The nitrate-nitrite-nitric oxide pathway in humans mechanisms and clinical cardiovascular effects

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The Nitrate-Nitrite-Nitric Oxide Pathway in Humans: Mechanisms and Clinical Cardiovascular Effects

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Dedication

This thesis is dedicated to my wife, Stephanie, for travelling this journey with me and encouraging me every step of the way.

Acknowledgements

I would like to thank all the members of the research teams at King's College Hospital, St Thomas' Hospital, and The James Black Centre, King's College London for their help and support over the course of this research. Additional thanks are due to the cardiac catheterisation lab team at King's College Hospital for facilitating the invasive studies. Modern clinical research is a team effort and cannot be done alone.

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Abstract

Background

Nitric oxide (NO) is a key signalling molecule in the human cardiovascular system. Relatively recently, it has been recognised that, in addition to NO synthesis from L-arginine by NO synthase enzymes, a second major pathway of NO production exists in humans i.e. the inorganic nitrate-nitrite-NO pathway. In the nitrate-nitrite-NO pathway, orally-ingested inorganic nitrate (e.g. as beetroot juice) is sequentially reduced, via nitrite, to NO.

Methods and Results

This thesis comprises a series of mechanistic experimental medicine studies in humans along two key themes. Firstly, I explored the *in vivo* effects of inorganic nitrite on left ventricular and coronary artery function. An additional experiment sought to identify the mechanism of action of nitrite-induced conduit artery dilatation. The key findings of these studies are that: nitrite enhances LV diastolic function through a combination of direct myocardial and indirect effects; nitrite dilates epicardial coronary arteries without a significant effect on flow or coronary resistance; and Na^+/K^--ATPase is not the channel responsible for nitrite-induced conduit artery dilatation.

Secondly, I performed two experimental studies to identify the mechanisms of altering the oral reduction of inorganic nitrate to nitrite, through co-administration of oral inorganic nitrate with grapefruit juice (as a CYP3A4 inhibitor) and through altering oral pH. These studies found that grapefruit juice enhanced the clinical effect of inorganic nitrate, although not via the hypothesised mechanism. Lowering oral pH also increased the clinical effect of inorganic nitrate.

Conclusions

This study has identified a number of important mechanistic findings relating to the nitrate-nitrite-NO pathway.

Firstly, inorganic nitrite has beneficial cardiac and vascular effects whose profile suggest therapeutic potential in heart failure states e.g. hypertensive heart disease, heart failure with preserved ejection fraction (HFpEF). Secondly, the clinical effects of dietary nitrate can be enhanced by altering conditions in the oral cavity and therefore manipulating the nitrate-nitrite-NO pathway. In addition to the clinical relevance of the findings, the studies within this thesis have also generated hypotheses for further work.

Publications arising from this work

Chapter 1

Modelling the complexity of heart failure with preserved ejection fraction.

O'Gallagher K, Shah AM

Cardiovasc Res 2018;114(7):919-921

Chapter 3

Inorganic Nitrite Selectively Dilates Epicardial Coronary Arteries

O'Gallagher K, Khan F, Omar SA, Kalra S, Danson E, Cabaco AR, Martin K, Melikian N, Shah AM, Webb AJ

J Am Coll Cardiol 2018;71(3):363-364

Chapter 4

Direct cardiac versus systemic effects of inorganic nitrite on human left ventricular function

O'Gallagher K, Cabaco A, Ryan M, Roomi A, Dancy L, Melikian N, Chowienczyk P, Webb A, Shah AM

Am J Physiol Heart Circ Physiol 2021; 321(1):H175-H184

Chapter 5

Grapefruit juice enhances the systolic blood pressure-lowering effects of dietary nitrate-containing beetroot juice

O'Gallagher K, Cardona SB, Hill C, Al-Saedi A, Shahed F, Floyd CN, McNeill K, Mills CE, Webb AJ

Br J Clin Pharmacol 2020 Jun 10. Doi 10.1111/bjcp.14420. PMID: 32520418

Prizes and nominations arising from this work

General

South London Cardiovascular Research Network Research Registrar of the Year (2018)

Finalist, Medical Research Council Max Perutz Science Writing Award (2018)

Chapter 4

Finalist, "Best of the Best" Abstract Competition, British Cardiovascular Society Annual Conference 2019.

Chapter 5

- British Journal of Clinical Pharmacology Annual Prize 2020. Awarded for best publication by an Early Career Researcher
- British Heart Foundation Award, 2018. Awarded for best abstract presentation at the 10th International Conference on the Biology, Chemistry and Therapeutic Applications of Nitric Oxide, Oxford, UK.
- Finalist, Young Investigator Prize, British & Irish Hypertension Society, 2018.

Chapter 1. Introduction

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1.1 The nitric oxide signalling cascade and the cardiovascular system

Since the discovery that the Endothelium-Derived Relaxing Factor (EDRF) was nitric oxide (NO)[1, 2], over the last four decades NO has been identified as a key signalling molecule in the cardiovascular system and multiple other organ systems. The importance of the discovery and identification of NO as EDRF is reflected by the award of the 1998 Nobel Prize in Medicine to Furchgott, Murad, and Ignarro[3].

NO is a free radical gas, with a half-life in the circulation of ~20ms[4]. In humans, it is produced via two key mechanisms: the "L-arginine-nitric oxide pathway" as originally described by Moncada[5] and the more recently discovered nitrate-nitrite-NO pathway[6].

The L-arginine-nitric oxide pathway involves the generation of NO via the conversion of L-arginine to L-citrulline by haem- and flavin-containing nitric oxide synthase (NOS) enzymes[7] in a process that requires dimerism of the NOS enzyme and the presence of the co-factors tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH) and O_2[8]. Three NOS isoenzymes have been identified in humans; endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). As will be discussed in detail below, eNOS and nNOS are constitutively expressed and have distinct roles in the regulation of cardiovascular function. By contrast, the role of iNOS is in relation to host response to infection and inflammation. While the production of NO by eNOS and nNOS is Ca^{2+}/calmodulin-dependent, production of NO by iNOS will continue until the enzyme is degraded, resulting in higher local concentrations of NO[9].

The nitrate-nitrite-NO pathway (also known as the enterosalivary pathway) describes the two-step process by which orally ingested inorganic nitrate (NO_3^-) is reduced to NO via inorganic nitrite (NO_2^-). The discovery of the nitrate-nitrite-NO pathway significantly altered the understanding of the role of inorganic nitrate and nitrite in relation to the cardiovascular system. Previously, they were considered to be inert by-products of NO oxidation. However, it is now recognised that dietary nitrate is converted, via nitrite, to NO[10, 11]. Due to the half-life of nitrite being significantly longer than NO in the blood, nitrite therefore represents a circulating pool of potentially bioavailable NO.

NO signalling in the vasculature

NO's role as a signalling molecule in the vasculature occurs primarily in a paracrine fashion i.e. NO produced by NOS (either eNOS in the vascular endothelium or nNOS in nitrergic nerves) diffuses into the vascular smooth muscle cell, whereby it binds to the haem moiety of soluble guanylate cyclase (sGC). This in turn leads to the activation of cyclic guanylate monophosphate (cGMP) and subsequently protein kinase G (PKG), relaxation of the smooth muscle and therefore vasodilatation. Additionally, s-nitrosylation (the NO-mediated modification of cysteine residues in proteins) affords a mechanism by which NO can exert a number of effects, including vasodilatation, in a cGMP independent manner. Examples include NO's effect on calcium-dependent potassium channels in vascular smooth muscle[12] and also the vasodilatory actions of the nitrosothiol S-nitrosoglutathione[13].

In the healthy human vasculature eNOS and nNOS have distinct roles: nNOS is responsible for basal regulation of microvascular tone (and therefore systemic

vascular resistance and peripheral blood pressure[14]), while eNOS is responsible for the dilatory response to increased shear stress or agonist stimulation[15, 16].

There are potential limiting factors to the ability of NO to diffuse from the vascular endothelium to adjacent smooth muscle. Firstly, as noted above, the half-life of NO is approximately 20ms in whole blood, due to NO's status as a reactive oxygen species. This innate reactivity limits NO to diffusion from adjacent cells rather than being transported in blood as free NO. Secondly, NO is avidly scavenged by haemoglobin in both its deoxy- and oxy- haemoglobin forms. The reaction of NO with oxyhaemoglobin to produce methaemoglobin and inorganic nitrate is described below:

Equation 1 $$HbO_2 + NO \rightarrow MetHb + NO_3^-$$

As pointed out by Kim-Shapiro *et al* in their review[17], this presents an apparent paradox: NO is produced by the vascular endothelium in extremely close proximity to a substance (the blood) which would prevent it enacting its role in relaxation of vascular smooth muscle, especially as any NO concentration gradient would, in fact, favour diffusion *away* from the vascular smooth muscle cell and into the bloodstream to be scavenged. Several mechanisms have been described which limit NO scavenging by Hb and therefore ensure that eNOS-derived NO can indeed diffuse from the vascular endothelium into the vascular smooth muscle and initiate relaxation. Firstly, haemoglobin is encapsulated within the erythrocyte, which decreases the magnitude of scavenging when compared with free Hb[18]. Secondly, due to the dynamics of blood flow within the vessel, there exists a "cell-free layer" in the blood immediately adjacent to the vascular endothelium (faster blood flow velocity in the middle of the vessel lumen leads to a pressure gradient within the

vessel and relative concentration of erythrocytes in the middle of the vessel), resulting in a 'barrier' between free NO and erythrocytes. Thirdly, in a moving/mixing fluid (in this case the blood) there exists an 'unstirred layer'[19] around a cell membrane (in this case the erythrocyte membrane) in which there is static fluid i.e. a region of adjacent to a cell membrane when there is low flow, which, like the cell-free layer acts as a barrier to diffusion.

The effect of NO on cardiac function

NO exerts an effect on cardiovascular function in a number of ways. From a vascular point of view, its role in the regulation of systemic arterial haemodynamics[14, 16, 20] affects cardiac loading conditions. NO also regulates cardiac function via a direct myocardial effect, through both paracrine (NO from the coronary endothelium[15]) and autocrine (NOS enzymes expressed within cardiomyocytes[21]) processes.

Endothelial paracrine regulation of left ventricular function

NO's role in the regulation of vascular tone and therefore blood pressure is well-established[22-24]. In addition to this role, NO released from vascular (and endocardial) endothelium exerts a paracrine effect on cardiac function, while NO generated within cardiomyocytes exerts an autocrine effect[25]. The paracrine has been demonstrated in both animal preparations and also in human *in vivo* experiments using substance P (a known stimulator of eNOS-derived NO release)[26]. Disruption of NO signalling can have deleterious effects on cardiac function. In dogs, prolonged NOS inhibition with L-N^G-nitro arginine methyl ester

(L-NAME) resulted in decreased LV compliance and markers of cardiac efficiency[27].

Effects of NO signalling at the myocardial level

In the myocardium, there is subcellular localisation of NOS isoenzymes (eNOS localised to caveolae[28], nNOS to sarcoplasmic reticulum[29]), which allows NO to have varying effects on myocardial function, including contractility[30].

NO stimulation of sGC results in increased levels of intracellular cGMP. cGMP in turn stimulates protein kinase G (PKG). Through PKG-mediated phosphorylation of L-type calcium channels[31] (decreasing calcium influx) and cardiac troponin I (decreasing myofilament calcium sensitivity[32]), NO exerts a negatively inotropic effect. Conversely, in response to stimulation by beta-agonism[33], nNOS-derived NO can exert a positively inotropic effect, via cAMP-mediated protein kinase A (PKA) action on ryanodine receptors and phospholamban, which has the net result of increasing sarcoplasmic reticulum calcium release[30]. In addition to sGC, cGMP is also produced in an NO-independent fashion by a membrane-bound guanylate cyclase isoform known as particulate guanylate cyclase (pGC) which acts as a receptor for natriuretic peptides[34].

Interaction with phosphodiesterase (PDE)

PDE are a family of cyclic nucleotide-hydrolysing enzymes, which play a key role in the regulation of second messenger signalling. Of the 11-member PDE 'superfamily', 7 myocardial PDEs have been reported[35]. cGMP interacts with members of the PDE family in a number of ways. PDE5 hydrolyses cGMP to 5'

GMP, therefore regulating NO-mediated PKG activity by exerting an inhibitor effect on cGMP. PDE5 inhibitors were initially developed as potential therapies for coronary artery disease but are currently established as effective treatments for erectile dysfunction[36, 37] and pulmonary arterial hypertension[38].

At the level of the cardiomyocyte PDE5 inhibition promotes negative inotropic effects. By increasing cGMP levels, PDE5 inhibition promotes increased PKG-mediated phosphorylation of both L-type calcium channels (decreasing calcium influx) and also troponin I (decreasing myofilament sensitivity), thus opposing beta-adrenergic stimulation. In mice, PDE5 inhibition with sildenafil blunts LV hypertrophic remodelling in response to transverse arterial constriction and reverses existing LV hypertrophy, all in the context of increased PKG-1 activity[39].

PDE2 and PDE3 provide a mechanism by which cGMP (and therefore NO signalling), interacts with cAMP (and therefore adrenergic signalling). PDE2 is cGMP stimulated, acting to hydrolyse cAMP, therefore inhibiting its activity[40]. Conversely, PDE3 hydrolysis of cAMP is competitively inhibited by cGMP, by virtue of PDE3 having a higher substrate affinity for cGMP than cAMP.

PDE9 is also expressed in the human heart and, unlike PDE5, does not require NOS activity. The actions of PDE9 therefore represent a pathway by which PDE regulates cGMP in an NO-independent manner. Based on animal data, PDE9 has been identified as a potential target in LV hypertrophy[41].

1.2 The Nitrate-nitrite-NO pathway: Dietary inorganic nitrate as an exogenous source of NO via the intermediary, inorganic nitrite

Metabolic balance studies provided important initial evidence that nitrate is biosynthesised in healthy humans[42] (with increased levels of nitrate biosynthesis in inflammatory states[43, 44]) and that L-arginine, the substrate for NOS, is the precursor in this process[45]. Accordingly, nitrate (and nitrite) were traditionally considered to be inert by-products of NO oxidation. However, it is now recognised that dietary nitrate is converted, via nitrite, to bioavailable NO[10, 11]. The discovery of the nitrate-nitrite-NO pathway significantly altered the understanding of the roles of inorganic nitrate and nitrite in the cardiovascular system.

Orally ingested inorganic nitrate is readily absorbed by the upper gastrointestinal tract with a bioavailability of ~100%[46, 47]. Following oral ingestion of dietary nitrate, peak plasma [nitrate] are seen after ~60 minutes, while nitrate has a half-life of 5 hours in the circulation[48]. Approximately 25% is concentrated in the salivary glands, with the majority of the remainder being excreted via the kidneys[49]. Nitrate in the oral cavity is then reduced to nitrite by anaerobic bacteria on the tongue[50] resulting in a salivary nitrite concentration >1000 times the plasma nitrite concentration[51]. A proportion of the nitrite in swallowed saliva is subsequently converted in the acidic stomach environment in a non-enzymatic fashion to NO i.e. nitrite disproportionation via nitrous oxide and dinitrogen trioxide[52] (which is also a mechanism of NO generation from nitrite in ischaemic tissues, where the local pH is low[53, 54]). Nitrite also enters the circulation due to absorption across the upper gastrointestinal tract[55].

The benefit of having a circulating pool of nitrite is reflected in its stability in whole blood. Nitrite has a half-life of approximately 50 minutes [56, 57] compared to the extremely short half-life of molecular NO (~20ms)[4]. This disparity in half-life therefore allows effective delivery of nitrite to the target tissue, whereby local reduction yields bioactive NO.

The physiological role of the nitrate-nitrite-NO pathway has been demonstrated by human *in vivo* studies. If an oral load of dietary nitrate is ingested, a decrease in blood pressure is seen after approximately 2-3 hours[58]. The importance of the salivary conversion of nitrate to nitrite is demonstrated by the finding that if subjects spit rather than swallow their saliva (and therefore expel salivary nitrate), the rise in plasma nitrite associated with ingestion of dietary nitrate is abolished[51]. The role of nitrate reducing bacteria is demonstrated in studies that have used mouthwash as a mechanism to eliminate the oral microbiome. In healthy volunteers receiving an oral nitrate load, gargling chlorhexidine mouthwash virtually abolished the increase in salivary [nitrite] seen in the control group. An increase in plasma[nitrite] was seen, but of a significantly smaller magnitude than in the control group: +72 ± 23 nmol/l [mean ±SEM] in the chlorhexidine group compared to +286 ± 62 nmol/l in the control group[59]. In the same study, tongue scrapings were taken from a subset of participants. Chlorhexidine mouthwash decreased mean bacterial counts by 80%, a finding that was associated with a significant decrease in nitrate-reducing capacity in incubated samples. Furthermore, the use of chlorhexidine mouthwash over a 7-day period in healthy volunteers resulted not only in interruption of conversion of salivary nitrate to nitrite (as demonstrated by the study above), but also an increase in systolic and diastolic blood pressure (+2.4 ± 0.9 mmHg and +2.2 ±0.8 mmHg

versus baseline mmHg respectively on ambulatory blood pressure monitoring). The change in blood pressure was evident within the first day of mouthwash use and was associated with the decrease in plasma [nitrite] (Δ-71 ± 15 nmol/L versus baseline, a decrease of approximately 25%)[60]. Animal models suggest that chlorhexidine mouthwash has no effect on the blood pressure decreasing effect of orally ingested sodium nitrite[61], thereby isolating the effect to a decrease in bacterial reduction of nitrate to nitrite.

Candidate species for the role of key nitrate-reducing bacteria include: *Veillonella, Actinomyces, Rothia, Staphlococcus, Propionibacterium*[50]. A recent study which utilised full metagenomic analysis of bacteria present in the tongue scrapings of human healthy volunteers also found *Veillonella* to be the most abundant nitrate reducing bacterium, but identified *Prevotella*, *Neisseria* and *Haemophilus* as more abundant than *Actinomyces.[62]*

When different types of mouthwash are compared, chlorhexidine gluconate has the greatest effect on both salivary [nitrite] and blood pressure[63]. Furthermore, chlorhexidine gluconate, but not povidine-iodine mouthwash inhibited the nitrate-reducing species *Varionella dispar* in the human oral cavity (although interestingly in this study, despite inhibition of *Varionella*, saliva [nitrite] did still increase from baseline in the chlorhexidine group)[64].

The symbiotic relationship of the oral microbiota and host human is not unique. The gut microbiota plays an important role in the production of vitamin K, an essential factor in the human clotting cascade[65]. Conversely, eliminating the normal gut

microbiota with broad spectrum antibiotics allows proliferation of pathogenic bacteria such as *Clostridium Difficile*[66]. More recently it has been recognised that the composition of the gut microbiome may have a role in influencing systemic blood pressure[67]. Various animal models have linked gut dysbiosis and/or individual bacterial species to hypertension. In a retrospective population-based study of 6953 Finnish individuals who underwent metagenome sequencing of the gut microbiota, it was demonstrated that hypertensive patients had changes in several genera compared with non-hypertensive patients; *Lactobacilllus* species were negatively associated with dietary sodium intake and blood pressure[68].

Dietary sources of inorganic nitrate

Inorganic nitrate is abundant in a range of vegetables, in particular the green leafy variety. Vegetables classified as high in nitrate i.e. >2mmol per 80g portion include spinach, lettuce, rocket and beetroot[11], while low levels are found in tap water (0.1 mmol/L). One particular food source that has been used extensively in clinical cardiovascular research is concentrated beetroot juice. Commercial preparations exist for use in clinical research that provide a fixed dose of nitrate (Beet-it, James White Drinks Ltd, UK).

Summary

In summary, I have discussed the role of dietary inorganic nitrate as an exogenous source of bioactive NO via the nitrate-nitrite-NO pathway. This pathway represents an attractive therapeutic target and raises important questions, including:

- Does increasing the input (dietary nitrate) result in an increase of bioavailable NO under physiological conditions?
- In patients with cardiovascular pathology related to decreased NO bioavailability, does increasing the input (dietary nitrate) result in amelioration of the cardiovascular pathology commensurate with increased NO bioavailability?
- Is it possible to manipulate stages of the pathway to increase the end-effect? It is this question which is investigated in the work presented in this thesis.

1.3 Inorganic nitrite

As discussed above, in addition to being formed from the reduction of dietary nitrate in the oral cavity, nitrite is also a by-product of NO metabolism: NO is oxidised in cells by cytochrome C oxidase and in blood by caeruloplasmin[69, 70]. Although the physiological effects of nitrite have only been described and recognised relatively recently (see below), the therapeutic potential of nitrite was first recognised in the 9th century AD, with Chinese documents remarking on its ability to relieve "acute heart pains, and cold in the hands and feet."[71]. Approximately 70% of circulating nitrite is derived from the L-arginine NO synthase pathway[72] (with the remaining 30% ingested as dietary nitrate and converted to inorganic nitrite via the nitrate-nitrite-NO pathway[73]).

Production of NO from nitrite by acidic disproportionation

In the acidic conditions of the human stomach nitrite is converted to NO via nitrous acid and nitrogen oxides (see equations 2-4 below):

Equation 2 $NO_2^- + H^+ \rightleftharpoons HNO_2$

Equation 3 $2HNO_2 \rightleftharpoons H_2O + N_2O_3$

Equation 4 $N_2O_3 \rightleftharpoons NO^{\cdot} + NO_2^{\cdot}$

The evolutionary basis for the development of the nitrate-nitrite-NO pathway has been suggested to include the prevention of gastrointestinal infection: concentrations of nitrite similar to physiological levels found in the acidic stomach of healthy humans are sufficient to inhibit the growth of both *Candida Albicans* and *Escherichia Coli*[74]. Indeed, the use of sodium nitrite in food preservation is due to its bacteriocidal action.

Enzymatic reduction of nitrite to NO

A large number of proteins have a role in the reduction of nitrite to NO. In tissues, the predominant method of NO production from nitrite is enzyme-facilitated, with acid disproportionation accounting for only ~20% of NO generation[54]. This process involves the transfer of an electron from the protein to nitrite[75], as shown in the general equation below:

Equation 5

$$NO_2^- + H^+ + Nitrite\ Reductase^{n+} \rightarrow NO + OH^- + Nitrite\ Reductase^{(n+1)+}$$

The proteins that have been demonstrated to perform nitrite reduction include: haem-associated globins (e.g. haemoglobin, myoglobin), mitochondrial enzymes, xanthine oxidoreductase, cytochrome p450, NOS. Conflicting data exists as to whether carbonic anhydrase possesses nitrite-reductase activity (which would potentially

explain the vasodilatory action of the drug) given its lack of redox centre, although it may produce S-nitrosothiols via an anhydrase reaction[76-78].

Strategies to increase local tissue concentrations of NO or to enhance NO-dependent signalling may have therapeutic potential. Inorganic nitrite (NO_2^-) is of interest in this regard as it can be reduced to NO and have effects similar to NO donors. In addition, until recently it has been considered that tolerance does not develop to the effects of nitrite with continued use, unlike the case with organic nitrates[6, 56]. However, this has been called into question with the publication of recent data showing that the blood pressure lowering effect of oral sodium nitrite diminished after 10-12 weeks of therapy, suggestive of the development of tolerance[79].

The Effect of Inorganic Nitrite on the Cardiovascular System – Vasodilatation

Despite data from as early as 1953 demonstrating that high concentrations of inorganic nitrite relax strips of rabbit aorta[80], inorganic nitrite was traditionally considered to be a biologically inert product of NO metabolism.

However, a series of studies at the start of the 21st Century identified nitrite's physiological role, culminating in human studies demonstrating that minimally supra-physiological concentrations of nitrite, when infused into the brachial artery, resulted in a significant increase of blood flow in the forearm skeletal muscle compartment[81]. The vasodilation was only seen with infusion of supraphysiological concentrations of nitrite (>0.1 mmol/L) and only under hypoxic conditions. Nitrite is therefore recognised as a hypoxia-dependent dilator of small resistance arterioles[81] [82].

In conduit arteries however, a paradoxical response is seen[83]: nitrite's action is maximal in normoxia and inhibited by hypoxia.

When given via an intravenous infusion, inorganic nitrite causes vasodilatation and a reduction in central blood pressure[83].

The Effect of Inorganic Nitrite on the Cardiovascular System – Ventricular Haemodynamics

Studies in the isolated rat heart mammalian model have demonstrated a concentration-dependent lusitropic effect of nitrite which is NO and cGMP/PKG-dependent, but NOS-independent[84]. Nitrite has also been shown to induce a concentration-dependent augmentation of the Frank Starling response and an increase in cardiac relaxation, both of which are NOS-independent and cGMP-PKG-dependent[85], similar to effects previously described with NO[86]. In patients with heart failure intravenous sodium nitrite is associated with lower measures of afterload and increased stroke volume and cardiac output[87].

1.3 Inorganic nitrite/dietary nitrate as therapeutic agents

The discovery of the nitrate-nitrite-NO pathway had led to investigations exploring whether exogenous oral (or inhaled, discussed later) inorganic nitrate/nitrite supplementation can be used to effect cardiovascular benefits in both healthy volunteers and patients with cardiovascular disease.

Hypertension

Systemic hypertension is the most prevalent cardiovascular risk factor with an estimated worldwide prevalence of 24% in men and 20% in women[88]. The first randomised evidence that inorganic nitrate decreases blood pressure was provided by Larsen *et al* who gave healthy volunteers sodium nitrate (at a relatively low dose of 0.1 mmol/kg/day for 3 days) and demonstrated a significant decrease in diastolic blood pressure (DBP) versus placebo (sodium chloride)[89]. This concept was extended to dietary inorganic nitrate in the form of beetroot juice: acute dosing of 22 mmol nitrate decreased both systolic blood pressure (SBP) and DBP in healthy volunteers versus placebo (water)[58]. Subsequent meta-analyses have confirmed the effect of nitrate on blood pressure[90, 91].

The finding that dietary nitrate decreases blood pressure in healthy volunteers led to studies exploring nitrate's effect in patients with hypertension. The first such study involved 15 drug-naïve hypertensive patients, who received a single dose of beetroot juice (3.3 mmol) or water in a crossover fashion. Despite the relatively low dose of nitrate compared to the aforementioned study of 22 mmol beetroot juice in healthy volunteers (Webb *et al.* [58]), the average peak systolic blood pressure decrease was 11.2 mmHg[92].

From the same group, Kapil *et al* performed a phase II, randomised, crossover placebo-controlled trial in 68 patients with hypertension (defined as daytime BP >135/85 on 24-hour BP monitoring)[93]. Patients were randomised to either 250 ml nitrate-containing beetroot juice (6.4 mmol) or 250 ml nitrate-depleted beetroot juice per day. In the nitrate-containing beetroot juice arm, blood pressure was significantly decreased (SBP -7.7mmHg, DBP -2.4mmHg), both on clinic and 24-hour blood pressure measurements. This decrease in blood pressure was associated with increases in plasma [nitrate] and [nitrite], as well as an increase in plasma [cGMP]. In addition to the improvement in blood pressure, nitrate-containing beetroot juice was also associated with beneficial changes in vascular function.

Subsequently, several studies have investigated the effects of nitrate on blood pressure in hypertensive patients, with treatment durations ranging from acute effects (2.5 hours) to 1 week[94-97]. These studies provide differing results with some demonstrating a significant blood pressure response and others no significant response. Aside from patient selection issues and methodological considerations (e.g. patients with blood pressure in normotensive range and/or with high circulating [nitrite][94]), it appears that although beetroot juice is effective in decreasing blood pressure in those with uncontrolled hypertension[95, 96], such an effect is not present in patients with hypertension who have better blood pressure control[94, 97].

Regardless of whether clinic blood pressure or ambulatory recordings were made, the above studies relied on measurement of peripheral -rather than central- blood pressure as a study endpoint. This is an important consideration because, in a randomised, double-blind, factorial-design intervention trial in hypertensive patients, beetroot juice was shown to have an effect on central but not peripheral blood pressure[98]; this is consistent with the findings with sodium nitrite described

above[83]. Additionally, independent of any effect on peripheral blood pressure, beetroot juice had significant beneficial effects on LV end-diastolic volume, LV end-systolic volume and LV end-diastolic mass/volume ratio[99], as well as decreasing arterial stiffness[98].

A recent larger scale (n=243) randomised, placebo-controlled trial compared the effect of inorganic nitrate (300 mg /day) versus placebo on blood pressure in patients with high-normal and grade I hypertension [100]. In this study, patients were randomised to either 300 mg green leafy veg, 300 mg potassium nitrate pills, or placebo for 5 weeks. Although treatment with potassium nitrate was associated with a significant decrease in clinic systolic blood pressure and pulse pressure, there was no significant change in the primary endpoint, 24-hour ambulatory blood pressure. This was despite good adherence: in the nitrate-supplemented groups, salivary [nitrate] and [nitrite] were significantly increased compared to placebo. Plasma [nitrite] was increased in the potassium nitrate pills arm, but not in the green leafy vegetable arm (plasma [nitrite] sampling was performed in the morning with the patient in the fasted state).

In summary, there is conflicting evidence from disparate studies for the role of inorganic nitrate as a blood pressure decreasing therapy in hypertension. As with more traditional pharmaceutical drug development, it is only with randomised control trials of large scale and long duration that the role of dietary nitrate supplementation as a treatment for hypertension (or adjunct to other drugs) will be defined. Given that the highest blood pressures are now seen in patients from low-income countries[88], cheap non-pharmaceutical therapies e.g. concentrated beetroot juice as a source of dietary inorganic nitrate are attractive.

Heart Failure with Preserved Ejection Fraction (HFpEF)

In 1984 Dougherty *et al* described a cohort of heart failure patients referred for radionucleotide ventriculography, of whom 36% had a normal ejection fraction (defined in this study as EF >0.45)[101]. The identification of a group of patients with HF but normal/near-normal systolic function therefore formed the basis for the syndrome of HFpEF.

HFpEF is a highly prevalent condition associated with significant morbidity and mortality. It is estimated to be at least as prevalent as heart failure with reduced ejection fraction (HFrEF)[102], accounting for approximately half of heart failure hospital admissions [103]. Mortality is high (23% at 3-years)[104] but there are no proven prognostic therapies[105].

The pathophysiological hallmark of HFpEF is an abnormal rise in left ventricular filling pressures during exercise despite a normal LV ejection fraction (LVEF, a marker of systolic function), leading to exertional breathlessness. However, the pathophysiology of HFpEF is incompletely understood. Traditionally, HFpEF was thought to be due to left ventricular diastolic dysfunction[106-108]. However it has become apparent that, in addition to structural and functional cardiac abnormalities leading to abnormal diastolic function [109], the pathophysiology of HFpEF also involves vascular and endothelial dysfunction, and abnormalities in the complex interaction between the left ventricle and the arterial tree through ventricular-arterial coupling (V-A coupling)[110-112].

One current hypothesis of HFpEF pathophysiology suggests that comorbidities – which are frequently seen in patients with HFpEF – promote systemic inflammation[113]. This results in vascular endothelial dysfunction due to an imbalance between reactive oxygen species (ROS) production and NO

bioavailability. Data from cardiac biopsies supports this theory (decreased PKG activity, decreased [cGMP], increased passive stiffness, abnormalities in titin[114-116]). As such, NO donors have been considered as potential therapies to increase NO bioavailability e.g. the nitric oxide donor sodium nitroprusside has previously been shown to have a beneficial effect on left ventricular distensibility[117].

In a multicentre, randomised, crossover intervention study (NEAT-HFpEF), the effect of daily isosorbide mononitrate (ISMN, an organic nitrate) on patient activity level was assessed. Contrary to hypothesis, ISMN therapy resulted in a deterioration of patient activity level. There were no significant effects on secondary endpoints; 6-minute walk test, quality of life, NT-proBNP[118]. Secondary analysis of data of the TOPCAT trial indicates that the use of organic nitrates in HFpEF is associated with increased risk of major adverse cardiovascular events[119].

Published animal data exists to support the concept of inorganic nitrate/nitrite exerting a cardiac effect. The hearts of mice whose drinking water is supplemented with sodium nitrate demonstrate increased contractility and faster rates of relaxation[120]. Several studies have assessed the effect of inorganic nitrate/nitrite in patients with HFpEF. An intravenous infusion of sodium nitrite has been shown to improve exercise haemodynamics[121], while a single dose of dietary nitrate (beetroot juice) has been shown to increase exercise capacity[122]. A single dose of inhaled sodium nitrite decreases biventricular filling pressures both at rest and during exercise in patients with HFpEF[123]. However, in a double-blind, placebo-controlled, crossover trial of patients with HFpEF (INDIE-HFpEF, n=105), 4 weeks treatment with TDS inhaled sodium nitrite did not result in any significant improvement in exercise capacity (primary endpoint), quality of life, activity levels, ventricular filling pressures, or biomarkers[124].

Using carotid tonometry to measure carotid (and therefore estimate aortic) haemodynamics, oral dietary inorganic nitrate in the form of 12.0mmol nitrate-rich beetroot juice, has been shown to reduce central arterial wave reflections in patients with HFpEF (n=16)[125], with inorganic nitrate inducing a larger effect than that seen with 0.4mg of sublingual GTN (n=26). Additionally, inorganic nitrate, but not GTN, improved the Buckberg index (ratio of diastolic to systolic pressure-time integrals[126]). This suggests that inorganic nitrate, but not GTN, improves the relationship between pulsatile haemodynamics and myocardial oxygen supply:demand matching. Of note, however, although GTN reduced central systolic blood pressure, inorganic nitrate had no significant effect.

The above data highlights several important differences between organic and inorganic nitrates in the context of HFpEF. The ventricular and arterial dysfunction and stiffening seen in HFpEF renders patients susceptible to hypotension in the setting of excessive peripheral vasodilation. Given inorganic nitrate/nitrite's relatively selective action on conduit versus microvascular arteries (and theoretically central over peripheral BP) in normoxia, this confers a potential advantage versus organic nitrate. Furthermore, chronic therapy with organic nitrates induces both rapid tolerance and endothelial dysfunction[127, 128].

There are several limitations of the current evidence base for inorganic nitrite in HFpEF. For example, on a mechanistic level, it is not clear whether systemic delivery of nitrite acts solely via changing cardiac loading conditions or whether there is a direct myocardial effect. Furthermore, on a therapeutic level, although dietary nitrite – in contrast to organic nitrates – has been shown to improve exercise capacity in patients with HFpEF, there is a lack of placebo-controlled data to suggest that the effects seen with acute dosing persist with extended duration of therapies.

Ischaemia-Reperfusion Injury

Ischaemia-Reperfusion Injury (IRI) describes the phenomenon of cell death resulting from reperfusion of ischaemic tissue. Pertaining to the heart, IRI is defined as "myocardial injury caused by the restoration of coronary blood flow after an ischaemic episode[129]." One aspect in the pathophysiology of cardiac IRI is the generation of oxidative stress, which mediates myocardial injury[130]. Increased levels of superoxide in the setting of IRI acts to decrease the bioavailability of NO. In animal models, increasing local NO bioavailability results in a decrease in infarct size[131]. Initial clinical evidence suggested that nitrite is protective in human models of IRI[132]. In a randomised placebo-controlled trial in 80 patients with acute STEMI, preconditioning with intracoronary nitrite prior to restoration of coronary flow reduced infarct size, albeit only in patients with TIMI 1 or 0 flow[133]. However, in a larger, multicentre, placebo-controlled trial assessing the effect of intracoronary nitrite on infarct size in acute STEMI (n=229), no significant effect on infarct size was seen [134].

The relationship between nitrite and remote ischaemic preconditioning (RIPC) has been studied in both animals and humans and may provide insights as to why RIPC clinical trials have not been successful e.g. RIPC in cardiac surgery[135]. Webb *et al*. demonstrated that plasma [nitrite] is decreased during the reperfusion phase following RIPC, both locally and systemically[136].

Pulmonary Hypertension

Along with the prostacyclin and endothelin pathways, NO signalling is a key therapeutic target in pulmonary arterial hypertension (PAH). When NO is delivered

via inhalation, it selectively dilates the pulmonary vasculature, decreasing pulmonary arterial pressure[137]. These selective effects of inhaled NO on pulmonary artery dilatation are also seen in human acute respiratory distress syndrome[138]. However, a Cochrane review of published data reports that although inhaled NO improves oxygenation, it has no effect on mortality and is associated with an increased incidence of renal failure[139].

Attention in recent years has therefore shifted to downstream NO signalling targets. Phosphodiesterase inhibitors (PDEi) block the degradation of cGMP, thereby increasing local concentrations. Compared with placebo, in patients with PAH, sildenafil increases exercise capacity, functional class and pulmonary haemodynamics[38]. Another class of drug acting on the NO signalling pathway, the sGC stimulators, also have clinical benefit in PAH[140].

Cerebral vasospasm following Sub-arachnoid haemorrhage (SAH)

Cerebral vasospasm is a potentially fatal complication affecting approximately 15% of cases of intracranial aneurysm rupture[141]. It is thought that, following SAH there is decreased NO bioavailability in the local cerebral artery territory, due to a combination of decreased NOS activity[142-144] and scavenging of NO by free haemoglobin. Both of these mechanisms can be attributed at least in part to the action of oxyhaemoglobin released from the SAH. In a primate model of cerebral vasospasm, intravenous sodium nitrite increased the concentration of nitrite in the cerebrospinal fluid, which was associated with successful prevention[145] and reversal[146]. Subsequently, safety and feasibility of nitrite infusions have been demonstrated in a phase IIa study in SAH[147], although no phase III studies have been published or registered on clinicaltrials.gov.

1.4 Limitations of inorganic nitrate and nitrite

Over and above the neutral/negative RCT evidence listed above, several limitations of inorganic nitrate/nitrite therapy must be considered.

Firstly, there are safety considerations, which can broadly be categorised as either acute side-effects or concerns regarding long-term risk of malignancy. Acute side-effects of inorganic nitrate/nitrite therapy depend on the mode of delivery. Dietary nitrate in the form of beetroot juice may be associated with discolouration of the urine or stool[58], but is not associated with the side-effects commonly seen with preparations of organic nitrate (e.g. isosorbide mononitrate), such as headache and dizziness. Although inorganic nitrate has not been demonstrated to cause significant hypotension[58], this can be seen with intravenous preparations of inorganic nitrite.
Methaemoglobinaemia is a possible adverse effect of nitrate/nitrite therapy. Methaemoglobin is formed when haemoglobin undergoes oxidation to methaemoglobin, which cannot carry oxygen[148]. This is only clinically significant when methaemoglobin levels exceed 5-10%, which has not been demonstrated with the doses to be used in the studies reported on in this document.
Levels of nitrite or nitrate levels in a standard diet are not typically of a level to have any acute effects at all. However, a recent published case report highlights sodium nitrite poisoning in three family members following consumption of homemade sausages with excessively high sodium nitrite levels (3.5g/kg), associated with methaemoglobin levels of up to 34%. Of the three affected individuals, there was one death[149].

Secondly, there have been concerns regarding risk of malignancy from inorganic nitrates. Nitrates/nitrite (in addition to salt) have been used for many centuries as a food preservative in the process of curing (and processing) meats. Nitrite has both an antibacterial role (acting against *Clostridium Botulinum*) and also a cosmetic one (oxidising myoglobin in the meat to provide the colouration that is considered characteristic for red meat). There has, however, been concern that inorganic nitrate/nitrite could be associated with gastrointestinal malignancy[150, 151], although definitive data is absent[152]. Indeed, some evidence exists to suggest an inverse relationship between inorganic nitrate consumption and the risk of gastrointestinal malignancy[153]. The explanation for such a polarisation of effect of nitrate consumption appears to be due to the competing effects of dietary nitrate found in green leafy vegetables (beneficial, protective) versus the nitrate/nitrite added as a food preservative (likely harmful). This is reflected in the guidance from the International Agency for Research on Cancer (IARC) guidance that it is nitrate/nitrite consumed under "*conditions that result in endogenous nitrosation*" that is probably carcinogenic to humans, rather than dietary nitrate/nitrite in themselves. Furthermore, it has been suggested that the gastric production of NO has a protective effect against various gut pathogens[74].

Thirdly, as discussed in detail in this introduction, the main mechanism of action of inorganic nitrate and nitrite is considered to be through increasing NO bioavailability, acting via soluble guanylate cyclase (sGC). sGC is a key component in NO signal transduction, generating cyclic guanosine monophosphate (cGMP) in the presence of NO stimulation. Vascular disease, e.g. hypertension, is associated with decreased expression and activity of sGC in rats [154-156]. sGC is itself under redox regulation[157] and exists in both reduced, haem-containing and oxidised,

haem-free forms. Haem-free sGC is unresponsive to NO. In the setting of oxidative stress, free radicals such as peroxynitrite oxidise sGC, resulting in an increased proportion of oxidised, haem-free sGC. A concept has therefore emerged which hypothesizes pathological NO resistance within cardiovascular disease states, in which oxidative stress results not only in free radical-induced decrease in NO bioavailability, but also in peroxynitrite-induced decrease in sGC function via an increased proportion of NO-unresponsive oxidised sGC[158]. As inorganic nitrate/nitrite are upstream of sGC in the NO signalling cascade, in the setting of NO resistance, their ability to effect NO-mediated responses would be limited.

Finally, both sodium nitrate and sodium nitrite are associated with side effects. When given orally, over 10% of patients taking sodium nitrate experience gastrointestinal side effects (nausea, vomiting, abdominal cramps)[159]. Headache and dizziness are the side effects most frequently attributed to sodium nitrite[160].

1.5 Summary

Our understanding of NO biology in humans has evolved from considering inorganic nitrate and nitrite as simple by-products of NO oxidation to the current position whereby the nitrate-nitrite-NO pathway is considered as a key physiological pathway with a role in regulating blood pressure. Accordingly, both dietary nitrate and nitrite are considered to have therapeutic potential in a range of conditions in which decreased NO bioavailability is seen e.g. hypertension, HFpEF.

Key lines of evidence that are still lacking include whether inorganic nitrate and nitrite decrease LV filling pressures through systemic effects (decreasing afterload and/or preload) and whether there is a direct myocardial relaxant effect. In the arterial system, nitrite is selective for conduit arteries over microvascular arterioles

in normoxic conditions. However, although nitrite's mechanism of action in the microvasculature is well established, this is not true for conduit vessels. Achieving a clearer understanding of nitrite's actions in conduit vessels and the heart is important for understanding nitrite's therapeutic potential in HFpEF, a HF syndrome in which central arterial (conduit artery) and myocardial stiffening are pathological hallmarks. A key question regarding the therapeutic potential of inorganic nitrate is whether the clinical effects of dietary nitrate can be enhanced through measures other than increasing the dose. For example, is it possible to increase the oral conversion of nitrate to nitrite?

1.6 Hypotheses & Aims

Hypothesis relating to inorganic nitrite

The hypothesis underpinning the inorganic nitrite studies in this thesis is that, in addition to its actions on conduit arteries (which are cGMP-independent), nitrite exerts a direct myocardial effect.

Hypothesis relating to dietary nitrate and the nitrate-nitrite-NO pathway

The hypothesis underpinning the dietary nitrate studies is that the conversion of inorganic nitrate to nitrite can be manipulated through intervening on physiological redox mechanisms.

Aims:

1. To investigate the mechanism of action of inorganic nitrite conduit artery dilatation.
2. To investigate the physiological effects of inorganic nitrite on coronary arteries and on left ventricular function.
3. To investigate the mechanisms that surround the conversion of salivary nitrate to nitrite in the human oral cavity.

Chapter 2. Methods

2.1 Ethical Approval

All studies were approved by the relevant local NHS Research and Ethics Committee (REC) and by the Trust R&D Department. The following REC references apply:

- Chapter 3. The effect of inorganic nitrite on conduit arteries. 11/H0802/4: Regulation of vascular tone in conduit and resistance vasculature. 12/LO/1066: An investigation of the effects of nitrite on coronary arteries.
- Chapter 4. The effect of inorganic nitrite on left ventricular function in the human heart. 12/LO/1067: An investigation into the effects of sodium nitrite on cardiac function in patients undergoing cardiac catheterisation
- Chapter 5: Enhanced blood pressure-lowering effects of a beetroot juice and grapefruit juice cocktail compared to beetroot juice alone: a randomised cross-over study. 10/H0802/52: Relationship between nitrate/nitrite handling and glucose tolerance, Grapefruit and Dietary Nitrate sub-study
- Chapter 6. The effect of altering oral pH on bioactivation of dietary inorganic nitrate. 10/H0802/52: Relationship between nitrate/nitrite handling and glucose tolerance, Chewing gum sub-study.

2.2 Participant Recruitment

For the studies "The effect of inorganic nitrite on conduit arteries, study 1: Intracoronary nitrite study" (**Chapter 3**) and "The effect of inorganic nitrite on left ventricular function in the human heart" (**Chapter 4**), participants were patients presenting for coronary angiography to exclude coronary artery disease. Patients were therefore approached for inclusion in the study in advance of their procedure. Exclusion criteria for both studies were:

- Significant coronary stenosis, either by angiographic estimation or by invasive assessment (Fractional Flow Reserve[161])
- Impaired left ventricular systolic function
- Hypotension at baseline (systolic BP <90mmHg)
- Glucose-6-phosphatase deficiency
- Inability to consent for themselves.

In addition, the study "The effect of inorganic nitrite on conduit arteries, study 1: Intracoronary nitrite study" (**Chapter 3**) excluded patients with a coronary vessel of interest of less than 2mm diameter and/or who had previously undergone percutaneous coronary intervention (PCI).

Healthy volunteers for the studies in **Chapters 3, 5** and **6** were recruited through approved internal King's College London advertisements (the KCL Fortnightly circular: Research Volunteer Recruitment) administered by the King's College London Department of Research Governance, Ethics and Integrity (rec@kcl.ac.uk).

2.3 Sodium nitrite infusions

Intracoronary infusions

Intracoronary infusions of sodium nitrite were delivered via a guiding catheter seated in the left main coronary ostium. For the intracoronary infusions in **Chapter 3 and 4**, two doses of intracoronary nitrite were used: 8.7 μmol/min and 26 μmol/min.

Intravenous infusions

In **Chapter 4**, a 7 minute infusion of sodium nitrite, at a dose of 50 μg/kg/min (as in Borlaug *at al*[121]) is used: this dose demonstrated significant effects on cardiovascular function. Prolonged intravenous infusions of sodium nitrite of up to

48 hours have been demonstrated to be safe[162], with a half-life of 23-40 minutes[55].

Intrabrachial infusions

For the intrabrachial infusions in **Chapter 3** a single dose of 2.6 μmol/min inorganic nitrite is used.

2.4 Pressure volume loop acquisition

When a continuous recording of left ventricular (LV) pressure is plotted against LV volume a pressure-volume (PV) loop is acquired. PV loop analysis is considered the "gold-standard" of assessing left ventricular function, due to its ability to derive relatively load-independent variables of function[163]. Early animal experimental models involved the placement of a pressure transducer through the apex of the left ventricle to measure instantaneous LV pressure[163]. LV volume was measured with a cardiometer i.e. an experimental set-up in which the heart is enclosed within an airtight chamber, which is connected to a pressure transducer. Changes in the air pressure within the chamber therefore reflect changes in LV volume.

In human experimental protocols the conductance catheter technique is employed[164]: a micromanometer-conductance catheter is inserted into the arterial tree via a percutaneous puncture and retrogradely advanced to the aortic root and then into the LV across the aortic valve. Typically, the puncture site is at either the radial or common femoral artery, the two most common sites of arterial access in coronary angiography and/or percutaneous coronary intervention.

In the PV studies in **Chapter 4**, we used a commercially available micromanometer-conductance catheter (CD Leycom, Netherlands). The catheter consists of a solid-

state micromanometer designed to sit in the mid-ventricle to obtain instantaneous LV pressure recordings. The catheter has 12 electrodes spaced equally apart to allow acquisition of volume data (by the conductance method as discussed below). A pigtail configuration at the distal tip allows the catheter to be placed in the LV in an atraumatic manner with the distal tip in the LV apex. The catheter is radiopaque, therefore allowing visualisation of the catheter position using fluoroscopy. Two sizes of catheter are commercially available: 4Fr (no lumen) and 7Fr (with lumen). Both sizes can be advanced across the aortic valve into the LV using standard manual catheter manipulation techniques, however the lumen allows the 7Fr catheter to be "railroaded" over a 0.025in exchange-length guidewire placed in the LV apex. Data acquisition and offline analysis are performed via the Intra-Cardiac Analyser (INCA) console (CD Leycom, Netherlands).

Principles of conductance catheter technique for volumetric assessment

The conductance catheter technique relies on electrical conductance of blood within the LV. The time-varying electrical conductance of blood within the LV follows an (approximately) linear relationship with the volume of blood in the chamber. Segmental conductance is assessed by measuring the conductance between pairs of equally spaced electrodes along the portion of the conductance catheter that is within the left ventricle. The estimates for segmental conductance are then summed to give an estimate of total volume of blood in the ventricle. Important considerations of this technique include:

- As the technique relies on measuring segmental conductance between pairs of electrodes, the volume of blood distal to the most distal electrode at the apex is excluded from the LV volume estimation.

- The sum of the segmental conductance measurements produces a total volume based on a "stacked cylinder" shaped model, compared to the spherical actual shape of the ventricle. This is corrected for by the slope factor, α, which represents the slope of the relation between conductance-derived and true volume. The slop factor is obtained by comparing the conductance-derived volume with an independent volume measure e.g. 3 dimensional echo, as is performed in all of our patients.
- The conductance catheter should be aligned along the longitudinal axis of the LV. Any alteration to this configuration in practice e.g. due to the angle of the ascending aorta in relation to the LV, has the potential to decrease the accuracy of the volume estimation.
- In the PV studies in **Chapter 4**, we have not accounted for parallel conductance i.e. electrical conductance due to structures other than the volume of blood within the LV (the LV myocardium, the blood within the RV, the lungs). Because these structures also conduct electricity, the electrical conductance being measured by the conductance catheter is not restricted solely to the blood. Parallel conductance can therefore lead to an overestimation of the blood pool and therefore of the LV volume. It is possible to adjust for parallel conductance by measuring the change in conductance induced by the injection of a hypertonic saline bolus through a balloon flotation catheter within the pulmonary artery[164, 165]. The small amount of hypertonic saline changes the conductance of the blood pool without significantly changing the volume or pressure. As a result of this change in conductance, there will be an apparent increase in LV volume (without a change in actual LV volume) following hypertonic saline bolus.

The offset between the baseline volume and the volume measurement following hypertonic saline bolus therefore allows estimation of the effect of parallel conductance on measured LV volume and calibration of the software to account for it. Rather than performing hypertonic saline injection (which would require an additional venous puncture to allow placement of a pulmonary artery catheter, we elected to calibrate for LV volume by ensuring that patients have a 3D transthoracic echocardiogram on the day of the study to measure LV volumes and therefore allow for off-line volume calibration[166]. We have therefore sought to reach a compromise between the relative accuracy of the technique with its less invasive nature (one fewer vascular access required).

The standard dataset acquired through PV analysis includes indices of LV systolic and diastolic function, as described below:

Indices of diastolic function:

- End-diastolic pressure volume relationship (EDPVR).

  The EDPVR represents the passive filling of the LV during diastole and is considered to be a load-independent marker. In contrast to the linear nature of the ESPVR, the EDPVR is curvilinear i.e. the increase in EDP is higher for a given EDV. Calculating its slope provides a measure of LV stiffness (and the inverse of the slope a measure of compliance). Due to the fact the EDPVR describes passive LV filling, its determinants are primarily those relating to LV structure i.e. the cardiomyocytes (size, mass and organisation, sarcomere structure) and extracellular matrix (ECM). The EDPVR will therefore be altered by any disease process that causes abnormalities in

cardiomyocytes and/or the ECM e.g. ischaemia, fibrosis, hypertrophy. In HFpEF, the EDPVR is shifted upward and to the right i.e. a higher EDP for any given EDV reflecting decreased compliance (the inverse of stiffness) in the HFpEF LV.

In our PV studies, we employ the single-beat method of estimation of EDPVR[167]. It is important to note that, when using the single-beat method of PV loop acquisition, it is not possible to describe the end-diastolic pressure volume relationship (EDPVR) in full detail, for a number of reasons. Firstly, because the EDPVR is curvilinear, deliberate acute changes in preload e.g. balloon inflation/deflation within the inferior vena cava are required to fully describe this relationship. Secondly, the single-beat method ignores any external forces on the ventricle and their influence on diastolic function e.g. pericardial constriction[110, 168]

- LV Electro-systolic time (LVEST, also referred to as "time to end-systole"). Calculated as the time from the onset of the first deflection of the QRS complex on the ECG to the point of dP/dt_{min}.
- Tau and dP/dt_{min}. Tau represents the time constant of pressure decay during isovolumic relaxation and is therefore a measure of lusitropy (rate of relaxation). Isovolumic relaxation is an active process, requiring ATP to uncouple cross-bridges. Tau and dP/dt_{min} can therefore be considered markers of active relaxation. dP/dt_{min}, the maximum rate of pressure decay is heavily load dependent.

Indices of systolic function

- End-systolic pressure-volume relationship (ESPVR). Unlike the EDPVR, the ESPVR is considered to be much more of a linear relationship. The ESPVR is defined by the relationship:

  Equation 6 $$P_{es}=E_{es}(V_{es}-V_0)$$

  Where P_{es} is the end-systolic pressure, V_{es} is the end-systolic volume. E_{es} is the end-systolic elastance, equivalent to the slope of the ESPVR. V_0 is the theoretical volume in the LV at the x axis intercept i.e. an LV pressure of 0 mmHg.

- End-systolic elastance (E_{es}). In a canine model, E_{es} has been shown to be independent of changes in preload and afterload[163]. In that same model E_{es} was shown to increase with positively inotropic drugs e.g. catecholamines and is therefore considered to be a (load-independent) marker of LV contractility. E_{es} increases with heart rate (the force-frequency relationship)[169], however the relationship between increase in heart rate and increase in E_{es} is not in itself linear. In the canine model, E_{es} increases significantly with an increase in heart rate from 60-120, then plateaus between 120-180, with further significant increase as the heart rate increased above 180 (however the changes seen with heart rate >180 was accompanied with changes in V_0, which had hitherto been relatively constant).

- dP/dt_{max}, the maximal rate of change of LV pressure is used as a marker of LV systolic function. It is load-dependent and therefore not a true marker of underlying myocardial contractility; a change in preload exerts a greater effect on dP/dt_{max} than an equivalent change in afterload[170]. In the original human experiments assessing the effect of loading conditions on dP/dt_{max}, it

was found that the percentage increase in dP/dt_{max} from an increase in preload was similar to the percentage increase in end-diastolic circumference[171]. Changes in preload can therefore be accounted for by correcting changes in dP/dt_{max} for changes in LV end-diastolic volume ($dP/dt_{max}/EDV$), also known as the Starling-Contractile Index (SCI).

- Stroke Work represents the area within the pressure volume loop and is calculated as the product of stroke volume and the peak LV systolic pressure. Stroke work, like dP/dt_{max} is dependent on preload, with a linear relationship between stroke work and end-diastolic volume[172], therefore preload recruitable stroke work can be calculated by normalising the stroke work to the LV end-diastolic volume.

Indices of afterload

The term 'afterload' represents the mechanical load against which the left ventricle must pump to ensure forward flow of blood in the arterial system. Assuming a structurally normal and well-functioning aortic valve, the mechanical load which the LV pump must overcome is that imposed by the arterial tree i.e. LV afterload is arterial load. Afterload is not, however, simply a measure of the pressure against which the LV must pump. Blood flow is an important consideration in afterload. For example, two patients with identical blood *pressures* may have completely different patterns of afterload due to difference in LV function and/or aortic size and therefore differences in blood *flow*.

It is important to consider afterload not as a single 'lumped' load on the ventricle, but something that varies with timing of the cardiac cycle. This loading sequence is

affected by arterial wave reflections from points of bifurcation in the vasculature. In the healthy cardiovascular system, with compliant conduit vessels, arterial wave reflections occur late in systole, with the reflected wave arriving back at the heart in diastole (increasing coronary perfusion). However, in patients with stiffened arteries, reflected waves are transmitted back to the heart at greater speed, tending to arrive at the heart in late systole, therefore both increasing the mechanical load on the heart and also altering the 'loading sequence' from the norm. Determinants of afterload include the resistive load (systemic vascular resistance, determined by resistance arterioles) and pulsatile load.

There are various measures of afterload, each of which measure and represent different aspects of afterload. Total peripheral resistance (TPR, also known as Systemic vascular resistance (SVR)) and total/systemic vascular resistance index (TVR/SVR corrected for body surface area) estimate lumped, non-pulsatile arterial load. This is reflected by the use of mean arterial pressure (MAP) in the calculation of these indices. As a marker of blood pressure, MAP disregards systolic and diastolic changes in blood pressure and therefore ignores any pulsatile element.

To take into account the pulsatile nature of afterload, one must incorporate intra-beat changes in blood pressure. The simplest such measure is pulse pressure:

Equation 7

$$Pulse\ pressure = Systolic\ blood\ pressure - Diastolic\ Blood\ Pressure$$

Pulse pressure depends not only on stroke volume (forward flow of blood) but also on aortic stiffness (restriction to forward flow of blood) and arterial wave reflections

(backward reflected waves). In general, the higher the PP, the higher the pulsatile afterload.

Total Peripheral Resistance (TPR)

TPR is largely a measure of systemic microvascular resistance. It can be calculated using the following equation:

Equation 8

$$Total\ Peripheral\ Resistance = \frac{Mean\ Arterial\ Pressure}{Cardiac\ Output}$$

Total Arterial Compliance (TAC)

As the name suggests, TAC is a measure of the compliance within the entire arterial tree, including both the large conduit vessels and the peripheral resistance arterioles. Under normal conditions, the arterial system acts as a reservoir for blood during systole, ensuring that an excessive pressure rise is avoided. Abnormalities in TAC have been shown to have adverse effects on both cardiac function and on long-term cardiovascular risk[173]. Data from animal models suggest that the proximal aorta accounts for 50-60% of total compliance in the arterial tree[174, 175].

The term compliance, the antonym of stiffness, represents the change in volume seen with a change in pressure:

Equation 9

$$Compliance = \frac{\Delta volume}{\Delta pressure}$$

TAC cannot be measured directly but is instead estimated through the relationship of central pulse pressure to cardiac output. There are three main

methods of estimating TAC, which show good correlation with each other (however values derived from the pulse pressure method are significantly lower)[176]:

- The Area Method

TAC is estimated from the area under the diastolic pressure wave.

- The Pulse Pressure Method (PPM)
- Ratio of Stroke Volume to Pulse Pressure (SV/PP)[177]

Utilising the patient's body surface area data, we can also substitute the stroke volume index (SVI) for SV, to give an estimation of the Total Arterial Compliance Index (TACI).

Effective Arterial Elastance (E_A*)*

Derived from data collected in the pressure-volume plan, E_A has been proposed as a marker of lumped resistive and pulsatile load on the LV. E_A can be represented as the ratio of End-systolic pressure (ESP) to stroke volume (SV).

Equation 10

$$Effective\ Arterial\ Elastance = \frac{End\ Systolic\ Pressure}{Stroke\ Volume}$$

E_A can also be estimated from the following equation:

Equation 11

$$E_A = \frac{R_c + R}{\left\{t_s + \tau\left[1 - \exp\left(-\frac{t^d}{\tau}\right)\right]\right\}}$$

Where R_c=Characteristic impedence, R=Arterial resistance, t_s=Systolic time, τ=Time constant of diastolic pressure decay, t^d=Diastolic time. However, this equation makes approximations regarding the area under the ventricular pressure curve[178].

The advantage of E_A lies in its simplicity. However, E_A has several disadvantages. In the first instance, because its calculation involves ESP as the only pressure measurement, it ignores the pulsatile nature of afterload, as demonstrated in human studies[179] and therefore also ignores the LV loading sequence. Furthermore, E_A is greatly dependent on heart rate and vascular resistance, with the latter determined mainly by small resistance arteries

Preload: Left Ventricular End Diastolic Pressure

In **Chapter 4**, the left ventricular end-diastolic pressure (LVEDP) is used as a primary outcome measure to assess the effect of inorganic nitrite on the LV, when delivered via either the intravenous or intracoronary route. LVEDP describes the pressure within the LV at the end of diastole i.e. just before left ventricular isovolumic contraction occurs at the onset of systole. This is consistent with the instant before the first deflection of the QRS complex. LVEDP thus represents a direct measure of LV filling pressures, in contrast to the pulmonary capillary wedge pressure (PCWP), which is only an indirect measure of LV filling pressures. In patients with pulmonary hypertension, PCWP has been shown to correlate poorly with LVEDP, providing only moderate discrimination between normal and high LVEDP[180].

2.5 First Phase Ejection Fraction (EF1)

EF1 is the proportion of blood ejected from the ventricular cavity from the opening of the aortic valve to the first peak of aortic flow. EF1 is calculated by the following equation:

Equation 12

$$EF1 = \frac{LVEDV - LV\ volume\ at\ time\ of\ peak\ aortic\ flow}{LVEDV} \times 100$$

Where LVEDV is left ventricular end-diastolic volume.

EF1 therefore differs from LVEF in that it records only the fraction of blood ejected until the point of maximal ventricular contraction (i.e. the first peak of aortic flow), rather than the fraction of blood ejected throughout the whole of systole.

It is well established that there can be significant abnormality in LV systolic function, whilst the LVEF remains "normal" i.e. LVEF >55%. For example, in HFpEF, although impaired myocardial systolic function is demonstrated by markers such as LV strain, the LVEF is by definition normal. Therefore, in such a condition, there is a clear need for a biomarker to identify abnormalities in systolic function to which LVEF is blind.

EF1 represents the fraction of blood ejected from the LV from the onset of systole to the point of maximal aortic flow, which in turn represents the point of maximal ventricular contraction and therefore maximal velocity of myocardial fibre shortening. Maximal myocardial fibre shortening is important in systo-diastolic coupling due to the concept of "shortening deactivation" which describes the phenomenon whereby myocyte shortening leads to smaller and more rapid muscle contraction compared to the isometric condition. On the cellular level, it has been proposed that sarcomeric length-dependent decreases in myofibrillar calcium sensitivity[181] and velocity-dependent decrease in myosin cross bridge attachment[182] (through mechanosensing[183]) are important mechanisms.

In the human heart, a rapid decrease in myocardial wall stress is seen at/around the point of maximal aortic flow (and therefore maximal velocity of myocardial fibre shortening) that occurs early in systole[184]. The early systolic peak and prompt relaxation of myocardial wall stress may facilitate effective diastolic relaxation. Abnormalities such as an increase in afterload can lead to a delay in the point of maximal fibre shortening velocity and therefore lead to a longer duration of sustained myocardial wall stress and therefore impaired cardiac relaxation. Therefore, although the overall fraction of blood ejected from the ventricle during each systole is within normal limits, the pattern of changes in flow and myocardial wall stress may be grossly abnormal and can lead to effects on the following diastole.

In patients with hypertension, those with echocardiographic evidence of diastolic dysfunction had significantly lower EF1 compared to those with normal diastolic function, even though LVEF was similar between groups[185]. In patients with aortic stenosis, EF1 identifies those with poor prognosis[186] and also predicts need for valve replacement[187].

EF1 is easily recorded, requiring echocardiographic images of left ventricular volume and continuous wave doppler flow across the aortic valve, both obtained throughout the cardiac cycle. Current published data suggests that it is possible to determine EF1 from ~90% of patients[186, 187].

2.6 Measurement of coronary artery diameter by Quantitative Coronary Analysis

Quantitative Coronary Analysis (QCA) is a method for measurement of coronary artery diameter from angiographic images. Coronary artery diameter is measured offline using an automated edge detection system (Philips). An area of interest (approximately 2.5-5mm in length) is chosen. The automated edge detection system provides a mean vessel diameter for the area of interest, using the diameter of the guiding catheter as a reference measurement. The Image Intensifier angle of projection is kept constant throughout the study protocol, as is the position of the Doppler wire.

In **Chapter 3**, QCA measurement is combined with coronary blood flow (CBF) assessment. In such studies, the area of interest is approximately 5mm distal to the tip of the Doppler wire. Positioning in this manner provides a balance between avoiding the intracoronary Doppler wire being erroneously identified by the edge-detecting software as the vessel wall and ensuring that the area of interest is anatomically approximate to where flow is being measured. This is important when measures of flow and diameter are used in estimations of coronary artery resistance.

2.7 Measurement of coronary artery blood flow by Doppler Analysis

CBF measurement is performed using a 0.014 inch intracoronary Doppler wire (FloWire, Volcano therapeutics, USA)[188]. The Doppler wire is introduced through a guiding catheter into the proximal portion of the vessel of interest; a straight segment of the vessel, away from bifurcations. A real-time spectral analysis interface (ComboMap, Volcano Therapeutics, USA) is used to record the average peak velocity (APV) of blood flow within the vessel. CBF (cm^3/s) is calculated by:

Equation 13

$$CBF = \left(\frac{APV}{2}\right)\pi r^2$$

Where r= vessel radius.

2.8 Measurement of coronary artery resistance

Using the principle of Ohm's law, coronary artery resistance (mmHg/cm^3/s) can be calculated using the mean arterial blood pressure (MAP) and the CBF.

Equation 14

$$Coronary\ Resistance = \frac{MAP}{CBF}$$

This calculation assumes an absence of obstruction to flow in the course of the vessel i.e. that the pressure in the distal portion of the artery (Pd) is equal to the aortic pressure (Pa). (it should be noted that other measures of coronary microvascular resistance are available, however these require use of either thermodilution techniques or a dual-tipped pressure and flow coronary wire[189]).

2.9 Measurement of forearm blood flow: Venous occlusion plethysmography

Venous Occlusion Plethysmography is a well-established method of determining Forearm Blood Flow (FBF)[190]. The utility of this measure is in the relation of FBF to resistance arteriolar tone, as will be described below.

Hagen-Poiseuille's law describes the relationship between (laminar) blood flow and vessel radius:

Equation 15

$$Q = \frac{Pd.\pi.r^4}{8\mu l}$$

Where Q = laminar flow, Pd = pressure difference, r = radius of vessel, l = vessel length, and μ represents a coefficient of fluid viscosity.

The principle of Venous Occlusion Plethysmography is that, if venous return from the forearm is prevented, the subsequent increase in blood volume within the compartment is proportional to the rate of arterial blood inflow[191]. Changes in circumference of the forearm, which represent the increase in volume and therefore FBF, can be made using mercury-in-silastic strain gauges[190, 192].

To obtain as accurate as possible picture of the blood flow within the resistance arterioles of the forearm compartment, it is important to exclude hand blood flow, for two key reasons. Firstly, under resting conditions, 60-70% of the blood flow within the forearm compartment represents blood flow within skeletal muscle[190, 192], with the remainder representing blood flow within the skin. Within the hand, a much higher proportion represents skin blood flow. Therefore, by excluding hand blood flow, the proportion of studied blood flow within skeletal muscle is higher[193]. Secondly, the hand compartment contains a greater number of arteriovenous shunts than the forearm compartment.

Description of method

The volunteer is asked to lie flat. The arms are positioned on cushions to ensure the forearm is above the level of the heart; this is to ensure the forearm veins are not fully distended. Blood pressure cuffs are placed above the elbows (‘upper arm cuffs’) to allow for occlusion of venous return without arterial occlusion when the

cuffs are inflated to 40mmHg. Blood pressure cuffs are placed at the wrists to allow for exclusion of hand blood flow when the cuffs are inflated to ~180mmHg. A blood pressure cuff is placed around the upper arm of the control arm to allow for non-invasive measurement of systemic blood pressure. Mercury-in-silastic strain gauges are placed around the forearm. The volunteer lies flat for 30 minutes before measurements are taken. The wrist cuffs are inflated to 180mmHg, 1 minute prior to measurement, to allow for stabilisation of FBF following occlusion of hand blood flow. Subsequently, the upper arm cuffs are inflated to 40mmHg for 10 seconds on 5 occasions, with 5 seconds deflation between inflations. Strain gauge voltages are recorded during upper cuff inflation (Powerlab, AD Instruments, Australia). FBF is calculated as the gradient of the linear portion of the strain gauge voltage vs time curve during upper cuff inflation.

Room temperature (24-26^{o}C), time of day, caffeine and alcohol limitation (none in previous 24 hours) are all standardised.

2.10 Determination of plasma and salivary [nitrate] and [nitrite]

At each collection point, 6ml of blood is drawn into a chilled syringe. It is immediately transferred into a chilled lithium heparin Vacutainer® (BD, Germany), then centrifuged at 4500rpm (2000x g) for 5 minutes at a temperature of 4^{o}C in a MIKRO 220R centrifuge (Hettich, Germany). Following centrifugation, the plasma is collected. Both plasma and saliva samples are stored at -80^{o}C until time of analysis. Analysis of [nitrate] and [nitrite] is performed by ozone chemiluminescence[194] using a Sievers 280i nitric oxide analyser.

Chapter 3. The Effect of Inorganic Nitrite on Conduit Arteries

3.1 Introduction

Nitrite is established as an exercise/hypoxia-dependent vasodilator of small resistance arterioles[81, 82]. Nitrite also exerts a selective, normoxia-dependent vasodilator effect in the conduit arteries at rest, with a similar selectivity as GTN for conduit versus small resistance arterioles [83]. The experimental model for these findings was the human forearm, with measurements of radial (conduit) artery diameter and forearm blood flow (an estimation of resistance arteriole response) following intrabrachial nitrite infusion. It is assumed, but not yet proven, that nitrite's selectivity for conduit versus resistance arteries in normoxic conditions applies to other local arterial beds.

Furthermore, although the mechanism of action of nitrite-induced dilatation of resistance arterioles is widely accepted to be via the cGMP-NO pathway, the mechanism of action of nitrite in conduit artery vasodilatation has not been clearly defined. Unpublished data from others in our research group suggested that nitrite-induced conduit artery dilatation is cGMP-independent: data from these studies is presented next as part of the background to the studies I conducted.

cGMP-independent vasodilatation of conduit vessels

8 healthy volunteers underwent a 3-visit crossover study to assess the effect of increasing plasma [cGMP] on nitrite-induced radial artery dilatation. On each study day, participants received an intra-brachial infusion of either sodium nitrite (2.6 μmol/min), sildenafil (a phosphodiesterase-5 inhibitor, to enhance cGMP-dependent

activity), or a nitrite-sildenafil co-infusion. Three doses of sildenafil were used: 30 μg/ml/min, 100 μg/ml/min, and 300 μg/ml/min.

Forearm blood flow (FBF), a measure of the effect of an intrabrachial infusion on the arterial microvasculature, was increased by the nitrite-sildenafil co-infusion compared to the individual infusions (Area Under Curve of 38% more than the sum of its parts, $p<0.0001$, see **Figure 1**). This was associated with a 3-fold increase in plasma [cGMP] with the nitrite-sildenafil co-infusion compared with nitrite alone (**Figure 2**). Taken together, these data confirmed the cGMP-dependent nature of nitrite-induced dilatation of resistance arterioles.

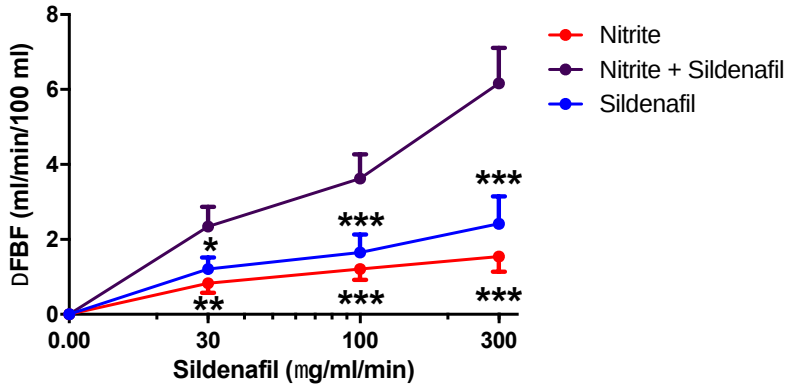

Figure 1. The effect of sildenafil, nitrite and a sildenafil-nitrite co-infusion on FBF in healthy volunteers. *$p<0.05$ vs Nitrite+Sildenafil, **$p<0.01$ vs Nitrite+Sildenafil,***$p<0.001$ vs Nitrite+Sildenafil. Data expressed as mean [SEM].

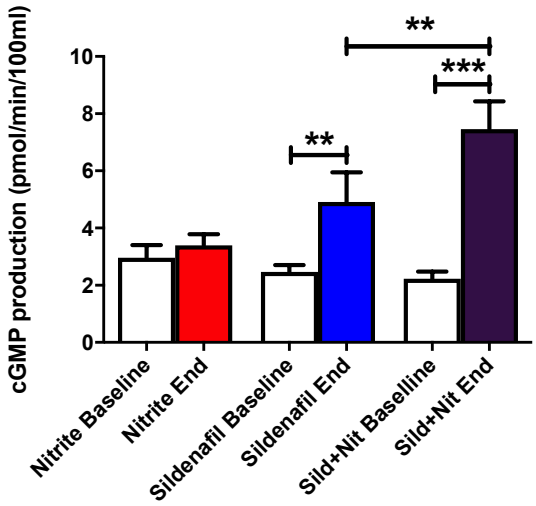

Figure 2. cGMP production at baseline and following intrabrachial infusion of nitrite, sildenafil, or nitrite+sildenafil co-infusion. **p<0.01, ***p<0.001. Data expressed as mean [SEM].

In contrast to the microvasculature, the level of conduit artery dilatation seen with the nitrite-sildenafil co-infusion was not significantly different to that seen with nitrite infusion alone, although the co-infusion did result in significant dilatation versus sildenafil alone (**Figure 3**). Furthermore, the sildenafil infusion had the effect of constricting the radial artery. These data suggested that, while nitrite's action in the resistance arterioles is mediated by cGMP, its action in the radial artery is not.

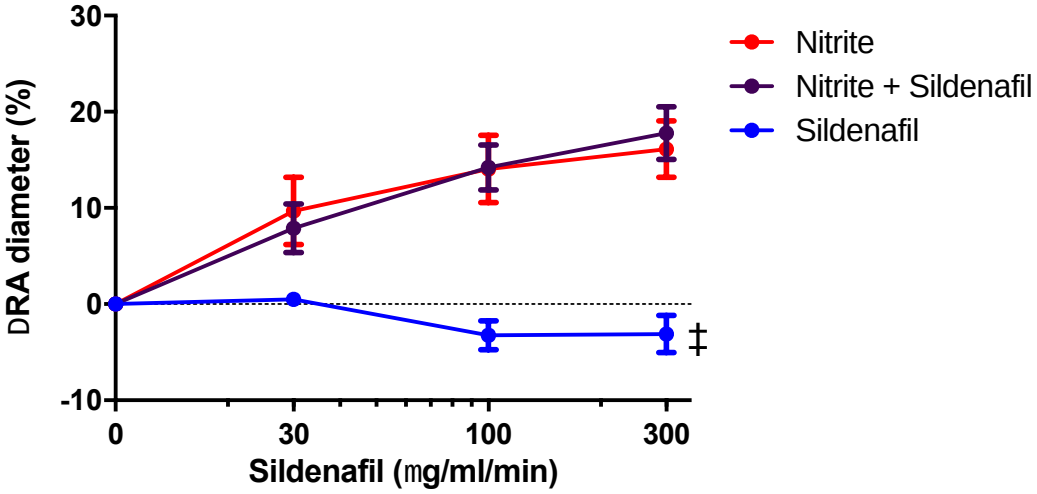

Figure 3. The effect of sildenafil, nitrite and a sildenafil-nitrite co-infusion on radial artery diameter in healthy volunteers. ‡$p<0.05$ versus baseline as measured by 1 way ANOVA for sildenafil infusion. Data expressed as mean [SEM].

Potassium channel mechanism

A further crossover study assessed whether nitrite's effects on conduit vessels could be mediated by potassium (K^+) channels. 8 healthy volunteers attended for 3 visits; on each visit they received an intrabrachial infusion of either inorganic nitrite (2.6 μmol/min), quinine (a non-selective K^+ channel antagonist), or a nitrite-quinine co-infusion. Quinine increased FBF, with the nitrite+quinine co-infusion increasing FBF compared to both the nitrite infusion and the quinine infusion ($p<0.001$, see **Figure 4**).

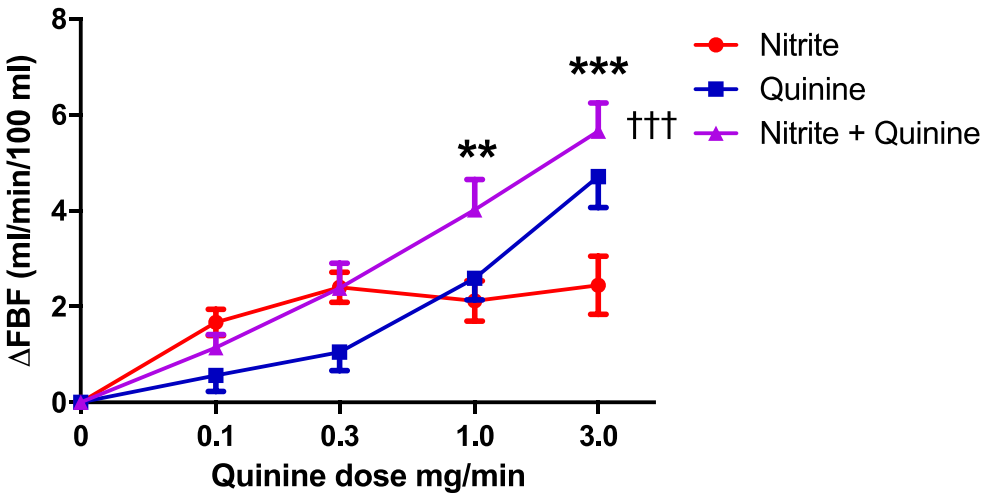

Figure 4. The effect of nitrite, quinine, and nitrite+quinine co-infusion on forearm blood flow. **p<0.01, ***p<0.001 for individual doses as analysed by 2 way ANOVA. †††p<0.001 for nitrite + quinine co-infusion versus baseline as analysed by 1 way ANOVA. Data expressed as mean [SEM].

However, quinine alone had no effect on the radial artery (**Figure 5**). Radial artery dilatation was significantly inhibited by adding quinine to nitrite i.e. the nitrite+quinine co-infusion resulted in significantly less dilatation of the radial artery than nitrite alone. Therefore, potassium channel inhibition via quinine had opposite effects on FBF (microvasculature) compared to the radial (conduit) artery.

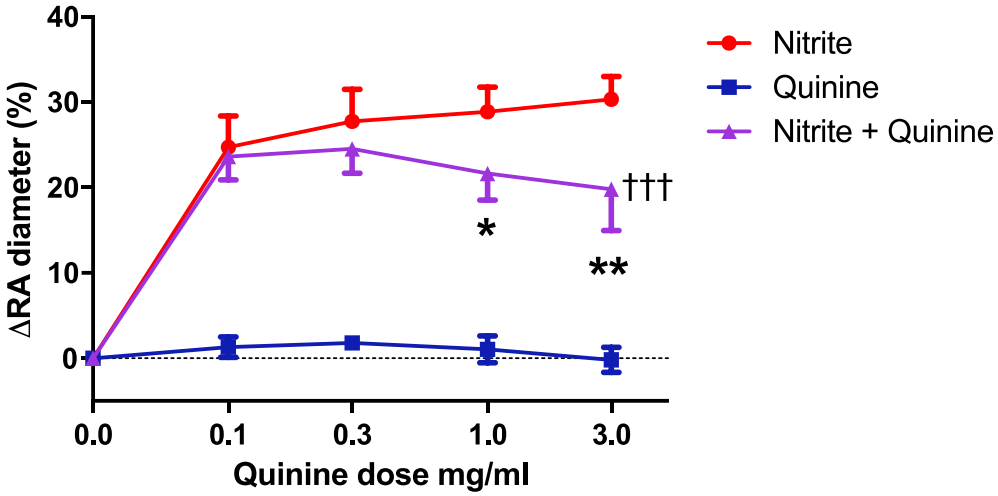

Figure 5. The effect of nitrite, quinine, and nitrite+quinine co-infusion on radial artery dilatation. *p<0.05, **p<0.01 for individual doses as analysed by 2 way ANOVA. †††p<0.001 as analysed by 1 way ANOVA.

Given that quinine is a non-specific K^+ channel inhibitor, further animal studies were then performed to assess the effect of a range of K^+ channel inhibitors on rat thoracic aortic strips, pre-constricted with phenylephrine. Of four K^+ channel inhibitors, both ouabain (Na^+/K^+-ATPase inhibitor) and barium (inhibitor of Kir2.1) inhibited nitrite-induced relaxation. TEA (inhibitor of K_{ATP}, BKCa, delayed rectifier K^+ channels, Kv1.2, Kv1.3, Kv1.6) and iberiotoxin (BKCa inhibitor) had no significant effect on nitrite-induced relaxation. The magnitude of effect seen with ouabain suggests that Na^+/K^+-ATPase plays a major role in the regulation of nitrite-induced vasorelaxation (**Figure 6**).

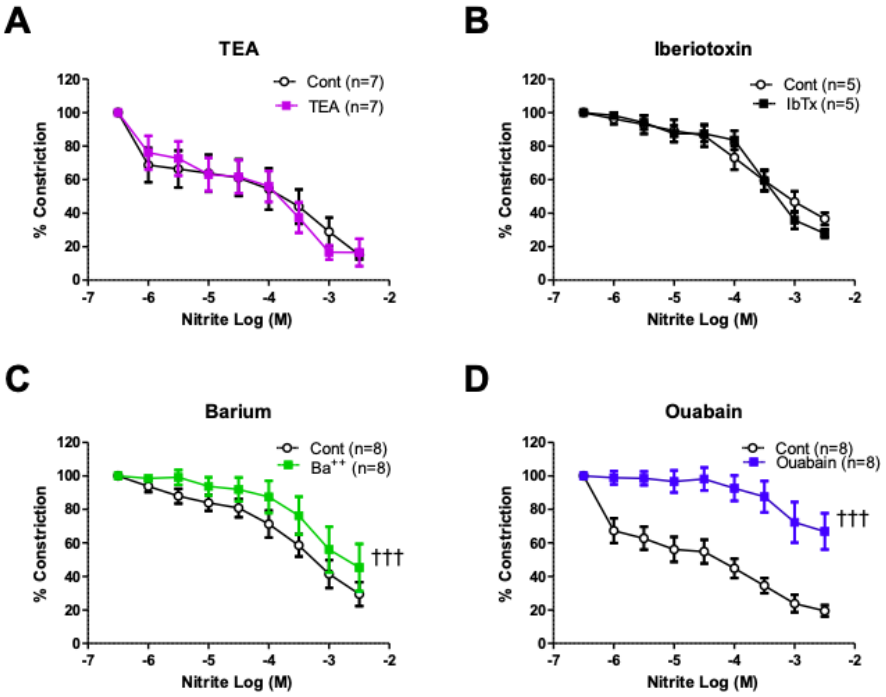

Figure 6. The effect of TEA (Panel A), iberiotoxin (Panel B), barium (Panel C) and ouabain (Panel D) on rat aortic rings pre-constricted with phenylephrine. †††$p<0.001$ vs control. Data expressed as mean [SEM].

Aims & Hypotheses

This chapter comprises two studies whose aims were as follows:

Study 1: We aimed to study the response of the coronary arteries to inorganic nitrite. Consistent with the effect seen in the forearm, we hypothesised that an intracoronary infusion of sodium nitrite would result in selective vasodilatation of the conduit epicardial coronary artery compared to its effects on the coronary microvascular resistance vessels. Furthermore, we hypothesised that nitrite would demonstrate a similar selectivity to GTN for conduit versus resistance vessels.

Study 2: We aimed to identify the cGMP-independent mechanism of nitrite-induced conduit artery dilatation, using a forearm experimental set-up in healthy human volunteers. Based on the pilot data presented above, we hypothesised that nitrite's effect on conduit arteries is mediated by Na^+/K^+ ATPase.

3.2 Methods

A description of methods is included in **Chapter 2.3, 2.6-2.9**. Patient selection, a brief summary of the infusion protocol, and statistical methods are described below.

Approvals

Ethical approval for Study 1 was obtained from the South East London Research Ethics Committee (REC) (10/H0802/52, PI: Professor Ajay M Shah) and the Research and Development department at King's College Hospital NHS Foundation Trust, London, UK. Ethical approval for Study 2 was obtained from St Thomas' Hospital REC (11/H0802/4, Regulation of vascular tone in conduit and resistance vasculature, PI: Professor Phil Chowienczyk) and the Research and Development department at Guy's & St Thomas' NHS Foundation Trust. Written informed consent was obtained from all participants prior to commencing any protocol-related procedures.

Study 1 (Intracoronary nitrite study): Participants, Protocol, and Statistical Analysis

Participants were patients who were due to undergo invasive coronary angiography for clinical indications. Participants were eligible for inclusion into the study if they had an epicardial coronary artery that was angiographically free of disease, with no previous percutaneous coronary intervention (PCI). Regular cardiac medications other than antiplatelet medications were omitted on the day of the procedure. Participants were excluded if they were unable to provide informed written consent in English; if they had previously undergone percutaneous coronary intervention to

the vessel which was to be studied; or if the vessel to be studied was <2mm in diameter by visual angiographic assessment.

Diagnostic coronary angiography was performed via the radial or femoral artery as defined by clinical requirements. Following completion of the diagnostic procedure, a 6Fr guiding catheter was placed at the ostium of the vessel to be studied, either the left main stem (to study the left anterior descending or the left circumflex) or the right coronary artery. A Doppler wire was positioned within the proximal artery. Sequential intra-coronary (i.c.) infusions of 0.9% saline (baseline), 2.6 μmol/min sodium nitrite, 26 μmol/min sodium nitrite, and 1 μg/min GTN were infused into the study artery via the guiding catheter, each over 5 minutes. The 26 μmol/min nitrite and GTN infusions were separated by a 5 min infusion of 0.9% saline as washout. At the end of each infusion, measurements of average peak velocity (APV) and invasive blood pressure were taken, with acquisition of coronary angiographic images. Quantitative Coronary Analysis (QCA) was subsequently performed offline.

Statistical analysis

Data were analysed using GraphPad Prism 7 (GraphPad Software Inc.). Baseline characteristics are presented as mean +/- standard deviation. Experimental data are presented as mean and 95% confidence intervals for parametric data and median [interquartile range] for non-parametric data. Experimental data are presented as change from the relevant baseline. Statistical analysis was performed using one-way ANOVA for repeated measures (paired analysis) with appropriate *post* testing for multiple comparison.

Study 2 (intrabrachial digoxin study): Participants, Protocol, and Statistical Analysis

Participants were recruited through an advertisement in a bi-weekly circular email from the King's College London Department of Research, Ethics and Integrity. Inclusion criteria: Healthy volunteers aged between 18-65. Exclusion criteria were:

- Subjects unable to give informed consent, either due to lack of capacity or due to poor understanding of verbal/written English
- Pregnant or lactating females
- Any known past history or current diagnosis of cardiovascular disease
- The presence of any risk factors for cardiovascular disease (see below). Apart from hypertension, the presence or absence of the risk factors listed below was established from screening questions. We did not specifically test serum lipids, blood glucose or HbA1c.
    - Cigarette smoking
    - Hypertension i.e. sustained elevation of blood pressure over 140/90
    - Hypercholesterolaemia (total cholesterol > 6 mmol/L)
    - Diabetes mellitus
    - BMI > 30 Kg/m^2
- Alcohol consumption > 30 units/week
- Use of recreational drugs
- Any long-term medication except the oral contraceptive pill, implant contraception, hormone replacement therapy and/or asthma inhalers
- Any other psychological or physical disorder at the discretion of the investigator

To test the hypothesis that the effect of nitrite on conduit arteries is mediated by Na^+/K^+ ATPase, we elected to assess the effect of digoxin (a selective inhibitor of the alpha2 subunit of Na^+/K^+ ATPase) on the vasodilator effect of inorganic nitrite. The infusion protocol for this study is shown in **Figure 7**. After a 5 min period of saline driver infusion, baseline readings were recorded. This was followed by a 7 min infusion of sodium nitrite 2.6 µmol/min. After a 30 min washout, an infusion of digoxin 1.7 µg/min was commenced. Following 53 min of digoxin infusion, a co-infusion of sodium nitrite and digoxin was administered for 7 min (2.6 µmol/min and 1.7 µg/min respectively). The duration of digoxin infusion was chosen based on a previous study in which digoxin was infused into the brachial artery at a dose of 1.7 µg/min over 60 mins[195]. In this study, digoxin had no significant effect on basal FBF, however it did increase the FBF response to methacholine.

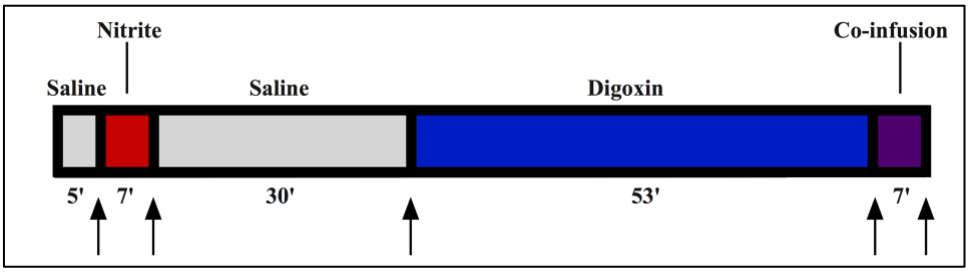

Figure 7. Diagram indicating order of intra-brachial infusions in study 2 protocol. Arrows indicate time points at which measurements were made.

At the end of each infusion (saline baseline; nitrite; washout; digoxin; and nitrite-digoxin co-infusion) the following recordings were made:

- Heart rate.
- Peripheral blood pressure (taken from the arm contralateral to the infusion).

- Measurement of radial artery diameter using 2D ultrasound with edge-detecting software.
- Forearm Blood Flow (FBF). For the purposes of this study, FBF was considered as the ratio of measured blood flow in the forearm compartment of the intervention arm versus the measured blood flow in the forearm compartment of the control arm. See **Chapter 2.9** for a description of the venous occlusion plethysmography method of estimating FBF.
- Measurement of forearm vascular resistance. Resistance was calculated by dividing mean arterial pressure by the absolute value for FBF in the intervention arm. Resistance is expressed as mmHg x min/ml per 100ml of tissue.

Statistical analysis

Baseline characteristics of subjects are presented as mean ± SD. Experimental data are presented as mean ± SEM or median [IQR] as appropriate. Changes in variables were analysed both as absolute values and also as percentage change from the relevant preceding baseline (or preceding infusion where appropriate). Changes in variables were compared by ANOVA for repeated measures (or non-parametric equivalent) with adjustment for baseline values where appropriate. The effect of the nitrite-digoxin co-infusion on the percentage change in variables was corrected for the effect of the digoxin infusion by considering the post-digoxin measurement as the baseline for the change in variable attributed to the co-infusion.

3.3 Results

Baseline Characteristics

Table 1 and **Table 2** display the baseline characteristics and average baseline variables for the study participants.

Age (years)	56 ± 11
Male gender (n)	5 (56%)
SBP (mmHg)	140.1 ± 28.9
DBP (mmHg)	71.5 [65.8, 80.2]
MAP (mmHg)	98.3 [76, 100.7]
Heart rate (bpm)	64.7 ± 11.9
Hypertension	7 (78%)
Hyperlipidaemia	6 (67%)
Diabetes	2 (22%)
Smoker	4 (44%)
LVEF (%)	51.7 ± 5.7
PAVP (cm/s)	20 ± 10.1
Coronary Diameter (mm)	2.5 ± 0.1
Coronary Blood Flow (cm^3/s)	0.49 ± 0.25
Coronary Resistance ($mmHg/cm^3/s$)	292 ± 179

Table 1. Baseline characteristics and baseline study variables for Study 1 (n=9).

Age (years)	25 ± 4
Male gender (n)	8 (100%)
SBP (mmHg)	118 ± 5
DBP (mmHg)	67 ± 8
MAP (mmHg)	84 ± 6
Heart rate (bpm)	61 ± 8
Radial artery diameter (mm)	2.47 ± 0.42
$FBF_{intervention\ arm}$ (ml/min per 100ml)	3.83 ± 1.21
Microvascular resistance (mmHg x min/ml per 100ml)	22 ± 7.7

Table 2. Baseline characteristics and baseline study variables for and Study 2 (n=8).

Patient medications

Table 3 displays the regular medications for Study 1 participants.

Medication	n (%)
Antiplatelet	5 (56)
Anticoagulant	1 (11)
Beta-blocker	5 (56)
Other rate-limiting drug	1 (11)
ACEi/ARB/MRA	5 (56)
Diuretic	2 (22)
Other anti-hypertensive drug	2 (22)
Statin	8 (89)
Diabetic medication	2 (22)
Proton pump inhibitor	2 (22)
Nitrate or other anti-anginal	2 (22)

Table 3 Study 1 participant medications. ACEi = Angiotensin Converting Enzyme Inhibitor, ARB = Angiotensin Receptor Blocker, MRA = Mineralocorticoid Receptor Antagonist.

Study 1:

Coronary Diameter

2.6 μmol/min nitrite had no significant effect on coronary artery diameter (mean [95% CI]: +3.9% [-2.8,+10.6], p=0.3). Both 26 μmol/min nitrite and GTN had a significant dilating effect (+8.9% [+2.0,+15.8], p=0.01 and +10.8% [+1.0,+20.5] p=0.03, respectively, **Figure 8 A**). Additionally, both 26 μmol/min nitrite and GTN had a significant dilating effect compared to the 2.6 μmol/min nitrite infusion

(+5.0% [+0.5,+9.5], p=0.03, and +6.9% [+1.1,+12.7] p=0.02). There was no significant difference in coronary dimeter change between 26 μmol/min nitrite compared to GTN (-1.9% [-5.6,+1.9] p=0.45).

Coronary Blood Flow

Both doses of nitrite (2.6 μmol/min and 26 μmol/min) lacked significant effect on coronary flow compared to baseline (median [interquartile range]): +12.0% [-10.2, +24.1] p>0.99 and +16.9% [+3.2, +64.4] p>0.99 respectively (**Figure 8 B**). GTN increased coronary flow relative to baseline: +23.9% [+6.3,+72.7], p=0.02. There was no significant difference in coronary flow following 26 μmol/min nitrite compared to 2.6 μmol/min nitrite (p>0.99). There was no significant difference in coronary flow following GTN infusion compared to either the 2.6 μmol/min nitrite or 26 μmol/min nitrite infusions (p=0.11, p=0.6 respectively).

Coronary Resistance

Neither dose of nitrite (2.6 μmol/min or 26 μmol/min) resulted in a significant reduction of coronary resistance compared to baseline (mean [95% CI]): -11.1% [-36.7,+14.5] p=0.52, and -19.0% [-43.4,+5.4] p=0.13, respectively) (**Figure 8 C**). There was no significant difference in coronary resistance following infusion of 26 μmol/min nitrite compared to 2.6 μmol/min nitrite (-7.9% [-29.6, +13.8], p=0.64). GTN lowered coronary resistance relative to baseline (-29.3% [-52.8,-5.9] p=0.02). GTN also lowered coronary resistance relative to both 2.6 μmol/min nitrite and 26 μmol/min nitrite (-18.3% [-34.1, -2.4] p=0.03, -10.4% [-18.3,-2.4] p=0.01, respectively.

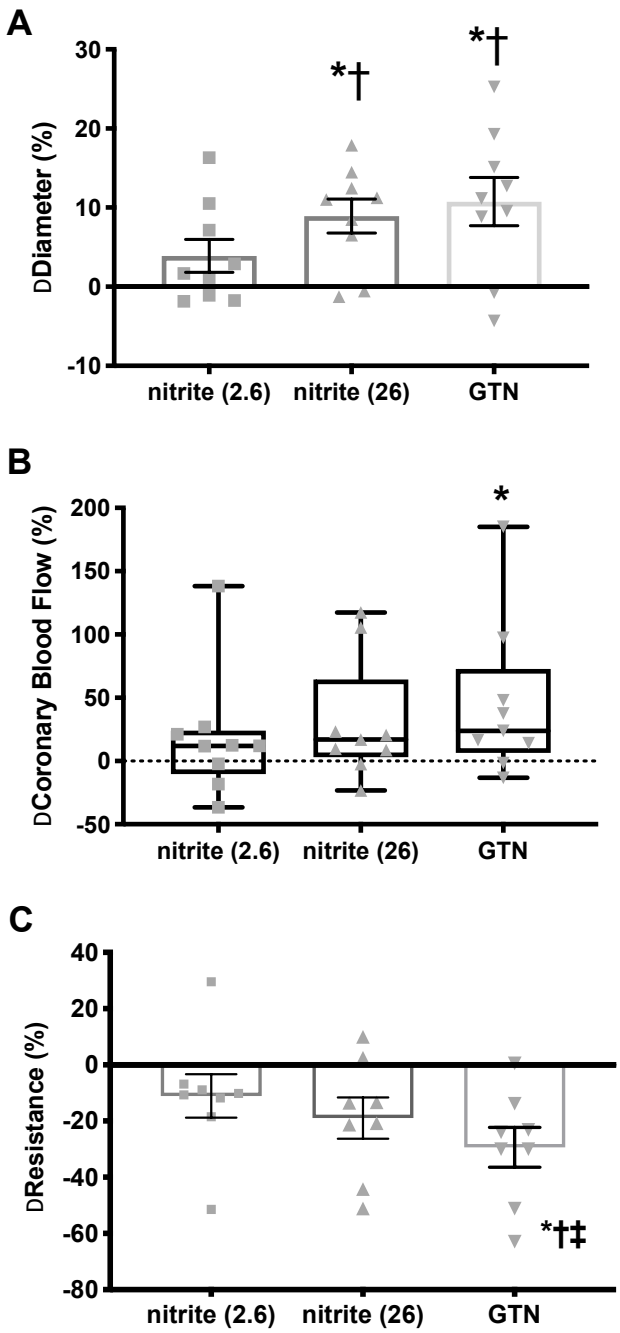

Figure 8. Change in coronary artery diameter (panel A), blood flow (panel B), and resistance (panel C) in response to intracoronary nitrite and GTN. Data in panels A and C expressed as mean ± SEM. Box and whisker plots in panel B demonstrate median [IQR] with error bars indicating range. *p<0.05 vs baseline, †p<0.05 vs 2.6 μmol/min nitrite, ‡p<0.05 vs 26 μmol/min nitrite.

Blood pressure and Heart rate

There were no significant changes in systolic blood pressure with nitrite or GTN versus baseline; however, SBP was lower with GTN versus nitrite: -9.5mmHg [-2.2,-16.8] p=0.01, and -9.8 mmHg [-1.8,17.8] p=0.02, with nitrite (2.6 μmol/min and 26 μmol/min), respectively. There were no significant overall differences in diastolic blood pressure, p=0.76, or mean blood pressure, p=0.09; however, mean blood pressure was significantly lower with GTN compared to nitrite (2.6μmol/min): -5.5mmHg [-0.8,-10.2], p=0.02 (**Figure 9 A**).

Nitrite (2.6 μmol/min) significantly reduced heart rate compared to baseline -3.1 bpm [-0.2,-6.1], p=0.03 and compared to GTN -6.1 bpm [-0.9, -11.3], p= 0.02 (**Figure 9 B**). Nitrite (26 μmol/min) and GTN had no significant effect on heart rate.

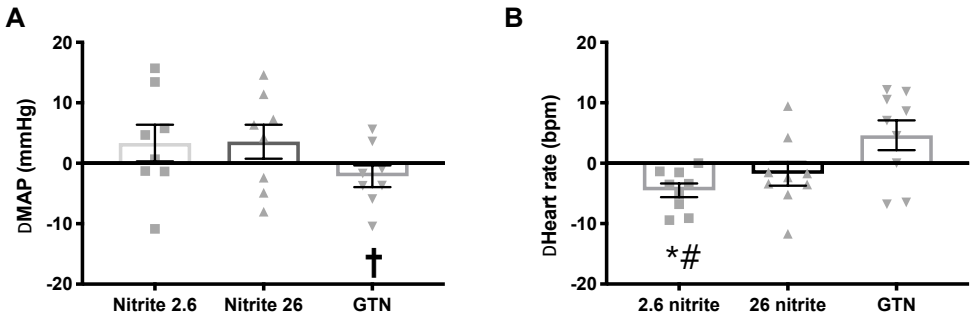

Figure 9. Change in mean arterial pressure (MAP, Panel A) and heart rate (Panel B) in response to intracoronary nitrite and GTN. Error bars indicate SEM. *p<0.05 vs baseline †p<0.05 vs 2.6 μmol/min nitrite, #p<0.05 vs GTN

Study 2:

Radial artery diameter: The average radial artery diameter at Baseline 1 was 2.47 ± 0.42 mm (mean±SD). Nitrite significantly dilated the radial artery compared to baseline: +0.4 mm [+0.27, +0.52], p<0.0001 (mean [95% CI]). The co-infusion also significantly dilated the radial artery: +0.43 mm [+0.28, +0.59], p=0.0001 versus the digoxin infusion. There was no significant difference in radial artery dilation seen between nitrite infusion and co-infusion: +0.02 mm [-0.18, +0.22], p>0.99. Digoxin was not associated with a decrease in radial artery calibre: -0.11 mm [-0.25, +0.03], p=0.14.

When expressed as percentage change, nitrite significantly dilated the radial artery versus baseline: +16.7% [11.2, 21.7] (median [IQR]), p=0.01, **Figure 10**. Digoxin had no significant effect on radial artery diameter (-4.3% [-8.4, -0.1], p=0.97) relative to baseline 2. The co-infusion significantly dilated the radial artery: +19.8% [16.1, 20.8], p<0.001 versus digoxin infusion.

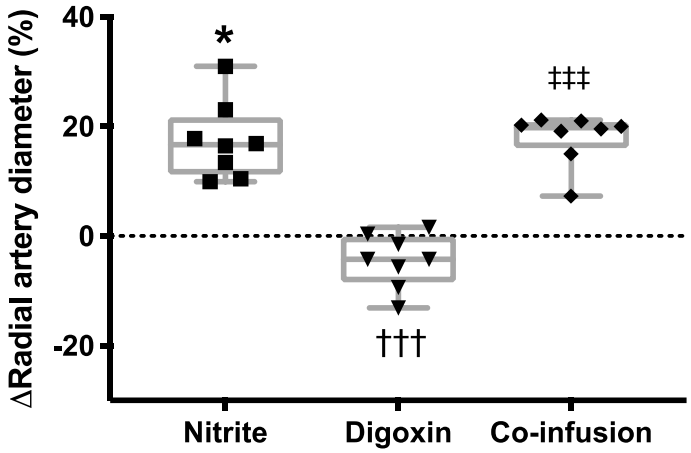

Figure 10. The effect of nitrite, digoxin, and co-infusion on radial artery diameter, expressed as percentage change in radial artery diameter. Data expressed as median

[IQR], with error bars indicating range. *p<0.05 vs Baseline, †††p<0.001 vs nitrite, ‡‡‡p<0.001 vs digoxin.

Forearm Blood Flow. Considering the ratio of FBF in the intervention arm compared to the control arm, expressed as percentage change, nitrite was associated with a significant increase in FBF: +64.1% [12.0, 116.2] (mean [95%CI]), p=0.02, **Figure 11**. The co-infusion significantly increased FBF vs digoxin : +87.5% [+12.6, +162.4], p=0.02. There was no significant difference in the % change between nitrite and co-infusion (p>0.99).

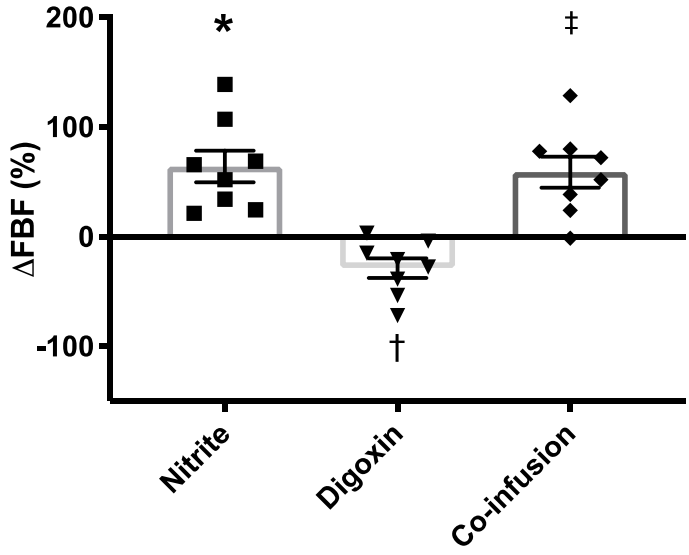

Figure 11. The Effect of nitrite, digoxin, and nitrite-digoxin co-infusion on forearm blood flow (FBF), calculated as the ratio of FBF in the intervention arm to the FBF in the control arm. *p<0.05 vs baseline, †p<0.05 vs nitrite, ‡p<0.05 vs digoxin. Data expressed as mean ± SEM.

Resistance in Forearm Vessel Compartment. Nitrite significantly reduced the resistance in the intervention arm forearm compartment: -32.6% [-50.3, -14.8] (mean [95%CI]), p=0.002, **Figure 12**. The co-infusion resulted in a significant decrease in resistance compared to digoxin infusion: -81.8% [-131.3, -32.3], p=0.004. There was no significant difference in change in forearm resistance between the nitrite infusion and the co-infusion (p=0.97).

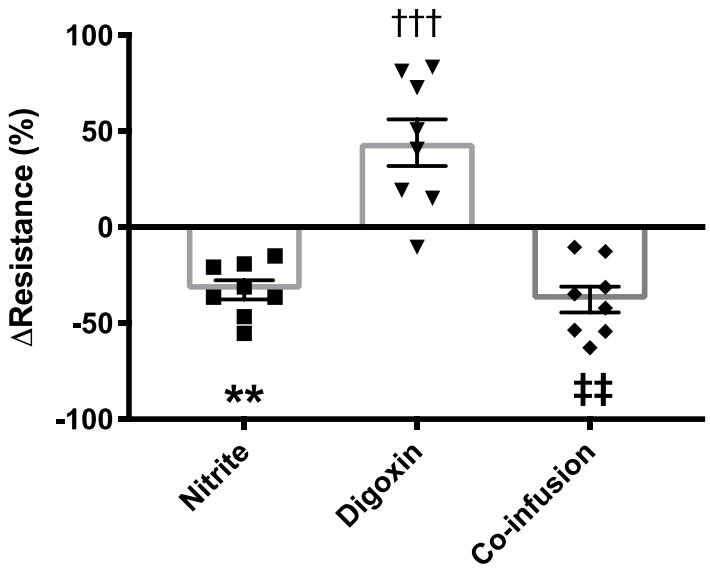

Figure 12. The effect of inorganic nitrite, digoxin, and a nitrite-digoxin co-infusion on microvascular resistance in the intervention arm. **p<0.01 vs Baseline 1, †††p<0.05 vs Baseline 2, ‡‡p<0.01 vs digoxin . Data expressed as mean ± SEM.

Mean Arterial Pressure (MAP)

There was no significant change in MAP

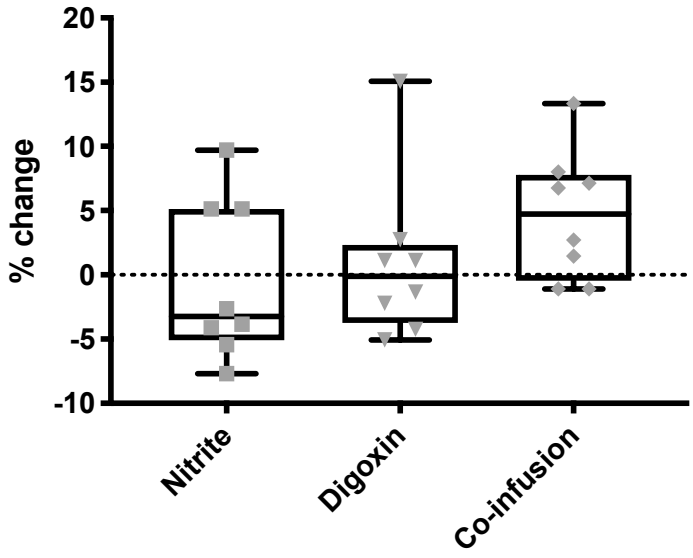

Figure 13 The effect of nitrite, digoxin, and co-infusions on mean arterial pressure. Data displayed as median [IQR].

Safety

No adverse events were experienced in this study. In particular, the local (i.e. intrabrachial) administration of digoxin was not associated with any significant change in heart rate. The mean heart rate at baseline was 61.2 ± 7.9, compared to 62.0 ± 10.6 at the end of the study.

3.4 Discussion

This is the first data to show that inorganic nitrite dilates coronary arteries and does so in a dose-dependent manner. The magnitude of epicardial dilatation was not significantly different to that seen with GTN. Consistent with data from the radial artery[83], nitrite did not cause any significant decrease in coronary flow or resistance, unlike GTN. In the absence of functionally significant epicardial coronary stenosis and in the presence of a stable perfusion pressure, coronary flow and resistance are determined mainly by the coronary microvasculature. The finding that nitrite has no significant effect on flow and resistance is consistent with what is considered to be nitrite's physiological action as a selective vasodilator of conduit arteries in normoxic conditions (as opposed to its action as a dilator of resistance arterioles in hypoxic conditions[82, 132]). It is of interest that nitrite exerts a selective effect on conduit vessels, even in the beating (and therefore exercising, metabolically active) heart, albeit under conditions of rest.

Pertaining to the mechanism of action of nitrite in conduit vessels, the pilot data presented in the introduction section of this chapter provides evidence that the dilating effect of inorganic nitrite on conduit arteries in humans is independent of cGMP and is mediated by potassium channels. Further experiments on rat aortic rings suggested that Na^+/K^+-ATPase may be the potassium channel responsible. In the data presented in the chapter, however, intra-brachial digoxin had no significant effect on radial artery dilatation due to sodium nitrite in healthy humans. Therefore, these data do not support the hypothesis that inorganic nitrite acts via Na^+/K^+-ATPase to dilate conduit vessels. Furthermore, digoxin had no effect on nitrite's

action in the resistance arterioles, in keeping with nitrite's well-described cGMP-dependent mode of action in the resistance vessel as cGMP mediated.

As discussed in **Chapter 1**, reduction of inorganic nitrite to NO results in vasodilation through the action of NO on soluble guanylate cyclase (sGC), leading to activation of protein kinase G (PKG) via an increase in [cGMP]. This pathway is promoted by hypoxic conditions. Potential mechanisms whereby inorganic nitrite may cause vasodilatation in a cGMP-independent manner include: Potassium channels other than Na^+/K^+-ATPase;
NO-independent PKG activation; and S-nitrosothiol formation, as discussed below.

Potassium channels other than Na^+/K^+-ATPase

Other potential potassium channels that could mediate a cGMP-independent effect include calcium-dependent potassium channels (BK_{Ca}), which are susceptible to cGMP-independent NO-mediated activation in rabbit aortic smooth muscle cells[12]. However in the pilot data for this study, the BK_{Ca} inhibitor TEA had no effect on nitrite-induced relaxation of pre-constricted rat aorta (see **Figure 6**).

NO-independent PKG activation

cGMP-activated PKG is a key component of NO signalling, resulting in vasodilation. However, the α1 subunit of PKG (PKG1α) is also itself under redox regulation. Oxidation of PKG1α results in disulfide bond formation between cysteine 42 residues on PKG1α, rendering PKG1α active, increasing enzyme activity without any increase in [cGMP][196]. Accordingly, hydrogen peroxide

(H_2O_2, a key component of the endothelium-dependent hyperpolarising factor response[197]) relaxes conduit arteries via disulfide-mediated activation of PKG1α [198]. Experiments in mice demonstrate a prolonged blood pressure reduction in response to nitrite, present in WT mice but not mice with inactive PKG (C42S PKG1α knock-in mice)[199]. Furthermore, neither the NO scavenger CPTIO (2,4-carboxy-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) nor the guanylate cyclase inhibitor ODQ (oxadiazolo[4,3,-a]quinxalin-1-one) had a significant effect on nitrite-induced vasorelaxation/vasodilatation. Although it is noted that the effects seen in the aforementioned study were observed in the resistance (mesenteric arteries) but not conduit (thoracic aorta) vessels, the proof-of-concept of nitrite-induced, PKG-mediated, yet NO-independent vasodilatation is relevant to this thesis.

S-nitrosothiol formation

An additional possible mechanism is via S-nitrosylation of PKA. S-nitrosylation describes the NO-mediated modification of cysteine residues on proteins, resulting in the formation of an S-nitrosothiol (SNO)[13]. In rat thoracic aortic rings, nitrocysteine (CysNO, a transnitrosylating NO donor) induced vasorelaxation that was unaffected by the adenylate cyclase inhibitor 2',5'-dideoxyadenosine and only partially inhibited by the sGC inhibitor ODQ. These responses were decreased by PKG inhibition and to a greater degree, PKA inhibition. It was therefore concluded that CysNO induces vasodilation independently of the classical NO-sGC-cGMP pathway, with s-nitrosylation of PKA and/or PKG as possible mechanisms[200].

Understanding the mechanism of action of nitrite-induced conduit artery dilatation is of value in that it may characterise a novel signalling pathway in vascular smooth muscle which – in theory – could represent a therapeutic target.

Limitations

Study 1: The study population, consisting of 9 patients, is small. One of the main conclusions of this study is that inorganic nitrite has no significant effect on coronary blood flow or resistance. However, there are non-significant trends to an effect for both of these outcomes. The higher dose of nitrite (26 μmol/min) resulted in a 16.9% increase in CBF and a 19% decrease in coronary resistance. In the setting of a small sample size this may represent type II error. Indeed, the forearm model allows comparisons between conduit and microvascular responses at rest, whereas the coronary arteries are supplying cardiac muscle that is constantly beating, even if the experimental setting was that of the resting heart rate. Therefore, in retrospect, we may have predicted more of an effect of nitrite on the microvasculature (an exercise-mediated effect) while at the same time observing the normoxia/resting effect on the conduit vessel.

Secondly, the calculation of coronary diameter, coronary blood flow, and coronary resistance rely on several assumptions. Measurement of coronary diameter was performed using QCA (see **Chapter 2.5**). This measurement technique relies on coronary angiography and assumes that the vessel cross sectional area is uniformly circular, which may not be the case. It could therefore be argued that invasive imaging techniques (e.g. intravascular ultrasound, IVUS) could potentially have provided more accurate assessment. Furthermore, the calculation of both CBF

and resistance use the QCA measure of vessel calibre. Given that the radius is squared to provide vessel cross-sectional area, any errors are therefore also squared, compounding any error in the coronary artery diameter measure. However, analysis of IVUS and QCA data from a randomised control trial demonstrates good correlation of coronary artery diameter measures between the two modalities[201]. Where the correlation weakens is in the setting of diseased vessels e.g. measurement of percent atheroma volume, which is not relevant to this study.

Thirdly, the calculation of coronary resistance used is a crude measure utilising central blood pressure (MAP) and CBF and relies on the assumption that the ratio of pressure in the distal vessel to the pressure at the left mainstem ostium is 1, which may not be the case in the setting of even minor, diffuse, angiographically-insignificant coronary artery disease.

Finally, it has been demonstrated that coronary blood flow varies with normal cardiac catheter laboratory stimuli, such as verbal commands [202]. It is therefore possible that instructions from the operators to the catheter laboratory team e.g. to record APV measurements, could have affected the measurements recorded. However, this phenomenon should be expected to affect the baseline and post-drug recordings equally.

Study 2: The sample size in this study is small, but consistent with sample sizes (~n=10) that have been used in other studies of this nature. An ideal inhibitor in a study such as this would be one which was free of its own vasoactive properties. Although the intrabrachial infusion of digoxin did not have significant effects on radial artery calibre, FBF, or forearm resistance in this study, there were non-

significant trends in FBF and resistance. This, in keeping with the small sample size, raises the possibility of Type 1 error.

Relating to the choice of study drug, we would ideally have preferred to use ouabain on the basis that it was ouabain that was used in the pilot data experiments on pre-constricted rat aortic rings. However, it was not possible to obtain ouabain for human use, which led to the choice of digoxin as an alternative Na^+/K^+-ATPase inhibitor, with a well-established safety profile in humans. Furthermore, the effect of digoxin on arterial blood flow has previously been studied: in patients with heart failure, an infusion of 1 μg/min digoxin into the femoral artery had no effect on lower limb blood flow (determined by plethysmography) or mean femoral arterial blood flow[203]. In this study, as expected with local arterial drug administration, there was no effect on peripheral blood pressure (recorded from the contralateral arm) or heart rate.

Clinical Implications

The finding that nitrite has no significant effect on coronary flow or resistance has important implications for other work. In **Chapter 4**, I will discuss data relating to the effect of inorganic nitrite on left ventricular function at rest. This study's finding of nitrite's lack of significant effect on coronary flow therefore suggests that any change in left ventricular performance following intra-coronary nitrite is due to a direct effect on the myocardium rather than any indirect effect from changes in myocardial perfusion. Furthermore, nitrite's action to selectively dilate large arteries highlights its potential as an agent to improve ventricular-arterial coupling, which is thought to be abnormal in conditions such as HFpEF. Nitrite therapy would

therefore appear to be advantageous in HFpEF compared to organic nitrate therapies (e.g. GTN, ISMN); although organic nitrates relax conduit vessels, they also induce endothelial dysfunction[204] and are associated with decreased activity levels in HFpEF[118].

Pertaining to study 2, the clinical implications of identifying a cGMP-independent mechanism of action of nitrite-mediated conduit artery relaxation relate to the potential for new therapies targeting such a novel mechanistic pathway. Obvious diseases of interest would include hypertension and HFpEF. Potential therapeutic strategies could involve either activation of the relevant potassium channel to enhance the effect of physiological concentrations of inorganic nitrite, combination therapies i.e. a combination of dietary nitrate/inorganic nitrite and a potassium channel activator, or targeting other potential causative mechanisms e.g. PKA activation.

Further work

Study 1: The intracoronary nitrite study was performed in patients with preserved LV function and structurally normal hearts, under resting conditions. Inorganic nitrite is of interest as a potential therapy in diseases such as HFpEF, where the heart is structurally abnormal, with an increase in left ventricular mass index and abnormalities in conduit artery and left ventricular stiffness. Accordingly, the proportion of microvasculature to conduit vessel length (and therefore the total volume of blood within the microvasculature versus the conduit vessels at any one time) would be expected to be different in patients with HFpEF compared to those patients with structurally normal hearts. Therefore, while the findings of this study are relevant to HFpEF, further investigation should be carried out to confirm the

exact effects of inorganic nitrite in the coronary vasculature of patients with HFpEF and other conditions exhibiting left ventricular hypertrophy.

Study 2: Studies were planned to further investigate the hypothesis that the effect of inorganic nitrite on conduit arteries is mediated by potassium channels other than Na^+/K^+-ATPase. However, the commencement of this part of the study was delayed by the COVID-19 pandemic and subsequently by the National Patient Safety Alert (NatPSA) regarding sodium nitrite[205]. Although the NatPSA was regarding cases of accidental and incorrect administration of sodium nitrite instead of sodium bicarbonate in the clinical paediatric setting, it necessitated the disposal of the departmental stock of sodium nitrite.

Chapter 4. The Effect of Inorganic Nitrite on Left Ventricular Function in the Human Heart

4.1 Introduction

Nitric oxide (NO) has important roles in the physiological regulation of cardiovascular function while dysfunction of endogenous NO production or NO-cyclic GMP (cGMP) signaling are implicated in the pathophysiology of several cardiovascular diseases[206, 207]. Accordingly, strategies to increase local tissue concentrations of NO or to enhance NO-dependent signaling may have therapeutic potential. Inorganic nitrite (NO_2^-) is of interest in this regard as it can be reduced to NO and have effects similar to NO donors but tolerance does not develop to its effects with continued use, unlike the case with organic nitrates[6, 56]. Inorganic nitrite is a vasodilator, affecting both arterial[81-83] and venous tone[82]. In the coronary bed, nitrite is selective for conduit versus resistance vessels, see **Chapter 3** (study 1)[208]. When given via intravenous infusion, nitrite causes vasodilatation and a reduction in central blood pressure[83]. Nitrite also inhibits platelet aggregation[209, 210] and can improve mitochondrial efficiency[211]. Previous experimental and clinical studies have therefore explored the potential therapeutic benefit of nitrite in conditions such as myocardial ischemia-reperfusion injury[134] [133], pulmonary hypertension[212], cerebral vasospasm[146] and impaired exercise capacity in heart failure[121, 124].

Left ventricular diastolic function describes cardiac relaxation and is mainly determined by two key factors. Firstly, myocardial inactivation, an active process involving removal of Ca^{2+} from the sarcoplasmic reticulum and myofilament cross-bridge detachment. Secondly, myocardial stiffness, which determines the passive relaxation properties of the ventricle. Myocardial stiffness is determined by both the

cardiomyocytes and also components of the extracellular matrix. In the cardiomyocytes, the giant cytoskeletal protein titin is considered to be important. Titin isoform shifts[115] and abnormalities in titin phosphorylation[116] can affect myocardial stiffness. Abnormalities in collagen – whether amount, the relative proportion of Type I collagen, or the amount of cross-linking - and other extracellular matrix proteins can lead to a decrease in the passive stiffness of the ventricle.

Clinically, abnormalities in diastolic function become more common as patients increase in age. In patients aged 60 years or older, the prevalence of isolated LV diastolic dysfunction in 36%, significantly higher than systolic dysfunction.[213]

Endogenously generated NO has direct acute effects on myocardial relaxation and diastolic function. A selective NO- and cGMP/protein kinase G (PKG)-dependent lusitropic effect that does not alter systolic function has been reported in isolated mammalian cardiomyocytes and isolated hearts[32, 214]. A similar effect was observed in human subjects *in vivo* after acute intracoronary infusion of substance P to trigger the endogenous release of NO[26]. Consistent with these myocardial relaxant effects, it has been suggested that dysfunction of NO-cGMP signaling contributes to left ventricular (LV) diastolic dysfunction both experimentally and in patients[115, 215, 216]. As such, the clinical utility of nitrite to enhance relaxation and diastolic function is of interest. However, the direct myocardial effect of nitrite in the human heart and the relationship to its systemic effects has not been established. In the studies in this chapter, the effect of intracoronary nitrite infusion on LV contractile function was investigated and compared to the effects of systemic intravenous infusion.

4.2 Methods

Participants

Invasive LV pressure-volume (PV) studies were performed on patients (n=40) referred for diagnostic coronary angiography who were known to have normal left ventricular systolic function. Written informed consent was obtained prior to cardiac catheterization and the research study was performed at the end of the diagnostic procedure if there was an absence of significant epicardial coronary artery disease (<50% stenosis by visual estimation on coronary angiography and/or a fractional flow reserve >0.80). Patients were also excluded if they had heart failure, clinically significant valve disease or a history of glucose-6-phosphate dehydrogenase deficiency. Patients were required to be in sinus rhythm at the time of assessment, with atrial fibrillation and ventricular bigeminy/trigeminy being considered excluding factors. The study complied with the *Declaration of Helsinki* and was approved by the local Research and Ethics Committee (REF:12/LO/1067, see **Chapter 2.1**).

Study protocols

We studied intracoronary and intravenous nitrite infusion (n=20 subjects each). Radial or femoral arterial access was used for coronary angiography at the discretion of the operator and a second arterial puncture was used for patients receiving intracoronary infusion. All patients received unfractionated heparin (5000 IU bolus), with additional doses as required to maintain an activated clotting time (ACT) of >250 s.

For intracoronary infusion studies, a 6Fr guide catheter was positioned at the ostium of the left main coronary artery. Patients received an intracoronary infusion

of sodium nitrite ($NaNO_2$, Tayside NHS, UK) at 8.7 μmol/min for 5 min, followed by 26 μmol/min for 5 min. This dose is estimated to achieve a maximal intracoronary concentration of approximately 1000 μM, using average resting coronary blood flow estimates as described in **Chapter 3** [208] and is equivalent to concentrations that when administered intra-arterially in the peripheral circulation are locally active (i.e. devoid of systemic effects)[83]. For intravenous infusion studies, sodium nitrite was administered at 50 μg/kg/min for 7 min via a canula in a large antecubital fossa vein. This dose was chosen to achieve physiologically significant reduction in systemic blood pressure and pulmonary capillary wedge pressure (i.e. both afterload and preload)[121]. The local concentration of nitrite in the coronary circulation after systemic infusion is estimated to be >100-fold lower than after intra-coronary infusion but achieves significant reduction in loading due to the generalized systemic actions. The direct myocardial actions of intracoronary nitrite could therefore be compared with the indirect effects (due to altered loading) of systemic nitrite. A micromanometer-conductance catheter (CD Leycom, Netherlands) was placed in the left ventricle to record steady-state LV PV relations via an Intra-Cardiac Analyser (INCA) console (CD Leycom, Netherlands). Measurements were also made of heart rate, central blood pressure and the ECG. All patients had a 3D transthoracic echocardiogram to estimate LV volumes, which were used for volumetric calibration. Dedicated software (CD Leycom Netherlands) was used for analysis of PV loop data including LV systolic and diastolic indices, as well as ventricular-arterial coupling (VA coupling, calculated as the ratio of arterial elastance to end systolic elastance, Ea/Ees)[167, 217]. Recordings were made at end-expiration. Ten beats were averaged to provide each data point.

Sample size and study end-points

Previous work reported that a bi-coronary infusion of sodium nitroprusside induced a decrease in LV end-diastolic pressure (LVEDP) from 18±5 mmHg to 12±3 mmHg[117], equating to an effect size of 1.37. We estimated that a single left coronary infusion of sodium nitrite might have an effect of two thirds of this magnitude, i.e. an effect size of 0.91. Therefore, with an alpha of 0.05 and power (1-beta) of 0.95, the required sample size was 18 for a primary end-point of reduction in LVEDP. 20 patients per group were recruited to allow for any drop-outs. Exploratory secondary end-points included other measures of LV systolic and diastolic function.

Statistical analyses

Analyses were performed using GraphPad Prism 8 (GraphPad Software Inc, USA). Shapiro-Wilk test was used to assess normality. Data are expressed as mean ± SEM unless otherwise stated. Intracoronary data were compared by repeated measures ANOVA with Tukey's post-test for multiple comparisons (or non-parametric equivalent). Student's t test was used to compare the intravenous data (change from baseline) as well as the effect of intracoronary versus intravenous nitrite on PV parameters. Linear regression analysis was used to test for correlation between measures of LV structure and changes in the primary end-point. $p<0.05$ was considered statistically significant.

4.3 Results

The baseline characteristics of the patients included in the study are shown in **Table 4**, while the patients' medications are listed in **Table 5.** All patients had a normal LV ejection fraction (EF). The studies were performed without clinical complications in any patient. **Table 6** shows baseline values for the measured variables. Baseline LVEDP was similar between the intracoronary and intravenous groups (11.0 mmHg [8.1, 14.3] vs 10.6 [4.7, 15.3] (median [IQR]) respectively).

The clinical indication for cardiac catheterisation in the patients enrolled in this study was to exclude obstructive coronary disease on the basis of symptoms and cardiovascular risk factors. Specifically, 33 patients described some form of chest discomfort, while 4 patients presented with a syncopal episode. A further 3 had palpitations. A single patient had shortness of breath (with added chest discomfort and palpitations) as their main presenting symptom, but with no other signs or symptoms to meet a clinical diagnosis of heart failure and a negative NTproBNP result. Of the 40 patients, 6 described breathlessness as a secondary symptom in addition to the primary symptom of chest discomfort. None of these patients had a clinical syndrome of heart failure nor a raised BNP or NTproBNP.

Characteristic	Intracoronary studies	Intravenous studies
Female (%)	8 (40)	7 (35)
Age (years)	64.8 ± 2.7	60.0 ± 2.3
BMI (kg/m^2)	30.8 ± 1.3	29.8 ± 1.0
Hypertension (%)	16 (80)	16 (80)
Smoking history (%)	7 (35)	10 (50)
Hypercholesterolaemia (%)	12 (60)	9 (45)
Diabetes (%)	4 (20)	4 (20)
Number of antihypertensives	1.8 ± 0.3	1.7 ± 0.3
Haemoglobin (g/dL)	135.0 [126.3, 142.8]	135.5 [124.3, 145.3]
Creatinine (mmol/L)	75.5 [64.8, 89.0]	82.5 [74.0, 93.0]
Left Ventricular Ejection Fraction (%)	58 [55.3, 62.2]	59.7 [56.05, 61.0]
LVESVI (ml/m^2)	19.8 [16.5, 24.1]	21.9 [15.4, 26.8]
LVEDVI (ml/m^2)	50.3 ± 3.3	51.0 ± 3.7
LV mass index (g/m^2)	84.3 ± 20.7	63.2 ±19.9
LA volume index (mm^3/m^2)	30.0 ± 9.1	26.3 ± 8.3
TAPSE (mm)	2.3 ± 0.3	2.2 ± 0.3
E/e'_{ave}	9.5 ± 3.6	8.5 ± 2.7

Table 4. Baseline characteristics of study participants. Parametric data are expressed as mean ± SEM, non-parametric data as median [IQR]. BMI, body mass index; LVESVI, LV end-systolic volume index; LVEDVI, LV end-diastolic volume index; TAPSE, tricuspid annular plane systolic excursion.

	Intracoronary, n (%)	Intravenous , n (%)
Antiplatelet agent	15 (75)	11 (55)
Anticoagulant	0 (0)	0 (0)
Beta-blocker	11 (55)	9 (45)
Other rate-limiting drug	1 (5)	1 (45)
ACEi/ARB/MRA	11 (55)	9 (45)
Diuretic	6 (30)	5 (25)
Other anti-hypertensive	10 (50)	4 (20)
Statin	15 (75)	7 (35)
Diabetic drug	4 (20)	7 (35)
Proton pump inhibitor	8 (40)	7 (35)
Oral Nitrate	0 (0)	0 (0)

Table 5. Medications of study participants, expressed as n (%). ACEi = Angiotensin Converting Enzyme inhibitor, ARB = Angiotensin Receptor Blocker, MRA = Mineralocorticoid Receptor Antagonist

	Intracoronary studies	Intravenous studies
Heart rate (bpm)	64.9 ± 2.4	62.3 ± 2.5
Mean Arterial Pressure (mmHg)	99.3 [89.7, 110.3]	93.0 [86.0, 107.0]
LVEDV (ml)	99.3 ± 8.0	104.7 ± 7.6
LVESV (ml)	42.2 ± 3.9	42.7 ± 4.2
LV End-systolic elastance	2.8 [1.7, 3.8]	3.1 [2.4, 6.3]
Stroke Work (cJ)	7043 ± 624	7998 ± 523
dP/dt_{max} (mmHg/s)	1288 ± 45	1347 ± 44
dP/dt_{min} (mmHg/s)	-1465 ± 61	-1415 ± 56
LVEDP (mmHg)	11.0 [8.1, 14.3]	10.6 [4.7, 15.3]
LVESP (mmHg)	138.3 ± 5.0	143.1 ± 6.4
EDPVR (mmHg/ml)	0.11 [0.08, 0.15]	0.08 [0.05, 0.15]
LVEST (ms)	425.7 ± 11.1	432.60 ± 10.0
Tau (ms)	33.5 [30.0, 35.8]	32.1 [29.6, 35.7]
LVEF (%)	65.7 ± 1.9	67.6 ± 1.9
EF1 (%)	22.5 ±2.1	23.0 ± 2.1
Starling Contractile Index	13.7 [10.1, 18.4]	13.7 [9.5, 16.6]
Cardiac Output (L/min)	4.4 ± 0.4	4.4 ± 0.2
Arterial Elastance	2.2 ± 0.2	2.1 ± 0.2
VA coupling	0.6 ± 0.03	0.6 ± 0.05

Table 6. Baseline data describing recorded variables for intracoronary and intravenous nitrite groups. Parametric data expressed as mean ± SEM, non-parametric data expressed as median [IQR].

Effects of intracoronary nitrite infusion

Intracoronary nitrite had no significant effect on heart rate or mean arterial blood pressure (MAP), consistent with a lack of systemic effect (Error! Reference source not found. **A-B**). Markers of LV systolic function, namely LV end-systolic elastance (Ees), stroke work and dP/dt_{max} were unaltered by intracoronary nitrite (Error! Reference source not found. **E-G**). However, there was a significant decrease in the primary end-point, LVEDP, following intracoronary nitrite (p=0.004) (Error! Reference source not found. **I**). When considered as change from baseline, the 26 μmol/min nitrite dose decreased LVEDP by 1.9 mmHg [-3.3, -0.5] (mean [95% CI]), p=0.006. Intracoronary nitrite also significantly decreased EDPVR, while the time to LV end-systole (LVEST) was decreased by 11 ms [-19, -4] (p=0.002) at the higher dose of nitrite (Error! Reference source not found. **J-K**). There were no significant changes in dP/dt_{min}, tau or LV volumes (**Error! Reference source not found. C-D,H,L**).

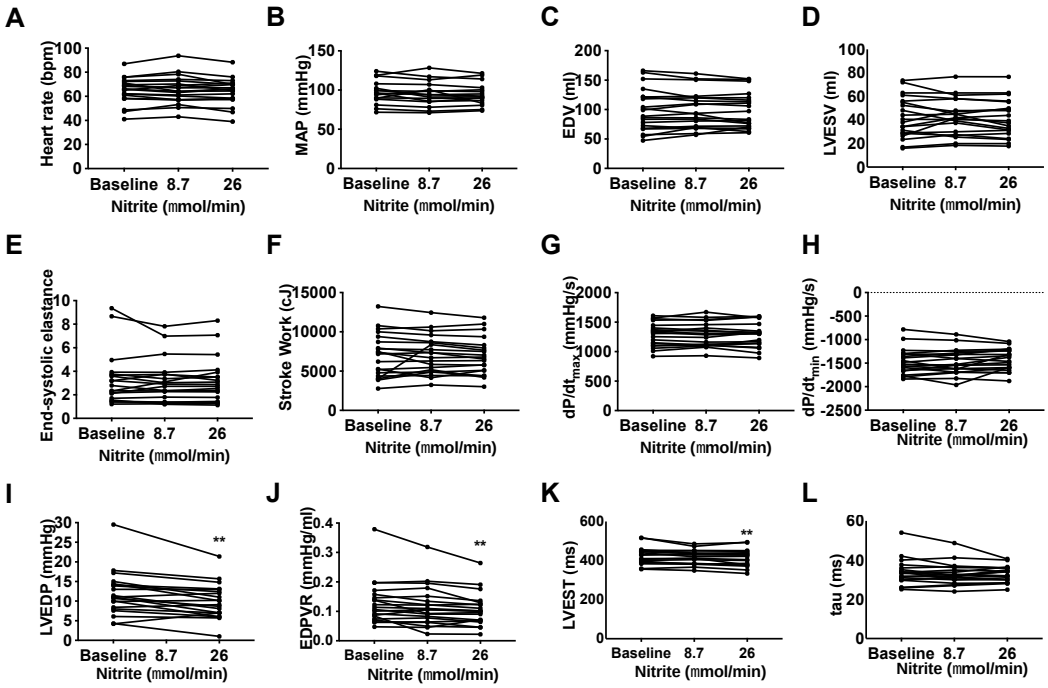

Figure 14. Effect of intracoronary nitrite on parameters of LV function. A: Hearate; B: MAP, mean arterial pressure; C: LVEDV, end-diastolic volume; D: LVESV, end-systolic volume; E: Ees, end-systolic elastance; F: SW, stroke work; G: dP/dt_{max}; H: dP/dt_{min}; I: LVEDP, LV end-diastolic pressure; J: EDPVR, end-diastolic pressure-volume relation; K: LVEST, time to LV end-systole (LVEST); L: tau. **p<0.01. n=17 for LVEST. N=20 for all other parameters.

Effects of intravenous nitrite infusion

Intravenous nitrite resulted in a significant decrease in MAP of 6.9 mmHg [-4.3, -9.5] (mean [95% CI]), p<0.0001, but had no effect on heart rate (**Figure 15 A**-**B**). Consistent with a reduction in afterload, the arterial elastance (Ea) decreased from 2.1 ± 0.7 to 1.9 ± 0.7 (p=0.002). There was no change in the total peripheral resistance: mean change -0.7 [-2.2, +0.7] (mean [95% CI]), p=0.3. Intravenous nitrite also induced a significant reduction in LVEDV (-8.3 ml [-15.4, -1.1] (mean [95% CI]), p=0.03), consistent with a decrease in preload (**Figure 15 C**). No changes were observed in Ees or dP/dt_{max} while stroke work decreased significantly: -829 centijoules (cJ) [-1327, -331] (mean [95% CI], p=0.003) (**Figure 15 E**-**G**). Intravenous nitrite caused a significant reduction in LVEDP from a baseline of 10.6 mmHg [4.7 – 15.3] (median [IQR]) to 5.2mmHg [2.9, 9.9], p<0.0001 (**Figure 15 I**). Intravenous nitrite also resulted in significant decreases in EDPVR, LVEST, dP/dt_{min} and tau (**Figure 15 H, J**-**L**). There was no significant change in ventricular-arterial coupling (Ea/Ees from 0.6 ± 0.2 to 0.5 ± 0.3, p=0.06).

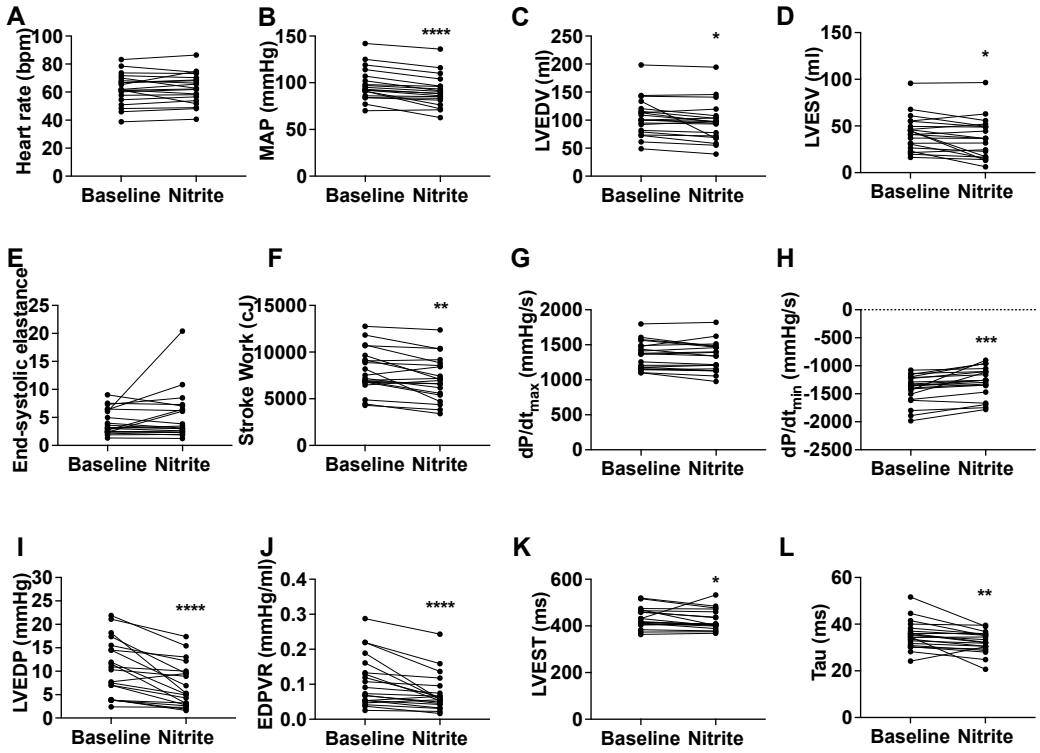

Figure 15. Effect of intravenous nitrite (50 μg/kg/min) on parameters of LV function. **A**: Heart rate; **B:** MAP, mean arterial pressure; **C:** LVEDV, end-diastolic volume; **D:** LVESV, end-systolic volume; **E:** Ees, end-systolic elastance; **F:** SW, stroke work; **G:** dP/dt_{max}; **H:** dP/dt_{min}; **I:** LVEDP, LV end-diastolic pressure; **J:** EDPVR, end-diastolic pressure-volume relation; **K:** LVEST, time to LV end-systole (LVEST); **L:** tau. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=19 for MAP and LVEST, n=20 for all other parameters.

Comparison of intracoronary versus intravenous nitrite

Representative PV loops showing the effect of intracoronary and intravenous nitrite infusion are shown in **Figure 16** and suggest that intravenous infusion had a larger effect. **Figure 17** shows a quantitative comparison of the effects of intravenous and intracoronary infusion. Intravenous nitrite had significantly greater effects than intracoronary nitrite on MAP (**Figure 17 I**), LV end-systolic pressure (LVESP) (**Figure 17 F**), and tau (**Figure 17 D**). While the mean decrease in LVEDP following intravenous nitrite was numerically greater than after intracoronary infusion (**Figure 17 E**), this was not statistically significant.

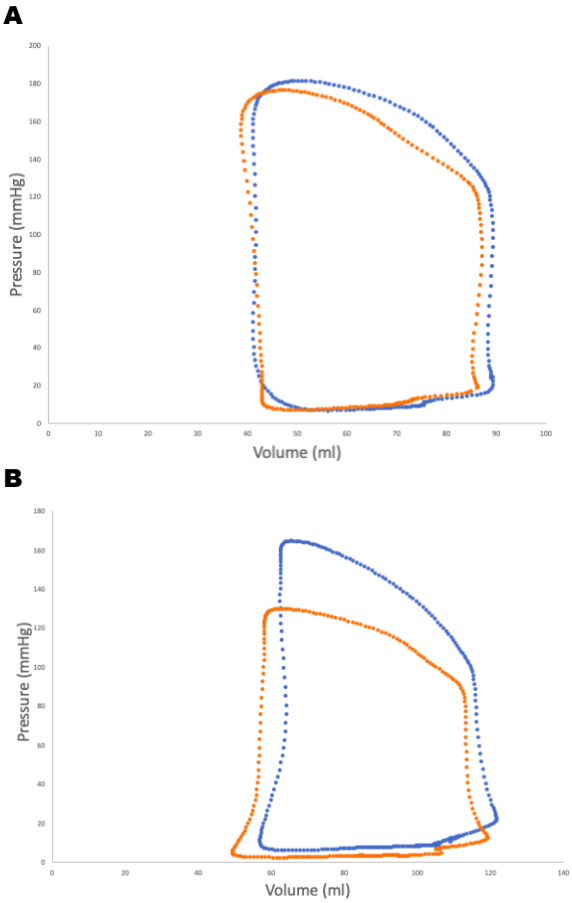

Figure 16. Representative pressure volume loops. **A**: Intracoronary nitrite. **B**: Intravenous nitrite. Blue loops represent baseline values. Orange loops represent response to inorganic nitrite.

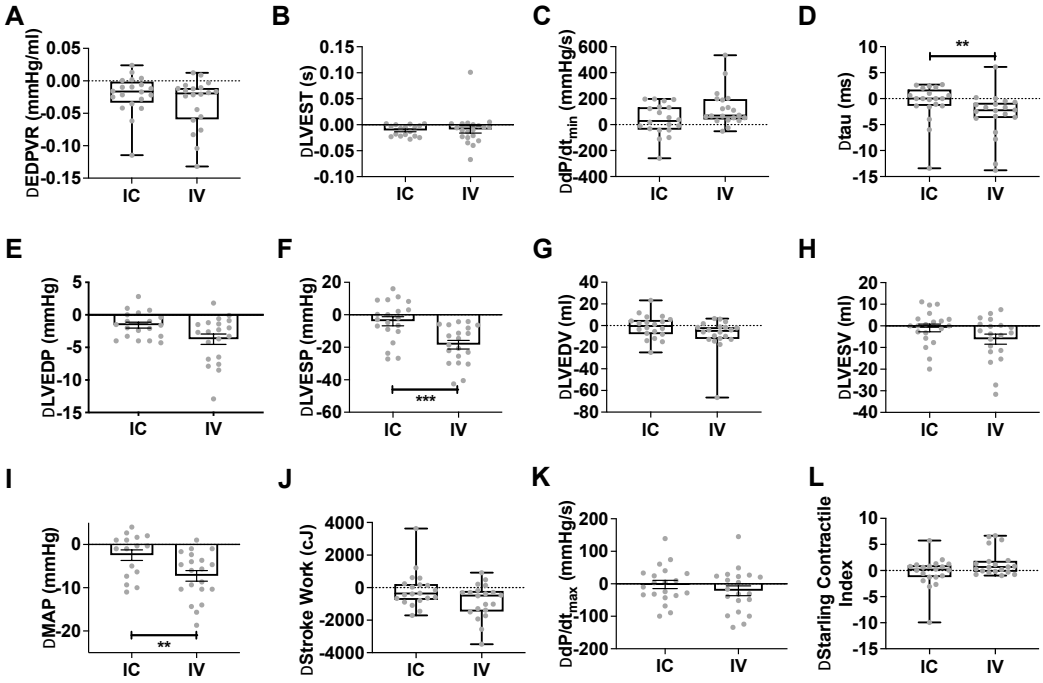

Figure 17. Comparison of effect between intracoronary and intravenous nitrite. **A:** EDPVR, End-diastolic pressure volume relationship; **B:** LVEST, time to LV end-systole; **C:** dP/dt_{min}; **D:** tau; **E:** LVEDP, LV end-diastolic pressure; **F:** LVESP, LV end-systolic pressure; **G:** LVEDV, LV end-diastolic volume; **H:** LVESV, LV end-systolic volume; **I:** MAP, Mean arterial pressure; **J:** Stroke work; **K:** dP/dt_{max}; **L:** Starling Contractile Index. IC= Intracoronary nitrite, IV= Intravenous nitrite. **p<0.01, ***p<0.001.

Recently, first-phase ejection fraction (EF1) - which represents the proportion of blood ejected from the LV from the onset of systole to the time of the first peak of LV pressure – has been suggested as an index that assesses systolic function early during contraction and reflects systolic-diastolic coupling[185, 186]. A reduced EF1 reflects impaired early systolic dysfunction and is typically accompanied by

impaired diastolic function. We therefore assessed the effect of intracoronary and intravenous nitrite on EF1 (**Figure 18**). While intracoronary nitrite had no significant effect on EF1 (p=0.5 by 1-way ANOVA) (**Figure 18 A**), intravenous nitrite induced a marked increase in EF1 (**Figure 18 B-C**). From a baseline of 23.0±2.1%, the EF1 post-nitrite was 34.2±3.1% - a relative increase of approximately 50% as illustrated by the representative traces in **Figure 18 D-E**.

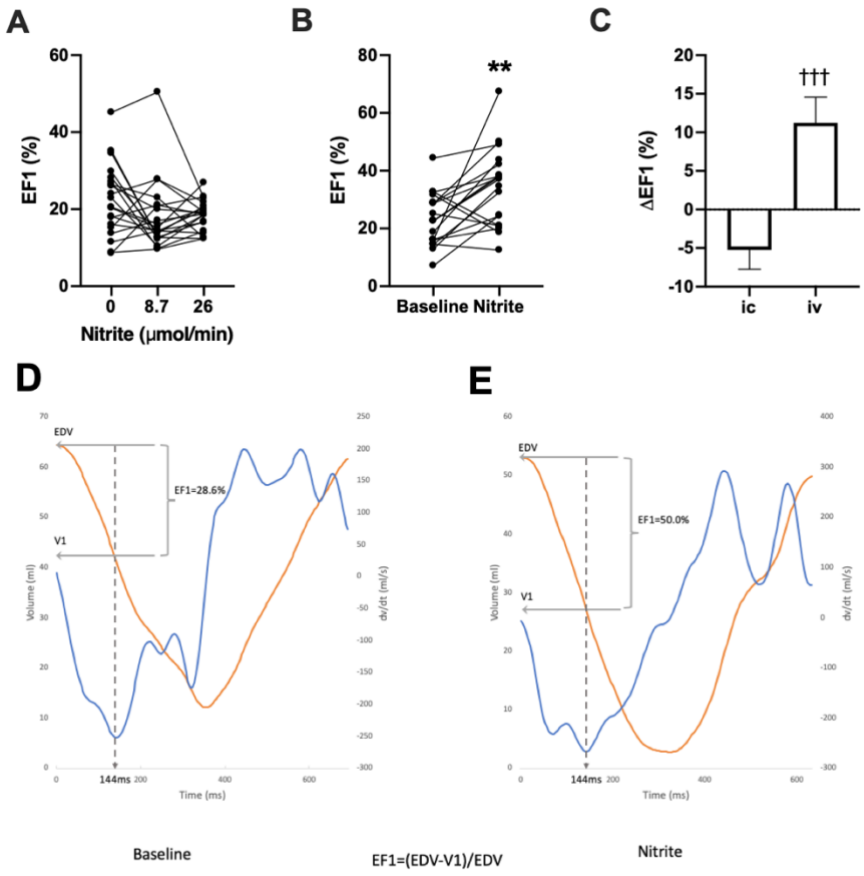

Figure 18. The effect of nitrite on EF1. **A**, Intracoronary nitrite (n=20). **B**, Intravenous nitrite (n=19). **C**, Comparison of peak change after intracoronary versus intravenous nitrite. **D,** Representative baseline trace of LV volume (orange) and dv/dt (blue) with EF1 calculation demonstrated. **E,** Representative trace of LV volume (orange) and dv/dt (blue) following intravenous nitrite with EF1 calculation demonstrated. **p<0.01 vs baseline, †††p<0.001 vs intracoronary nitrite.

Association between baseline LV structure and function and the effect of nitrite

To assess whether inter-individual variation in the response to nitrite might be related to baseline cardiac structure, we determined the association between LV mass (LVMI) and the magnitude of change in LVEDP but found no significant correlation either in the intracoronary or intravenous nitrite groups (**Figure 19 A-B**). We also assessed whether the magnitude of reduction in LVEDP was related to baseline LV EDPVR (as a marker of end-diastolic stiffness). There was a significant association between the nitrite-induced decrease in LVEDP and baseline EDPVR for both the intracoronary group (R^2=0.33, p=0.008) (**Figure 19 C**) and the intravenous group (R^2= 0.38, p=0.004) (**Figure 19 D**).

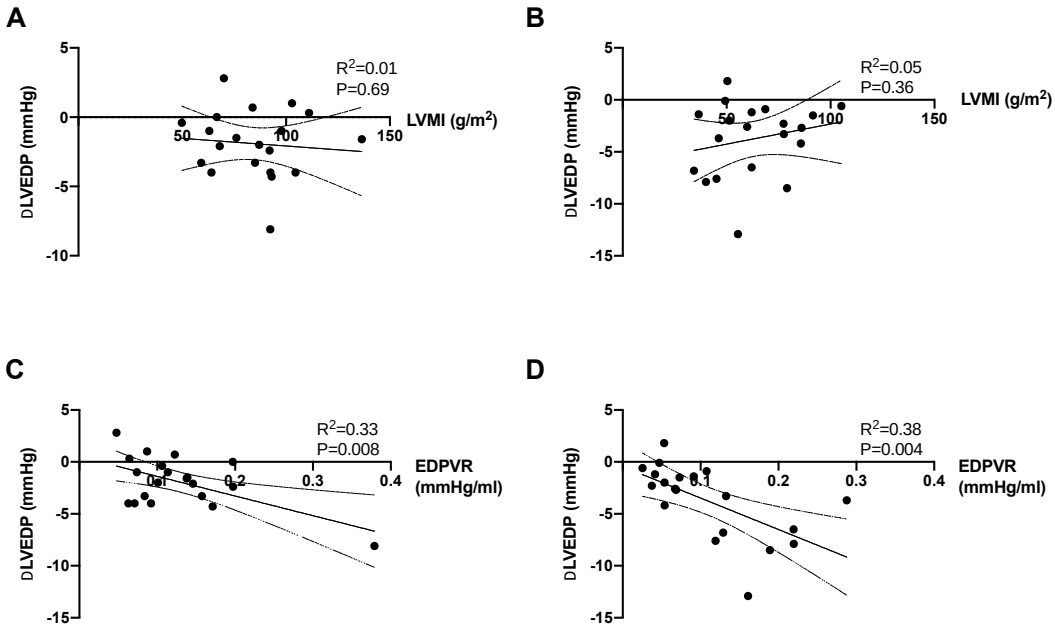

Figure 19. Correlation between LV mass and change in LVEDP (**A, B**) and between baseline EDPVR and change in LVEDP (**C, D**). A and C show data for intracoronary nitrite and B and D show data for intravenous nitrite.

Accounting for outlier data

Three patients in the intracoronary studies and 5 patients in the intravenous studies had a baseline LVEDP >15 mmHg. We therefore performed a further analysis to assess whether excluding these patients had any effect on the primary end point. Excluding patients with LVEDP >15mmHg, intracoronary nitrite still significantly decreases LVEDP: -1.4mmHg [-2.7, -0.01] (mean [95% CI]), $p<0.05$, n=16 and intravenous nitrite also still significantly decreases LVEDP: -2.0 mmHg [-0.9, -4.2], $p=0.001$, n=15, see **Figure 20**.

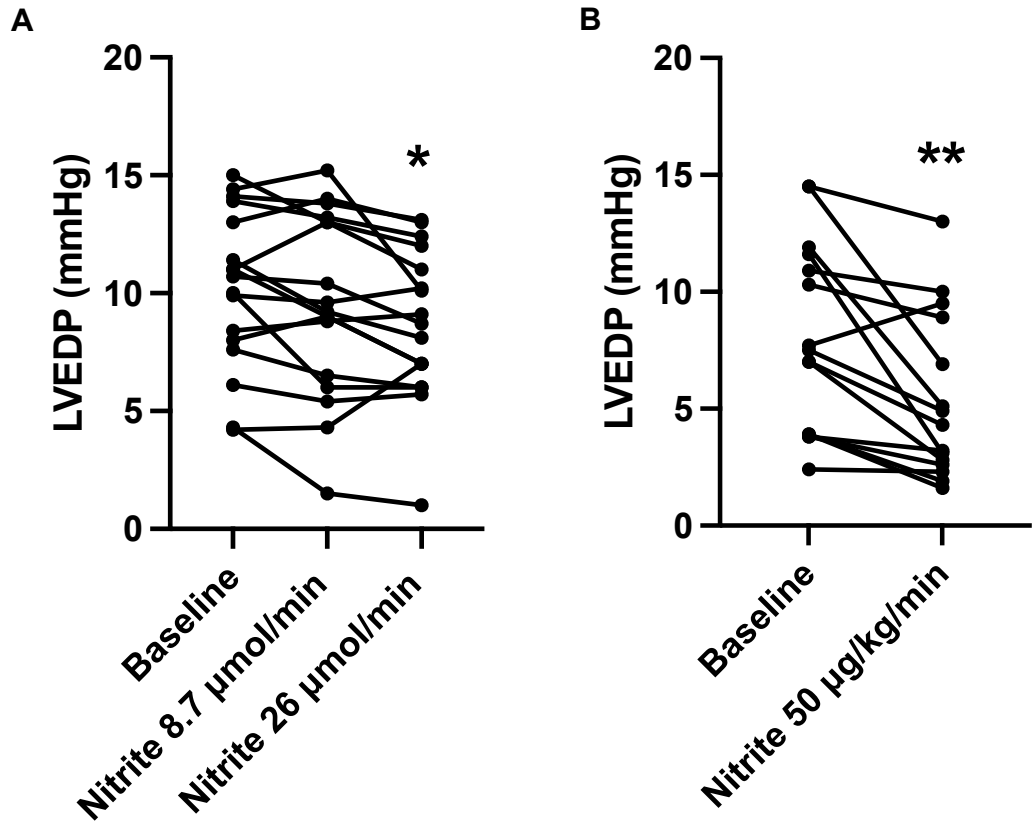

Figure 20. The effect of intracoronary (Panel A) and intravenous (Panel B) on LVEDP. *$p<0.05$ vs baseline (analysed by one way ANOVA), **$p<0.01$ (analysed by t-test).

4.4 Discussion

In this study, the direct and indirect acute effects of inorganic nitrite on contractile function of the human heart have been examined in detail. Several important findings are demonstrated that may have relevance to the potential therapeutic use of nitrite.

Firstly, inorganic nitrite delivered via the intracoronary route results in a small but nonetheless statistically significant decrease in LVEDP and EDPVR as well as hastening the onset of LV relaxation (i.e. decreasing LVEST). Although the magnitude of alterations in diastolic properties may not be considered to be clinically important, the findings do provide important mechanistic insight into nitrite's actions on the heart: these effects are not accompanied by any change in blood pressure or heart rate, consistent with a local action on the heart. Furthermore, the nitrite-induced changes in LV diastolic function occur without any alteration in indices of LV systolic function, indicating a selective effect on the diastolic properties of the heart. The data from **Chapter 3 (study 1)** demonstrates that the dose of intracoronary nitrite used in this study does not significantly alter coronary blood flow[208]. Taken together, these data suggest that nitrite has a direct and selective action on the myocardium to reduce ventricular stiffness and hasten the onset of relaxation. No change in tau or LV dP/dt_{min} is observed following intracoronary nitrite, indicating that although nitrite improves LV compliance (a passive property), there is a lack of effect on active (ATP-dependent) myocardial relaxation. This pattern of effect on LV contractile function is consistent with prior studies reporting selective effects of NO donors and NO-cGMP signaling on the onset of relaxation and diastolic stiffness, both in isolated preparations and in humans in vivo[26, 117, 214, 218]. At a mechanistic level, such actions are considered to involve

cGMP/protein kinase G (PKG)-mediated phosphorylation of troponin I and titin in cardiomyocytes[32, 219-221]. However, other NO-mediated mechanisms such as altered S-nitrosylation of contractile proteins or proteins involved in sarcoplasmic reticulum calcium uptake could also be involved[13, 222]. The current study was not designed to test such mechanisms.

Secondly, the results of the intravenous infusion studies indicate that systemic administration of nitrite reduces LV preload (LVEDV) and afterload (MAP and Ea) and is associated with more marked effects on LV diastolic function than with intracoronary infusion. In addition to decreases in LVEDP, EDPVR and LVEST, intravenous nitrite significantly accelerates tau and reduces LV dP/dtmin. These effects are likely to be indirect rather than direct myocardial since the local intracoronary concentration of nitrite achieved with systemic infusion is estimated to be substantially lower than with intracoronary infusion. Therefore, nitrite-induced alteration in loading conditions has a greater impact on LV diastolic function than direct myocardial actions in this patient group. The pattern of nitrite's effect on afterload, with a significant decrease in arterial elastance and MAP but no effect on total peripheral resistance, is consistent with nitrite exerting a preferential effect on conduit arteries over resistance arterioles. Indeed, nitrite is known to be an arterial dilator with selectivity for conduit arteries versus the microvasculature[83], and to decrease pressure wave reflections in the arterial tree and thereby decrease the late-systolic load on the LV[223]. The reduction in preload by intravenous nitrite is likely to be due to an effect on venodilation and venous capacitance, and is consistent with prior knowledge that nitrite reduction to NO is facilitated by hypoxia (as in the venous circulation)[81, 82, 224].

Thirdly, intravenous nitrite infusion significantly increases EF1 - a hemodynamic index that describes the proportion of LV ejection that occurs up to the time of maximal rate of ventricular contraction[185]. A decreased EF1 has recently been shown to reflect early systolic dysfunction, even in patients in whom the overall EF is within the normal range, and to strongly correlate with abnormal LV diastolic function as indexed by an elevated E/e' ratio on echocardiography.[185] EF1 has therefore been proposed as an index that reflects coupling between systolic and diastolic function. Furthermore, a decreased EF1 appears to predict a worse prognosis in patients with aortic stenosis or heart failure[186, 187]. In the present study, the intravenous nitrite-induced increases in EF1 suggest that an improvement in early ejection phase systolic function is seen in addition to the changes in diastolic function. Given that no change in EF1 was seen with intracoronary infusion of nitrite, it is likely that the increase in early ejection phase systolic performance seen following intravenous nitrite infusion is due to improved cardiac loading conditions, rather than a direct effect on myocardial systolic function.

A careful assessment of the systemic versus direct actions of NO donors on cardiac contractile function has not previously been undertaken. It was reported that intracoronary infusion of the NO donor sodium nitroprusside decreased LVEDP and LVEST (similar to the current study) but that investigation did not involve measurement of PV loops nor the assessment of the effects of systemic administration[117].

There has been considerable interest regarding nitrite's therapeutic potential in conditions associated with decreased NO bioavailability. One such condition is HFpEF, in which comorbidities such as hypertension and diabetes/obesity drive a

pro-inflammatory state leading to peripheral and coronary endothelial dysfunction. The subsequent dysfunctional NO signaling leads to vascular and myocardial stiffening. In the heart, decreased myocardial PKG activity and therefore decreased titin phosphorylation result in decreased myocardial compliance[225]. Furthermore, patients with HFpEF have several systemic abnormalities e.g. altered cardiac loading and abnormal vascular reserve, which would be expected to be beneficially influenced by systemic interventions[226]. Recent studies have focused on the potential clinical utility of nitrite to improve exercise hemodynamics in patients with HFpEF or to reduce pulmonary hypertension and filling pressures in such patients. Borlaug et al. reported that systemic nitrite infusion[121] reduced pulmonary capillary wedge pressure (PCWP) during exercise. Furthermore, both Borlaug *et al* and Simon *et al* reported that inhaled nitrite reduced PCWP and pulmonary pressures in patients with HFpEF who had pulmonary hypertension[123, 212]. While these studies provided an assessment of pulmonary hemodynamics and LV filling pressures (from the PCWP), they did not directly assess LV contractile function nor the relative impact of systemic versus local nitrite actions. This may be relevant because a recent randomised multi-centre clinical trial of inhaled nitrite in 105 patients with HFpEF failed to show any benefit with respect to echocardiographic filling pressures or exercise capacity[124]; inhaled nitrite may be considered more analogous to local than systemic delivery. In the current study, using comprehensive LV PV analyses, we find that the acute hemodynamic effects of nitrite in subjects with normal LV function are significantly more pronounced with intravenous (systemic) administration than local cardiac administration, with the enhanced effect driven by changes in preload and afterload. Whether the same would be the case in patients with congestive heart failure or HFpEF requires further study. It is of

interest that patients with congestive heart failure are reported to demonstrate arterial hyper-responsiveness to an intra-arterial infusion of sodium nitrite[224], while patients with HFpEF demonstrate blood pressure hyper-responsiveness to sodium nitroprusside compared to HFrEF, but for a lesser increase in stroke volume and cardiac output, suggesting that excessive vasodilatation may be deleterious in HFpEF[227]. In the arterial system, sodium nitroprusside acts mainly on small resistance arterioles, while nitrite is selective for large conduit arteries[83, 208]. Nitrite may therefore be better suited to target abnormalities in central arterial stiffness and therefore ventricular-vascular interaction that are seen in HFpEF, while avoiding adverse excessive vasodilatation. This hypothesis should be tested in comparative studies.

Study limitations

This study involved recruitment of consecutive patients firstly to the intracoronary studies and then to the intravenous studies. As such, the lack of randomisation is a key limitation. However, given the need for an additional point of arterial vascular access for the intracoronary studies (for the coronary guide catheter) versus the intravenous studies, it was not considered appropriate to randomise patients: some patients may not have been willing to consent to be randomised when there was potential for a further arterial puncture. We studied a relatively small number of subjects in total, many of whom had risk factors for cardiovascular disease and were on medications. Furthermore, some of the patients had increased LV mass or raised LV filling pressures at baseline. As such, our study population is not considered to consist of "healthy volunteers". The possibility that the magnitude or pattern of observed effect may vary depending on risk factors cannot therefore be assessed.

The average LV mass index was within the normal range. However, due to the fact that some patients had a raised LV mass index (and therefore had a structurally abnormal heart), we did look for any correlation between LV mass and the magnitude of effect on LVEDP. We found no significant relationship. However, the magnitude of reduction in LVEDP was related to the baseline EDPVR, suggesting that patients with diastolic dysfunction may be more responsive to the effects of nitrite. The current study only looked at the acute effects of a single nitrite infusion at rest. It is possible that the effects may be larger upon exercise and the results also cannot necessarily be extrapolated to the effects of chronic administration. EDPVR was assessed using the single beat method, rather than through vena cava occlusion to induce a loading change, but the single beat method is known to reliably detect the acute effects of interventions. As is common in clinical studies, our dataset contained a number of outlier data points. However, repeating the analyses without inclusion of such outliers did not alter the pattern of results.

A previous landmark study assessing the effect of an NO donor, sodium nitroprusside, on LV function in humans involved drug delivery through a bi-coronary infusion i.e. a guide catheter at the left main coronary artery ostium and one at the right coronary artery ostium[117]. A bi-coronary infusion of sodium nitrite would have required a second guide catheter and therefore a third arterial access site: two guide catheters and the LV conductance catheter. We felt that a research study that required three arterial access sites, only one of which was for clinical reasons (the original clinically indicated coronary angiogram) with the additional two being purely for research reasons, would have been difficult to justify, particularly as each additional arterial puncture is associated with an increase in bleeding risk. We also felt that potential participants may have felt that three arterial

punctures would be too much of a burden and this would possibly affect their willingness to consent for the study and therefore impede timely recruitment of the study sample. We chose to infuse the sodium nitrite infusion down the left main coronary artery to ensure we were able to deliver the study drug to as large a proportion of the myocardium as possible. Taking the above reasons together, we feel that using a single guide catheter to deliver the study drug down the left main coronary artery represents a balance between different aspects of study feasibility.

In conclusion, we have undertaken a comprehensive characterisation of the acute effects of intracoronary and intravenous nitrite on human cardiac contractile function. Our findings demonstrate that nitrite induces selective effects on LV diastolic function and the onset of LV relaxation which involve both direct effects on the heart and effects secondary to altered cardiac loading. This profile of effect could be beneficial in conditions characterized by LV diastolic dysfunction whether due to cardiac pathology or secondary to abnormal cardiac loading.

Chapter 5. Enhanced blood pressure-lowering effects of a beetroot juice and grapefruit juice cocktail compared to beetroot juice alone: a randomised cross-over study

5.1 Introduction

The studies presented so far have addressed the physiological actions of inorganic nitrite in the cardiovascular system in humans. In this Chapter, the work focuses on potential ways of enhancing nitrite levels. Dietary inorganic nitrate (NO_3^-) is found in beetroot and green leafy vegetables. Beetroot juice has been shown to produce beneficial cardiovascular effects[228], decreasing blood pressure (BP) in both healthy volunteers[58] and in patients with hypertension[90, 93, 229]. Furthermore, independent of peripheral BP, in hypertensive patients with or at risk of diabetes mellitus, chronic treatment with beetroot juice over 6 months results in beneficial changes in cardiac chamber volume[99]. Dietary nitrate also has additional benefits on platelets, endothelial function and mitochondrial efficiency[10, 11].

Much of the recent focus on the nitrate-nitrite-NO pathway has been on the mechanisms of bioactivation: nitrate reduction to nitrite (i.e. via the enterosalivary circulation and lingual bacterial nitrate reductases[50, 52]), and nitrite reduction to NO (e.g. by deoxyhaemoglobin[81]). However, the redox nature of these metabolic processes permits re-oxidation of NO back to nitrite and then nitrate. Indeed, before the enterosalivary pathway was characterised, nitrate and nitrite were thought to be merely biologically inert by-products of NO oxidation, rather than key components of an important NO synthase (NOS)-independent mechanism of NO production.

The cytochrome P450 (CYP) enzymes, particularly CYP3A, present in gut enterocytes and the liver [230] play a major role in drug metabolism and may also be involved in nitrite oxidation. Using troleandomycin to inhibit CYP3A4, Curtis et al. recently demonstrated inhibition of nitrite re-oxidation back to nitrate in rat liver homogenates[231]. Grapefruit juice is recognised as interacting with many drugs via inhibition of CYP3A4[232], attributed to furanocoumarins in the juice, particularly 6',7'-dihydroxybergamottin (DHB)[233]. Thus, it is plausible that the co-ingestion of grapefruit juice with beetroot juice would decrease CYP3A4-mediated oxidation of nitrite to nitrate, resulting in an increased plasma nitrite concentration.

We hypothesised that grapefruit juice, when co-ingested with beetroot juice, would potentiate beetroot juice's BP-lowering effect, via increased plasma nitrite concentration due to furanocoumarin-mediated CYP3A4 inhibition of nitrite oxidation.

5.2 Methods

Approvals

Ethical approval for this study was obtained from the South East London Research Ethics Committee (REC) (10/H0802/52). Written informed consent was obtained from all volunteers prior to commencing any protocol-related procedures.

Participants

Participants were healthy volunteers aged 18-45 years old, with normal BP (SBP 90-140 mmHg and DBP <90 mmHg), a body mass index (BMI) of 18-40 kg/m^2 and without any recent illness or regular systemic medication (other than the oral contraceptive pill).

Study Design

A 3-visit randomised (for the two active beetroot juice visits), single-blind (with respect to Active versus Placebo beetroot juice), placebo-controlled crossover intervention design was used. The 3 different visits involved the consumption of either:

- Active beetroot Juice (nitrate-containing: ~0.4 g) and golden grapefruit juice (Active Beet+GFJ) i.e. 70 ml of nitrate-containing beetroot juice shot ('Beet It' James White Drinks, UK) with 250 ml of grapefruit juice (Golden Grapefruit Juice, Tropicana UK Ltd, UK).
- Active beetroot Juice and Water (Active Beet+H_2O) i.e. 70 ml of nitrate-containing beetroot juice shot + 250 ml of low-nitrate water (<0.1 mg/l, Buxton Mineral Water, UK).

- Placebo beetroot Juice (nitrate-depleted) and grapefruit juice (Placebo Beet+GFJ) i.e. 70 ml of nitrate-depleted beetroot juice shot (James White Drinks, UK) + 250 ml of grapefruit juice. The placebo beetroot juice is identical in appearance, taste, and smell to the active beetroot juice. This visit was performed once the volunteer had completed the two Active beetroot juice visits.

Each of the visits lasted 7 hours. For the duration of the visit, participants sat in an examination chair with their feet resting on a stool (to avoid changes in posture affecting plasma [nitrite])[234]. Blood pressure was measured in triplicate every 15 minutes from 1 hour (T=-1) before ingestion of the juice intervention at (T=0) and until 6 hours post-ingestion. The BP for an individual at a given time point was taken as the average of the three (triplicate) readings. The BP readings from timepoints T=-1 to T=0 were averaged to produce a baseline reading. Blood pressure measurements were taken according to guidelines, using an automated BP monitor (Intellisense 705IT, Omron, UK). To avoid the action of BP recording interfering with the measurement of plasma [nitrate]/[nitrite][136, 235], BP recordings were taken from the arm contralateral to that from which blood samples were taken. Recordings of heart rate were taken alongside BP recordings.

The room in which the study took place was not actively temperature regulated, however the room temperature was monitored throughout the study protocol.

Volunteers were asked to fast (including avoidance of caffeine) for 12 hours before the study and to avoid high nitrate containing food, strenuous exercise and nicotine (smoking) for 24 hours before the study. To ensure participants remained hydrated

during the study visit, we adopted an "optimised hydration protocol" whereby at every hour following the juice intervention, participants consumed 250 ml of low-nitrate water (<0.1 mg/l, Buxton Mineral Water, UK). Participants were given two slices of toasted Hovis wholemeal thick brown bread just after T=3 h.

Volunteers were asked to rate the taste of each intervention by giving it a score of between 1-10 (disgusting-delicious), immediately after drinking the intervention.

Sample Collection

Blood and saliva samples were taken immediately prior to ingestion of each of the juice interventions, then at 30-minute intervals for the first 3 hours, and hourly for the final 3 hours. At time points where BP and blood samples were taken, BP recordings were taken prior to blood sampling. Urine samples were collected every hour, and the pH and volume recorded. A urine dipstick was performed upon collection of the first urine sample to exclude the possibility of high nitrite concentrations due to the presence of a urinary tract infection.

At each blood draw, 6 ml of venous blood was drawn into a chilled syringe then transferred to a chilled green lithium heparin tube (Vacutainer®, BD). Samples were immediately centrifuged at 4^{o}C and 2000x *g* for 5 minutes (MIKRO 220R, Hettich, Germany), after which plasma was collected.

Prior to collection of saliva, volunteers were asked to avoid swallowing saliva for 2 minutes. They were then asked to drool all saliva into a collecting tube. The volume of saliva collected was recorded.

All plasma, saliva, and urine samples were divided into two chilled 2ml tubes and stored at -80^{o}C.

Sample Analysis

Plasma and saliva samples were analysed for nitrate and nitrite concentrations using chemiluminescence, as described previously[194]. The quantification of nitrate and nitrite in plasma and saliva was performed by an investigator who was blinded to the treatment allocation.

Data Analysis

Data was analysed using GraphPad Prism 8.0 (GraphPad Software Inc.). All data are expressed as mean±SEM unless otherwise stated (e.g. non-parametric statistics (median [IQR]) for non-normally distributed data). Data were compared by 2-way ANOVA and/or 1-way ANOVA as appropriate, with Fisher's LSD post-test. $p<0.05$ was considered statistically significant.

5.3 Results

A total of 11 volunteers were enrolled in the study (of 11 potential volunteers screened, all 11 were eligible and randomised). Volunteer baseline demographics for the 11 participants are shown in **Table 7**. Of the 11 volunteers, 9 completed all 3 visits (the other 2 volunteers did not attend the Placebo Beet+GFJ visit). There were no adverse events attributed to study participation.

N	11
Gender (n, male)	8
Age, years	23± 4
Weight, kg	60.9 ±9.8
BMI, kg/m^2	21.3 ± 1.9
HR, bpm	80 ± 14
SBP, mmHg	122 ± 8
DBP, mmHg	75 ± 8

Table 7. Clinical parameters of participants, taken at time of screening. BMI is body mass index; HR is heart rate; SBP is systolic blood pressure; DBP is diastolic blood pressure. Data are mean ± SD

Plasma

The addition of Grapefruit juice to Active beetroot Juice (Active Beet+GFJ) had no effect on plasma [nitrate] compared to Active beetroot Juice with water (Active Beet+H_2O); p=0.38 (**Figure 21 A**). Plasma nitrate was not measured for the Placebo Beet + GFJ intervention.

As anticipated, both Active Beet+GFJ and Active Beet+H_2O significantly increased plasma [nitrite] compared to Placebo Beet+GFJ (both $p<0.0001$). Comparison between the two Active Beet interventions found that the addition of Grapefruit juice decreased mean plasma [nitrite] by ~14%; p=0.006 (**Figure 21 B**).

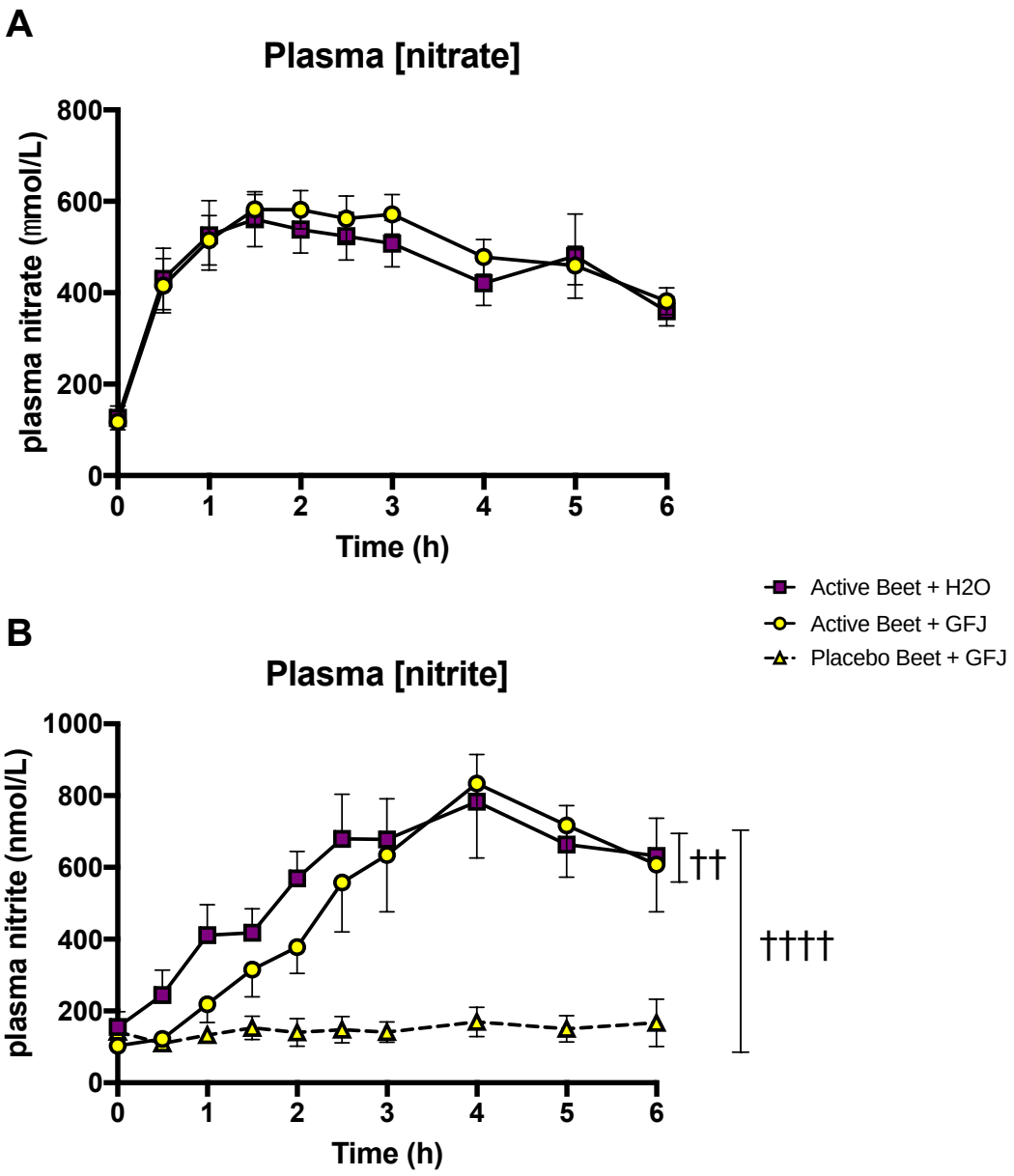

Figure 21. The effect of grapefruit juice and beetroot juice (given at T=0h) on plasma [nitrate] and [nitrite]. **A**: Plasma [nitrate], *n*=11, (note: not measured for the Placebo Beet + GFJ intervention) **B**: Plasma [nitrite] *n*=11 for Active Beet + GFJ and Active Beet H_2O, *n*=9 for Placebo Beet + GFJ. Data shown as mean ± SEM. Statistical significance shown as, $^{††}p<0.01$, $^{††††}p<0.0001$ as analysed by 2-way ANOVA between the curves.

Saliva

Grapefruit juice decreased nitrate secretion into the mouth (measured as total salivary nitrate and nitrite production), which was lower in Active Beet+GFJ than Active Beet+H_2O (p=0.02), with the peak difference occurring at T=1.5 h: -437 μmol/h (95% CI -844 to -30); see **Figure 22 A**. Salivary nitrite production was markedly decreased by grapefruit juice: i.e. lower with Active Beet+GFJ than Active Beet+H_2O (p=0.002); see **Figure 22 B**. Salivary [nitrate]:[nitrite] was higher with Active Beet+GFJ versus Active Beet+H_2O (p=0.01), with the peak difference occurring at T=0.5 h: +7.4 (95% CI +1.5 to +13.3); see **Figure 22 C**.

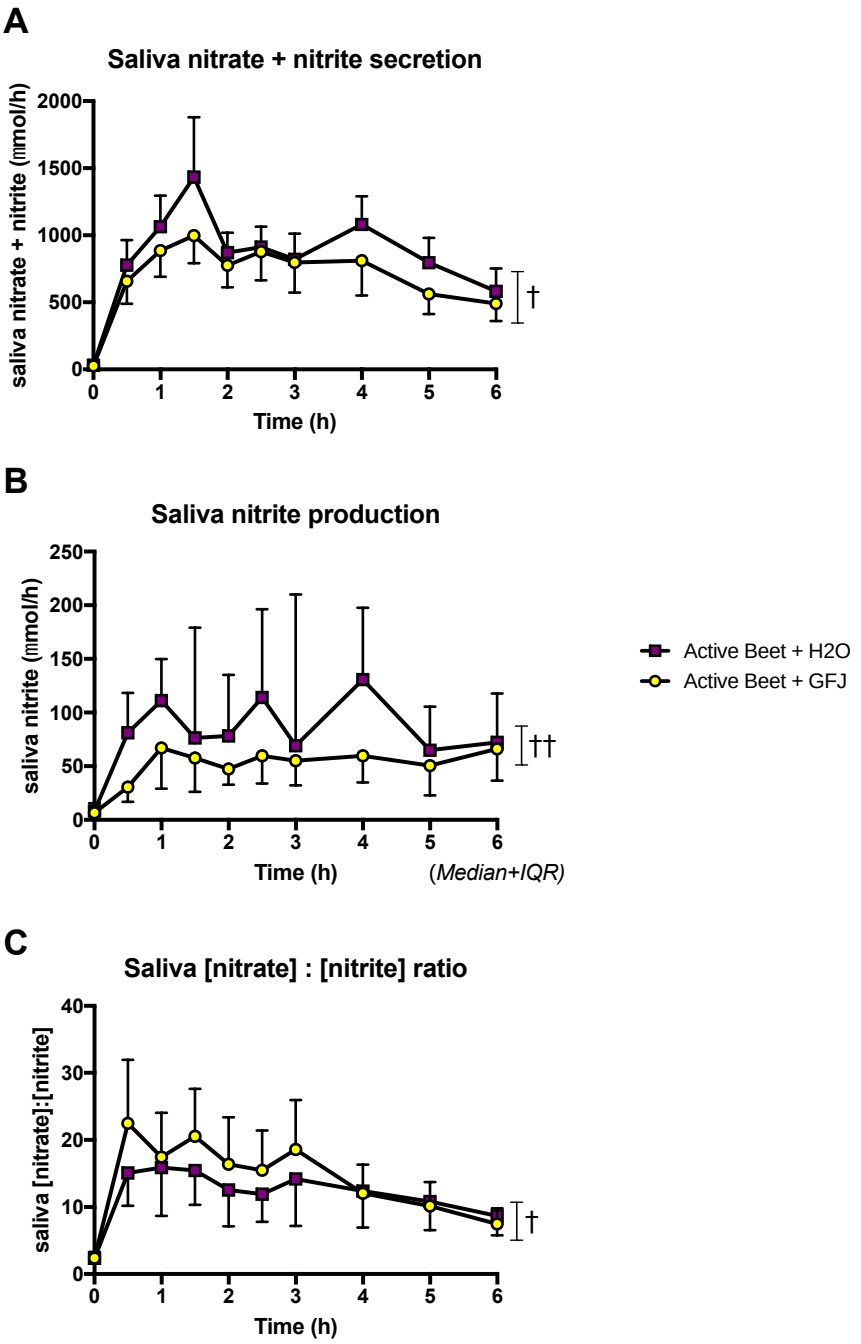

Figure 22. The effect of grapefruit juice and beetroot juice (given at T=0h) on salivary nitrate secretion and metabolism**. A:** Total salivary nitrate secretion (i.e. amount of salivary nitrate and amount of salivary nitrite), *n*=9. Data shown as mean ± SEM. **B:** Salivary nitrite production, *n*=11. Data shown median ± IQR. **C:** Saliva [nitrate]:[nitrite] ratio, *n*=9. Data shown as mean ± SEM. Statistical

significance shown as †p<0.05, ††p<0.01 as analysed by 2-way ANOVA between the curves. (Placebo Beet + GFJ data not presented)

Dietary nitrate (Active Beet+H_2O) increased salivary flow from baseline, p=0.02 overall, and by 0.38 ml/min at 4 h (95% CI 0.16 to 0.61); see **Figure 23 A**. Whilst grapefruit juice (Placebo Beet+GFJ) did not significantly decrease salivary flow relative to baseline, p=0.7, grapefruit juice appeared to exert an astringent effect compared to Active Beet+H_2O: salivary volume was decreased with Placebo Beet+GFJ (p<0.0001) and Active Beet+GFJ (p=0.04) with the peak difference occurring at T=4 h: -0.34 ml/min (95% CI -0.05 to -0.68); see **Figure 23 A**.

Grapefruit juice significantly increased the salivary pH when combined with dietary nitrate (p=0.005) for Active Beet+GFJ versus Active Beet+H_2O (**Figure 23 B**). Dietary nitrate also increased salivary pH compared to grapefruit juice: Active Beet+H_2O versus Placebo Beet+GFJ (p<0.0001) with the peak difference occurring at T=3 h: ΔpH 0.37 (95% CI 0.16 to 0.58). Grapefruit juice without dietary nitrate resulted in decreased salivary pH: Placebo Beet+GFJ versus Active Beet+GFJ (p<0.0001) with the peak difference occurring at T=3 h: ΔpH 0.53 (95% CI 0.26 to 0.80).

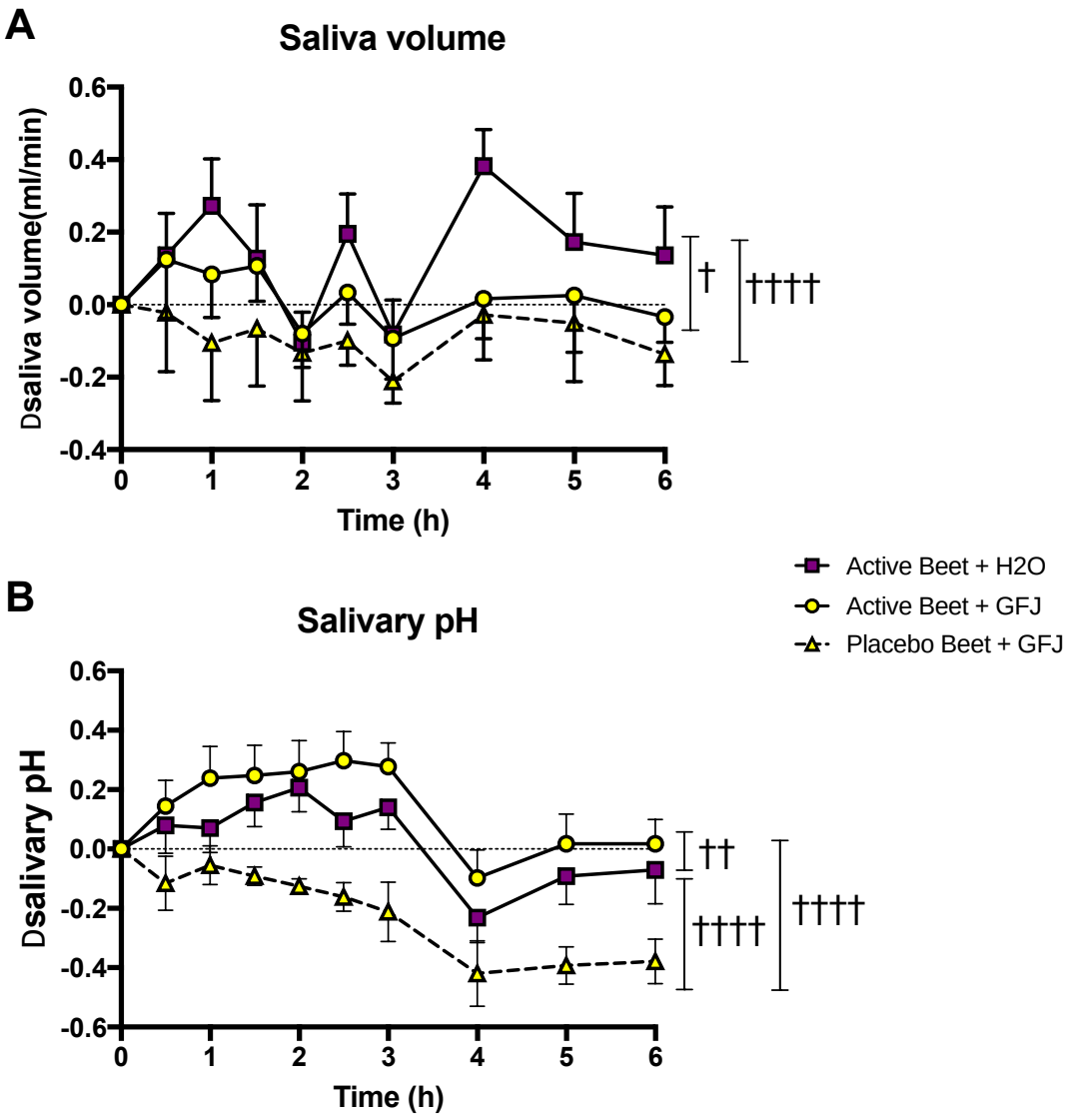

Figure 23. The effect of grapefruit juice and beetroot juice (given at T=0h) on saliva volume and pH. **A:** Salivary volume, *n*=11 for Active Beet + GFJ and Active Beet H_2O, *n*=9 for Placebo Beet + GFJ. **B**: Salivary pH values, *n*=11 for Active Beet + GFJ and Active Beet H_2O, *n*=7 for Placebo Beet + GFJ. Data shown as mean +/- SEM. Statistical significance shown as, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger\dagger}p<0.0001$ as analysed by 2-way ANOVA between the curves.

Blood Pressure

Baseline BP data is presented in **Table 8.**

	Active Beet + GFJ	Active Beet + H_2O	Placebo Beet + GFJ
SBP (mmHg)	108.1 ± 7.5	107.7 ± 8.2	106.5 ± 8.7
DBP (mmHg)	67.6 ± 5.6	66.2 ± 6.2	67.2 ± 6.1
MAP (mmHg)	83.8 ± 6.1	82.8 ± 6.2	83.0 ± 6.9
PP (mmHg)	40.5 ± 4.0	41.5 ± 7.0	39.3 ± 4.8
PPi	0.48 ± 0.04	0.50 ± 0.09	0.47 ± 0.05

Table 8. Baseline BP parameters (taken as an average of BP readings T=-1 to T=0). SBP is systolic blood pressure; DBP is diastolic blood pressure; MAP is mean arterial pressure; PP is pulse pressure; PPi is pulse pressure index. Data are mean ± SD

The addition of grapefruit juice to (active) beetroot Juice resulted in a lower SBP: Active Beet+GFJ versus Active Beet+H_2O (P=0.02), with the peak mean difference in SBP seen at T=5 hours: -3.3 mmHg (95% CI -6.43 to -0.15); see **Figure 24 A**. As expected, active nitrate-containing beetroot juice (Active Beet+GFJ) also lowered SBP versus Placebo Beet+GFJ (p=0.0005). However, no difference in SBP was seen between Active Beet+H_2O and Placebo Beet+GFJ. Relative to baseline, SBP was decreased by the active nitrate-containing beetroot juice combinations: Active Beet +GFJ (P=0.02) and Active Beet +H_2O (P<0.01) but not by Placebo+GFJ (p=0.09).

In contrast to SBP, grapefruit juice tended to increase diastolic BP (DBP), i.e. Active Beet+GFJ versus Active Beet+H_2O (p=0.04); see **Figure 24 B**. Moreover, Placebo Beet+GFJ resulted in a significant decrease in DBP versus Active Beet+GFJ (p=0.002) but not Active Beet+H_2O (p=0.1). Relative to baseline, DBP was not decreased by any of the interventions (all p=0.2).

Pulse pressure (PP) was decreased by Active Beet+GFJ versus Active Beet+H_2O (p=0.0003), with the peak mean difference in PP seen at T=2.5 hours: -4.2 mmHg (95% CI -0.3 to -8.2); see **Figure 24 C**. Active Beet+GFJ decreased PP versus Placebo Beet+GFJ (p<0.0001), with a peak mean difference in PP seen at T=4 hours: -6.6 mmHg (95% CI -2.0 to -11.2). Similarly, Active Beet+H_2O decreased PP versus Placebo Beet+GFJ (p=0.006), with a peak mean difference in PP seen at T=6 hours: -4.2 mmHg (95% CI -0.4 to -8.0).
Relative to baseline, PP was decreased by the active nitrate-containing beetroot juice combinations: Active Beet +GFJ (P=0.04) and Active Beet +H_2O (both p=0.03).

Mean Arterial Pressure (MAP, calculated as DBP+0.4xPP) was not significantly changed by Active Beet+GFJ versus either Active Beet+H_2O (p=0.6) or versus Placebo Beet+GFJ (p=0.7); data not shown. Relative to baseline, MAP was significantly decreased by Active Beet+GFJ (p=0.04), but not by Active Beet+H_2O (p=0.1) or Placebo Beet+GFJ (p=0.3); data not shown.

Pulse Pressure index (PPi, the ratio of the pulse pressure to MAP[236]) was significantly decreased by Active Beet+GFJ versus both Active Beet+H_2O (p=0.001) and Placebo Beet+GFJ (p<0.0001). Active Beet+H_2O decreased PPi versus Placebo

Beet+GFJ (p=0.01). Relative to baseline, PPi was decreased by Active Beet+GFJ (p=0.03) and Active Beet+H_2O (p=0.01) and increased by Placebo Beet+GFJ (p=0.01).

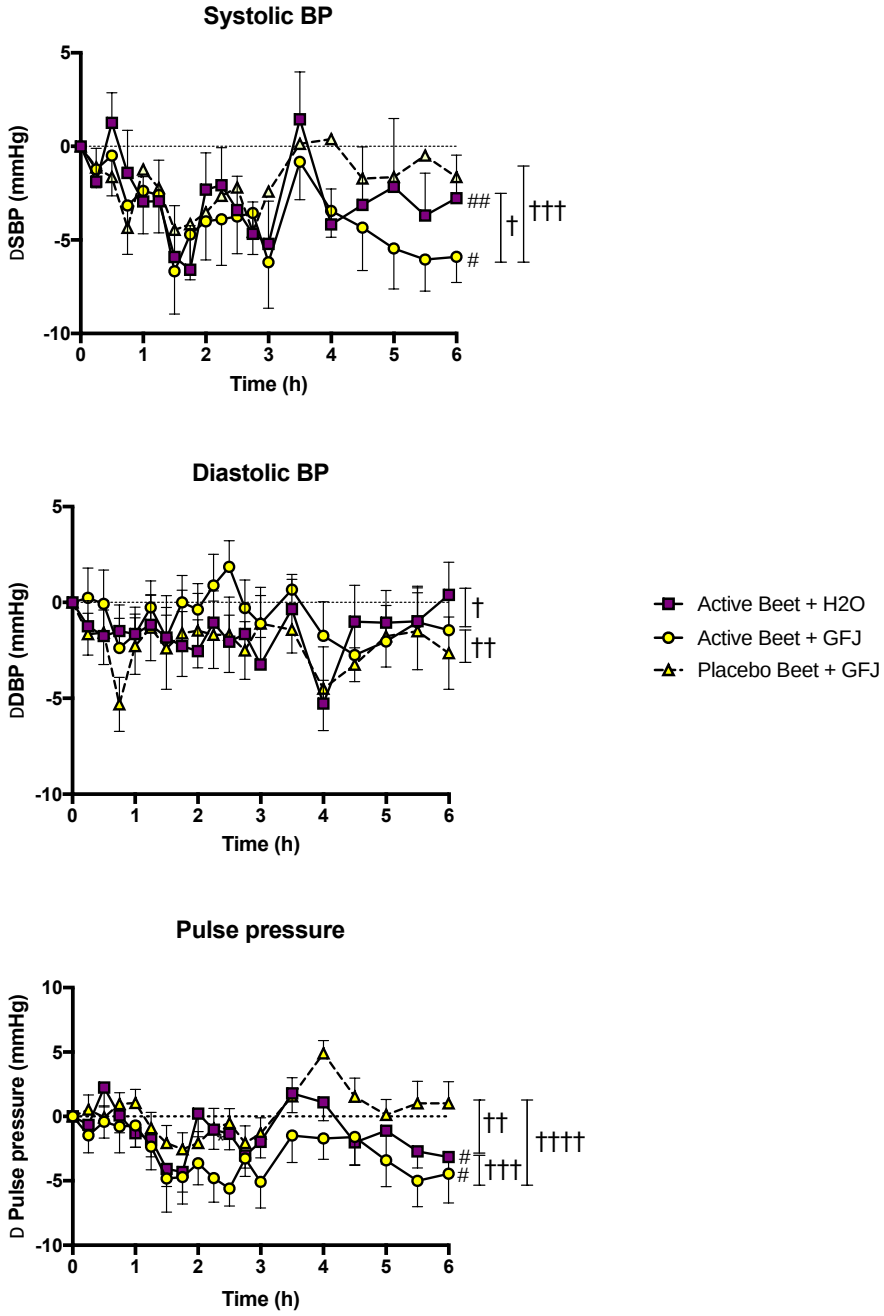

Figure 24. The effect of grapefruit juice and beetroot juice (given at T=0h) on blood pressure (BP). **A:** Systolic BP (SBP). **B**: Diastolic BP (DBP). **C**: Pulse pressure (PP). n=9 for all interventions and parameters assessed. Statistical significance

shown as †p<0.05, ††p<0.01, †††p<0.001, †††† p<0.0001 as analysed by 2-way ANOVA between the curves and #p<0.05, ##p<0.01 by 1-way ANOVA vs baseline.

Heart rate

The addition of grapefruit juice to (active) beetroot juice resulted in a higher heart rate: Active Beet+GFJ vs Active Beet+H_2O (p=0.0009), however without any significant change at individual timepoints; see **Figure 25**. Both Active Beet+GFJ and Active Beet+H_2O increased heart rate compared to Placebo Beet+GFJ (p<0.0001 and p=0.0006 respectively). Relative to baseline, heart rate was decreased by Active Beet+H_2O (p=0.03) and Placebo Beet+GFJ (p=0.03), but not by Active Beet+GFJ (p=0.09).

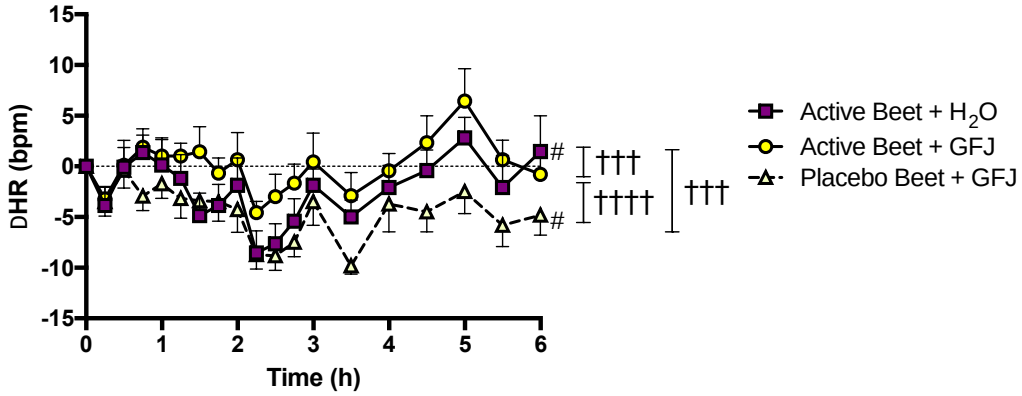

Figure 25. The effect of grapefruit juice and beetroot juice (given at T=0h) on heart rate. n=9 for all interventions and parameters assessed. Statistical significance shown as †††p<0.001, †††† p<0.0001 as analysed by 2-way ANOVA between the curves and #p<0.05 by 1-way ANOVA vs baseline.

Urine

Grapefruit juice as Placebo Beet + GFJ increased urinary pH versus Active Beet + H_2O ($p<0.0003$) but not versus Active Beet + GFJ ($p=0.1$; **Figure 26**).

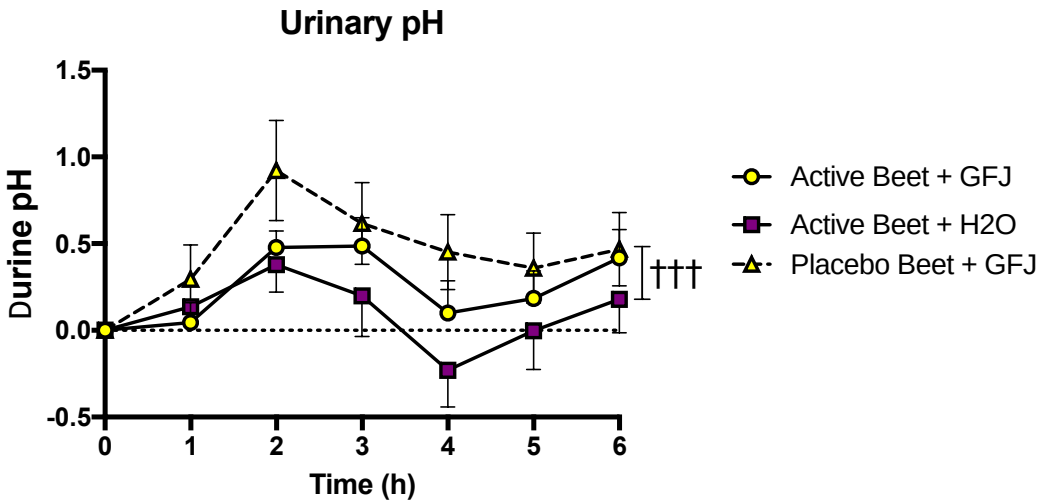

Figure 26. The effect of grapefruit juice and beetroot juice (given at T=0h) on urinary pH, *n*=11 for Active Beet + GFJ and Active Beet H_2O, *n*=9 for Placebo Beet + GFJ. Statistical significance shown as $^{\dagger\dagger\dagger}p<0.001$ as analysed by 2-way ANOVA between the curves.

Taste Score

The addition of grapefruit juice to Active beetroot Juice resulted in a significantly higher taste score, suggesting a greater palatability compared to beetroot juice and water: Active Beet+GFJ versus Active Beet+H_2O: +1.4 (95% CI 0.15 to 2.58), p=0.03, and Placebo Beet+GFJ versus Active Beet+H_2O: +1.56 (95% CI 0.16 to 2.95), p=0.03. There was no significant difference in taste score between the two grapefruit juice-containing interventions: Active Beet+GFJ and Active Placebo Beet+GFJ (p=0.7) (**Figure 27**).

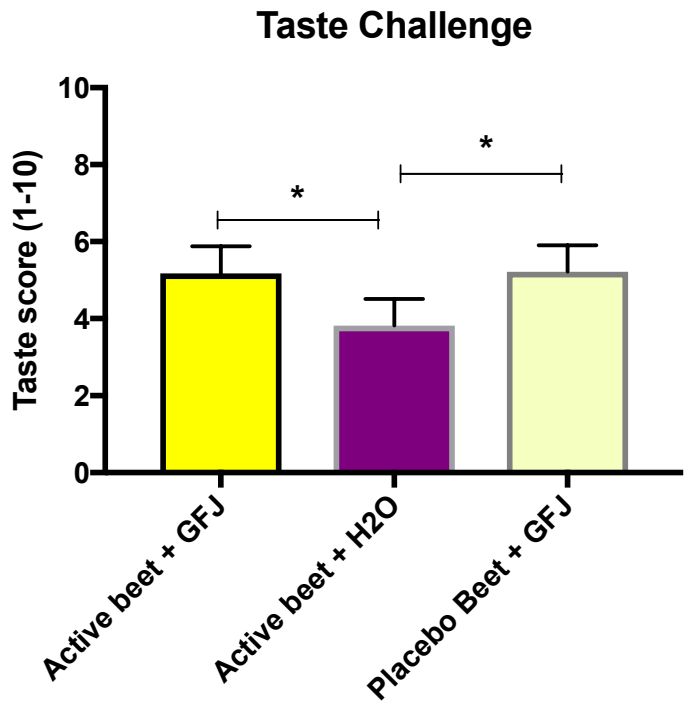

Figure 27. Taste Challenge: mean taste scores for each cocktail containing grapefruit and/or beetroot juice, *n*=11 for Active Beet + GFJ and Active Beet H_2O, *n*=9 for Placebo Beet + GFJ. Data expressed as mean±SEM. Statistical significance shown as $^{*}p<0.05$

5.4 Discussion

We have demonstrated that co-ingestion of grapefruit juice with dietary nitrate results in a potentiation of dietary nitrate's effect in lowering SBP and PP, albeit without the hypothesised increase in plasma [nitrite]. Therefore, the effect on SBP was unlikely to have been via furanocoumarin-mediated CYP3A4 inhibition of nitrite oxidation. Indeed, plasma [nitrite] was lower with Active Beet+GFJ compared to the Active Beet+H_2O (though DBP was slightly higher with Active Beet+GFJ versus Active Beet+H_2O).

The potential mechanism(s) by which grapefruit juice (in the Beet+GFJ intervention) decreases plasma [nitrite] versus Beet+H_2O will be considered by the kinetic processes (absorption, distribution, metabolism and secretion/excretion) at different anatomical sites.

In the oral cavity, grapefruit juice appears to have inhibited the metabolic conversion of nitrate to nitrite, as indicated by the increased salivary [nitrate]:[nitrite] ratio. This was associated with an unexpected increase in salivary pH with grapefruit juice. whilst previous studies have suggested that the relationship between salivary pH and nitrate reduction is in the opposite direction[237, 238], these are models, or only checked at a single timepoint of 30 minutes, rather than full physiological/kinetic studies. Our finding requires confirmation in further detailed mechanistic studies.

A potential additional contributing factor is the lower total salivary volume with GFJ. Whilst the astringent effect of grapefruit (juice) may account for the *absolute*

decrease in the total salivary nitrate secretion (measured as the total amount of salivary nitrate and nitrite: a secretory/excretory kinetic mechanism), this would not account for the change in rate of conversion of nitrate to nitrite (a metabolic mechanism) as indicated by the *relative* salivary [nitrate]:[nitrite] ratio. It is therefore likely that both kinetic processes – decreased metabolism and secretion - contribute to the decreased *absolute* salivary nitrite production.

Grapefruit juice *per se* decreased salivary pH (Placebo Beet+GFJ) from baseline and compared to the other two interventions. Dietary nitrate tended to increase salivary pH, which has been reported previously[239], and in combination with grapefruit juice resulted in a further increase in salivary pH. The mechanism for this interaction is not currently clear, though it could relate to carbonic anhydrase: quercetin, a flavonol found in grapefruit juice, has been demonstrated to inhibit human carbonic anhydrase in vitro [240]. Grapefruit juice also acted to increase the urinary pH, further supporting an effect on carbonic anhydrase.

In the stomach, grapefruit juice might impact gastric pH, modifying the chemical reactions contributing to a lower plasma [nitrite]. Despite being a citrus fruit, ingestion of a similar volume of grapefruit juice as our study (180 ml) has been found to more than double gastric pH (from 1.39 ± 0.4 to 3.20 ± 0.3; $p<0.05$), albeit in the presence of indinavir [241]. Nitrite is reduced to NO and other NO species by acid disproportionation (with the remaining nitrite being absorbed into the systemic circulation) in the stomach: accordingly gastric NO production is inhibited by proton-pump inhibitors[52]. In rats, increasing gastric pH with a proton-pump inhibitor (PPI) diminished the BP-lowering effect of orally-ingested nitrite

independently of plasma [nitrite][242]. Similar results with a dissociation between plasma [nitrite] and BP following PPI administration have been found in the study by Montenegro and colleagues in humans [243]. In their study, the plasma [nitrite] at 60 min following oral sodium nitrite ingestion, was significantly greater with the PPI esomeprazole compared to placebo. However, the nitrite-induced BP decrease with placebo was blocked by esomeprazole. Therefore, the relationship between plasma nitrite and BP was comparable to our data. These data have important implications: stomach pH appears to be an important modulator of the nitrate-nitrite-NO pathway. However, dietary nitrate's BP-lowering effect is not fully explained by an increase in plasma [nitrite] alone. Such uncoupling of the plasma [nitrite] from the clinical response suggests that other NO species may be involved which were not measured in this study e.g. thiocyanates in beetroot juice interacting to form S-nitrosothiols (increasing gastric pH with esomeprazole did not decrease S-nitrosothiol levels in the study of Montenegro et al. [243]). Altered gastric NO species may also have an effect on splanchnic blood flow, which may in turn impact systemic BP.

This study was designed to assess the effect of grapefruit juice, with the mechanism postulated to be mediated by furanocoumarins in the juice. However, grapefruit juice also contains a number of other substances that may play a role through mechanisms other than CYP3A4. For example, grapefruit juice is abundant in compounds which inhibit OATP (organic anion-transporting peptide)[244] and therefore potentially inhibits the uptake of organic anions; whether absorption of inorganic ions (such as nitrite) would be affected is not known. In addition to OATP, p-glycoprotein represents another possible mechanism by which grapefruit juice may affect gut absorption[245].

Grapefruit juice also contains reducing agents, such as ascorbic acid, which could reduce nitrite to NO in the gut, diminishing nitrite absorption into the plasma (however the preparation of beetroot juice used also contains 2% lemon juice which itself will contain ascorbic acid). Other potential reducing agents found in grapefruit juice are flavonoids, naringin, kaempferol and quercetin. In the stomach nitrite and quercetin react to form NO[246, 247], a process that is favoured by acidic conditions. Whilst the short half-life of NO would suggest that NO produced in the stomach would have only direct local effects, this has the potential to indirectly affect systemic BP via splanchnic blood flow as suggested above. Currently there is no evidence of flavonols reducing nitrite systemically.

The enhanced SBP-lowering effects of dietary nitrate and grapefruit juice co-consumption could represent a synergistic dynamic effect. Placebo Beet + GFJ was associated with a trend to decrease SBP ($p=0.09$). It is therefore possible that grapefruit juice has its own independent BP-lowering effect, however this study was not designed to measure such an effect. Combining the BP-lowering effect of beetroot juice with grapefruit juice would therefore be expected to result in an enhanced BP-lowering effect compared to beetroot juice alone. Indeed, grapefruit juice contains several vasoactive compounds including naringin and quercetin[248]. Naringin, when given daily for 6 weeks as grapefruit juice, has been shown to have a beneficial effect on arterial stiffness compared to treatment with a naringin-free grapefruit juice control[249], and a single dose of quercetin has been shown to increase brachial artery diameter [250]. Actions of these vasoactive compounds

might therefore explain the decreased PP observed for Active Beet+GFJ versus Active Beet+H_2O.

The results of this study have interesting potential clinical implications. The combination of beetroot juice with grapefruit juice decreased SBP and had a small but nonetheless significant effect on increasing DBP. Therefore, the combination of the juices reduced PP (and PPi) but not MAP. Raised PP is highly prognostic for adverse cardiovascular events[251]. Furthermore, given the adverse effect of a low DBP in patients with isolated systolic hypertension[252], an intervention which decreases SBP without decreasing DBP has potential clinical utility.

Limitations

Limitations of this study include the small number of participants; the single blind nature of the study; the use of peripheral BP measurement; and the use of interventions (BRJ and GFJ) that contain bioactive substances other than those whose effect is being measured in the study. These limitations are discussed below.

Possibly the key limitation of this study lies in the blinding methodology. In this study the volunteers were blinded (to the Active versus Placebo Beet juice cocktails), but not to the presence of absence of GFJ. Although blinding to GFJ would be difficult, strategies such as drinking from a covered cup and through a straw (to hide colour/appearance) could have been adopted. Furthermore, the investigators were not blinded. In retrospect, it would have been preferable to have blinded the person recording the data and analysing the samples, to reduce the risk of bias as much as

possible. Any future studies assessing the therapeutic potential of a GFJ-Beet juice cocktail in disease groups (see below) should be double-blinded to reduce this risk.

This was a pilot study and therefore the participant sample size was not determined by a power calculation based on previous data. This sample size (n=11) is small in absolute terms, but is broadly consistent with samples sizes in other similar experimental medicine pilot studies[58, 92].

In this study BP was measured as peripheral BP. Given that inorganic nitrite is known to be an arterial dilator with selectivity for medium and large arteries over smaller resistance arterioles[83, 208] the effects on central BP may have been more marked than those on peripheral BP[83] (as seen in the VaSera study, where 6 months treatments with beetroot juice was shown to decrease central, but not peripheral BP in hypertensive patients with/at risk of type 2 diabetes[253, 254]). It would therefore have been preferable to have recorded measures of central BP in addition to peripheral BP, for example by estimating central pressures through the use of a non-invasive device such as the SphygmoCor system (Atcor, Australia), Finometer (Finopress Medical Systems, The Netherlands), or Arteriograph (Tensiomed Ltd, Hungary).

A further limitation of the study design is that both juices contain bioactive substances other than those being studied, including ascorbic acid and flavonoids in the grapefruit juice, and ascorbic acid and thiocyanates in the beetroot juice, therefore limiting the study's ability to describe mechanistic aspects of the enterosalivary circulation over and above the clinical end-point of BP decrease as a

result of combining the two juices. Paradoxically, although the complex mix of bioactive juices is a limitation, so too is the simplicity of this study as it is unclear whether the clinical effects seen in this tightly controlled study are reproducible when added to a standard, mixed diet.

Two aspects of the study methodology limit the scope of the results. Firstly, the study involved acute doses of BRJ and GFJ. It remains to be determined whether or not the clinical effect seen by combining active BRJ with GFJ persists with repeated dosing. Secondly, the participants were all healthy volunteers, specifically without evidence of hypertension. Further studies are required to explore whether the effect seen in this study is also evident in patients with hypertension and/or other vascular disease who may benefit from BP lowering.

Conclusion

In summary, our study found that grapefruit juice potentiated the SBP-lowering effect of beetroot juice, despite a decrease in plasma [nitrite]. Grapefruit juice appears to have inhibited the metabolic conversion of nitrate to nitrite, as indicated by the increased salivary [nitrate]:[nitrite], associated with an increase in salivary pH with grapefruit juice. The BP-lowering effect may have been due to other NO species not measured in this study, such as S-nitrosothiols. These findings have implications for maximising the clinical benefit of dietary nitrate and also in further exploring mechanisms of dietary nitrate bioactivation. Given that the taste was improved by grapefruit juice, this combination has potential for use as a dietary approach to improve BP.

Chapter 6. The Effect of Altering Oral pH on bioactivation of dietary inorganic nitrate

6.1 Background

As discussed in **Chapter 1**, the reduction of nitrate to nitrite within the oral cavity is facilitated by commensal bacteria that possess nitrate reductases not known to be present in eukaryotes. The essential role of these bacteria in the nitrate-nitrite-NO pathway was demonstrated by Govoni *et al.* who found that rinsing the mouth with anti-bacterial mouthwash before a dietary nitrate load abolished the conversion of nitrate to nitrite and therefore attenuated the rise in plasma [nitrite] seen in the control group[59]. This abolition of enterosalivary nitrite production is clinically significant, with mouthwash use leading to increased blood pressure; the rise in blood pressure is correlated with the decrease in plasma [nitrite][60]. Furthermore, it has been demonstrated that the maximal activity of reductase-containing bacteria in the oral cavity occurs at an acidic pH i.e. 6.5-7 [255]. Dietary nitrate has a role in regulating the oral microbiota[256]. This may be related to its ability to increase the oral pH (vs placebo) from 7.0 to 7.5, giving rise to the hypothesis that nitrate has beneficial effects on dental health[257].

Given that interventions to decrease the activity of the nitrate-reducing oral microbiome have been demonstrated to abolish the clinical effects of inorganic nitrate, this study proceeded upon the hypothesis that an intervention to promote the activity of the nitrate-reducing oral microbiome would therefore potentiate the clinical effects of inorganic nitrate. In **Chapter 5**, the combination of golden grapefruit juice and (active) beetroot juice significantly increased salivary pH compared to: i) the combination of golden grapefruit juice and placebo beetroot

juice; and ii) active beetroot juice with water. This increase in salivary pH was associated with a decrease in salivary nitrite production and the ensuing plasma [nitrite]. The focus of this study was therefore to confirm the relationship between salivary pH, salivary nitrite production and plasma [nitrite] and the blood pressure-lowering effect of dietary nitrate, by altering pH using sugar-containing chewing gum. It has previously been demonstrated that sugar-containing chewing gum significantly decreases the salivary pH compared to both baseline and to sugar-free chewing gum[258].

Hypothesis

Sugar-containing chewing gum will – by decreasing salivary pH and enhancing the nitrate reductase activity of oral bacteria – increase the oral conversion of inorganic nitrate to nitrite and in turn potentiate the BP-lowering effect of inorganic nitrate.

We therefore performed a crossover study in healthy volunteers to assess the effect of changes in oral pH on plasma [nitrite] as well as clinical effects i.e. blood pressure.

6.2 Methods

Approvals

Ethical approval for this study was obtained from the South East London Research Ethics Committee (REC) (10/H0802/52) and from Guy's and St Thomas' Research & Development Department. Written informed consent was obtained from all volunteers prior to commencing any protocol-related procedures.

Study Design

A 2-visit randomised, crossover intervention design was used. On each visit, volunteers consumed a 70ml shot of concentrated beetroot juice containing ~0.4g inorganic nitrate ('Beet It' James White Drinks, UK). Volunteers were asked to chew gum during each study visit. The type of gum was randomised to either sugar-containing acidic chewing gum (Seriously Strawberry Hubba Bubba ("Hubba Bubba"), Wrigley Company, Plymouth, UK) or sugar-free non-acidic chewing gum (Extra Peppermint ("Extra"), Wrigley Company, Plymouth, UK) as control. The sugar-free was considered as control given its neutral effect on oral pH[258]. Volunteers were asked to chew gum throughout most of the duration of the experiment, changing the piece of gum every 20-30 minutes (each piece is designed to be chewed for at least 20 minutes according to the packaging label). Volunteers were allowed to take short breaks from chewing the gum (~5 minutes per hour) after they had provided salivary samples and during blood pressure measurements (see below for details).

Blood pressure was measured in triplicate every 15 minutes from 1 hour (T=-1) before ingestion of the juice intervention at (T=0) and until 3 hours post-ingestion. The blood pressure data value for a given time point was recorded as the average of the three triplicate readings. While the BP was being recorded, the volunteer was asked to keep their legs uncrossed and refrain from chewing gum, to avoid any background interference during BP recording (it has previously been shown that chewing gum increases mean arterial pressure[259]).

Sample Collection

Blood and saliva samples were taken immediately prior to ingestion of beetroot juice, then at 30-minute intervals for 3 hours, and hourly for a further 3 hours. At time points where blood pressure and blood samples were taken, blood pressure recordings were taken prior to blood sampling.

At each blood draw, 6 ml of venous blood was drawn into a chilled syringe then transferred to a chilled green lithium heparin tube (Vacutainer®, BD). Samples were immediately centrifuged at 4^{o}C and 2000x *g* for 5 minutes (MIKRO 220R, Hettich, Germany), after which plasma was collected.

Prior to collection of saliva, volunteers were asked to avoid swallowing saliva for 5 minutes. They were then asked to drool all saliva into a collecting tube. The volume of saliva collected was recorded.

All plasma, saliva, and urine samples were divided into two chilled 2ml tubes and stored at -80^{o}C until biochemical analysis.

Sample Analysis

Plasma and saliva samples were analysed for nitrate and nitrite concentrations using chemiluminescence, as described previously[194].

Data Analysis

Data was analysed using GraphPad Prism 8.0 (GraphPad Software Inc.). All data are expressed as mean±SEM unless otherwise stated. Data were compared by 2-way ANOVA and/or 1-way ANOVA as appropriate, with Fisher's LSD post-test. $p<0.05$ was considered statistically significant.

Sample size and duration of study protocol

In an iterative experimental medicine approach to this exploratory study, we recruited an initial 8 volunteers and, at that point, reviewed the data to guide the overall sample size required. Based on the data, we elected to recruit a total of 14 volunteers.

It was initially uncertain whether volunteers would tolerate chewing gum near-continuously for the full 6 hours allowed in the protocol. For the first 8 volunteers we therefore collected saliva, plasma and BP data up to T=3, but in dietary nitrate studies, changes in plasma [nitrite] are typically only seen at around ~2.5-3 h following ingestion of dietary nitrate. Therefore, as no problems were encountered with chewing gum for an extended duration, we elected to collect saliva, plasma and BP data until T=6 for the final 6 volunteers.

6.3 Results

Participant Characteristics

14 participants completed both visits. Participant and Baseline Characteristics are shown in **Tables 9** and **10**.

N	14
Gender (n, male)	6
Age, years	21 [19.75, 22]
Weight, kg	60 [59.0, 78.7]
BMI, kg/m^2	21.7 [19.8, 23.1]

Table 9. Participant characteristics. Data expressed as median [IQR]

Baseline Characteristics

	Sugar-free gum	Sugar-containing gum
SBP, mmHg	116 ± 11	118 ± 11
DBP, mmHg	71 ± 6	73 ± 6
MAP, mmHg	86 ± 7	88 ± 7

Table 10. Baseline clinical parameters of study participants. N=14. Data expressed as mean ± SD.

The initial results of the first 8 volunteers can be seen in **Appendix 6.1**. Below are presented data for n=14.

Plasma

There was no significant difference in plasma [nitrate] between sugar-containing vs sugar-free gum (p=0.22) see **Figure 28 A**. Plasma [nitrite] increased significantly

with sugar-containing vs sugar-free gum (p<0.0001), with the peak difference at

T=2.5h: +406 nmol/L (95% CI 132 to 698) p=0.004, see **Figure 28 B**.

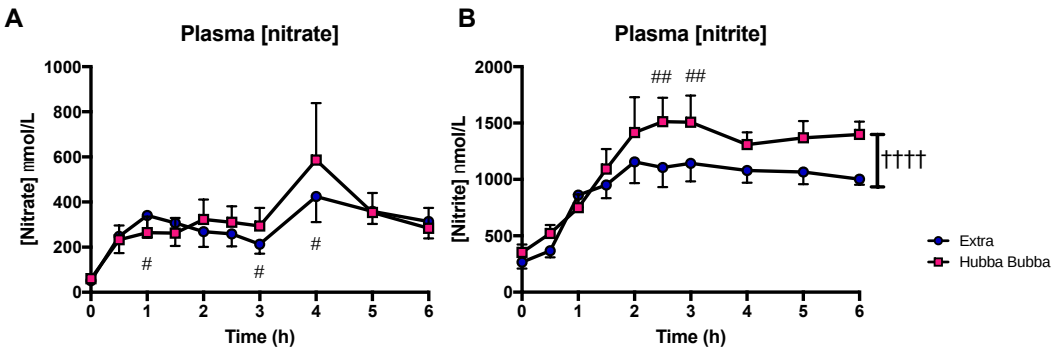

Figure 28. The effect of sugar-free and sugar-containing gum on plasma [nitrate] (Panel A) and [nitrite] (panel B). ††††p<0.0001 as analysed by 2 way ANOVA. #p<0.05, ##p<0.01 for individual time point. Data expressed as mean±SEM.

Saliva

Salivary pH was decreased with sugar-containing vs sugar-free gum (p<0.0001), with the peak difference seen at T=5h: -1.9 [-2.6, -1.2], p<0.0001, **Figure 29 A**.

Salivary volume was increased with sugar-containing vs sugar-free gum (p=<0.0001), with the peak difference seen at T=2h: 7.6 ml [4.7, 10.7], p<0.0001, **Figure 29 B**.

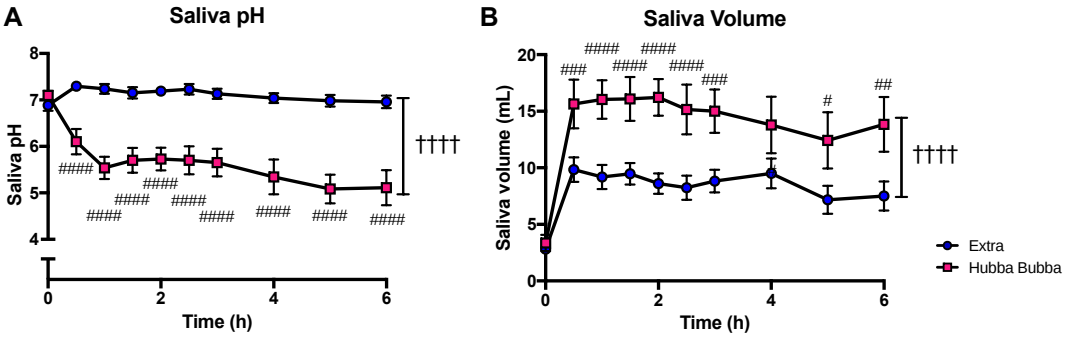

Figure 29. The effect of sugar-free and sugar-containing gum on salivary pH (Panel A) and volume (Panel B). ††††p<0.0001 as analysed by 2 way ANOVA. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 for individual time points. Data expressed as mean±SEM.

The total salivary nitrite amount (over each 5 minute-long collection period) was increased for sugar-containing vs sugar-free gum (P<0.01) with the peak difference seen at T=2.5 h: +9.9 nmol [5.2, 14.7], p<0.0001, see **Figure 30.**

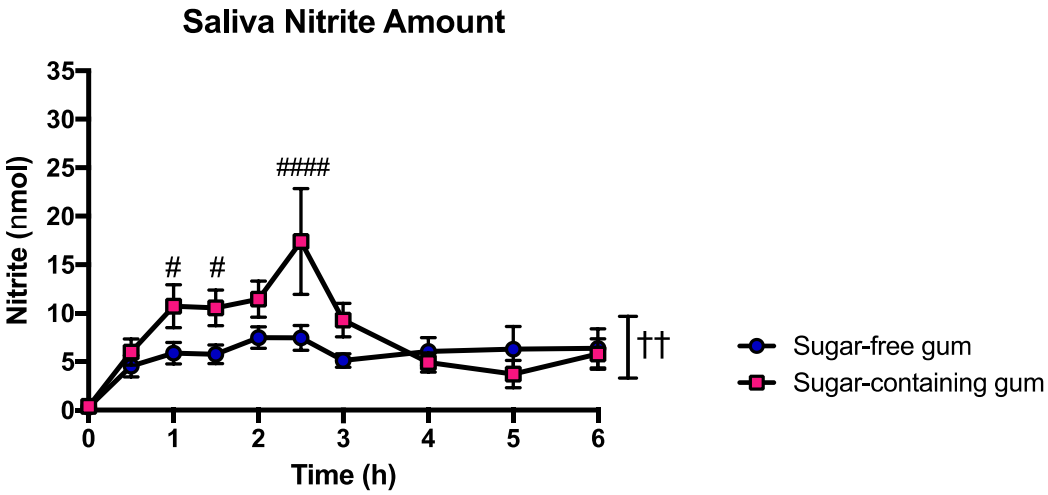

Figure 30. The effect of sugar-free and sugar-containing gum on salivary nitrite production (over 5 min); ††p<0.001 as analysed by 2 way ANOVA. #p<0.05, ####p<0.0001 at individual time points expressed as mean±SEM.

Blood pressure

Blood pressure data is shown in **Figure 31**. SBP was significantly decreased with sugar-containing vs sugar-free gum ($p<0.0001$) with the peak difference seen at T=2 h: -5.6 mmHg [-10.1, -1.1], p=0.01. DBP was also significantly decreased with sugar-containing vs sugar-free gum ($p<0.0001$), with the peak difference see at T=1.5 h: -4.5 mmHg [-8.0, -1.1], p=0.01. MAP was significantly decreased by sugar-containing vs sugar-free gum ($p<0.0001$), with the peak difference see at T=2 h: -4.6 mmHg [-8.0, -1.2], p=0.01.

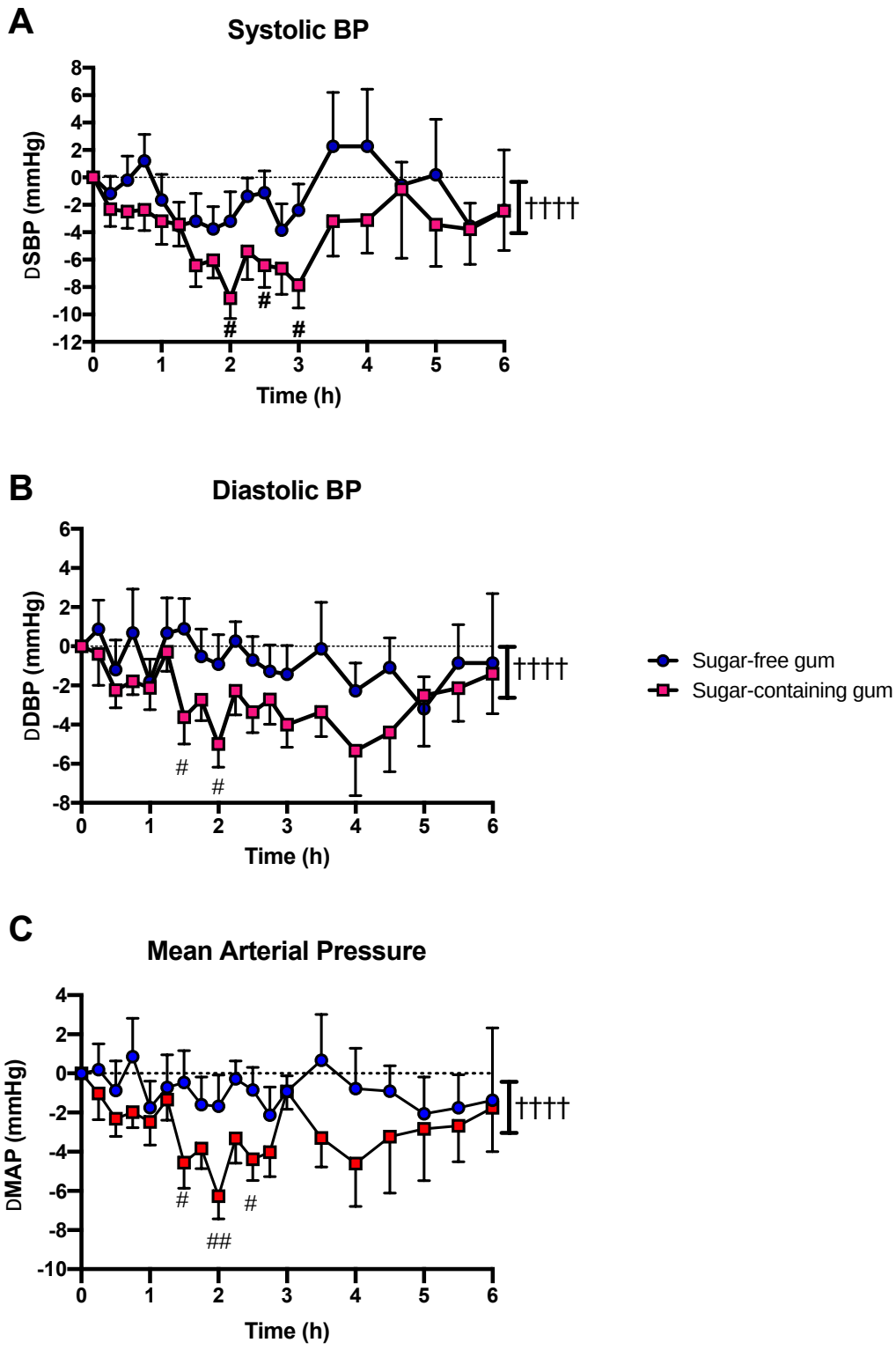

Figure 31. The effect of sugar-free and sugar-containing gum on changes from baseline in blood pressure parameters. Panel A: Systolic blood pressure, Panel B: Diastolic blood pressure, Panel C: Mean arterial pressure. ††††p<0.0001 as analysed by 2 way ANOVA, #p<0.05, ##p<0.01 at individual time point. Data expressed as mean±SEM.

6.4 Discussion

These data suggest that, as hypothesised, chewing sugar-containing gum results in an elevated salivary nitrite production, associated with an increased plasma [nitrite] and an enhanced BP-lowering effect of dietary nitrate.

The effect seen with sugar-containing gum appeared to be mediated by two mechanisms. Firstly, it increased salivary volume, therefore increasing the amount of substrate (nitrate) available to commensal bacteria for reduction to nitrite. Secondly, it significantly decreased the salivary pH within 30 mins which, as discussed in the introduction, has an effect on the activity of nitrate reductase-containing commensal bacteria. This was reflected by the significant increase in salivary nitrite content, which occurred following T=1 h.

The findings of this study have several implications. Firstly, as discussed in **Chapter 5**, it is possible to manipulate the nitrate-nitrite-NO pathway to bring about an increased clinical effect from a dietary nitrate load. Further studies are required to assess whether the previously identified beneficial effects of beetroot juice on athletic performance can also be potentiated by co-administration with sugar-containing, acidic, chewing gum; though the utility of this strategy may be limited by the risk to dental health associated with persistently decreased salivary pH (athletes already represent a group with a high prevalence of poor oral health[260]). The limitations of using a sugar-containing chewing gum also apply to any potential application in patients with cardiovascular disease such as hypertension or HFpEF, who have a high prevalence of co-existing diabetes mellitus.

Limitations

The study involved healthy volunteers. Therefore, the results must be considered only in the context of physiological mechanisms and any extrapolation to the potential effects in patients with hypertension is hypothesis-generating.

As presented in the methods section, we recruited an initial 8 volunteers before deciding on a final sample size of 14. Furthermore, for the final 6 volunteers, we elected to collect data until T=6, based on the initial findings. Although we consider an iterative approach to be valid for an exploratory, mechanistic, non-CTIMP experimental medicine study such as this, it does carry limitations. For example, the final sample size was not defined *a priori*, but was decided based on data from the first 8 volunteers. As stated by Jones *et al*, this approach should be used with caution as in theory it can lead a researcher to discontinue a study at the point of interim analysis if the results are statistically significant at that time (which may have occurred due to chance)[261].

We have not presented data for salivary nitrate amount or concentration. The baseline salivary [nitrate] for volunteers 9-14 were extremely high (values up to 44,000 μmol/L), such that we consider them to be lab errors. In **Appendix 6.1**, we demonstrate that in volunteers 1-8 the saliva [nitrate]:[nitrite] decreases with sugar-containing gum compared to sugar-free gum, suggesting that the effect is not simply due to increased saliva volume, but also due to increased metabolic conversion of nitrate to nitrite in the saliva. We had planned to re-analyse these samples following servicing of the NO analyser. However, due to changes in working practices arising from COVID-19, this was not possible before submission of this thesis.

In this study, to provide a constant pH-altering mechanism, we used chewing gum, which has multiple ingredients e.g. acesulfame-K and aspartame. Aspartame has

been shown to decrease systolic blood pressure in rats[262], however no data exists to support a similar effect in humans (a prospective randomised study protocolised BP measurement, but fails to report the data within the manuscript or supplemental data[263]). However, both chewing gums used in this study (Hubba Bubba, Wrigley's Extra) contain both acesulfame-K and aspartame, so any BP effect is controlled for.

Appendix 6.1

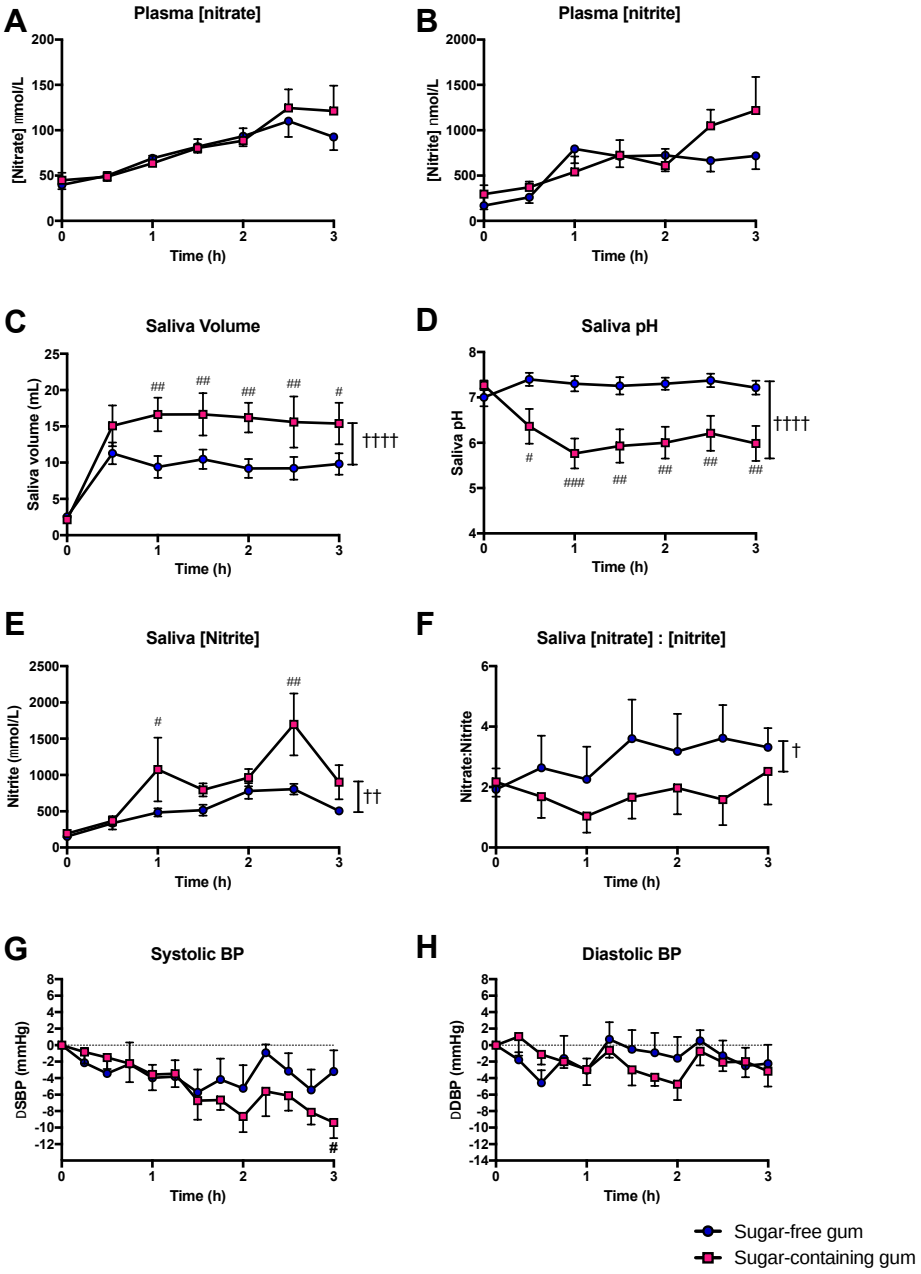

Figure 32. The effect of sugar-free and sugar-containing gum on plasma [nitrate] (panel A); plasma [nitrite] (panel B); salivary volume (Panel C); salivary pH (panel D); salivary [nitrite] (panel E); salivary [nitrate]:[nitrite] (panel F); Systolic BP (Panel G); Diastolic BP (Panel H) for the first 8 volunteers recruited to the study. Data expressed as mean ± SEM. †p<0.05, ††p<0.01, ††††p<0.0001 as analysed by 2 way ANOVA. #p<0.05, ##p<0.01, ###p<0.001 at individual time points.

Chapter 7. Discussion

The series of studies outlined in this thesis demonstrate several pieces of new data regarding the *in vivo* effects of inorganic nitrite and dietary nitrate in humans. The data in **Chapter 3** describes for the first time the effect of nitrite on coronary artery calibre, flow and resistance: I found that, as in the forearm, nitrite in the coronary bed is a selective arterial dilator with preference for conduit versus resistance vessels. **Chapter 4** presents data to describe in detail the effect of inorganic nitrite on invasively measured LV function in humans. The observed changes indicate that nitrite's effect in previously published studies[121, 123] involves both alterations in loading conditions and a direct myocardial effect of nitrite. The finding that nitrite has no significant effect on coronary flow is also relevant to nitrite's effect on LV function i.e. the direct myocardial effect identified above cannot be explained solely by changes in myocardial blood flow. The giant cytoskeletal protein titin represents a possible mechanism through which nitrite's response is mediated, as discussed in the introductory chapter (**Chapter 1**).

The introduction to **Chapter 3** outlines a series of *in vivo* experiments in humans and also *ex vivo* animal data using rat aortae, from which was derived the hypothesis that nitrite's mechanism of action of arterial dilation in conduit vessels is via potassium channels, namely Na^+/K^+-ATPase. The data presented in **Chapter 3** does not support this hypothesis: digoxin failed to inhibit nitrite's vasodilatory effect on the radial artery. The difference between the effect of digoxin in the human conduit artery and the effect of ouabain in the rat aorta could be explained by a number of reasons. Firstly, interspecies differences could play a role e.g. in the effect of cardiac glycosides. Secondly, the study relies on both the rat aorta and the human

radial artery being equivalent models for the study of the effect of various drugs on conduit vessels – this may not be the case. Further forearm studies are planned to assess the effect of selective inhibition of other potassium channels.

The Nitrate-Nitrite-NO pathway

To the best of my knowledge, the study in **Chapter 5** is the first published evidence that the effect of dietary nitrate can be enhanced through manipulation of the nitrate-nitrite-NO pathway[264], although it is accepted that this effect was observed in a manner contrary to the proposed hypothesis (there was no increase in plasma [nitrite]). **Chapter 6** provides data on enhancing the clinical effect of dietary nitrate by altering the oral pH, showing that a decrease in oral pH results in a potentiation of nitrate's BP lowering effect. Taken together, these two experimental medicine studies provide an important 'proof of concept' i.e. the clinical effect of dietary nitrate can be increased by mechanisms other than increasing the dose. However, it is clear that further studies are required to confirm the mechanisms behind these changes. Such studies could also, in addition to providing important mechanistic data regarding the nitrate-nitrite-NO pathway, potentially lead to future development of 'combination therapies', whereby dietary nitrate is given with another agent which interacts with the nitrate-nitrite-NO pathway in such a way as to potentiate nitrate's effect on the end-point in question. Despite the findings in **Chapter 5**, it is unlikely that a cocktail of golden grapefruit juice and beetroot juice would be a combination that could be easily translated to clinical practice, given the wide range of medication interactions seen with grapefruit juice. Finally, as noted in **Chapters 5** and **6**, there is a large interest in the effects of dietary nitrate within the context of

sport. Further research is required to assess whether manipulation of the nitrate-nitrite-NO pathway can potentiate dietary nitrate's effect on sporting performance.

Dietary nitrate: Do all roads lead to NO?

The studies reported in **Chapters 5 & 6** also provide important insights into another important theme, namely the metabolic fate of dietary nitrate in relation to its clinical effect. The accepted key mechanistic pathway, as described throughout this thesis, is the Nitrate-Nitrite-NO pathway whereby dietary inorganic nitrate undergoes sequential reduction – via the intermediary nitrite – to bioactive NO. However, multiple lines of evidence exist to suggest that this simplified concept does not fully represent the complex range of metabolic fates of dietary nitrate, and that further research should be performed to fully understand the role of signalling via S-nitrosylation and other NO-independent mechanisms. Indeed, in **Chapter 5**, the potentiation of dietary nitrate's BP-lowering effect by co-administration of grapefruit juice was seen in the absence of any increase in [nitrite], suggesting alternate mechanisms at play. While it must be accepted that S-nitrosothiols are somewhat difficult to measure in human experimental medicine studies[265], further characterisation of the metabolic fate of dietary nitrate has, in turn, the potential to identify new therapeutic targets.

The oral microbiota and the Nitrate-Nitrite-NO pathway

Bacteria in the oral cavity are associated not only with poor dental health e.g. halitosis, gingivitis and periodontitis[266], but also with cardiovascular disease: in the Oral Disease and Vascular Disease Epidemiology Study (INVEST), periodontal bacterial burden was associated with carotid intimal thickness[267], while tooth loss

(a marker of previous periodontal disease) was associated with carotid artery plaque burden[268]. The role of the oral microbiota in the nitrate-nitrite-NO pathway therefore calls into question the concept that all bacteria in the oral cavity are undesirable. This challenge to orthodoxy is matched by the finding that dietary nitrate has beneficial cardiovascular effects: previously it was considered that inorganic nitrate/nitrite had deleterious health effects via their conversion to S-nitrosamines, something that has been hotly debated over the past 5 decades[269, 270].

Clinical translation

Many of the studies exploring the clinical effects of dietary nitrate and inorganic nitrite (see **Chapter 1**) have small samples size while the interventions have in general been over short durations. However, therapies for chronic conditions such as hypertension and HFpEF will necessarily need to be long-term therapies. Therefore, future clinical studies focussed on chronic conditions will require longer durations of treatment to be studied, reflecting the chronic nature of treatment that would be required in the relevant condition. In summary, therefore, with notable exceptions of studies such as the Vasera study[98, 99], the field of inorganic nitrate/nitrite therapeutics has yet to progress to large scale, long-duration randomised control trials, a step that will be necessary if these therapies are to become widely adopted in clinical practice.

EF1 and relevance to disease states

In **Chapter 4**, EF1 was used as a secondary end-point. The results indicated that an intravenous infusion of sodium nitrite led to a significant increase in EF1, while

intracoronary sodium nitrite had no effect on EF1. These data would suggest therefore that the effects of nitrite on EF1 are due to effects on loading conditions rather than a direct myocardial effect.

The mechanisms by which nitrite alters loading conditions have been discussed in the relevant chapters, but in brief, nitrite alters afterload via its selective effects on large arteries, decreasing central arterial pressure and decreasing arterial wave reflections. In the venous system, it is proposed that the hypoxic environment results in reduction of nitrite to NO (facilitated by deoxyhaemoglobin) and therefore venodilation with resultant decrease in preload.

EF1 is an attractive measure in that it appears to be more sensitive to early LV systolic dysfunction than LVEF. This ability to detect abnormalities in systolic performance in patients who have a normal LVEF despite the presence of LV contractile dysfunction may provide a role for EF1 in a number of conditions. In patients with aortic stenosis and preserved LVEF, an EF1 <25% is predictive of requirement for valve replacement and for heart failure and death[186]. In the majority of patients who undergo aortic valve replacement, there is an improvement in EF1 of approximately 11%[187].

Another condition of interest is HFpEF. By definition, in HFpEF the LVEF is normal or "preserved". However, it is widely recognised that patients with HFpEF do demonstrate abnormalities in systolic function e.g. LV longitudinal strain is decreased in patients with HFpEF compared to controls[271] and carries prognostic significance[272]. Therefore, the use of EF1 in the diagnosis and management of HFpEF requires investigation.

There are, however, limitations surrounding the use of EF1. Firstly, there is no published dataset defining the normal range of EF1. From the hypertensive and

aortic stenosis populations, we can identify that the patients least affected by disease have higher EF1 values. However, there is discordance between the populations. Hypertensive patients with a normal LVEF and no evidence of diastolic dysfunction (defined as E/e' < 6.44) had an average EF1 of 20%. In one cohort of aortic stenosis patients, those with mild aortic stenosis (mean AV gradient <20 mmHg) and a normal LVEF had an average EF1 of 30%. In a second cohort of aortic stenosis patients, those with moderate valve disease, preserved LVEF (and no AVR/heart failure/mortality) had an average EF1 of 35%. Although all three of these average EF1 values were significantly higher than the 'severe disease' group in each population, taken together these data are problematic: the EF1 of 20% in the mild disease (hypertensive) group is below the cut-off that predicts mortality in the aortic stenosis group (25%). Furthermore, in a recent study exploring the effects of marathon running in a small sample of healthy volunteers, the average EF1 value at baseline (before the start of the marathon) was 16%[273], which rose to 23% immediately after the completion of the marathon. In summary, the current body of EF1 samples represents discrete pools of data in conditions with particular haemodynamic considerations. The need for larger datasets of EF1 containing patients with different cardiovascular diseases is therefore necessary, as is more data on healthy patients without cardiovascular disease (endurance runners may not necessarily represent a truly healthy group from a cardiac point of view[274]).

Proposed further work

Nitrite

The studies in **Chapter 4** suggest that a systemic infusion of sodium nitrite changes cardiac loading conditions, with changes in preload and afterload. To attempt to

quantify the effect on preload, recording of pressure-volume relations in response to nitrite infusion with and without leg cuffs inflated could be performed. Inflating leg cuffs to supra-diastolic pressures would exclude a large portion of venous return; by measuring the effect of leg cuff inflation on the change in markers of preload (LVEDP, LVEDV) due to nitrite, the proportion of nitrite's effect that is attributable to preload reduction can be established. The working hypothesis would be that a large proportion of the effect of systemic nitrite is due to preload reduction, with deoxyhaemoglobin in the venous compartment facilitating reduction of nitrite to NO.

Additional ongoing work

I had planned to present data from two additional studies in this thesis. Unfortunately, recruitment had to cease due to the COVID-19 pandemic. Both studies involve patients with HFpEF (see **Chapter 1**). One study is an invasive PV loop study in which the aim is to assess the effect of inorganic nitrite on left ventricular function during exercise in patients with HFpEF. We hypothesise that nitrite will blunt increases in left ventricular filling pressure during exercise and also blunt deleterious changes in other markers of left ventricular diastolic function e.g. tau, EDPVR, dP/dt_{min}. We further hypothesise that intravenous (systemic) nitrite will have a larger effect than intracoronary nitrite, due to systemic nitrite's effect on loading conditions. In this study, patients with HFpEF are randomised to receive either sodium nitrite or saline control immediately prior to a period of exercise (supine bicycle or hand-grip). In addition to randomisation between nitrite and control groups, patients are also allocated to either an intravenous group or an intracoronary group (non-randomised allocation). The primary end-point is the effect of nitrite on change in LVEDP during exercise. Secondary end points are

other markers of LV diastolic and systolic function, markers of afterload and of ventricular-arterial coupling. See **Figure 33** for a study flowchart. Although previous studies have determined the effect of inorganic nitrite on cardiac function during exercise, these involved measuring right heart pressures, rather than the invasive PV loop methodology I am using. PV loop analysis will provide a much more detailed assessment of how inorganic nitrite affects parameters of LV function – and in particular diastolic function – during exercise in patients with HFpEF.

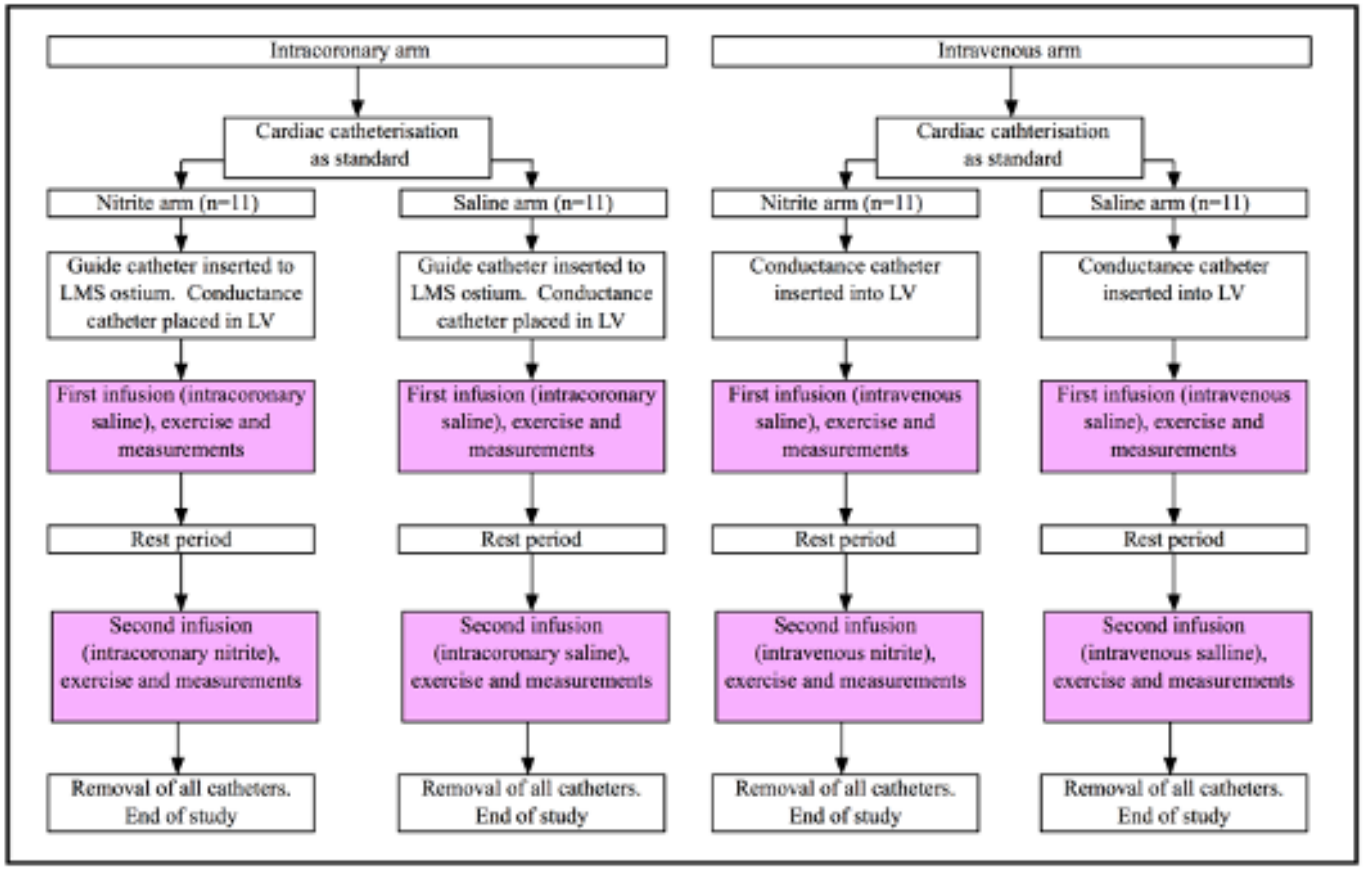

Figure 33. Flow diagram of study design of invasive catheter lab study of the effects of inorganic nitrite in HFpEF

A second ongoing study is a placebo-controlled, randomised, crossover intervention study, assessing the effect of a two-week course of beetroot juice (as dietary nitrate) on exercise capacity and LV diastolic function in 25 patients with HFpEF (see **Figure 34** for study flow chart). Previous studies have demonstrated that dietary nitrate improves exercise haemodynamics, but no placebo-controlled data has been published for dosing regimens other than acute dosing. In addition to providing mechanistic insights into the effect of inorganic nitrate on exercise in patients with HFpEF, this study will also produce important data on whether the acute effects of inorganic nitrate are sustained with prolonged dosing.

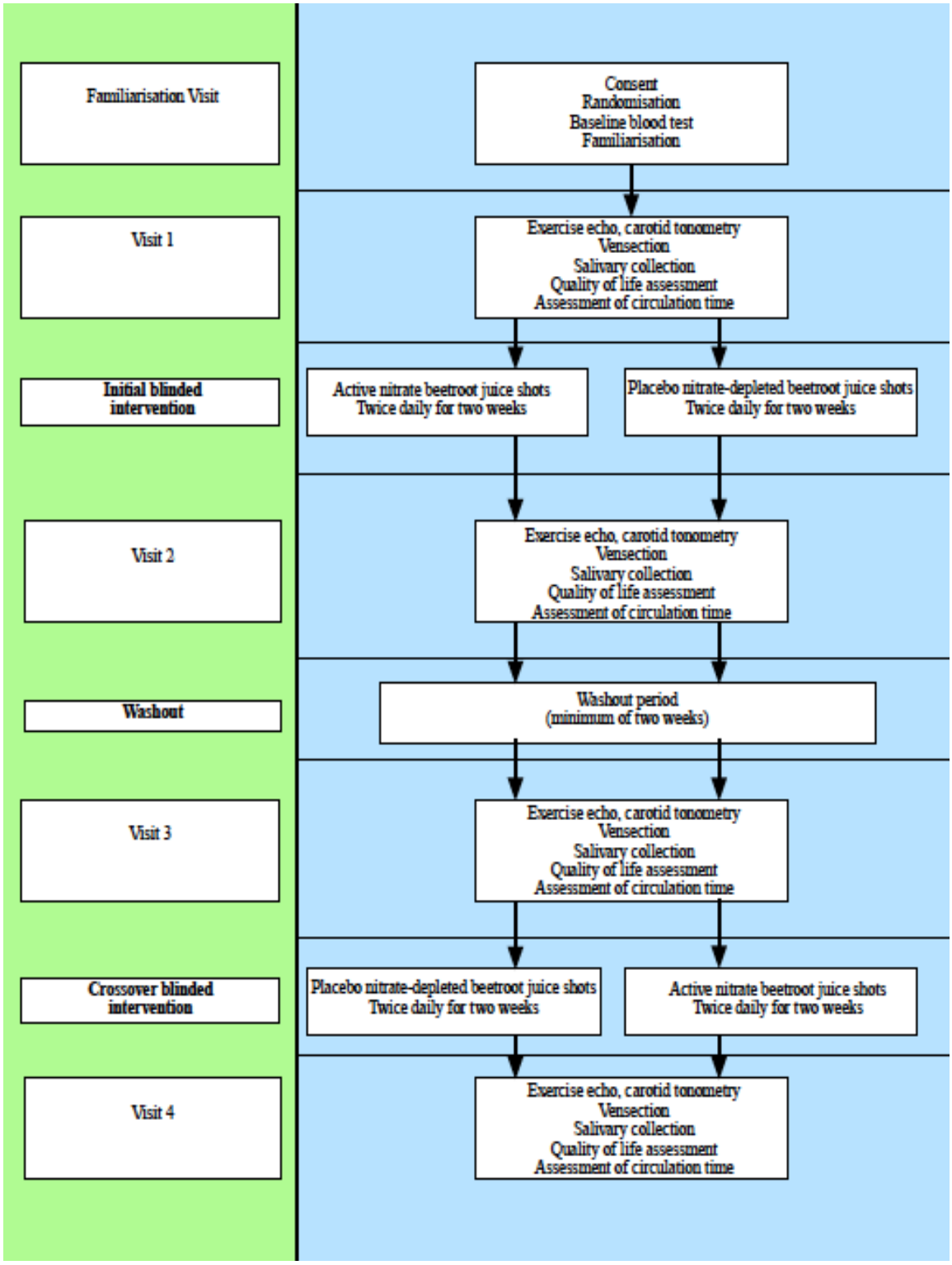

Figure 34. Study flow chart for dietary nitrate HFpEF study

Conclusion

The data contained in this thesis provides insights into both the physiological mechanisms of the nitrate-nitrite-NO pathway and the potential therapeutic applications of inorganic nitrate and nitrite. The major findings were: i) inorganic nitrite has a selective vasodilator effect on conduit versus microvascular coronary arteries, ii) inorganic nitrite improves left ventricular diastolic function via both direct myocardial and systemic effects, and iii) the blood pressure-lowering effect of dietary nitrate can be enhanced through manipulation of the nitrate-nitrite-NO pathway.

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