protein S proteoglycans, thrombomodulin, annexin V & II, ectonucleotidase, tissue plasminogen activator (tPA), tissue factor pathway inhibitor, NO and $PGI_2$. Platelet activation factor (PAF) and von Willebrand factor (vWF) is the predominant prothrombotic factor present on the endothelium surface.

Prostacyclin ($PGI_2$) and nitric oxide (NO) are important factors in the prevention of platelet adhesion and aggregation, through the increase in cAMP and cGMP in platelets (18). The ectonucleotidase on the liminal surface hydrolyses ATP and ADP into AMP and adenosine, thereby minimising the extent of platelet aggregation.

Tissue plasminogen activator (tPA) is a powerful thrombolytic agent secreted by endothelial cells in response to vasopressin, norepinephrine, and thrombin, which allows conversion of plasminogen to plasmin which breaks the fibrin network in the thrombus (19). Protein C in complex with protein S inactivates factor VIIIa and Va cofactors which are essential for coagulation. Thrombomodulin prevents thrombin from interaction with fibrinogen. Platelet activation factors, thrombin, ADP and ATP activate the release of prostacyclin from endothelium thus limiting the extent of plug formation (20).

Endothelial cells are activated in the event of a vessel wall injury or exposure to thrombin, histamines and cytokines (TNF-α, IL-1, IL-6). Activated endothelial cells synthesize PAF and vWF. PAF is a potent platelet activator which promotes platelet adhesion to endothelial cells (21). Whereas vWF is primarily stored in Weibel-Palade bodies within endothelial cells. It is mobilized rapidly upon stimulation, which binds platelets to exposed extracellular matrix as a result of vessel injury (22).

### 1.2.2 Vascular tone

Endothelial cells regulate vascular smooth muscle contraction and vascular tone, thereby regulating blood flow and blood pressure within the vasculature. This effect is mediated through the release of endothelial derived relaxing factors such as NO, $PGI_2$, endothelium derived hyperpolarising factor (EDHF), and vasoconstrictor molecules like thromboxane ($TXA_2$), superoxide ($O_2^-$) and endothelin-1(ET-1). NO is the predominant vasodilator released from endothelial cells and it has several additional beneficial effects such as inhibition of platelet aggregation, smooth muscle proliferation, inhibition of leukocyte adhesion and anti-atherosclerotic effects (23-25). These effects are discussed in detail in section 1.5.

$PGI_2$ is formed from arachidonic acid by the enzyme cyclooxygenase (COX). Cyclooxygenase-1 (COX1) is the main isoform involved in the production of $PGI_2$ which is primarily expressed in endothelial cells. $PGI_2$ acts on IP receptors on smooth muscle cells, causing an increase

in intracellular levels of cyclic adenosine monophosphate (AMP) through stimulation of adenylate cyclase, affecting vascular smooth muscle relaxation. $PGI_2$ and NO work synergistically to facilitate vascular smooth muscle relaxation. NO inhibits phosphodiesterase enzyme that degrades cyclic AMP hence prolonging the cyclic AMP mediated effect of $PGI_2$ in smooth muscle cells. $PGI_2$ enhances NO release from endothelial cells (26). Upon entering the blood stream $PGI_2$ inhibits platelet aggregation (27, 28).

Potassium ions, hydrogen peroxide, and epoxyeicosatrienoic acids have all been proposed as the endothelial derived hyperpolarising factor (EDHF), which causes a relaxation of the vascular smooth muscle by hyperpolarization (29). EDHF is thought to take precedence where NO activity is limited, and as such is predominantly active in smaller vessels/arterioles lacking smooth muscle.

Endothelin is an extremely potent vasoconstriction factor. There are 3 isoforms of endothelin -ET1, ET2 and ET3. ET1 and ET2 are more potent agonists than ET3. ET1 is synthesised by the vascular endothelial cells and is the most abundant isoform. ET1 synthesis is enhanced in the presence of hypoxia, Ischaemia, thrombin, vasopressin, catecholamine, interleukin-1 and transforming growth factor 1 (30-32). ET1 acts on endothelin receptor A and B (ETA and ETB) located mainly on the smooth muscle and endothelial cells, thereby regulating vascular tone and provoking proinflammatory and mitogenic reactions, ET1 play an essential role in various cardiovascular diseases such as systemic hypertension, pulmonary hypertension and atherosclerosis(33). ET2 has a key role in ovarian physiology, it can also mimic the actions of ET1 by acting on ETA and ETB(34). ET3 is important for development of neural crest-derived cell lineages, such as melanocytes and enteric neurons(35).

Superoxides, causes vasoconstriction by scavenging the NO, sensitising the vascular smooth muscle for calcium ion and direct oxidative disruption of normal signalling mechanisms in the endothelium and vascular smooth muscle cells. Superoxide radicals are formed in response to a rise in blood pressure and endothelial agonists (36). Superoxide, is an anion, produced by the one-electron reduction of molecular oxygen, and is formed in all living organisms that come in contact with air. Its level is regulated in vivo primarily by superoxide reductase and superoxide dismutase (as well as other cellular antioxidants). Superoxide can act as a signalling agent, a toxic molecule or a harmless intermediate, depending upon its biological context (37) (38).

### 1.2.3 Endothelial activation and endothelial dysfunction

Endothelial cells are dynamic, with the ability to adapt their phenotype according to the nature of the local milieu (39). Alteration of the normal endothelial function constitutes endothelial activation and can lead to endothelial dysfunction.

#### 1.2.3.1 **Endothelial activation**

The participation of endothelial cells in the acute inflammatory response induced by infection or injury has been long appreciated. The classical signs of inflammation such as rubor, dolor, pallor and tumor are due to the endothelial mediated effects on microvascular tone, permeability and leukocyte adhesion. These acute responses are collectively termed as endothelial activation type 1 (40).

Endothelial activation can be considered a proinflammatory, pro-coagulative and proliferative state. Bacterial endotoxins, interlekin-1, tumor necrosis factor (TNF) and interferon-gamma stimulates a more sustained endothelial activation referred to as type 2 activation. Endothelial activation causes a loss of vascular integrity, change in phenotype from antithrombotic to prothrombotic with expression of leukocyte adhesion molecules, upregulation of HLA molecules and cytokine production. The hall mark of this form of response is the activation of pleiotropic transcription factors such as nuclear factor-kappa-B (NF-κB). Once activated, NFκB is transported into the nucleus where it binds promoter genes which are upregulated in endothelial activation, resulting in the expression of various effector proteins with pathophysiological consequences (41, 42). The effector proteins include inducible endothelial-leukocyte adhesion molecules such as VCAM-1 and ELAM-1 (E-selectin), chemokines such as interleukin-8 and procoagulant molecules such and tissue factor. VCAM-1 and chemokines such as IL-8, IL-1, and MCP-1 are important links between endothelial activation and vascular atherogenesis (39, 43). Endothelial activation promotes all stages of atherogenesis (44).

The presence of infection, inflammation and cardiovascular risk factors increases the activity of NADPH oxidases causing an increase in ROS in the tissues. ROS molecules target NF-κB and phosphates leading to endothelial cell activation. ROS mediated endothelial activation is

a host defense response in infections or inflammation and a pathophysiological response in the presence of cardiovascular risk factors.

#### 1.2.3.2 **Endothelial dysfunction**

Endothelial dysfunction is characterized by impaired vasodilation, increased pro-thrombotic and a pro-inflammatory state. This is typically the result of diminished production and/or availability of vasodilators, predominantly NO, with or without an imbalance in the relative production of other endothelium derived relaxing and constricting factors (45).

Endothelial dysfunction is associated with traditional cardiovascular risk factors such as smoking, ageing, hypercholesterolemia(46, 47), hyperglycemia, hypertension, along with other risk factors like chemotherapy(anthracyclines) (48) and radiation (49).

These risk factors have a well-established association with alteration in endothelial function resulting in a chronic inflammatory process leading to a loss of antithrombotic factors and an increase in vasoconstrictor and prothrombotic effects associated with increased cardiovascular events (Figure 1.2) (47, 50, 51).

More recently, endothelial dysfunction has also been associated with obesity (52), chronic systemic infection (53), chemotherapy (48), radiation (49) and post heart transplantation.

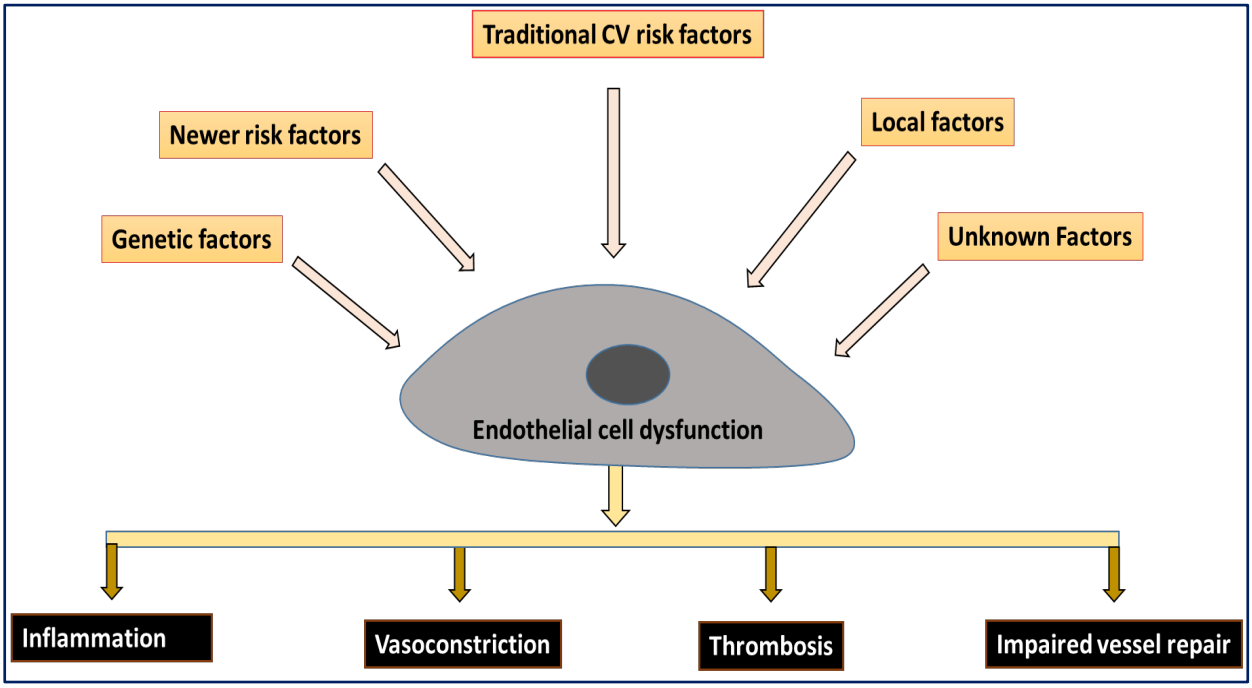



*Figure 1.2: Diagrammatic representation of various factors affecting the ECs causing endothelial dysfunction and its consequences. Adopted from (54)*

These cardiovascular risk factors and pro-inflammatory conditions cause an increase in the activity of NADPH oxidases, leading to increased reactive oxygen species (ROS) production. ROS at low concentrations function as signalling molecules of fundamental cell activities like growth and cell adaptations. At higher concentration where there may be an imbalance between ROS generation and antioxidant defences, when ROS can cause vascular oxidative stress and cell injury. Vascular oxidative stress causes an alteration in the function of endothelial nitric oxide synthase (eNOS). There is upregulation of eNOS and NADPH oxidase expression. The combination of both enzymatic products (NO and superoxide, respectively) increases the amount of peroxynitrite (ONOO-). Peroxynitrite in turn causes eNOS uncoupling by oxidising the eNOS co-factors ($BH_4$ and zinc) causing eNOS to produce further superoxide instead of NO. Prolonged impairment of NO production and chronic ROS production leads to further endothelial dysfunction (38) (37).

eNOS is the main source of NO in the vasculature (55) It has been reported that 70% of resting plasma nitrite is derived from eNOS activity in humans and other mammals (56). An

imbalance in the production and consumption of NO is critical in endothelial dysfunction (is discussed in section 1.5.3).

Endothelial dysfunction is an independent predictor for the occurrence of cardiac events. It plays a crucial role in the pathogenesis of acute coronary syndromes. It precipitates the plaque destabilisation process through a complex interplay of cellular plaque components and proinflammatory mediators (51). Impaired NO production enhances the endothelial expression of several adhesion molecules and inflammatory mediators that increases plaque vulnerability (25, 57, 58). Endothelial dysfunction is also referred to as a barometer of vascular health, a unified overall effect of risk factors and intrinsic defence mechanisms.

#### 1.2.3.3 **Assessment of endothelial dysfunction**

Endothelial dependent vasodilatory function is commonly evaluated in the coronary and peripheral circulations.

The potential detection of endothelial dysfunction as an early marker of atherosclerosis makes these assessments an extremely useful tool for early stratification of patients at risk of cardiovascular events.

In recent years numerous invasive and non-invasive methods of measuring endothelial function were studied and validated. An invasive quantitative coronary angiographic method of evaluation of the changes in diameter of coronary arteries in response to intracoronary infusion of acetylcholine remains the “gold standard” to assess the endothelium-dependent vasodilation (59). However, the invasive nature of these methods limits its widespread clinical application.

Non-invasive evaluation of brachial-artery flow mediated dilation using ultrasound is a technique that has been widely used and is a clinically applicable method for the study of NO dependent endothelial function. This technique examines the change in forearm blood flow in response to direct administration of agonists in the brachial artery. The arterial compliance and wave form morphology provide a marker of vascular well-being (60).

Circulating biomarkers such as NO metabolites, inflammatory cytokines, and adhesion molecules can be measured as surrogate indicators of endothelial function. These techniques

are often difficult and expensive to measure, with a risk of erroneous interpretation and many confounding factors.

#### 1.2.3.3.1 Endothelial dysfunction and cardiovascular risk factor modification

The cardiovascular risk factors modification approach, such as weight reduction, smoking cessation, cholesterol lowering, antihypertensive therapy, ACE inhibitor therapy, supplementation with folic acid and physical exercise have all been shown to improve endothelial function (61, 62). Obesity is a major risk factor for endothelial dysfunction and atherosclerosis, with recent evidence suggesting that obesity is associated with accelerated coronary atherosclerosis and premature aging, in adolescent and young adults. Control of obesity is important in the prevention of endothelial dysfunction, atherosclerosis and its sequelae (63-65)

Smoking is a well-known health hazard, both active and passive smoking led to endothelial dysfunction, hypertension, and increased ROS generation and decreased NO bioavailability, thereby contributing significantly towards CV morbidity and mortality. The full effect of smoking on CV health is not fully understood, and there exists a smoking paradox which is nicely shown in a recent study that shows patients who continued to smoke following PCI had fewer repeat coronary interventions(66) (67).

### 1.2.4 Atherosclerosis

Atherosclerosis is the most common pathological process causing arterial thickening and luminal narrowing leading to ischaemia of the organ supplied by that vessel. This is commonly associated with endothelial dysfunction.

Atherosclerosis is the pathological basis of almost all of CAD resulting in myocardial ischaemia and infarction. The earliest lesions of atherosclerosis develop in a distinctive, non-random pattern at branch points and at regions of altered blood flow supporting the role of haemodynamic forces. It begins in childhood, with silent progression over the years appearing as fatty streaks, which are not clinically significant but serve as a precursor of formation of more complex plaques, containing a necrotic lipid rich core with smooth muscle accumulation.

These atherosclerotic plaques cause a luminal narrowing resulting in tissue ischaemia producing typical symptoms such as angina or claudication. Clinical events such as MI/CVA are initiated when a plaque ruptures exposing its vasoactive core which culminates in a downstream thrombotic occlusion (Figure 1.3).

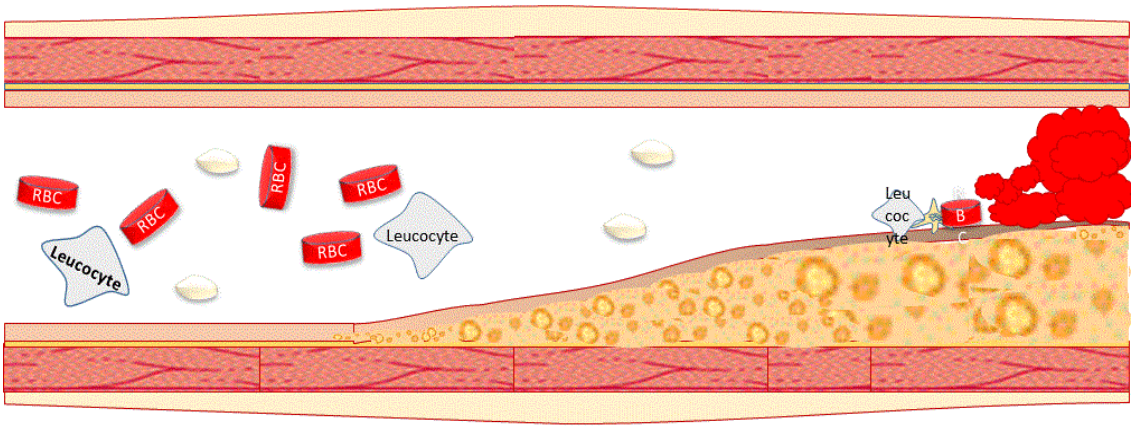



*Figure 1.3: Diagrammatic representation of the timeline for progression of atherosclerosis over time in the vasculature, RBC represent red blood cells*

Atherosclerosis is regarded as a systemic inflammatory disease. Lipid lowering therapy with statins has been shown to inhibit progression of coronary plaques, by reducing the size and alteration of cellular and chemical composition (68). Lipid lowering therapy in combination with antiplatelet therapy remains the mainstay therapeutic approach to effecting a significant reduction of cardiovascular events in patients with atherosclerotic CAD (69, 70).

## 1.3 Role of platelets in coronary artery disease

Platelets are anucleate disc shaped circulating cells which play a crucial role in vascular haemostasis, repair of injured vascular endothelium and prevent blood loss after vascular injury. These cells are derived from bone marrow megakaryocytes, and they carry parent RNA for expression of protein required in haemostasis. They contain numerous granules and a wide range of surface receptors.

Platelets usually circulate in an inactive state. They are activated when there is an injury to endothelium or alteration in blood flow in the vasculature. An intact endothelium secretes NO, prostacyclin and ADPase, which inhibits platelet activation and shape change.

The major platelet functions include

a) Adherence
b) Activation and secretion
c) Aggregation
d) Binding with coagulation factors

### 1.3.1 Platelet activation and thrombosis

A break in the endothelial cell integrity exposes the connective tissue matrix which lies underneath the endothelial layer. Platelets adhere to the exposed collagen fibrils and von Willebrand factor, and in the presence of other mediators like ADP, thromboxane and adrenaline, platelets get activated. A change in shape occurs at this stage which increases the expression of glycoprotein IIb-IIIa receptors promoting a cross linking with other activated platelets via fibrinogen. Activated platelets release ADP and thromboxane A2 causing further recruitment of circulating platelet activation, platelet aggregation and thrombus formation (Figure 1.4) (71).

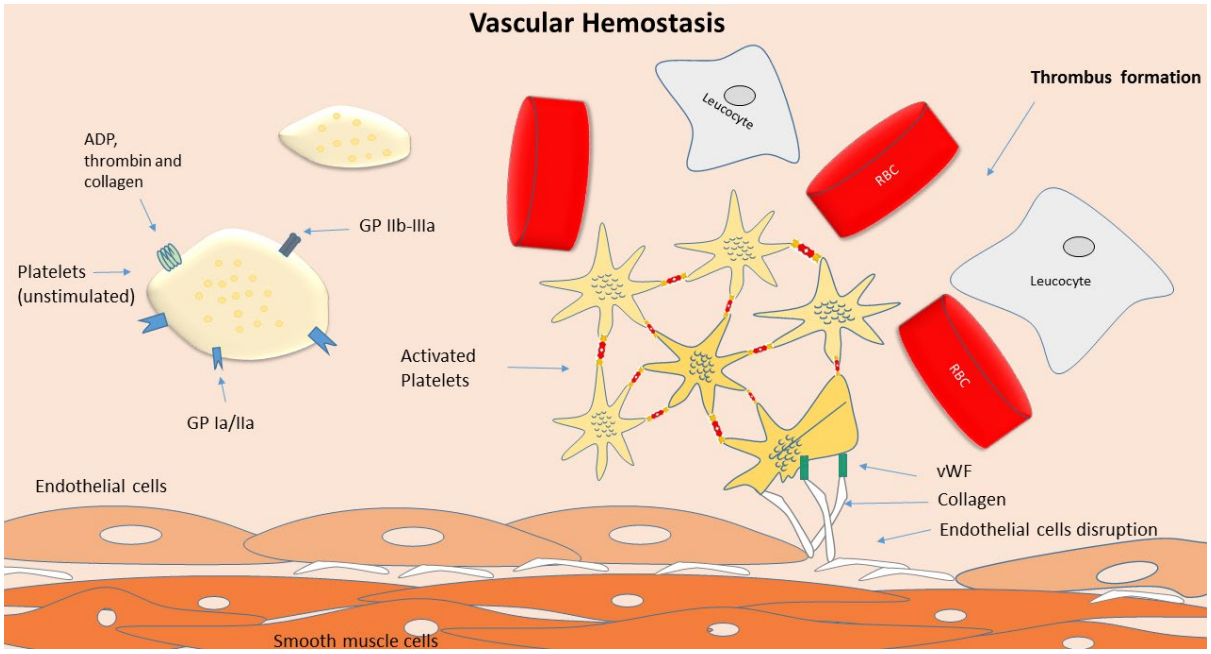



*Figure 1.4: Diagrammatic representation of the process of platelet activation and its role in haemostasis and vessel repair, RBC : red blood cells, ADP: Adenine diphosphate, GP: glycoprotein*

Platelets play an integral role in thrombus formation. Atherosclerotic-thrombotic occlusions of the arteries is the most common cause for coronary and cerebrovascular events.

Platelets are known to contribute to the pathogenesis of atherosclerosis. Activated platelets enhance smooth muscle proliferation via the release of vasoactive substances (72, 73). The phagocytized platelet can act as a source of lipids, promoting atherosclerotic plaque and thrombi formation.(74, 75)

Platelets contain numerous intracellular granules and a wide range of receptors on their surface, which play a critical role in the adhesion-activation-aggregation-coagulation cascade. Platelet surface P-selectin is considered as a gold standard marker of platelet activation. However, Michelson *et al*, demonstrated that circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than P-selectin (76).

There is an increase in degranulated platelets with greater monocyte platelet aggregation in patients with stable coronary artery disease (CAD) (77, 78), suggesting a central role in the pathogenesis of stable CAD.

Collagen, thrombin, ADP, epinephrine, and thromboxane A2 are well recognised platelet agonists. Most agonists stimulate the cell receptors present on the platelet membrane (Figure 1.5). These receptors interact with the G proteins at their cytosolic surface. These G proteins contain a GTP binding alpha subunit and Beta-gamma heterodimers. In its inactive state GDP is bound to the G protein. The stimulated receptor displaces GDP with GTP, causing G protein activation with increase in intracellular calcium, thereby stimulating the platelets.

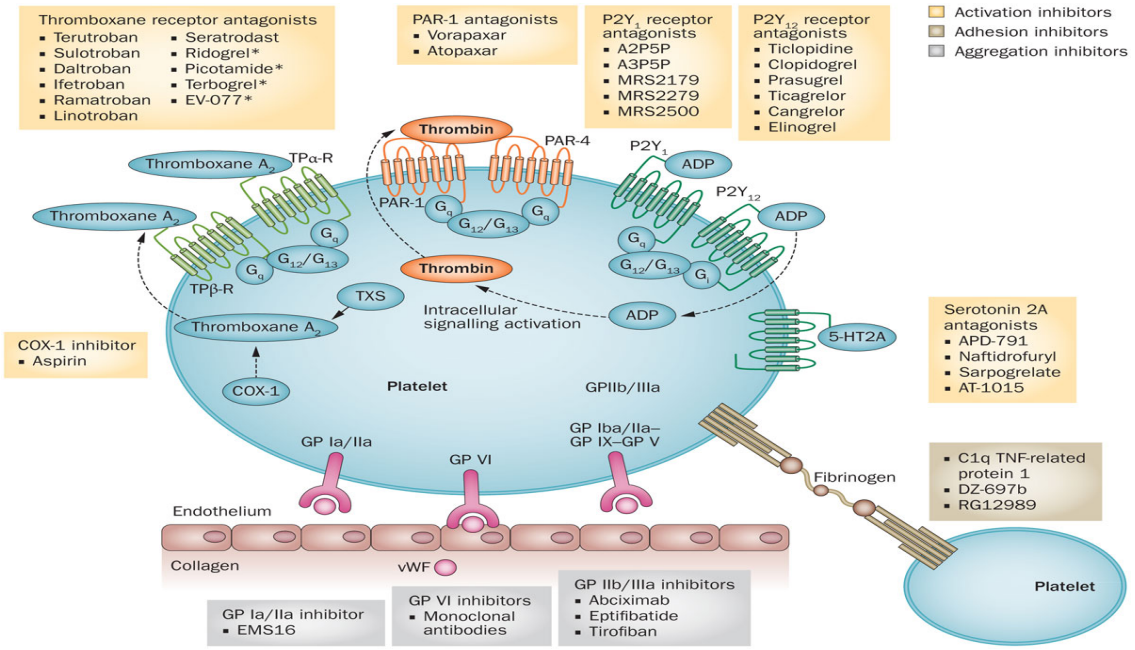



*Figure 1.5: Diagrammatic depiction of the known receptors, with their respective agonists and blocking agents involved in platelet participation in haemostasis and thrombotic process. Adapted from (79).*

## 1.4 Antiplatelet medications

Antiplatelet medications are designed to alter platelet function, primarily targeting activation or propagation of the aggregation cascade. They form an integral therapeutic component in management of CAD and cerebrovascular disease.

Antiplatelet drugs that are currently prescribed can be broadly classified into the following three groups based on their mechanism of action.

a) Cyclooxygenase inhibitors: Aspirin
**b)** GP IIb/IIIa Inhibitors: Abciximab, tirofiban.
c) $P2Y_{12}$ receptor blockers: Thienopyridine (ticlopidine, clopidogrel and prasugrel) and non-thienopyridine (ticagrelor and cangrelor).

### 1.4.1 Aspirin and GPIIB/IIIA receptor blockers

Aspirin was the first drug to be used widely as an effective antiplatelet agent. It binds to COX1 and inhibits thromboxane A2 ($TxA_2$) mediated platelet activation. Platelets then lack the ability to synthesise $TxA_2$ for rest of their life span (approximately 7 to 10 days). Lack of synthesis of $TxA_2$ also impairs platelet stimulation by other agonists like ADP and thrombin. Aspirin blocks platelet aggregation more than adhesion.

Numerous randomised trials and meta-analysis have confirmed statistically significant and clinically noticeable benefits of use of aspirin in CAD (80-83). Aspirin is an essential component of antiplatelet therapy in patients undergoing PCI (84, 85) and coronary artery bypass grafting (CABG) (86, 87).

Aspirin therapy has noticeable adverse effects, the more common adverse effect is bleeding, specifically gastro-intestinal bleeding (88, 89) and less frequently, haemorrhagic stroke. (90)

Despite being on aspirin therapy, patients do suffer from recurrent cardiovascular events. Although Aspirin blocks one of the many mechanisms of platelet activation, there are many other pathways through which platelets get activated leading to thrombotic events (Figure 1.5). An ongoing search for a more comprehensive, potent and efficient antiplatelet drug has been pursued in the last 3 decades. Despite these, aspirin remains the first line of antiplatelet therapy due its low cost and relative proven safety and efficacy. The newer antiplatelet drugs are used as an added therapy along with aspirin to enhance platelet inhibition in patients at high risk of CV recurrent events or used as substitutes in patient's intolerant or high risk for aspirin therapy.

GPIIB/IIIA drugs such as abciximab, tirofiban, eptifibatide inhibit the final common pathway of platelet aggregation, preventing the cross binding of platelets to fibrinogen. These drugs cause a potent and effective platelet inhibition, with increased risk of bleeding. Current medications

are available in intravenous form and are being used in ACS patients undergoing PCI (91-93). These drugs are used as an additional antiplatelet agent along with aspirin and $P2Y_{12}$ receptor blockers (94).

### 1.4.2 $P2Y_{12}$ receptor Blockers

$P2Y_{12}$ is a G protein coupled purinergic receptor found mainly on the platelet surface. It is a chemoreceptor for adenosine diphosphate (ADP) (95). ADP acts as a potent, broad-spectrum, physiological agonist which activates $P2Y_1$, $P2Y_{12}$ and $P2X_1$.

$P2Y_{12}$ receptor activation by ADP forms an important step in platelet activation and thrombus formation. $P2Y_{12}$ receptors also potentiate platelet activation by other agonist like collagen, and thromboxane A2 (96).

$P2Y_{12}$ receptors has been studied extensively and numerous agents aimed at blocking this receptor have been developed successfully and are used in clinical settings (Figure 1.6). $P2Y_{12}$ receptor blocking agents have shown a significant reduction in thrombotic vascular events in patients with ACS and in those undergoing PCI. Currently available $P2Y_{12}$ receptor blocking drugs are classified as thienopyridines or non-thienopyridines, based on their chemical structure.

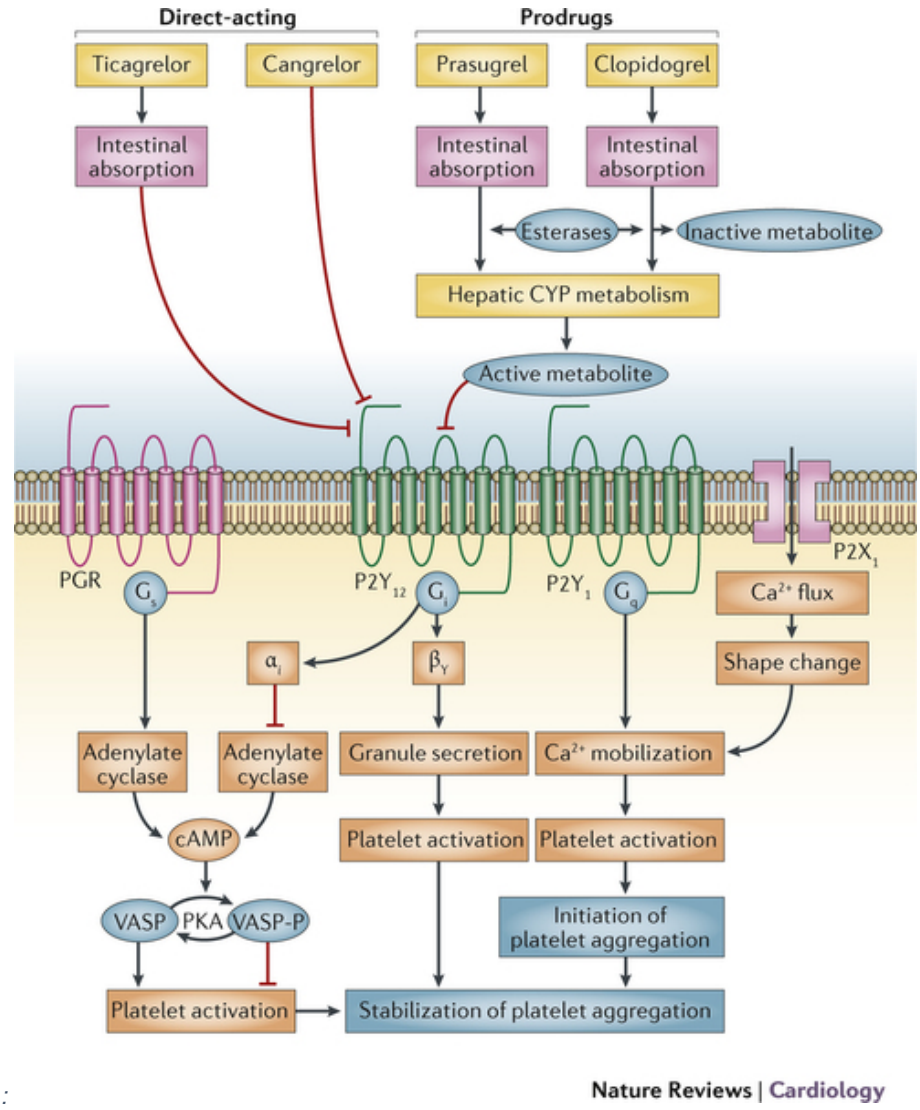



*Figure 1.6: Adapted diagram depicting the process of $P2Y_{12}$ receptor mediated activation of platelets and currently available $P2Y_{12}$ receptor blocking drugs, with their metabolic process (97).*

### 1.4.2.1 **Thienopyridines**

Thienopyridines (Ticlopidine, Clopidogrel and Prasugrel) act by binding to the cysteine residue of the $P2Y_{12}$ receptor irreversibly and modifies the receptor configuration, thereby preventing ADP induced activation of the receptor.

Ticlopidine was the first drug used in this class in the 1970's. It is a prodrug, requiring metabolism by cytochrome p450 enzymes to form an active compound(98). This drug has demonstrated a clear effectiveness in reducing thrombotic events in patients with CVD(99) and was used in combination with aspirin, especially in patients undergoing PCI (100). Its use has been limited due to rare but severe side effects causing neutropenia (> 1%) and thrombotic thrombocytopenic purpura (0.2%) with a 50% fatality. Clopidogrel was developed to overcome these adverse effects. Clopidogrel is synthesised by an addition of a substituted ester linkage to the Ticlopidine base molecule.

#### 1.4.2.1.1 Clopidogrel

Clopidogrel is the most commonly prescribed antiplatelet drug across the globe and has become the cornerstone therapy for secondary prevention of ischaemic cardiovascular events in patients with CAD (101-104) . Clopidogrel is an inactive compound and forms active metabolites in the liver via two-step metabolism (Figure 1.7).

Upon absorption 85% of the drug forms a carboxylic acid derivative of clopidogrel which is a major but inactive metabolite. About 15% of absorbed clopidogrel gets transformed to a thiol metabolite via a host of CYP enzymes in the liver, which is an active metabolite (Figure 1.7).

The active metabolite irreversibly inhibits platelet aggregation by selectively inhibiting the binding of ADP to the platelet $P2Y_{12}$ receptors; thereby interfering with subsequent ADP mediated activation of glycoprotein IIb-IIIa signalling for the lifetime of the platelet.

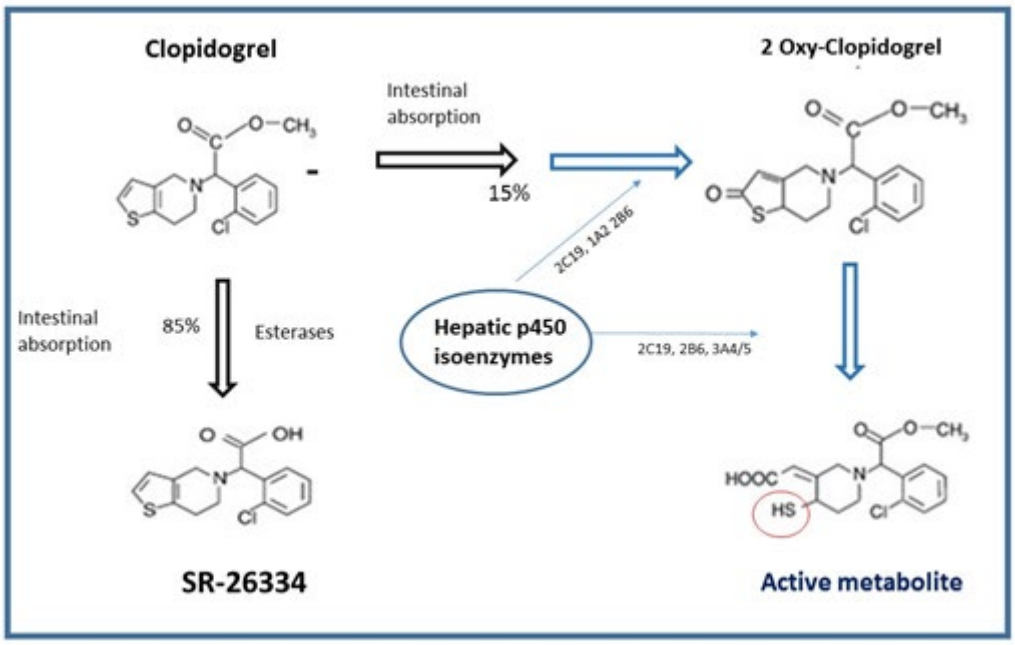



*Figure 1.7: Clopidogrel molecular structure and metabolism*

The clopidogrel active metabolite peaks at approximately 90 minutes post ingestion, achieving a maximum antiplatelet effect within 2 hours. The peak active metabolite concentration can be further increased by administering “loading” clopidogrel doses up to 600 mg and has become routinely used in clinical practice to ensure greater rapidity of action and improved clinical efficacy.(105) Following this a regular dose of 75 mg once daily to maintain steady state platelet inhibition is typically administered.

Clopidogrel is used as an additional antiplatelet agent along with aspirin in high risk conditions such as ACS(102) and PCI (97). Clopidogrel has morbidity and mortality benefit in the prevention of ischemic complications in patients with ACS (101, 103, 104) and/or after PCI (103).

Drug eluting stents are used in the majority of PCI procedures performed currently. Stent thrombosis (ST) is a serious but uncommon complication of coronary artery stenting. Dual antiplatelet therapy with aspirin and platelet $P2Y_{12}$ receptor blocker (clopidogrel) significantly reduces the risk of stent thrombosis and Ischemic events(106).

The benefits of pre-treatment with clopidogrel in patients undergoing elective PCI is well established. In the CREDO trial, 2100 patients were randomised to 300mg of clopidogrel or placebo. A significant reduction was observed in the combined end point of MI, stroke and death in patients loaded with 300mg of clopidogrel. The benefit was maximum when given at least 24 hours prior to PCI (105). The limitation of very early initiation of clopidogrel therapy (>15hrs) was overcome by giving an additional 300mg of Clopidogrel to the dose used in the CREDO trial.

The ARMYDA-2 trial compared 600mg of clopidogrel vs 300mg of clopidogrel, given 4-8 hours prior to elective PCI. This demonstrated safety and significantly reduced periprocedural MI in patients pre-treated with 600mg of clopidogrel (107). There was no significant difference in 30-day incidence of cardiac death, MI or unplanned target vessel revascularisation or bleeding in patients given 600mg of clopidogrel 4 to 8 hour or immediately prior to PCI (108).

A loading dose of 600mg of clopidogrel produces a maximal antiplatelet effect within 90-120 minutes and maintains a sustained effect with a daily 75mg dose. Similar antiplatelet effects are observed with ingestion of 75mg of clopidogrel for at least three consecutive days (106).

At the University Hospital of Wales, we typically prescribe a loading dose 600mg of clopidogrel in clopidogrel naïve patients undergoing PCI with coronary stenting. No additional loading dose of clopidogrel is administered prior to procedure for the patients who are already receiving long term clopidogrel therapy.

**Clopidogrel non-responsiveness**

Adverse cardiovascular events still occur despite patients being on a recommended dual antiplatelet therapy (aspirin and clopidogrel). Clopidogrel has its limitations, in terms of delayed onset of action, modest antiplatelet action, and significant variation in responsiveness in patients.

It has been estimated that about 15-50% of patients have high platelet reactivity while on clopidogrel therapy; this is termed as “high on treatment platelet” reactivity (HPR) (or equally “Resistant” or “non-responsive” with some variation in their definitions) (109). Several observational studies have demonstrated a strong link between HPR/resistance and recurrent Ischaemic events in post PCI patients(110, 111).

The possible causes for inter individual variation and hypo responsiveness remain largely unexplained but include

a) Clopidogrel metabolism:
    - Variable absorption of drug (101, 109)
    - Genetic polymorphisms have been identified in individuals which interfere with the CYP2C group of enzymes required for activation.(112)
b) Drug interactions
    - Drugs enhancing or inhibiting CYP activity or competing with Clopidogrel for enzyme can affect the metabolism.
    - Proton pump inhibitors, Statins, calcium channel blockers(113-116)
c) Other conditions like diabetes mellitus, renal failure, cessation of smoking, and non-compliance (117-119).

Platelet function testing (LTA, Multiplate, PFA-100, VerifyNow) and genetic testing are frequently used methods of assessing responses to clopidogrel. No single method is validated or developed as a robust clinical tool for use. Most studies testing platelet inhibition using the verifyNow $P2Y_{12}$ point of care test have shown an increase risk of thrombotic occlusion in patients who have poor response to clopidogrel treatment. It is more commonly seen in patients undergoing PCI. The major adverse event being acute stent thrombosis which can be fatal in a few cases (120, 121). The suggested approaches of managing poor responses are, altering the dose of clopidogrel, changing to alternate, newer, and more potent agents like Ticagrelor or Prasugrel, and/or stent optimization.

However a recent large randomised trial failed to demonstrate an improved outcome in patients with high platelet reactivity when given high dose of clopidogrel therapy (122). There were several possible reasons for a lack of beneficial effect of high dose clopidogrel; there was only a modest reduction in platelet reactivity with high dose of clopidogrel therapy and possibly had no effect on carriers of CYP2C19 allele, who exhibit high on treatment reactivity. The frequency of high on treatment reactivity decreased in both groups over the initial 30-day period and beyond, which meant there were fewer patients with high on platelet reactivity in both arms of the trial. This in conjunction with low event rate observed in this trial could have had a significant effect on the outcome. Even though this trial was a large randomised study, it had inherent limitations such as high-risk patients were underrepresented, and a fixed high

dose clopidogrel was administered rather a dose adjusted for platelet reactivity, which could affect the outcome of this study.

### Pleiotropic effects of clopidogrel

Recent studies have shown novel pleiotropic properties and effects of clopidogrel in addition to platelet $P2Y_{12}$ receptor inhibition. These actions could be partly due to its effect on the non-platelet $P2Y_{12}$ receptors which can be found in several different tissues of the body including the vascular smooth muscle (123), macrophages, leukocytes, macrophages, microglial and dendritic cells (123-125).

Clopidogrel therapy is shown to be associated with reduced leukocyte counts (PLATO trial) (126), CD40 ligand, CRP, P-selectin and other inflammatory markers associated with activated platelets, which play a significant role in the atherosclerotic process (127, 128).

Non- $P2Y_{12}$ receptor dependent mechanisms of action of these drugs are also being hypothesised and researched. Clopidogrel and Ticlopidine have a proven vasomodulatary activity in animal models. Jakubowski *et al*. have demonstrated a NO-dependent vasodilation in the isolated guinea pig heart(129). Warnholtz *et al.* for the first time demonstrated *in vivo,* clopidogrel dose-dependent improvement in endothelial dysfunction (130). This effect is only seen with a loading dose of clopidogrel and lost with continued use of clopidogrel over 28 days (131). Clopidogrel has also been shown to improve endothelial NO bioavailability in patients with CAD (132).

Our research (James *et al*) group have successfully shown in previous studies that clopidogrel has a potential to form RSNO compounds in the presence of nitrite at low pH. This is a potential novel mechanism, which is independent of endothelial mediated NO action, and is purposed to form a circulating store of RSNO that can donate NO-like activity (133).

Importantly, Clopidogrel improves endothelial NO availability (132) and shows greater improvement of endothelial dysfunction at higher clopidogrel dose (107) with improved vascular reactivity (134). Taken with the above, it is not unreasonable to suggest these effects could be the result of its ability to form RSNO.

#### 1.4.2.1.2 Prasugrel

Prasugrel is a newer and potent antiplatelet drug belonging to the thienopyridine group and is found to be more effective than clopidogrel. The TRITON-TIMI 38 trial showed a significantly reduced rate of ischemic events and stent thrombosis in patients treated with prasugrel in comparison to clopidogrel in ACS patients (135, 136). Unsurprisingly, there was an increased incidence of bleeding with prasugrel therapy proving its higher potency albeit with increased risk.

Like clopidogrel, prasugrel is a pro-drug and requires a one-step metabolism by the cytochrome P450 system to form an active metabolite. Prasugrel readily undergoes nitrosation reactions similar to clopidogrel (Figure 1.8) (137).

Thornhill *et al* have successfully shown an acute increase in the circulating RSNO and nitrite levels following a single loading dose of prasugrel to CAD patients(137). This rise is not maintained with chronic prasugrel treatment. The RSNO formed can potentially act as a NO donor.

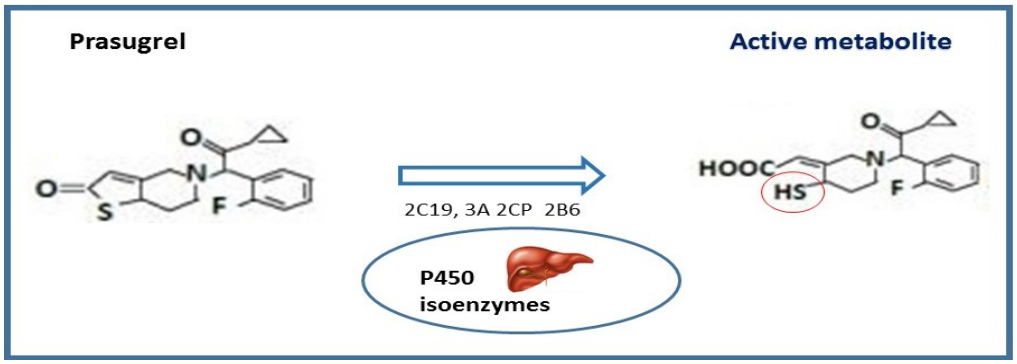



*Figure 1.8: Molecular structure of prasugrel and its metabolism*

### Pleiotropic effects of Prasugrel

In addition to its inhibitory effect on platelets, Prasugrel metabolites can also cause an inhibition of neutrophil activation, thereby modulating the inflammatory process. This effect is

believed to be independent of $P2Y_{12}$ receptor influence (138). Totani *et al.* demonstrated that treatment of mice with prasugrel resulted in inhibition of TXB2 production, inhibition of P-selectin expression, platelet –PMN adhesion, reduction of tumour necrosis factor α synthesis and increased NO metabolites in endotoxin treated mice *in vivo* (139). Thereby limiting the platelet mediated inflammatory process.

### 1.4.2.2 **Non-Thienopyridines**

#### 1.4.2.2.1 **Ticagrelor**

Ticagrelor is a novel oral antiplatelet medication licenced for use in ACS. It belongs to a new chemical class of drugs called cyclopentyltriazolopyrimidines. Cangrelor was the first agent to be developed in this class, which has a chemical structure like adenosine. Cangrelor does not require prior metabolism and is a short acting drug and is available only in intravenous form.

Ticagrelor was developed by modifying the chemical structure of cangrelor (Figure 1.9). Ticagrelor is marketed as a more stable drug which can be administered orally, making it a preferred antiplatelet agent in its class.

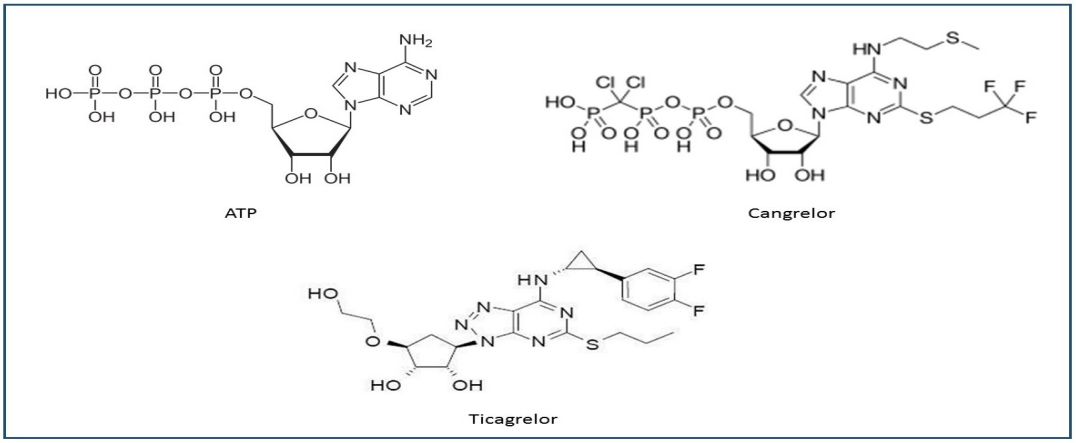



*Figure 1.9: Molecular structure of Adenosine triphosphate, Cangrelor and Ticagrelor*

Ticagrelor is a very potent direct acting $P2Y_{12}$ receptor antagonist. It is an active drug, does not need prior metabolism, and binds reversibly and non-competitively with the $P2Y_{12}$ receptor (140, 141). This changes the conformation of the $P2Y_{12}$ receptor and inhibits ADP induced G protein activation (142).

Ticagrelor is rapidly absorbed orally with bioavailability of ~36% and reaches a peak concentration ($T_{max}$) in 1.3-2 hours (143, 144). AR-C124910XX is a main metabolite formed quickly via CYP3A4 metabolism. Ticagrelor and its metabolites are both highly protein bound (99.7%) and pharmacologically active. Ticagrelor has a rapid (30min) onset of antiplatelet activity. It has a moderate half-life of 6.7 to 9 hours, hence a twice per day regime is recommended.

Ticagrelor therapy is typically initiated at a higher dose of 180mg (loading dose) and then reduced to a continued therapy at 90mg twice daily. Ticagrelor causes maximum platelet inhibition (80%) within 1hr and peak inhibitory platelet aggregation achieved ~ 2 hours in comparison to 7.8 hours with clopidogrel. (145)

Ticagrelor blocks adenosine diphosphate (ADP) receptors of the $P2Y_{12}$ subtype like the thienopyridines (clopidogrel and prasugrel). It has a different binding site from ADP, hence an allosteric antagonist (146). 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. This results in reversible, concentration dependent inhibition of the receptor (147).

The Platelet inhibition and patient outcome (PLATO) trial evaluated the efficacy of ticagrelor (180mg loading/90mg BD) against clopidogrel (600/300mg loading and 75mg OD) in prevention of cardiovascular events in patients with ACS. There was a significant reduction in cardiovascular death and MI at 30 days and 12 months with ticagrelor compared to clopidogrel. There were no differences in major bleeding in both groups. The benefit was noted across all groups of patients in this study, irrespective of patient's age, type of ACS, type of therapy (conservative or invasive therapy) (148, 149).

Finally, the PEGASUS-TIMI 54 trial was a randomised placebo-controlled study which tested the feasibility of long-term usage of ticagrelor with aspirin in patients who had previous MI. There was significant reduction in risk of CV death, MI and stroke with ticagrelor therapy at 3

years. The bleeding rates were higher in ticagrelor treated patients compared to placebo. There is no clear consensus for use of long-term ticagrelor use in high risk ACS patients at this moment (150).

### Pleiotropic effects of ticagrelor

There was a significant reduction in all-cause mortality with ticagrelor therapy compared to clopidogrel in the PLATO trial (148) and in a sub group analysis of CABG patients in PLATO ticagrelor therapy had a reduction in vascular and infection related death (151), which was an unexpected finding. This finding fuels the possibility that ticagrelor does have pleiotropic effects independent of receptor blockage and this may have contributed to mortality benefit. Patients on ticagrelor in PLATO had fewer incidences of chest infection, sepsis and infection related deaths. It has been hypothesised that ticagrelor could potentiate the effect of adenosine on neutrophil chemotaxis and phagocytosis. Alsharif *et al* showed an inhibition of cellular adenosine reuptake by ticagrelor potentiates the adenosine mediated effect on neutrophil chemotaxis and phagocytosis, with a potential influence on host defence against bacterial infections (152). However, the PEGASUS trial did not show a reduction in infection related death in the ticagrelor group compared to placebo, which could be due to the different populations studied.

Clopidogrel and ticagrelor reduce the release of pro-inflammatory cytokines TNF-α but ticagrelor also reduces interleukin 6 (IL-6) in a human sepsis model (128).

## 1.5 Nitric oxide

Nitric oxide (NO) is a key mediator in a variety of physiological processes (153). NO was first identified in 1777 by Joseph Priestly. Until late 1970's it was considered an atmospheric pollutant. Dr Furchgott in 1980, identified a molecule which caused smooth muscle to relax. He called the molecule endothelium derived relaxing factor (EDRF)(154). Dr Murad in separate research found that nitro-glycerine, a drug that contains nitrate groups and known to be an effective anti-anginal, works by releasing NO and subsequently relaxing vascular smooth muscle(155). In 1986 Dr Ignarro demonstrated by biochemical analysis that EDRF

was identical to NO. After more than a decade later(1992) NO was fully recognised and named the molecule of the year, and the three US scientists who discovered it received the Nobel prize for physiology and medicine in 1998.(156)

NO is a diatomic colourless odourless gaseous molecule consisting of oxygen and nitrogen bound together by covalent bonds. NO is a free radical with a free unpaired electron between the two atoms. It is highly reactive and has a short life (milliseconds)(153). Due to these features its radius of effect is limited only up to 100 μm or less from its source of origin. Overall, NO concentration is a balance between its rate of formation and its rate of reaction/decomposition.

In humans, NO is formed via endogenous synthesis and through exogenous oral nitrate intake(157).

Endogenous NO is synthesised from the amino acid L- arginine by the enzyme nitric oxide synthase (NOS). There are three main isoforms of NOS:

a) Neuronal type (nNOS, NOS1) primarily regulates neurotransmission and neurotoxicity, with some evidence of vasomotor regulation.
b) Macrophage or cytokine inducible type (iNOS, NOS2) which regulates immune responses.
c) Endothelial type (eNOS, NOS3) involved in vasomotor regulation.(158)

Mitochondrial NOS (mtNOS) is a subtype of NOS found in mitochondria, generating NO, which is a crucial biochemical regulator of mitochondrial function (159).

Endothelial NOS (eNOS) is present predominately on the vascular endothelium. eNOS is a calcium/calmodulin dependent oxidoreductase dimer. Shear stress as a result of blood flow activates $\mathrm{NF\kappa B\ and}$ promotes eNOS expression by serine phosphorylation (160). eNOS catalyses generation of NO in vascular endothelium from L-Arginine. It is a two-step reaction that starts with hydroxylation of L Arginine using two electrons from nicotinamide adenine dinucleotide phosphate (NADPH) and one molecule of oxygen ($O_2$) to form N-hydroxy-L-Arginine. N-hydroxy-L-Arginine forms NO and L-Citruline by utilising a second oxygen molecule and one electron from NADPH. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydro-l-biopterin ($BH_4$) are essential cofactors. The cyclic

conversion of cofactor $BH_4$ to trihydrobiopterin radical ($BH_3^-$) is essential for eNOS activity (Figure 1.10) (161, 162).

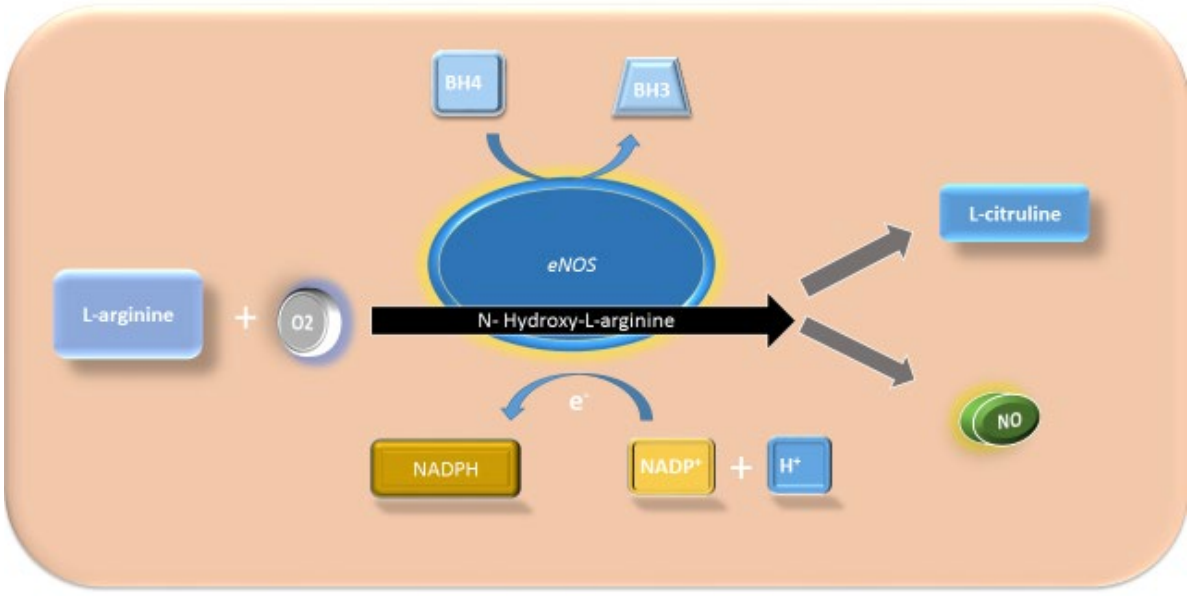
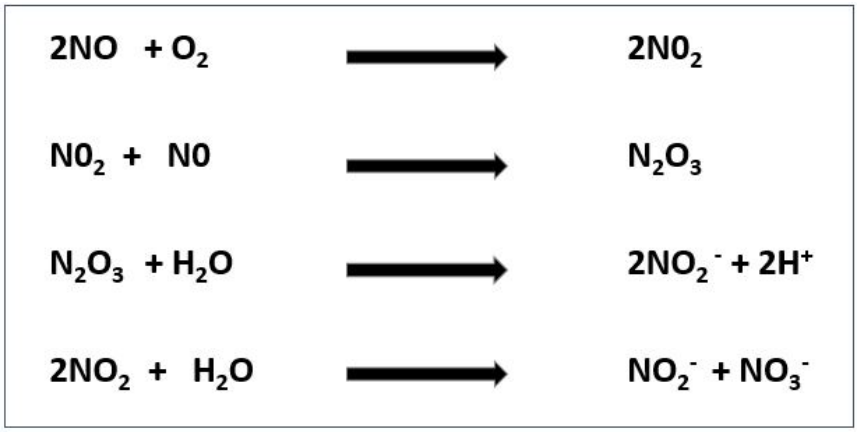



*Figure 1.10: Endogenous synthesis of NO via eNOS*

NO has a short half-life (milliseconds), it instantaneously reacts with reactive oxygen radicals (ROS) like superoxide ($O_2^{*-}$) in cells and in blood at almost diffusion limited rates. NO in aqueous solution reacts more slowly with oxygen and water to form a series of nitric oxides which are stable endocrine molecules (Equation 1.1). Nitrogen dioxide ($NO_2$) is hydrolysed to equimolar amounts of nitrite ($NO_2^-$) and nitrate ($NO_3^-$). In human blood the half-life of $NO_2^-$ and $NO_3^-$ is 110 seconds and 5-8 hours, respectively (163).




*Equation 1.1: NO is auto-oxidised with oxygen in aqueous solution to nitrogen dioxide($NO_2$),(1) which further reacts with NO to form nitrogen trioxide ($N_2O_3$)(2) . These oxides are further hydrolysed to nitrite and nitrate.*

NO reacts extremely quickly with ROS, produced by xanthine oxidase and NADPH oxidase present on the vessel wall. ROS are formed from oxygen metabolism, they are potentially harmful to cellular proteins, lipid, and DNA (164). ROS are highly reactive due to the presence of unpaired electrons. NO reacts with ROS to form damaging peroxynitrate ($ONOO^-$) which can under certain conditions act as an alternative source of nitrite. ROS and $ONOO^-$ are elevated in disease conditions, thus contributing to reduce NO effectiveness.

In blood, NO reacts with both oxygenated and deoxygenated haemoglobin forming methaemoglobin and nitrosylhaemoglobin respectively. This process is believed to be the main mode of degradation of NO. Under physiological conditions nitrosyl-haemoglobin (HbNO) has limited significance as an NO donor due to its high affinity (1500 times higher than CO in hypoxia) and slow disassociation rate. NO can bind to a cysteine thiol group of haemoglobin, forming an S-nitroso haemoglobin (HbSNO), which is more stable and can travel to more distal part of circulation and release NO to regulate microvascular blood flow in deoxygenated tissues (165). Myoglobin has a similar nitrite bio-activation role in low oxygen conditions, especially in myocardial ischaemia/reperfusion injury (166).

NO forms s-nitrosothiol (RSNO) through reacting with reduced thiols on protein (mainly cysteine residues) and other molecules (Equation 1.2).

$$N_2O_3 + R\text{-}SH \longrightarrow H^+ + NO_2^- + RSNO$$

*Equation 1.2; Nitrogen trioxide reacts with molecules containing a thiol group to form nitrosothiols and nitrites, RSNO are more stable bi products of NO metabolism with a half-life of 1-40min.*

It is currently not possible to measure the amount of NO directly in humans due to its high reactivity and very short half-life. Nitrite, nitrate and RSNO are relatively stable metabolites of NO with half-life of 110 seconds, 8 hours and 1-40 minutes respectively, hence these metabolites are measurable (163, 167, 168).

NO is eventually metabolised to nitrate and excreted in urine. NO metabolites including nitrite, nitrate and RSNO are now considered not only as a direct measure of physiological activity of NO, but also as a biologically active NO reservoir. These intermediate molecules act as storage pools of NO in plasma and tissues (mitochondrial, endothelium)(157, 163).This can be recycled back to NO under appropriate physiological and pathological conditions.

### 1.5.1 Nitric oxide in the cardiovascular system

NO readily penetrates the membranes of neighbouring cells and through binding with soluble guanylate cyclase converts guanosine monophosphate (GMP) to cyclic GMP (cGMP). cGMP along with cAMP acts as an important second messenger in the smooth muscle relaxation(169) and subsequent vasodilation. The physiological effects of cGMP are predominantly mediated through activation of cGMP-dependent protein kinases, cyclic nucleotide-gated (CNG) ion channels and the activation or inhibition of phosphodiesterase (PDEs). NO can also exert its effects via cGMP-independent signalling, such as S-nitrosylation of target proteins, activation of sarco/endoplasmic reticulum calcium ATPase and production of cyclic inosine monophosphate (cIMP)(170).

NO within the vascular lumen exerts a potent inhibitor effect on platelet aggregation and adhesion via both cGMP dependent and cGMP independent mechanisms (162). It also promotes angiogenesis, inhibits endothelial cell apoptosis (133, 171-173), and several other critical roles in maintaining normal vascular homeostasis.

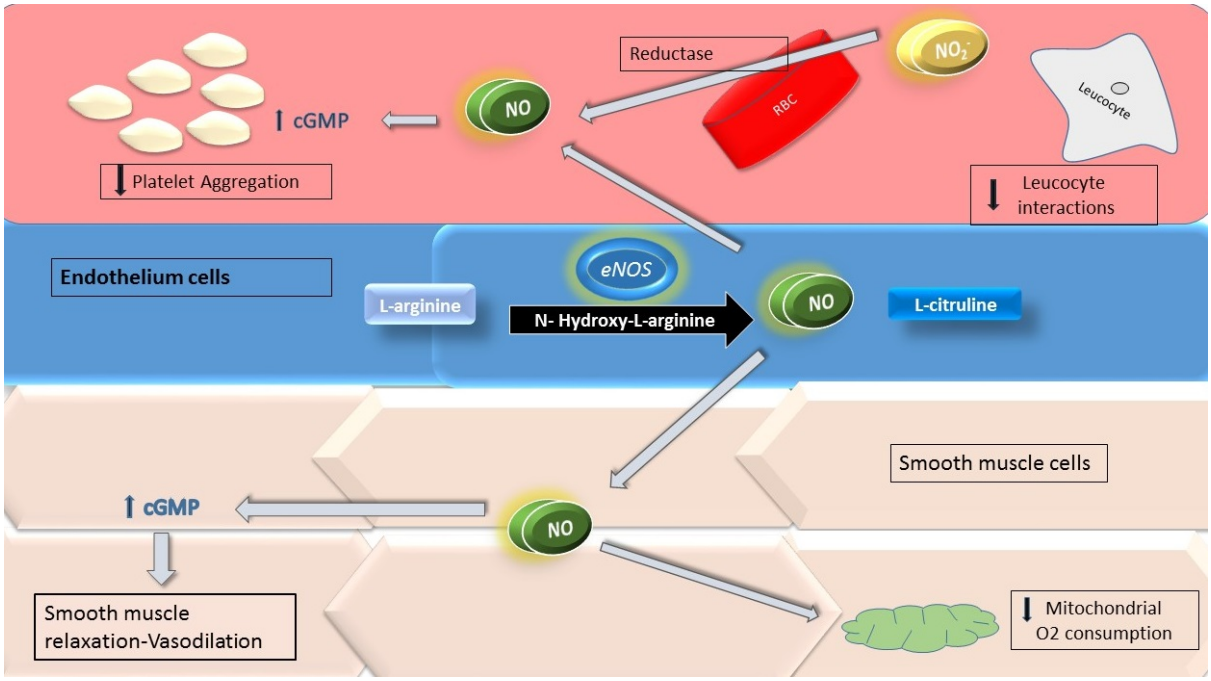



*Figure 1.11: Physiological actions of NO on the vasculature and its crucial role in cardiovascular physiology*

### 1.5.2 Nitrosothiols

Nitrosothiols (RSNO) are endogenous metabolites of NO, exhibiting similar biological functions as NO in vivo as well as in vitro. RSNO are ubiquitous molecules formed by nitrosation of nitrosonium cation ($NO^+$) and thiol anion ($RS^-$). Plasma contains ~600 μM thiol groups, majority of which are albumin, GSH, and cysteine. The electrophilic interaction of these thiols with oxides of nitrogen generates RSNO (Equation 1.2).

They have the general formula of RSNO, R representing the protein side chain of a peptide containing cysteine. The type of protein and R group determines the RSNO formation kinetics and chemical properties. RSNO can be formed by endogenous NO produced by eNOS, from dietary sources, or from exogenous sources like S-nitrosoglutathionine (GSNO) (170, 174). Freely available RSNOs include S-nitrosocysteine, S-nitrosoglutathione and S-nitrosoalbumin. Plasma S-nitrosoalbumin levels are positively correlated with NOS activity (170). Typically large protein RSNO is more stable than low molecular weight RSNO(175).

RSNO have longer half-life than NO and are considered to be NO donors, and transfer of $NO^+$ across the plasma membrane via protein disulphide isomerases(176). RSNO are stable at $37^0$ C and pH of 7.4 in the presence of transition metal ion chelators (177). NO release from RSNO is facilitated by numerous factors such as light, heat, superoxide, transition metals, and enzymes like xanthine oxidase (178), superoxide dismutase and various dehydrogenases (179, 180). NO can be transferred from a donor S-nitrosylated protein to an acceptor S-nitrosylation substrate and this is referred as transnitrosation (equation below). Transnitrosation of thiol represents a potential mechanism of modification of protein and enzyme activity.


RSNO can react with thiolate anion forming a disulfide and nitroxyl anion at intracellular level in response to oxidative stress. This process of S-thiolation is more stable than S –nitrosation. The fate and effect of by-products in vivo is unknown(181).


RSNO activate soluble guanylyl cyclase (sGC) by being converted to dinitrosyl ion complexes which give $NO^+$ to the sGC haem moiety. Thiol groups on haemoglobin does bind to $NO^+$ to form nitrosohaemoglobin (HbSNO). S-nitrosation of haemoglobin increases oxygen affinity at low oxygen concentrations (182, 183). S-nitroso-N-acetyl penicillamine can directly stimulate guanylyl cyclase via interacting with the enzyme haem (184). GSNO can act as a substrate for several enzymes that utilize glutathione (185).

RSNO exhibits potent vasodilatory (186-188) and platelet inhibitory effects (189). These effects are largely attributed to NO release and direct binding to sGC (177, 187). HbSNO in the presence of glutathione facilitates hypoxia mediated vasodilation (182).The platelet inhibition effect is achieved at lower concentrations than those required for vasodilation (190, 191). RSNO also exhibits cytoprotective effects against cellular toxicity associated with oxidative stress (192) and immunosuppressive effects.

The role of endogenous RSNO in normal cellular function and physiology is not well known. There are many potential clinical uses of RSNO which are yet to be fully elucidated and exogenous RSNO compounds have been extensively researched for their therapeutic potential. Exogenously administered nitrosylated bovine serum albumin has shown a coronary vasodilatory and antiplatelet effect in animal studies (193, 194). In humans, GSNO can inhibit platelet aggregation without affecting vascular tone. The platelet specificity is related to a mechanism by which platelets metabolize GSNO and this effect can be significantly decreased by use of copper specific chelators. Platelets have cell surface copper containing proteins that promote the release of NO from GSNO, and platelets can also concentrate GSNO and release upon activation by neutrophils. Human studies with GSNO administration as an antiplatelet agent showed a marked reduction in thrombotic embolization after carotid endarterectomy surgery (195), reduced clot formation in coronary artery bypass grafts (196) and normalised platelet function in an aggressive form of preeclampsia (197). RSNO have shown to improve ischaemia/reperfusion injury in the heart (198) and liver (199), relax bronchial smooth muscles and has antimicrobial effects in animal model studies.

RSNO have great potential as therapeutic agent. They are a useful alternative to organic nitrate due to their lack of development of tolerance. RSNO is available only in intravenous form and currently only used in a small number of animal and human studies(168, 200-204).

Antiplatelet medications belonging to the thienopyridine class of drugs (Clopidogrel and Prasugrel) exhibit a critical thiol group. These active metabolites bind to the $P2Y_{12}$ receptors on the platelet surface, inhibiting platelet activation and clot formation *in vivo.* Our research group successfully demonstrated an in vitro formation of thienopyridine-SNO with pharmaceutical grade preparations of clopidogrel (133) and prasugrel (137).

### 1.5.3 Nitric oxide and endothelial dysfunction

As described above, NO plays a central role in the regulation of vascular tone and the maintenance of vascular haemostasis. Impaired production of endogenous NO is a hallmark of endothelial dysfunction. Cardiovascular risk factors like smoking, age, hypertension, hypercholesterolemia, diabetes and family history of Ischemic heart disease are associated

with endothelial dysfunction (50, 51, 205). Endothelial dysfunction is a vital step in atherosclerosis, leading to development of coronary artery disease.

Endothelial dysfunction occurs as a consequence of progressive oxidative stress and inflammation, causing an abnormality of G protein signalling, elevated asymmetric dimethyl arginine, decrease in tetrahydrobiopterin and a deficiency in L-arginine. In this sense decreased NO is the result of either decreased NO production or increased NO denaturation.

In CVD there is parallel enhancement of NADPH oxidases and eNOS. $O_2^-$ derived from NADPH oxidases and/or xanthine oxidases may combine with NO· and increase the formation of peroxynitrite ($ONOO^-$). $ONOO^-$ oxidises $BH_4$ to biologically inactive products such as $BH_3$· radical or $qBH_2$. $ONOO^-$ also causes oxidative damage to eNOS resulting in destabilization of the dimer interface. As a consequence of this, eNOS is uncoupled, becomes dysfunctional and is converted into a superoxide-generating enzyme (206, 207) adding to a vicious cycle of oxidative insult (Figure 1.12).

Lack of endogenous NO synthesis is a critical component in CVD. Recently there has been a great interest in improving the NO bioavailability in patients with endothelial dysfunction through exogenous (dietary) sources.

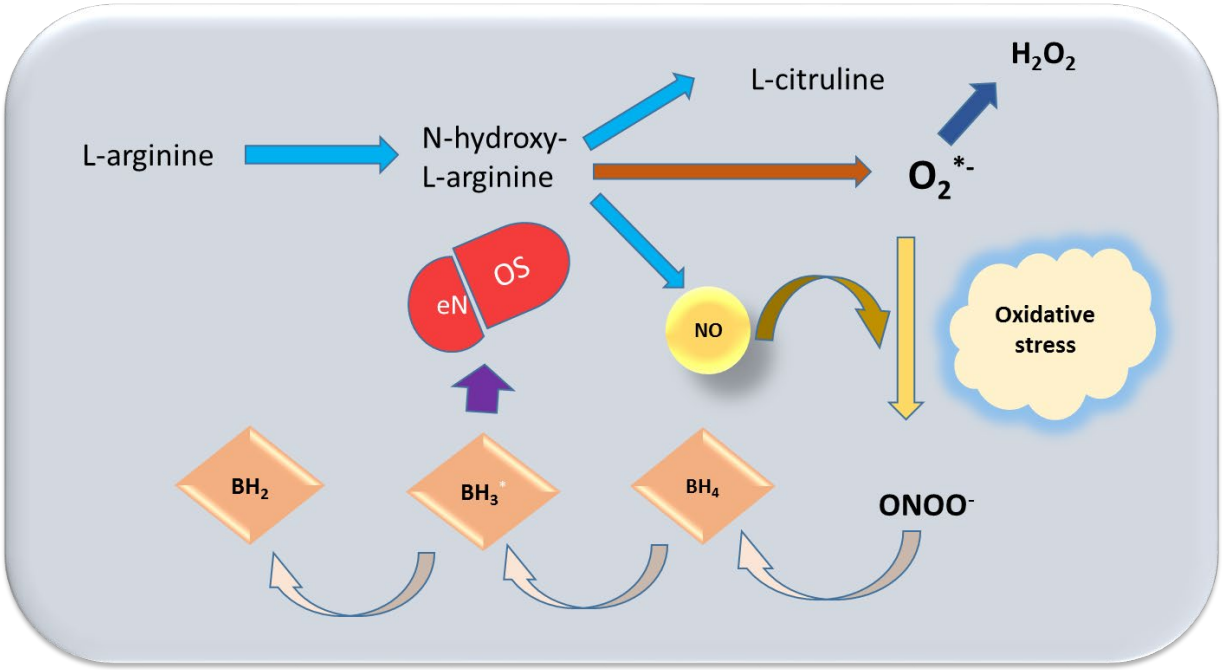



*Figure 1.12: Diagrammatic description of the effect of endothelial dysfunction and oxidative stress on the functioning of eNOS system*

### 1.5.4 NO resistance and platelet function

NO is an important down regulator of platelet function, largely through activation of soluble guanylyl cyclase and subsequent cGMP formation as described above. There are some reports of intrinsic expression of eNOS in platelets(208, 209), thereby generating their own NO. However this remains a contentious issue(210).

NO resistance has been well described in CAD patients, but the exact mechanism is not known(211, 212). NO resistance is likely due to NO scavenging in oxidative conditions or impaired NO-sGC activity. It has been considered as an independent predictor of mortality and morbidity in CVD patients. Borgognone et al showed platelet NO resistance is an intrinsic phenomenon, which was successfully circumvented by nitrite causing a direct activation of sGC and phosphorylating VASP serine 239 independent of NO in patients with heart failure with preserved ejection fraction (HFpEF)(213). This study proves that inorganic nitrite substitution through diet could overcome the impairment in the NO-cGMP pathway (NO

resistance) in CVD and could improve outcomes. It is well established that nitrite forms RSNO as described above, and RSNO has a dual role of being a potent NO donor and a direct stimulator of sGC. It could be postulated that the ability of nitrite to circumvent NO resistances could also be due to its role in RSNO formation.

## 1.6 Dietary Nitrate (inorganic nitrate) supplementation.

Nitrate in humans originates from two sources, first via oxidation of endogenous eNOS derived NO and second through diet.

Dietary nitrate is an important exogenous source of nitrate; nitrates are normal constituents of our daily diet and vegetables provide 60-80% of daily nitrate intake in the typical western diet (214, 215). A vegetarian diet contains about 4.3 mM nitrate whereas a normal diet has approximately 1.2mM of nitrate. The cardiovascular benefits of a Mediterranean and Japanese diet is mainly attributed to high fruit and vegetable intake which are high in dietary nitrate(216-218).

Vegetables can generally be grouped into 5 groups based on their nitrate content as shown in Table 1.1. Examples of high nitrate vegetables includes, beetroot, spinach, swiss chard, rhubarb, lettuce.

| Nitrate (mg/100g weight) | Vegetables |
|---|---|
| **Very Low (<20mg/100g)** | Asparagus, broad bean, eggplant, garlic, onion, green bean, mushroom, pepper, potato, pea, sweet potato, tomato, watermelon |
| **Low (20-49mg/100g)** | Broccoli, cauliflower, cucumber, carrot, chicory, pumpkin |
| **Medium (50-99mg/100g)** | Cabbage, turnip, dill |
| **High (100-249mg/100g)** | Chinese cabbage, fennel, celeriac, endive, fennel, leek, parsley |
| **Very high (>250mg/100g)** | Cress, chervil, lettuce, red beetroot, Swiss chard, rhubarb |

*Table 1.1 : Classification of fruits and vegetables based on their nitrate content.*

Inorganic nitrates have a long history in the treatment of CAD, its value was earliest recognized by the Chinese in 700 AD (219) Inorganic nitrates and nitrites were prescribed indiscriminately to treat CVD and other conditions such as lung disease, epilepsy, and edema. There was an increase in reports of nitrite toxicity in the 1950's causing it to fall out of favor. There were concerns of carcinogenesis, teratogenesis, and infantile methemoglobinemia. Stringent regulations were introduced for the last 50 years by governments and by WHO to maintain lower nitrate concentration in food and drinking water. However recent epidemiological studies have failed to prove this association and in fact have been shown to have positive beneficial effect in CVD (220). Indeed, in large scale prospective studies the association with carcinogenesis could also be associated with pre-existing medical conditions (for example gastric ulcer or infection).

### 1.6.1 Nitrate-Nitrite-NO cycle

Lundeberg *et al*, Zweier *et al*, and Benjamin *et al*, in the mid-90's demonstrated NOS-independent formation of NO in biological tissue from inorganic nitrate and nitrates (221, 222) These discoveries lead to enormous interest in this area and in particular research into the mechanism and its possible benefits in CVD. Dietary in-organic nitrate and nitrites are explored as a viable and safe source of NO augmentation in humans especially in patients with endothelial dysfunction.

The current focus is on fruits, green-leafy vegetables like lettuce, cabbage, and spinach and in super-foods like beetroot and garlic as a means of improving cardiovascular health.

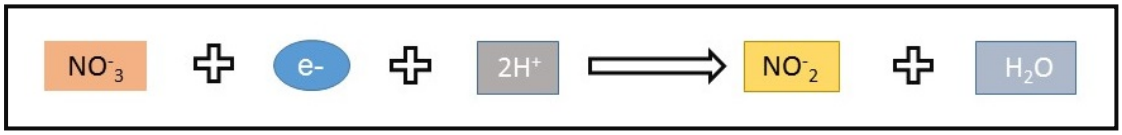
The nitrate components in these foods is believed to exert cardio-protective effects by augmenting the nitrate-nitrite-NO axis in health and CVD.

#### 1.6.1.1 **Dietary nitrate metabolism**

##### 1.6.1.1.1 Nitrate to Nitrite conversion

Nitrate on its own has relatively few direct effects in vivo, and first needs to be metabolised to nitrite, which is the biologically active product. Orally ingested nitrate is rapidly absorbed in the proximal gastrointestinal tracts into the blood stream, mixing with endogenously produced nitrate. The nitrate concentration in plasma peaks within 60 minutes of ingestion and the half-life is approximately 5 hours (215, 223).

The majority (~75%) of nitrate is excreted via the urine, and the rest is excreted mainly in the saliva and sweat. Active salivary concentration of nitrate is 10 times (200-1000 μM) higher than plasma (224-226). The commensal bacteria (facultative anaerobes) present in the GI tract (highest concentration in the oral cavity), effectively reduce nitrate to nitrite (Equation 1.3). Human cells intrinsically lack this nitrate reductase. Bacterial presence is therefore essential in this process. Gram negative bacteria *Veillonella* Spp present on the dorsum of the tongue accounts for >50% of nitrate reductase activity (215). Gram positive bacteria *Actinomyces* is the second most abundant bacteria causing nitrate reduction (157). This process of reduction of nitrate to nitrite was found to be reduced in healthy volunteers given antibacterial mouthwash (215, 227).




*Equation 1.3: Equation depicting the conversion of nitrate($NO^-_3$) to nitrite($NO^-_2$) via bacterial nitrate reductase present in the oral cavity.*

The nitrite produced in the oral cavity from salivary nitrate is then swallowed and enters the stomach, in acidic conditions some of the nitrite is converted to NO and other N-Oxides, the rest gets absorbed into the systemic circulation as nitrite.

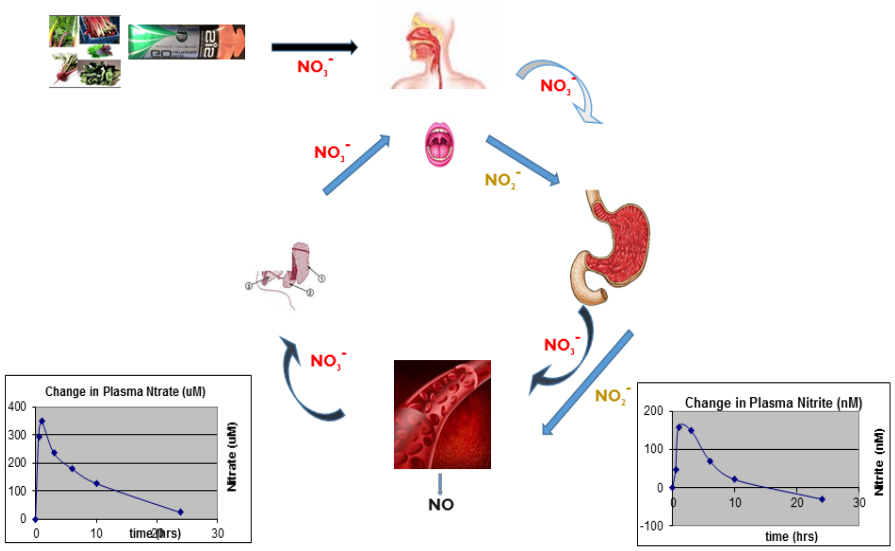



*Figure 1.13: Enterosalivary circulation of orally ingested nitrate source. The graphs on the left and right side of the diagram shows the rise in the plasma nitrate and nitrite levels respectively with time upon ingestion of dietary bolus in the form of Swiss chard containing gel (SIS® ltd)*

Our group investigated the rise in plasma nitrate and nitrite levels in 10 healthy volunteers after ingestion of a dietary nitrate supplement (a single bolus of SIS® gel containing 4.8mmol nitrate) which showed similar pharmacokinetic pattern (Figure 1.13) as demonstrated by Kapil *et al* (227).

Plasma nitrate levels rise to a significant level at 60-90 minutes and reaches a peak in 2 to 2.5 hrs of ingestion of dietary nitrate. There is a slight delay in the rise of plasma nitrite level compared with nitrate due to the requirement for entero-salivary reduction of nitrate to nitrite. It reaches a peak in 2.5 to 3 hours.

#### 1.6.1.1.2 Nitrite to NO reduction

There are numerous eNOS independent pathways that generate NO from the reduction of nitrite. In biological systems this is largely an enzymatic reduction process through allosteric reactions catalysed by nitrite reductase. Examples include xanthine oxidase (XOR), the Heme moiety of haemoglobin/myoglobin, polyphenols, cytochrome P450 and protons(228-230). In physiological conditions, globin acts as primary nitrite reductase source. In pathological conditions like acidosis and hypoxia, xanthine oxidase dominates as a nitrite reductase and generates NO (231, 232). In strong acidic environments, nitrite chemically disproportionated to NO – a factor that we utilise to good effect during the measurement technique (see Methods section).

CVD like hypertension, atherosclerosis, and CVA are associated with endothelial dysfunction and reduced NO production. Under hypoxic and ischaemic conditions, nitrite conversion to NO via chemical acidification or by enzymatic conversion can facilitate vasodilatory, platelet inhibition, angiogenesis, and endothelial cell proliferation.(133, 171) NO also provides protection against ischaemia/reperfusion injury in myocardial and cerebral vasculature (233, 234).

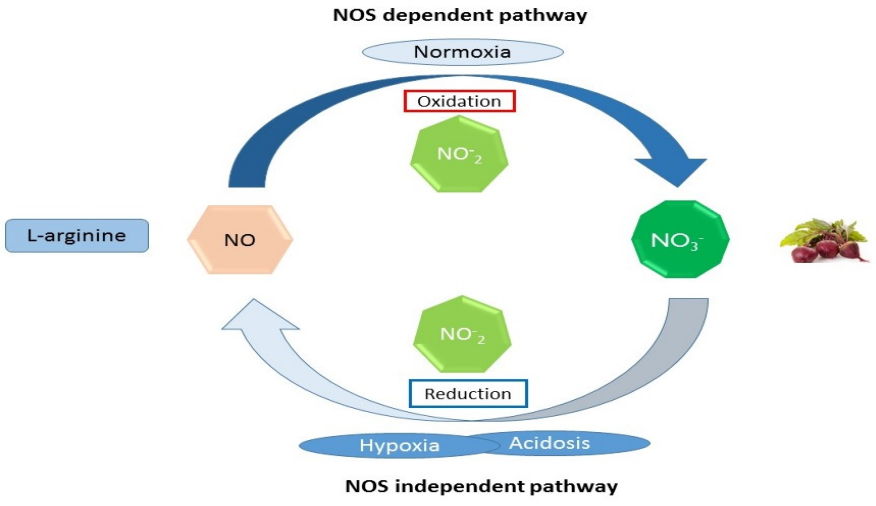



*Figure 1.14: Endogenous NO generation via eNOS dependent and eNOS independent mechanisms under different conditions, adapted from O Lundberg et al., 2008. (32).*

It is now well established that there are primarily two pathways to generate bioactive NO. The Classical pathway of conversion of L- Arginine and oxygen through NO synthases (NOS). This is oxygen dependent and NO generation becomes limited as oxygen levels fall. Under conditions of reduced oxygen, the NOS-independent pathway for NO generation is enhanced. This pathway is also known as the nitrate-nitrite-NO pathway (Figure 1.14)

The Nitrate-nitrite-NO axis complements the conventional endogenous generation of NO by NO synthases (NOS) (235, 236). Parallel actions of both pathways can ensure a sufficient amount of NO within the tissues.

#### 1.6.1.1.3 Clinical application of in-organic nitrate therapy

Supplementation of diet with inorganic nitrate is being studied extensively as a cost-effective therapeutic intervention to augment intravascular NO level in patients with endothelial dysfunction and healthy individuals participating in competitive sports.

A clinically relevant blood pressure lowering effect of orally ingested dietary nitrate supplement is well established in trials conducted both in healthy volunteers and hypertensive patients (226, 227, 237).

Larsen *et al* first demonstrated an enhance exercise performance in well trained male cyclist upon ingestion of dietary nitrate supplements. There was significant lower oxygen consumption ($VO_{2peak}$) with no increase in blood lactate concentration(238). Several studies thereafter showed a variable effect on exercise performance. A recent meta-analysis of 76 eligible trials concluded a positive outcome on endurance exercise capacity with less likely effect on time-trial performance (239).

Oral administration and intravenous infusion of nitrite in mice showed a protection against myocardial and hepatic ischaemia reperfusion injury and improved cardiac performance in the setting of heart failure (233). A recent clinical study in patients with ST elevation MI demonstrated a reduction in infarct size in patients who had intracoronary infusion of sodium nitrite (240). Interestingly, there was no change in infarct size in patients who were given intravenous sodium nitrite(241). Nitrate rich beetroot juice supplementation in patients with

heart failure demonstrated improved exercise capacity and endurance, likely due to improved skeletal muscle activity (242-244).The role of NO in cerebrovascular disease has also been well studied, with several studies suggesting a prospect of attenuation of ischaemic and reperfusion brain injury after stroke with nitrate or nitrite administration (245-247).

Dietary nitrate supplementation has been shown to reduce platelet reactivity in healthy human volunteers (248). However, there is a lack of published data on the effects of nitrate supplementation on platelet reactivity in patients with known coronary artery disease. Hence it is clear there is a need to understand the role of naturally occurring supplements in diseased states. This thesis addresses this directly and aims to establish the influence of nitrate supplement on platelet inhibition and nitrate metabolism in CAD patients.

#### 1.6.1.1.4 Commercially available dietary nitrate products

Nitrates are fast gaining a reputation as a health and performance enhancing nutritional supplement. Beetroot juice is the most commonly used nitrate supplement across the published studies and is well-known for its high nitrate content (>250mg/100g fresh vegetable). Dietary nitrate products from natural sources are commercially available for general population use. These products are very popular among individuals participating in sports. Some of the more commonly available dietary nitrate products include, Holland & Barrett beetroot capsules, bio-synergy power beet-beetroot capsules, Raspberry extract, Beet it, and Science in sport (SIS®) Go plus gel (*SIS® Go+)*.

*SIS® Go+ nitrate gel* (SIS® Limited, UK), is a natural inorganic nitrate supplement gel, based on Swiss chard, manufactured and marketed in UK by SIS® limited. It is available as a 60 ml sachet in all leading superstores as an over the counter product. The cardio-beneficial effects are well studied in the field of sports science. *SIS® Go+ nitrate gel* is a widely available sports gel with inorganic nitrate extracted from natural resources. This form of nitrate supplement is very popular amongst cyclists and other endurance athletes. Celebrated sports personalities like Sir Chris Hoy, Helen Jenkins and Team GB rowing athletes have endorsed and used this product to help them achieve improved muscular effort during performance (249).

”Beet- it” is another popular product readily available in most supermarkets across UK. Beetroot products have been shown to enhance physical stamina and recovery in trained athletes (0.5l/day for 6days) (250). The constituents of SIS® Go+ Nitrate and Beet-it are described in Table 1.2 for comparison.

The plasma nitrate and nitrite levels are enhanced upon ingestion of these dietary nitrate supplements, and our group studied the pharmacokinetics of SIS® Go$^{+}$ Sports gel in healthy human volunteers. 10 healthy volunteers were given 2 sachets of SIS® Go$^{+}$ nitrate Gel. Blood samples were collected for up to 24 hours after ingestion of the gel. Isolated plasma samples were analysed for nitrite and nitrate levels and were plotted against time. (Figure 1.15). The peak concentration of nitrite and nitrate occurred at 90 minutes and 120 minutes respectively.

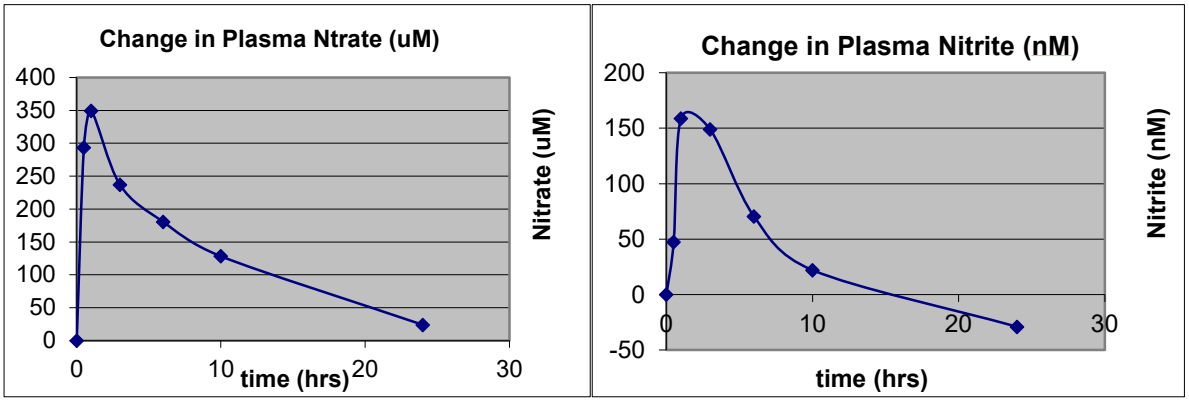



*Figure 1.15: Plasma levels of nitrite and nitrate with time upon ingestion of 120ml of SIS® Go$^{+}$ nitrate supplement. Plasma nitrate levels and plasma nitrite levels measured in micromolar and nanomolar respectively.*

| Commercial product-Ingredients | |
|---|---|
| ***SIS® Go⁺ nitrate gel* (60ml/Sachet)** | |
| Swiss chard juice concentrate (15%) |   |
| Maltodextrin (from maize) (5.5%) | |
| Rhubarb juice concentrate (4%) | |
| Acidity regulator (citric acid, sodium citrate) | |
| Preservatives (potassium sorbate, sodium benzoate | |
| Sweeteners (acesulfame K, sucralose) | |
| Flavoring | |
| Folic acid | |
| Gelling agents (xanthan gum, gellan gum) | |
| Water | |
| ***Beet It® ( 500 ml)*** | |
| Pressed organic beetroot juice (90%) |   |
| Pressed organic apple juice (10%) | |

*Table 1.2: Ingredients and constituents of the leading commercially available nitrate supplements*

Luke *et al*. demonstrated a similar extent of increase in plasma nitrate, nitrite levels upon ingestion of beetroot juice and chard gel (SIS® Go⁺) in their study on healthy human volunteers, intriguingly there was a greater increase in RSNO with ingestion of beetroot juice in comparison to chard gel, which was believed to due to higher content of phenols in the chard gel (251).

## 1.7 Antiplatelet therapy and Nitric oxide

### 1.7.1 Clopidogrel and nitric oxide metabolism

It is considered that the clinical benefit of clopidogrel is due largely to its antithrombotic and antiplatelet activity. Recently, off target effects have emerged including improvement in endothelial dysfunction in stable CAD patients via a mechanism independent of platelet function (107, 130, 132, 252) , and improvement in brachial artery vasodilation independent of endothelial NO production with higher clopidogrel doses (107, 132, 252).

*In vivo*, the active metabolite of clopidogrel (as with all Theinopyridines) exhibits a critical thiol group that governs its interaction with platelets. This prompted investigation at the WHRI into whether active clopidogrel might form nitrosothiol (-SNO) derivatives. Indeed, the James group have shown direct nitrosothiol formation from clopidogrel, prasugrel, and ticlodipine formulations when under acidic conditions and in the presence of background nitrite. The RSNO formed exhibits direct anti-platelet and vasodilator activity (133). This occurs without the need for prior metabolism. The group has since demonstrated increased NO metabolites in CAD patients following an acute or chronic dose of clopidogrel (253). Clopidogrel-SNO also causes an endothelium independent vasodilatory effect. Clopidogrel SNO also participate in transnitrosation reaction with other thiol groups (133). Taken together, this implies clopidogrel induced RSNO formation might represent an important and yet unstudied pathway in CAD patients.

Clopidogrel-SNO can exert its antiplatelet activity potentially via three mechanisms. Firstly, it activates the cGC via $NO^+$ causing an inhibition of intracellular calcium influx, thereby dampening the $P2Y_{12}$ mediated activation(254). Secondly, it can directly inhibit the activation of P12K pathway by TRAP (255). Finally, by nitrosation of proteins like tyrosine residue of COX1 enzyme within the platelets, which inhibits the conversion of arachidonic acid to TXA (256).

It is noteworthy that ideal conditions exist in humans for formation of thienopyridine RSNO. Orally ingested clopidogrel enters the acidic environment of a fasting stomach, and encounters nitrite present in saliva and the stomach (<20μM) favouring expression of thiol groups on the parent drug and formation of clopidogrel RSNO (Figure 1.16). In *vitro* studies have showed

that formation of SNO is dependent on nitrite concentration, the dependence is linear up to the point of saturation of free thiols and the availability of clopidogrel substrate.

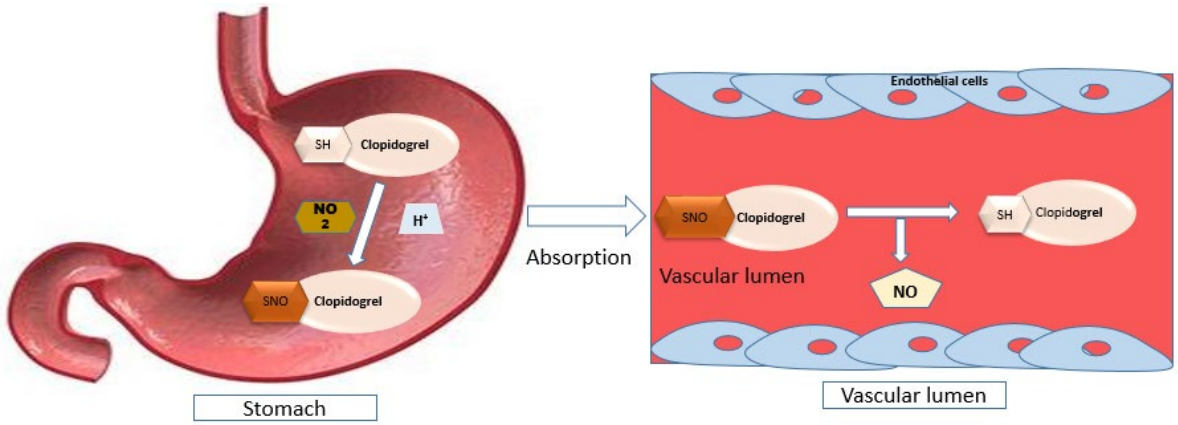



*Figure 1.16: Clopidogrel SNO formation in vivo in the stomach and its absorption into the blood vessel where it acts as a NO donor.*

### 1.7.2 Prasugrel and nitric oxide

James *et al* have successfully shown that prasugrel can also readily form nitrosothiol. Prasugrel exhibits a higher number of free thiols than clopidogrel and ticlopidine, and consequently forms SNO more readily *in vitro* (133),

In CAD patients our group have recently demonstrated an increase in circulating SNO store following prasugrel in the acute and chronic setting (137). More importantly, the occurrence of plasma SNO following prasugrel was found to depend on co-treatment with PPI (which alters stomach pH and thus SNO formation) which was inversely associated with the extent of platelet inhibition in patients.

### 1.7.3 Thienopyridine-SNO

Clopidogrel and prasugrel readily undergo nitrosation, and form SNO in the presence of nitrite and in low $P_H$ without need for in vivo metabolism. Prasugrel-SNO is more stable in comparison with Clopidogrel-SNO (137).

The potential for antiplatelet drugs, such as clopidogrel and prasugrel, to influence this circulatory NO pool and enhance 'pleiotropic' non-platelet effects of these drugs in the circulation are as yet poorly characterised.

These findings warranted a further investigation to understand the formation and the role of clopidogrel-SNO in patients with stable CAD and their association with nitrate supplements.

### 1.7.4 Non-thienopyridine-SNO

Ticagrelor lacks a free thiol group with the sulphydryl group contained within the ring structure of the molecule (as shown in **Error! Reference source not found.** ), hence it was predicted that ticagrelor will not have the ability to form an S-nitrosothiol products.

*In vitro* experiments conducted in our laboratory by Dr Thornhill as a part of his thesis showed ticagrelor formed RSNO efficiently as the pH dropped below 3 in the presence of background nitrite and to a greater degree compared with RSNO formation with the thienopyridine drugs clopidogrel, ticlodipine and prasugrel (133). This was an important and unexpected finding. In addition to this finding, when subjected to a pH of below 3 the parent drug lost its ability to inhibit platelet aggregation completely in response to activation by either ADP or TRAP agonist in platelet rich plasma (137, 257). Commercially available ticagrelor tablets have a waxy coating, and there was no difference in time for the whole table to dissolve in simulated gastric medium at various pH levels (within 4 minutes). Extrapolated to the *in vivo* patient scenario, ticagrelor molecules would be fully exposed to the acidic environment in the human stomach within 5 minutes of its ingestion which would render the ticagrelor parent drug ineffective at inhibiting platelets. This was unexpected given the clear effectiveness of ticagrelor as a potent antiplatelet agent in CAD patients.

Furthermore, in the PLATO trial proton pump inhibitors (PPIs, which typically rise the gastric pH levels in patients to >5) therapy was associated with increase adverse outcomes (including mortality) with both clopidogrel and ticagrelor (258).

Given the above findings of a direct effect of pH on the capacity for ticagrelor to inhibit platelet aggregation and adverse clinical outcome, along with a novel evidence of Ticagrelor-induced RSNO formation, merits further investigation.

## 1.8 Overall Thesis Aims

The benefits of antiplatelet therapy in patients with established coronary artery disease are well established. Dual antiplatelet therapy of aspirin with either thienopyridine or non-thienopyridine agents is a standard treatment for patients undergoing percutaneous angioplasty to lower the risk of stent thrombosis and myocardial infarction. Despite this, adverse events still occur.

It is well established that the beneficial effect of thienopyridine and non-thienopyridine drugs is due to their ability to block $P2Y_{12}$ receptor mediated platelet activation. There is novel but limited evidence to suggest that thienopyridine agents have additional, $P2Y_{12}$ independent effects. Clopidogrel and prasugrel exhibit vasomodulatory properties in addition to the established $P2Y_{12}$ inhibition. Newer non-thienopyridine agents also show non- $P2Y_{12}$ receptor mediated effects in trials but the exact mechanism is not established.

Dietary nitrates form an important source of exogenous nitrate, thereby forming an imperative and alternate source of NO via nitrate-nitrite-NO cycle in humans. There is considerable evidence suggesting that dietary nitrate supplementation enhances platelet inhibition in healthy volunteers and a decline in vasomotor tone in healthy human volunteers and hypertensive patients. CAD patients are known to have impaired endothelial NO production. Yet there is no study to date that describes the effect of these supplements in patients with established CAD, specifically in terms of platelet inhibition, the mechanisms involved and how this relates to nitrate metabolism.

This thesis aims to further investigate the effect of dietary nitrate supplement with or without clopidogrel therapy on NO metabolites and platelet inhibition in patients with stable CAD.

Newer more potent antiplatelet agents like ticagrelor have developed some of the same limitations of clopidogrel therapy, and they have also shown non- $P2Y_{12}$ receptor mediated effects in trials, but the exact mechanism is not established. This thesis will also explore whether ticagrelor can form RSNO compounds in patients with stable CAD and how this relates to platelet inhibition.

### 1.8.1 Specific aims

- To characterise and develop an *in vitro* model of conversion of dietary nitrate to nitrite upon ingestion of oral dietary nitrate.

- To investigate the influence and relationship between NO metabolite formation with ingestion of dietary nitrate in CAD patients.

- To investigate the effect of dietary nitrate ingestion on the ADP and TRAP mediated platelet inhibition in stable CAD patients on and off clopidogrel treatment.

- To investigate the ability of non-thienopyridines (ticagrelor) to form nitrosothiol under laboratory condition and in stable CAD patients.

### 1.8.2 Hypothesis

- Commercially available dietary nitrates undergo enterosalivary metabolism and participate in the Nitrate-Nitrite-NO cycle.

- Concurrent ingestion of clopidogrel and dietary nitrate augments the level of circulating nitrite, nitrate and RSNO in stable CAD patients.

- Elevated RSNO levels secondary to clopidogrel therapy with dietary nitrate will augment platelet inhibition.

- Ticagrelor has no ability to from RSNO due to the lack of a critical thiol group in its chemical structure.

# 2 General methods:

## 2.1 Patient Recruitment

Patients for this study were recruited from the cardiology outpatient clinics and cardiac day-case unit at the University Hospital of Wales (UHW).

The cardiac day unit at UHW is a 15-bed unit, with the specific remit of delivery and support of elective cardiac procedures. Patients typically undergo a pre-procedural assessment and evaluation a week prior to their proposed procedure date.

Patient's participation was voluntary. Full ethical approval for the study was sought and approved by the Local Research Ethics Committee (LREC) for Wales (IRAS Project ID 102427) (appendix i), and the study was conducted with the recommendations for physicians involved in research on human subjects adopted by the 18$^{th}$ World Medical Assembly, Helsinki 1964 (and later revisions).

Patients with known stable CAD were identified and approached for study participation. The study details were discussed with the potential participants in person, and a study information leaflet (appendix ii) was given. After a minimum of 3 days we re-approached the participants and sought their informed consent for participation.

On the day of procedure, the patients arrive at 8 am and pre-procedural checks were conducted by the clinical team. Patients, who are for coronary interventions, would be given a loading dose of antiplatelet drugs (clopidogrel 600mg or ticagrelor 180mg or prasugrel 60mg) as per clinical indication and preference of the Lead/Consultant clinician.

Patients who had agreed for voluntary participation in the pre-assessment appointment were approached, the study details were re-discussed with emphasis on addressing the patient's concerns. An informed consent (Appendix iii) is obtained and each patient was assigned a research code.

The following inclusion and exclusion criteria were applied for the recruitment.

#### 2.1.1.1 Inclusion Criteria

i. Adults over the age of 18 years with Stable CAD admitted to the University hospital for planned coronary angiogram ± PCI
ii. All patients to be taking aspirin
iii. Patients due to be given or already on clopidogrel or ticagrelor therapy for clinically approved condition
iv. Patients were fasted for >6 hours

#### 2.1.1.2 Exclusion Criteria

i. Contraindication to or patients not able to take clopidogrel/ticagrelor medication
ii. Patients unable to provide informed consent
iii. Patients with acute coronary syndrome (STEMI, NSTEMI, Unstable angina)
iv. Patients on long term oral anticoagulant drugs
v. Patients on non-steroidal anti-inflammatory drugs
vi. Patients receiving intravenous or subcutaneous anti thrombin therapy

### 2.1.2 Patients groups

The patients were grouped according to the antiplatelet therapy, as follows

#### 2.1.2.1 Clopidogrel and dietary nitrate study

***A) Clopidogrel naive group***

These patients were on aspirin alone and no other antiplatelet drugs.

***B) Acute loading group (Group A):***

These patients were on aspirin and no previous $P2Y_{12}$ therapy. They were prescribed with a loading dose of clopidogrel 600mg as per clinical guidelines on the day prior to their coronary intervention.

***C) Chronic clopidogrel (Group B):***

These patients were on dual antiplatelet therapy of aspirin and clopidogrel (75mg once a day) for more than 1 month and scheduled for a day case coronary intervention.

This cohort of patients included both diabetic and non-diabetic patients.

#### 2.1.2.1.1 Dietary nitrate supplementation and study design

SIS® Go+ nitrate supplements are used as the dietary nitrate source. SIS® Go+ nitrate supplements are available as a gel in a 60 ml sachet. An identical sachet of placebo gel was prepared which contained all ingredients except the nitrate (Swiss chard). These gels are prepared, marketed and sold by Science in Sport Ltd which is based in UK.

A randomised double blinded placebo control design was applied for the dietary nitrate and clopidogrel study. Patients were randomly allocated with either active nitrate gel or placebo gel. The recruiter and patient were blinded regarding the form of gel being administered.

**Clopidogrel naive study:** This was a paired cross over randomised double blinded placebo-controlled study, with cross over after a wash out period of 7 days as shown in Figure 2.1.

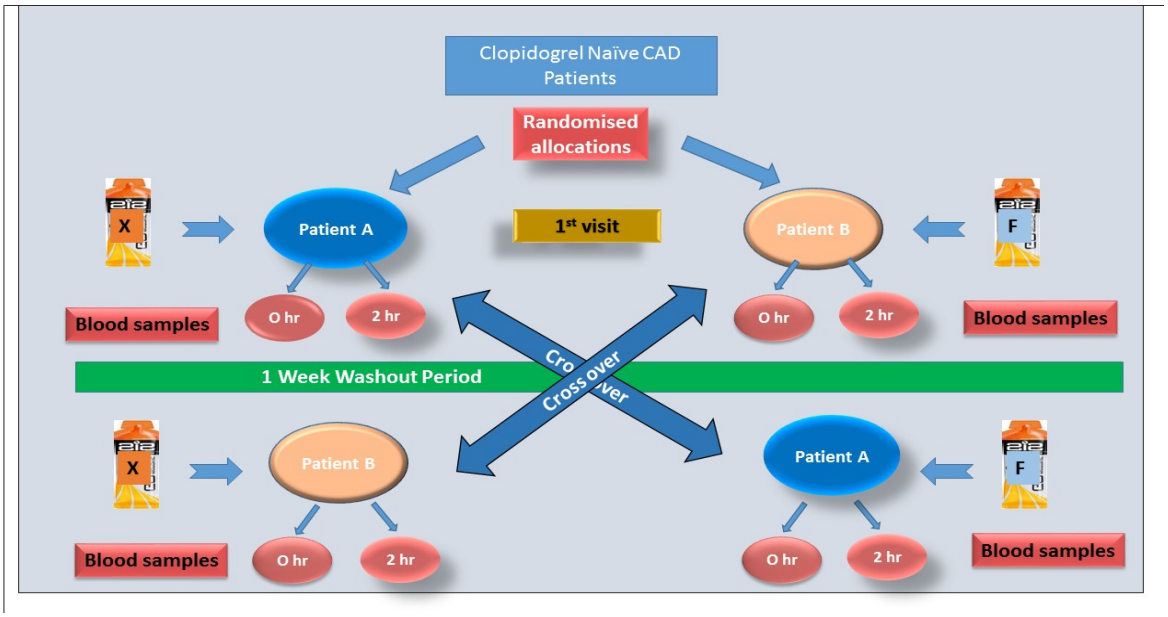


*Figure 2.1: Clopidogrel Naïve patient flow chart showing patient selection, randomisation and allocation to either X(placebo) or F(nitrate) gel at their first visit, and then at 2nd visit after a 1 week wash out period the patient is crossed over to the other gel. Blood samples were collected at 0 hours and 2 hours post ingestion on both occasions.*

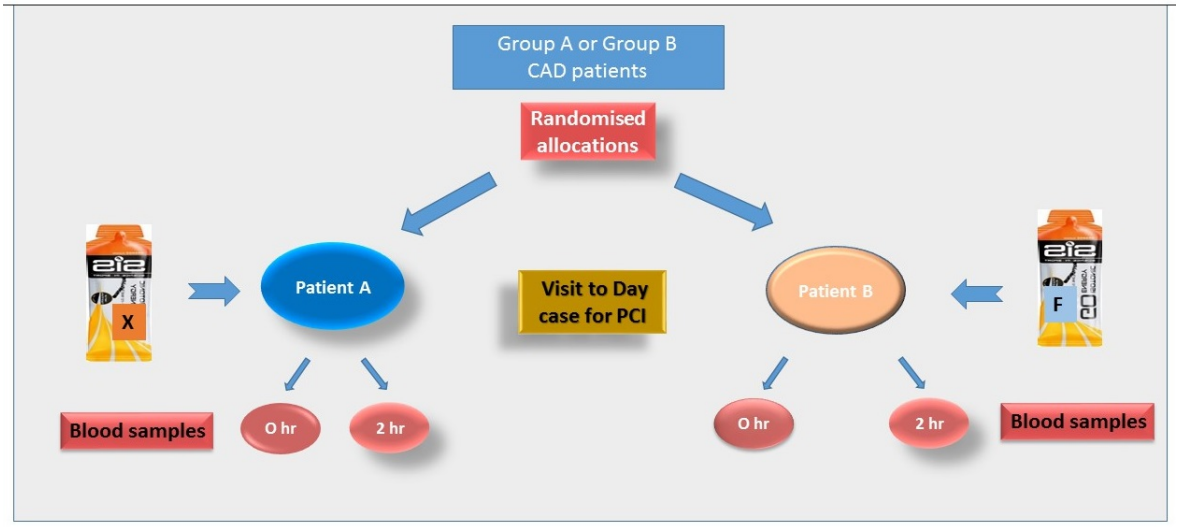



*Figure 2.2 : Group A and B patients were randomly given either nitrate or placebo gel, which are coded F and X respectively. These patients were not paired.*

**The Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg) study**: This was designed as a randomised double blinded placebo controlled, unpaired study, as shown in Figure 2.2.

The nitrate gel and placebo gel sachets were coded as F or X gel. The un-blinding of the codes was done at the stage of writing the thesis. The F and X gels were nitrate and placebo supplements respectively. Each patient was randomly administered either 2x60ml (9.6mmol total nitrate dose) of active supplement or placebo supplement, as shown in Figure 2.1 and Figure 2.2.

A zero-hour blood sample was collected just before supplementation with dietary nitrate in all groups as described in section 2.2. This sample provided the baseline measurement of the NO metabolites and platelet function. The second sample was collected 120 minutes post ingestion of dietary nitrate in the same patients in all groups. This was based on the pilot study, evaluating the pharmacokinetics of SIS® Go+ Sports gel in healthy human volunteers. The peak concentration of nitrate and nitrite occurred at 90 minutes and 120 minutes respectively

(Figure 1.15). This timing was also practically applicable for patients who were waiting for their PCI, both samples were collected before the coronary angiogram +/- PCI, thereby limiting the confounding effect of drugs such as heparin used during the procedure.

#### 2.1.2.2 **Ticagrelor Group**

A) ***Acute Ticagrelor loading group***

These patients were on aspirin therapy listed for coronary intervention and they were prescribed with Ticagrelor 180mg (loading dose) prior to their intervention as per clinical guidelines.

***B) Chronic Ticagrelor group***

This group of patients were dual antiplatelet therapy of aspirin 75mg once a day and ticagrelor (90 mg twice a day) for at more than one month.

Patients who were prescribed either with a loading dose of ticagrelor 180mg or maintained on a dose of ticagrelor 90mg BD were enrolled for this study. A zero hour and 2 hour blood sample were collected in ticagrelor (180mg) loading group as described in section 2.2. The second sample was collected at 2-hour to match with the median Tmax of ticagrelor in the plasma (1-4 hours) and cause minimal interruption for the patient's clinical management who are due for coronary angiogram +/- PCI. A single blood sample was collected in patients on maintenance ticagrelor therapy (90 mg twice a day).

## 2.2 Blood collection

### 2.2.1 Blood sampling process

The blood sample collection was in accordance with Cardiff and Vale University Health Board policy and guidelines after obtaining a valid consent. An 18-gauge intravenous (IV) cannula was inserted into the subjects' antecubital fossa under strict aseptic precautions. Blood was directly drawn into vacutainers via the cannula. A total of 15ml of blood collected in vacutainers

(Vacuette Greiner Bio-One$^{TM}$). The IV cannula was flushed with 10ml of 0.9% saline solution. The same cannula was used for collecting the 2$^{nd}$ sample after an interval of 120 minutes. 10ml of blood is drawn in a syringe and discarded, to ensure there are no clots and no contamination. A further 15ml of blood collected into the vacutainers and the cannula was re-flushed with 10ml of normal saline solution.

Blood samples for antiplatelet study were collected into 3ml vacutainer tubes containing hirudin. For NO metabolites (nitrate, nitrite and RSNO) study the blood samples were collected in a 4ml $K_3$EDTA vacutainer tube. The 4ml $K_3$EDTA blood tubes were immediately placed into an airtight ice box and hirudin tubes were kept at room temperature in a well-padded box to prevent agitation and minimise the activation of platelets during the transportation to the laboratory.

### 2.2.2 Storage of blood samples

In the laboratory, the 4ml $K_3$EDTA blood tubes were centrifuged at 3000 g for 10 minutes at 4$^{o}$C. Plasma and cells are separated forming platelet poor plasma (PPP). The PPP samples were allocated into 500 µl aliquots and immediately snap frozen in liquid nitrogen. The frozen samples are stored at -80$^{0}$C and used for batch analysis of NO metabolite at a later date.

Prof James's Group at WHRI has previously shown that total plasma NO metabolites remain relatively stable after snap freezing (259) for 7-10 days. We were able to recruit 2-3 patients in one session, and it was not practically feasible to immediately analyse NO metabolites. The process of freezing and storage of the samples allowed us to analyse them at a later date. This was undertaken in batches of approximately 10-15 samples per day.

## 2.3 Measurement of nitric oxide metabolites

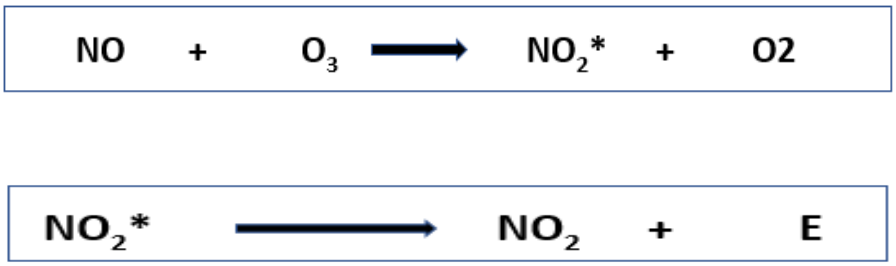
### 2.3.1 Ozone based chemiluminescence

Numerous methods are described in the literature for measurement of NO metabolites in human biological fluids; colourimetry, spectrophotometry, fluorescence, chemiluminescence,

gas and liquid chromatography, electrophoresis and mass spectrometry are a few of the well described methods (260). The colorimetric method using the Griess reagent is a well described and popular method. However, this has the limitation of not being sufficiently sensitive to measure physiological levels of nitrite (<1 μM) present in bodily fluids (261). The fluorescence assay based on Griess improves sensitivity significantly with detection of nitrate and upper levels of nitrite possible, but physiological levels of nitrite are hampered by background plasma fluorescence.

The Ozone based chemiluminescence (OBC) method is a highly sensitive and most accurate technique for measuring NO metabolites at physiologically relevant concentrations (nanomolar – micromolar). This technique involves a chemical cleavage of the NO moiety from the NO metabolites to form free NO in a radical, gas form. The free NO gas is carried by oxygen free nitrogen gas (inert gas at a constant flow rate 100-150 $cm^3$/min) into the Nitric oxide analyser (NOA) for NO measurement.

NOA generates ozone ($O_3$) from oxygen in its reaction cell where it reacts with the sample derived NO carried to an excited form of Nitrogen di-oxide ($NO_2^*$) and oxygen ($O_2$). $NO_2^*$ is an unstable state and it returns to its original state with release of its energy as a photon (Equation 2.1).




*Equation 2.1: NO analyser reaction equation*

The released photon of energy is amplified into an electrical signal by the NOA photomultiplier tube (PMT) and it is recorded in millivolts (mV).

The Sievers NOA 280i (GE Analytix, Durham, UK) is used in our laboratory. These are periodically tested and maintained by the certified GE technical team, ensuring a limitation in errors, variations and system failures.

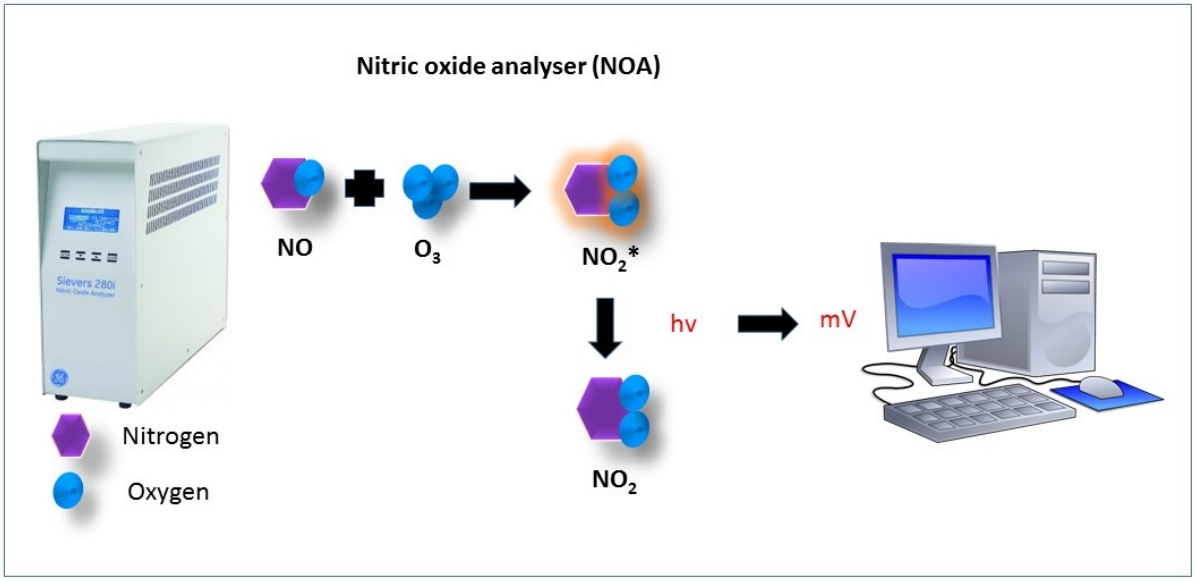



*Figure 2.3: Ozone based chemiluminescent technique of measurement of NO metabolites using NOA analyser.*

### 2.3.2 Chemical cleavage

Chemical cleavage is an essential step in the measurement of NO and its metabolites from biological samples. In our laboratory we use (i) tri-iodide in glacial acetic acid, (ii) vanadium chloride in HCl, and (iii) cuprous chloride/cysteine (2c's), as reagents for chemical cleavage of NO from nitrite, nitrate and RSNO, respectively (Figure 2.4). The systems are custom designed and have been validated in the James laboratory and are detailed in multiple manuscripts(133, 253, 262-264). The limitations are well understood, and we have established intra- and inter-assay coefficients of variation.

A pre-specified volume (shown below) of reagent is placed in a glass purge chamber which has a side port covered with rubber septum for injection of samples. Typically, 200 µl of test sample are drawn into a glass Hamilton syringe (Fisher Scientific, UK) and injected into the purge chamber. The sample interacts with the cleavage reagent generating NO.

The reaction chambers are connected to the NO analyzer via a NaOH (40ml) trap to filter impurities and contaminants. The NOA create a recordable millivolt signal and construct a trace on the computer.

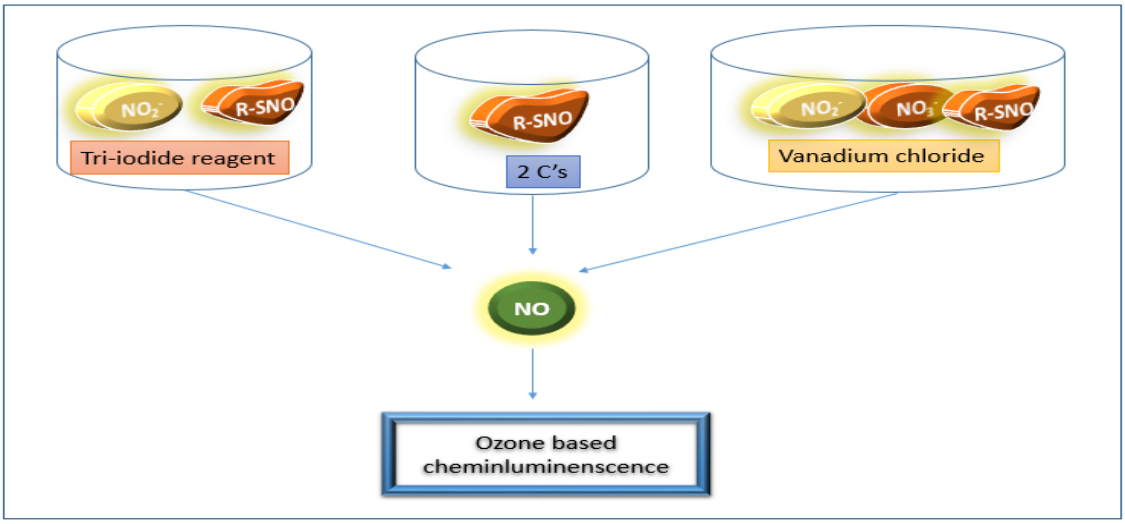



*Figure 2.4: Chemical cleavage reagents, Tri-iodide, vanadium chloride, and cuprous chloride/cysteine (2C's) reagents are used to cleave NO molecule from nitrite, nitrate and nitrosothiols as above. The NO moiety is detected through OBC.*

### 2.3.3 Measurement of nitrate level

Vanadium chloride ($VCl_3$) reagent was prepared by adding 785 mg of vanadium chloride into a mixture of 20 ml of HPLC grade water with 80 ml of hydrochloric acid (HCL). The cloudy turquoise blue reagent formed was stirred for 10 minutes and then filtered through a millipore filter; giving a clear mixture.

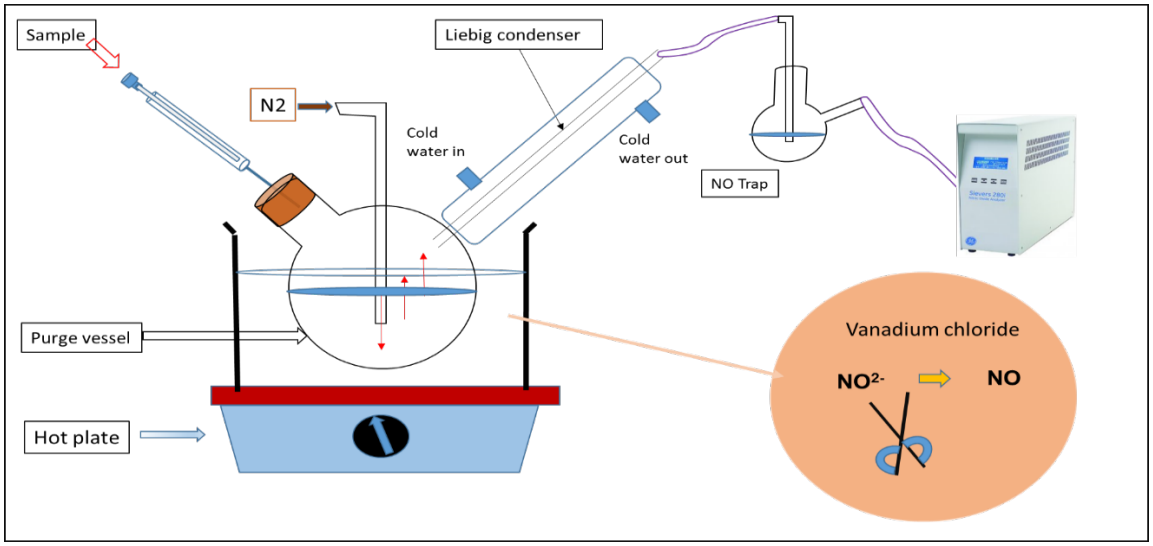


*Figure 2.5: Schematic diagram of setup of Nitrate measurement using Vanadium chloride reagent and OBC. The purge vessel contains 30 ml of vanadium chloride reagent kept at 85° C via a thermostatically controlled water bath. The test sample is injected into the purge cell through a rubber septum injection port. The cleaved NO is carried by inert $N_2$ gas into NO-Analyzer where reacts with ozone ($O_3$) to form an electrical signal detected by the photomultiplier tube for the measurement of NO.*

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

*Equation 2.2: Chemical reaction depicting the reduction of nitrate using Vanadium trichloride ($VCL_3$) and Hydrochloric acid (HCL). The end products are Vanadium pentachloride ($VCL_5$), water ($H_2O$) and Nitric oxide (NO)*

Vanadium chloride is a strong reducing agent, it predominantly reduces nitrates, but has ability to reduce nitrite and some RSNO (Equation 2.2). Hence the amount measured by this method is a total of nitrate+nitrite+RSNO released form the sample.

### 2.3.3.1 **Calibration of the NOA using sodium nitrate**

Sodium nitrate standards of 100.00, 50.00, 25.00, 12.50 and 6.25 µM concentrations are prepared and used for NOA calibration using vanadium chloride to cleave nitrate (Figure 2.6 & Figure 2.7).

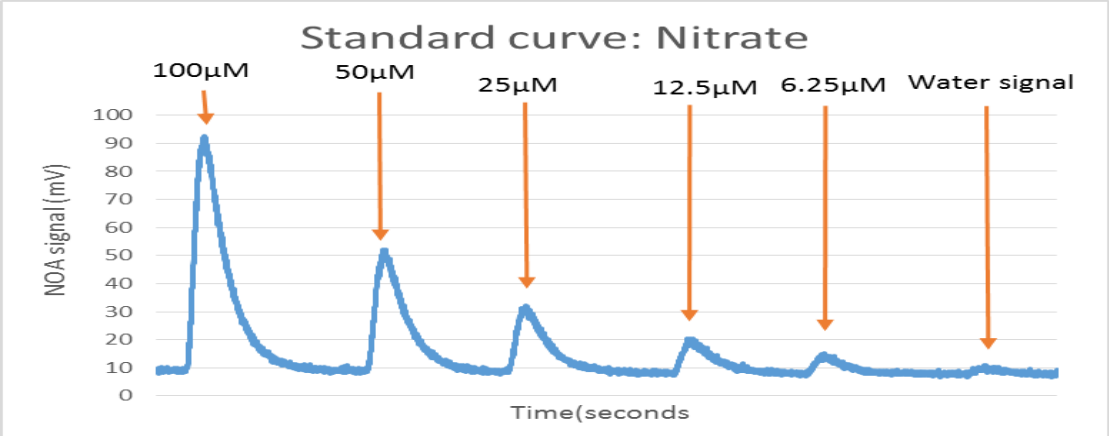


*Figure 2.6: Computer recorded trace obtained from injection of serial dilutions of nitrate in HPCL graded water, the area under curve (AUC) is calculated. The AUC of water is subtracted from each of the serial nitrate measurements, thereby eliminating the water background signal.*

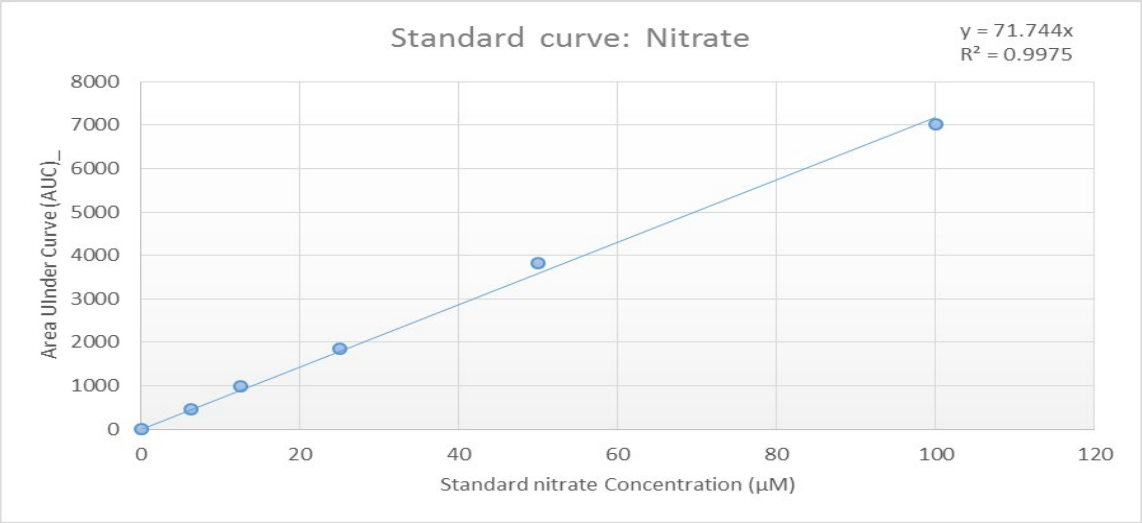



*Figure 2.7: The corrected AUC values are plotted against the known concentration of nitrate and a straight-line correlation is plotted. An R value of more than 0.95 is considered acceptable for the experiment.*

### 2.3.4 Measurement of plasma nitrite and nitrosothiols

Tri-iodide ($I_3$) reagent was prepared by mixing 1g of potassium iodide in 10 ml of HPLC grade water with 650mg of iodine in 70ml of glacial acetic acid. The prepared solution was kept at room temperature with constant stirring for 30 minutes (Figure 2.8)

The acidic iodine solution readily cleaves both nitrite and nitrosothiols present in plasma. Nitrite was converted to nitrous acid and NO in the presence of excess acid (Equation 2.3).

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

*Equation 2.3: Chemical reaction of nitrite in triiodide solution*

The free iodine in the presence of potassium iodide reduces nitrosothiols to NO and water (Equation 2.4). This reaction gives a total of plasma nitrite and nitrosothiols.

$$I^{2} + I^{-} \rightarrow I^{-}_{3}$$

$$I^{-}_{3} + 2RSNO \rightarrow RS\text{-}SR + 2NO^{*}$$

$$2NO^{*} + 2\,I^{-} + 2H^{+} \rightarrow 2NO + I2 + 2H_2O$$

*Equation 2.4: Chemical reactions of free iodine and RSNO in the triiodide solution.*

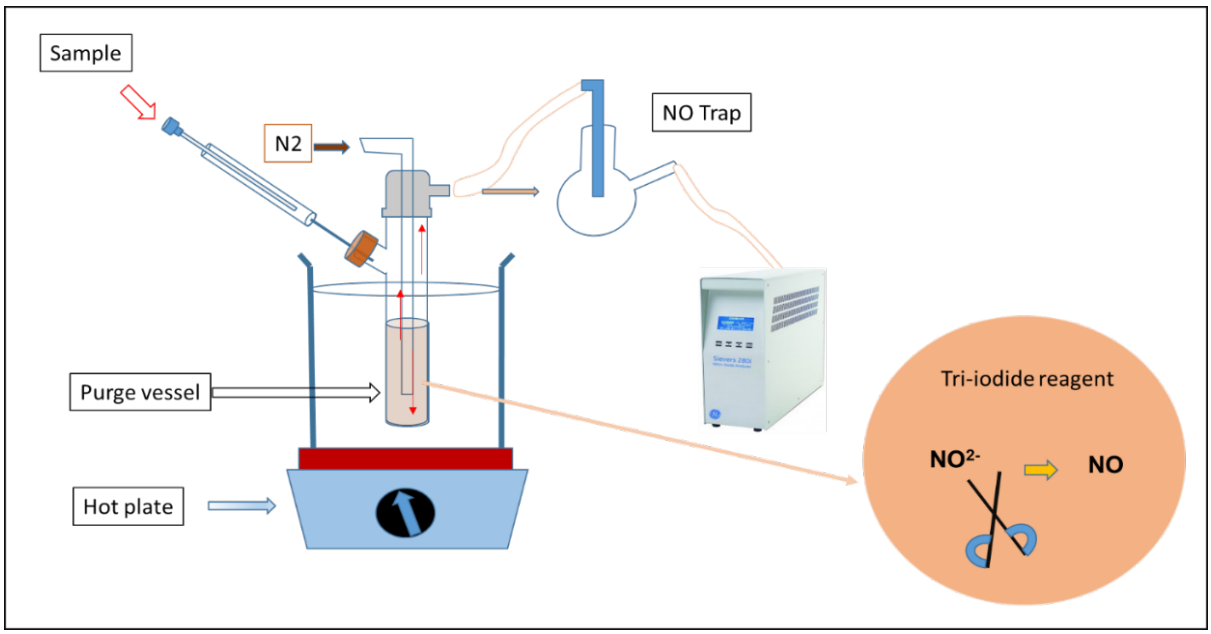



*Figure 2.8: Schematic diagram showing the practical setup for nitrite and nitrosothiol measurement using Tri-iodide reagent and OBC. The purge vessel contains 5ml of reagent kept at $50^{0}$ C in a thermostatically controlled water bath. The test sample is injected into the purge cell through a rubber septum injection port. The cleaved NO is carried by inert $N_2$ gas into NO-Analyzer where reacts with ozone ($O_3$) to form an electrical signal detected by the photomultiplier tube for the measurement of NO.*

#### 2.3.4.1.1 Selective measurement of plasma nitrosothiols

Acidified sulphanilamide pre-treatment of the sample renders the plasma nitrite undetectable by the above tri-iodide method. This technique would allow a selective measurement of the remaining nitrosothiol in the plasma. Acidified sulphailamide is prepared by dissolving 500mg of sulphanilamide in 10ml of 1M Hydrochloric acid.

Typically, 540µl of plasma sample was mixed with 60µl of acidified sulphanilamide and the mixture kept in the dark for a period of 15 minutes to reduce all the nitrite. 400µl of this mixture was injected into the tri-iodide purge chamber and the cleaved NO detected via OBC, thereby quantifying the amount of nitrosothiol in the sample. The RSNO signals are ~40 times smaller compared with nitrite signals, hence double the volume injections are typically used to enhance the signal amplitude (typically 400 ul). The final results are divided by 2 to get a corrected value.

### 2.3.4.2 Calibration of the NOA using sodium nitrite

Sodium nitrite standards of 1000.0, 500.0, 250.0, 125.0 and 62.5 nM concentrations are prepared and tested for NOA calibration using tri-iodide to cleave nitrite on each day of the experiment (Figure 2.9 & Figure 2.10).

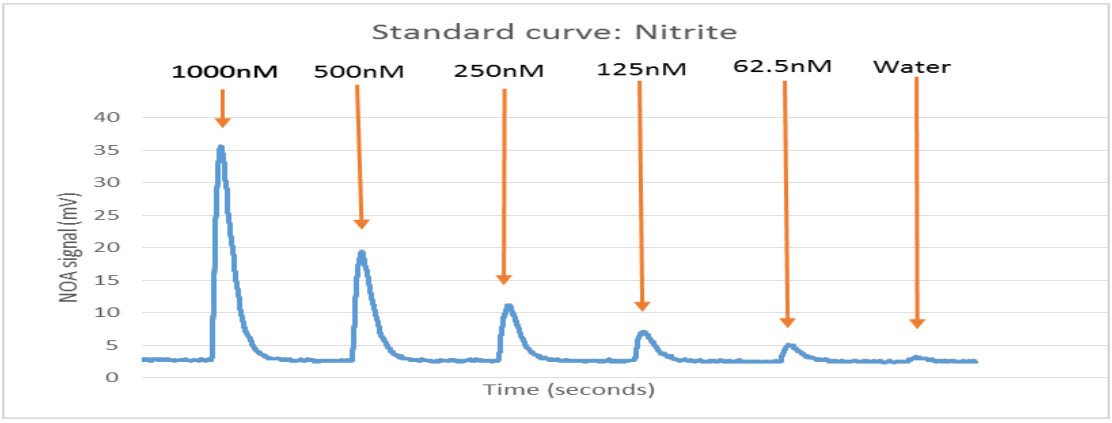



*Figure 2.9: Computer recorded trace obtained from injection of serial dilutions of nitrite and HPCL graded water, the area under curve (AUC) is calculated. The AUC of water is subtracted from each of the serial nitrate measurement, thereby eliminating the water contaminants*

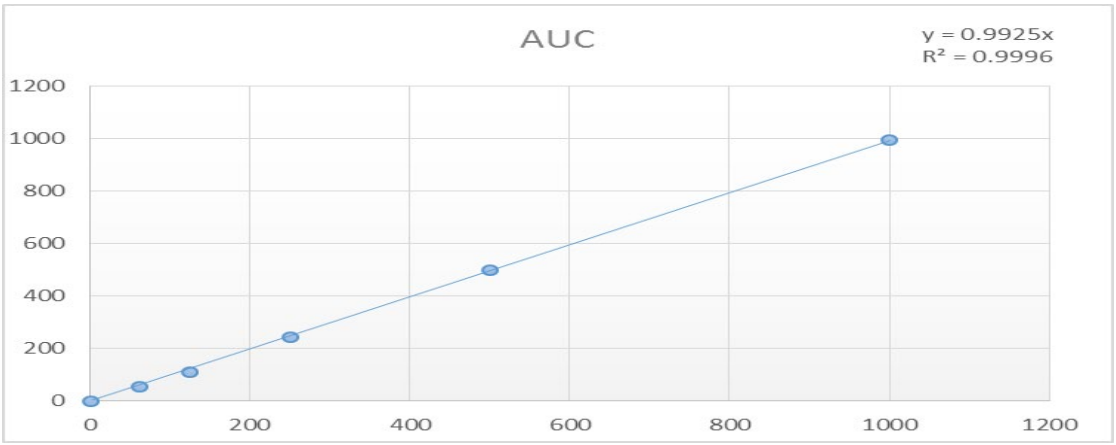



*Figure 2.10: The corrected AUC values are plotted against the known concentration of nitrite and a straight-line plotted. An R value of more than 0.95 is considered acceptable for the experiment.*

### 2.3.5 Measurement of laboratory synthesized R-nitrosothiols

Cuprous (I) chloride/cysteine (CuCl/CSH) reagent (2C reagent) was prepared by adding 47.25 mg of cysteine into 390 ml of HPLC grade water. 39.59 mg of cuprous chloride was then added into 10 ml of high-pressure liquid chromatographic (HPLC) grade water, which creates a solution of 40 mM concentration. This is then diluted further by 1/10 (4 mM concentration). 10 ml of the latter solution was added to 390 ml solution containing cysteine

The 2C reagent is highly sensitive and specific for RSNO detection. The selectivity is pH dependent; nitrates and nitrates are undetected at pH >6. Maintaining pH is crucial in this method. Cysteine can readily undergo auto-oxidation to cystine leaving the assay ineffective therefore care must be taken to ensure this chemical's viability.(265, 266). The $Cu^{+}$ ions selectively cleave the RS-NO bond and cysteine enhances the NO formation (259).

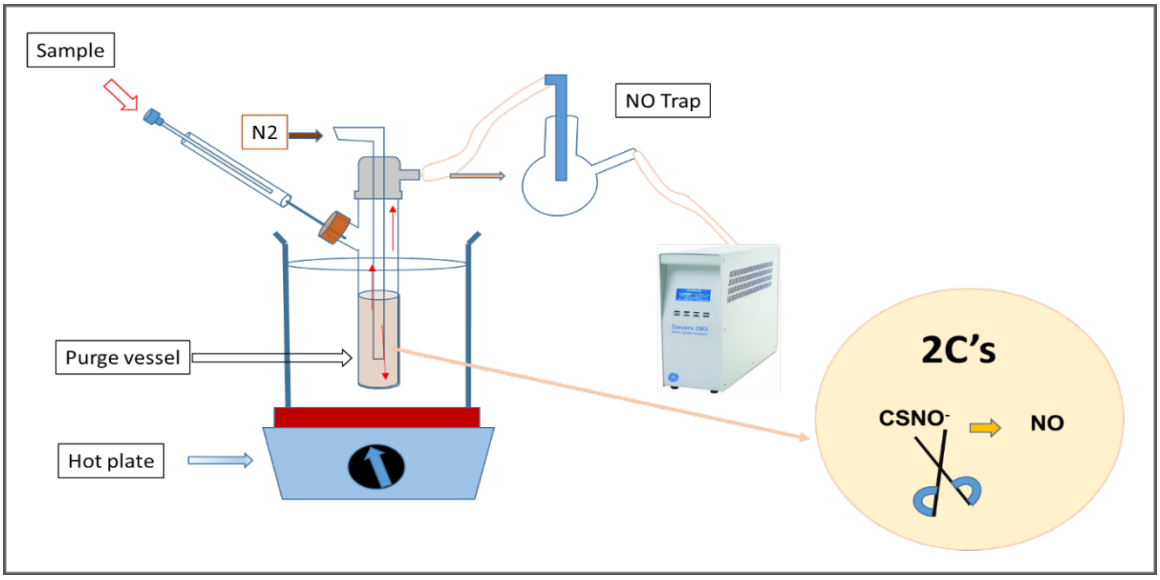



*Figure 2.11: Diagram showing the set up for the measurement of nitrosothiols using OBC. The purge vessel contains 5 ml of 2C's cleavage reagent kept at $50^0$ C via thermostatically controlled water bath. The test sample is injected into the purge cell through a rubber septum injection port. The cleaved NO is carried by inert $N_2$ gas into NO-Analyzer where reacts with ozone ($O_3$) to form an electrical signal detected by the photomultiplier tube.*

### 2.3.5.1 **Calibration of the NOA using acetyl-cysteine-SNO**

N-Acetyl-cysteine-SNO (NACSNO) was prepared by mixing 1.63 g of N-acetyl cysteine (1 M NAC) in 10 ml of hydrochloric acid (1 M). 759 mg of sodium nitrite (1.1 M) and was then mixed in 10 ml HPLC grade water. 500 µl of NAC was pipetted into a brown bottle with an injection port covered with a rubber septum top. 500 µl of sodium nitrite was then injected through the rubber septum. The mixture bubbles and turns red. NACSNO was subject to thermal and photochemical decomposition so is kept in the dark, on ice (267).

NACSNO was calibrated by dilution (1/200) using HPLC grade water and analysed by light spectrophotometry using a single cell holder spectrophotometer (6705 UV/Vis. Spectrophotometer, Jenway, UK), using HPLC grade water as a blank.

Maximal light absorbance was read at a 334 nm wavelength (expected result is ~1.5-2). The concentration of NACSNO (mM) was calculated using the equation [Light absorbance/

Absorption coefficient (ε = 727)] x 200 (expected result is ~0.4-0.5 mM) (Figure 2.12).

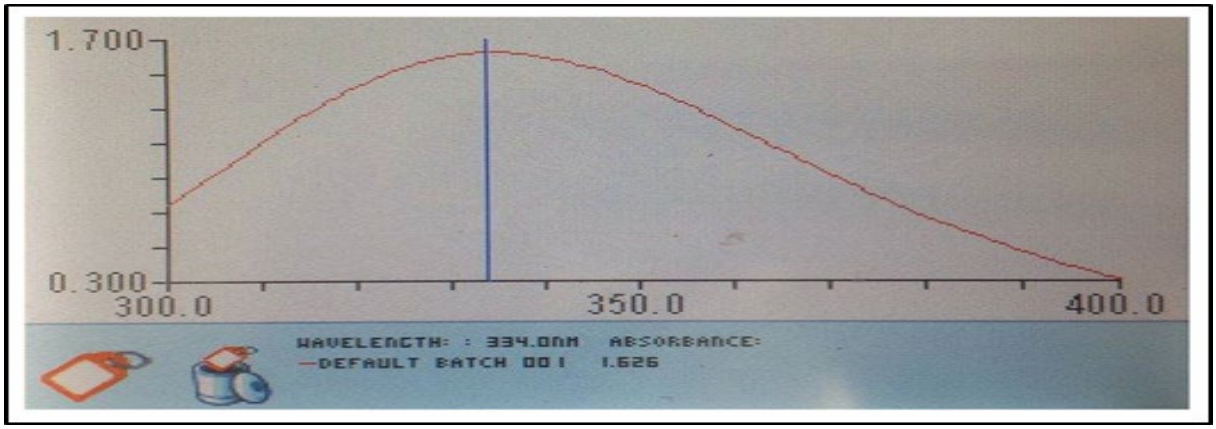



*Figure 2.12: Representative spectrophotometric reading of NACSNO. The peak absorbance is read at 334nm (blue line) giving a value of 1.69 in this case.*

Acetyl-cysteine-SNO (NACSNO) standards of 4.3, 2.15, 1.07, 0.53, 0.26 µM and HPLC graded water are used for calibration using 2 C's to cleave RSNO (Figure 2.13 and Figure 2.14).

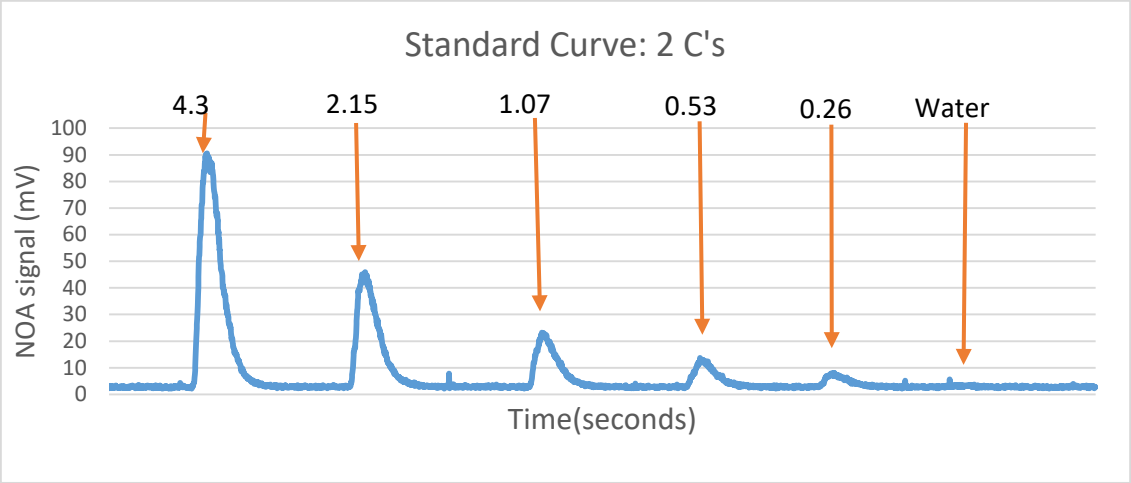



*Figure 2.13: Computer recorded trace obtained from injection of standards dose of NACSNO to generate a calibration curve with OBC, The area under the curve is calculated by the NOA analysis Liquid version 3.2.1 software.*

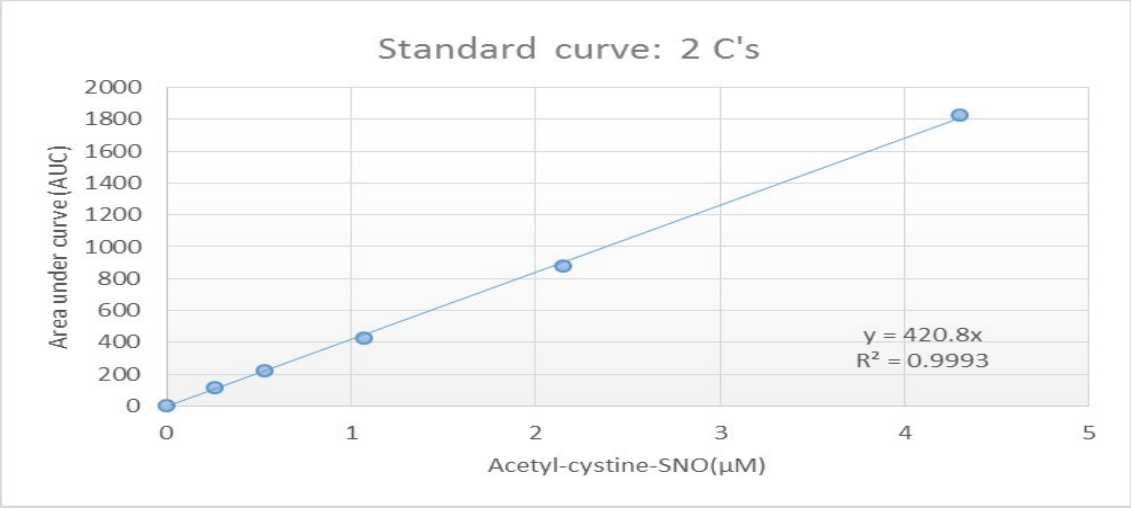



*Figure 2.14: The area under curve of the standards is plotted against the actual NACSNO concentrations. A Pearson correlation is calculated to ensure linear relationship. R value of > 0.95 is considered acceptable for experimental purposes.*

### 2.3.6 Standardisation of measurements

All reagents are prepared fresh on the day of experiments. These were stored as per specified laboratory guidelines.

Standard curve calibrations were performed prior to measurement of nitrate, nitrite and nitrosothiols on each day as described in the above sections. There is variation in the detection of NO by NOA on a daily basis that is well established. In our laboratory we have noticed a day to day variation in the area under curve of the standard curve and the actual NO metabolite strength. The coefficient of variation of measuring the same NO metabolite in our lab from experience is in the order of 4% for nitrates, 7% for nitrites and 7% for nitrosothiols. These variations are due to changes in the cooling temperature of the photomultiplier tube, supply pressure of oxygen, and room temperature.

Daily standard curve measurement is essential to limit these variations. The room temperature and the oxygen flow pressures are closely monitored through the day and stable laboratory conditions (air conditioning etc) are maintained.

Based on previous plasma analysis performed in the laboratory at our institution, typical nitrite, RSNO and nitrate levels measured by ozone based chemiluminescence (OBC) are 160 nM, 25nM and 30 μM respectively. 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%(261). Marley *et al* measured, the mean concentration of plasma nitrosothiol levels in venous sample of healthy humans was 21-35nM with high sensitivity, an ability to detect levels down to 5nM using the above method(268).

## 2.4 Measurement of platelet aggregation

A variety of platelet function tests are available to detect platelet dysfunction and monitoring of antiplatelet therapy. Platelets are influenced by several agonists which act on various receptors present on the platelet surface making it practically difficult to develop one test which measures all platelet functions. Evaluating platelet function has a clinical relevance in patients who are taking anti platelet drugs because there is considerable variation in the extent of response to antiplatelet therapy in patients. Impaired response or resistance to antiplatelet therapy is known to be associated with further adverse cardiovascular events (269). In the last decade, there is an increase in number of coronary interventions worldwide, in UK has risen from 800 per million population in 2002 to 1488 per million in 2014(270). Most of these patients received drug eluting stents (DES) which require long term dual antiplatelet therapy. Decreased platelet inhibition or resistance has shown to be associated with increased risk of stent thrombosis and high mortality (271-273). Approximately 10% of cardiovascular deaths after stent implantation are attributed to stent thrombosis (ST) (274). Newer antiplatelet drugs such as prasugrel (135) and Ticagrelor (148) have a lower incidence of ST compared to clopidogrel. Thus, it is imperative that a method be developed for routine analysis of platelet activity. Platelet aggregation tests have been established to test the ability of platelets to aggregate in response to an external agonists such as arachidonic acid (AA), collagen, epinephrine (EPI) adenosine diphosphate (ADP) and Thrombin activating protein (TRAP)(275).

### 2.4.1 Classical aggregometry

Light transmission aggregometry (LTA) of platelet rich plasma was first described by Born in the year 1960(276). It is the most widely used and has been a gold standard method for the assessment of platelet aggregation. Platelet rich plasma is an optically dense solution. Addition of an agonist to PRP triggers platelets aggregation and results in a less turbid solution. The LTA technique measures the rate and maximum percentage of increase in light transmission through the PRP sample upon addition of an agonist (increase in light transmission=decrease in turbidity). The signals are converted automatically into a graph and are readily quantified. Different platelet activation pathways could be tested with this method using various agonists. This method has been used in clinical trials in monitoring antiplatelet therapy and prediction of major adverse cardiovascular events in patients at high risk of thrombotic events (277, 278). This technique has its own challenges and limitations. It is highly dependent on pre-procedural and procedural factors like platelet counts, plasma lipids, anticoagulants used, preparation techniques of PRP. It also does require a high degree of skill and expertise among staff performing and applying this test. Its major drawback is that it cannot be applied to whole blood samples.

### 2.4.2 Impedance aggregometry

Impedance aggregometry is a whole blood aggregometry technique assessing platelet function in an anticoagulated whole blood sample. This is based on the principle that activated platelets adhere to the surface of two electrodes immersed in the whole blood. The platelet adherence onto the electrodes changes their impedance with time (279). Platelet aggregation is measured by detecting the increase in the impedance and recorded in Ohms (280, 281). This a well-established method for diagnosis of platelet dysfunction and monitoring of antiplatelet therapy. A specific agonist is added to stimulate the platelets and assess respective platelet receptor function. Arachidonic acid (AA) and Adenine di-phosphate (ADP) agonist are typically used for testing efficacy of aspirin and clopidogrel respectively.

The whole blood aggregometry technique does not require pre-preparation of samples limiting artificial platelet activation. The platelet function is assessed under more physiological conditions allowing the contribution of other blood substances such as ADP derived from

RBC's which may affect platelet aggregation. This is a rapid and simple method of analysis of platelet function (282).

Single electrode aggregometry consisting a pair of reusable electrodes was first invented by Cardinal and Flower in the 1980s. Multiple electrode impedance aggregometry with two pairs of disposable electrodes was developed in 2006(283).

#### 2.4.2.1 **Multiple electrode aggregometry (MEA)**

Multiple electrode aggregometry (multiplate platelet function analyser-Dynabyte-Roche Diagnostic, Mannheim Germany) (Figure 2.15) is the latest device for whole blood impedance aggregometry available across the world. This device is used as a point of care device for evaluation of platelet function and monitoring of antiplatelet drug therapy. It is a five-channel computerised device with an automated pipetting unit and disposable cuvettes each with two independent sensor units and magnetic stirrer. Platelet aggregation is measured simultaneously by each of the units independently and calculated as area under curve (AUC). This device could test different agonists induced platelet response, similar to LTA. Comparative studies of MEA with classical aggregometry (LTA) showed similar results in evaluating platelet dysfunction and outperformed when monitoring anti-platelet therapy (284-286). Impedance whole blood aggregation is more sensitive in detecting the effect of clopidogrel on platelet inhibition when compared to LTA (287). MEA testing has been used to identify patients who are not responding or hyper-responsive to antiplatelet drugs, thereby identifying those patients who are at risk of thrombotic events or bleeding (288, 289). This Multiplate analyser device allows rapid, reliable assessment of platelet function at the point of care. The machine features an automated work pattern and step by step method with clear instruction needing minimum technical knowledge and training. It does not require a specialised laboratory setup and can be easily used in a general laboratory or in clinical areas (282, 290).

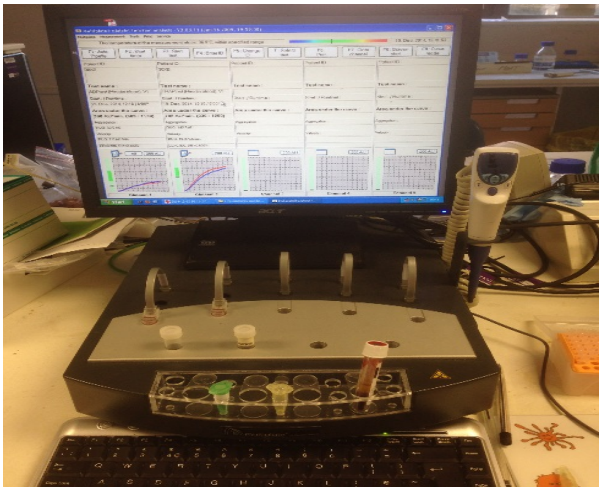


*Figure 2.15: Multiplate® analyser from Roche, Germany. It has 5 channels for simultaneous measurement of different sample/agonist with an electronic pipette with predefined pipette programs for tests.*

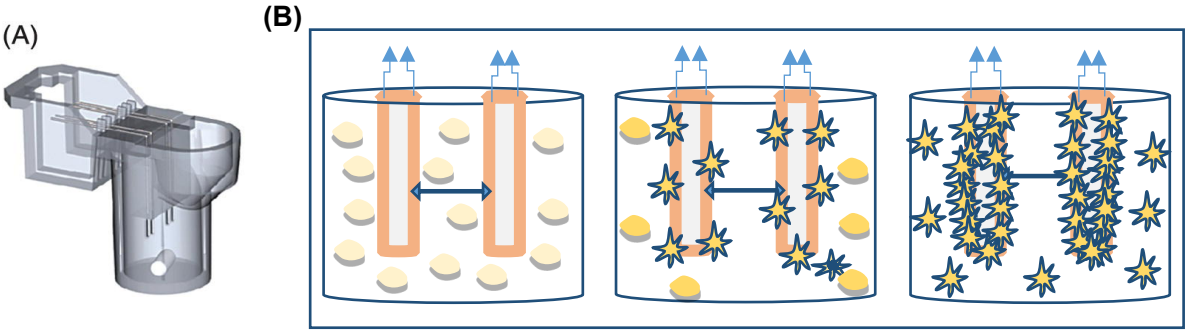



*Figure 2.16: Disposable cuvettate with two pairs of electrodes and a magnetic stirrer (A). The principle of platelet aggregation relies upon activation with an agonist and increase in impedance detected over time.*

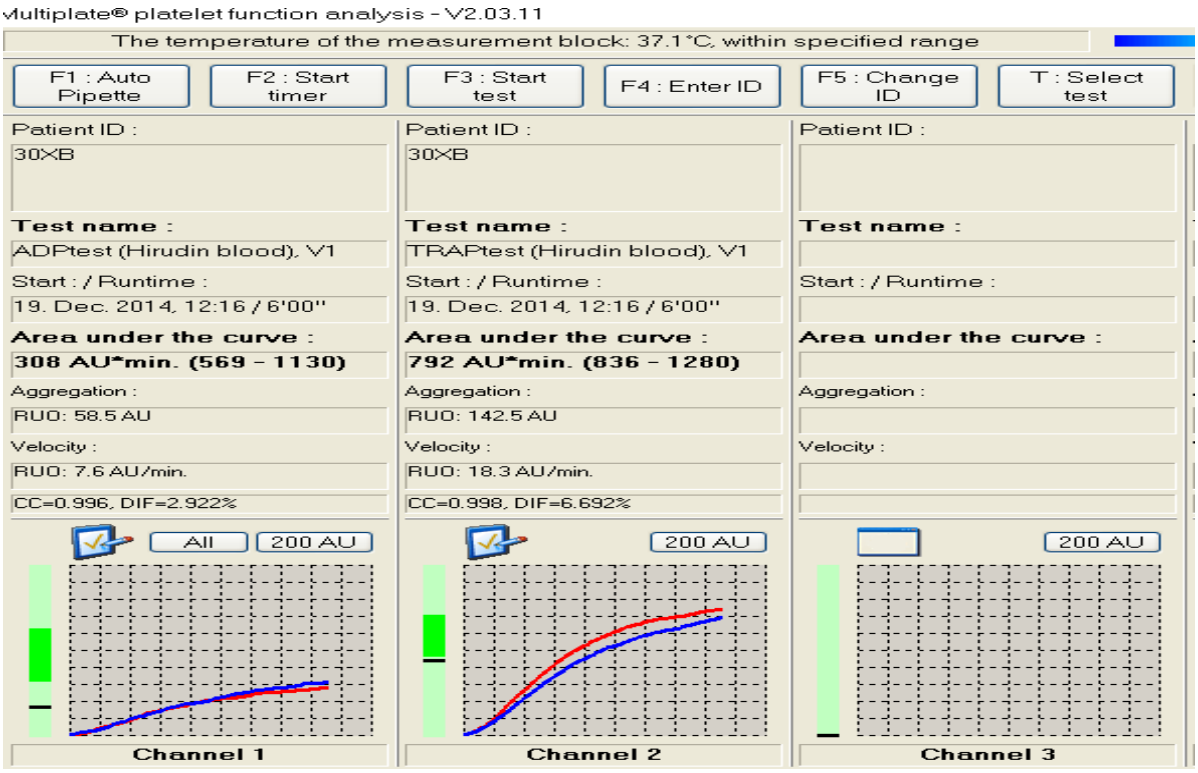



*Figure 2.17: Screenshot image of the computer-generated data sheet showing typical platelet aggregation in response to ADP and TRAP, the value is expressed as area under curve.*

The Multiplate analyser is auto calibrated every day prior to blood sample analysis. Disposable cuvettes are inserted into the wells and connected to the respective channels. Sample ID and the agonist are entered into specified fields on the data sheet for each channel. An auto pipetting program is activated and provides on screen step wise prompts.

300µl of normal saline is added into each test cuvette and, left to incubate for 2 minutes. Then 300µl of whole blood sample is pipetted into the test cuvette and incubated for a further 2 minutes, 20 µl of ADP or TRAP reagent is then added to the above mixture and left for 6 minutes. The increase in the impedance of electrodes due to platelet adhesion and aggregation (Figure 2.16) is recorded in real time by the computer software. At the end of 6 minutes an automatic calculation of the area under curve is done and the value is displayed as platelet reactive units (PRUs) (Figure 2.17). The impedance of each pair of electrodes are measured and displayed separately as blue and red curves. A variation of <25% between two pairs of electrodes is acceptable.

## 2.5 In vitro dietary nitrate and clopidogrel study: materials and methods

### 2.5.1 Materials

| | Material | Source | Concentration |
|---|---|---|---|
| **NADPH** | Beta-Nicotinamide adenine dinucleotide2'-phosphate reduced tetrasodium salt hydrate | Sigma-Aldrich, UK | 25 mg |
| **G6PD** | Glucose-6-phosphate Dehydrogenase from baker's yeast (S.cervisiae) | Sigma-Aldrich, UK | 250 units |
| **G6P** | D-Glucose 6-phosphate sodium salt | Sigma-Aldrich, UK | 500 mg |
| **Iodine** | Iodine, 99.8%, A.C.S. reagent | Sigma-Aldrich, UK | 500 g |
| **Vanadium Chloride** | Vanadium (III) Chloride 97% | Sigma-Aldrich, UK | 100 g |
| **Potassium Iodide** | Potassium Iodide, 99.8% A.C.S. reagent | Sigma-Aldrich, UK | 100 g |
| **Acetic Acid glacial** | Acetic Acid glacial Analytic reagent grade | Fisher Scientific, UK | 2.5 L |
| **Hydrochloric Acid** | Hydrochloric acid 1M, Stabilised | Fisher Scientific, UK | 2.5 L |
| **Nitrate Reductase** | Nitrate Reductase (NAD[P]H) from *Aspergillus Niger* | Sigma-Aldrich, UK | 10 units |
| **Sodium Nitrate ($NaNO_3$)** | Sodium nitrate 99.99+% metals basis | Sigma-Aldrich, UK | 10 g |
| **Sodium Hydroxide (NaOH)** | Sodium Hydroxide Trap, Volumetric solution | Fisher Scientific, UK | 1 M |
| **Peptone** | Casein, from bovine milk | Sigma-Aldrich, UK | 500 g |
| **D-Glucose** | D-Glucose anhydrous Analytic reagent grade | Fisher Scientific, UK | 2 kg |

| | | | |
|---|---|---|---|
| **Sodium Chloride (NaCl)** | Sodium chloride (Laboratory reagent grade) | Fisher Scientific, UK | 3 kg |
| **Potassium dihydrogen orthophosphate ($KH_2PO_4$)** | Potassium dihydrogen orthophosphate (Analytic reagent grade) | Fisher Scientific, UK | 500 g |
| **Calcium Chloride ($CaCl_2$)** | Calcium chloride dihydrate (Analytic reagent grade) | Fisher Scientific, UK | 500 g |
| **Potassium Chloride (KCl)** | Potassium chloride Laboratory reagent grade | Fisher Scientific, UK | 500 g |
| **Porcine Bile** | Bile extract, porcine | Sigma-Aldrich, UK | 100 g |
| **Lysozyme** | Lysozyme from chicken egg white | Sigma-Aldrich, UK | 5 g |
| **Pepsin** | Pepsin from porcine gastric mucosa powder, > or = 250g units/mg solid | Sigma-Aldrich, UK | 25 g |
| **Resazurin** | Resazurin sodium salt | Sigma-Aldrich, UK | 1 g |
| **N-Acetyl-L-cysetine** | N-Acetyl-L-cysetine | Sigma-Aldrich, UK | 50 g |
| **Copper Chloride** | Copper(I) chloride | Sigma-Aldrich, UK | 10 g |
| **L-Cysteine Hydrochloride** | L-Cysteine hydrochloride anhydrous, minimum 98% TLC | Sigma-Aldrich, UK | 10 g |
| **High-performance liquid chromatography (HPLC) grade water** | ELGA Lab-Water | Elga-veolia. UK | Reservoir 25 L |
| **Clopidogrel (Actavis™) 28 tablets** | Clopidogrel hydrogen sulphate | Bristol-Myers Squibb, UK | 75 mg per tablet |
| ***Beet It*®** | BEET IT® | James White Farm, UK | 100 mg/100 ml |
| ***SIS® Go+ nitrate gel*** | Science-in-Sport® GO Plus Nitrate Gel | Science-in-Sport (SIS®) Limited, UK | 250 mg/60 ml |

*Table 2.1 : Materials are reagent-grade quality and used without further purification.*

## 2.5.2 Methods

### 2.5.2.1 Enzymatic conversion of nitrate to nitrite

A technique described and applied in a PhD project by Alexandra B Milsom in Prof James's laboratory at the Wales Heart Research Institute was used as a starting point (291). A well-known assay for the measurement of nitrate and nitrite in solution involves the initial reduction of all nitrate to nitrite prior to measurement. This procedure (using a bacterial nitrate reductase source) was adapted enabling in-vitro conversion of nitrate to nitrite. Laboratory grade sodium nitrate, Beet it and SIS® Go+ nitrate gel was used as the nitrate substrates for catalytic conversion of nitrate to nitrite by bacterial nitrate reductase. Nitrate reductase was derived from *Aspergillus niger* (A. Niger)

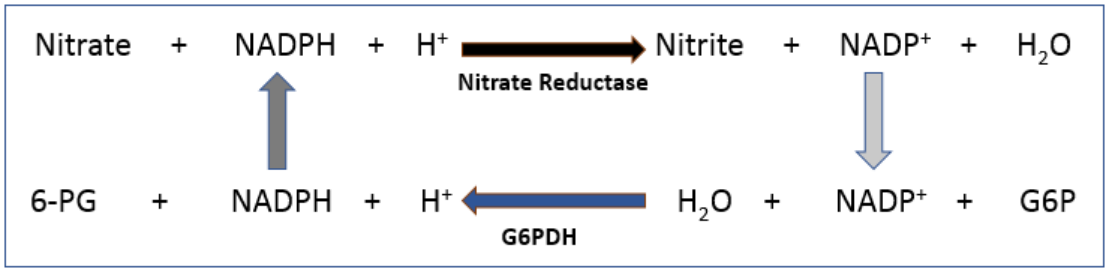



*Figure 2.18 : Enzymatic conversion of nitrate to nitrite in the presence of co-factors*

### 2.5.2.2 Nitrate reductase (NR)

Nitrate reductases (NRs) are molybdenum dependent ubiquitous enzymes. Anaerobic denitrifying microflora from the posterior aspect of the tongue and gut are the principle source of nitrate reductase in humans. *A. niger* is a fungus that produces NR that catalyzes the reduction of nitrate *in vitro* and is available in commercial form.

### 2.5.2.3 Stock preparations

All working solutions were prepared fresh on the day of the experiment. Dilutions are made using HPLC grade water.

| Nitrate source | Nitrate concentration | Storage |
|---|---|---|
| Sodium nitrate | 10(nM) | Room temperature |
| *SIS® ® Go+ nitrate* gel | 250 mg/60 ml | Room temperature |
| *Beet It®* | 100 mg/100 ml | Stable in fridge at -4°C |

*Table 2.2: Source of nitrate used in the experiments with their concentration and stability features*

| Components of Nitrate reductase enzyme mixture | Manufacturer strength | Dilutions | Stock solution |
|---|---|---|---|
| NADPH | 25 mg | Add 3.0000 ml of PBS to bottle | 10 mM |
| G6P | 500 mg | Add 7.0892 ml of PBS to 100 mg of bottle contents | 50 mM |
| G6PD | 250 units | Add 2.5000 ml of PBS to bottle | 100 U/ml |
| Nitrate Reductase | 10 units | Add 1.6666 ml of PBS to bottle | 6 U/ml |

*Table 2.3: Nitrate reductase and cofactor stock preparations*

| Components of Nitrate reductase enzyme mixture | Stock solution | Dilutions | Desired working solution |
|---|---|---|---|
| NADPH | 10 mM | Add 1800 µl of HPLC grade water to 200 µl stock solution | 100 or 1000 µM |
| G6P | 50 mM | Add 1800 µl of HPLC grade water to 200 µl stock solution | 5 mM |
| G6PD | 100 U/ml | Add 840 µl of HPLC grade water to 160 µl stock solution | 16 or 160 U/ml |
| Nitrate Reductase | 6 U/ml | Add 1800 µl of HPLC grade water to 200 µl stock solution | 0.6 U/ML |

*Table 2.4 : Contents of enzyme mixture for working solution*

| Sample components | Volume in ??mixed in sample (µl) | Ratio of mix to be maintained | Standard sample volume injected into the redox chamber (µl) |
|---|---|---|---|
| Nitrate source | 200 | 0.5 | 200 |
| NADPH | 40 | 0.1 | |
| Enzyme mix | 160 | 0.4 | |
| Total | 400 | 1.0 | |

*Table 2.5: Composition of enzyme mixture*

200 µl of known concentration of nitrate solution is mixed with 40µl of 10 µM NADPH. Equal quantities of G6PD (1.6 U/ml), G6P (5 mM) and NR (6 U/ml) were mixed and made up to 160 µl. The above two mixtures were mixed together to create a total sample volume of 400 µl in a 1.5 ml Eppendorf.

### 2.5.2.4 **Artificial stomach media**

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*(292) .

| Composition of materials for the gastric juice medium | Manufacturer strength | Desired working mass |
|---|---|---|
| Peptone | 500 g | 4.15000 g |
| D-Glucose | 2 kg | 1.75000 g |
| Sodium Chloride (NaCl) | 3 kg | 1.02500 g |
| Potassium dihydrogen orthophosphate ($KH_2PO_4$) | 500 g | 0.30000 g |
| Calcium Chloride ($CaCl_2$) | 500 g | 0.05500 g |
| Potassium Chloride (KCl) | 500 g | 0.37000 g |
| Bile Extract | 100 g | 0.02500 g |
| Lysozyme | 5 g | 0.05000 g |
| Pepsin | 25 g | 0.00665 g |
| Resazurin Sodium Salt | 1 g | 0.00100 g |

| Final mixture of gastric juice | pH of gastric juice | Temperature of gastric juice hot plate (℃) |
|---|---|---|
| All weighed materials are mixed in 500 ml of HPLC-grade water | Gastric Juice pH maintained at 1-2 by correction using hydrochloric acid(HCL) | 37±1  |

*Table 2.6: Constituents of artificial gastric juice*

Gastric juice is prepared fresh on the day of experiment. All materials are weighed using Mettler Toledo AE 50 Analytical Balance, UK. It is kept at 37±1℃ using a thermostatically controlled hotplate (Stuart® US152, UK). The final mixture is acidified to pH≤2 using hydrochloric acid; a colour change from purple to pink is noted with change in pH.

#### 2.5.2.5 **Clopidogrel dissolved in gastric medium**

One 75 mg tablet is dissolved in 25 ml of gastric juice (8.75mM of clopidogrel) and eight 75 mg tablets in a separate 25 ml of gastric juice (70mM of clopidogrel). Both mixtures are prepared fresh and continuously mixed on the day of experiment. They are maintained at 37±1℃ using a thermostatically controlled hotplate (Stuart® US152, UK). This model is adopted to mimic the patient status where 75mg of clopidogrel (typical maintenance dose) or 600mg of clopidogrel (a typical loading dose) is ingested into a fasting stomach (25-30mL).

The average volume of gastric volume is estimated about 25-30 ml and the typical pH of gastric fluid is ≤ 2 in healthy, pre-operative and fasting patients (293). A pH ≤2 is optimal for clopidogrel-SNO generation; pH of each mixture was checked prior to use (133).

200 µl of this “reduced nitrite” (from *SIS® Go+ nitrate* gel or beet it) was added to 200 µl of the gastric medium, and another 200 µl of reduced nitrite stock added to 200µl of gastric medium and clopidogrel mixture, separately.

200 µl samples of the above mixtures were drawn into a glass Hamilton syringe and injected into the purge vessel containing the 2C’s reagent to measure RSNO generation. Samples were test immediately and following an incubation period of 20 minutes at 37 ± 1 ℃. This time point was previously established as optimum for RSNO production from nitrite in aqueous solutions (133, 253).

## 2.6 In vitro experiments with ticagrelor

### 2.6.1 Materials and methods

#### 2.6.1.1 Preparation of ticagrelor solutions

Ticagrelor (Brilique™, Astra-Zeneca, London, UK) film-coated tablets containing 90mg ticagrelor were crushed individually and mixed with 30 ml HPLC/double distilled water to create a stock 5.74 mM/L milky solution. Pure ticagrelor was unavailable hence crushed Brilique™ tablets were used.

| Ingredients of Ticagrelor (Brilique™) | |
|---|---|
| Tablet core | Mannitol (E421)<br>calcium hydrogen phosphate dehydrate<br>Magnesium stearate (E470b)<br>Sodium starch glycolate type A<br>Hydroxypropyl-cellulose (E463) |
| Tablet coating | Talc<br>Titanium dioxide (E171)<br>Iron oxide yellow (E172)<br>Macrogol 400<br>Hypromellose (E464) |

*Table 2.7: Ingredients of commercially available ticagrelor tablet (Developed and marketed by Astra-Zeneca, London, UK).*

Whilst the effects of Briligue™ excipients (Table 2.7) 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. Dr Thornhill and Prof James tested the purified Ticagrelor (made available via Multiplate supplier) in their experiments that confirmed the effect of pH and potential to form RSNO in the presence of nitrite (137).

#### 2.6.1.2 **Simulated gastric fluid with ticagrelor mixture.**

SGF was freshly prepared on each day of experimentation, and two beakers were filled with 30ml of SGF and labelled as solution G and solution TG. Whole tablets (90mg) of ticagrelor were mixed in 30ml of simulated gastric fluid with adjusted pH of 2, mimicking the physiological state of a patients who is fasted overnight, prior to a coronary angiography procedure. The solution was kept at 37ºC (± 1ºC) on a thermostatically controlled hotplate with constant stirring.

Varying concentrations of nitrite were mixed with the above solutions and incubated for 10 minutes at 37ºC in a hot water bath. Samples were withdrawn and neutralised with 1 M NaOH. The neutralised sample was immediately injected into the purge vessel containing 2Cs reagent and the baseline RSNO level was measured using OBC techniques.

It is well established that proteins containing free thiol groups readily form RSNO with nitrite. Casein, pepsin and lysozymes are main protein components of the SGF.

To test the contribution of individual SGF components in the formation of RSNO, a baseline solution with all ingredients except resorium, lysozyme, pepsin and casein was prepared. To this mixture resorium, lysozyme, pepsin and casein were added in a step wise fashion. Each sample was mixed with 500µmol/L of nitrite and incubated as in previous experiments.

## 2.7 Data collection and integration

The NOA signals are recorded digitally by the Liquid software on a computer and the area under the curve (AUC) was measured. This value was divided by the gradient of the line of transection from the standard curve performed on the day of the experiment. Nitrate, nitrite and RSNO were measured in Molar concentration and percentage of conversion was determined by comparing to the original known concentration.

The RSNO trace typically displayed a prolonged tail, often did not return completely to baseline (compared with nitrate and nitrite traces), which impairs accurate measurement of results. Hence the signal recording was stopped after 20 minutes for each sample analysed to maintain signal time-point comparison of the AUC. Mean values were used to minimise bias.

## 2.8 Data analysis and statistics

### 2.8.1 Power calculation:

The change in the RSNO levels with antiplatelet therapy and dietary nitrate has been used as primary outcome measure.

A power calculation based on prior results from CAD patients from our laboratory showed that for studies of dietary nitrate supplement in CAD, 13 subjects would provide 80% power for detecting a 50% difference in RSNO between placebo and $NO_3^-$ supplementation, assuming 10% variation, with α=0.05. A lower number were needed in this group as the patient were their own controls as it is a paired study.

For studies of dietary nitrate supplementation and clopidogrel therapy in CAD, 25 subjects in each placebo and active arm would provide 80% power for detecting a 50% difference in RSNO between placebo and $NO_3^-$ supplementation, assuming 10% variation, with α=0.05.

The ticagrelor study would require recruiting 25 subjects that would provide 80% power for detecting a 50% difference in RSNO between pre and post ticagrelor therapy, assuming 10% variation, with α=0.05.

GraphPad Prism Version 6.0 software was utilized for statistical analyses. Data are expressed as mean ± standard error of mean; error bars represent the standard error of the mean and *n*= x represents the number of repeated experiments. Two tailed student's T test or analysis of variance (ANOVA) test was used to analyze the differences between means of experimental groups. A one-way or two-way ANOVA as appropriate was applied for comparison of three or more groups. Differences were considered statistically significant where $P \leq 0.05$.

# 3 Results

## 3.1 Results 1: In vitro model of conversion of physiological in-organic nitrate from dietary source to nitrite and nitrosothiols.

### 3.1.1 Background and relevance

The role of the nitrate-nitrite- NO cycle upon ingestion of commercially available dietary nitrate supplements is not fully understood in humans. There is a need and relevance for understanding the processes involved in enzymatic conversion of the dietary supplements upon ingestion and its potential conversion to NO and RSNO. An in-vitro model would be ideal for studying the kinetics and factors that may affect the production of NO and other metabolites with these supplements in vivo.

A double blinded randomised placebo-controlled study was designed (which is a part of this current thesis (Chapter 4 to 5)), to study the effect of dietary nitrate on NO metabolites and platelet aggregation in patients with CAD. *SIS® Go+ nitrate* gel was used as the dietary nitrate supplement to enhance plasma levels of nitrate in patients with CAD who were on or would receive clopidogrel therapy.

This sub-project was designed to establish an *in vitro* model under physiological conditions to investigate the conditions affecting nitrate-nitrite-NO (RSNO) formation. This model would allow us to study possible RSNO formation under in vivo conditions, and the potential influence of introducing clopidogrel under controlled conditions. This was aimed at mimicking the supplementation of dietary nitrate to CAD patients.

Elements of this study were undertaken with the assistance of Dr Genna Logue ($5^{th}$ year medical study, joined our group for her intercalated BSc project)

### 3.1.2 Aims and hypothesis

#### 3.1.2.1 Hypothesis

An *in-vitro* model which facilitates the enzymatic reduction of nitrate to nitrite (hence RSNO and NO) from a dietary source will be successfully established and it would be in line with the in vivo process of Nitrate-Nitrite-NO (RSNO) cycle.

#### 3.1.2.2 Aims

a) Develop an *in vitro* model for enzymatic reduction of nitrate (salt) to nitrite.
   Test and optimise the reaction catalyzed by nitrate reductase from *Aspergillus niger* (Sigma Aldrich, UK)
b) Convert nitrate present in *SIS® Go+ nitrate gel* and *Beet It®* to nitrite, using the above model.
c) Test the ability of nitrite formed from the *SIS® Go+ nitrate gel* to form RSNO with gastric proteins.
d) Test the ability of nitrite formed from the *SIS® Go+ nitrate gel* to form RSNO with anti-platelet (clopidogrel) treatment when added in gastric stomach medium.

### 3.1.3 Results

#### 3.1.3.1 Conversion of sodium nitrate salt to nitrite

##### 3.1.3.1.1 Nitrate to nitrite conversion using E coli Nitrate reductase

Initial experiments were conducted with nitrate reductase from *E.Coli* (Sigma Aldrich, UK), An enzyme mix of 0.6 U/ml of NR, 5 mM of G6P, 16 µM of G6PD and 100 µM of NADPH was used as described in Alexandra B Milsom's technique (294), results are shown in Figure 3.1 and Figure 3.2.

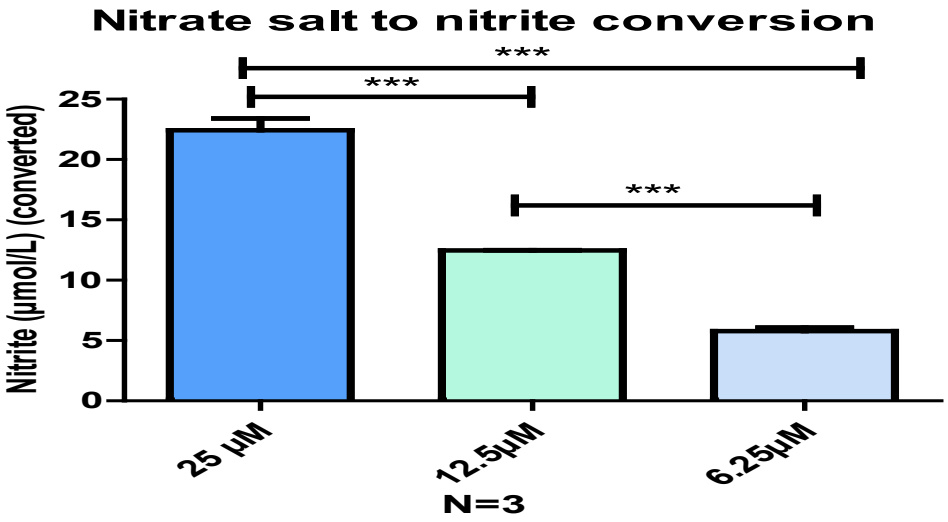



*Figure 3.1: Graphical demonstration of the amount of nitrate salt reduced to nitrite with NR derived from E Coli, x axis represent the amount of nitrate salt added to the mixture, Y axis represent the amount of nitrite reduced. An ANOVA test showed a significant concentration dependent increase in nitrite formed from nitrate salt (*** represents p<0.0001).*

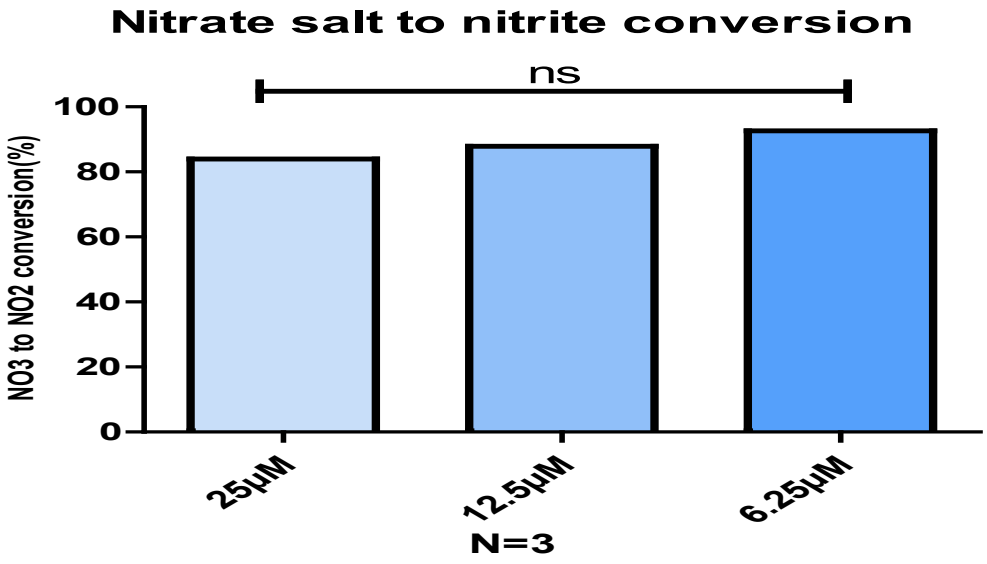



*Figure 3.2: Graphical demonstration of the percentage of nitrate salt reduced to nitrate with NR derived from E Coli, x axis represent the amount of nitrate salt added to the mixture, Y axis represent the percentage of nitrite reduced. An ANOVA test showed no significant change in extent of conversion*

The conversion of nitrate to nitrite was consistent, the conversion rate was above 80% and increased to >90 % at lower starting nitrate concentrations. We were satisfied with the model of nitrate reductase mediated conversion of nitrate to nitrite.

However, NR from E. Coli (Sigma Aldrich, UK), was commercially discontinued and was no longer available for further experiments. Nitrate reductase from other sources were explored and NR from Aspergillus Niger (Sigma Aldrich, UK) was tested.

#### 3.1.3.1.2 Nitrate to nitrite conversion using NR from Aspergillus niger

An enzyme mix (0.6 U/ml of NR, 5 mM of G6P, 16 µM of G6PD and 100 µM of NADPH) that was used in initial experiment was tested with NR from Aspergillus niger. 25 µM of sodium nitrate was added to the enzyme mix. Samples were left at +17 ± 1 °C for 45 minutes. Results are shown in Figure 3.3 and Table 3.1

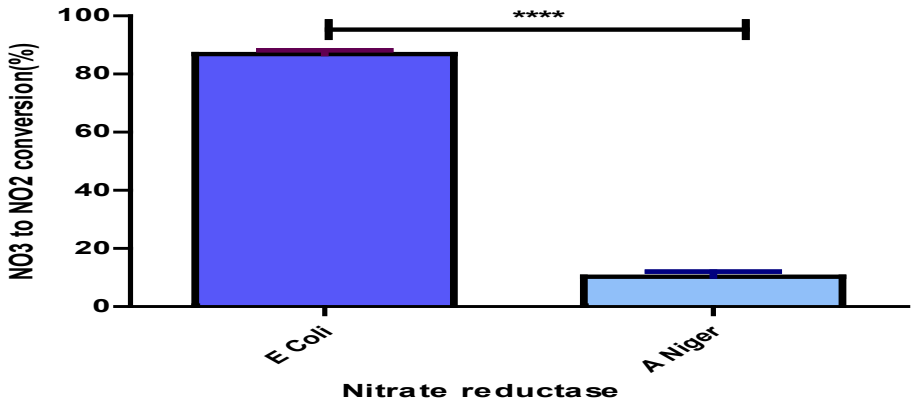



*Figure 3.3 : Reduction efficacy of NR from E Coli and A Niger on nitrite production, an Unpaired t test performed showed a P value of < 0.0001(****).*

| Enzyme Source | Mean (%) | Std. Error |
|---|---|---|
| E Coli $NO_3 \rightarrow NO_2$ (%) | 86.83 | 1.4 |
| A Niger $NO_3 \rightarrow NO_2$ (%) | 10.33 | 1.726 |
| Difference | 76.50 | ****(P<0.0001) |

*Table 3.1: Data presented as mean and standard error of mean*

A total of 6 experiments (n=6) were performed on separate days. Nitrite production was tested with NR derived from E-Coli and A Niger.

Nitrite production was significantly reduced using NR from *A. niger* (10.33%) compared to nitrite produced from preliminary work on *E. Coli* (86.83%) in the presence of similar enzyme mixes containing 0.6 U/ml of NR, 5 mM of G6P, 16 µM of G6PD and 100 µM of NADPH.

#### 3.1.3.1.3 Optimisation of enzymatic cofactors

Experiments on reduction efficacy of NR from *A. niger* were then undertaken with modification to co-factors in the enzyme mix. Varied concentrations of G6PD and NADPH were evaluated in a new enzyme mix and added to 25 µM of sodium nitrate. A maximum yield of 75.11 ± 4.34% achieved with a combination of 1000 µM of NADPH and 160 µM of G6PD and was therefore used in future experiments. Results are shown in Figure 3.4.

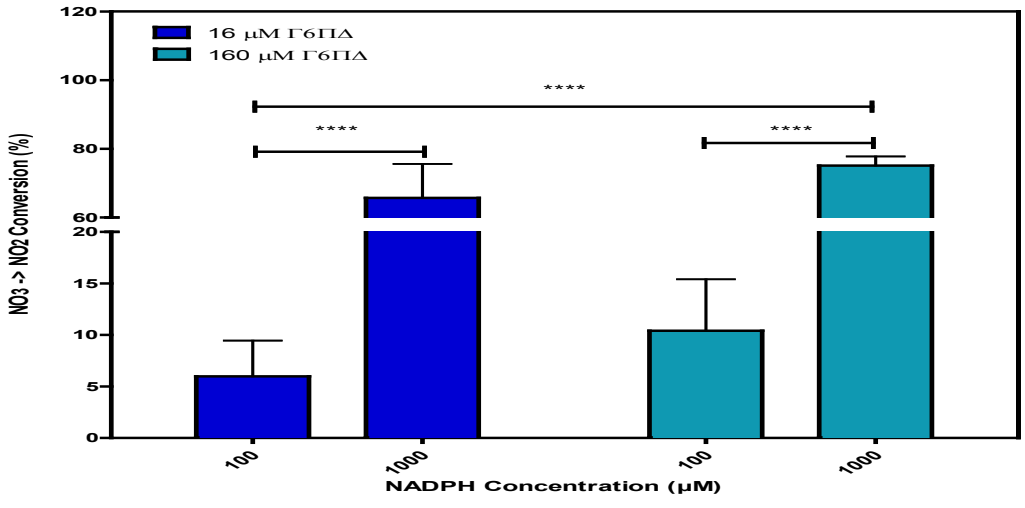



*Figure 3.4: Optimal Enzymatic Cofactor Concentrations for Nitrate Reductase activity.*

Each group represents 3 experiments performed on separate days (n=3). Two-way ANOVA with Tukey's multiple comparisons test was used, *P<0.05. (100:16 µM vs. 100:160 µM of NADPH: G6PD) (Table 3.2). There was no significant difference in nitrite production between 16 and 160 µM of G6PD in the presence of 100 µM of NADPH (P=0.7824) (100:16 µM vs.

1000:160 µM of NADPH: G6PD). A significant increase in nitrite production was observed between 100 and 1000 µM of NADPH in the presence of 160 µM of G6PD, **** (*P*<0.0001).

#### 3.1.3.1.4 Conversion of sodium nitrate to nitrite in molar concentrations

A total of 3 experiments were performed on separate days (n=3). The concentration of starting nitrate was now increased. A control of 100 µM of sodium nitrate in water without NR in the enzyme mix and a second control of enzyme mix in HPLC-grade water without nitrate was tested, these produced negligible nitrite.

Test samples contained sodium nitrate concentrations of 10, 25, 50, 100 and 1000 µM. there was linear positive correlation between the Molar concentrations of nitrite produced and molar concentrations of sodium nitrate in the test sample when NR was added. Results are shown in Figure 3.5 and Table 3.2..

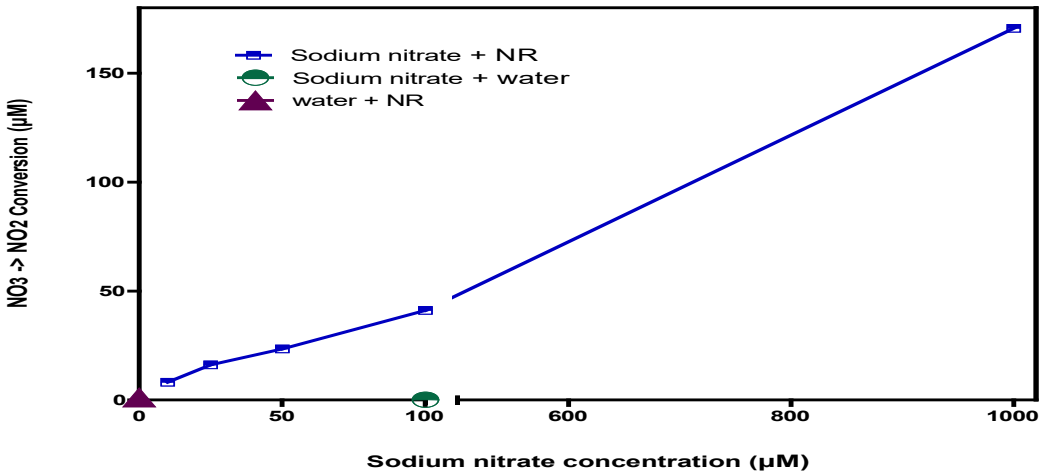



*Figure 3.5: Graph depicting the Influence of increasing the molar concentration of sodium nitrate (µM) against the molar concentration of nitrite (µM) produced.*

| Sodium Nitrate ($NaNO_3$) Tested | Converted $NO_2$ (µM) |
|---|---|
| **10** | 8.17±1.61 |
| **25** | 16.14±1.14 |
| **50** | 25.44±2.05 |
| **100** | 41.16±1.89 |
| **1000** | 170.61±2.33 |

*Table 3.2: Nitrate conversion data presented as mean ± SEM, ↑↓ represents the direction of change*

#### 3.1.3.1.5 Effect of increasing concentration of nitrate on reduction efficacy of nitrate reductase

To test the reduction efficacy of NR with increasing concentration of initial nitrate, the same test samples of sodium nitrate concentration were utilised and the percentage yield of nitrite produced calculated. The results are shown Figure 3.6 and Table 2.3

There is a linear relationship between 10 µM and 1000 µM of sodium nitrate creating a negative correlation against efficiency of nitrite produced.

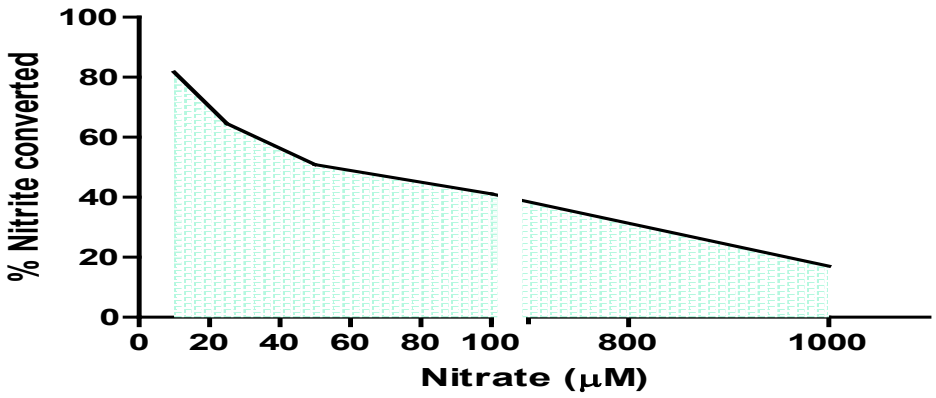



*Figure 3.6: Effect of increasing the molar concentration of sodium nitrate (µM) against nitrite produced (%).*

| Sodium Nitrate ($NaNO_3$) Tested | Converted $NO_2$ (%) |
|---|---|
| **10** | 81.70% |
| **25** | 64.56% |
| **50** | 50.88% |
| **100** | 41.16% |
| **1000** | 17.06% |

*Table 3.3: Nitrate conversion data presented as mean ± SEM, ↑↓ represents the direction of change*

#### 3.1.3.1.6 Time course of sodium nitrate conversion to nitrite by nitrate reductase

A fixed concentration of nitrate was mixed with the enzyme mix and kept at room temperature; the sample mix was injected at regular intervals of 15 minutes for a total investigation period of 1 hour 45 minutes. Mean values of sodium nitrate conversion to nitrite at each time point were recorded. Minimal and maximal nitrite production occurred at 0 and 45 minutes ± 30 seconds, respectively. Results are shown in Figure 3.7 and Table 3.4.

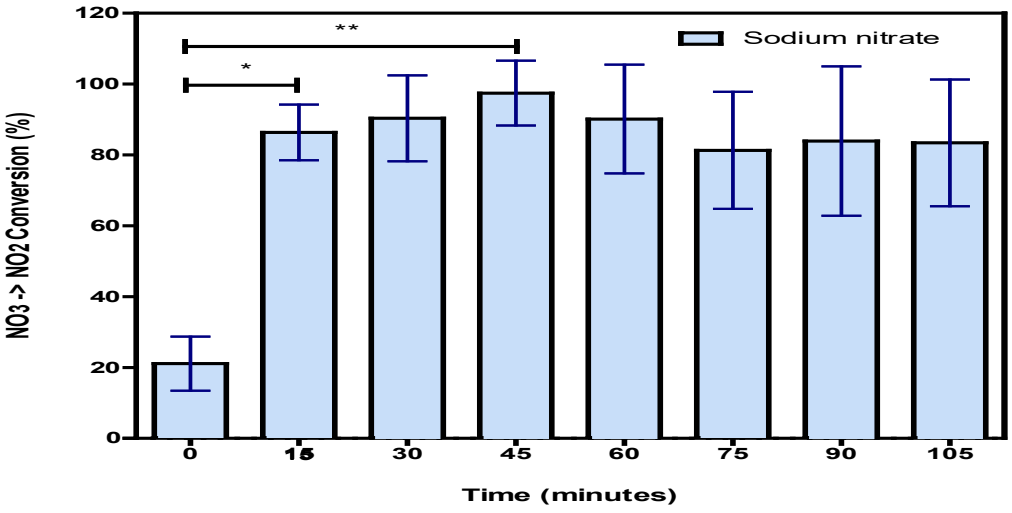



*Figure 3.7: Percentage of nitrite converted from fixed amount of nitrate with time*

| Time start (minutes ± 30 seconds) | $NO_3 \rightarrow NO_2$ (%) |
|---|---|
| 0 | 17.76 |
| 15 | 86.34 |
| 45 | 97.44 |
| 105 | 83.42 |

*Table 3.4: Nitrate conversion efficiency rate with change in time*

A total of 3 experiments performed on separate days (n=3). There was a significant increase in nitrite production between 0 and 15 minutes (*$P$<0.05) and between 0 and 45 minutes, ** ($P$=0.0050). There was no further significant increase in nitrite production after 45 minutes ($P$=0.9952).

The above experiments successfully established an in-vitro model of enzymatic conversion of laboratory grade sodium nitrate to nitrite (>95%) at 17± 1 $^0$C to serve as a standard method for future experiments with dietary nitrate supplements.

Samples were left at 17± 1 $^0$C for 45 minutes thereafter before testing (unless time was the dependent variable under investigation).

Additionally, nitrate salt conversion to nitrite was measured on each day of future experimentation to ensure the enzyme model was functioning efficiently prior to testing the commercial products. This served as a control and standardized the experiments.

#### 3.1.3.1.7 Pilot study to convert nitrate within SIS® Go+ nitrate gel and Beet It® using the above model

The typical NOA signal from 25 µM sodium nitrate following reduction to nitrite showed a peak of ~840 mV and the AUC representing the quantity of nitrite generated as shown in Figure 3.8.

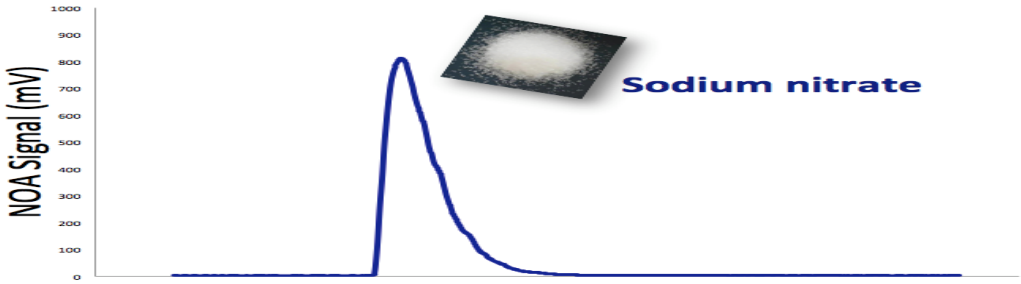



*Figure 3.8: Nitrite production from sodium nitrate (25 µM) in relation to time*

The experiment was then conducted to reduce nitrate contained within *SIS® Go+ nitrate gel* sachets in its commercially available original form using the successful model developed. A typical trace from reduction of the gel (containing 4.8M of starting nitrate) showed a peak of ~50 mV and the AUC represented the quantity of nitrite present and or generated (Figure 3.9).

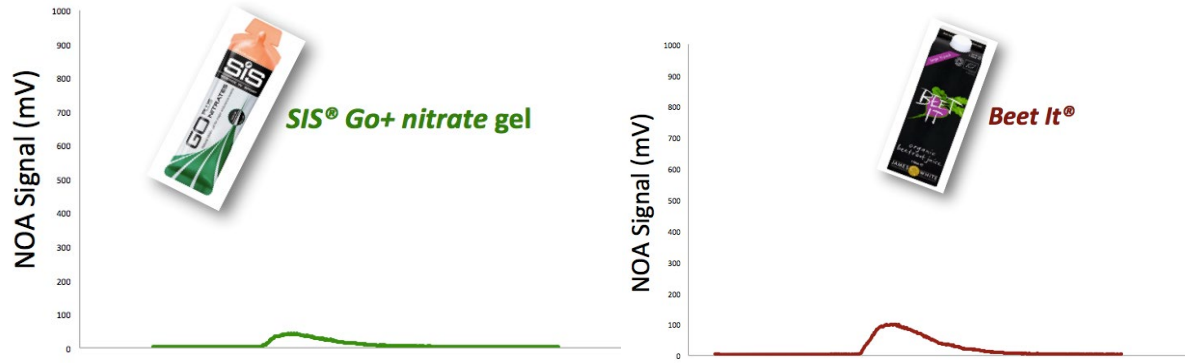



*Figure 3.9: NOA signal from SIS® Go+ nitrate gel and beet it, following incubation with the nitrate reductase enzyme mixture for 45 minutes*

Similar experiments reducing nitrate contained within '*Beet it'* were also undertaken using the successful model developed for sodium nitrate. The typical NOA trace showed a peak of 120 mV and the area under the curve is measured to quantify the nitrite generated (Figure 3.9)

| Nitrate Source | Final Nitrate Concentration injected | Measured Nitrite (µM) (Amount) | Nitrite Conversion (%) |
|---|---|---|---|
| Sodium Nitrate | 25 µM | 24.74671883 | 98.99 ↑ |
| *SIS® Go+ nitrate* gel | 250 mg/60 ml | 1.148235938 | 0.01 ↓ |
| *Beet it®* | 100 mg/ 100 ml | 0.4930258 | 0.01 ↓ |

*Table 3.5; Data presented in mean and percentage of the nitrite converted from the test sample of nitrate. The nitrate concentrations of commercial products are specified at the manufacturer's labeled value (4.8 mM for SIS® Go⁺ and 6.5mM for Beet it).*

There is minimal nitrite generated from the nitrate present in the commercial products, in their commercially available original form, in contrast with nitrate salt when using the model of nitrate reduction (Table 3.4)

### 3.1.3.2 **Optimisation of conversion of nitrate in commercial products**

#### 3.1.3.2.1 Conversion of nitrate in diluted SIS® Go+ nitrate gel to nitrite

The SIS® Go+ nitrate gel was a thick concentrate. It was diluted by factors of x4, x8, x40, x80, x400 and x800, and applied to the successful NR enzyme model. The resulting amount of nitrite generated was measured. Results are displayed in tabular form (Table 3.6) and graph (Figure 3.10)

A total of 7 experiments were performed on separate days. There was no significant difference in nitrite produced between 1/4 and 1/40 dilution, ($P$>0.9999). There was a significant increase in nitrite produced between 1/40 and 1/80 dilutions of the gel, *($P$=0.0168). A prominent increase occurs between 1/80 and 1/400 dilutions and a further marked increase between 1/400 and 1/800 dilutions, **** ($P$<0.0001) (Table 3.6).

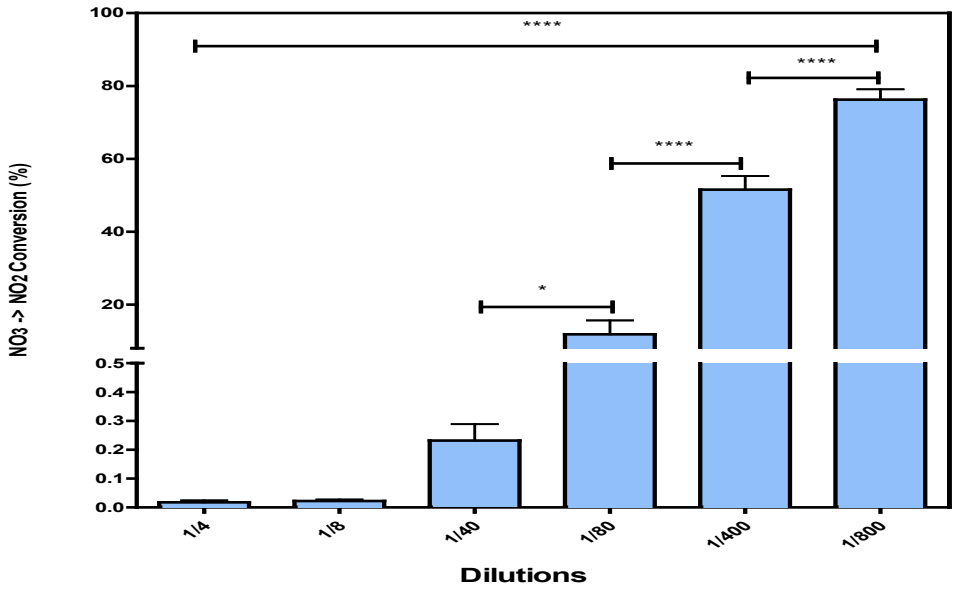



*Figure 3.10: Nitrite production from diluted concentrations of SIS® Go+ nitrate gel. A one-way ANOVA and Tukey's multiple comparison test was employed. * denotes p ≤ 0.05, *** denotes p < 0.0001.*

| Dilution | Difference of $NO_3$ → $NO_2$ (%) | Significance |
|---|---|---|
| 1/4 → 1/40 | 0.21 ± 3.20 ↑ | *ns* |
| 1/40 → 1/80 | 11.82 ± 3.38 ↑ | *P*= 0.0168 |
| 1/80 → 1/800 | 64.41 ± 3.47 ↑ | *P*<0.0001 |
| 1/400 → 1/800 | 24.72 ± 3.30 ↑ | *P*<0.0001 |

*Table 3.6: Statistical comparison of data presented as mean ± SEM, ↑↓ represents the direction of change*

#### 3.1.3.2.2 Conversion of nitrate in diluted Beet It® to nitrite

Beet it is supplied as a semi-concentrated juice. The juice was diluted by factors x2, x10, x20, x100, and x200 and the nitrite formed was measured. Results are presented in and Table 3.7.

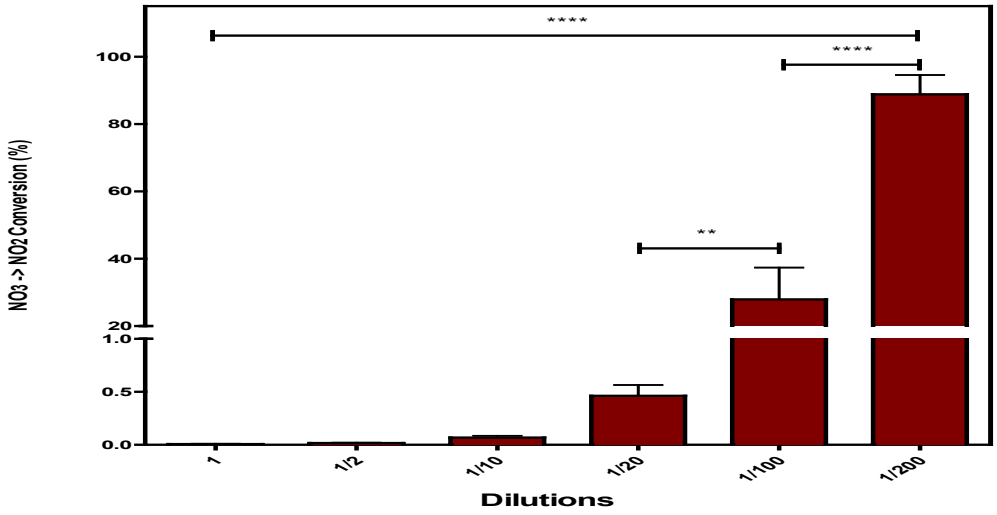



*Figure 3.11: Effect of dilution of Beet it on nitrite production catalysed by NR. One-way ANOVA test with tukey post test showed (1 vs. 1/200) ** denotes P<0.05. **** denotes P<0.0001.*

| Dilution factor of *Beet It*® | Final Nitrate Concentration Injected (µM) | Measured Nitrite Concentration (µM) | Conversion (%) |
|---|---|---|---|
| 1 | 6586.00 ↓ | 0.49 ↑ | 0.01↑ |
| 1/2 | 3293.00 ↓ | 0.62↑ | 0.02↑ |
| 1/10 | 658.50 ↓ | 0.47↑ | 0.07↑ |
| 1/20 | 329.50 ↓ | 2.51↑ | 0.76↑ |
| 1/100 | 66.00 ↓ | 50.59↑ | 76.65↑ |
| 1/200 | 33.00 ↓ | 35.13↑ | 106.45↑ |

*Table 3.7; Data presented as mean ± SEM, ↑↓ represents the direction of change*

| Dilution | Difference of $NO_3$ → $NO_2$ (%) | Significance |
|---|---|---|
| 1 → 1/20 | 0.21 ± 3.20 ↑ | *ns* |
| 1/20 → 1/100 | 27.47 ± 6.746 ↑ | *P*= 0.0073 |
| 1/100 → 1/200 | 60.88 ± 6.400 ↑ | *P*<0.0001 |

*Table 3.8: Statistical comparison of data presented as mean ± SEM, ↑↓ represents the direction of change*

#### 3.1.3.2.3 Measurements of nitrate and converted nitrite

A total measure of nitrate, nitrite and RSNO in a *SIS® Go+ nitrate* gel and Beet *it* samples were measured using the $VCl_3$ assay. The sample was then added to an enzyme mix and incubated for 45 minutes at $17^0$ C. Total nitrites produced was measured using the $I_3$ assay. Nitrite recovered from the nitrate sample was then calculated. Results are shown in Table 3.2.

| Nitrate source | Dilution | $NO_3 + NO_2 +$ RSNO* (µM) | Total $NO_2$ (µM) | Total $NO_3$ (µM) | Conversion $NO_3 \rightarrow NO_2$ (%) |
|---|---|---|---|---|---|
| *SIS® Go+ nitrate* gel | 1/400 | 95.29 | 36.86 | 58.43 | 63.08 ↑ |
| | 1/40 | 842.33 | 2.43 | 839.91 | 0.29 ↓ |
| *Beet It®* | 1/100 | 99.58 | 41.50 | 58.07 | 71.46 ↑ |
| | 1/10 | 1067.18 | 0.44 | 1066.74 | 0.04 ↓ |

*Table 3.9: SIS® Go+ nitrate gel and Beet it samples Nitrate and nitrite levels*

#### 3.1.3.2.4 A Time course of SIS® Go+ nitrate gel conversion to nitrite

A fixed concentration of 200 µl of the sample containing *SIS® Go+ nitrate* gel (1/800 dilution) and Beet it (1/100 dilution) was mixed with the enzyme mix as separate experiments and kept at room temperature, the sample mix was injected at regular intervals of 15 minutes for a total investigation period of 1 hour 45 minutes (Figure 3.12).

A total of 4 experiments were performed on separate days. There was a significant increase in nitrite production between 0 and 15 minutes (P=0.0003) and between 0 and 45 minutes

(*P*<0.0001)). There was a non-significant increase in nitrite production after 45 minutes (*P*=0.2727).

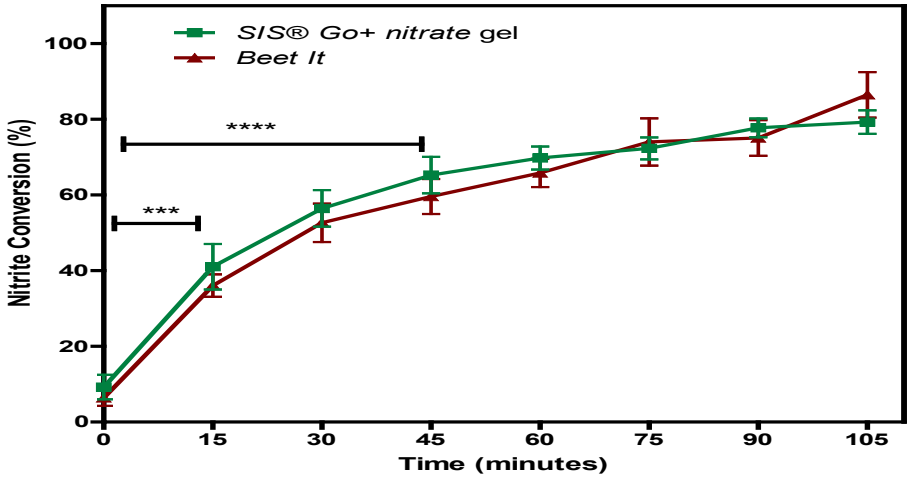



*Figure 3.12: Time effect on nitrite produced from of SIS® Go+ nitrate gel and Beet it. One-way ANOVA and Tukey's multiple comparison test was used. *** denotes p<0.001, ****denotes P<0.0001.*

### 3.1.3.3 **Effect of ingredients in the gel on nitrate conversion to nitrite**

The *SIS® Go+ placebo gel* contains identical ingredients to *SIS® Go+ nitrate* gel, except a negligible nitrate content. To test the influence of ingredients in the gel on nitrate to nitrite conversion with the NR model developed, varied dilutions of the placebo gel were also prepared similar *to SIS® Go+ nitrate dilutions).* Sodium nitrate salt was introduced into 1/4, 1/40 and 1/400 dilutions of placebo gel, so as to achieve final concentrations of 500, 50 and 5 µM. 200 µl of an enzyme mix was added to 200 µl of placebo gel and sodium nitrate mixtures. The standard procedure developed was followed and nitrite production was measured. Conversion of sodium nitrate mixed within placebo gel to nitrite

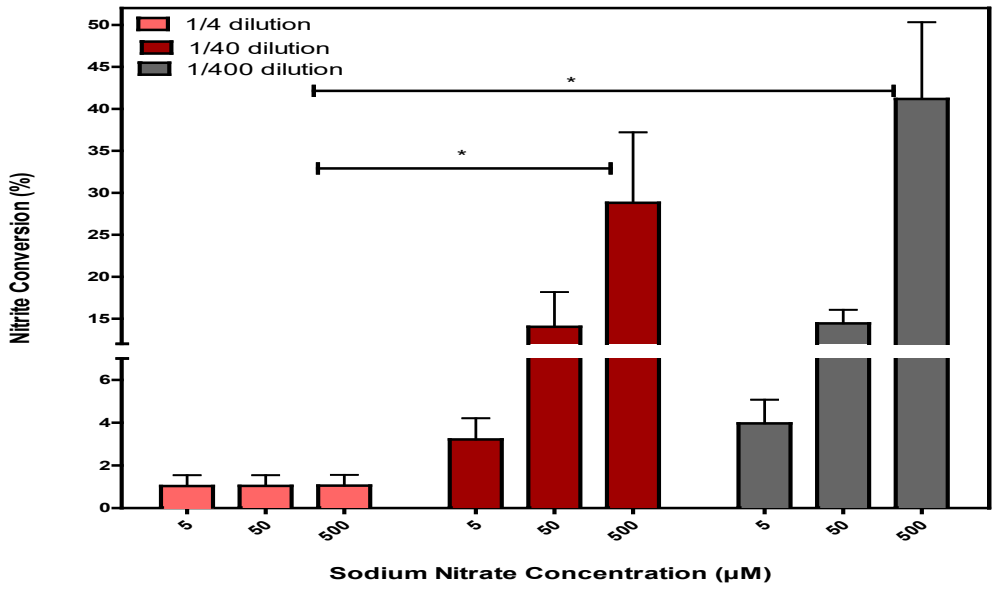



*Figure 3.13: Effect on nitrite production from known concentrations of sodium nitrate (5, 50, 500 µM) contained in diluted concentrations of placebo gel. Two-way ANOVA and Tukey's multiple comparison test was applied, *P<0.05. (1/4 vs. 1/40 and 1/400 placebo). * denotes P<0.05.*

A total of 3 experiments were performed on separate days. There was a significant difference in nitrite production between 1/4 vs. 1/40 and 1/400 dilutions of the placebo gel mixed with 500 µM of sodium nitrate, ****(*P*<0.0001). There was no significant difference in nitrite production between 1/40 and 1/400 dilutions of the placebo gel mixed with 500 µM of sodium nitrate, (*P*=0.6210). There was no significant difference in nitrite production between 1/40, 1/40 and 1/400 dilutions of the placebo gel mixed with 5 or 50 µM of sodium nitrate. Control experiments using each dilution of the placebo gel and NR show negligible nitrite production. HPLC-grade water and NR also show negligible nitrite production.

In summary, 1/4 dilution of the gel has an inhibitory effect on sodium nitrate reduction and consequently minimal nitrite yield. With increasing dilution there was an increase in percentage of nitrite produced. Results are shown in Figure 3.13.

50 µM of sodium nitrate was tested against placebo gel. Nitrite yield was 91.28 ± 6.44% in the absence of placebo gel. In the presence of 1/40 and 1/400 dilutions of the placebo gel nitrite production significantly decreased, as shown in Table 3.10.

| | Dilution | $NO_3$ from sodium nitrate (µM) | Conversion $NO_3 \rightarrow NO_2$ (%) | Difference |
|---|---|---|---|---|
| *Placebo gel* | 0 | 50 | 91.28 ± 6.44 | |
| | ¼ ↓ | 50 | 1.04 ± 0.50 ↑ | 13.41 ± 1.70 ↑ |
| | 1/400 ↓ | 50 | 14.45 ± 1.62 ↑ | |

*Table 3.10: Percentage of nitrite conversion with diluted placebo and 50µM nitrate mix*

### 3.1.3.4 **RSNO generation from Nitrite in gastric medium**

### 3.1.3.5 **Conversion of nitrite formed from sodium nitrate reduction to RSNO**

Using the model described above, the formation of RSNO in simulated gastric fluid from nitrite (produced by NR catalysis of 50 µM sodium nitrate) was investigated. The yield was 4.41 ± 3.05% of RSNO as shown in Figure 3.14

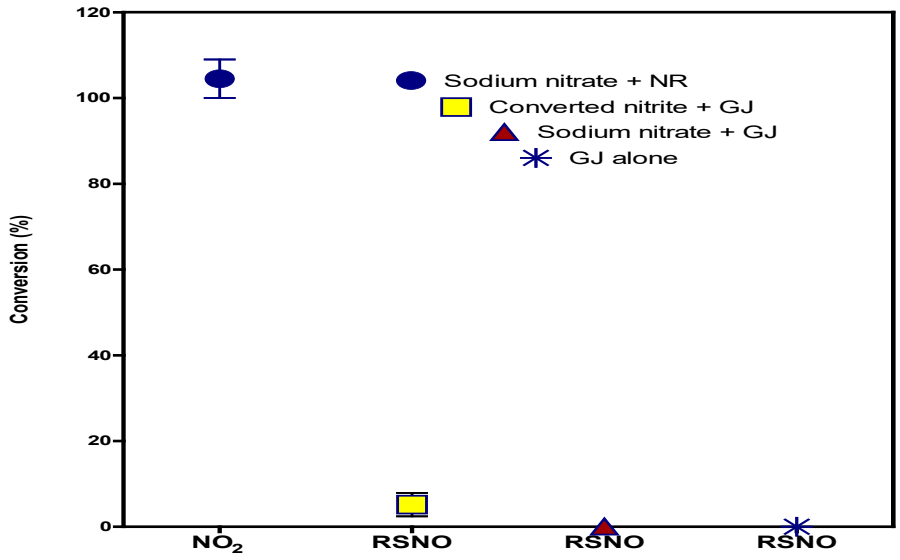



*Figure 3.14 : Nitrite produced from sodium nitrate (catalyzed by NR) and subsequent RSNO formation in gastric medium.*

A total of 3 experiments was performed on separate days. 104.49 ± 4.48% of nitrite was produced from 50 µM of sodium nitrate. Of the nitrite produced, 4.41 ± 3.05% formed RSNO in the presence of gastric proteins. Control experiments using 50 µM sodium nitrate plus Gastric juice (GJ) and GJ alone showed negligible RSNO production.

### 3.1.3.6 **Conversion of nitrite formed from *SIS® Go+ nitrate* gel to RSNO**

Using the model described above, the formation of RSNO in simulated gastric fluid from nitrite produced from NR catalysis of a 1/400 dilution of *SIS® Go+ nitrate* gel was investigated. An yield of 36.71 ± 13.01% of RSNO was measured (Figure 3.15).

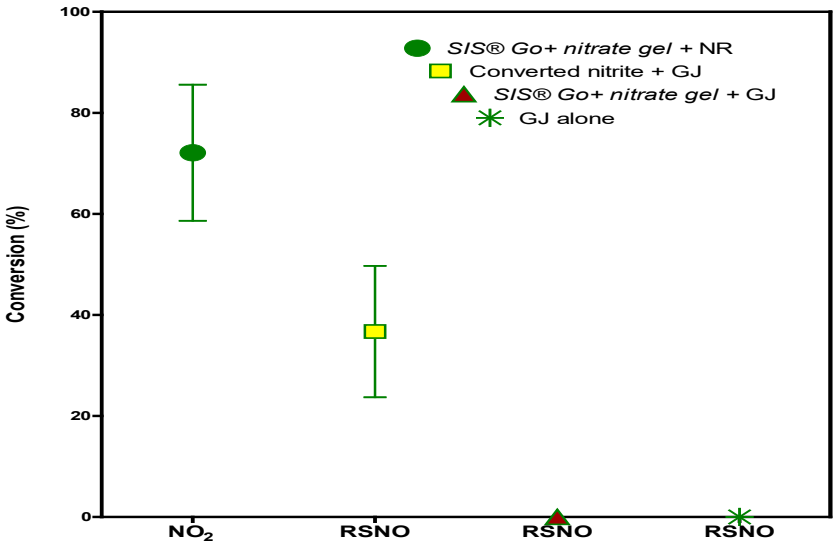



*Figure 3.15: Nitrite produced from SIS® Go+ nitrate gel (catalyzed by NR) and subsequent RSNO in gastric medium.*

A total of 3 experiments were performed on separate days. 72.11 ± 13.48% of nitrite was produced from 1/400 dilution of *SIS® Go+ nitrate* gel. Of the nitrite produced, 36.71 ± 13.01% formed RSNO in the presence of gastric proteins. Control experiments using 1/400 dilution of *SIS® Go+ nitrate* gel plus GJ and GJ alone show negligible conversion to RSNO.

### 3.1.3.7 RSNO formation from nitrite formed from SIS® Go+ nitrate gel in the presence of thienopyridine

An experiment similar to the above was performed to investigate the influence of clopidogrel on the formation of RSNO in the gastric juice mixed with nitrite reduced from SIS® Go+ nitrate gel, from the NR activity.

Clopidogrel 75mg and 600mg added separate samples of artificial gastric juice and nitrite converted from 1/800 diluted of SIS® Go+ nitrate gel

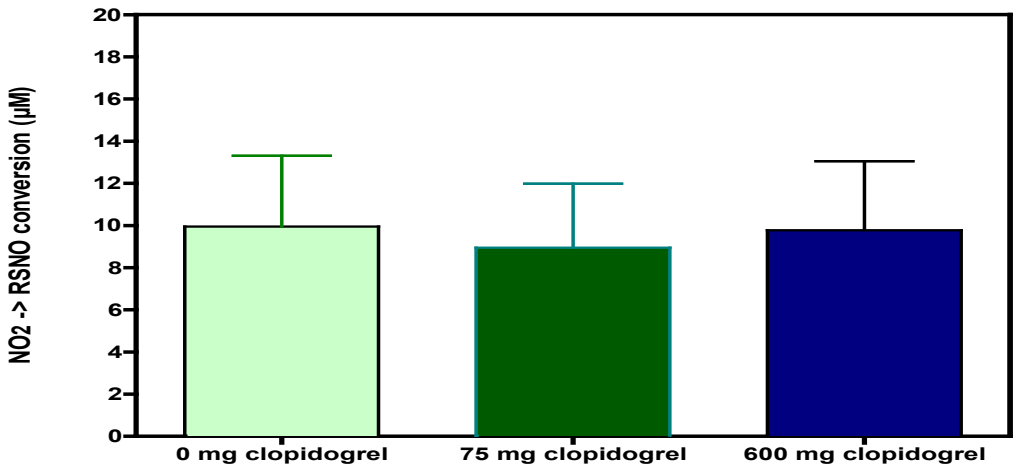



*Figure 3.16 : RSNO formation from nitrite that is produced from 1/800 dilution of SIS® Go+ nitrate gel in gastric juice proteins and clopidogrel. One-way ANOVA and Tukey's multiple comparison tests, (0 mg vs. 75mg vs. 600 mg clopidogrel).*

A total of 3 experiments were performed on separate days. There is no significant difference in RSNO formation between the 3 groups. Summary (*P*=0.9731). Control experiments using GJ in the absence or presence of 75 mg or 600 mg of clopidogrel are shown as black symbols and indicate RSNO production.

| Anti-platelet therapy | Dose (mg) | $NO_2$ from 1/400 dilution of *SIS*$^{\ominus}$ $^{\ominus}$ *Go+ nitrate* gel (µM) | Conversion $NO_2$ → RSNO (µM) | Significant difference |
|---|---|---|---|---|
| *Clopidogrel* | 0 | 41.67 ± 6.48 | 13.26 ± 0.75 | *ns* |
| | 75 | | 11.93 ± 0.82 | |
| | 600 | | 13.03 ± 0.52 | |

*Table 3.11: Data presented as mean ± SEM*

There is no significant difference in RSNO production in the absence or presence of clopidogrel (75 or 600 mg) in the gastric medium with similar starting nitrite concentrations as shown in Figure 3.16, and Table 3.11.

### 3.1.4 Summary of principal findings

- ✓ An enzyme mix composition of 0.6 U/ml of NR from *A. niger,* 5 mM of G6P, 1000 µM of NADPH and 160 µM of G6PD resulted in optimum conversion of nitrate to nitrite.

- ✓ A positive relationship occurs between increasing the starting molar concentrations of laboratory grade sodium nitrate and the nitrite produced.

- ✓ Nitrate present within the commercial products, *SIS® Go+ nitrate gel* and *Beet It ®* (in their original commercially available forms) produced limited nitrite, when using the above model.

- ✓ Dilution of the *SIS® Go+ nitrate* gel concentration by 400 and *Beet It®* concentration by 100 enhanced the nitrate to nitrate conversion.

- ✓ Optimally 45 minutes was required to improve nitrite production from nitrate within *SIS® Go+ nitrate gel* and *Beet It®.*

- ✓ The placebo gel (in its commercially available form) inhibited nitrite production from sodium nitrate, under standard conditions.

- ✓ RSNO was produced in gastric medium which was converted from sodium nitrate and *SIS® Go+ nitrate* gel.

- ✓ A greater amount of RSNO was formed from the nitrite converted within the *SIS® Go+ nitrate* gel, compared to that converted from sodium nitrate.

- ✓ There was no significant difference in RSNO produced in the absence or presence of 75 mg and 600 mg of clopidogrel within gastric medium.

## 3.2 Results 2: Dietary Nitrate supplement in Stable CAD Patients

### 3.2.1 Background

Dietary nitrate administration among healthy volunteers has shown a considerable reduction in platelet reactivity and increase in NO metabolites in the circulation. Endogenous sources of NO and its metabolites are impaired in patients with CAD, and exogenous sources through diet may present a promising and feasible alternative to maintaining NO biology in humans, thereby regulating vasomotor function and haemostasis. There is no study published to date, which has tested the effect of dietary nitrate supplements in patients with established CAD, other than the work of our own research group, where a portion of this data, along with other parallel work was published recently (263). This section presents the results of dietary nitrate supplementation on platelet function and NO metabolites in patients with stable angina.

### 3.2.2 Hypothesis

We hypothesised that administration of dietary nitrate supplement in patients with stable CAD would

- Enhance the circulatory NO metabolites, hence increase NO bioavailability.
- Reduce platelet activation via ADP and TRAP stimulation.
- Result in an inverse correlation between the NO metabolite levels and platelet reactivity.

### 3.2.3 Results

Fifteen patients with established CAD consented to take part in this study. Patients attended the cardiology day care unit, as part of their normal clinical care. All patients were randomised to receive either 2x60ml placebo coded nitrate supplement, followed by a wash out period then the active nitrate supplement or vice versa. A seven-day wash out period was adopted in line with several other studies (295). The study design is illustrated in Figure 2.1.

### 3.2.3.1 **Patient characteristics**

Of the 15 patients recruited to this study, 13 were male. The mean age of the group was 62.7 ± 3.2 years, 4 had previous MI and undergone percutaneous intervention, 4 were diabetic, 12 had hypertension and majority of them had hypercholesterolemia. There was a mixture of cardiovascular risk factors in the group, depicting the general presentation of the population of CAD patients (Table 3.12).

| Participant Characteristics | Naïve group (n=15) |
|---|---|
| Age(mean) | 62.7 ± 3.19 |
| BMI (kg/m2) | 29.9 ± 1.49 |
| **Male** | 13(87%) |
| **Cardiovascular Risk Factors** | |
| Diabetes Mellitus | 1 (7%) |
| Past/Current Smoking | 7 (46%) |
| Hypertension | 12 (80%) |
| Dyslipidaemia | 15 (100%) |
| Stroke/TIA | 1 (7%) |
| Peripheral Vascular Disease | 1 (7%) |
| History of MI | 4 (27%) |
| **Previous Revascularisation** | |
| PCI | 4 (27%) |
| CABG | 2 (14%) |
| **Medications** | |
| Aspirin | 13 (86%) |
| Clopidogrel | 0 (0%) |
| Proton Pump Inhibitor | 9 (60%) |
| Beta Blockers | 7 (46%) |
| ACE inhibitors/ARB | 10 (66%) |
| Statins | 15(100%) |
| Thyroxin | 1 (10%) |
| NSAIDs | 0 (0%) |

*Table 3.12: Baseline characteristics of patients, summary sheet including age, cardiovascular risk factors and medications.*

### 3.2.3.2 Effect of nitrate and placebo gel supplementation on NO metabolites

#### 3.2.3.2.1 Nitrate levels

The mean plasma concentration of nitrate rose significantly from a baseline of 30.92 µM (± 10.9) to 266.1 µM (± 90) following intake of nitrate gel (Figure 3.17A), whereas there was no rise in mean nitrate concentration with a baseline of 29.20 µM (±12.6) to 31.71 µM (±13.8) following intake of placebo gel (Figure 3.17B). There was a statistically significant (P<0.0001) increase in the nitrate level 2hrs following ingestion of nitrate gel in comparison to placebo gel.

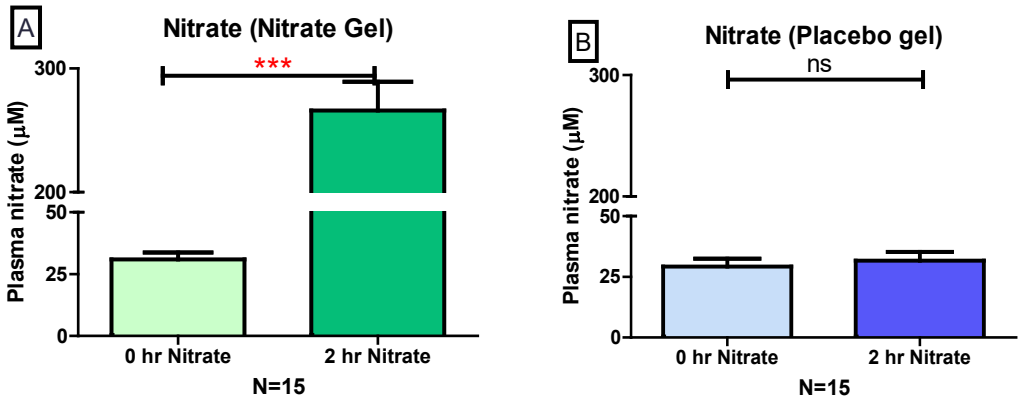



*Figure 3.17: Mean plasma concentration of nitrate at baseline and 2 hours post nitrate gel ingestion and placebo gel respectively. *** represents p<0.001 and ns represents no significance.*

The mean difference in the rise of nitrate levels from baseline to 2-hour post ingestion was 235.2µM (±89) and 2.5 µM/L (±6) after ingestion of active and placebo gel respectively. There was a statistically significant (P<0.0001) increase in the nitrate level upon ingestion of nitrate gel in comparison to placebo gel (Figure 3.18).

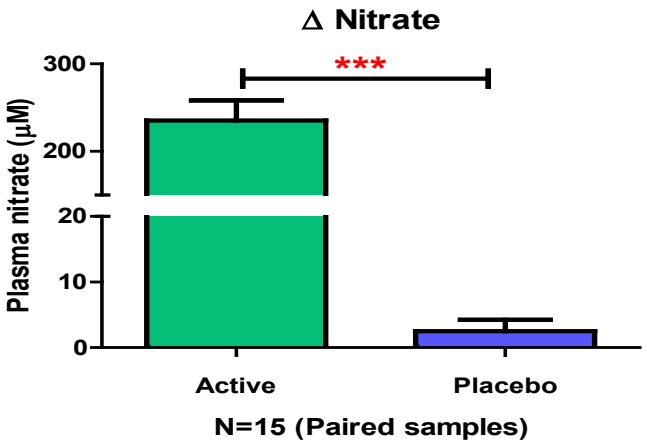



*Figure 3.18: The mean difference derived from nitrate levels before and after ingestion of nitrate and placebo gel. *** represents p<0.001*

### 3.2.3.2.2 Nitrite levels

The mean plasma concentration of nitrite rose significantly (P<0.0001) from a baseline of 115.9nM (± 68) to 330nM (±193) following intake of nitrate gel (Figure 3.19A), whereas there was a small non-significant rise in mean nitrite concentration with a baseline of 137nM (±159) to 167nM (±198) following intake of placebo Gel (Figure 3.19B).

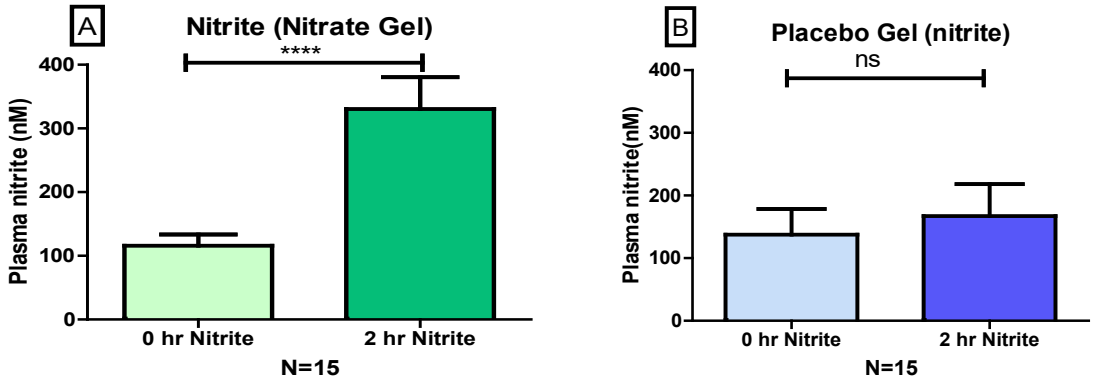



*Figure 3.19: Effect of the dietary supplement of nitrate gel (A) and placebo gel (B) on plasma nitrite at 0 hour and two-hour post ingestion. Data are expressed as mean ± standard error of mean. Student paired T-test is applied for test of significance.*

The mean difference in the rise of nitrite levels from baseline to 2-hour post ingestion was 214.6nM (±144) with 95% confidence interval of 134.8 to 294.4 for nitrate gel. For the placebo gel the mean difference was 29.8nM with 95% confidence interval of -6.9 to 66.4. A paired T test showed a statistically significant (P=0.0002) change in the nitrite levels with ingestion of nitrate gel in comparison to placebo gel (Figure 3.20)

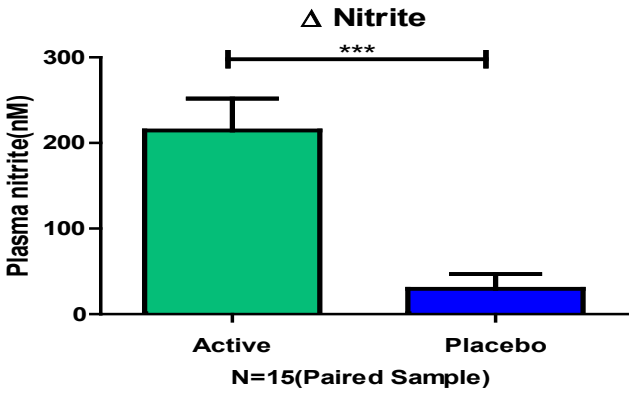



*Figure 3.20: A comparison of the change (Δ) in the nitrite levels from baseline to 2 hours post ingestion nitrate gel and placebo gel measured in nmol/L. Data are expressed as mean (difference) ±SEM.*

#### 3.2.3.2.3 RSNO levels

The mean plasma concentration of RSNO rose significantly from a baseline of 8.3 nM (± 4.5) to 18.6 nM (±14.6) following intake of nitrate gel (Figure 3.21A), whereas there was no rise in mean RSNO concentration with a baseline of 10.89nM (±7.4) to 10.95nM (±7.4) following intake of placebo gel (Figure 3.21B). A paired student T test showed a statistically significant (P=0.049) increase in the RSNO levels measured two hours post ingestion of nitrate gel (Figure 3.21A).

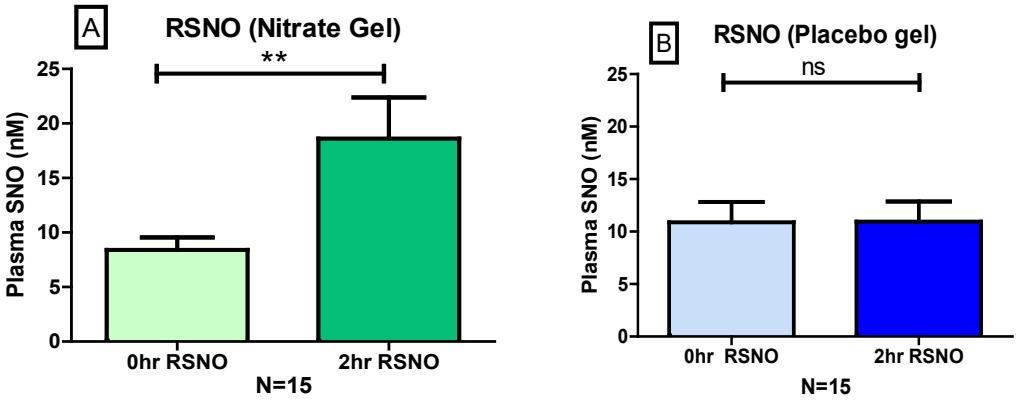



*Figure 3.21: Mean plasma concentration of RSNO at baseline and 2 hours post nitrate gel ingestion (graph A) and placebo gel (graph B) respectively.*

The mean difference in the rise of RSNO levels from baseline to 2-hour post ingestion was 10.38 nM at 95% confidence interval of 3.8 to 17 after ingestion of nitrate gel and with placebo gel ingestion the mean difference in from baseline to 2 hours was 0.05 nM with 95% confidence interval of -1.9 to 2. A paired t test confirmed a statistically significant (P=0.014) increase in the RSNO levels following ingestion of nitrate gel (Figure 3.22).

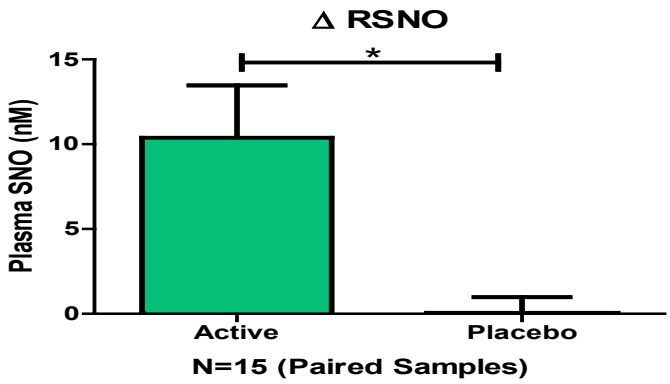



*Figure 3.22: Mean difference in RSNO levels before and after ingestion of active and placebo gel. * represents P<0.05*

### 3.2.3.3 Effect of nitrate and placebo gel supplementation on platelet reactivity

#### 3.2.3.3.1 ADP induced platelet reactivity

The mean platelet response to ADP was measured using multiple electrode aggregometry (Multiplate®). Applying a Kolmogorov-Smirnov test and D'Agostino & Pearson omnibus normality testing confirmed Gaussian normal distribution of the data. A paired t-test was used for analysis of significance. The platelet reactivity was 527.7 (±156.8) AU/min at baseline and 574.6 (± 239) AU/min at 2 hours following the intake of nitrate gel (Figure 3.23A), with a P value of 0.32. With the intake of placebo gel the platelet reactivity was 530.4(±223) AU/min at baseline and 635.8 (± 239) AU/min at 2 hours (Figure 3.23B)with a P value of 0.06115.

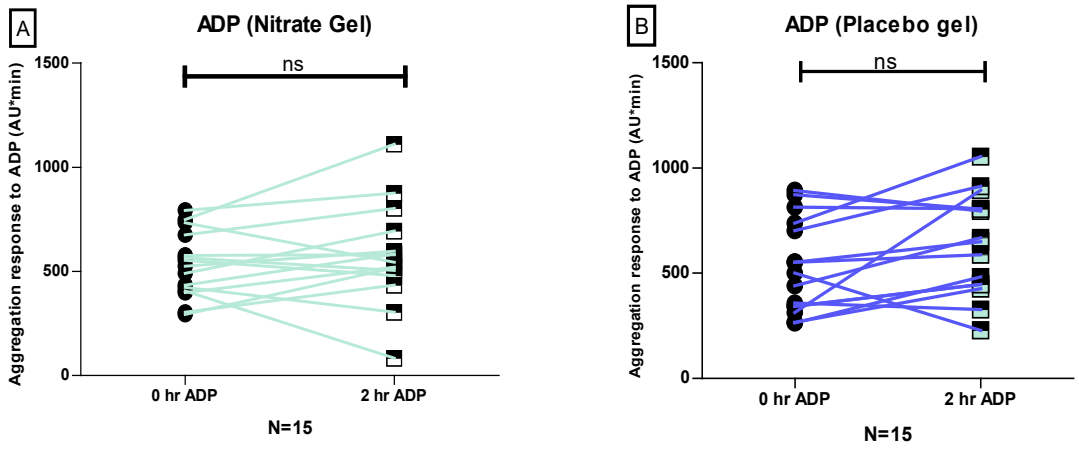



*Figure 3.23: Platelet reactivity upon stimulation with ADP agonist, Measure at baseline (0 ADP) and two-hour post ingestion of F (graph A) and placebo gel (graph B), measured in AU*min. ns represents statistical no significance.*

The mean difference in the platelet reactivity from baseline to 2-hour post ingestion was -48.87 (±176.1) AU/min and -105.4 (±200) after ingestion of active and placebo gel respectively. There was an apparent decrease in the platelet reactivity with nitrate gel compared to placebo gel, however there was no statistical difference (P=0.29) (Figure 3.24).

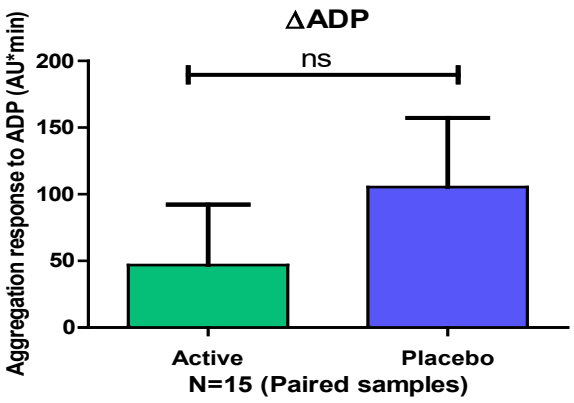



*Figure 3.24 : Mean difference in measured Platelet reactivity from baseline to 2 hours upon stimulation with ADP agonist*

#### 3.2.3.3.2 TRAP induced platelet reactivity

The platelet reactivity to TRAP was 917.7 (±214) AU/min at baseline and 1065 (± 264) AU/min at 2 hours following the intake of nitrate gel (Figure 3.25A), with a P value of 0.02. With the intake of placebo gel the platelet reactivity was 917.8(±276) AU/min at baseline and 981.5 (± 286.5) AU/min at 2 hours (Figure 3.25B), with a P value of 0.354. A student paired t test showed a statistically significant increase in platelet reactivity at 2 hours with ingestion of nitrate gel.

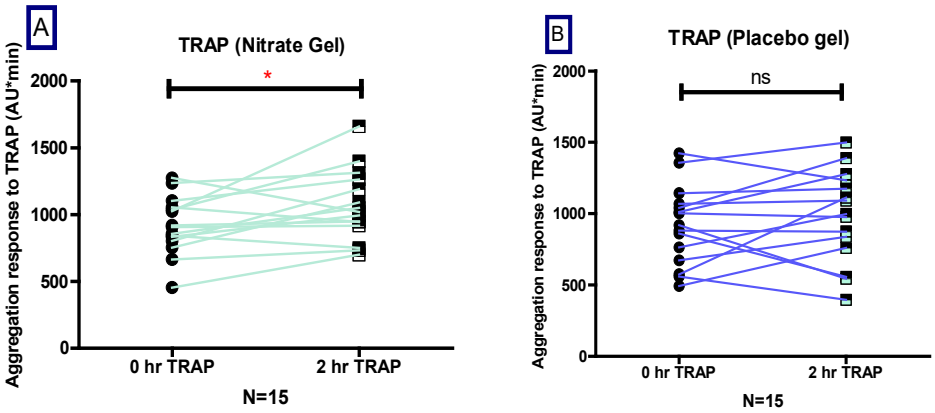



*Figure 3.25: Platelet reactivity upon stimulation with TRAP agonist, Measure at baseline (0 TRAP) and two hour( 2-TRAP) post ingestion of nitrate (graph A) and placebo gel(graph B), measured in AU*min. * represents p<0.05 and ns represents no significance*

The mean difference in the platelet reactivity from baseline to 2-hour post ingestion was 147.5 (±225.7) AU/min and 63.67 (±210) after ingestion of active and placebo gel respectively. There was no statistically significant variation (0.42) in the platelet reactivity to TRAP with nitrate gel or placebo gel intake (Figure 3.26).

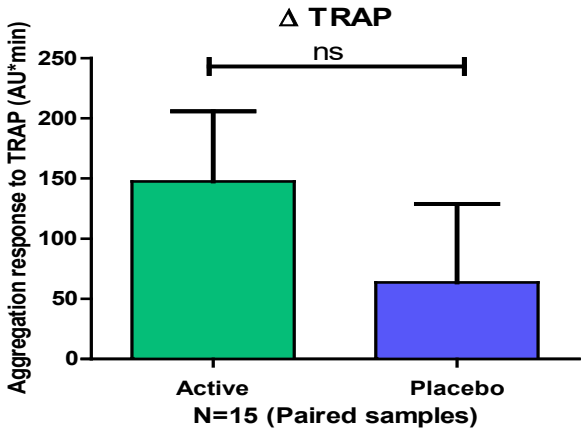



*Figure 3.26: Mean difference in measured Platelet reactivity from baseline to 2 hours upon stimulation with TRAP agonist,*

### 3.2.5 Summary of principle findings

- ✓ Ingestion of active SIS® Go$^+$ nitrate supplement is associated with a significant rise in the plasma nitrate levels in CAD patients

- ✓ CAD patients have lower level of plasma nitrite at baseline compared with healthy volunteers

- ✓ Ingestion of active SIS® Go$^+$ nitrate supplement is associated with a significant rise in the plasma nitrite levels within two hours in CAD patients

- ✓ There was also a significant rise in the plasma RSNO levels in patients after ingestion of SIS® Go$^+$ nitrate supplement.

- ✓ The finding of generation of nitrite and RSNO following intake of nitrate supplement is consistent with *in vitro* experiments described earlier in chapter 3.

- ✓ There was no effect on ADP mediated platelet aggregation with SIS® Go$^+$ nitrate supplement in CAD patients.

- ✓ There is a significant change in the TRAP mediated platelet aggregation from baseline to 2 hours, with SIS® Go$^+$ nitrate supplement in CAD, however this effect becomes non-significant when compared with placebo supplementation.

## 3.3 Results 3: Dietary nitrate supplementation with clopidogrel therapy in stable CAD patients

### 3.3.1 Background:

Clopidogrel therapy is commonly prescribed in patients with ACS and or in patients undergoing PCI. This section reports the results of a double-blinded, randomised placebo-controlled study of concomitant intake of dietary nitrate supplementation and clopidogrel in patients with stable CAD. This is the first known study to explore a combined effect of clopidogrel and nitrate therapy.

### 3.3.2 Hypothesis

In this chapter, we hypothesised that administration of dietary nitrate supplement along with clopidogrel therapy in patients with stable CAD would

- Enhance the circulatory NO metabolites, hence increase NO bioavailability.
- Promote Clopidogrel reaction with exogenous derived nitrite *in vivo* and form clopidogrel-SNO.
- Cause additional platelet inhibition independent of $P2Y_{12}$ receptor blockage.
- Influence RSNO formation and platelet aggregation in patients on concomitant proton pump inhibitor therapy (PPI).

#### 3.3.2.1 **Subgroups:**

1. Acute Clopidogrel Loading group (clopidogrel 600mg): **Group A**

   These patients are on aspirin alone and no other antiplatelet therapy. They are prescribed with a loading dose of clopidogrel 600mg as per clinical guidelines on the day of their coronary intervention procedure.

2. Chronic clopidogrel (clopidogrel 75mg): **Group B**

   These patients are on aspirin and Clopidogrel therapy (75mg) for more than 1 month and listed for a day case coronary intervention.

Dietary nitrate or placebo supplementation was given in randomised fashion as shown in Figure 2.2 of Materials and Methods (Chapter 2).

### 3.3.3 Results

A total of 104 patients were recruited for this study. They were divided into **Group A** (clopidogrel 600mg) (n=50) and **Group B** (n=54) based on the clopidogrel treatment regime as explained in the Methods.

#### 3.3.3.1 **Patient characteristics**

**Group A: SCAD patients with acute clopidogrel loading**

Patients in this group were diagnosed to have stable angina and were scheduled for elective coronary angiogram and PCI. As a part of established evidence based clinical practice, these patients received a loading dose of 600 mg of clopidogrel prior to their procedure.

A total of 50 patients met the inclusion criteria and gave voluntary consent for participation. Three patients were excluded; 1 because of inability to collect the 2-hour sample, 2 due to analysis issue. The final number of patients was 47, with 24 patients were randomly assigned to nitrate gel Group A (clopidogrel 600mg) and 23 patients to placebo gel group. 3 Baseline blood samples were collected. Patients were given 2 sachets of study gel (active or placebo) for ingestion, along with 600 mg of clopidogrel. A second blood sample was collected 2 hours later, as described in the Methods.

There was no significant difference in the age, gender, clinical presentation, past medical history (e.g. hypertension, diabetes, cigarette smoking, cardiac procedures) or other medical

conditions and prescribed medications comparing patients receiving nitrate gel versus placebo gel (Table 3.13).

| Demography | Nitrate gel (n=24) | placebo gel (n=23) | P value |
|---|---|---|---|
| **Age** | 67 (± 29) | 63(±20) | ns |
| **Male** | 16 | 15 | ns |
| **chest pain** | 26 | 24 | ns |
| **SOB** | 17 | 16 | ns |
| **Myocardial infarction** | 7 | 6 | ns |
| **Previous PCI** | 10 | 5 | ns |
| **Cardiac surgery** | 2 | 1 | ns |
| **Diabetes** | 8 | 5 | ns |
| **Hypertension** | 20 | 19 | ns |
| **Hyperlipidaemia** | 17 | 19 | ns |
| **Smoker** | 16 | 17 | ns |
| **Thyroid** | 1 | 0 | ns |
| **Respiratory problem** | 6 | 3 | ns |
| **CVA/TIA** | 3 | 1 | ns |
| **Renal history** | 2 | 0 | ns |
| **Medications** | | | |
| **Aspirin** | 26 | 24 | ns |
| **Beta blockers** | 20 | 15 | ns |
| **ACE inhibitors** | 13 | 9 | ns |
| **Statins** | 22 | 20 | ns |
| **Diabetic drugs** | 9 | 5 | ns |
| **CCB** | 5 | 5 | ns |
| **Thyroxin** | 1 | 0 | ns |
| **NSAID** | 13 | 10 | ns |
| **PPI** | 13 | 10 | ns |

*Table 3.13: Demographic and clinical characteristics of Group A patients, including list of current medication. Aspirin was prescribed in all patients*

### Group B: SCAD Patients with chronic clopidogrel treatment

Patients in this group were diagnosed to have stable angina and are on long-term aspirin and clopidogrel therapy. They are scheduled for elective coronary angiogram and PCI on the day of recruitment.

A total of 53 patients met the inclusion criteria and gave a voluntary consent for participation. 28 patients were randomly assigned to nitrate gel and 25 patients to the placebo gel group. Baseline blood samples were collected. Patients were given 2 sachets of study gel for ingestion (active or placebo). A second blood sample was collected 2 hours later.

| Demography | Nitrate gel(n=28) | placebo gel (n=25) | P value |
|---|---|---|---|
| **Age** | 66 (± 29) | 65(±20) | ns |
| **Male** | 23 | 21 | ns |
| **chest pain** | 23 | 15 | ns |
| **SOB** | 12 | 9 | ns |
| **Myocardial infarction** | 22 | 16 | ns |
| **Previous PCI** | 18 | 17 | ns |
| **Cardiac surgery** | 6 | 5 | ns |
| **Diabetes** | 4 | 2 | ns |
| **Hypertension** | 24 | 19 | ns |
| **Hyperlipidaemia** | 25 | 19 | ns |
| **Smoker** | 18 | 17 | ns |
| **Thyroid** | 2 | 2 | ns |
| **Respiratory problem** | 5 | 3 | ns |
| **CVA/TIA** | 3 | 2 | ns |
| **Renal history** | 0 | 0 | ns |
| **Medications** | | | |
| **Beta blockers** | 17 | 14 | ns |
| **ACE inhibitors** | 13 | 14 | ns |
| **Statins** | 23 | 20 | ns |
| **CCB** | 6 | 5 | ns |
| **Thyroxin** | 1 | 2 | ns |
| **NSAID** | 2 | 2 | ns |
| **PPI** | 15 | 13 | ns |

*Table 3.14: Summary of patient's characteristic, collected at the time of recruitment of group B. There is no statistically significant variation between two groups.*

### 3.3.3.2 Effect of nitrate and placebo gel supplementation on NO metabolites

**Nitrate levels**

There was no significant difference in the baseline levels of plasma nitrate between nitrate supplement and placebo therapy in either groups [Group A : Nitrate:-28.7(±9) vs 33.98(±17), P>0.05 and [Group B: Nitrate:- 28.7(±9) vs 33.98(±17), P>0.05] as shown in Figure 3.27.

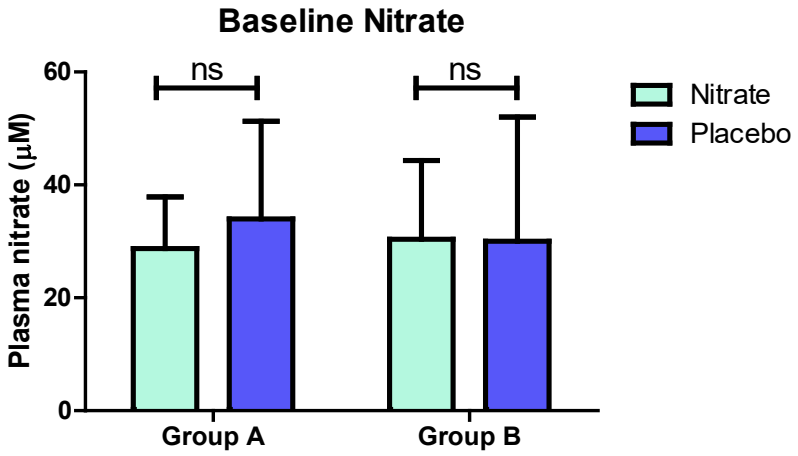



*Figure 3.27: Baseline Nitrate measured in Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg)*

The mean plasma concentration of nitrate rose significantly (P<0.0001) from a baseline of 28.77 µM (± 9) to 288.9 µM (± 85.64) in Group A (clopidogrel 600mg) (Figure 3.28A) and from a baseline of 30.40 µM (± 13) to 307.3 µM (± 100) in Group B (clopidogrel 75mg) following intake of nitrate gel (Figure 3.29A)

There was a non-significant small rise in mean nitrate concentration from the baseline of 33.98 µM (±17.27) to 36.04 µM/L (±20.62) (Figure 3.28B) in Group A (clopidogrel 600mg) and from a baseline of 30.03 µM (±16.54) to 31.25 µM (±49) (Figure 3.29B). Group B respectively following intake of placebo gel

D'Agostino & Pearson omnibus normality test of data showed a non-Gaussian distribution, a Wilcoxon matched pairs test of significance showed a statistically significant (P<0.0001) rise

in nitrate levels with nitrate gel and a non-significant rise (P>0.5) with placebo gel in these groups, respectively.

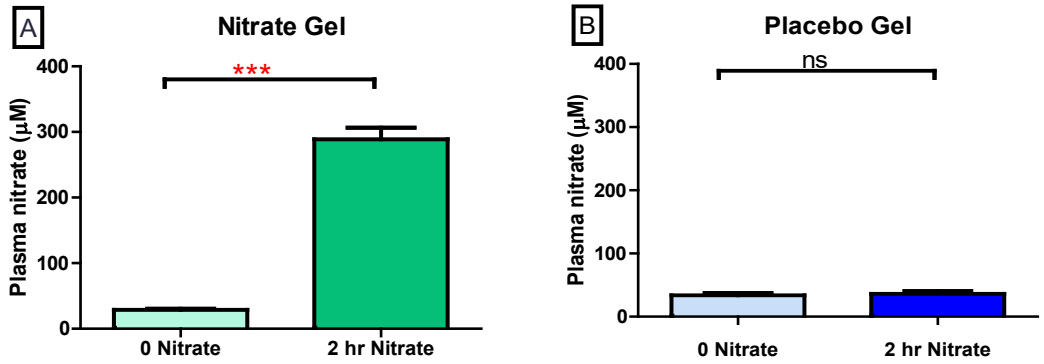



*Figure 3.28: Mean plasma concentration of Nitrate at baseline and 2 hours post Active and placebo respectively in Group A (clopidogrel 600mg). *** represent p <0.001 and ns represent no significance*

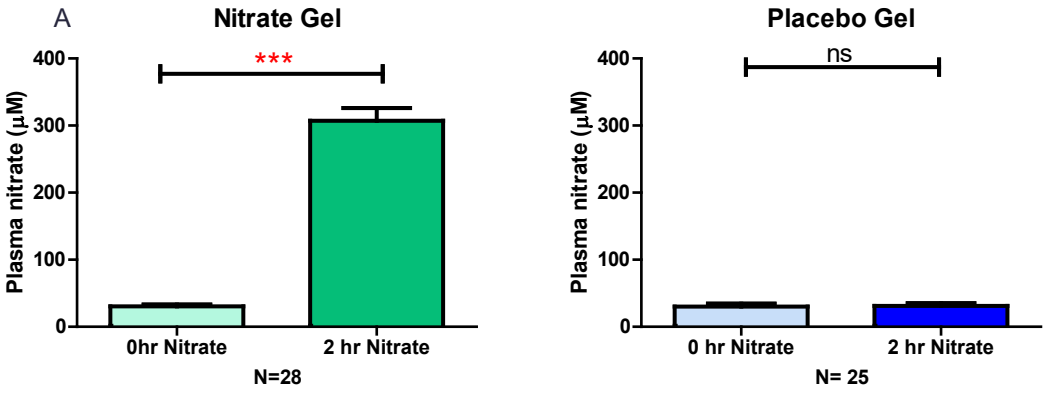



*Figure 3.29 : Mean plasma concentration of Nitrate at baseline and 2 hours post Nitrate gel and placebo respectively in Group B (clopidogrel 75mg). *** represent p <0.001 and ns represent no significance*

In Group A (clopidogrel 600mg), the mean difference in the rise of nitrate levels from baseline to 2-hour post ingestion was 260.1 µM (with 95% confidence interval of 225 to 295.2) and 2.05 µM (with 95% confidence interval of -7.25 to 11.37) for nitrate gel and placebo gel, respectively (Figure 3.28).

In Group B (clopidogrel 75mg), the mean difference in the rise of nitrate levels from baseline to 2 hour post ingestion was 276.9µM (with 95% confidence interval of 239.6 to 314.2) and

1.213 µM (with 95% confidence interval of -3.431 to 11.25) for nitrate gel and placebo gel respectively (Figure 3.29).

A statistically significant (P<0.0001) change in the plasma nitrate level following ingestion of nitrate gel in comparison to placebo gel in both groups was observed (Figure 3.30).

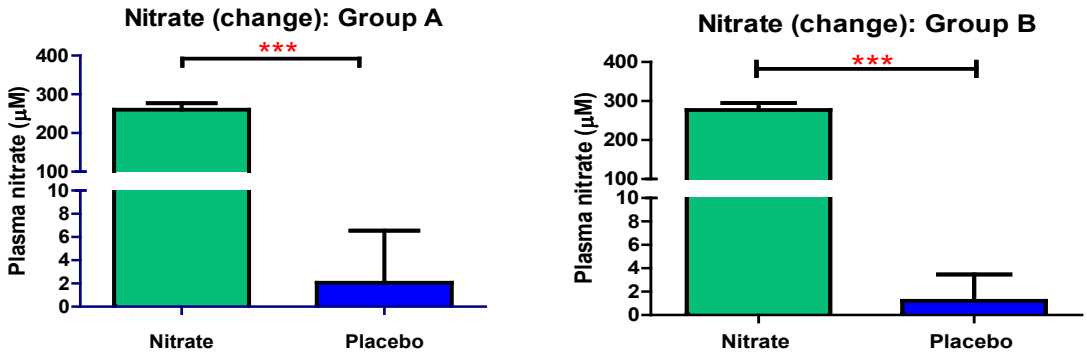



*Figure 3.30: Mean difference in nitrate levels between baseline and 2 hour following ingestion of dietary nitrate supplement and placebo gel in Group A and Group B. A Mann-Whitney t test showed a statically significant change in both groups (*** Denotes a p ≤ 0.001, data presented as mean with error bars representing standard of error).*

There was an identical rise in the level of nitrate following the ingestion of active nitrate supplement in both groups (Figure 3.31 ).

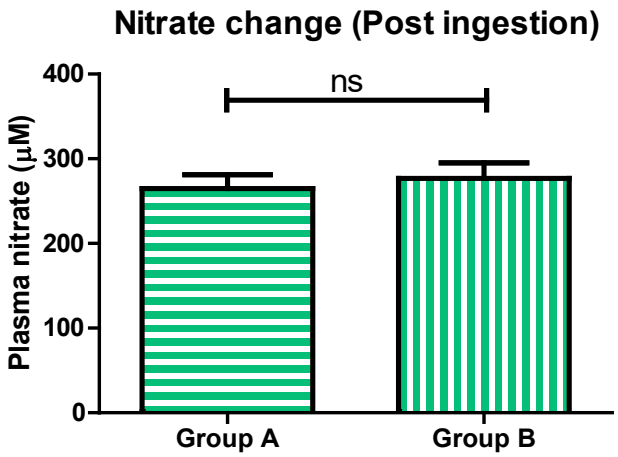


*Figure 3.31: A comparative graph of the rise in nitrate levels at two hours post ingestion of dietary nitrate supplementation gel in Group A (clopidogrel 600mg) and Group B (Clopidogrel 75mg) patients.( ns represent no significance Data presented as mean with error bars representing standard of error).*

**Nitrite levels**

There was no significant difference in the baseline level of plasma nitrite between nitrate gel and placebo gel in either groups [Group A (clopidogrel 600mg): Nitrite: - 220.1nM (±145) vs 246.6(±146), P>0.05] [Group B: Nitrite:- 220.1nM (±145) vs 246.6(±146), P>0.05] as showed in Figure 3.32.

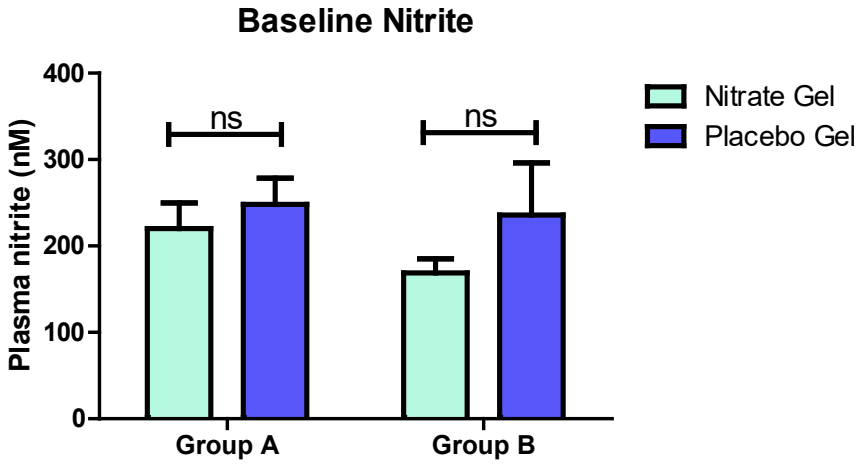



*Figure 3.32 : Baseline nitrite measured in Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg), Data presented as mean with error bars representing standard of error.*

The mean plasma concentration of nitrite rose significantly (p=0.0001) from a baseline of 220.1nM (± 145) to 455.1nM (±266) (Figure 3.33a) in Group A (clopidogrel 600mg) and from a baseline of 168.7nM (± 88) to 368.8nM (±238) (Figure 3.34A) in Group B following intake of nitrate gel.

There was a non-significant small rise in mean nitrite concentration from a baseline of 248.6nM (±146) to 294.1nM (±282) in Group A (clopidogrel 600mg) [P<0.0001] (Figure 3.33B) and a small non-significant drop in mean nitrite concentration with a baseline of 235nM (±303) to

173.1nM (±127) [P>0.05] following intake of placebo gel in Group B (clopidogrel 75mg) (Figure 3.34B).

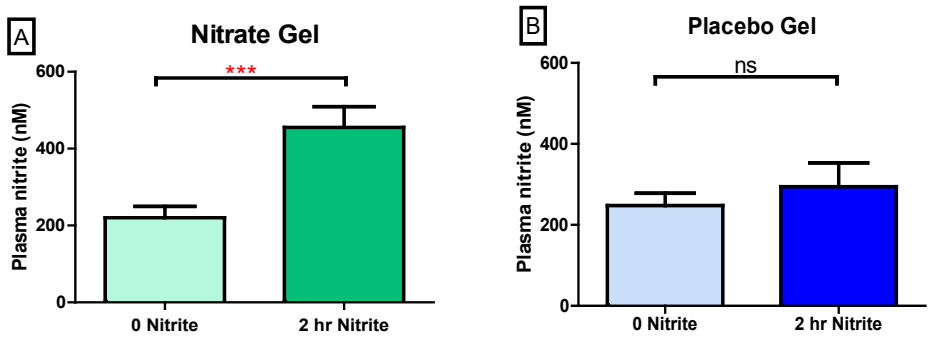



*Figure 3.33: Mean plasma concentration at baseline and 2 hours post nitrate gel (Graph A) and placebo gel (graph B) respectively in Group A (Acute clopidogrel 600mg). A Wilcoxon matched pair test showed a statistically significance (P<0.0001) rise with dietary nitrate supplement and a non-significance (P>0.05) with placebo gel ingestion*

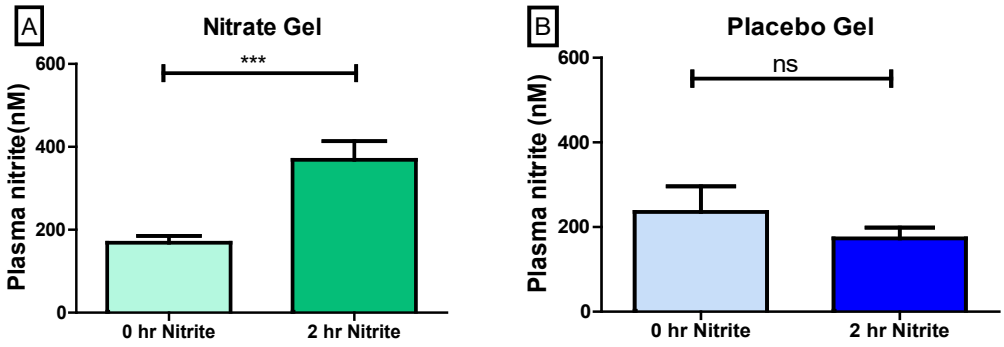



*Figure 3.34 : Mean plasma nitrite concentration at baseline and 2 hours post nitrate gel ingestion (graph A) and placebo gel (graph B) respectively in Group B (Chronic Clopidogrel 75mg). A Wilcoxon matched pair test showed a statistically significance (P<0.0001) rise with dietary nitrate supplement and a non-significance (P>0.05) with placebo gel ingestion*

The mean difference in the rise of nitrite level from baseline to 2-hour post ingestion was 235.0 nM (with 95% confidence interval of 142.6 to 327.5) for dietary nitrate gel and for the placebo gel the mean difference was 46.13nM (95% CI of -55.34 to -144.6) in Group A (clopidogrel 600mg) patients.

The mean difference in the rise of nitrite level from baseline to 2-hour post ingestion was 200.0nM (with 95% confidence interval of 107.3 to 292.7) for dietary nitrate gel and with the placebo gel the mean difference was -69.53nM (with 95% confidence interval of -178.1 to 39.03) in Group B (clopidogrel 75mg) patients

There is a statistically significant (P<0.01) change in the nitrite level following ingestion of dietary nitrate gel in comparison to placebo gel in both groups (*Figure 3.35*)

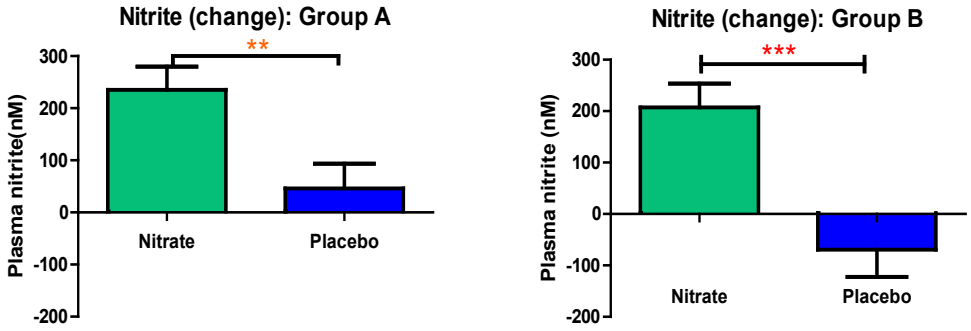



*Figure 3.35: Mean difference in nitrite levels following ingestion of dietary nitrate and placebo gel in Group A (clopidogrel 600mg) and Group B, A Mann-Whitney t test showed a statistically significant (P<0.01) change in the nitrite levels in upon ingestion of dietary nitrate gel in comparison to placebo gel in both groups (Data presented in mean with standard of error of mean, ** denotes p=0.001, *** denotes P<0.001)*

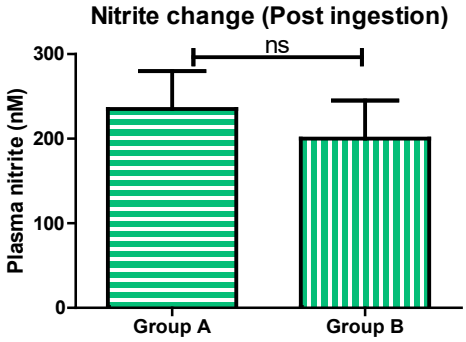



*Figure 3.36: A comparative graph of the rise in nitrite level at two hours post ingestion of dietary nitrate supplementation gel in Group A (clopidogrel 600mg) (Clopidogrel 600mg) and Group B (Clopidogrel 75mg) patients. (Data presented in mean with standard of error of mean)*

There was a numerically higher nitrite measured at 2 hours in Group A (clopidogrel 600mg) patients in comparison with and Group B (Clopidogrel 75mg) patients, but this was not statistically significant (Figure 3.36).

**RSNO levels**

There was no significant difference in the baseline levels of plasma RSNO between nitrate supplement and placebo therapy in either groups [Group A (clopidogrel 600mg): RSNO-: 15.8nM (±13) vs 26.2(±23), P>0.05] and [Group B: RSNO-: 36.38nM (±42) vs 23.8(±32)] as shown in Figure 3.37

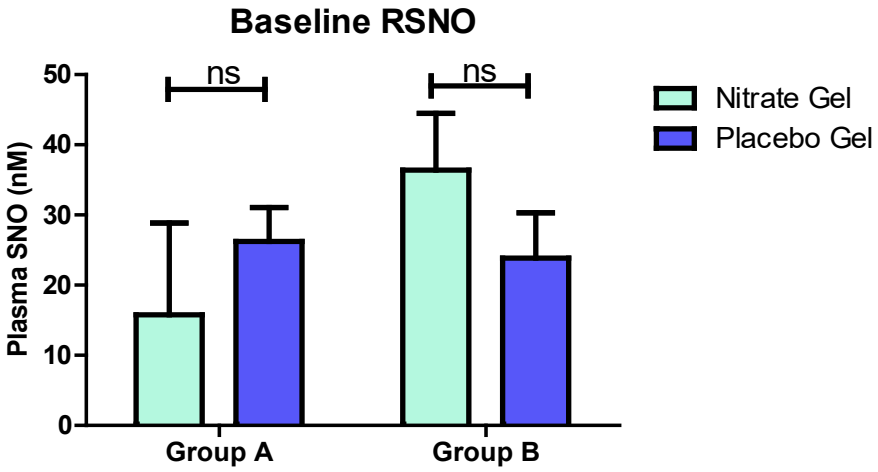



*Figure 3.37: Baseline RSNO levels in Group A and Group B patients, Unpaired t test showed a non-significance. Data represented as mean with SEM as error bars.*

The mean plasma concentration of RSNO rose significantly (P= 0.0001) from a baseline of 15.79 nM (± 13) to 32.83 nM (±30.) at 2 hours following intake of dietary nitrate gel (Figure 3.38A) whereas there was no significant change in the mean RSNO concentration from a baseline of 26.24nM (±23) to 21.18nM (±13) at 2 hours following intake of placebo gel in Group A (clopidogrel 600mg) patients (Figure 3.38B)

The mean plasma concentration of RSNO rose significantly from a baseline of 36.38 nM (± 42) to 50.04 nM (±45) at 2 hours following intake of nitrate gel (Figure 3.39A) and There was a no change in the mean RSNO concentration from a baseline of 23.8nM (±32.3) to 23.25nM (±31.4) at 2 hours following intake of placebo gel (Figure 3.39B) in group B (Clopidogrel 75mg) patients.

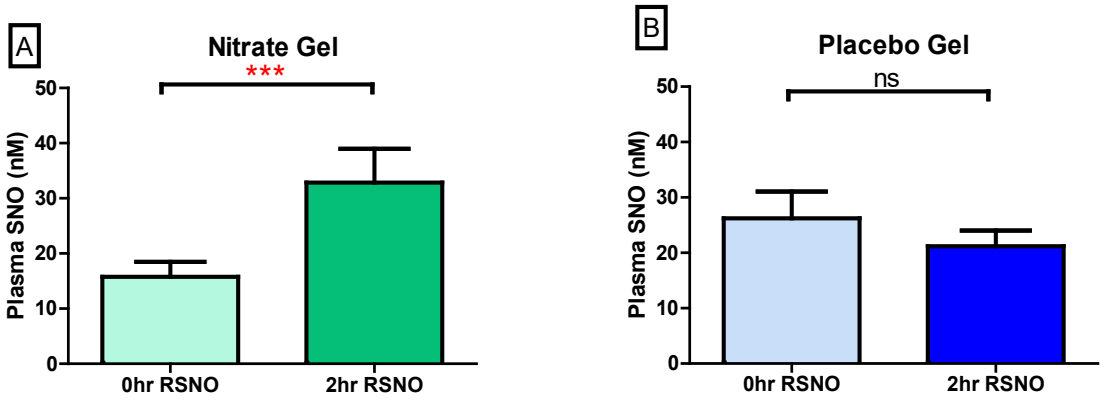



*Figure 3.38: Mean plasma concentration of RSNO at baseline and 2 hours post Dietary nitrate gel ingestion and placebo gel respectively in Group A (clopidogrel 600mg), D'Agostino & Pearson omnibus normality test of data showed a non-Gaussian distribution, and the applied Wilcoxon matched pair test showed a statistically significant *** (P=0.0001) increase in the RSNO levels in upon ingestion of nitrate gel (A) and no significant change (0.14) with ingestion of placebo gel(B).*

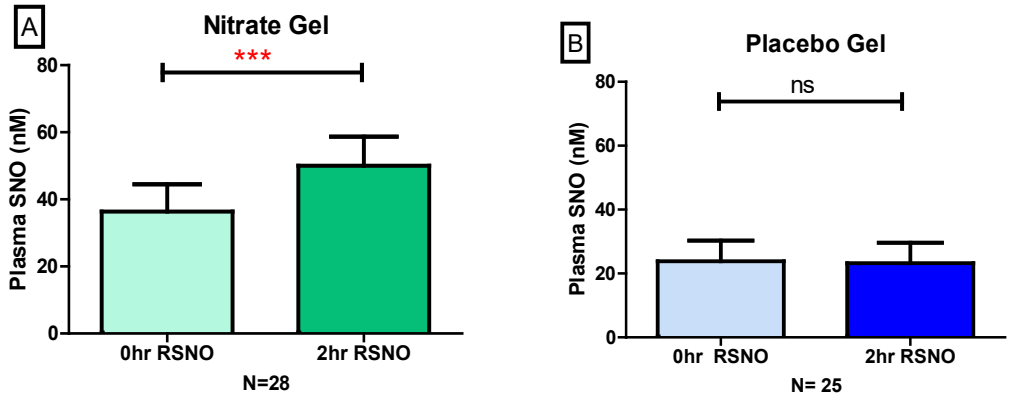



*Figure 3.39: Mean plasma concentration of RSNO at baseline and 2 hours post Dietary nitrate gel ingestion and placebo gel respectively in group B (Clopidogrel 75mg), D'Agostino & Pearson omnibus normality test of data showed a non-Gaussian distribution, and the applied Wilcoxon matched pair test showed a statistically*

*significant *** (P=0.0001) increase in the RSNO levels in following ingestion of nitrate gel (A) and no significant change (0.14) with ingestion of placebo gel (B)*

In Group A (clopidogrel 600mg), the mean difference in the rise of RSNO levels from baseline to 2 hours post ingestion of the nitrate gel was by 17.05nM (95% CI: 8.25-25.84nM). Following the placebo gel, RSNO levels decreased by 5.05nM (95% CI: –12.94-2.83nM) (Figure 3.40A).

In Group B (clopidogrel 75mg), the mean difference in the rise of RSNO levels from baseline to 2 hour post ingestion was 13.67 nM (95% CI; 7.423 to 19.91) following nitrate gel and following the placebo gel the mean difference was –1.51nM (95% CI; –12.19 to 9.17) as showed in Figure 3.40B.

There is a statistically significant (P=0.001) change in the RSNO levels in upon ingestion of nitrate gel in comparison to placebo gel in both groups (*Figure 3.40*)

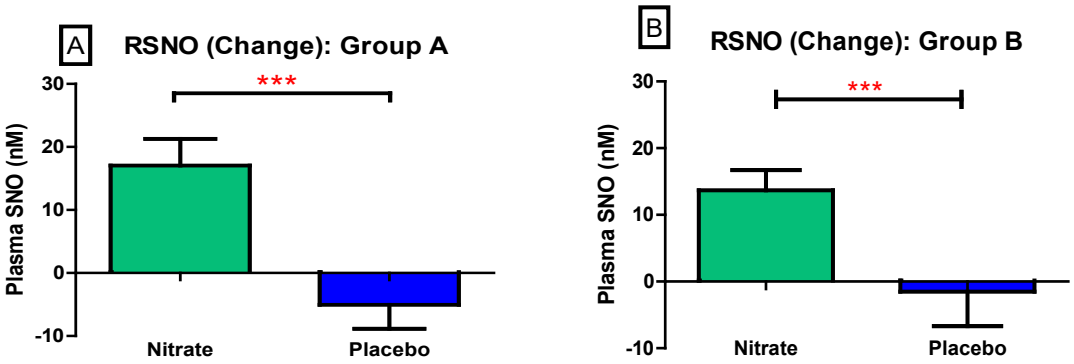



*Figure 3.40: Mean difference (delta) in change in RSNO levels following ingestion of dietary nitrate and placebo in Group A (clopidogrel 600mg and Group B (Clopidogrel 75mg), A Mann-Whitney t test showed a statistically significant (P=0.001) change in the RSNO levels in upon ingestion of nitrate gel in comparison to placebo gel in both groups (Data presented in mean with standard of error, ** denotes p=0.001, *** denotes P<0.001)*

There was a numerically higher change in RSNO level (17nM vs 14nM) formed 2 hours post dietary nitrate ingestion in Group A (clopidogrel 600mg) compared to Group B (Clopidogrel 75mg), but this was not statistically significant (Figure 3.41).

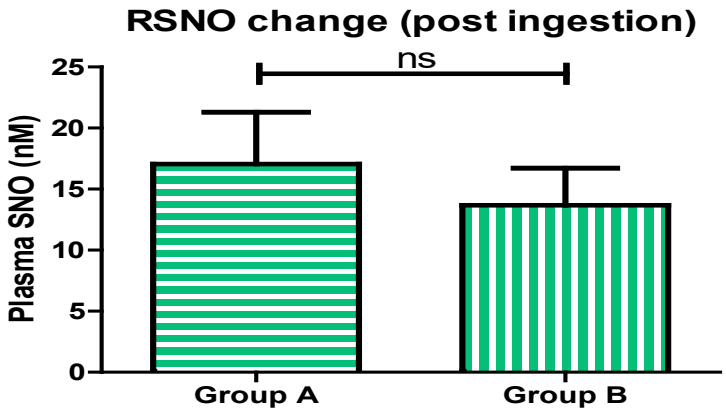



*Figure 3.41: A comparative graph of the rise in RSNO levels at two hours post ingestion of dietary nitrate supplementation gel in Group A (clopidogrel 600mg)) and Group B (Clopidogrel 75mg) patients.*

### 3.3.3.3 **Effect of nitrate and placebo gel supplementation on platelet reactivity**

### 3.3.3.4 **ADP induced platelet reactivity**

#### 3.3.3.4.1 Group A (clopidogrel 600mg)

The mean platelet response to ADP was measured using Multiplate® electrode aggregometry. The platelet reactivity was 636.2 (±239.9) AU/min at baseline and 368.8 (± 213.7) AU/min at 2 hours following the intake of dietary nitrate gel and clopidogrel 600mg (Figure 3.42A), with a paired t test, P value = <0.0001. The mean platelet aggregator response to ADP with the intake of placebo gel and clopidogrel 600mgs was 578.4(±191) AU/min at baseline and 345.2 (± 185) AU/min at 2 hours (Figure), with a P value of <0.0001 in Group A (clopidogrel 600mg) (Figure 3.42B)

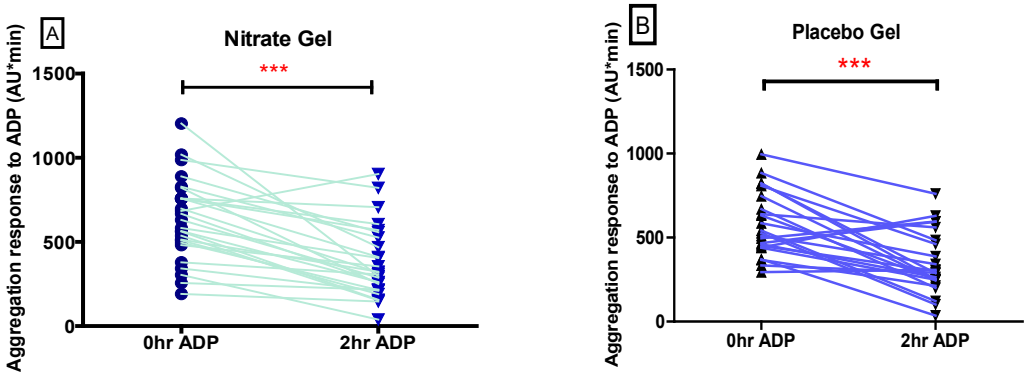



*Figure 3.42: Platelet reactivity upon stimulation with ADP agonist, measured at baseline (0 ADP) and two hour post ingestion of dietary nitrate gel and placebo gel, measured in AU*min. *** Denotes a p ≤ 0.0001*

#### 3.3.3.4.2 Group B (clopidogrel 75mg)

The platelet reactivity was 411.5 (±221) AU/min at baseline and 356.2 (± 158) AU/min at 2 hours following the intake of nitrate gel (Figure 3.43A), with a P value of <0.0795. With the intake of placebo gel the platelet reactivity was 338.4(±146) AU/min at baseline and 344.4 (± 194) AU/min at 2 hours (Figure), with a P value =0.882(Figure 3.43B)

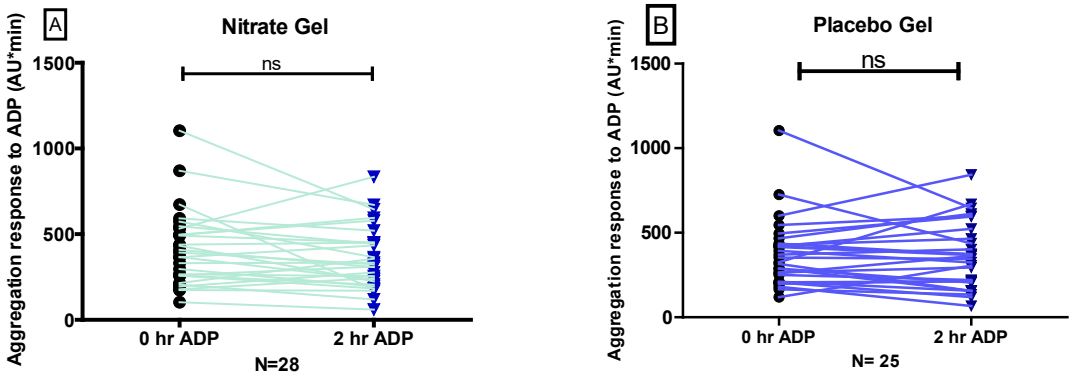



*Figure 3.43: Platelet reactivity upon stimulation with ADP agonist, measure at baseline and two hour post ingestion of nitrate (A) and placebo (B) gel, measured in AU*min. A paired T test as applied for testing the significance*

The mean difference in the platelet reactivity from baseline to 2-hour post ingestion was -267.4 AU/min (with 95% confidence interval of -351.5 to -183.3) and -233.3 AU/min (with 95% confidence interval of -327.1 to -183.3) for dietary nitrate gel and placebo gel administered to

Group A (clopidogrel 600mg), respectively. There was a minor increase in platelet inhibition with nitrate gel compared to placebo gel but this did not reach statistical significance (P=0.57) using unpaired t test (*Figure 3.44*A).

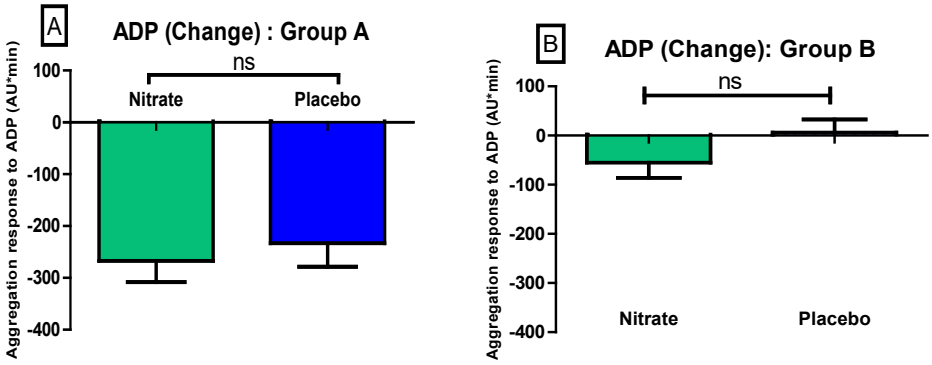



*Figure 3.44: Mean difference in measured platelet reactivity from baseline to 2 hours upon stimulation with ADP agonist Data presented as mean with standard of error of mean.*

The mean difference in the platelet reactivity from baseline to 2-hour post ingestion was -55.36 AU/min (with 95% confidence interval of -118.6 to 7.86) and 5.96 AU/min (with 95% confidence interval of -49.06 to 60.98) for nitrate gel and placebo gel in Group B, respectively. There was a minor increase in platelet inhibition with nitrate gel compared to placebo gel, but this did not reach statistical significance (P=0.20) (Figure 3.44B above).

### 3.3.3.5 **TRAP induced platelet reactivity**

#### 3.3.3.5.1 Group A (clopidogrel 600mg)

The platelet reactivity was 1050.0 (±234.1) AU/min at baseline and 927.3 (± 298) AU/min at 2 hours following the intake of nitrate gel and clopidogrel 600mg, this was statistically significant (P = 0.019) as showed in Figure 3.45A. With the intake of placebo gel and clopidogrel 600mg the platelet reactivity was 1048(±267) AU/min at baseline and 943 (± 226) AU/min at 2 hours, which was not significant (P= 0.056) (Figure 3.45B).

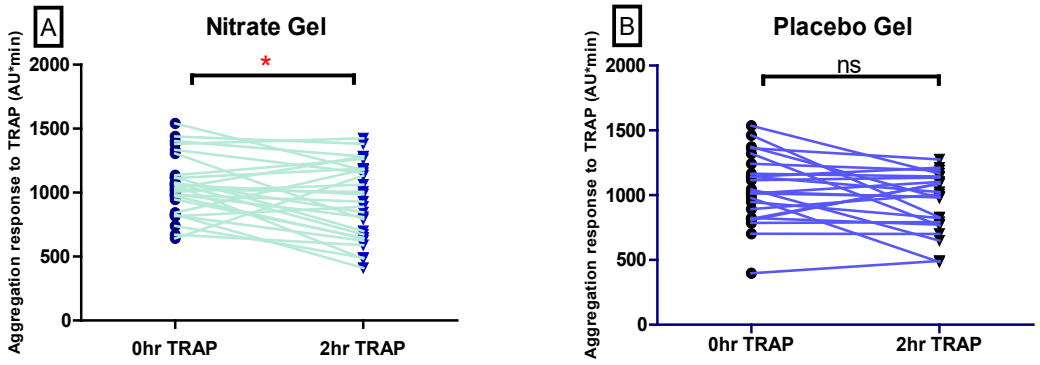



*Figure 3.45 : Platelet reactivity upon stimulation with TRAP agonist, measured at baseline (0 TRAP) and two hours ( 2-TRAP) post ingestion of nitrate and placebo gel, measured in AU*min. A paired T test as applied for testing the significance. (* denotes P<0.01 and ns denotes non-significance)*

### 3.3.3.5.2 Group B (clopidogrel 75mg)

The platelet reactivity was 924.4 (±304) AU/min at baseline and 887.4 (± 286) AU/min at 2 hours following the intake of dietary nitrate gel (Figure 3.46A) with a paired test P value of 0.39. With the intake of placebo gel the platelet reactivity was 930.1(±287) AU/min at baseline and 893.5 (± 279) AU/min at 2 hours, with a P value of 0.52. (Figure 3.46B)

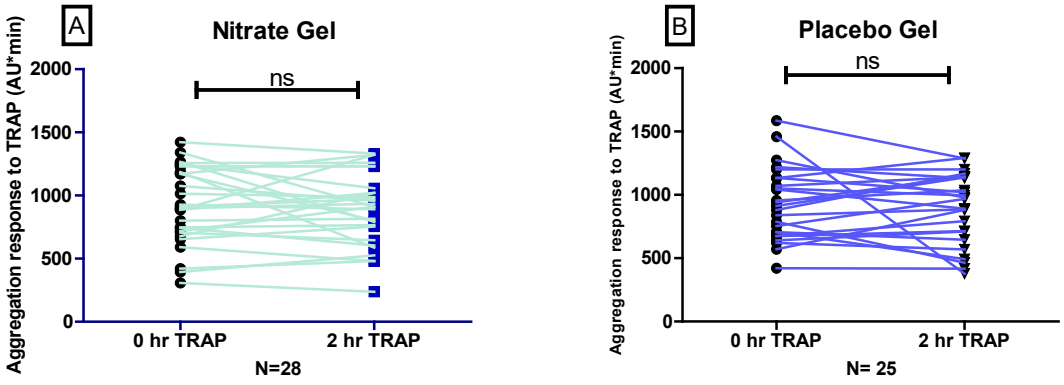



*Figure 3.46 : Platelet reactivity upon stimulation with TRAP agonist, measured at baseline (0 TRAP) and two hours ( 2-TRAP) post ingestion of dietary nitrate and placebo gel, expressed in AU*min. (ns denotes non-significance)*

The mean difference in the TRAP induced platelet reactivity from baseline to 2 hour post ingestion was -122.5 AU/min (with 95% confidence interval of –224.4 to -20) and -105.1 AU/min (with 95% confidence interval of –213.3 to 3.165) for nitrate gel and placebo gel in Group A (clopidogrel 600mg), respectively (Figure 3.47A).

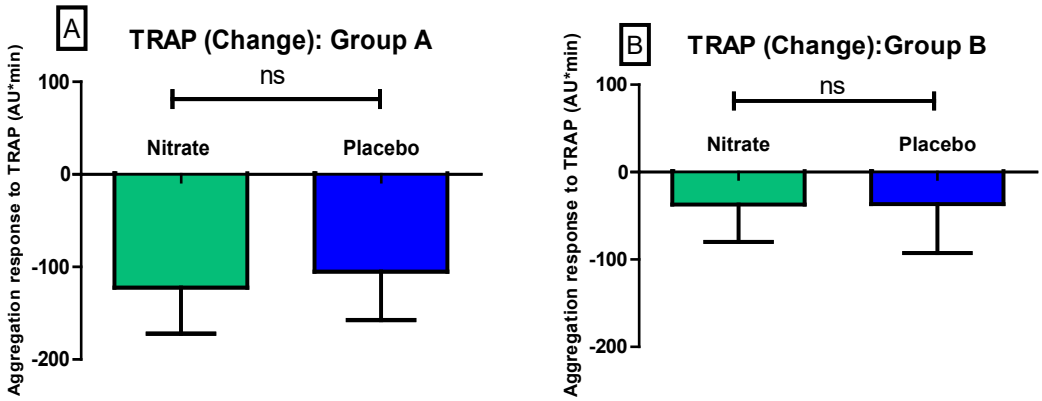



*Figure 3.47: Mean difference in measured platelet reactivity from baseline to 2 hours upon stimulation with TRAP agonist in Group A (clopidogrel 600mg) and group B (clopidogrel 75mg) patients expressed in AU*min. (ns denotes non-significance)*

The mean difference in the platelet reactivity from baseline to 2-hour post ingestion was -37.07 (±226) AU/min and 36.56 (±280) AU/min after ingestion of active and placebo gel, respectively. There was no increase in platelet inhibition with nitrate gel compared to placebo gel, P value =0.65 (Figure 3.47B).

### 3.3.4 Combined results

#### 3.3.4.1 Overall effect of nitrate supplement on NO metabolites in patients with stable CAD

This section is a comparison of results of the mean change in values (from baseline to 2 hours) comparing ingestion of nitrate supplement in the three patient groups (studied in Chapter 4 and in the current chapter): CAD patients naive to clopidogrel, group A (Acute clopidogrel 600mg), and group B (Chronic clopidogrel 75mg). A one-way ANOVA (non-parametric) statistical analysis method was applied while comparing the 3 groups.

**Nitrate**

The mean change in plasma concentration of nitrate following nitrate gel was 235.2(±88) μM, 276.9 (±96) μM and 264.9 (±84) μM in clopidogrel naïve (CAD only), Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg). respectively. There was no statistically significant difference (P=0.56) noted between these three groups. (Figure 3.48A)

**Nitrite**

The mean change in plasma concentration of nitrite following nitrate gel was 214.6(±144) nM, 235 (±219) and 207.4 (±240) nM in clopidogrel naïve (CAD only), Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg), respectively. There was no statistically significant difference (P=0.69) noted between these three groups. (Figure 3.48B)

**RSNO**

The mean change in plasma concentration of RSNO following nitrate gel was 10.4(±11) nM/L, 17.1 (±21) nM/L and 13.7 (±16) nM/L in clopidogrel naïve (CAD only), Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg), respectively. There was no statistically significant difference (P=0.58) noted between these three groups. (Figure 3.48C)

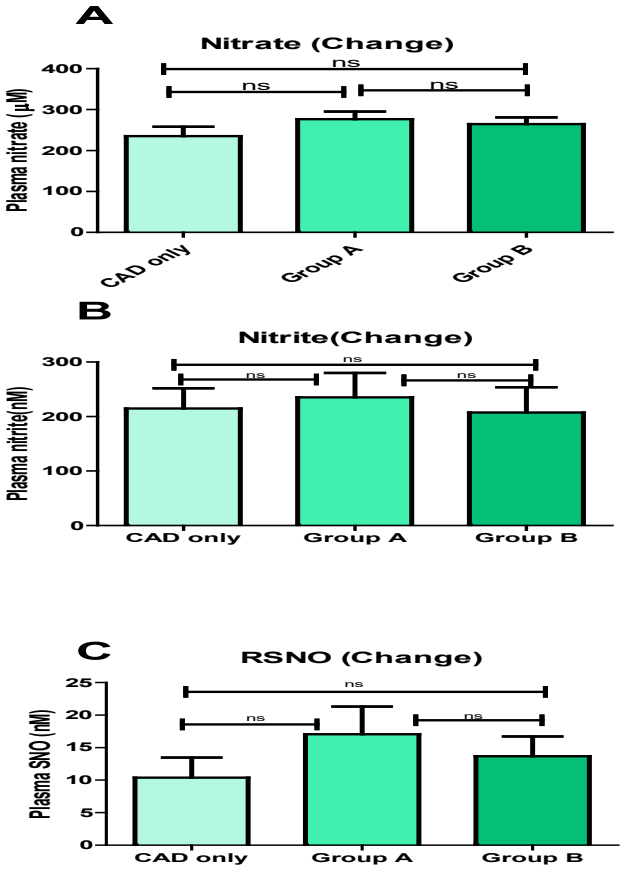



*Figure 3.48: A Comparative chart of mean difference in the nitrate, nitrite, and RSNO levels 2 hour after ingestion of nitrate supplementation in stable coronary artery disease patients with and without clopidogrel therapy. These are unpaired samples, data represented as mean with standard error of mean. CAD only: stable CAD patients not on clopidogrel therapy. Group A (clopidogrel 600mg): SCAD patients on Clopidogrel loading (600mg), Group B: SCAD patients on Chronic clopidogrel therapy (75mg)., ** denotes p=0.001, *** denotes P<0.001.*

## Platelet aggregation

### ADP

The change in platelet reactivity to ADP following nitrate gel was 46.87 (±176) AU/min, -267.4 (± 212) AU/min and -55.36 (±163) AU/min in clopidogrel naïve (CAD only), Group A (clopidogrel 600mg) and Group B (clopidogrel 75mg), respectively. There was a significant difference (P<0.001) noted between these CAD vs Group A and Group A vs Group B (Figure 3.49 A)

**TRAP**

The change in platelet reactivity to TRAP following nitrate gel was 147.5 (±225) AU/min, -122.5 (± 257) AU/min and -37.1 (±227) AU/min in clopidogrel naïve (NC), acute clopidogrel loading and chronic clopidogrel loading group respectively. There was a statistically significant difference (P=0.01) noted between these CAD vs Group A (clopidogrel 600mg) and B. (Figure 3.49 B)

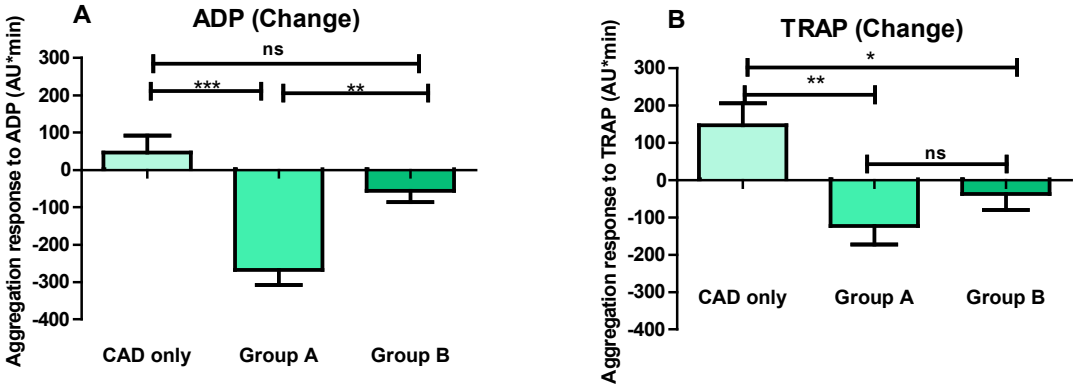



*Figure 3.49:A Comparative chart of mean difference in the ADP and TRAP inhibition 2 hour after ingestion of nitrate supplementation in stable coronary artery disease patients with and without clopidogrel therapy. These are unpaired samples, data represented as mean with standard error of mean. CAD only: stable CAD patients not on clopidogrel therapy. Group A (clopidogrel 600mg): SCAD patients on Clopidogrel loading (600mg), Group B: SCAD patients on Chronic clopidogrel therapy (75mg)., * denotes p<0.05, ** denotes P<0.01. and ***p<0.001.*

### 3.3.5 Correlations between NO metabolites and platelet inhibition.

In this section a relationship between change in RSNO levels with change in platelet reactivity was investigated by collating the data of all three patient groups.

There was a significant correlation between the change in RSNO levels and ADP/TRAP mediated platelet inhibition. No similar correlations were observed between the other NO metabolites (nitrate, nitrite) and platelet inhibition (Table 3.15)

| Correlation Matrix | | | | | | |
|---|---|---|---|---|---|---|
| | | Delta Nitrate | Delta Nitrite | Delta RSNO | Delta ADP | Delta TRAP |
| **Delta Nitrate** | Pearson Correlation | | .076 | -.196 | .016 | -.007 |
| | Sig. (2-tailed) | | .541 | .112 | .900 | .953 |
| | N | | 67 | 67 | 67 | 67 |
| **Delta Nitrite** | Pearson Correlation | .076 | | .061 | -.046 | -.181 |
| | Sig. (2-tailed) | .541 | | .623 | .710 | .143 |
| | N | 67 | | 67 | 67 | 67 |
| **Delta RSNO** | Pearson Correlation | -.196 | .061 | | -.194 | **-.312*** |
| | Sig. (2-tailed) | .112 | .623 | | .116 | **.010** |
| | N | 67 | 67 | | 67 | **67** |
| **Delta ADP** | Pearson Correlation | .016 | -.046 | -.194 | | **.595**** |
| | Sig. (2-tailed) | .900 | .710 | .116 | | **.000** |
| | N | 67 | 67 | 67 | | **67** |
| **Delta TRAP** | Pearson Correlation | -.007 | -.181 | **-.312*** | **.595**** | |
| | Sig. (2-tailed) | .953 | .143 | **.010** | **.000** | |
| | N | 67 | 67 | **67** | **67** | |
| ***. Correlation is significant at the 0.05 level (2-tailed).** | | | | | | |
| ****. Correlation is significant at the 0.01 level (2-tailed).** | | | | | | |

*Table 3.15: A correlation matrix demonstrating the relationship of Change(delta) in the NO metabolites and Platelet inhibition, significant correlations are highlighted in bold.*

There is a negative correlation between RSNO levels and platelet reactivity with ADP and TRAP agonist. The association was strongest with statistical significance between increase in RSNO and the decrease in the TRAP mediated platelet inhibition (P=0.01) as shown Figure 3.50.

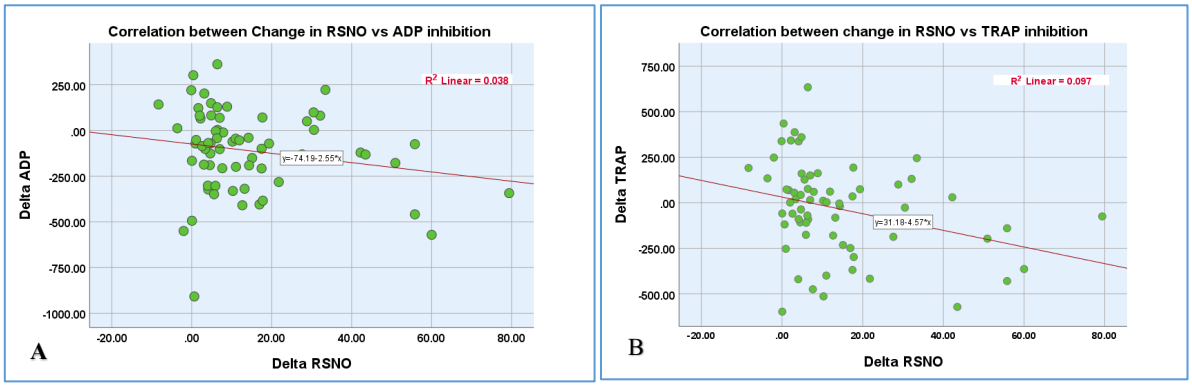



*Figure 3.50: The correlation of change in the RSNO levels in relation to platelet inhibition. Graph A, demonstrates there is a negative association between ADP inhibition and RSNO with R value of 0.0038, Pearson correlation coefficient of -0.194 and p>0.05. Graph B shows a significant negative correlation between the change in RSNO and change in TRAP mediated platelet inhibition with a R value of 0.097, Pearson correlation coefficient of -0.312 and a P<0.05.*

### 3.3.6 Summary of principle findings

- ✓ There was no significant difference in the baseline levels of plasma nitrate and nitrite between nitrate supplement and placebo therapy in both groups (Group A and Group B)

- ✓ The baseline plasma RSNO levels were significantly lower in the nitrate receiving group compared to the placebo group in patients given 600mg of clopidogrel (Group A). No such difference seen in Group B patient with nitrate or placebo therapy.

- ✓ There was a significant rise in the plasma nitrate, nitrite and RSNO levels 2 hrs post ingestion of the nitrate gel in both groups.

- ✓ There was no significant difference in NO metabolite's levels 2-hour post ingestion of placebo gel in either groups.

- ✓ There was a significant drop in the platelet aggregation simulated via ADP receptors both in nitrate and placebo gel groups given 600mg of clopidogrel (Group A). No such difference was observed in Group B patients receiving nitrate or placebo therapy.

- ✓ The platelet aggregation due to simulation via TRAP was significantly lower in patients given nitrate gel given 600mg of clopidogrel (Group A). No such difference seen in Group B patient with nitrate or placebo therapy.

- ✓ There was a significant correlation between the change RSNO metabolite and change in TRAP mediated platelet aggregation.

## 3.4 Results 4: The role of newer antiplatelet drugs (ticagrelor) in Nitrate-nitrite-RSNO metabolism

### 3.4.1 Background

Ticagrelor is a newer potent P2Y12 receptor blocker. Its potency is primarily attributed to its $P2Y_{12}$ receptor blocking effect. The $P2Y_{12}$ independent effects are recognised but exact mechanism is not established.

### 3.4.2 Hypothesis

In this chapter we hypothesised that

- Ticagrelor, would not form ticagrelor-SNO in the acidic milieu of a gastric environment using a simulated stomach media in presence of nitrite.
- Ticagrelor therapy would not have an effect on plasma RSNO levels in patients undergoing PCI for CAD and thereby beneficial effects of enhanced NO metabolites.
- Concomitant use of PPI with ticagrelor has an effect on RSNO formation and platelet inhibition in patients with CAD

### 3.4.3 In vitro study -ticagrelor- RSNO formation in simulated gastric fluid.

In this section we sought to investigate whether ticagrelor could form ticagrelor induced RSNO in an artificial simulated gastric medium in the presence of nitrite. We also studied the interplay of various gastric constituents' and different nitrite concentration on the resulting RSNO formation.

#### 3.4.3.1 **Baseline RSNO levels**

Simulated artificial gastric fluid (SGF), ticagrelor solution, and SGF-ticagrelor mixtures were freshly prepared on each day of the experiments as described in the methods chapter.

200µL of each solution were incubated separately at $37^0$C for 10 minutes and then neutralised with 1M NaOH. The neutralised sample was immediately injected into the purge vessel containing 2Cs reagent and the baseline RSNO level was measured. A total of 3 experiments were performed on different days. Negligible amount of RSNO was detected in the SGF (0.21nM/L) ticagrelor only solution (0.22nM/L) and SGF-ticagrelor mixture (0.24nM/L) (Figure 3.51). There was no statistically significance difference in baseline RSNO levels in the three tested mixtures.

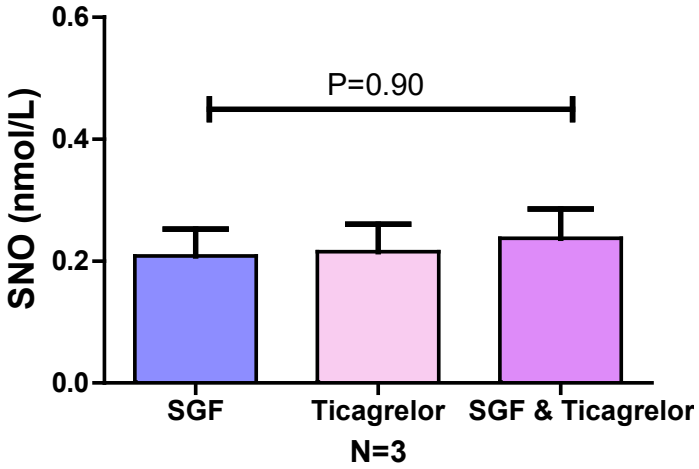



*Figure 3.51: RSNO level measured in Gastric juice (GJ), Ticagrelor solution, and Ticagrelor mixed in gastric Juice without any added nitrite.*

### 3.4.3.2 Ticagrelor induced RSNO formation in simulated gastric media in the presence of nitrite

RSNO generation in SGF both with and without ticagrelor was measured. A total of 5 experiments were conducted on separate days. Adding nitrite at varying concentrations to gastric media alone resulted in the formation of RSNO from the endogenous proteins within the media (Figure 3.52). Furthermore, RSNO formation was augmented when ticagrelor was added to gastric media with similar amounts of added nitrite P=0.0084 at 5000 µmol/L and P=0.0360 at 500 µmol/L.

This effect was only modest at physiological levels of nitrite (Figure 3.53), and it was nevertheless measurable at the low doses. There was no statistically significant change at low does with a p value of 0.3146 (ns), 0.1982 (ns) and 0.2612 (ns) at 50 µmol/L, 25 µmol/L, and 12.5 µmol/L respectively.

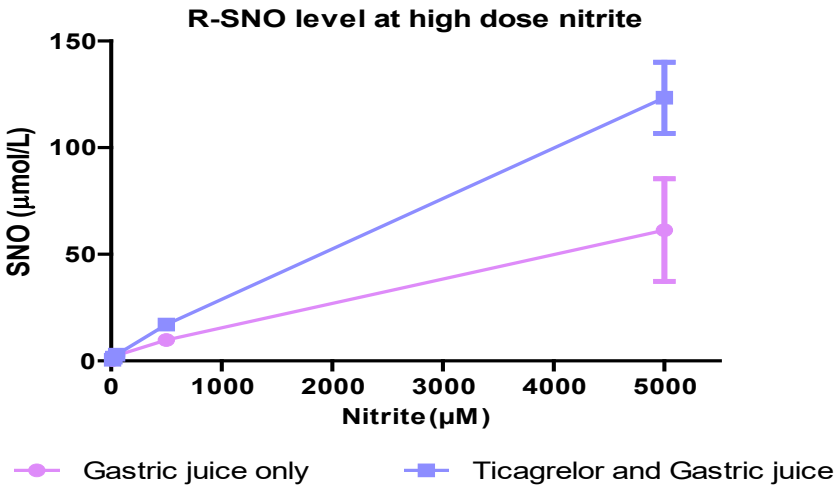



*Figure 3.52: Drug-SNO formation in gastric medium and ticagrelor gastric medium mixture against high dose of nitrite, showing an augmented RSNO production with drug in gastric medium. There is a statistically significant change with a P=0.0084 at 5000 µmol/L and P=0.0360 at 500 µmol/L.*

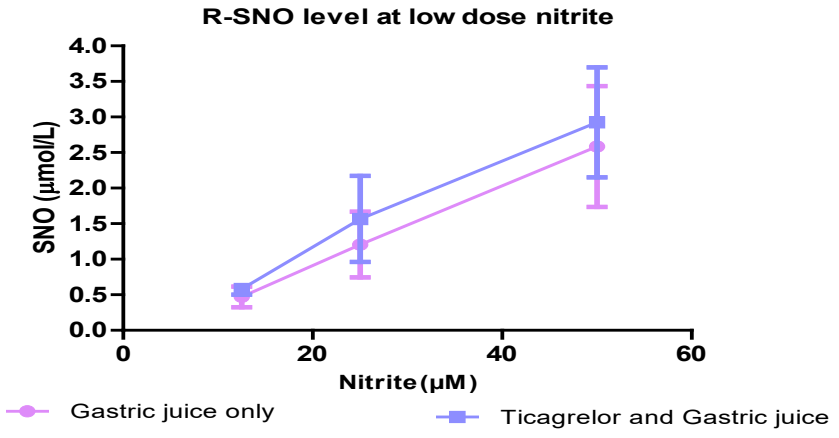



*Figure 3.53: Drug-SNO formation in gastric medium and ticagrelor gastric medium mixture across a low physiological range of nitrite, showing an augmented RSNO production with drug in gastric medium.*

### 3.4.3.3 **Effect of simulated gastric fluid contents on RSNO formation**

The above experiments clearly show RSNO formation in acidified gastric medium in the presence of nitrite.

There was negligible RSNO measured in the baseline sample and a considerable stepwise increase in RSNO formation with stepwise addition of resorium, lysozyme, pepsin, casein and ticagrelor to the baseline sample (Figure 3.54 & Table 3.16).

| Solution | RSNO (µmol/L) |
|---|---|
| **Solution 1= NACL, D GLUCOSE, KCL, CACL, KHPO4** | 0.158343 |
| **Solution 2= Solution 1+ Resorium** | 1.814479 |
| **Solution 3= Solution e 2+lysozyme** | 2.073342 |
| **Solution 4 = Solution 3+ pepsin** | 3.200252 |
| **Solution 5= Solution 4+ casein** | 4.168032 |
| **Solution 6= Solution 5+Ticagrelor** | 6.054435 |

*Table 3.16: RSNO formation with stepwise addition of constituents of simulated gastric fluid upon addition of 500µmol/L of nitrite.*

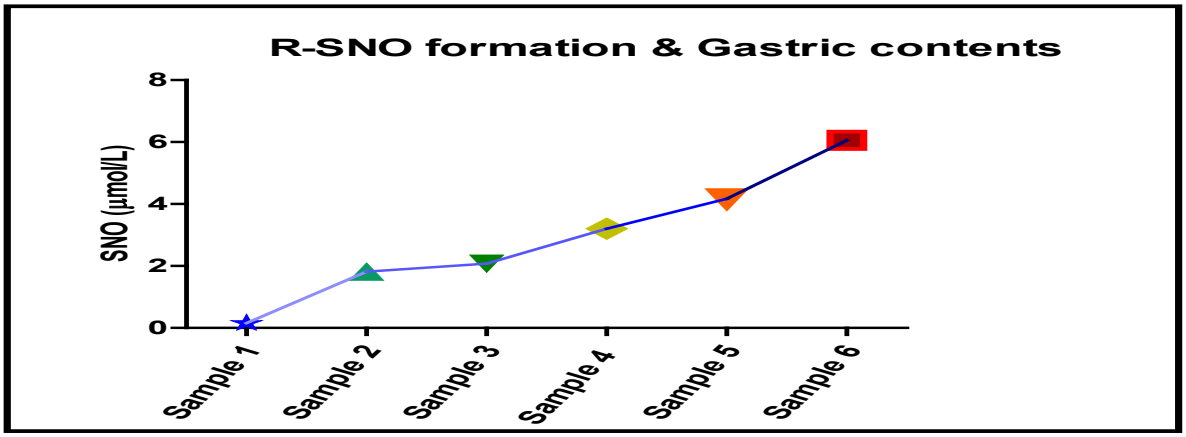



*Figure 3.54: RSNO formation in relationship with constituents of simulated gastric juice upon addition of 500µmol/L of nitrite, Sample 1= NACL, D GLUCOSE, KCL, CACL, KHPO4. Sample 2= sample 1+ Resorium. Sample 3= Sample 2+lysozyme, Sample 4 = sample 3+ pepsin, Sample 5= sample 4+ casein Sample 6= sample 5+Ticagrelor. There is a linear increase in the level of RSNO formation and it further enhanced with the addition of ticagrelor to the SGF (sample.*

### 3.4.4 Effect of ticagrelor therapy on NO metabolites and platelet reactivity in patients with CAD undergoing PCI

A total of 47 patients were recruited for this study from the pre-assessment unit, who were scheduled for coronary intervention (PCI) for stable CAD. The first group of 24 patients (Loading group) who were given a first dose of ticagrelor loading (180mg) and a second patient group of 23 patients (chronic group) who were already on a maintenance dose of ticagrelor (90mg daily) for over 28 days.

#### 3.4.4.1 Patient groups and characteristics

There were no significant differences in the clinical characteristics of the acute loading and chronic therapy patient groups including hypertension, hyperlipidaemia, cigarette smoking, family history of Ischemic heart disease and drug treatment, except for a prevalence of previous myocardial infarction and PCI in the chronic Ticagrelor group (Table 3.17).

| Characteristics | Loading(N=24) | Chronic(N=23) | T Test |
|---|---|---|---|
| **Myocardial infarction** | 4 | 12 | NS |
| **Previous PCI** | 4 | 23 | NS |
| **Cardiac surgery** | 2 | 0 | NS |
| **Diabetes** | 4 | 6 | NS |
| **Hypertension** | 11 | 14 | NS |
| **Hyperlipidaemia** | 10 | 13 | NS |
| **Smoker** | 16 | 13 | NS |
| **Thyroid** | 3 | 2 | NS |
| **Respiratory problem** | 2 | 0 | NS |
| **CVA/TIA** | 0 | 0 | NS |
| **Renal history** | 0 | 0 | NS |
| **Medications** | | | |
| **Aspirin** | 13 | 10 | NS |
| **Clopidogrel** | 2 | 1 | NS |

| | | | |
|---|---|---|---|
| **Beta blockers** | 7 | 10 | NS |
| **ACE inhibitors** | 8 | 12 | NS |
| **Statins** | 23 | 23 | NS |
| **Diabetic drugs** | 1 | 16 | NS |
| **Nitrates** | 3 | 0 | NS |
| **CCB** | 0 | 5 | NS |
| **Thyroxine** | 3 | 1 | NS |
| **NSAID** | 0 | 0 | NS |
| **PPI** | 9 | 6 | NS |

*Table 3.17: Patients demography and characteristics of patients on acute Ticagrelor loading and chronic Ticagrelor therapy*

### 3.4.4.2 **Influence of acute ticagrelor loading on NO metabolites**

The concentration of plasma RSNO rose significantly (p=0.0072) from a baseline of 23.6 (±3.3) nM to 35.4 (±5.8) nmol/L following Ticagrelor loading (Figure 3.55). Two hours after a loading dose plasma nitrite concentration remained unchanged (p=ns) from 211.1 (±24.3) to 217.7 (±25.4) nmol/L (Figure 3.56). Plasma nitrate concentration also remained unchanged from 30.4 (±1.9) to 28.8 (±1.4) µmol/L (p=ns) (Figure 3.57)

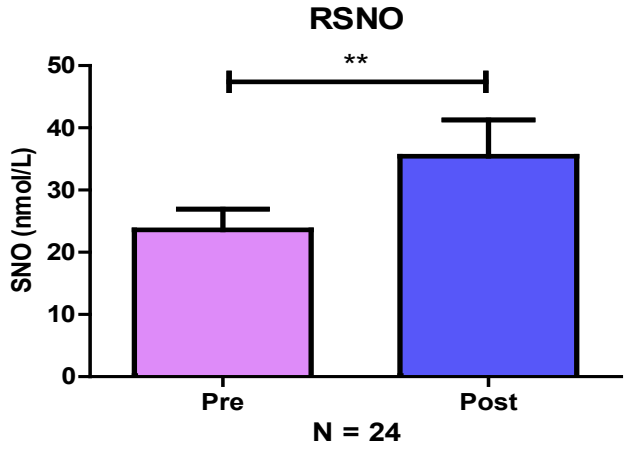



*Figure 3.55: Mean plasma concentration of RSNO at baseline and 2 hours post 180mg of Ticagrelor ingestion, A paired T test was applied with values are presented as mean with SEM.*

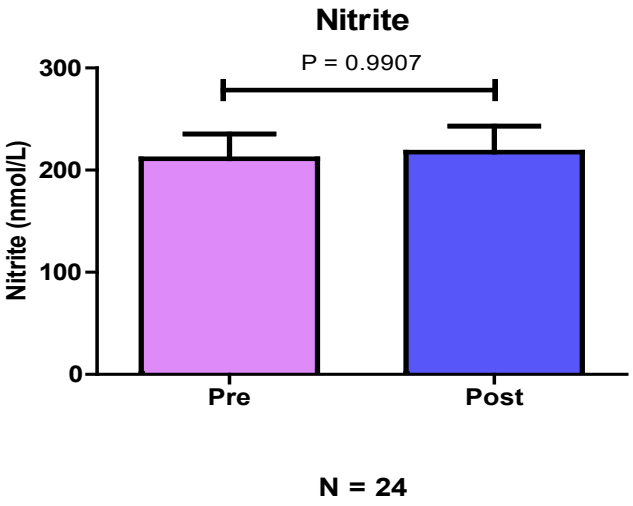



*Figure 3.56: Mean plasma concentration of nitrite at baseline and 2 hours post 180 mg of Ticagrelor ingestion, A paired T test was applied with values are presented as mean with SEM.*

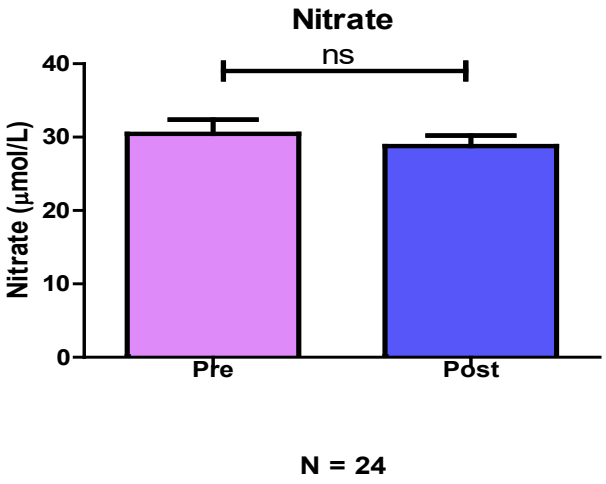



*Figure 3.57: Mean plasma concentration of nitrate at baseline and 2 hours post 180 mg of Ticagrelor ingestion, A paired T test was applied with values are presented as mean with SEM.*

### 3.4.4.3 Comparative effect of chronic treatment vs acute effect post loading of ticagrelor on NO metabolites

In comparing patients on chronic ticagrelor therapy (90mg bid) to patients receiving an acute loading dose of ticagrelor (180mg) we observed the following changes in NO metabolites.

Plasma RSNO levels (35.41 nmol/l ± 27) at 2 hours post ingestion of a loading dose of ticagrelor (180mg) were significantly higher (P=0.0043) compared with patients on chronic Ticagrelor therapy (17.07 nmol/L ± 7) (90mg BD). The RSNO levels on chronic therapy were not significantly different compared with the baseline levels (23.59 nmol/L ± 16) in the acute loading group (Figure 3.58 a).

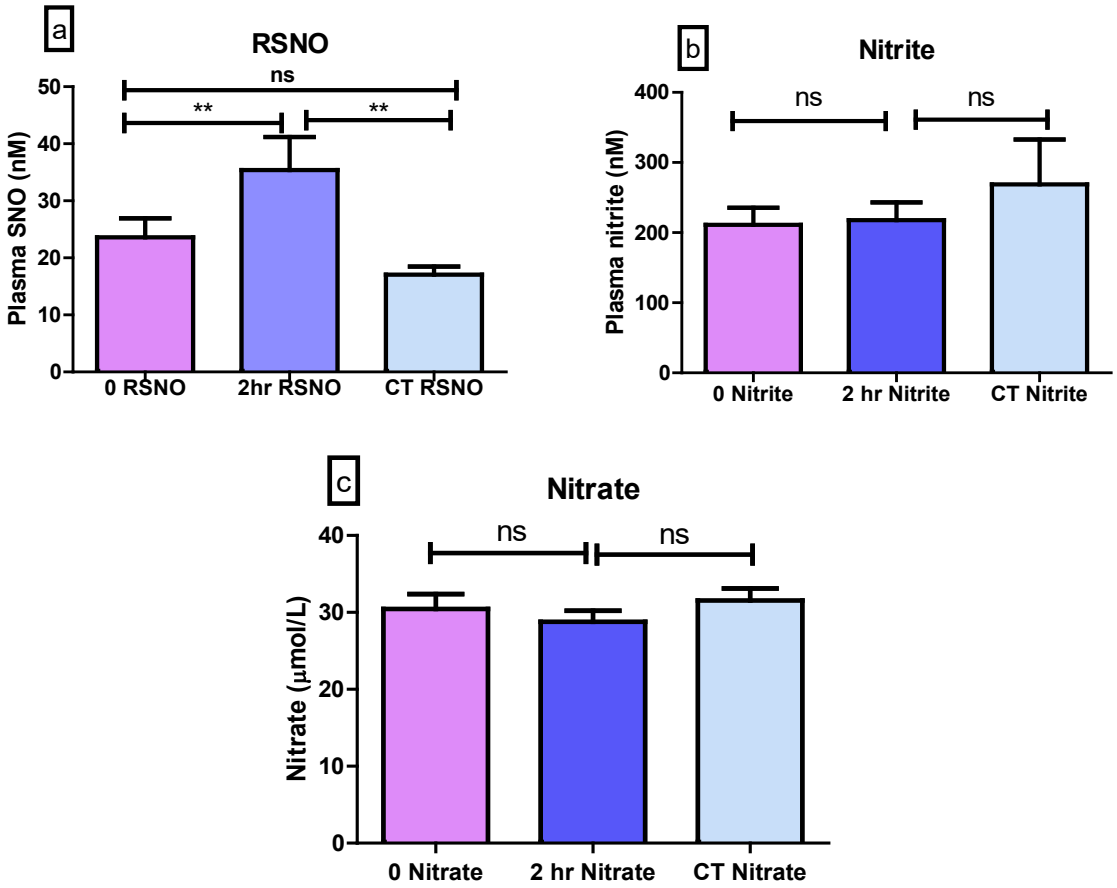



*Figure 3.58: Mean RSNO (a), Nitrite(b), and Nitrate (c) levels at baseline, 2 hours post ticagrelor loading of 180mg and chronic ticagrelor therapy (CT), which is measured at two hours after intake of regular dose of 90mg of Ticagrelor*

Plasma nitrite levels (268.8 nmol/L ± 63) were marginally higher in chronic Ticagrelor patients in comparison to levels at baseline (217.7 nmol/L ± 25) and at 2 hours post ingestion (211.1 nmol/L ± 24) of an acute loading dose, however this was not statically significant (P= 0.45) (Figure 3.58b).

Plasma nitrate level was unchanged in patients receiving chronic ticagrelor therapy when compared to patients at baseline and 2-hour post-acute loading of ticagrelor (Figure 3.58c). The mean nitrate levels were 30.44 µmol/L, 28.8, and 31.54 at baseline, 2hour post-acute ticagrelor loading and in chronic ticagrelor therapy patients, respectively, there was no statistical significance established between the results (p=0.20).

### 3.4.4.4 **Effect of acute ticagrelor loading on platelet aggregation**

MEA confirmed a mean platelet response to ADP of 659 (±53.6) AU*min before loading with Ticagrelor and a mean of 168 (± 14.9) AU*min 2 hours following administration (p<0.0001) (Figure 3.59). Platelet response to TRAP was 1024 (±47.2) AU*min at baseline and 874 (±59.5) AU*min following Ticagrelor (p<0.0002) (Figure 3.60)

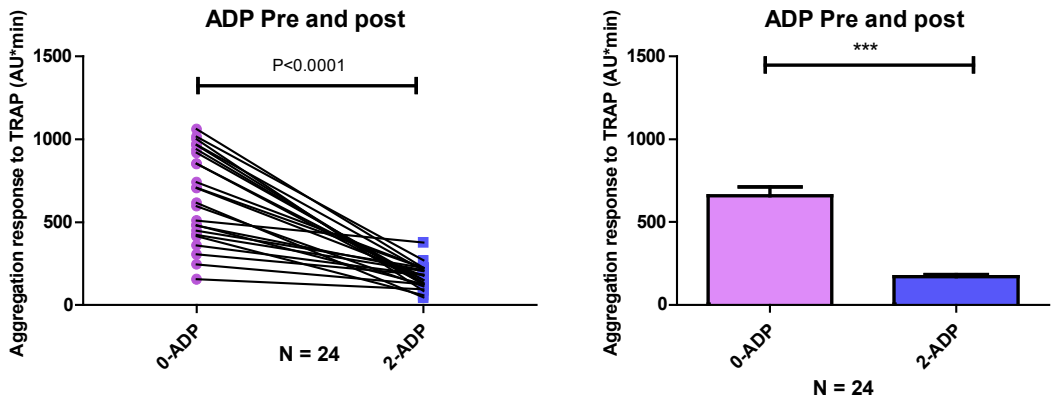



*Figure 3.59: Representing individual and group data on the change in platelet reactivity to ADP comparing 0 hr (0-ADP) to 2 hr (2-ADP) post ingestion of ticagrelor, measured in AU*min. A paired T test was applied with values are presented as mean with SEM,*

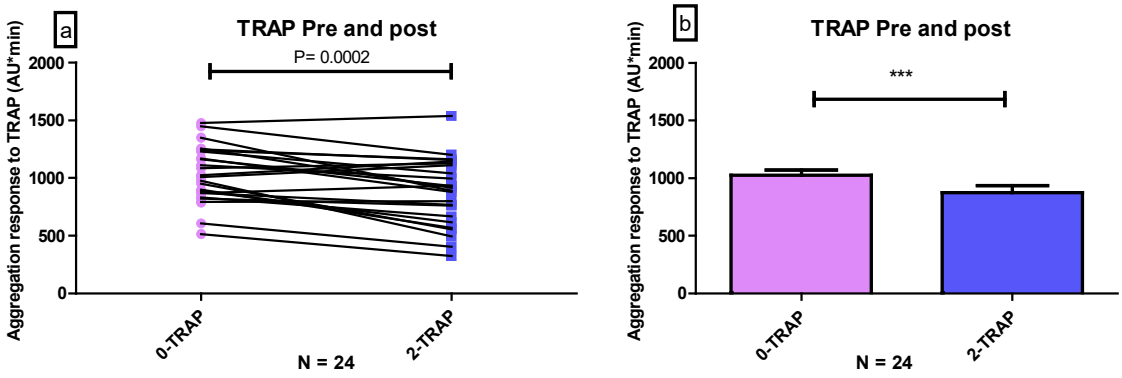



*Figure 3.60: Representing individual and group data on the change in platelet reactivity to TRAP comparing 0 hr (0-TRAP) to 2 hr (2-TRAP) post ingestion of ticagrelor, measured in AU*min. A paired T test was applied with values are presented as mean with SEM.*

### 3.4.4.5 Comparative effects of acute post ticagrelor loading therapy and chronic ticagrelor therapy on platelet aggregation

Mean ADP response was 168.8 (±72.92) AU*min 2 hours following a ticagrelor loading dose (180mg) which was not significantly changed at 212.6 (+/-99.44) AU*min) in patients after chronic therapy of 90mg twice daily (p=0.09) (Figure 3.61). Similarly, the mean TRAP response was 874.9 ± 59.53 AU*min 2 hours following a ticagrelor loading dose (180mg) which was higher (1024 ± 57.17 AU*min (p=0.0789)) after chronic therapy (Figure 3.62).

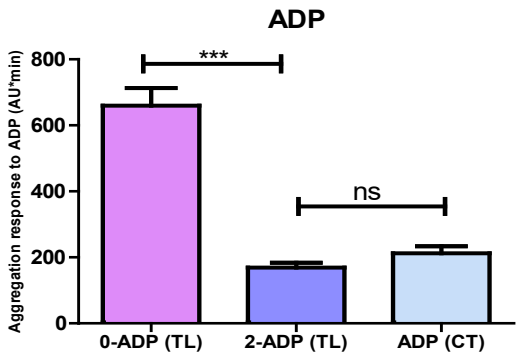



*Figure 3.61: Mean platelet aggregation response to ADP at 2 hours post ticagrelor loading (**TL**) and Mean platelet aggregation response to ADP with chronic ticagrelor therapy (**CT**), which is measure approximately two hours after intake of regular dose of ticagrelor. One-way ANOVA test of significance was applied.*

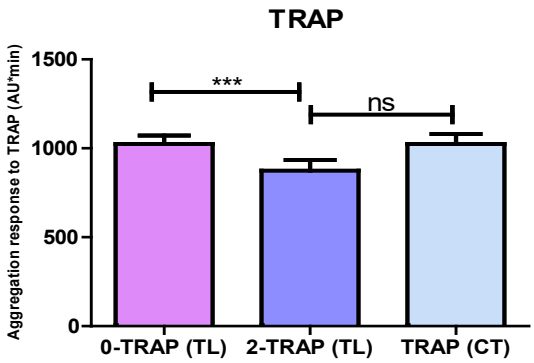



*Figure 3.62: Mean platelet aggregation response to TRAP at 2 hours post ticagrelor loading of 180mg and chronic ticagrelor therapy (CT), which is measure approximately two hours after intake of regular dose of 90mg of Ticagrelor, One way ANOVA test of significance was applied.*

### 3.4.4.6 The effect of ticagrelor therapy on platelet reactivity with or without a proton pump inhibitor

#### 3.4.4.6.1 Effect of acute ticagrelor loading on platelet reactivity with or without a proton pump inhibitor

When differentiating the acute ticagrelor loading group into those taking a proton pump inhibitor (n=16) compared to those not (n=10), (Figure 3.63 a&b) using Mann-Whitney test, there was no significant impact on platelet reactivity to ADP with or without concomitant PPI therapy at 2 hours post ingestion (Median of 164 vs 187, Mann-Whitney U =59.50, $n_1$ =16, $n_2$ =10, P=0.89) (Figure 3.63a)

Similar outcomes were also seen in the platelet aggregation responses to TRAP with or without a PPI with a mean of 865.8 ±284 on a PPI and 893.1 ± 286.7 AU*min not taking a PPI at 2 hours. Using unpaired t test, there was a non-statically significant change (p=0.81) in response to TRAP-induced aggregation with mean difference of -27.5±115 with 95% confidence interval of -264.6-209.9 (Figure 3.63b).

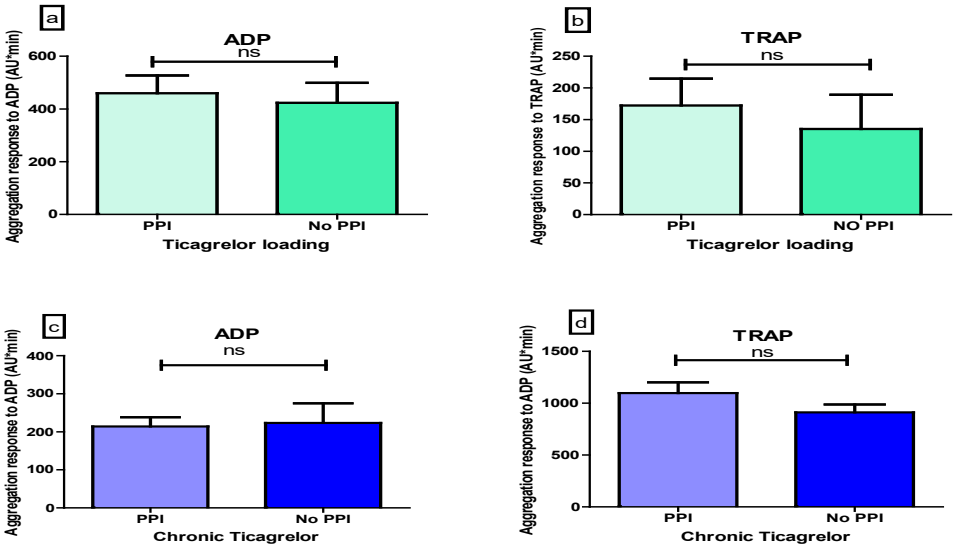



*Figure 3.63: ADP and TRAP induced platelet aggregation with or without PPI in ticagrelor loading and chronic ticagrelor therapy. (a) & (b) ADP and TRAP response in acute ticagrelor loading respectively, (c) & (d) ADP and TRAP response in chronic ticagrelor therapy respectively.*

#### 3.4.4.6.2 Effect of chronic ticagrelor loading on platelet reactivity with or without a proton pump inhibitor

Similarly, in the chronic ticagrelor group, upon differentiation into those taking a proton pump inhibitor (n=6) compared to those not (n=8), (Figure 3.63c &d) using Mann-Whitney test, there was no significant impact on platelet reactivity to ADP with or without concomitant PPI therapy (Median of 221 vs 183, Mann-Whitney U =18, $n_1$ =6, $n_2$ =8, P=0.49) (Figure 3.63)

The mean of platelet aggregation responses to TRAP with or without a PPI was 1096 ±258 on a PPI and 910.6 ± 218.5 AU*min respectively. Using unpaired t test, there was also no statically significant change (p=0.17) in response to TRAP-induced aggregation with mean difference of 185±127 with 95% confidence interval of –91.9 to 463.3 (Figure 3.63 d).

### 3.4.4.7 The effect of acute ticagrelor loading and chronic ticagrelor therapy on RSNO levels with or without a PPI

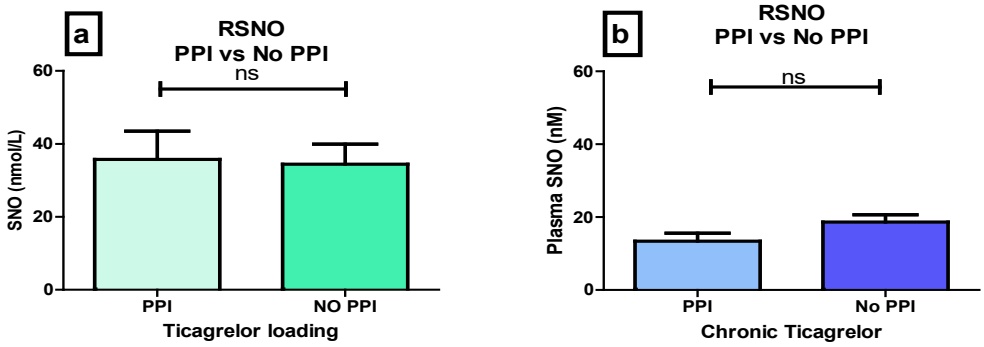



*Figure 3.64: comparative graphs of Plasma RSNO levels measure at 2 hours post Ticagrelor loading between patients on PPI and not on PPI (a) and similar comparison in patients on chronic Ticagrelor (b).*

There was a consistent rise in plasma R-SNO in patients with or without concomitant PPI therapy [(∆11.37±5.2 nmol/L (p=0.0422) with a PPI vs ∆7.4±5.3 nmol/L (p=0.0197) without PPI (p=ns between groups)] (Figure 3.64a) in the ticagrelor loading group. There was no

statistical significance difference in the levels of RSNO (∆13.44±5.2 nmol/L vs ∆18.62±5.7 nmol/L (P=ns)) with or without PPI (Figure 3.64b).

### 3.4.5 Summary

- ✓ Ticagrelor has an ability to form drug-SNO in an acidic environment in the presence of nitrite, despite the lack of a free thiol group in its original chemical structure.

- ✓ There is a higher proportion of ticagrelor SNO formed with higher dose of nitrite compared with low dose nitrite

- ✓ Ticagrelor is a potent antiplatelet drug suggested by a significant ADP and TRAP mediated platelet inhibition with loading dose.

- ✓ There is a significant increase in plasma RSNO post ingestion of 180mg of Ticagrelor

- ✓ There is no effect of concomitant PPI therapy on platelet inhibition or NO metabolites with acute ticagrelor loading or chronic ticagrelor therapy.

# 4 Discussion

The focus of this thesis is on the comprehensive evaluation of the influence of dietary nitrate supplementation and clopidogrel therapy on NO metabolites and platelet aggregation in patients with known stable CAD. The initial portion of study focused on understanding the detailed role of nitrate reductase enzyme, and the process of nitrate-nitrite-RSNO-NO cycle with respect to the dietary nitrate supplement and antiplatelet drug interaction under laboratory settings. This part of the study complemented the second part of the thesis involving a placebo-controlled, cross over trial in stable CAD patients where the NO metabolites and platelet aggregation response were measured following intake of the same dietary nitrate supplement. Thirdly, this thesis for the first time explores the effects of co-administration of clopidogrel and dietary nitrate supplement in CAD with emphasis on the RSNO formation and platelet inhibition. Additionally, my studies identified the ability of ticagrelor, a non-thienopyridine antiplatelet agent to form RSNO directly in patients.

CVD is associated with endothelial dysfunction with impaired NO bioavailability. The cardiovascular role(s) of NO have been well described in numerous published articles since its discovery in 1980. It is a vital signalling molecule in maintaining vascular homeostasis and a healthy cardiovascular system. It acts as a primary substrate for the sGC enzyme mediated vasomodulation and inhibition of platelet aggregation with thrombus formation. Reduced bioavailability of NO as seen in patients with CAD is associated with endothelial dysfunction, results in vasoconstriction, thrombosis, inflammation and vascular hypertrophy. A drug or molecule capable of delivering NO or augmenting NO levels in these patients therefore has a clear therapeutic benefit.

Dietary sources of inorganic nitrate are a subject of major interest in research because of their potential role in promoting cardiovascular health. Discovery of the nitrate-nitrite-NO pathway as an alternate and promising process to replenish NO levels in tissues has opened a new avenue of research in the area of using dietary nitrate supplements as NO augmenting therapies, and there are numerous studies published exploring their preventive and therapeutic potential.

It is now well established that the cardio protective effect of a fruit and vegetable rich diet is attributed largely to their nitrate content, with orally ingested nitrate now a well-established exogenous source of nitrite.

## 4.1 In vitro experiment of Nitrate-Nitrite-RSNO(Clopidogrel-SNO) formation.

Nitrite is formed through the reduction of nitrate present in the dietary supplement via the reduction by NR. Facultative anaerobes present in the oral cavity and GI tract facilitates this conversion via NR. The in vitro study of this process of enzymatic conversion of nitrate to nitrite and its subsequent NO and RSNO formation under controlled laboratory conditions provides a valuable model in which to gain better insight into the process of dietary nitrate metabolism and kinetics in patients.

Enzyme mixes containing NR derived from *E Coli* facilitated a maximal (~96%) nitrate to nitrite conversion from laboratory grade sodium nitrate in preliminary experiments, however upon adopting the same mix with NR from *A Niger* (due to cessation of commercial availability) yielded only a 8.8% conversion. The initial cofactor concentration was modified and tested at different ratios. At 1000 µM of NADPH and 160 µM of G6PD in the enzyme mix, a significantly enhanced reductive potential of NR was established (100 µM of NADPH and 16 µM of G6PD). NADPH acts as an essential electron donor for this reaction and G6PD acts to scavenge NADP+ and regenerate NADPH. Enhanced NADPH amplified the nitrate reduction process and simultaneously an increased NADPH in the enzyme mix requires heightened levels of G6PD to maintain a high NADPH/NADP+ ratio thereby facilitating the enzyme kinetics (296-298).

*In vitro* experiments showed that the nitrite generation is dependent on the sodium nitrate salt concentration, suggesting increasing nitrate enhances NR's turnover of the substrate. The reduction efficacy of NR enhances up to >90% (NR concentration was constant in each sample) at high concentration. The capacity for NR to reduce nitrate eventually reaches its maximum and a plateau was reached. In vivo, nitrite concentration in saliva positively

correlates with increased dietary nitrate (299), implying the capacity of the bacterial NR system in humans is unlikely exceeded.

In the oral cavity, nitrate reduction occurs on the dorsum of the tongue in an aqueous solution, facilitated by the colonizing bacteria. In vivo only ~20% of salivary nitrate is reduced (5% of ingested nitrate) to nitrite by anaerobic oral bacteria. A significant amount of nitrate remains unreduced(299), due to a short transit time of the ingested nitrate in the oral cavity and rapid transfer of the majority of nitrate through the alimentary canal. Nevertheless, significant levels of nitrate are accumulated in the salivary glands where NR can reduce the available nitrate to nitrite. The in vitro model showed a maximum nitrate accumulation occurred at 45 minutes and although nitrate reduction occurs within minutes, this implies nitrate reduction may be a relatively slow process.

Nitrate contained within *SIS® Go+ nitrate gel* and *Beet It®* was inefficiently converted to nitrite when in its original form with this model. *SIS® Go+ nitrate gel is a viscous gel-like solution of an array of ingredients (Table 3.1.4). Beet It®*, despite being a thin liquid composed of pressed organic beetroot and apple juice had a similar property of not being reduced to nitrite. Regardless of their consistencies, nitrate ion contained in the *SIS® Go+ nitrate gel and Beet It®* was not freely accessible for NR. Progressive dilution improved the conversion of nitrate to nitrate. A possible explanation for this is that the nitrate molecules present within the gel are physically bound to an ingredient in the gel or vegetable extract in the juice such that only a modest component of nitrate is available to the enzyme, which potentially may also block the binding of nitrate and the active site of NR. Diluting the original supplements may have overcome this blockage, facilitating the diffusion of the nitrate through the gel or vegetable extract to bind to the active site of NR. There may also be a strong cross-linked pore, limiting the anions mobility and interaction. Dilution of the supplements possibly caused a strong electrostatic matrix interaction(300) and hydrodynamic forces(301) that encourage enzymes and substrates to move towards one another, thus potentially explaining increased reduction efficacy The *SIS® Go+* nitrate gel and *SIS® Go+* placebo gel had identical ingredients except for nitrate. We found insufficient nitrate to nitrite conversion by NR when known amounts of laboratory grade sodium nitrate was added to the placebo gel. This confirms under laboratory condition components of the gel either prevent or limit nitrate reduction from taking place or prevent the availability of nitrate present in swiss chard.

A further testing of the second cycle of the entero-salivary Nitrate-nitrite-NO cycle, which is the 2 hours after ingestion of the *SIS® Go+ nitrate* gel, nitrate is accumulated in the blood stream via absorption from the intestinal tract and there is active sequestration of nitrate by the salivary glands, The commensal bacteria present in the oral cavity converts the nitrate to nitrite via NR and the majority of this is swallowed forming the main source of nitrite in the acidic gastric environment (302). This nitrite, upon entering the gastric cavity encounters thiol groups on gastric proteins and possibly other thiol containing molecules like thienopyridines (clopidogrel) allowing nitrosation to occur.

RSNO formation occurs in acidic gastric medium in the presence of nitrite. In vitro, SGF has no background nitrite, hence there was no RSNO measured in the gastric medium or mixture of the *SIS® Go+ nitrate* and SGF. The nitrite which is converted from SIS® Go+ nitrate by NR does form RSNO when mixed in SGF suggesting that the thiol groups on gastric proteins are readily subject to nitrosation and create RSNO(157, 303).

There was a greater percentage of RSNO generated from nitrite converted within *SIS® Go+ nitrate* gel, compared to that converted from sodium nitrate. This is an interesting finding which indicates that adjuvant ingredients in the swiss chard gel exhibit additional free thiol groups that can bind nitrite and form RSNO. There is also a transnitrosation reaction between proteins in the gastric juice and/or ingredients in the gel. This is a measure of total RSNO, and it is not possible to categorize individual types of RSNO.

The presence clopidogrel tablets (75mg or 600mg) did not affect the RSNO yield in gastric medium with nitrite derived from SIS® Go+ nitrate. The possible explanation for this is that the amount of nitrite generated from diluted *SIS® Go+ nitrate* gel was minimal (43.75 µM) which is 20 times less than Bundhoo et al. recommended. An estimate of more than 10mM of nitrite is need for detectable clopidogrel-RSNO generation, and it is dependent on the quantity of background nitrite (133) (79). The inability of nitrate present in the undiluted *SIS® Go+ nitrate* gel to be converted to nitrite was a major hurdle, that need to be addressed in future experiments.

*In vitro* experiments (Chapter 3 results 1) demonstrated a need for an optimum mixture of co-factors such as NADP, G6PD and *A Niger* derived nitrate reductase in order to efficiently convert nitrate to nitrite. The dietary nitrate supplement (*SIS® Go+ nitrate gel)* tested in this thesis required significant dilution prior to its reduction demonstrating that the supplement in

its original form is not able interact instantaneously with nitrate reductase (NR) enzyme derived from bacterial commensals under laboratory conditions. This implies that in vivo, nitrate present in the supplements first needs absorption into the blood stream, then is actively concentrated in the salivary glands and subsequently the nitrate rich saliva in the mouth interacts with bacterial NR for conversion of nitrate to nitrite to take place. This then subsequently can enter the alimentary canal, be absorbed into the circulation, or interact with protein and other components contained within the stomach. This is confirmed by our studies in CAD patients where we observed a mean rise of nitrite level of 214nM following ingestion of dietary nitrate gel, confirming a functioning entero-salivary cycle. Interruption of this cycle would prevent nitrate reduction to nitrite and subsequent NO formation, as shown in experiments using antibacterial mouthwash and salivary spitting techniques (304).

A successful in vitro model was established for the enzymatic reduction of nitrate from laboratory grade sodium nitrate, SIS® Go+ nitrate gel and Beet It® to nitrite catalysed by nitrate reductase from Aspergillus niger. RSNO formation occurred within a simulated acidic gastric medium and in the presence of background nitrite. The ingredients within the gel, in its concentrated commercial form, limit the accessibility of nitrate anions for nitrate reductase to catalyse a reaction. Dilution of the gel with HPCL graded water overcomes this obstacle. Interestingly RSNO experiments suggested proteins within the gel exhibit thiol groups that enhance nitrosation with nitrite. These products can also act as NO metabolite-delivery vehicles. This project gives significant insights into the understanding of nitrate-nitrite-RSNO-NO cycle and understanding the results of my clinical research trial on the study of nitrate supplementation in patients on clopidogrel therapy.

## 4.2 Dietary nitrate supplementation in stable CAD patients

Dietary nitrate supplementation in healthy volunteers and hypertensive subjects is known to exhibit a vasodilatory effect, significant blood pressure reduction and noticeable antiplatelet effect (226, 236, 248, 305). Little was known regarding their use in humans with established CAD who are known to have impaired endogenous NO levels associated with endothelial dysfunction.

Clopidogrel is the most commonly prescribed $P2Y_{12}$ receptor blocking antiplatelet drug across the globe. It is utilised with aspirin in patients with acute coronary syndrome and in most

patients undergoing PCI. Clopidogrel still remains the number one choice of antiplatelet therapy in this group of patients despite the availability of newer and more potent antiplatelet agents like ticagrelor and prasugrel. This could be explained due to an established trust and experience of its use by clinicians for more than 15 years, along with being a cost effective $P2Y_{12}$ blocker. It is also prescribed in patients with cerebrovascular and peripheral vascular disease and it has a proven, long-standing record of efficacy and safety in a wide variety of patients. Despite having universal acceptability, it has its own limitations, mainly variability in antiplatelet response and interaction with other commonly prescribed drugs such as PPI and calcium channel blockers.

In our research group, Bundhoo *et al* conducted *in vitro* experiments leading up to this thesis and showed that clopidogrel has $P2Y_{12}$ independent modes of action. It has an ability to actively form RSNOs in its original inactive prodrug form when in the presence of inorganic nitrite and in an acidic environment.

However, initial *in vivo* studies by our group in CAD patients did not find a significant rise in the overall plasma RSNO with clopidogrel loading (600mg) or maintenance therapy (75mg daily). This could be explained by insufficient RSNO formation above baseline with physiological levels of nitrite (100-200nM), such that these RSNO concentrations would be close to the detectable limit by OBC. We therefore hypothesized *in vivo* RSNO formation could potentially be enhanced following elevation of nitrate and nitrite in the acidic stomach milieu when given exogenous nitrate. Indeed, this is the first study which shows that administration of a single dose of dietary nitrate supplement along with clopidogrel therapy results in significant increase in the plasma NO metabolites, particularly RSNO, at 2 hours in patients with stable CAD.

The baseline levels of plasma nitrate before the nitrate or placebo gel supplementation was 29.93 ± 1.41 µmol/L & 31.27±2.29 µmol/L respectively. These values are similar to the plasma nitrate levels measured previously in healthy volunteers in our laboratory using OBC(306). Basal nitrate production from endogenous sources is in the range from 1.2 to 2mmol/per day whereas the dietary nitrate intake contributes from 75-150mmol/day. The amount of nitrate measured in plasma or urine is therefore significantly influenced by the diet. Patients in our group were fasting for > 6 hours, limiting the influence of recent dietary intake on the measured plasma nitrate values.

There was a noticeably lower level of basal plasma nitrite measured in this patient group prior to intake of the supplement on both occasions of the study (nitrate (175.3 ±13.94nM) and placebo gel (194.1±17.8 nM)) compared with the values typically observed in healthy individuals (200-300 nM) which we have reported previously (28).

Previously, Kehmeier *et al*., had shown similar lower levels of whole blood nitrite in patients with myocardial infarction(167). The findings of low levels of nitrite in CAD patients may reflect and supports the concept of reduced systemic NO bioavailability and prevalence of endothelial dysfunction in CAD patients.

Supplementation of SIS® Go+ nitrate gel caused a 10-fold rise (29.93 μM to 291.49 μM) in the circulating levels of nitrate, indicating that the nitrate content within the gel is efficiently absorbed from the gut, entering the blood stream. The plasma nitrite level rose by 2.3-fold from baseline (175nM to 391nM), confirming an active conversion of a proportion of the ingested nitrate to nitrite via an intact entero-salivary circulation. The observed rise in nitrite level with SIS® Go+ nitrate gel (9.6 mmol nitrate dose) in CAD patients is marginally higher in comparison (3-fold vs 1.6-fold), with intake of beetroot juice (5.5mmol nitrate dose) in healthy volunteers. This difference in rise in the nitrite levels is difficult to assess because this could be due to use of gel versus beetroot concentrate or the fact our study was in CAD patient vs previous studies in Healthy volunteers. It may be also be implied that CAD patients have efficient nitrate to nitrite conversion when compare to healthy volunteers. Nitrite can also mediate its effects directly (307) without need for conversion to NO, or indirectly through the production of SNO (308) or nitroso-fatty acids(309). Impaired platelet response to NO is a known characteristic of many cardiovascular disease, chircov et al in 2013 demonstrated that nitroxyl by its virtue of not being inactivated by ROS, does induce platelet inhibition in platelet NO resistance subject(310). Madhani et al group recently showed that nitrite overcomes the platelet resistance in patients with heart failure preserved ejection fraction and chronic AF and. NO scavenging agents failed to revert the platelet inhibitor effect of high concentration of nitrite, indicating a NO independent effect of nitrite on platelet inhibition(213). An exponential rise in the level of nitrite upon ingestion of the SIS® Go+ nitrate gel in this study should augment platelet inhibition independent of its NO donor property. It can also be postulated that this effects are mediated by a s-nitrosation or s-thiolation.

My study demonstrates a significant increase in RSNO levels in all groups (CAD only, Group A (clopidogrel 600mg) and Group B (Clopidogrel 75mg) following dietary nitrate supplementation, confirms that enhanced nitrite levels facilitates S-nitrosation and s-thiolation, hence enhanced formation of RSNO. This effect is not seen in CAD patients who were on regular oral organic nitrate medication (ISMN, ISDN) suggesting that dietary nitrates have a high bioavailability, and an intrinsic potential to form RSNO (137). This is a significant positive feature, along with other known advantages of dietary nitrate and nitrite, compared to the negatives associated with organic nitrate therapies, for example, nitrate tolerance and high first past metabolism.

RSNOs are more stable and exhibit longer half-life than NO. They actively undergo transnitrosation reactions, participating in potential post-translational mechanisms of modification of protein and enzyme activity. RSNO are NO donors, with an additional characteristic of direct binding to sGC there by facilitating antiplatelet and antithrombotic effects (177, 187). The ability of nitrite to overcome platelet resistance in CVD could be potentially due to RSNO formation via nitrate-nitrite-RSNO-NO axis.

Parental GSNO infusion has shown a highly platelet selective effect with an inhibition of platelet activation with GSNO infusion 10 minutes before PCI and a resulting decrease in stent thrombosis (190). In a similar way, enhanced RSNO formed with dietary nitrate supplementation within 2 hours could potentially offer synergistic platelet inhibition along with antiplatelet drugs in patients undergoing PCI and therefore has clinical relevance. The patients in this research study are ideal as they are all listed for elective PCI and require optimum platelet inhibition prior to prevent thrombosis following implantation of stents. These findings make a strong case for future research projects that are specifically designed and adequately powered to address the potential longer-term benefits of dietary nitrate on clinical outcomes like stent thrombosis and myocardial infarction in a similar patient cohort.

It was not possible to differentiate the sub-types of RSNO in the plasma in this study. The majority of RSNO are protein bound and a small portion are from non-protein thiol containing molecules(133). The total amount of RSNO measured here is a composite of all RSNO species present in plasma at a given time. A specific quantification of the amount of Clopidogrel-SNO formed was not possible because of its transnitrosation reactions, platelet sequestration and its metabolism to several oxidative products. Indirect evidence of possible

Clopidogrel-SNO formation is derived from a comparison of the amount of RSNO formed in patients with CAD only compared to patients with CAD on clopidogrel therapy, however only a marginal increase in RSNO was observed that was not statistically significant (6.7nM in Group A & 3.4nM in Group B). Interestingly patients who had a loading dose of Clopidogrel (600mg) formed numerical higher amounts of RSNO compared to those receiving 75mg daily, which could be explained by Clopidogrel-SNO formation. However, this study was not adequately powered to test this finding.

Despite an intrinsic ability of clopidogrel prodrug to form RSNO as shown in *in vitro* studies, there was no rise in RSNO level in patients on clopidogrel only therapy (placebo group), suggesting further increases in nitrate/nitrite may be required for formation of noticeable clopidogrel-SNO. This might be explained by the relative low levels of baseline nitrite found in patients with CAD due to impaired NO synthesis/increased inactivation of NO. Interestingly in the active nitrate group, there was no significant rise in the RSNO levels in CAD patients with intake of either a loading dose of clopidogrel (600mg) or a long-term maintenance clopidogrel (75mg). This was also evidenced in my *in vitro* experiments described in chapter 3 where small amounts of converted nitrite from SIS go+ gel failed to produce significantly more clopidogrel-SNO and is in close agreement with the findings of Dr Bundhoo previously (133).

A study on prasugrel loading therapy (60mg) in a similar cohort of patients undertaken concomitantly by a colleague in our laboratory showed a significant rise in RSNO levels at physiological levels of nitrite/nitrate (i.e. without dietary nitrate supplementation), suggesting that prasugrel exhibits a greater capacity for formation of RSNO (109). This is certainly in line with *in vitro* studies and is an interesting observation, in that enhanced Prasugrel associated RSNO formation hypothetically could be one of the reasons for greater platelet inhibition with prasugrel therapy compared to clopidogrel therapy as observed clinically in patients - this warrant further investigation.

This study demonstrates that dietary nitrate supplement actively increases circulation nitrate-nitrite and RSNO levels, via the Nitrate-Nitrite-NO pathway and, thereby replenishes the depleted NO pool in CAD, which is associated with endothelial dysfunction.

Previous studies by Ingram *et al* from our research group have demonstrated than an acute increase of nitrite levels as little as 200nM is associated with an improvement in myocardial ischaemia and can induce localised vasodilation under hypoxia, as observed in CAD patients

(311). The elevated nitrite level can protect against the ischaemia reperfusion injury, especially in patients have acute arterial occlusion in myocardial infarction or stroke. The findings of elevated nitrite and RSNO in this thesis could hypothetical minimise myocardial injury/ischaemia caused by transient coronary arterial occlusions during balloon dilation and stent implantations in SCAD patients who are undergoing complex coronary intervention. In this study, post procedural measurement of CK or Troponin (markers of myocardial injury) was not performed, limiting the evaluation of this effect.

ADP and thrombin activate platelets by binding to the cell surface $P2Y_{12}$ receptor and the protease activated receptor (PAR) respectively, causing an intracellular influx of calcium stores from the sarcoplasmic reticulum, resulting in a series of secondary reactions causing platelet aggregation. A key secondary reaction is the activation of the phosphoinositide 3 kinase pathway (PI3K) which plays a crucial role in the cytoskeletal reorganisation of activated platelets. The PI3K pathway is directly activated via TRAP (312, 313). RSNO by virtue of being a NO donor, directly inhibits the activation of P13K pathway and activates sGC causing an inhibition of intracellular calcium influx thereby inhibiting platelet aggregation.

Interestingly we did observe an overall increase in platelet reactivity with the ADP and TRAP agonists, from baseline to 2 hours measured at both intervention points in stable CAD patients not on clopidogrel therapy. This is potentially explained by the naturally occurring diurnal variation in the platelet reactivity in humans. We did not account for this in our studies which were largely undertaken at the beginning of the working day using a similar protocol/timeline. Ischemic events such as myocardial infarction and stroke tend to occur more often in the mornings than at other times (314-316) and this has been attributed to the increase in platelet aggregation, platelet count, haematocrit and catecholamine in the morning. It is also linked with physical activity after waking up (317, 318). There was a non-statistically significance in the change in platelet reactivity to ADP or TRAP upon administration of the dietary nitrate supplement in this group of patients.

The platelet aggregation to ADP following a loading dose of Clopidogrel (600mg) was significantly decreased following both nitrate gel and placebo, which is unsurprising due to the large dose of clopidogrel received, and this is consistent with other platelet function studies reported (319-321).

Importantly the platelet aggregation response to TRAP was significantly impaired with dietary nitrate supplementation therapy and not with placebo in patients given a loading dose of 600mg of clopidogrel. It is well known that clopidogrel primarily acts via ADP associated $P2Y_{12}$ receptor mediated inhibition and theoretically has no effect on thrombin mediated inhibition. However, inhibition of TRAP mediated platelet activation by clopidogrel in patients with ACS was reported but the exact mechanism was not explained.

It is worth noting that there was a significant correlation between rise in the RSNO level with the drop in TRAP mediated platelet reactivity found upon combining all the three patient groups who ingested dietary nitrate supplement. This inverse correlation it taken to reflect a rise in RSNO levels dampens platelet activation. Diminishing platelet activation via a non-ADP-receptor mediated mechanism would significantly benefit patients with CAD and especially those undergoing percutaneous coronary interventions. Taking together the findings of this study and earlier in vitro evidence it is reasonable to hypothesize that the enhanced TRAP inhibition is due to RSNO formation, which in turn can be stored in platelets and can potentially directly inhibit the TRAP mediated aggregation (Figure 4.1)

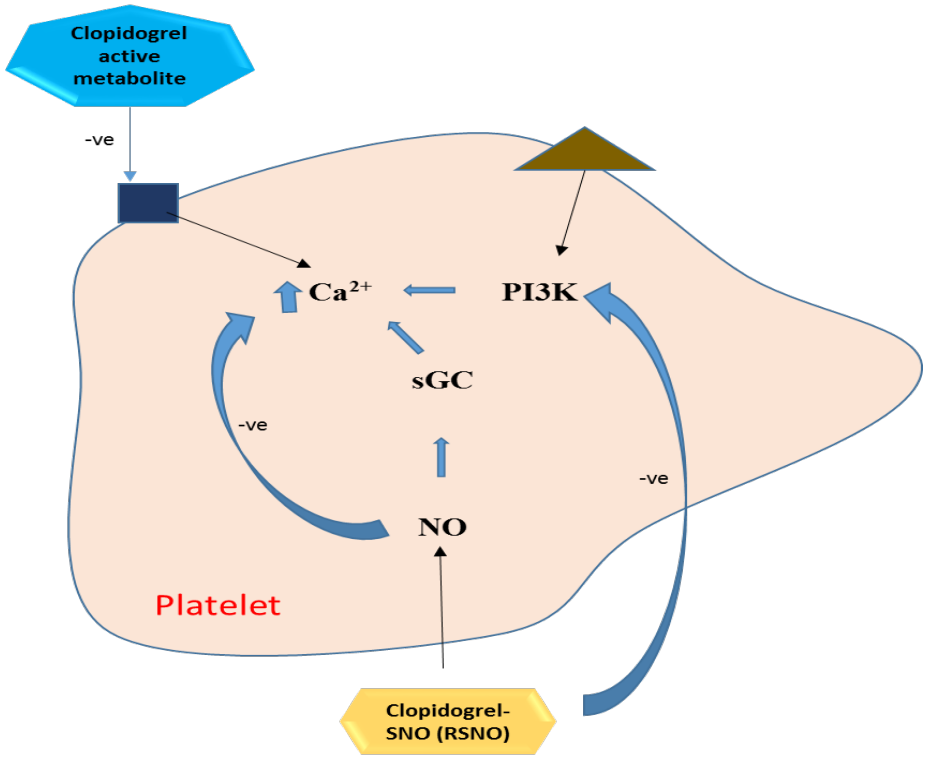


*Figure 4.1: Diagrammatic depiction of ADP ($P2Y_{12}$) receptor and TRAP receptor (PAR) stimulation, resulting in increased intra cellular calcium causing a change in platelet shape and aggregation. The active Clopidogrel metabolite blocks the $P2Y_{12}$ receptor and RSNO can inhibit platelet activation via $P2Y_{12}$ independent mechanisms via donation of NO resulting in activation of sGC, and via a direct action on PI3K receptors. (-ve represents a negative effect)*

Conversely, there was no statistically significant effect of dietary nitrate therapy on overall platelet reactivity against ADP or TRAP in patients receiving chronic clopidogrel therapy (Group B). This could be due to reduced amount of RSNO formed in this group or, intra-individual variation and underpowering of the study.

Interestingly, there is a trend towards numerically higher platelet inhibition in both Group A (clopidogrel 600mg) (ADP: -352 Vs -327 & TRAP: -123 vs -105) and Group B (ADP: -119 Vs 6 & TRAP: -37 vs -36) patients given a dietary nitrate supplement. This does strengthen the hypothesis that dietary nitrate supplementation with clopidogrel therapy could facilitate additional platelet inhibition through augmenting NO metabolites (RSNO) which is $P2Y_{12}$ independent, and this will need to be addressed in studies with greater patient numbers in each patient group.

It was anticipated that a rise in RSNO level should translate into a decrease in platelet responsiveness in individual groups. The lack of a statistically significant response could be due to several potential reasons. This may be due to a relatively small number of patients, as the study was not primarily powered for measuring a change in platelet reactivity.

There were multiple independent variables in the study cohort, which could potentially impact on the baseline platelet reactivity and its response to dietary nitrates. The placebo control study design was employed to limit these confounding effects and there were no significant difference in the variables such as diabetes, hypertension, smoking between the active group and placebo group (Table 3.12, Table 3.14 & Table 3.17 ). Despite this, these factors may still have influenced the outcome, especially diabetes.

There were noticeably number of diabetic patients recruited in this study, which are in line with the everyday clinical practice. A combined total of 13 and 8 patients with diabetes enrolled in nitrate supplement and placebo arms, respectively. They were all type 2 diabetics, with the majority on oral anti diabetic medication (Table 4.1).

| Diabetic patient enrolled | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Nitrate gel | | | | Placebo gel | | | |
| | Diet | Oral | Insulin | Total | Diet | Oral | Insuli n | Total |
| **Clopidogrel Naïve group (paired)** | 0 | 1 (7%) | 0 | 1 (7%) | 0 | 1 (7%) | 0 | 1 (7%) |
| **Acute clopidogrel loading (unpaired)** | 2 (8%) | 5 (19%) | 1 (4%) | 8 (31%) | 1 (4%) | 4 (17%) | 0 | 5 (21%) |
| **Chronic clopidogrel therapy (unpaired)** | 0 | 4 (14%) | 0 | 4( 14%) | 0 | 2 (8%) | 0 | 2 (8%) |

*Table 4.1: Summary of patients with diabetes enrolled in the study. The clopidogrel Naive study was a paired study hence had 1 patient in both arms.*

It is well known that in diabetic patients hyperglycaemia leads to oxidative stress, via production of advance glycation end products, enhanced polyol protein kinase C and hexosamine pathways, leading to aberrant NADPH oxidases (NOX) activation contributing to uncoupling of eNOS and endothelial dysfunction(322). NOX activity is significantly increased in both type 1 diabetic and type 2 diabetics(323). The excessive ROS scavenges NO, further reducing its bioavailability. A significant association between type 2 diabetes and eNOS gene polymorphisms is also known suggesting a link in NO production and insulin resistance states (324, 325). Type 2 diabetes has been associated with both increased and decreased level of circulating nitrate/nitrite (326, 327). However, CAD patients with diabetes have typically shown a higher nitrate/nitrite level(328) which might be a compensatory response to elevated insulin and oxidative stress(329).

Organic nitrate therapy has proven to be ineffective in patient with diabetes and metabolic syndrome due to its paradoxical ability of inducing endothelial dysfunction. It worsens the intrinsic impaired NO bioavailability and NO resistance seen in this group of patients. (330, 331). Exogenous supplementation of dietary nitrate has great potential as a beneficial therapeutic intervention in type 2 diabetic patients, attributed to its ability to convert to nitric oxide. In vivo work on dietary nitrate conducted in animal models has shown promising results of improved fasting glucose levels, reduced insulin concentration, improved insulin signalling via restoration of NO dependent pathways(332, 333), enhanced antioxidant capacity, and

protection against renal dysfunction(334). Human studies of high nitrate beetroot juice had no significant effect on insulin resistance, in type 2 diabetes(335) and no effect on postprandial plasma glucose and insulin levels in obese men(336). Dietary nitrate supplementation could improve vascular stiffness, and have other cardioprotective effects like anti-platelet activity, anti-inflammatory, hypolipidemic, improved flow in hypoxic and ischemic tissue(337-339). These effects are yet to be confirmed in diabetic patients(340).

These studies were limited due to a short intervention period. The antiplatelet effect and blood pressure lowering effect seen in healthy volunteers would be mild in diabetic patients, due to the impaired metabolic pathways and NO resistance. The presence of numerically higher number of diabetic patients in the nitrate supplement arm might have attenuated the beneficial effect of dietary nitrate, though this is not definite as nitrite and theoretically RSNO could potentially overcome the nitrate resistance as shown in HFpEF patients. A further randomised study of large number of patients with and without diabetes would test this effect.

The lack of antiplatelet effect could also be due to a relatively small effect of nitrate may be relatively small compared to the large inhibition found with clopidogrel itself, and this is further complicated by significant variation in the platelet responsiveness noted in this study. 22% (6 out of 27) of patients in Group A (clopidogrel 600mg) were clopidogrel non-responsive [exhibited less than 10% inhibition of ADP mediated aggregation at 2 hours (341)] whereas 36% (10 out of 28) had high on-treatment platelet reactivity [HPR, Multiplate ADP >468AUC(109)] in Group B (clopidogrel 75mg, chronic therapy) patients supplemented with dietary nitrate gel. This finding is consistent with the published data on clopidogrel resistance (342). There was a greater degree (-109 AUC vs -25 AUC) of platelet inhibition observed in patients who had higher platelet reactivity compared to those who were responsive which is a very interesting finding, which was also noticed in the GRAVITAS randomized trial (122) and this additional inhibition is possibly due to the clopidogrel-SNO formation and its effect as a NO donor. This hypothesis needs more exploration.

Other possible explanation could be due a delayed platelet effect (beyond the period measured in this study), an augmented baseline platelet reactivity in CAD patients compared to healthy volunteers, or a dose effect where levels of nitrite achieved were not adequate to show a demonstrably significant(although we think this unlikely given that beneficial effects of exogenous nitrate administration have been observed using a similar dose).

This study demonstrates enhanced formation of NO metabolites with a noticeable trend towards the attenuation of platelet reactivity against ADP mediated platelet reactivity with supplementation of a single dose of nitrate supplement in patients with CAD. Importantly, it demonstrates for the first-time in vivo formation of circulating RSNO from exogenous nitrate, and when taking all patients, we confirm RSNO level attained is inversely associated with platelet reactivity.

Exploring these pleiotropic effects of thienopyridines is where our group has focussed attention. This is a very novel and encouraging finding which encourages future studies to evaluate the option of using dietary nitrates, perhaps at elevated doses than used herein, as an alternative therapeutic option for attenuation of platelet reactivity and, thereby reducing thrombotic events in patients at high risk such as CAD and cerebrovascular disease.

The clinical studies successfully demonstrate that in patients with established CAD, dietary $NO_3^-$nitrate gel (SIS® $Go^+$) is absorbed at 2 hours into the blood stream and is reflected by a significant rise in the plasma nitrate levels measured 2 hours post ingestion. This clearly confirms an intact entero-salivary pathway, with active nitrate-nitrite-NO conversion in CAD patients, with clear evidence for a significant rise in plasma RSNO levels. There is no significant platelet inhibition observed but given the relative selectivity of RSNO for platelets, this might point the way forward to achieving therapeutic levels of circulating RSNO, and significant platelet inhibition. There was no placebo effect on the NO metabolites or ADP mediated platelet aggregation.

## 4.3 Ticagrelor and NO metabolites

Ticagrelor is a more potent antiplatelet in comparison with clopidogrel. Ticagrelor has shown pleiotropic effects which are independent of $P2Y_{12}$ inhibition, which is not entirely explained. Ticagrelor's ability to form Ticagrelor-SNO when mixed with nitrite salt, despite lacking a free thiol group, as demonstrated by Thornhill et.al in his in-vitro studies was a most intriguing finding. It can be postulated that some of the pleiotropic effects seen in early ticagrelor studies could be attributed to this feature.

In my in-vitro experiments, ticagrelor readily forms RSNO molecules in an acidic environment in the presence of nitrite, such as shown in artificial stomach medium, which is analogous to the human stomach. This appears over and above the amount of RSNO formed with proteins normally present in the stomach medium. Ticagrelor-SNO formation was significantly augmented with increasing the nitrite level in the gastric medium. All these findings suggest that ticagrelor generates additional thiol substrate when dissolved in the acidic stomach milieu, thereby enhancing the formation of SNO with nitrite at both physiological and excess levels. RSNO compounds are known to be potent vasodilators and inhibitors of platelet aggregation. Combining these findings with earlier results of *in vitro* studies conducted by Thornhill *et al* raises the exciting possibility that the anticipated mode of action of ticagrelor via $P2Y_{12}$ receptor inhibition may not be as expected in vivo and is actually lost in the acidic condition (pH<3) of the stomach. It also follows the anti-platelet effect of ticagrelor in vivo relies upon the formation of Ticagrelor induced RSNO as an extremely potent antiplatelet agent. This may go some way to explaining the observation that proton pump inhibitors (via raising stomach pH) blunted the clinical effectiveness of this drug in the PLATO trial (258).

Consistent with this hypothesis, the clinical study in this chapter interestingly showed a single 180mg ticagrelor dose (without concomitant administration of exogenous nitrate) results in a significant rise in plasma RSNO at 2 hours in addition to a profound platelet inhibition in patients with stable CAD. There was a significant inhibition of both ADP and TRAP mediated platelet activation. The inhibition of TRAP mediated activation suggests a non-$P2Y_{12}$ receptor effect, warranting further investigations and explanation. These findings were consistent regardless of concomitant PPI therapy. This significant rise in plasma RSNO was however not maintained in patients during chronic ticagrelor use (90mg bid), and there was a modest accompanying rise in platelet reactivity compared with acute loading group. This may reflect a dose dependent change in platelet inhibition where a 180mg dose is given acutely whereas the chronic dose is 90mg daily. Other plasma NO metabolites (nitrite and nitrate) did not change with ticagrelor loading or during chronic therapy.

Our in vitro data had shown that there was a pH dependent effect on formation of ticagrelor-SNO (137, 257) . However in *vivo* clinical data appears to show no confounding effect of concomitant proton pump inhibitor with ticagrelor on acute RSNO formation., with a consistent elevation in plasma RSNO acutely in both groups (on versus off a PPI). There were however more subtle changes in other NO metabolites with a significant decrease of plasma nitrate

post loading dose in subjects not taking a PPI implying utilization (consumption) of nitrate in this cohort, and this aligns with the increase in RSNO levels in both groups. This data may not mimic the pH dependent effect of RSNO generation seen in vitro as PPI therapy in humans does not produce a consistent rise in stomach pH ; during any 24 hour period the stomach pH on PPI still varies between 2 and 6 (343) (344). Therefore although the patients are taking PPIs the stomach pH is likely to remain at ~pH5, sufficient to support efficient RSNO formation as we demonstrated in vitro (345).

Our data also showed that PPI therapy had no effect on platelet inhibition after a Ticagrelor loading dose by either ADP or thrombin stimulation, despite recently published real life registry data demonstrating a tendency for higher risk of adverse events similar to that in the PLATO trial with concomitant use of Ticagrelor and PPI in ACS patients. However, the impact on clinical outcome was non-significant (346)

The significance of acute elevation in RSNO after Ticagrelor loading doses remains unclear. This ability to generate RSNO is 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 in vivo, it is likely that it becomes available following the breakdown of Ticagrelor in the acidic stomach environment. Typical gastric emptying half-life is 20-40 minutes in patients, Ticagrelor is not protected from this harsh gastric environment by its waxy coating due to its rapid dissolution at all pH levels within 4 minutes(137). This is likely to be the case clinically and is potentially prolonged in patients with delayed gastric emptying, a feature seen in acute myocardial infarction(347). Conversely crushing Ticagrelor in humans would be expected to increase the ability of Ticagrelor to form RSNO in the acidic milieu in the stomach by increasing the availability of drug to proceed with chemical reaction. Indeed this approach has shown to increase the onset of platelet inhibition in patients (within the 1$^{st}$ hour) loaded with Ticagrelor in STEMI (348).

The acute elevation in RSNO levels in CAD patients after Ticagrelor has many potential important consequences. RSNO, and therefore potentially Ticagrelor-SNO, can exhibit platelet anti-aggregatory properties similar to biologically occurring RSNOs like Glutathione-SNO(133). 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(254). Secondly, by acting as a source of NO, thrombin-induced platelet activation is decreased via direct inhibition of PI3K pathway activation by TRAP(255). This could be the likely mechanism for the inhibition of TRAP mediated activation, seen in patients loaded with ticagrelor. The third target involves nitrosation reactions in platelets, specifically protein tyrosine residues of the $COX_1$ enzyme which inhibit the conversion of arachidonic acid to thromboxane-$A_2$(256). 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(349).

Nitroso-vasodilation can also occur with RSNO via NO (or more correctly, $NO^+$) donation which can induce relaxation of vascular smooth muscle, mediated via classic sGC signal transduction. This phenomenon has been previously observed in patients as soon as 2 hours 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 (254, 262).

The demonstration of increased plasma RSNO with Ticagrelor loading may also be of relevance to some of the 'pleiotropic' effects shown recently in the microvascular circulation in patients where it augments adenosine induced microvascular vasodilation compared to placebo – an effect seen with the parent drug rather than the metabolite (350).

Furthermore, in vitro studies show that Ticagrelor can inhibit $P2Y_{12}$-mediated vasoconstriction in small human arteries *(351).*

Once formed, the fate of ticagrelor induced RSNO in vivo is unclear. We have previously shown that clopidogrel derived nitrosothiols can participate in transnitrosation reactions with bovine and human albumin with the potential to shuttle around the human circulation (133). 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. The tissue effects of all RSNOs are largely determined by their ability to release NO, Localised platelet inhibition and nitro-vasodilatation are likely sequelae to nitrosothiols in vivo and this is the first demonstration of a freely available antiplatelet mediated rise in plasma levels seen in CAD patients.

A 180mg Ticagrelor dose results in a consistent and significant rise in plasma RSNO at 2 hours in addition to a profound platelet inhibition in patients with stable CAD. These findings were consistent regardless of concomitant PPI therapy. Elevated levels of RSNO at the time of coronary intervention is likely to be responsible for some of the early putative pleiotropic effects of this medication seen in coronary and peripheral vasculature, in addition to its potent antiplatelet effect. However, these effects are not sustained and therefore may not contribute to the longer-term effects of Ticagrelor.

## 4.4 Limitations to these studies

In addition to the factors mentioned above, several important limitations and mitigating actions are considered.

The measurement of NO metabolites via OBC method utilizing NOA is highly sensitive to atmospheric temperature and the cell pressure changes, which is subject to day-to-day variation. Maintenance of a steady temperature between -15 °C and -18 °C, the cell pressure of 102000 Pa and the supply pressure of 41000 Pa is essential. The baseline value can be altered with variation in these conditions, affecting the reproducibility of experiments. The equipment is kept in an air-conditioned laboratory at a set temperature, with regular checks of gas supply pressures to minimize variation. Daily calibration of the NOA was performed prior to experimentation and is further repeated at the end of the day. An average of the two standards would more precisely account for changes in NOA sensitivity throughout the day (60). Importantly, change in baseline does not affect NOA sensitivity to given NO per se, and given that AUC is measured (as opposed peak height) these limitations can be considered negligible.

There is the potential confounding effect of other additives present within the dietary gels, which could not be evaluated fully. There is evidence suggesting that folate improves NO-dependent vasodilation and reverses endothelial dysfunction *in vivo*. Food preservatives such as benzoate and sorbate inhibit the growth of fungi and certain bacteria (85). Further biochemical tests to assess the pharmacologically active ingredients within the gel like citrate, folic acid, ascorbic acid and their effect on nitrite production would be very useful in future when considering use in patients. Nevertheless, these studies compared all results to a placebo control which was matched precisely.

The in vitro RSNO generation in presence of clopidogrel might have been enhanced if we could increase the amount of nitrite generated from SIS® Go+ nitrate. We were hampered by the lack of availability of commercial sources of NR and this limited the range of this study.

Ours patient studies administering dietary nitrate evaluates the effect on NO metabolites and platelet aggregation at a single time point post ingestion (2hrs). There is a considerable intra- and inter-individual variation and diurnal variation in the circulating nitrate level and platelet reactivity(306). Despite patients being fasted more than 6 hours prior to the study, a residual effect of previous dietary intake might influence the measured metabolites. Therefore, a low nitrate diet for more than 4 days, and then applying a prolonged course of daily administration of nitrate might show a sustained effect on NO metabolites facilitating a greater (statistically significant) platelet inhibition. A strict diet and medication compliance chart would have been ideal for accurate evaluation of results and this was not undertaken. In previous studies we have shown a 12-hr nitrate free diet is sufficient to reduce plasma nitrate variation considerably, and we were keen in the present study not to affect the routine clinical care plan of the patient. Given that the baseline variation in plasma nitrate was not excessive and was in line with previous measures by our group following a nitrate limited diet, we believe this not to be a significant confounder in these studies.

Even though there was a dampening effect of ADP and TRAP mediated aggregation in both groups with dietary nitrate therapy, statistical significance was not achieved. This could have been addressed more suitably if the patients/samples could have been paired, enhancing the power of the study. It was clinically and ethically not possible for achieving a paired sample approach in Group A, as this would mean giving a loading dose of 600mg of clopidogrel given on two separate occasions on two different days in the same individual. A paired sample could have been applied in Group B who are on a regular daily maintenance dose of 75mg of clopidogrel.

A retrospective power analysis shows that in the order of 610 and 170 patients in each arm of Group A (clopidogrel loading) and Group B (clopidogrel 75mg) respectively would be required in order to demonstrate a significant difference of 5% based on the means and variance of ADP induced platelet aggregation we have measured.

Numerous platelet activation markers have been described which could be measured using flow cytometry. A subgroup of patient (clopidogrel naive and Group B (clopidogrel 75mg)) from

my current study had undergone a further evaluation where blood samples were assessed for circulating plasma extracellular vesicles (EV) by Dr Burnley-hall as a part of his PhD work. Dietary nitrate supplementation in group B patients caused a significant reduction in the platelet derived EV, which is likely to be mediated by the concomitant increase in RSNO formation. This decrease in EV was associated with a reduction in platelet activation and platelet marker CD41. This effect was not seen in clopidogrel naïve patients(263). It was not practically feasible to extend these measurements to the other patient groups due to limitation in the time and requirement of additional assistance in running a test simultaneously to prevent sample degradation. This study was solely managed by me with a tight time frame. In retrospect, a measurement of other platelet activation markers such as P-selectin, cyclic GMP would have provided additional information to validate my hypothesis but due to above reasons was not feasible

Although there was a consistent rise in plasma RSNO levels after a loading dose of ticagrelor we have not shown a direct correlation with the degree of platelet inhibition seen in each individual patient. This may be due to the swamping effect by the profound overall platelet inhibition seen with ticagrelor, where the influence of nitrate administration may have been masked. Furthermore, we have not undertaken detailed analysis (such as mass spectrometry) to 100% characterise the species of RSNO produced and whether this is ticagrelor-SNO or other circulating RSNO species.

## 4.5 Future work:

Future in vitro studies should investigate a method to increase the generation of nitrite from concentrated *SIS® Go+ nitrate* gel. A modified experimental apparatus enabling it to hold larger volumes of diluted *SIS® Go+ nitrate* gel can be developed, thereby increasing the total nitrite concentration and promote clopidogrel-SNO formation. Another suggestion is to crush clopidogrel tablets prior to mixing with gastric medium for future work and evaluate the effect of coating on clopidogrel-SNO formation per se.

The beneficial role of dietary nitrate supplementations in cardiovascular disease is well known. Overall, this study shows that dietary nitrate supplementation has the potential to enhance nitrosothiol metabolism and influence NO availability in patients with CAD. This study

measured the effect of nitrate supplement on NO profile and assessed platelet reactivity at two hours post intervention. This study might be extended to include multiple sampling points following the ingestion of nitrate and clopidogrel in these patients to fully understand the exact kinetic interaction of these components. However, it was not practically possible to sample blood at multiple times in this group of patients as they were scheduled for a PCI procedure and would be administered with further anticoagulation as part of their routine clinical care which could confound the results. Similarly, the trend towards augmented platelet inhibition at 2 hours post ingestion of nitrate with or without clopidogrel therapy, could be further explored to establish whether there would be further inhibition with time in CAD patients.

Kapil *et al* had shown the antihypertensive effect of dietary nitrate supplementation upon administration for a prolonged period (4weeks) (237). Expansion of these findings along with our results which showed a trend towards inhibition of platelet aggregation with a single intake of nitrate supplement suggests that long-term intake of regular nitrate supplement might augment platelet inhibition in CAD patients. Thus, future studies might focus on conducting a similar placebo-controlled study to assess the effect of these supplements and clopidogrel therapy in CAD patients for prolonged periods where one might also consider monitoring of dietary intake with specific focus on daily nitrate intake.

This could present a feasible and acceptable therapeutic intervention especially in patients who are on clopidogrel therapy as it is well established that a large proportion of the patients are non-responders. It is crucial they are afforded optimum platelet inhibition for the prevention of adverse cardiac events. Regular intake of dietary nitrate along with clopidogrel could potentially enhance the platelet inhibitory efficacy of clopidogrel through formation of RSNO derivatives, thereby minimising the lack of classic response.

The active concentration of nitrate within the salivary glands following the ingestion of these supplements could be quantified. A simple measure of salivary nitrate concentration and its correlation with the plasma nitrate would provide valuable information on the entero-salivary cycle and its influence on the net amount of circulatory NO metabolites. This would be a very simple and useful test to apply in future studies.

The vasomodulatory effect of dietary nitrate and clopidogrel therapy in CAD patients could be quantified with a quantitative controlled assessment (QCA) of coronary artery diameter using coronary angiographic images and a further simple test of forearm plethysmography and

arterial waveform (these reflecting local coronary versus systemic effects, respectively) Evaluation of coronary vasodilation following these supplements would be an ideal test of its beneficial effects, especially in patients undergoing coronary angioplasty. However, the QCA method of evaluation of coronary size is affected by the image quality and has an inherent subjective bias.

A more comprehensive study of platelet activation to include markers such as EV, cyclic GMP, P-selectin and platelet macrophage aggregates would be useful in fully evaluating the antiplatelet effect of dietary nitrate in CAD patients.

Prasugrel is the most potent antiplatelet of the thienopyridine class of drugs  It has shown a potential for RSNO formation at physiological concentration of nitrite both in-vitro and in vivo (137, 257).  It would very interesting to study the influence of concurrent intake of prasugrel and dietary nitrate supplementation on the NO metabolites and platelet inhibition. There is a possibility of further enhancing the pleiotropic benefits of decreased rates of MI in patients on prasugrel seen in the TRIOLOGY ACS-trial. However, care must be taken because inherent to Prasugrel treatment is the potential risk for harm due to increased risk of bleeding.

The ability of ticagrelor to form RSNO despite lacking an available thiol is an unexpected and fascinating finding. In vitro experiments demonstrated an increase in ticagrelor-SNO formation with an increase in nitrate content. In vivo we observed a significant increase in circulating RSNO following nitrate administration. Therefore, it would be intriguing to study if this effect could be seen in patients when they were given a dietary supplement rich in nitrates.

The laboratory studies clearly demonstrated the influence of pH on the formation of drug-SNO with clopidogrel, prasugrel and ticagrelor. However, in patient studies, there does not appear to be an effect of PPI on RSNO formation in patients given concomitant clopidogrel or ticagrelor. This could be due to multiple reasons as discussed in chapter 4, section 4.3. It would be useful if the actual gastric pH of the patients was measured and this could be correlated with the formation of RSNO in this group. However, it is practically very difficult to ascertain the gastric pH in patients. ACS patients on antiplatelet therapy, who are ventilated and have a nasogastric tube in-situ, could be an ideal group in whom to measure gastric pH and RSNO formation. This would be a very challenging study with practical and ethical issues to overcome.

# 5 Appendix

## 5.1 Appendix I Research 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

**Research Ethics Committee for Wales**
Sixth Floor, Churchill House
17 Churchill Way
Cardiff CF10 2TW

Telephone : 029 2037 6829
Fax : 029 2037 6824
E-mail : corinne.scott@wales.nhs.uk
Website : www.nres.nhs.uk

05 November 2013

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** |
| **Amendment number:** | **letter from Dr Laurence Thornhill, Wales Heart Research Institute** |
| **Amendment date:** | **28 October 2013** |
| **IRAS project ID:** | **102427** |

Thank you for your letter of 28 October 2013, notifying the Committee of the above amendment.

The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

**Documents received**
The documents received were as follows:

| Document | Version | Date |
|---|---|---|
| Notification of a Minor Amendment | letter from Dr Laurence Thornhill, Wales Heart Research Institute | 28 October 2013 |
| Protocol | 4.2 | 07 October 2013 |

**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.

| | |
|---|---|
| **12/WA/0290:** | **Please quote this number on all correspondence** |

GIG NHS Wales | Bwrdd Iechyd Addysgu Powys | Powys Teaching Health Board

Cynhelir Cydweithrediad Gwyddor Iechyd Academaidd y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd gan Fwrdd Addysgu Iechyd Powys

The National Institute for Social Care and Health Research Academic Health Science Collaboration is hosted by Powys Teaching Health Board

Ariennir gan Lywodraeth Cymru | Funded by Welsh Government

Yours sincerely

**Mrs. Helen Williams**
**Coordinator**

E-mail: helen.williams19@wales.nhs.uk

Copy to: Professor Julian Halcox, Cardiff and Vale University Health Board
Dr Laurence V Thornhill, Cardiff and Vale University Health Board

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
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**Research Ethics Committee (REC) for Wales**
Sixth Floor, Churchill House
17 Churchill Way
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E-mail : corinne.scott@wales.nhs.uk

Website : www.nres.nhs.uk

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.

## 5.2 Appendix II 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 chosen 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 are 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 sachet of dietary nitrate supplement product to swallow about 2 hours before you have your stenting procedure. Before giving you

the dietary supplement. 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.

You will be invited to attend a follow up clinic. This will usually be between 1 and 12 months after your coronary stenting procedure. On that day we give you another sachet of supplement, one further blood sample from a vein taken after two hours. All that will be required at this final visit is. 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 and dietary nitrate supplement. 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 other 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 Abdul 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 Fairoz Abdul 029 2074 4192, email fairoz@doctors.org.uk or write to:

Dr. Fairoz B Abdul
Clinical Research Fellow in Cardiology
Wales Heart Research Institute, Heath Park, Cardiff, CF14 4XN

## 5.3 Appendix III-PATIENT CONSENT FORM

**Patient Identification Number for this trial**:

The Effect of Inorganic Nitrate and Antiplatelet Drugs on NO Metabolites and Platelet Reactivity in Patients with Stable Coronary Artery Disease (updated)
Name of researchers: **Dr Philip James, Dr Richard Anderson, Dr Fairoz B Abdul**

Please initial each box

| | | |
|---|---|---|
| 1. | I confirm that I have read and understood the information sheet dated 08/09/2013 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 |

## 5.4 Appendix IV-Patient Data Sheet

| | | | |
|---|---|---|---|
| Research code: | | Hospital ID | |
| Date of birth | | | |
| Date of procedure | | | |
| Nitrate Gel code | | | |
| Next date of App | | | |

Symptoms:

Chest pain ☐ SOB ☐ Palpitation ☐

Cardiac History:

| Medical history | Y/N | Details | |
|---|---|---|---|
| Myocardial infarction | | | |
| Previous PCI | | | |
| Cardiac surgery | | | |
| Diabetes | | Diet ☐ Tablets ☐ Insulin ☐ | |
| Hypertension | | | |
| Hyperlipidaemia | | | |
| Smoker | | Non ☐ Ex ☐ Current ☐ | |
| Thyroid | | | |
| Respiratory problem | | Asthma ☐ COAD ☐ O2 ☐ Inhalers ☐ | |
| CVA/TIA | | | |
| Renal history | | | |
| Other medical history | | | |
| **Medications** | **Y/N** | **Duration** | **Details** |
| Aspirin | | | |
| Clopidogrel | | | |
| Beta blockers | | | |
| ACE inhibitors | | | |
| Statins | | | |
| Nitrates | | | |
| Thyroxin | | | |

| | | | |
|---|---|---|---|
| NSAID | | | |
| PPI | | | |
| Anti-coagulants | | | |

| | | |
|---|---|---|
| Pulse | Pre: | 2 hr. Post: |
| Blood pressure | Pre: | 2 hr. Post: |

## 5.5 Appendix V Patient Quick Reference Guide

**Study Title:** The effect of inorganic nitrate and antiplatelet drugs on no metabolites and platelet reactivity in patients with stable coronary artery disease (updated)

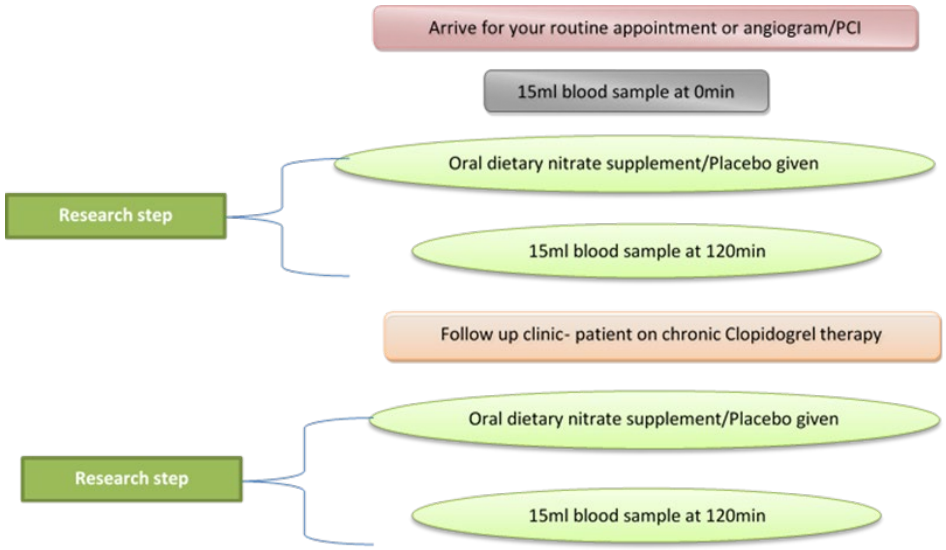
You have been chosen to participate because you will be given a medication called clopidogrel by your heart specialist in addition to your other medication.

Clopidogrel will keep your blood thin and this would improve and maintain good blood flow to your heart, hence reducing a risk of heart attack. This a standard form of treatment given to majority of patients who suffered a heart attack and for all patients who undergo coronary stenting.

Previous studies have shown that patient have variable blood thinning response to this medication. The blood thinning effect of Clopidogrel was found to improve with food containing nitrates.

In Our study we would give you two sachets of Food Product containing nitrate. We will collect your blood sample for measuring the blood thinning effect and additional beneficial effects on the blood vessel wall. We expect an improvement in the effect.

This diagram would illustrate our study plan;



Your participation is entirely voluntary, you could approach the following members of team for any further information:

Dr Fairoz B Abdul: Email: fairoz@doctors.org.uk

Ph No. 07738706675

Dr Richard Anderson: Email: Richard.anderson@wales.nhs.uk

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