A Pilot Study of Dietary Nitrate Supplementation in Anaemic Patients

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Submitted by Dr David Alistair Veale to the University of Exeter
as a thesis for the degree of
Doctor of Medicine
In September 2019

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Dr David Veale
28/09/2019

ABSTRACT

Introduction

Anaemia causes debilitating symptoms in people with cancer, partly through reduced tissue oxygenation. Nitrate supplementation, via reduction to nitrite then nitric oxide, attenuates the effects of systemic hypoxia on muscle metabolism. Nitric oxide also influences cerebral blood flow, neurotransmission and platelet aggregation.

Aims

To examine the feasibility of recruiting patients with cancer-related anaemia to a pilot study, and to estimate differences in outcome measures and predict resources required for a larger study investigating how nitrate supplementation affects thrombogenicity, muscle phosphocreatine recovery, exercise tolerance, cognition and quality of life.

Methods

This prospective, balanced randomised crossover study recruited 33 participants. Cycle ergometry, bloods, ^{31}P-magnetic resonance spectroscopy and quality of life & cognition questionnaires were completed at two baseline visits and two visits post-supplementation with either nitrate-rich (BR) or nitrate-depleted (PL) beetroot juice.

Results

85% of 33 screened patients were enrolled. 26 completed all visits. Plasma nitrate concentration was 8.4±58.8μM (mean±SD) at baseline and 78±33.5μM post-BR (p=<0.001*). Nitrite was 142±79nM (baseline) and 923±1006nM post-BR (p=0.000*).

Haemoglobin concentration was 111.4±9.8g/l (baseline), 109.1±10.9g/l post-BR but 114.6±12g/l post-PL (p=0.028*). FACT-An quality of life score was 146±20 at baseline and improved to 152±24 post-PL and 150±25 post-BR (p=0.025*). Baseline FACT-cog cognitive function was 105±23 and improved to 109±24 post-PL and 108±23 post-BR (p=0.03*). Gas exchange threshold was53±11W post-PL; baseline was 51±12W, BR 50±12W, p=0.0238*. Baseline systolic blood pressure was 122±15mmHg, higher than post-BR (117±15mmHg) and post-PL (116±15mmHg), p=0.0081*). Oxygen uptake, peak power, phosphocreatine recovery, diastolic blood pressure and platelet aggregometry were not significantly affected.

Conclusion

This pilot study recruited and retained participants well. The enterosalivary circulation of nitrate was intact. The study was not powered to prove or disprove its hypotheses, but demonstrated that clinical factors (particularly haemoglobin concentration which varied because of chemotherapy and cancer progression/response) in this unstable population should influence future study design.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ACKNOWLEDGEMENTS

I feel very fortunate to have benefitted from the vast expertise of my MD supervisory team Dr Paul Kerr, Professor Paul Winyard, Dr Anni Vanhatalo and Professor Andy Jones. Dr Vanhatalo and Professor Jones have helped turn an initial hypothesis into a viable study furthering their world-renowned research group's knowledge of the effects of inorganic nitrate supplementation. Professor Winyard, who also works closely with this group, has vastly improved my understanding of the biochemical basis of this area of investigation, whilst also helping me to develop my skills in laboratory techniques and in the critical analysis of experimental results. Dr Kerr has given me great guidance, drive, vision and enthusiasm from the moment the idea behind this study was first conceived, and has been a constant source of critical appraisal and unfailing encouragement.

I am hugely grateful for the financial support received from the Exeter Leukaemia Fund without whom this study would not have been possible, along with the Royal Devon and Exeter Foundation NHS Trust, the National Institute for Healthcare Research, the University of Exeter and the University of Exeter Medical School.

I have received great assistance from the fantastic team of research nurses based at the Exeter Clinical Research Facility, particularly including Jill Melhuish, Kim Rowden and Chris Holgate. I am also grateful for the support received from Dr Jon Fulford, who kindly supervised all MRI investigations and also assisted with interpretation of the data acquired. Jamie Blackwell was hugely helpful in supervising the laboratory testing of many blood samples, while Dr Steven Bailey, Dr Lee Wylie, Dr Chris

Thompson and Dr Sinead McDonagh all kindly helped teach me how to perform exercise physiology tests, randomised litres of beetroot juice and checked my interpretation of exercise physiology data. I am also very grateful that Mohammed Abu Al Aghayth, PhD student at the University of Exeter, gave me permission to include a summary of the further analysis he has performed on plasma samples from participants in the current study (see section 4.7: Future Work).

The group of people to whom I am most indebted are the incredible participants themselves. Despite a number of significant health problems, these wonderful people kindly gave their time, dedication and enthusiasm to this study in an attempt to improve understanding of human physiology and in turn, to reduce the burden of symptoms for the patients of the future.

DEDICATION

I dedicate this thesis to Georgie, my wife, whose unending support and encouragement made its completion possible, and my children Benjamin and Jemima.

SYMBOLS AND ABBREVIATIONS

*	Statistically significant on post hoc analysis
2,3-DPG	2,3 Diphosphoglyceric acid
20-HETE	20-Hydroxyeicosatetraenoic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BOLD	Blood oxygen level dependent
BR	Nitrate-rich beetroot juice
Ca^{2+}	Calcium
CaM	Calmodulin
cGMP	Guanosine 3’5’-monophosphate
CI	95% confidence interval
CNS	Central nervous system
COSHH	Control of substances hazardous to health
COX	Cytochrome c-Oxidase
CPD	Citrate phosphate dextrose
CREB	Cyclic adenine monophosphate response element-binding protein
CRF	Cancer-related fatigue
CRFs	Case report forms
CV	Coefficient of variation
DeoxyHb	Deoxyhaemoglobin
DNICs	Dinitrosyl iron complexes
EDTA	Ethylenediaminetetraacetic acid

EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FACIT	Functional Assessment of Chronic Illness Therapy
FACT-An	Functional Assessment in Cancer Therapy – Anaemia
FACT-Cog	Functional Assessment in Cancer Therapy – Cognition
FAD	Flavin adenine dinucleotide
FasL	Fas ligand
FMN	Flavin mononucleotide
GET	Gas exchange threshold
GTP	Guanosine 5’-triphosphate
H^+	Hydrogen
Haem	Iron protoporphyrin IX
Hb	Haemoglobin
HNO_2	Nitrous acid
Hz	Frequency in Hertz
IMS	Intermitochondrial membrane
iNOS	Inducible nitric oxide synthase
IQ	Intelligence quotient
IRP-1	Iron regulatory protein 1
IRP-2	Iron regulatory protein 2
LSD	Fisher’s least significant difference
MetHb	Methaemoglobin
MID	Minimum important difference
MIM	Mitochondrial inner membrane
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid

n	Number
N_2O_3	Dinitrogen trioxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaI	Sodium iodide
NaOH	Sodium hydroxide
NMDA	N-methyl-D-aspartate
$NO\cdot_2$	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
$^{\cdot}NO$	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
O_2	Oxygen
ONOO-	Peroxynitrite
^{31}P MRS	^{31}Phosphate magnetic resonance spectroscopy
P/O ratio	Mitochondrial oxidative phosphorylation efficiency
PCr	Phosphocreatine
PGE_2	Prostaglandin E2
P_i	Inorganic phosphate
PKG	cGMP-dependent protein kinase
PL	Nitrate-depleted beetroot juice
PLA_2	Phospholipase A2
P/O ratio	Mitochondrial oxidative phosphorylation efficiency
RBC	Red Blood Cell
RFs	Radio frequencies
RMR	Resting metabolic rate
RNI	Reactive nitrogen intermediate

ROS	Reactive oxygen species
RSNOs	S-Nitrosothiols
S	Seconds
SD	Standard deviation
sGC	Soluble guanylyl cyclase
SNO-Hb	S-nitrosohaemoglobin
T_{lim}	Time limit of exercise tolerance
TNFα	Tissue necrosis factor α
UCPs	Uncoupling proteins
UIN	Unique identifying number
VCl	Vanadium (III) chloride
VCO_2	Carbon dioxide output
VE	Minute ventilation
VO_2	Oxygen uptake
$VO_{2\ max}$	Maximum rate of oxygen uptake when exercising maximally
$VO_{2\ peak}$	Peak rate of oxygen uptake
W	Power in Watts
WCC	White cell count

CHAPTER 1: INTRODUCTION

The Nobel Prize in Physiology and Medicine 1998 was awarded jointly to Robert Furchgott, Louis Ignarro and Ferid Murad for their discoveries regarding nitric oxide (˙NO) as a signalling molecule in the cardiovascular system. Working independently, Furchgott described an unknown molecule, which he termed 'endothelial derived relaxation factor' (EDRF); this physiological activator produced by the vascular endothelium was found to elicit vasodilation (Furchgott and Zawadzki 1980). In unrelated experiments in 1977, while investigating the effect of nitroglycerin, Murad discovered that organic nitrates release ˙NO, which relaxes smooth muscle cells through activation of guanylate cyclase and increase in guanosine 3':5'-cyclic monophosphate levels (Arnold, Mittal et al. 1977). In 1988, Ignarro's experiments concluded that EDRF was in fact ˙NO (Ignarro, Buga et al. 1987). Since that discovery, this simple gas molecule previously thought to be merely an atmospheric pollutant, has been found to have a multitude of effects in the regulation of human physiology, prompting it to be named 'molecule of the year' in 1992 by Science journal (Koshland 1992). It is now known that ˙NO plays a fundamental role in mitochondrial respiration (Larsen, Schiffer et al. 2011), vasodilation (Ignarro, Buga et al. 1987, Bond, Curry et al. 2013, Carlstrom, Liu et al. 2014), glucose uptake (Kingwell, Formosa et al. 2002), muscle contractile efficiency and force (Vanhatalo, Fulford et al. 2011), exercise efficiency (Bailey, Fulford et al. 2010, Bescos, Rodriguez et al. 2011), platelet aggregation (Alheid, Frolich et al. 1987, Radomski, Palmer et al. 1987), control of cerebral blood flow (Aamand, Dalsgaard et al. 2013) and as a neurotransmitter (Kuriyama and Ohkuma 1995, Garthwaite 2008).

Humans derive ˙NO through two means; either through the L-arginine – nitric oxide synthase (NOS) pathway (Moncada and Higgs 1993), or through sequential reduction of dietary nitrate (NO_3^-) through its enterosalivary circulation (Benjamin, O'Driscoll et al. 1994, Lundberg, Weitzberg et al. 1994). The latter pathway employs facultative anaerobic bacteria on the dorsum of the human tongue, which reduce NO_3^- to nitrite (NO_2^-), which is subsequently further reduced to ˙NO upon exposure to gastric acid as outlined below.

An increasing body of research has attempted to manipulate human physiology through dietary supplementation of NO_3^- and the resultant upregulation of ˙NO bioavailability. The majority of this work has been conducted with healthy individuals but such supplementation is now being investigated as a possible therapeutic intervention in a multitude of disease states, including peripheral artery disease (Allen, Giordano et al. 2012), diabetes (Gilchrist, Winyard et al. 2014), hypertension (Bond, Curry et al. 2013), pulmonary hypertension (Baliga, Milsom et al. 2012), and protection against cardiac ischaemia-reperfusion injury (Bryan, Calvert et al. 2007).

The incidence and prevalence of cancer is increasing, with 330,000 new diagnoses in the UK in 2011 (C.R.U.K. 2015). Cancer-related fatigue is a multifactorial problem which affects 39-90% of people with cancer and can have a profound effect on their quality of life (Ludwig, Van Belle et al. 2004). One of the causes of cancer-related fatigue is anaemia, which has an incidence of 54% in patients with cancer. Anaemia contributes to significant reduction in performance status of patients with malignancy (Ludwig, Van Belle et al. 2004); however, physicians only have two effective treatments of anaemia, exogenous erythropoietin (EPO) injections or blood transfusions. EPO can improve quality of life (Crawford, Cella et al. 2002) but has

been associated with an increase in patient mortality in some disease populations (Leyland-Jones, Semiglazov et al. 2005), so must be used with caution for patients with cancer-related anaemia. Blood transfusions provide some subjective improvement in patient quality of life, dyspnoea and fatigue. Unfortunately, the duration of this benefit is unexpectedly shorter than the duration of objective increase in that patient's haemoglobin concentration (Mercadante, Ferrera et al. 2009). This may in part be explained by alterations in blood during its 'shelf-life' of permitted storage, which can affect oxygen carrying ability, ˙NO bioavailability and red blood cell morphology (D'Alessandro, Kriebardis et al. 2014). Dietary nitrate supplementation might counter some of this reduction in ˙NO bioavailability, and could potentially reduce some of the adverse symptoms and physiological adaptations associated with anaemia in patients with cancer through its effect on vascular tone, mitochondrial respiration, calcium handling, muscle contractile force and efficiency.

Chemotherapy-related cognitive impairment, sometimes referred to as 'chemobrain' by patients and their treating physicians, was first described in the 1970s (Weiss, Walker et al. 1974), affecting between 17 and 70% of patients with malignancy (Myers 2009). The pathophysiology of this debilitative condition is poorly understood but likely to be multifactorial. Firstly, DNA damage can occur through endocrine therapy-induced changes in antioxidant function (Ahles, Root et al. 2012). For example, Tamoxifen is genotoxic as it is metabolically activated via alpha-hydroxylation and sulphate conjugation to give reactive species that bind to DNA at the N(2) position of guanine, producing pro-mutagenic genetic lesions. Secondly, impairment of hippocampal neurogenesis by anti-cancer therapies can cause cognitive impairment (Monje, Vogel et al. 2007). Robust generation of new neurones

in this area of the brain occurs throughout life in healthy individuals, and is important in the generation of new memories. Cranial irradiation can inhibit this formation by perturbation in the neurogenic microenvironment by microglial inflammation, although anti-inflammatories can partially restore neurogenesis after radiation exposure. The presence of apolipoprotein E4 allele (APOE) (Ahles, Root et al. 2012) predisposes to cancer treatment – induced cognitive deterioration. APOE is a complex glycoprotein which facilitates the uptake, transport and distribution of lipids and plays a role in neuronal repair and plasticity after injury. The APOE4 allele has been associated with Alzheimer's disease, aging and brain trauma. However, patients with at least one APOE4 allele who had survived cancer and chemotherapy had a significant reduction in their cognitive function compared to those survivors who did not carry this allele (Ahles, Root et al. 2012). Finally, cancer treatment factors such as the type, timing and duration of treatment (Myers 2009) can have a significant influence on the likelihood that a patient may develop cognitive impairment as a result of that treatment. Self-renewing, lineage-committed neural progenitor cells and non-dividing mature oligodendrocytes (myelin-forming cells) are the most vulnerable cell populations to chemotherapeutic agents. Repetitive exposure to chemotherapeutic agents can exceed cellular repair potential and result in long-term suppression of cell division and apoptosis in the subventricular zone, hippocampus, and major white matter tracts of the central nervous system in animal models (Myers 2009), thus producing chemotherapy-induced cognitive impairment.

Pathological conditions can substantially upregulate the production of ˙NO by inducible nitric oxide synthase (iNOS) in activated macrophages and neutrophils (Beckman and Crow 1993). iNOS is one of three key enzymes generating nitric oxide from L-arginine (Lirk, Hoffmann et al. 2002). INOS-derived ˙NO plays an

important role in numerous physiological and pathophysiological conditions, making iNOS a mediator of unspecific host defence and central in the clearance of bacterial, viral fungal and parasitic infections. The concentration of ˙NO generated can be sufficient to produce nitrogen dioxide and thus greatly increase the toxic effects of ˙NO, linking iNOS to tissue damage and organ dysfunction, for example in the hypotensive and vasoplegive state characteristic of septic shock. ˙NO contains an unpaired electron, so readily participates in free radical processes of pathological relevance. For example, macrophages produce superoxide ($O_2^{\cdot-}$) which reacts with ˙NO to produce the powerful oxidant, peroxynitrite ($ONOO^-$) (Beckman and Crow 1993):

$$O_2^{\cdot-} + {}^{\cdot}NO \rightarrow ONOO^-$$

This potent biological oxidant has been implicated in free radical-induced tissue injury, and thus might be an important mediator of central nervous system injury following chemotherapy treatment (Tangpong, Cole et al. 2007). Hence, dietary nitrate supplementation may have an impact on the cognitive function of cancer patients.

Patients with cancer have an increased thrombotic risk due to a variety of factors such as immobility, infection and inflammation (Lee and Levine 2003). ˙NO inhibits platelet aggregation and hence, dietary supplementation with nitrate may provide some protection against thromboembolic events in this population of patients.

This introduction will outline current knowledge of nitric oxide synthesis and activity, and the effect dietary nitrate supplementation has on these biochemical pathways and human physiology both in health and in disease, investigating any potential clinical applications.

1.1 ˙NO SYNTHESIS

Nitric oxide is a free radical gas molecule which was initially thought to be merely an atmospheric pollutant. Its pharmacological properties were discovered in the late 1970s during studies of the pharmacology of glyceryl trinitrate (Arnold, Mittal et al. 1977); ˙NO was found to be a potent vasodilator and inhibitor of platelet function and leucocyte adhesion. Endothelial-derived relaxing factor, an endogenous vasodilator produced by the blood vessel was described in 1979 (Furchgott and Zawadzki 1980, Ignarro 2002), but was identified as ˙NO in 1987 (Ignarro, Buga et al. 1987, Palmer, Ferrige et al. 1987). There are two main pathways through which ˙NO is synthesised in humans; the Nitric Oxide Synthase pathway and the sequential reduction of Nitrate.

1.1.1 THE L-ARGININE-NITRIC OXIDE PATHWAY

Endogenous ˙NO is synthesised from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). Active NOS functions as a tetramer consisting of two identical NOS monomers bound to two calmodulins (Alderton, Cooper et al. 2001). Each NOS monomer can be functionally and structurally divided into two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain. The former contains binding sites for one molecule each of nicotinamide adenine dinucleotide phosphate (NADPH), and the flavins, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), in close homology with the cytochrome P-450 reductase. The N-terminal oxidase domain binds iron protoporphyrin IX (haem) and the NOS

cofactor, tetrahydrobiopterin (BH_4), as well as the substrate L-arginine. Between these two regions lies the calmodulin (CaM) binding domain. NOS catalyses a complex five-stage oxidation reaction involving the transfer of electrons from NADPH to FAD and FMN via CaM in the carboxy-terminal reductase domain, to the haem in the amino-terminal oxidase domain, where L-arginine is oxidised to L-citrulline and ˙NO (Andrew and Mayer 1999). This complex reaction is calcium- and oxygen-dependent, while also relying on the availability of five essential substrates to maintain constant ˙NO production (Crabtree, Tatham et al. 2009). Deficiency of NADPH, FAD, FMD, haem or BH_4 causes a reduction in ˙NO synthesis.

Three diverse isoforms of NOS exist, which differ in their structure and function; endothelial and neuronal NOS (eNOS and nNOS) are expressed constitutively in endothelial cells and neurones respectively, and can be collectively referred to as constitutive NOS. These are usually calcium ion (Ca^{2+}) dependent, although they can be activated in a Ca^{2+} independent manner. Inducible NOS (iNOS) is expressed at high levels only after induction by cytokines or other inflammatory agents, and its activity is independent of an increase in calcium (Andrew and Mayer 1999). The three NOS isoforms display high homology in their oxidase and reductase domains, but each isoform exhibits distinctive features which reflect their specific *in vivo* function in the vascular endothelium, the central nervous system and in white blood cells (Andrew and Mayer 1999).

1.1.2 THE ENTERO-SALIVARY CIRCULATION OF NITRATE

This pathway is the second major source of ˙NO synthesis, and describes the sequential reduction of NO_3^- to NO_2^- then ˙NO. The UK population consumes foodstuffs containing 57 mg of NO_3^- daily on average. Vegetables are the main dietary source of this NO_3^-, contributing approximately 70% of the total dietary exposure to NO_3^- of the general UK population (Ysart, Miller et al. 1999). The estimated mean NO_3^- intake reported by Ysart Miller et al (1999) was 52 mg per day per person in the United Kingdom, although individual dietary preferences and variability would significantly affect this intake. In particular, cruciferous and green leafy vegetables, and beetroot contain higher NO_3^- concentrations than other foods. NO_3^- is taken up by plants from soil and the use of nitrogen-based fertilisers may further increase NO_3^- concentrations in crops. Nitrite (NO_2^-) is also found in some foods such as meats, where it is used as a food additive to reduce risk of botulism and enhance its appearance. Environmental sources of NO_2^- include cigarette smoke, car exhausts and other environmental pollutants (Lundberg, Weitzberg et al. 2004).

NO_3^- is rapidly and completely absorbed into the bloodstream through the proximal gastro-intestinal tract following ingestion (Florin, Neale et al. 1990) (Figure 1). Sialin, an electrogenic NO_3^-/H^+ transporter found in the plasma membrane of salivary acinar cells (Qin, Liu et al. 2012), actively concentrates plasma NO_3^- into the salivary glands at 10-20 times higher than those levels found in the plasma (Lundberg 2012). Upon salivation, this is secreted into the oral cavity where a proportion of it is reduced into NO_2^- by facultative anaerobic bacteria located posteriorly on the dorsal surface of the

tongue (Doel, Benjamin et al. 2005). In this hypoxic setting, bacterial genera such as *Actinomyces* and *Veillonella* use NO_3^- as a terminal electron acceptor and produce NO_2^- as a by-product, a reaction catalysed by bacterial nitrate reductase (Lundberg, Weitzberg et al. 2004). A small proportion of this NO_2^- is further reduced to $^{\cdot}NO$ in the oral cavity through bacteria or periodontal acidity (Gilchrist, Winyard et al. 2010), but the majority of NO_2^- is swallowed and, when exposed to stomach acid, produces a complex array of nitrogen oxides including nitrous acid (HNO_2), nitrogen dioxide ($^{\cdot}NO_2$), dinitrogen trioxide (N_2O_3) and $^{\cdot}NO$ (Lundberg, Weitzberg et al. 2004):

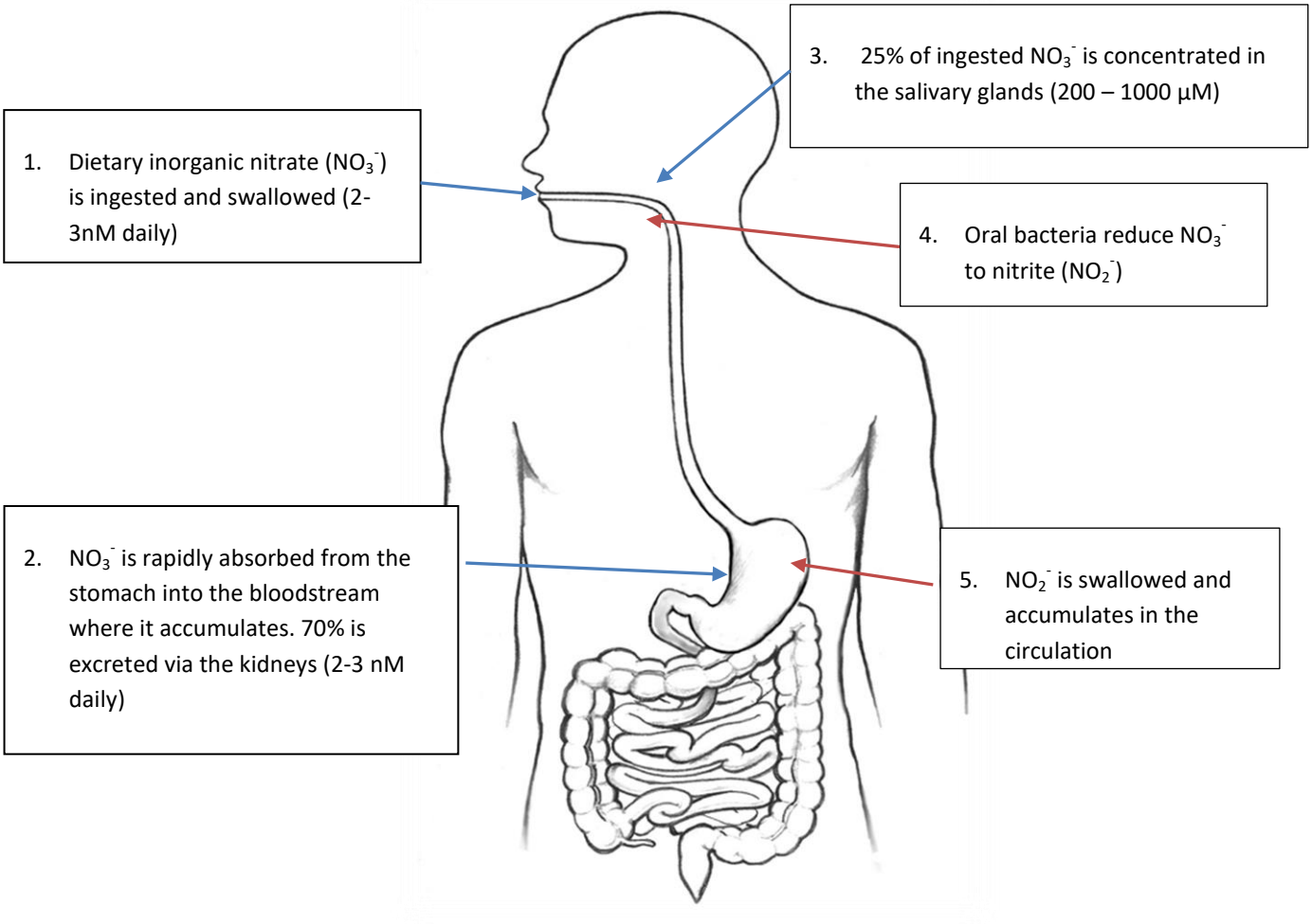
$$NO_2^- + H^+ \leftrightarrow HNO_2$$

$$2HNO_2 \rightarrow N_2O_3 + H_2O$$

$$N_2O_3 \rightarrow {}^{\cdot}NO + {}^{\cdot}NO_2$$

The chemical nature and reactivity of these, and other, reactive nitrogen intermediates (RNIs) formed in humans depends on ambient factors of the environment in which they are produced, including oxygen tension, acidity, proximity to haem-containing proteins, redox state and concentration of thiols. Within the stomach and gastrointestinal tract, these RNIs contribute to killing of ingested pathogens in the stomach, and the maintenance of gastric mucosal integrity through promotion of mucous production and regulation of gastric mucosal blood flow (Lundberg, Weitzberg et al. 2004). More distant effects are achieved through absorption of NO_2^-, and to a lesser extent $^{\cdot}NO$, from the gut into the circulation.

Figure 1: The Enterosalivary Circulation of Dietary Nitrate (Lundberg, Weitzberg et al. 2004)



NO_3^- ingestion increases plasma NO_2^- levels in humans (Bailey, Winyard et al. 2009, Vanhatalo, Fulford et al. 2011, Kelly, Fulford et al. 2013). This increase in plasma NO_2^- is attenuated through the use of antimicrobial mouthwashes (Govoni, Jansson et al. 2008) and through spitting of saliva rather than swallowing (Webb, Patel et al. 2008) after ingestion of inorganic NO_3^-, underlying the importance of the enterosalivary circulation and the role of oral facultative bacteria on the reduction of NO_3^-.

1.2 THE EFFECTS OF ˙NO IN HUMAN PHYSIOLOGY

˙NO gas is extremely unstable, being oxidised within seconds to nitrogen dioxide and higher oxides of nitrogen. In neutral aqueous solution, ˙NO exposed to oxygen has a half-life of less than 10 seconds (Ignarro 1989). Each NOS enzyme increases local ˙NO production in the vascular endothelium, white blood cells and the central nervous system according to local stimuli. Bioavailability of ˙NO produced through the L-arginine-nitric oxide synthase pathway or the enterosalivary circulation of nitrate may be regulated by formation of NO-containing compounds such as S-nitrosothiols, N-nitroso proteins and iron-nitrosyl complexes, along with NO_2^- (Allen, Giordano et al. 2012). These NO-derived species can be transported through the circulation in either the plasma or in red blood cells, and ˙NO is liberated in hypoxic conditions. This vascular storage pool essentially buffers the concentration of ˙NO (Stamler, Jaraki et al. 1992), although controversy exists regarding its nature. Some have proposed the theory that the reservoir of ˙NO donors is in the form of S-nitrosothiols (Foster, McMahon et al. 2003) while others suggested it is in the form of NO_2^- (Cosby, Partovi et al. 2003).

While investigating how nitrogen oxide-containing vasodilators such as glyceryl trinitrate exert their effect, Ignarro et al (1981) discovered that they react with cysteine residues to form S-nitrosocysteine through S-nitrosation (Ignarro, Lippton et al. 1981). S-nitrosation is the post-translational modification of a protein or amino acid. This term describes the reversible, covalent attachment of nitrogen monoxide to the thiol side chain of free cysteine, residues, or low molecular weight thiols such as glutathione with a one-electron oxidation of the ˙NO radical. This forms an S-

nitroso group (RS-NO) on the protein or amino acid (Hess, Matsumoto et al. 2005, Smith and Marletta 2012). RSNOs have long been known to circulate in the plasma and to have bioactivities similar to those of ˙NO but with half-lives in the order of hours (Stamler, Jaraki et al. 1992). Formation and degradation of RSNOs is a dynamic process which is largely influenced by the prevailing redox environment, oxygen and metal ion availability, and thiol reactivity (Miersch and Mutus 2005). Their formation can only proceed in the presence of an electron sink which enables oxidation in human physiology. An electron sink is an electrophilic atom or group of atoms that can capture an electron from another part of an electrical system (Ducluzeau, van Lis et al. 2009). This sink is either anaerobic metabolism, or transition metals in human physiology (Smith and Marletta 2012). RSNO formed through the mechanisms described above can then be transferred to small molecule or protein molecules through transnitrosation reactions, in which the ˙NO group from an RSNO is transferred to an acceptor cysteine thiol on another protein or small molecule (Foster, McMahon et al. 2003, Hess, Matsumoto et al. 2005). S-nitrosation of a wide range of proteins may therefore enable ˙NO to exert its ubiquitous effect on cellular function as outlined in section 1.2.1 below, while also buffering ˙NO concentrations in plasma.

Dinitrosyl iron complexes (DNICs) are bio-organic complexes of ˙NO which have also been implicated in RSNO formation. DNICs spontaneously form in aqueous solution containing ˙NO, ferrous iron and many anionic species. In biological systems, the reaction of ˙NO with iron-sulphur centres of intracellular proteins such as mitochondrial aconitase involved in electron transport forms high molecular mass DNICs (Henry, Lepoivre et al. 1993). Such protein-bound dinitrosyl-iron-dithiolate complexes are characterised by electron paramagnetic response spectra with g =

2.04 and g = 2.015 (Vanin, Men'shikov et al. 1992). ˙NO can also react with free cellular iron to form low molecular mass DNICs with cysteine or glutathione as the major ligands (Giannone, Takeda et al. 2000). Low molecular weight DNICs appear to have a significant role in the control of iron-dependent cellular processes. They are present in inflammatory cells (Pellat, Henry et al. 1990, Vanin 2009), tissues expressing iNOS (Muller, Kleschyov et al. 1996), and upon exposure to ˙NO-releasing compounds (Watts, Hawkins et al. 2006). In comparison to other ˙NO-derived adducts such as S-nitrosothiols, DNICs quantitavely represent the largest intracellular pool (Hickok, Sahni et al. 2011), although their relative importance is yet to be determined. However, they have a long half-life and are found in most tissues within most biological systems, and will probably be formed under all cellular settings of ˙NO production. The *in vitro* rate of formation of DNICs in murine macrophages is determined by the concentration and duration of ˙NO exposure, while the absolute quantity of DNIC formed is equivalent to or just in excess of the intracellular concentration of iron (Hickok, Sahni et al. 2011). The disappearance of DNICs once they are formed has proven impossible to fully characterise. Similarly, the effect of anaemia on intracellular low molecular weight iron has not been fully established, although it seems likely, albeit not proven, that levels of intracellular low molecular weight iron would fall in anaemia. If this were indeed the case, the availability of iron to form DNICs would be impaired by anaemia, which would have a series of adverse consequences on ˙NO bioavailability and its functions in healthy individuals.

˙NO metabolism is also likely to be affected by iron overload. Iron is usually closely regulated by a number of homeostatic mechanisms in order to provide an adequate intracellular level of this micronutrient whilst preventing its accumulation and toxicity (Abbaspour, Hurrell et al. 2014). In healthy individuals, upon infection, macrophages

phagocytose excess iron, thereby preventing free radical injury and reducing iron availability for the invading microorganism (Weinberg 2000). However, iron can accumulate in humans as a result of pathological conditions such as genetic haemochromatosis, but also as a result of blood transfusion programs. In such a scenario, accumulated iron can generate oxidative stress by acting as a catalyst of free radical reactions, increasing the rate of hydroxyl production ($^{\cdot}HO$) (Galleano, Simontacchi et al. 2004) with resultant tissue damage. A complex relationship exists between iron and $^{\cdot}NO$, and is yet to be fully characterised. In response to infection, iron-rich macrophages produce vast quantities of $^{\cdot}NO$ through the activity of iNOS, which in turn is dimerised and activated by heme iron (Stuehr 1999). Hence, the phagocytosis of iron by activated macrophages may be intended to allow this rapid production of $^{\cdot}NO$ in response to invading pathogens, although controversy remains regarding the influence and significance of iron on inflammatory $^{\cdot}NO$ production. Iron dextran, a preparation of intravenous iron used to treat patients with iron deficiency anaemia, administered to rats along with lipopolysaccharide from *Escherichia coli*, was found to cause increased iron uptake in the liver Kuppfer cells. This then led to an increased plasma $^{\cdot}NO$ concentration through increased iNOS activity within the Kuppfer cells (Galleano, Simontacchi et al. 2004). $^{\cdot}NO$ also plays a role in modulating the response of iron regulatory proteins such as iron regulatory protein 1 (IRP-1) and IRP-2. These two cytoplasmic iron regulatory proteins modulate the translation and stability respectively of ferritin and transferrin receptor messenger ribonucleic acid (mRNA) (Cairo, Recalcati et al. 2002), thus controlling the homeostasis of iron. $^{\cdot}NO$ increases IRP-1 activity while IRP-2 is inactivated by $^{\cdot}NO$. The latter effect accounts for the increased ferritin synthesis in cytokine-stimulated macrophages producing $^{\cdot}NO$ and $ONOO^{-}$, thus explaining the observed

sequestration of ferritin in macrophages under inflammatory conditions (Recalcati, Taramelli et al. 1998). It remains unclear if this effect can be used to minimise free radical formation in the diseased state. However, further exploration of the relationship between iron and ˙NO would be of benefit in improving our understanding of inflammatory conditions such as haemophagocytic lymphohistiocytosis, the detrimental effect of iron accumulation, and the effect of anaemia on iron metabolism and its interaction with ˙NO at a cellular level. Having been previously considered biochemically inert, NO_2^- appears to play an important role in ˙NO metabolism as part of a complex hypoxia-sensitive redox signalling biochemical system (Dezfulian, Raat et al. 2007). It exists at concentrations between 150 - 1000 nM in plasma and >10 μM in aortic ring tissue (Gladwin, Shelhamer et al. 2000), and has a half-life of tens of minutes (Bryan, Fernandez et al. 2005). This potential ˙NO reservoir is in vast excess compared to plasma RSNO concentrations, conjectured to be approximately 10 μM (Stamler, Jaraki et al. 1992). As described in section 1.1.2, ˙NO can be endogenously synthesised in acidotic conditions through reduction of NO_2^- by non-enzymatic acidic disproportionation or through the catalytic effect of enzymes (Samouilov, Kuppusamy et al. 1998, Dezfulian, Raat et al. 2007). NO_2^- disproportionation, or spontaneous reduction and oxidation, is described in the equation below:

$$3NO_2^- + 2H^+ \leftrightarrow 2^{\cdot}NO + NO_3^- + H_2O$$

Most biochemical reactions which produce ˙NO favour conditions of low oxygen tension and low pH, such as hypoxic vascular beds or ischaemic tissues. NOS activity is impaired in such conditions (Webb, Patel et al. 2008) but NO_2^- reduction can occur independent of these enzymes through disproportionation, or other

catalysts of reduction of NO_2^- to $^{\cdot}NO$ such as xanthine oxidase (Zhang, Naughton et al. 1998), aldehyde reductase (Li, Cui et al. 2008), deoxyhaemoglobin (Cosby, Partovi et al. 2003), myoglobin and mitochondrial cytochrome oxidase (Aamand, Dalsgaard et al. 2009, Gilchrist, Winyard et al. 2010). Deoxyhaemoglobin appears to be of particular importance in this context as it reduces NO_2^- along the physiological oxygen gradient, synthesising more $^{\cdot}NO$, enabling greater blood flow through vasodilation, and hence maximising oxygen delivery in hypoxic tissue beds (Cosby, Partovi et al. 2003, Dezfulian, Raat et al. 2007). This mechanism was supported by the demonstration of an arterio-venous gradient of NO_2^- in the presence of pharmacological inhibition of NOS (Gladwin, Shelhamer et al. 2000). This group reported a reduction in plasma NO_2^- in studies of forearm blood composition between arterial and venous samples, a gradient which was exaggerated by exercise of the limb. This suggests that there is a net consumption of NO_2^- in the hypoxic, more acidic capillary bed, with subsequent liberation of $^{\cdot}NO$ and hence, vasodilation. Interestingly they noted no gradient in the concentration of RSNOs.

The role of deoxyhaemoglobin in NO_2^- reduction is somewhat controversial. Haemoglobin is a highly efficient transporter of oxygen; a conformational change between its high oxygen affinity relaxed or R-state and its low oxygen affinity T-state improves this efficiency through an allosteric mechanism. Fully oxygenated haemoglobin in the R-state has long been recognised as a destroyer of $^{\cdot}NO$ (Ignarro 1989); in combination they form the ferric (Fe) form of haemoglobin, methaemoglobin (MetHb) and NO_3^- through the dioxygenation reaction;

$$HbO_2 + {}^{\cdot}NO \rightarrow MetHb + NO_3^-$$

This reaction occurs extremely quickly with a rate constant of 6-8 x 10^7 M^{-1} s^{-1} (Doyle, Pickering et al. 1981), meaning the half-life of ˙NO in the oxygenated red blood cell (RBC) is 0.5 μs, enough time for it to only diffuse approximately 0.02 μm. This effectively means that ˙NO would be trapped within the oxygenated RBC. Deoxygenated ferrous (Fe^{2+}) haemoglobin, however, appears to preserve the activity of ˙NO through the formation of iron-nitrosyl Hb (Sharma and Ranney 1978).

$$Hb + {}^{\cdot}NO \rightarrow HbNO$$

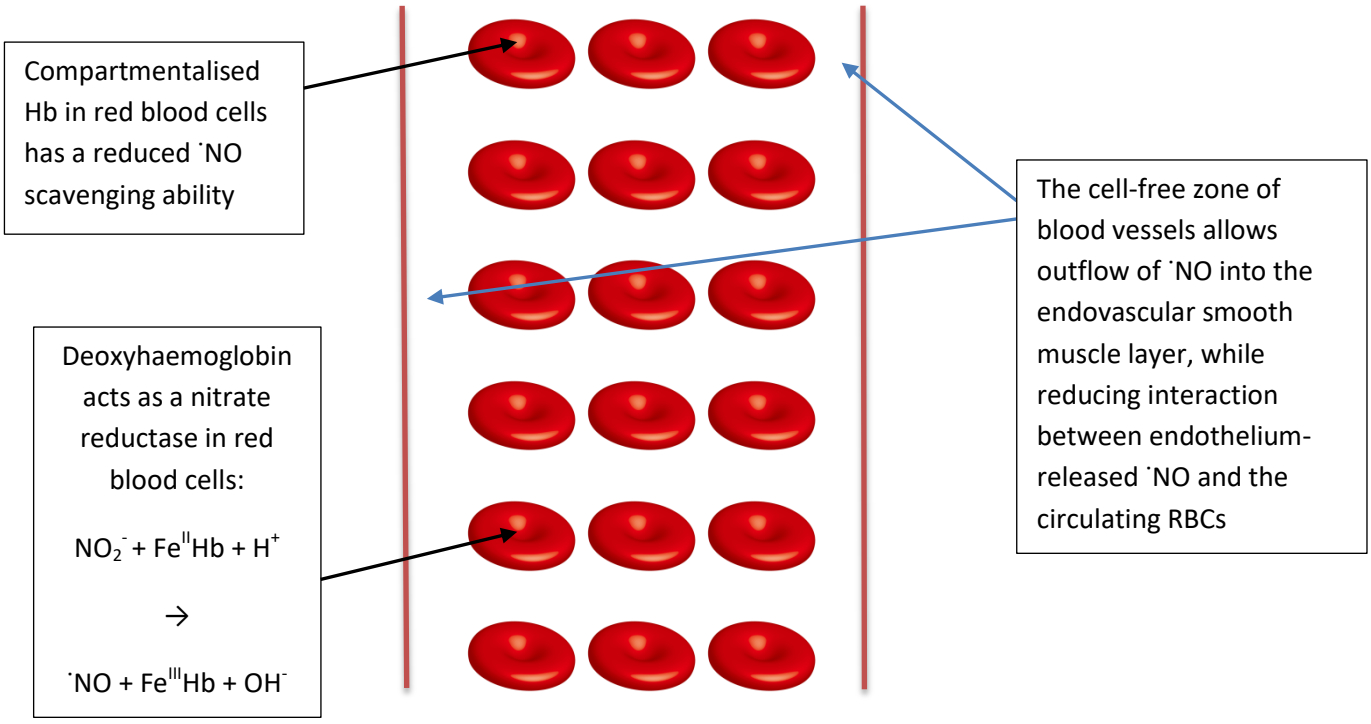
This reaction can occur at any of the four haem groups in the haemoglobin tetramer when it is in its deoxygenated state. However, subsequent release of ˙NO is slow, and when it is released, it would be rapidly subject to the dioxygenation reaction and therefore RBC release of ˙NO could be prevented.

While on the above evidence, haemoglobin would appear to be an ˙NO destroyer, its role is rather more complex, and several mechanisms have been proposed to enable it to contribute to ˙NO signalling. Firstly, compartmentalisation of haemoglobin within the RBC reduces its ability to scavenge ˙NO (2). When Hb is encapsulated within a RBC, it scavenges ˙NO up to 1000 times slower than when the Hb is not within a RBC, possibly through the effect intravascular flow and shear stress has on particles, and the subsequent formation of a cell-free zone (Liao, Hein et al. 1999). This RBC-free zone exists near the blood vessel wall, can be as much as 20-25% of the vessel's diameter, and allows the outflow of ˙NO into the endovascular smooth muscle layer, while reducing interaction between endothelium-released ˙NO and the circulating RBCs (Butler, Megson et al. 1998, Liao, Hein et al. 1999). Secondly, deoxyhaemoglobin (deoxyHb) preserves ˙NO bioavailability through its reaction with

NO_2^-, acting as a nitrite reductase to produce methaemoglobin (MetHb, $Fe^{III}Hb$) and $^{\cdot}NO$ (Huang, Keszler et al. 2005).

$$NO_2^- + Fe^{II}Hb + H^+ \rightarrow {}^{\cdot}NO + Fe^{III}Hb + OH^-$$

Figure 2: Effects of red blood cell deoxyhaemoglobin on ˙NO availability



This nitrite reductase activity of deoxyhaemoglobin produces ˙NO under allosteric control. This can be explained by the quaternary structure of Hb; in its deoxygenated T state, haemoglobin reacts with a single NO_2^- to form MetHb and an iron-nitrosyl-haem. These products have an R state-stabilising effect which lowers the redox potential of between two and six other haemoglobin chains, hence producing autocatalysis as R state haems form more R state haems, increasing the rate of NO_2^- reduction to ˙NO (Huang, Shiva et al. 2005). This could be allowed to escape the RBC through the existence of a membrane-associated nitrite-reductase metabolon, a supramolecular complex of sequential metabolic enzymes. This metabolon consists of deoxyHb, metHb, anion exchange protein, carbonic anhydrase, aquaporin and rhesus channels, hence bringing together nitrite, proton,

deoxyhaemoglobin, and highly hydrophobic channels which could concentrate the lipophilic ˙NO at the membrane complex (Cosby, Partovi et al. 2003, Huang, Shiva et al. 2005). The lipophilic nature of ˙NO allows it to readily diffuse across the red blood cell membrane following production.

No conclusive evidence exists to back either nitrite or S-nitrosothiols as the major vascular storage pool of NO; however, three factors suggest NO_2^- plays this role in human physiology. Firstly, NO_2^- is present in plasma, RBCs and tissues in greater concentration than RSNOs (Gladwin, Shelhamer et al. 2000). Secondly, it is more stable than RSNOs as it is not easily reduced by intracellular reductants such as glutathione (Gladwin, Wang et al. 2002); the reductive intra-erythrocytic environment decomposes SNO-Hb and prevents its accumulation as a reservoir of ˙NO. Thirdly, a gradient of NO_2^- concentration has been observed between the arterial and the venous elements of capillary beds indicating NO_2^-consumption between the two areas, with no difference in RSNO concentration (Gladwin, Wang et al. 2002).

While controversy exists regarding the storage and transport of potential endogenous ˙NO donors, there is no dispute regarding the multitude of potentially beneficial effects ˙NO exerts in both local and systemic human homeostatic mechanisms and hence, dietary supplementation with inorganic NO_3^- may provide a therapeutic tool in combating a number of disease states.

1.2.1 CIRCULATION

It has long been recognised that consumption of a diet plentiful in fruit and vegetables is associated with reduction in blood pressure and adverse cardiovascular events (Webb, Patel et al. 2008). In particular, the dietary intervention which affords greatest protection against cardiovascular disease is to increase consumption of green leafy vegetables such as spinach. After initial hypotheses that this benefit was derived from an increase in antioxidants, large trials have not confirmed their role; however, green leafy vegetables and root vegetables such as beetroot have a high nitrate content and their consumption increases ˙NO bioavailability, hence exerting cardioprotective and blood pressure lowering effects (Webb, Patel et al. 2008, Kelly, Fulford et al. 2013).

˙NO exerts this blood pressure-lowering effect through vasodilation and a reduction in peripheral vascular resistance (Bond, Curry et al. 2013). It acts as a local modulator of vascular tone, a role enabled by its lipophilic nature and hence its ability to penetrate tissues to exert its effect in the smooth muscle cells beneath the vascular endothelium (Ignarro 2002). The vasodilatory effect of ˙NO occurs in vascular smooth muscle due to its interaction with the iron haem in guanylate cyclase, activating this enzyme and hence catalysing the production of guanosine 3’5’-monophosphate (cGMP) from guanosine 5’-triphosphate (GTP). cGMP activates several protein kinases which cause calcium extrusion from the smooth muscle cell and increased uptake by the sarcoplasmic reticulum. This reduction in cytoplasmic calcium in turn causes smooth muscle relaxation and subsequent blood vessel dilation (Allen, Giordano et al. 2012).

˙NO can be endogenously synthesised through reduction of circulating NO_2^- by non-enzymatic acidic disproportionation as mentioned above (Samouilov, Kuppusamy et al. 1998, Dezfulian, Raat et al. 2007), thus mediating vasodilation.

Most biochemical reactions which produce ˙NO favour conditions of low oxygen tension and low pH, such as hypoxic vascular beds or ischaemic tissues. NOS activity is impaired in such conditions (Webb, Patel et al. 2008) but NO_2^- reduction can occur independent of these enzymes through disproportionation. Xanthine oxidase (Zhang, Naughton et al. 1998), aldehyde reductase (Li, Cui et al. 2008), deoxyhaemoglobin (Cosby, Partovi et al. 2003), myoglobin and mitochondrial cytochrome oxidase (Gilchrist, Winyard et al. 2010) can all catalyse the reduction of NO_2^-. Deoxyhaemoglobin is of particular importance in this context as it reduces NO_2^- along the physiological oxygen gradient, synthesising more ˙NO, enabling greater blood flow and hence maximising oxygen delivery in hypoxic tissue beds (Cosby, Partovi et al. 2003, Dezfulian, Raat et al. 2007).

˙NO synthesis via NO_2^- reduction conveys protection against hypoxic ischaemia/reperfusion injury in the heart (Bryan, Calvert et al. 2007), brain, liver and kidneys (Dezfulian, Raat et al. 2007) of animal models. NO_2^- itself can directly nitrosate thiols to form S-nitrosothiols (RSNOs) *in vitro* (Bryan and Grisham 2007) which have also been shown to be protective in the setting of ischaemia/reperfusion (Dezfulian, Raat et al. 2007).

Similar to the controversy regarding the nature of the ˙NO-donating vascular storage pool, an alternative mechanism has been proposed through which ˙NO matches regional blood flow with metabolic demand, involving S-nitrosohaemoglobin (SNO-Hb). SNO-Hb forms when ˙NO binds the cysteine residue in the 93rd amino acid

position of the β subunit of haemoglobin in concert with the oxygenation-induced allosteric reaction of haemoglobin, and dissociates to dispense equivalents of S-nitrosothiol (RSNO) in proportion to the degree of hypoxia of the tissue it is perfusing. This enables hypoxic vasodilation and hence, matching of oxygen supply and metabolic demand (Bennett-Guerrero, Veldman et al. 2007). However, this SNO-Hb hypothesis has been challenged by the results of further research involving murine models. Isbell *et al* (2008) created mouse models that expressed exclusively either human wild type haemoglobin, or human haemoglobin in which the β93cys residue was replaced by alanine (hence preventing formation of SNO-Hb). This substitution did not cause any alteration in red cell mediated hypoxic vasodilation, and no deficits in pulmonary or systemic haemodynamics. This suggests that SNO-Hb is in fact not essential for the physiologic coupling of RBC deoxygenation and increased ˙NO-bioactivity *in vivo* (Isbell, Sun et al. 2008). They conjecture that in fact, the mediator of vasodilation in hypoxic capillaries is ATP release from hypoxic red blood cells. This ATP binds to purinergic receptors on the endothelium and thus stimulates NO˙ production through endothelial nitric oxide synthase, resulting in vasodilation. To support this alternative mechanistic theory, the same group found that hypoxic capillary vasodilation was restricted by more than 90% in the presence of an eNOS inhibitor. However, the extrapolation of this murine model to explain human physiology may be misleading and further studies are required to further explore this question.

Platelet activity is governed by a variety of positive and negative stimuli that act to precisely regulate the process of homeostasis. Positive stimulators of platelet aggregation include subendothelial collagen, thrombin generated from the coagulation cascade, ADP and thromboxane A_2. The major inhibitory factors

dictating platelet function are prostacyclin and ˙NO, which are generated by the vascular endothelium (Radomski, Palmer et al. 1987, Apostoli, Solomon et al. 2014). ˙NO exerts its effect through activation of cGMP which results in reduced free cytoplasmic calcium, inhibition of platelet phosphoinositide (PI3) kinase, and hence reduction in the number and affinity of the platelet membrane fibrinogen-binding site Glycoprotein IIb/IIIa (Loscalzo 2001, Wallis 2005). Dietary nitrate supplementation with beetroot juice has also been shown to inhibit *ex vivo* platelet aggregation to ADP and collagen 2.5 hours after ingestion (Webb, Patel et al. 2008). While attempting to improve understanding of the physiological mechanism of this effect, Velmurugan et al (Velmurugan, Kapil et al. 2013) discovered that NO_3^- supplementation only reduced platelet reactivity in males, not females in a small study of healthy human volunteers. This was found to be caused by an elevation of circulating NO_2^- in male participants, and hence an elevation of platelet cGMP which was not demonstrated in females. They conjectured that dietary NO_3^- supplementation could convey some protection against cardiovascular disease without the bleeding risk associated with drug treatment such as aspirin or other antiplatelet agents. This theory was supported in an experiment using eNOS knockout mice to mimic conditions of endothelial dysfunction (Apostoli, Solomon et al. 2014). They reported that dietary NO_3^- supplementation exerts an antiplatelet effect, and hence, that this intervention could reduce platelet hyperactivity in endothelial dysfunction. This impact is currently being explored by the NITRATE-OCT randomised double-blind study investigating the effects of inorganic nitrate supplementation on vascular function, platelet reactivity and restenosis of coronary arterial stents in patients with stable angina (Rathod, Jones et al. 2016). It is yet to report its findings but hypothesises that rates of myocardial infarction, death,

cerebrovascular event and coronary arterial stenosis in those patients receiving oral nitrate is lower than placebo as a result of the effects of such supplementation on endothelial function and platelet activity.

1.2.2 RESTING METABOLIC RATE AND EXERCISE PERFORMANCE

Resting Metabolic Rate (RMR) is the minimum energy required to sustain vital body functions in the resting state during fasting conditions. RMR may be reduced by acute dietary nitrate supplementation in healthy individuals (Larsen, Schiffer et al. 2014), although this conclusion does not support the previous findings reported by Kelly in 2013 (Kelly, Vanhatalo et al. 2013).

Classical exercise physiology dictates that, regardless of training status, age, or diet, only minor fluctuations occur in an individual's oxygen consumption at a given exercise workload. However, recent research has questioned this belief, demonstrating that acute dietary nitrate supplementation can reduce this consumption in healthy individuals (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Lansley, Winyard et al. 2011). While dietary NO_3^- supplementation was not demonstrated to reduce oxygen consumption in walking exercise in a small study of 12 older participants aged between 60 and 70 years old (Kelly, Fulford et al. 2013), a speeding of VO_2 kinetics was observed on initiation of exercise.

The reason for this observed improvement in exercise efficiency following dietary NO_3^- supplementation is multifactorial, but probably either reflects an effect of NO_3^- or its metabolites on the mitochondria, or on cellular handling of calcium.

1.2.2.1 EFFECT OF NO_3^- SUPPLEMENTATION ON MITOCHONDRIA

Mitochondria are double membrane-bound organelles found in large numbers in most eukaryotic cells. They house the biochemical processes of respiration and produce energy in the form of adenosine triphosphate (ATP). Acute NO_3^- supplementation may improve exercise efficiency either by improving the efficiency of mitochondrial ATP production, or through reducing their oxygen consumption not directly related to ATP production. In order to explain this effect, it must be appreciated how mitochondria generate ATP during oxidative phosphorylation, the final stage of respiration. Electrons stripped from oxidisable substrates such as glucose, fatty acids etc., pass down a series of electron carriers in the mitochondrial respiratory chain to reduce molecular oxygen to water (Figure 3). This process of electron transfer generates energy which drives the endergonic (energy-consuming) proton pumping activities of respiratory complexes I, III and IV (also known as cytochrome c oxidase), which in turn create a protonmotive force across the inner mitochondrial membrane. Dissipation of this force through ATP synthase drives adenosine diphosphate (ADP) phosphorylation and hence, ATP is produced (Divakaruni and Brand 2011).

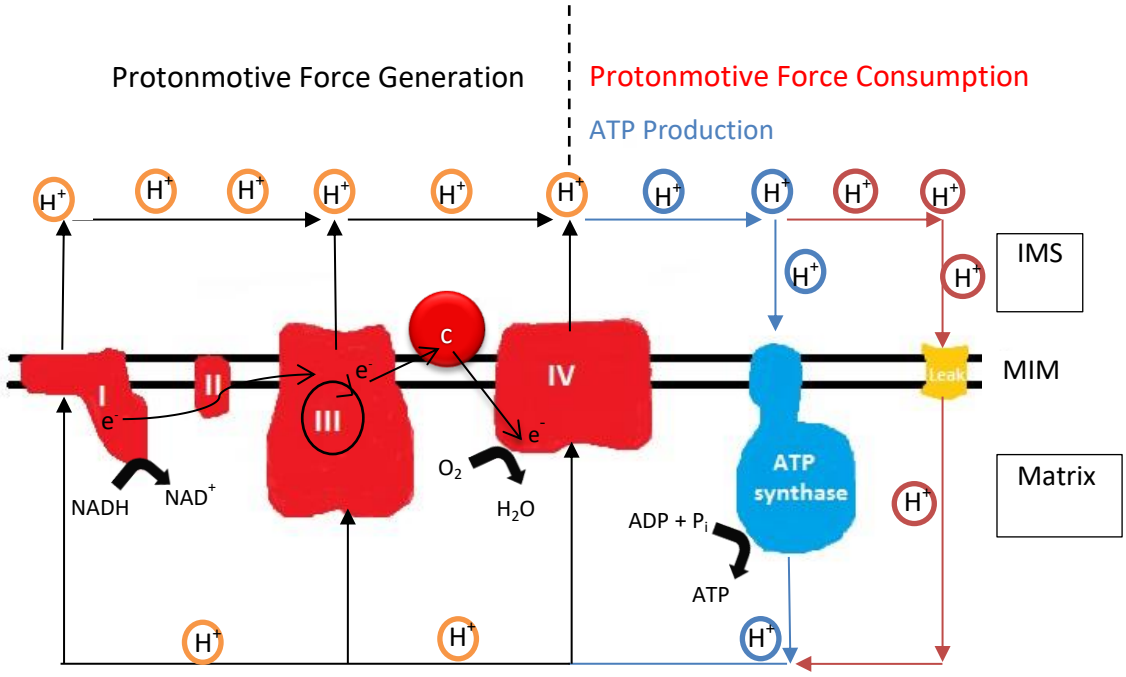


Figure 3: Generation and Consumption of Mitochondrial Protonmotive Force

Electrons (e^-) released by oxidised substrates pass through the respiratory chain. They drive proton pumping via hydrogen (H^+) from the mitochondrial matrix across the MIM (mitochondrial inner membrane) into the IMS (intermitochondrial membrane space) by respiratory complexes I, III and IV in an exergonic process. This creates an electrochemical gradient which is dissipated either through generation of ATP by ATP synthase, or through proton leak which does not generate ATP. Figure adapted from (Divakaruni and Brand 2011) and (Granger and Kvietys 2015). C is cytochrome c. I – IV are respiratory complexes I – IV.

Mitochondrial efficiency is limited by a number of factors, although proton leak appears to have the most significant effect on uncoupling of oxidation of substrates and ATP synthesis. Protons can return across the inner mitochondrial membrane independently of ATP-synthase, relieving the protonmotive force generated by oxidation without producing ATP (Divakaruni and Brand 2011). Proton leak occurs in two ways; a basal proton conductance occurs at all times, predominantly through

adenine nucleotide translocase (ANT) in the inner mitochondrial membrane, while this basal leak can also be regulated and induced by catalytic uncoupling proteins (UCPs) (Divakaruni and Brand 2011). Proton leak has some beneficial physiological function in maintenance of homeostasis including the following: Thermogenesis in brown fat which, for example, is of critical importance in homeostasis of human newborn babies (Ricquier and Bouillaud 2000); Maintenance of carbon flux (or transfer of energy in carbon fuels to electron potential energy despite low ATP demand); Modulation of the nutrient response in glucose-sensing pancreatic β cells. These cells usually respond to rising blood sugar by increasing oxidative metabolism, leading to an increased ATP/ADP ratio in their cytoplasm with a subsequent influx of calcium and eventual secretion of insulin (Fridlyand and Phillipson 2011). The majority of this ability to sense glucose concentration is derived from glucokinase, found in high concentrations in these cells. This facilitates phosphorylation of glucose to glucose-6-phosphate thus triggering the insulin response to rising blood glucose concentration. Mitochondrial proton leak dissipates protonmotive force, thus restricting ATP production and glucose-stimulated insulin secretion (Li, Stojanovski et al. 2012).

Mitochondrial proton leak may provide further benefit as it may also protect against oxidative tissue damage caused by reactive oxygen species (ROS) by tempering the protonmotive force generated and hence mitochondrial superoxide production (Divakaruni and Brand 2011).

Increasing controversy exists regarding the mechanism of the observed improvement in exercise efficiency following dietary NO_3^- supplementation. This beneficial effect on exercise efficiency and resting metabolic rate may be, in part,

due to the impact of NO_3^- supplementation on mitochondrial oxidative phosphorylation efficiency. Termed P/O ratio, this is typically measured as the amount of oxygen consumed per unit of ATP produced (Larsen, Schiffer et al. 2011). A number of potential mechanisms have been suggested by which NO_3^-, NO_2^- and ˙NO may affect the P/O ratio, although with each new pathway suggested, further reports are published countering the projected hypotheses. ˙NO partially inhibits mitochondrial respiration by rapidly and reversibly inhibiting cytochrome c oxidase (COX), the terminal enzyme acceptor of the electron transport chain, as demonstrated in mitochondria isolated from rat skeletal muscle (Cleeter, Cooper et al. 1994). This binding of ˙NO to COX is regulated by oxygen, and may also serve to control reactive oxygen species (ROS) signalling and to regulate tissue oxygen gradients (Thomas, Liu et al. 2001). Dietary NO_3^- supplementation can improve coupling between respiration and oxidative phosphorylation in human skeletal muscle as evidenced by an increase in both the respiratory control ratio and the P/O ratio compared to placebo (Larsen, Schiffer et al. 2011). This group demonstrated an increase in the maximal rate of mitochondrial ATP production and in oxygen affinity, indicating a reduction in proton leak in the electron transfer chain. This was achieved through a reported downregulation of adenine nucleotide translocase (ANT) protein levels and hence, reduced leak. Contrary to pre-existing research conclusions, Whitfield *et al (Whitfield, Ludzki et al. 2015)* displayed an improvement in the exercise VO_2 of healthy adults following beetroot juice supplementation, but no change in the key parameters of mitochondrial efficiency. Specifically, muscle biopsies taken from their participants showed no alteration in mitochondrial leak respiration, the content of proteins associated with uncoupling (uncoupling protein 3 [UCP3], ANT1, ANT2), maximal substrate-supported respiration, or ADP sensitivity.

There was no alteration in mitochondrial P/O ratio, respiratory control ratio or membrane potential. Nisoli reported an increase in rat skeletal muscle mitochondrial biogenesis following dietary NO_3^- supplementation (Nisoli, *et al* 2003), yet Larsen found that, after 3 days of supplementation, no demonstrable effect on mitochondrial biogenesis was found (Larsen, Schiffer et al. 2011).

1.2.2.2 EFFECT OF NO_3^- SUPPLEMENTATION ON ATP COST AND CALCIUM FLUX

An alternative explanation for the observed improvement in exercise efficiency following dietary NO_3^- supplementation is the effect of such intervention on cellular ATP turnover and handling of calcium. The ATP cost of contraction in skeletal myocytes is the sum of ATP consumption via the interaction between actin and myosin (actomyosin-ATPase) and by calcium pumping in the sarcoplasmic reticulum (Ca^{2+}-ATPase). Membrane depolarisation (Na^+-K^+-ATPase) makes a further small contribution to the total myocyte ATP turnover (Barclay, Woledge et al. 2007). NO_3^- supplementation, through an increase in the bioavailability of $^{\cdot}NO$, may regulate the ATP cost of force production. $^{\cdot}NO$ has been demonstrated to slow actin myosin cross-bridge cycling kinetics, reduce ryanodine activity, and hence reduce Ca^{2+} release and inhibit Ca^{2+}-ATPase activity (Bailey, Fulford et al. 2010). Ryanodine receptors are a class of intracellular calcium channels in the endoplasmic reticulum which are responsible for the release of calcium from intracellular stores during excitation / contraction coupling in skeletal and cardiac muscle (Lanner, Georgiou et al. 2010). Any inhibition of their activity through $^{\cdot}NO$ would therefore induce significant change in the calcium flux and ATP utilisation of muscles. Hence, this

mechanism would enable NO_3^- supplementation to reduce VO_2 for the same work rate in an individual by reducing total ATP turnover, without affecting mitochondrial P/O ratio. This hypothesis was proven in a murine model by Hernández *et al* (Hernández, Schiffer et al. 2012), who provided nitrate in the drinking water of experimental mice for 7 days. They reported a resultant increase of contractile force at low stimulation frequencies, as fast twitch muscles were activated at a lower frequency, achieving equivalent force output but requiring reduced ATP cost. As a result of this increased efficiency of contraction, fewer motor units were recruited in order to achieve the same force output, indicating that dietary nitrate supplementation could reduce the ATP cost of muscle contraction.

1.2.3 OXYGEN DELIVERY IN CONDITIONS OF HYPOXIA

Low barometric pressure at high altitude causes lower arterial oxygen content (both total saturation, and percentage of haemoglobin saturation) in Tibetan highlanders. Erzurum (Erzurum, Ghosh et al. 2007) found that this population's oxygen use at rest and during exercise was similar to that of a sea-level dwelling population. The reduced arterial oxygen component is offset through an increase in bioactive forms of ˙NO such as plasma and red blood cell nitrate and nitroso proteins, and plasma nitrite. Tibetan highlanders also have a lower concentration of iron-nitrosyl complexes in red blood cells. This alteration in regulation of metabolic pathways causes vasodilation, increased blood flow and hence improved oxygen delivery. Furthermore, mammalian erythropoiesis is stimulated by the release of erythropoietin in response to hypoxia in order to meet tissue oxygen demands. However, at

altitude, prolonged exposure to hypoxic atmospheric conditions or severe chronic respiratory pathologies such as cystic fibrosis or chronic obstructive pulmonary disease can lead to such marked elevation in haematocrit that a detrimental increase in blood viscosity may occur. An increased haematocrit may not, therefore, substantially increase oxygen delivery when atmospheric oxygen and tissue oxygen saturation are low. Ashmore et al (Ashmore, Fernandez et al. 2015) describe a pathway whereby erythropoiesis is suppressed by dietary nitrate supplementation in male Wistar rats in both hypoxia and normoxia. Improved tissue oxygenation and downregulation of tissue hypoxia markers in the liver of these animal models leads to reduced hypoxia-driven hepatic erythropoietin release. This effect was reversed at higher nitrate doses where the renal expression of erythropoietin increased, probably as a result of relative anaemia. Hence, dietary nitrate supplementation appears to decrease blood viscosity while matching oxygen supply to demand, whereas renal oxygen sensing may act as a brake, averting a potential detrimental fall in haematocrit.

In an experimental extrapolation of these observed findings, Vanhatalo *et al* (Vanhatalo, Fulford et al. 2011) reported that dietary supplementation with inorganic NO_3^- ameliorated the detrimental effects of systemic hypoxia on exercise tolerance (T_{lim}) in healthy individuals. This group found that subjects given nitrate-rich beetroot juice (9.3 mmol nitrate) had no deterioration in T_{lim} in hypoxia compared to normoxia, while those participants given nitrate depleted placebo beetroot juice had the expected deterioration. The exercise tests were performed within the bore of a ^{31}P magnetic resonance spectrometer (^{31}P-MRS), utilising this non-invasive method of

recording high-energy phosphorylated metabolites and pH changes before, during and after exercise. Simply speaking, the use of this magnetic resonance spectrometer allows real-time assessment of muscle ATP and pH during exercise without the requirement for muscle biopsies, allowing investigation into the effect of exercise and various interventions on skeletal muscle physiology. During exercise in hypoxia, ATP is resynthesized from anaerobic metabolism through phosphocreatine (PCr) breakdown and glycolysis (Bogdanis, Nevill et al. 1996) enabling ongoing muscle contraction. Intracellular pH correlates with glycolytic flux, or the rate at which molecules pass through the pathway of glycolysis, the fundamental pathway through which humans produce energy. The PCr to inorganic phosphate (Pi) ratio correlates with energy available as ATP for muscle activity. Phosphocreatine, the phosphorylated form of creatine, is primarily found in the skeletal muscles of vertebrates. There, it serves a critical role as a rapidly acting energy buffer for muscle cell actions such as contractions via its ability to regenerate adenosine triphosphate (ATP) from adenosine diphosphate (ADP). During muscle contraction, PCr is depleted to maintain constant ATP levels, while Pi accumulates. The ratio of PCr to Pi is therefore a measure of depletion of PCr and of production of ATP, while it also indicates reduction in cellular pH. Post-exercise PCr resynthesis is inversely proportional to the rate of oxygen consumption and hence, is an index of oxidative ATP production and mitochondrial function (Roussel, Bendahan et al. 2000). Vanhatalo *et al* (Vanhatalo, Fulford et al. 2011) reported that participants given placebo had more intramuscular metabolic changes during exercise in hypoxic conditions compared to those supplemented with beetroot juice in hypoxia, or those in normoxia. Specifically, muscle PCr concentration, P_i concentration and pH changed faster in the placebo arm in hypoxia than either the normoxia arm or the

beetroot juice arm in hypoxia. This indicates a reduction of muscle dependency on anaerobic metabolism when satisfying energy demand at a given work rate after nitrate supplementation, and an improvement in the matching of oxygen delivery and oxygen demand.

1.2.4 COGNITION

In the central nervous system, nNOS catalyses the production of ˙NO, although the proposed mechanism through which it exerts this effect has recently been called into question. Previously it has been proposed that ˙NO is produced via the nitrite anhydrase activity of carbonic anhydrase (Aamand, Dalsgaard et al. 2009). However, further evaluation of this proposed mechanism using enzyme kinetics, vascular myography and carbonic anhydrase knockout mice demonstrated that purified bovine carbonic anhydrase failed to catalyse NO_2^- reduction to ˙NO *in vitro* or *in* vivo (Wang, Sparacino-Watkins et al. 2019). Hence, future studies are required to explore other potential NO_2^- reductases which may have physiological significance in the human central nervous system.

Within the central nervous system, ˙NO plays a number of roles contributing to neurotransmission and to neurovascular coupling. The receptor proteins specialised to detect ˙NO are coupled to cGMP formation and provide exceptional amplification of even brief, low amplitude ˙NO signals (Garthwaite 2008).

As in the vascular endothelium, ˙NO stimulates guanylyl cyclase to form cGMP, which activates cGMP-dependent protein kinase (PKG), and phosphorylation of the transcription factor cyclic adenine monophosphate response element-binding protein (CREB). This has a number of effects on the mammalian brain. Firstly, it contributes to long-term memory formation (Lu, Kandel et al. 1999). This group studied the effect of electrical stimulation on post-mortem mouse hippocampus cells in the presence of ˙NO. They delivered 3 and 4 trains of electrical stimulation directly into the stratum radiatum of the hippocampus at 100 Hz for one second in the presence and absence of an nNOS inhibitor. They found that late long-term potentiation, a long-term form of synaptic plasticity, was blocked by the addition of an inhibitor of nitric oxide synthase in three trains of tetanic stimulation and reduced in four trains of stimulation. Synaptic plasticity is the biological process by which specific patterns of synaptic activity between neurones in the brain result in changes in synaptic strength and is thought to contribute to learning and memory. With further biochemical and immunofluorescent assays on the mouse hippocampi, they demonstrated that ˙NO, cGMP and PKG have a crucial role in producing long-term memory in response to stimuli.

Further evidence to support the crucial role ˙NO has in long-term memory formation was published by Kemenes et al (Kemenes, Kemenes et al. 2002). They reported an experiment in which the snail *Lymnaea stagnalis* was subjected to one trial appetitive conditioning in amyl acetate followed by sucrose solution. This induced them to demonstrate specific feeding behaviours upon further exposure to amyl acetate as a result of long-term memory formed following their conditioning. However, some snails were also exposed to three different agents which caused ˙NO depletion, NOS inhibition and inhibition of the soluble guanylyl cyclase (sGC) were used to interfere

with the ˙NO-cGMP pathway thought to be important in long-term memory formation. They discovered that those snails who were exposed to the above agents interfering with ˙NO-cGMP displayed no evidence of long-term memory formation. They also demonstrated that consolidation of such long-term memory is also dependent on an intact ˙NO-cGMP pathway. Hence, ˙NO is demonstrated to play a vital role in animal behaviour, synaptic plasticity and long-term memory formation.

Through both calcium-dependent and calcium-independent pathways, ·NO stimulates release of the neurotransmitters acetyl choline, dopamine, norepinephrine, along with the neuroactive amino acids glutamate, gamma-aminobutyric acid and taurine (Kuriyama and Ohkuma 1995).

The term ‘neurovascular coupling’ describes the coupling of local neuronal activity to regulation of blood flow in the brain, ensuring homeostasis of the cellular environment (Attwell, Buchan et al. 2010). This is controlled via forward feedback, whereby neuronal activity causes an increase in local cerebral blood flow and hence increased energy supply to those active neurones. Disruption of the mechanisms linking the neurovascular unit causes brain dysfunction and disease (Piknova, Kocharyan et al. 2011). The amino acid glutamate plays a central role in both the normal and abnormal functioning of the central nervous system (CNS). It is the main excitatory neurotransmitter in the CNS, estimated to be released at up to half of the synapses within the brain. It exerts its excitatory effect through three receptors, one of which is N-methyl-D-aspartate (NMDA). This receptor has been extensively implicated in CNS diseases, both through hyperstimulation such as in hypoxia – ischaemia of the CNS, and chronic hypostimulation as recorded in AL amyloidosis and Alzheimer’s disease. In health, glutamate is released in the presynaptic

membrane and activates neuronal NMDA receptors (Figure 4), causing calcium influx and activation of neuronal NOS releasing ˙NO. This directly mediates vessel dilation in the cerebellum but not in the cortex, where ˙NO modulates astrocyte signalling to cause vessel dilation. It exerts this effect through inhibition of astrocyte enzymes that synthesise the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE). This mechanism of vasodilation is independent of that mediated by cGMP. This allows preferential production of the vasodilatory prostaglandin and epoxyeicosatrienoic acid (EET) derivatives of arachidonic acid in the astrocytes, causing an unopposed effect on the arteriole diameter (Attwell, Buchan et al. 2010). Hence, unlike most neurotransmitters, ˙NO acts not only as a line of communication between neuronal cells, but between them and non-neuronal cells (Garthwaite 2008). Thus, through the action of ˙NO, the neurovascular unit, made up of neurons, glial cells and blood vessels, tightly couple local neuronal activity at the cellular level to control local cerebral blood flow (Attwell, Buchan et al. 2010).

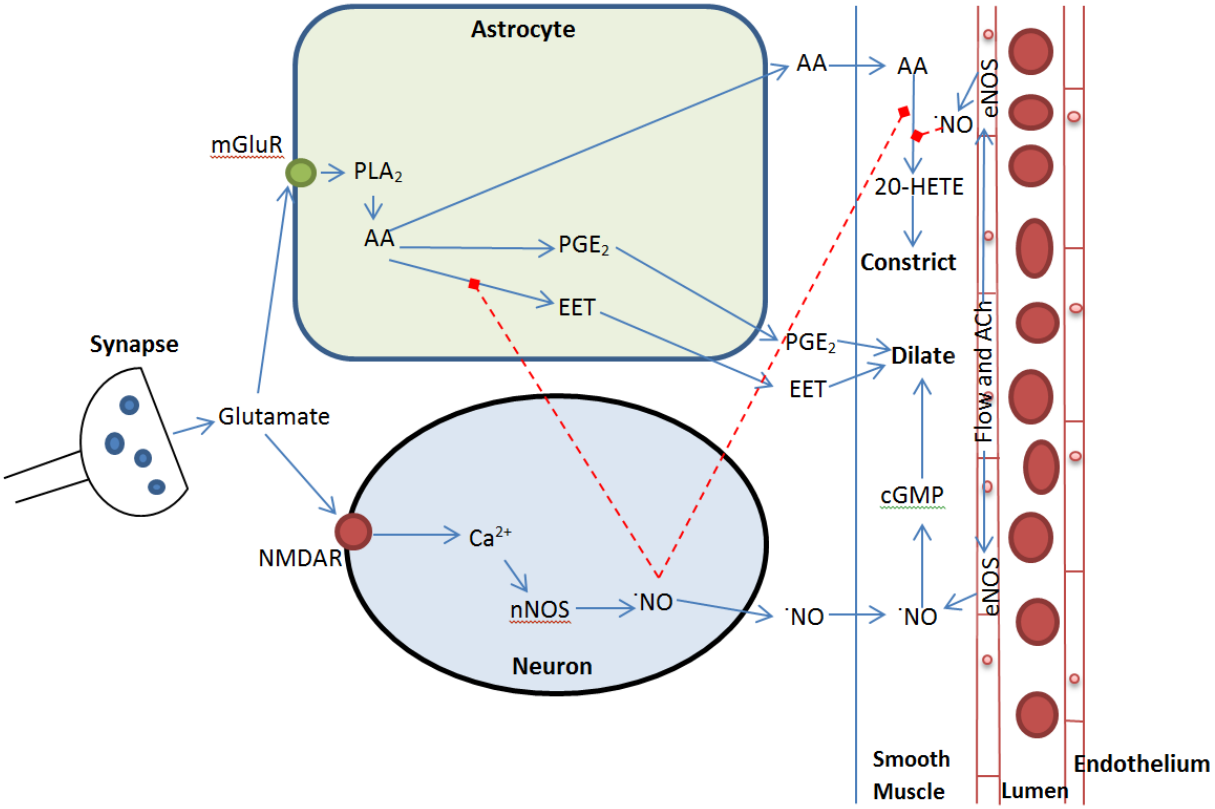


Figure 4: Neurovascular Coupling. Glutamate is released in the presynaptic membrane. In the cerebellum, it causes an influx of calcium ions through the NMDA receptor which in turn activates nNOS, releasing ˙NO. This is released by the neuron and directly mediates relaxation of the smooth muscle walls of the microvessels and therefore vasodilation within the cerebellum. In the cortex, ˙NO exerts a similar effect by inhibiting astrocyte signalling pathways (depicted by red hashed arrows) and reducing production of 20-HETE, opposing vasoconstriction. The net result of each of these actions is that the neuroexcitatory amino acid, glutamate, stimulates neuronal activity and this induces vasodilation and therefore improved delivery of glucose and oxygen to those nerves that are most active. Figure adapted from (Attwell, Buchan et al. 2010). PGE_2 is prostaglandin E2, while PLA_2 is phospholipase A2.

Global impairment of blood flow within the brain has long been linked with disease. Meyer et al (Meyer, Rogers et al. 1988) measured cerebral blood flow in elderly patients with either multi-infarct dementia or Alzheimer's Disease, and compared it with age-matched controls whilst longitudinally monitoring both groups' cognitive function. They reported a steady deterioration in cognition and vascular flow over

time in patients with Alzheimer's, compared to a more stepwise and fluctuating course of deterioration in those with multi-infarct dementia. Each group had a significantly reduced cerebral blood flow compared to the control participants. In order to counter this observed effect, a number of groups have attempted to improve cerebral blood flow and neurovascular coupling by harnessing the effect of dietary NO_3^- supplementation. Presley et al (Presley, Morgan et al. 2011) compared the effect of high and low nitrate diets on cerebral perfusion in 16 older adults using arterial spin labelling magnetic resonance imaging. They found that, while there was no observed difference in global cerebral perfusion, nitrate supplementation was linked with improvement in regional cerebral perfusion of frontal lobe white matter, particularly the dorsolateral prefrontal cortex and the anterior cingulate cortex. These areas are of crucial importance, providing much of the brain's higher executive functioning, yet are at risk of chronic ischaemia (Nordahl, Ranganath et al. 2006). Hence dietary nitrate supplementation may provide some protection against this very common and often debilitating effect of the aging process. Furthermore, dietary supplementation with inorganic nitrate alters the haemodynamic response to visual stimulation through improvement in neurovascular coupling (Aamand, Dalsgaard et al. 2013). This was demonstrated using 20 healthy volunteers, who were given either sodium nitrate or sodium chloride (placebo) supplementation in a crossover study. Participants were then exposed to visual stimuli during high resolution functional magnetic resonance imaging of their cerebral blood flow, which assessed their blood oxygen level dependent (BOLD) response in cerebral blood flow. While this demonstrated no change in the baseline cerebral blood flow, participants' BOLD response was faster and smaller after nitrate supplementation, and oral nitrate induced a reduction in the variation in the local cerebral cortex. This was consistent

with enhanced neurovascular coupling during elevated nitrate concentration, with a fast but small increase in blood flow isolated to areas of the brain pertinent to the visual stimuli.

Hence, these studies indicate that dietary inorganic nitrate supplementation may be of therapeutic benefit in central nervous system functioning in health and in the pathological state.

1.2.5 THE POTENTIALLY HARMFUL EFFECTS OF ˙NO

While there are a large number of beneficial effects attributable to ˙NO as outlined above, its role in cancer may not be entirely advantageous. Its role in tumour biology is complex given its combination of facilitatory and inhibitory effects in cellular processes. This role is dependent on intracellular conditions such as the cell's genetic make-up, the local concentration of ˙NO, and the presence of other regulators such as ˙NO scavengers (Lala and Chakraborty 2001). Most human carcinogenesis is caused by somatic mutations which either activate oncogenes or inactivate tumour suppressor genes. The development of these mutations is dictated by the chemicals within the cellular microenvironment and to a lesser extent, by genetic predisposition of the individual. ˙NO is a free radical and therefore has mutagenic properties as a result of its potential to both cause DNA damage and to hinder DNA repair. These effects are either directly attributable to the impact of ˙NO itself, or through its interaction with superoxide forming peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3) (Bonavida, Khineche et al. 2006). It can also increase

angiogenesis (blood vessel formation) within tumours, promoting metastatic spread and tumour growth, while ˙NO can also reduce chemotherapy-induced inflammation of tumour cells, thus contributing to limited efficacy of these agents in the treatment of some tumours (Bonavida, Khineche et al. 2006).

Intrinsic nitric oxide synthase (iNOS) has a compelling relationship with cancer progression and metastasis of tumours. Expression of iNOS is correlated with poor outcomes in terms of overall survival in a number of tumours including breast cancer (Loibl, Buck et al. 2005) and malignant melanoma (Ekmekcioglu, Ellerhorst et al. 2000). Similarly, patients with stage III ovarian cancer had better responses to first line of chemotherapy if iNOS was not present (Raspollini, Amunni et al. 2004). The explanation for these observed effects is poorly understood, and in fact many experiments investigating the effects of increased iNOS concentrations in cancer cell lines *in vitro* have reported a reduced metastatic potential (Hickok and Thomas 2010).

There is evidence of a dichotomy of effects of ˙NO on cancer metabolism and outcomes. While the papers outlined above describe detrimental effects of ˙NO, further studies describe the role of ˙NO in chemosensitisation and immunosensitisation of cancer to induction of apoptosis, or programmed cell death. An increase in NOS activity within tumour cells can trigger p53 accumulation, a potent tumour suppressor which induces cell cycle arrest and apoptosis (Ambs, Hussain et al. 1997). Chemotherapy-induced tumour cell death is detrimentally affected by hypoxia; the killing of human breast cancer cells and mouse melanoma cells by chemotherapy was impaired by ˙NO inhibition (Matthews, Adams et al. 2001), indicating that the role of ˙NO in matching blood flow with hypoxia is crucial in

ensuring efficacy of these agents. The sensitivity of cells to immune-mediated chemotherapy is affected by ˙NO, via its effect on transcription, causing upregulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins. For example, the expression of Fas receptor, a receptor to the apoptotic protein fas ligand (FasL) in aortic ring smooth muscle was increased by exposure to ˙NO, thus mediating increased sensitivity of those cells to apoptosis (Bonavida, Khineche et al. 2006). Inteferon gamma upregulates NOS activity and has sensitised human ovarian cell lines to FasL mediated apoptosis.

Hence ˙NO has a number of complex and contrasting roles within cancer. It can promote tumorigenesis and can affect risk of progression and metastasis of tumours. However, it can also be targeted to sensitise patients to both chemotherapies which affect cell cycle, and to immunotherapy.

1.2.6 ˙NO BIOLOGY SUMMARY

˙NO is an extremely important regulator of human physiology, exerting a broad range of effects on a number of target organs and organ systems. It plays an essential role in the maintenance of endothelial function and vascular tone, hence controlling vasodilation, blood pressure and oxygen delivery to hypoxic tissue beds, whilst affording some protection against hypoxic ischaemia/reperfusion injury. It affects mitochondrial respiration, myocyte calcium handling and ATP turnover, hence contributing to the efficiency of resting metabolic rate, and muscle function in conditions of normoxia and hypoxia. ˙NO is implicated in the neurovascular pairing of

cerebral blood flow and neuronal activity, long term memory formation and learning. It also controls platelet aggregation. It has an array of potential effects in cancer, some of which are proven to be beneficial but some of which are detrimental to patient outcomes.

1.3 CANCER, FATIGUE AND ANAEMIA

The incidence and prevalence of cancer is increasing throughout the world. More than 331000 people in the UK were diagnosed with a form of cancer in 2011, which equates to 910 people every day (C.R.U.K. 2015). Cancer-related symptoms vary according to the tumour, its site and its treatment, but its burden is significant and can have a major impact on quality of life, and may affect clinical treatment. Cancer-related fatigue (CRF) affects 39–90% of patients with malignancy; this is a subjective feeling of tiredness, weakness or a lack of energy, which is not relieved by rest (Stone and Minton 2008). The aetiology of this phenomenon is multifactorial and poorly understood, but contributory factors include psychological stress, malnutrition, infection, chronic pain, downregulation of the hypothalamo-pituitary-adrenal axis, inactivity, dysregulation of central serotonin metabolism and anaemia. There has been a distinct lack of well-designed randomised controlled trials of drug interventions to improve cancer-related fatigue. Only three agents have any significant published research. The psychostimulant methylphenidate shows no statistically significant effect, while the haematopoietic growth factors erythropoietin and darbepoietin both showed an improvement in cancer-related fatigue (Crawford, Cella et al. 2002, Wagner and Cella 2004, Stone and Minton 2008). Haematopoietic

growth factors must be used with caution in this patient population however, as outlined below (Leyland-Jones, Semiglazov et al. 2005).

Anaemia is usually defined in terms of a reduction in the concentration of haemoglobin or the number of red cells in the peripheral blood. The symptoms and severity of anaemia depend on the degree of anaemia, rapidity of its onset, and the age and co-morbidities of the patient. Reduction in oxygen delivery to the vital organs causes symptoms of cold skin, dizziness, lethargy, palpitations, and can develop into pulmonary oedema, heart failure, depression and severe impairment of cognitive function (Ludwig and Strasser 2001). There are a number of publications reporting poor quality evidence for the co-existence of anaemia and cognitive deterioration (Stivelman 2000, Peters, Burch et al. 2008, Terekeci, Kucukardali et al. 2010). However, there is a lack of reference in the literature to the phenomenon often observed by clinicians; most patients with severe chronic anaemia do not exhibit symptoms of cognitive impairment. The lack of published evidence may be a result of publication bias and the lack of enthusiasm to publish negative findings.

Anaemia is a commonly occurring consequence of cancer and its treatment, affecting 39% of cancer patients at diagnosis and 67% of them at some point during their illness and treatment (Ludwig, Van Belle et al. 2004). In this study of 15,000 European cancer patients, Ludwig *et al* described an incidence of anaemia of 54%, and reported a significant association between anaemia and deterioration in objective measures of performance status. Caro (Caro, Salas et al. 2001) found a correlation between anaemia and poorer survival rates in patients with lung carcinoma, cervico-uterine carcinoma, head and neck carcinoma, prostate carcinoma, lymphoma and myeloma. This link between anaemia and increased

mortality is very likely to be multifactorial and therefore, cannot be solely attributed to the anaemia itself: for example, more advanced, metastatic carcinomas or lymphomas may cause anaemia through infiltration of the bone marrow and through chemotherapy, and are associated with deterioration in survival compared to localised disease. A recent Danish study reported that one- and 5-year survival of patients with prostate carcinoma was 87% and 56% in those without bony metastases at diagnosis, but 47% and 3% in those who did have skeletal metastases (Nørgaard, Jensen et al. 2010).

Despite the questionable causality of evidence linking anaemia and deteriorating prognosis in patients with cancer, more conclusive evidence exists demonstrating that a lower haemoglobin level correlates with a poorer quality of life in this patient group (Crawford, Cella et al. 2002). While the primary objective of most cancer research is the prolongation of survival for this cohort of patients, quality of life is also extremely important. Supportive and palliative therapies that do not directly impact survival can have important positive effects on day-to-day physical, mental and social functioning, and self-perceived overall quality of life. Regardless of this, 61% of patients included in the European Cancer Anaemia Survey (Ludwig, Van Belle et al. 2004) received no treatment for their anaemia. It seems likely that the level of anaemia of those patients was deemed insufficiently depressed to warrant treatment; mean haemoglobin at the point of treatment initiation was 97 g/l, while 29.3% of patients had mild anaemia with Haemoglobin levels of between 100 g/l and 119 g/l. The decision to treat patients with a lower haemoglobin (Hb) concentration reflects poorer World Health Organisation performance status at each level (Table 1).

Table 1: World Health Organisation Performance Status Score

WHO Performance Status Score	Functional Ability
0	Normal activity
1	Symptoms but fully ambulant
2	Requiring bed rest for less than 50% of the day
3	Requiring bed rest for more than 50% of the day
4	Unable to get out of bed

The WHO Performance Status is a standardised measure of functional ability giving a score of 0 to 4 (Zubrod, Schneiderman et al. 1960). WHO scores of 2-4 were recorded for 50.7% of patients with Hb <80g/l, 40% of those with Hb 80-99g/l, and 24.8% of those with mild anaemia (Hb 100-119g/l) (Ludwig, Van Belle et al. 2004). This indicates that 70% of patients with mild anaemia do not receive treatment for anaemia but a quarter of this group of patients experience significant deterioration in WHO performance status.

1.4 TREATMENT OF CANCER-RELATED ANAEMIA

Physicians treating patients with cancer-related anaemia can prescribe one of two main supportive therapies to improve their haemoglobin level; transfusion of packed red cells, or exogenous erythropoietin (EPO) injections.

EPO injections given to anaemic patients with cancer have been shown to improve quality of life measures in several studies. Demetri *et al* (Demetri, Kris et al. 1998) treated 2370 non-myeloid cancer patients with EPO which brought about improvement in Hb and quality of life irrespective of baseline Hb, tumour type and disease response but not performance status. Crawford *et al* (Crawford, Cella et al. 2002) reported that EPO treatment of this cohort of patients can improve subjective quality of life scores up to an Hb concentration of 140 g/l. Each of these studies is potentially flawed by their design, as neither included a control group treated with placebo, while they were also open label trials. These factors allow both reporter bias and placebo effect. EPO treatment can also be associated with significant side effects including an increased risk of mortality (Leyland-Jones, Semiglazov et al. 2005): in an attempt to improve quality of life and survival, 939 patients with metastatic breast cancer with a Hb concentration between 120 and 140 g/l were given EPO weekly from the start of their chemotherapy. The Independent Data Monitoring Committee recommended early cessation of the trial because of higher mortality in the group being treated with EPO (12 month overall survival of 70% vs 76%). While the reason for a difference in mortality could not be determined, there was an association with increased rate of thromboembolic disease in the intervention

group. Hence EPO is not deemed a suitable treatment for those patients with mild anaemia.

Blood transfusion in anaemic patients almost always increases their haemoglobin concentration in the absence of active bleeding or haemolysis; however, patients with cancer have been found to derive limited symptomatic benefit from this therapeutic approach to their anaemia. Mercadante (Mercadante, Ferrera et al. 2009) transfused cancer patients with an Hb concentration of 80 +/- 5 g/l. This improved their dyspnoea (breathlessness) and fatigue at day 1 post-transfusion but the benefit was reduced by day 15 post-transfusion despite maintained Hb concentration.

Hence, neither exogenous erythropoietin injections nor blood transfusions provide an entirely effective or safe means of treating cancer-related anaemia and associated symptoms of fatigue. While the patient's malignancy, treatment and comorbidities may explain some of the limitation of the symptomatic benefit of transfusion on their symptoms, the biochemical and morphological changes within stored blood might be a contributory factor.

1.5 THE EFFECTS OF STORAGE ON BLOOD

Joint UK Blood transfusion and Tissue Transplantation Services Professional Advisory Committee guidelines state that, after donation, leukocyte-depleted packed red cell units can be stored for up to 35 days before transfusion (N.B.S. 2013). Blood is preserved by collection into sealed bags containing 63ml of citrate phosphate

dextrose (CPD) anticoagulant and its core temperature is maintained at 4 +/- 2°C. However, significant alterations occur within the sealed units during storage. These include slowing of cation transport, depletion of ATP and 2,3 diphosphoglyceric acid (2,3-DPG) reservoirs with resultant change in oxygen transport, accumulation of lactate, oxidative damage of proteins and lipids, and morphological changes, all of which contribute to progressive accumulation of oxidative stress and in turn, oxidative lesions to proteins and lipids (D'Alessandro, Kriebardis et al. 2014). While the unexpected limitation in the clinical benefit of transfusion is likely to be multifactorial, changes in oxygen transport and red blood cell morphology appear to be of particular relevance, in part through their effect on ˙NO bioavailability.

1.5.1 THE OXYGEN TRANSPORT DEFICIT OF STORED BLOOD

Adenosine triphosphate (ATP) and 2,3-DPG are consumed by, and rapidly depleted in stored blood (Gelderman, Yazer et al. 2010). This impairs the stability of deoxyhaemoglobin, which consequently has a higher affinity for oxygen (O_2), causing a steady increase in haemoglobin oxygen saturation up to 99% on day 42 of storage (Bennett-Guerrero, Veldman et al. 2007). This increase in affinity of haemoglobin is countered by glycolysis which, during storage, causes acidification of the intracellular environment. Through the Bohr effect, acidification causes the O_2 affinity of haemoglobin to fall again, although through negative feedback, acidification causes inhibition of glycolysis and hence limits the effect of this pathway. However, the net effect is an increase in oxygen affinity of haemoglobin in older stored red blood cells (D'Alessandro, Kriebardis et al. 2014). Storage also

causes deregulation of S-nitrosation of Hb, potentially reducing the bioavailability of ˙NO. In turn, this may limit the RBC's ability to induce hypoxic vasodilation and hence, reduce its ability to match O_2 supply to metabolic demand (Reynolds, Bennett et al. 2013).

This potential effect is somewhat controversial; two schools of thought exist regarding the nature of the ˙NO reservoir, both of which have equally credible backing: ˙NO gas is extremely unstable, oxidised within seconds to nitrogen dioxide and higher oxides of nitrogen. In solution, ˙NO exposed to oxygen has a half-life of less than 10 seconds (Ignarro 1989) so, in order to exert an effect when required, a reservoir must exist which releases ˙NO in order to exert its effect, essentially buffering its concentration (Stamler, Jaraki et al. 1992). As described above, this vascular storage pool is advocated to either be in the form of S-nitrosation (Foster, McMahon et al. 2003) or as NO_2^- (Cosby, Partovi et al. 2003).

Organic nitrites are potent vasodilators *in vitro (Ignarro, Lippton et al. 1981)*. While both organic and inorganic nitrates and nitrites mediate their principle effects *in vivo* via nitric oxide, there a number of important differences between them. Inorganic nitrate and nitrite have simple ionic structures, are produced endogenously and are also present in the diet, whereas their organic counterparts are far more complex, and, with the exception of ethyl nitrite, are all medicinally synthesised products (Omar, Artime et al. 2012). As a result, they have differing bioavailability and metabolic profiles, while their pharmacodynamic profiles are more markedly different still. Organic nitrates have potent acute effects causing vasodilation, whereas inorganic nitrates' effects are more subtle and depend on certain conditions. NO_2^- exists *in vivo* at concentrations of 150 - 1000 nM in plasma and >10 μM in aortic ring

tissue (Gladwin, Shelhamer et al. 2000), although both plasma and vascular endothelial NO_2^- concentrations are probably in vast excess compared to plasma S-nitrosothiol concentrations, conjectured to be approximately 10 μM (Stamler, Jaraki et al. 1992).

S-nitrosothiols, have long been known to circulate in the plasma and to have bioactivities similar to those of ˙NO but with half-lives in the order of hours (Stamler, Jaraki et al. 1992). While investigating how nitrogen oxide-containing vasodilators exert their effect, Ignarro discovered that they react with cysteine to form S-nitrosocysteine. They found that this is a potent activator of guanylate cyclase, stimulating cGMP accumulation and hence smooth muscle relaxation.

The most characterised and established mechanism by which ˙NO acts as a second messenger in signal transduction is its reaction with the haem centre of soluble guanylate cyclase and a consequential increase in cGMP, with a subsequent effect on vasoactivity (Foster, Hess et al. 2009) (see section 1.2.2.1 above). Further mechanisms must exist explaining some of the post-translational effects of ˙NO, as haems generally do not elicit cellular signalling involving post-translational modification of proteins. The process of S-nitrosation may enable ˙NO to exert its ubiquitous effect on cellular function through mechanisms analogous to phosphorylation as outlined above (Hess, Matsumoto et al. 2005).

1.5.2 CHANGES IN MORPHOLOGY OF STORED RED BLOOD CELLS

Following donation, stored RBCs become progressively smaller and more dense as a result of ATP depletion and lipid loss, forming Hb-containing microparticles

(Haradin, Weed et al. 1969) particularly after the second week of storage. Oxidative stress to lipids in the red blood cell membrane causes transformation of the RBC morphology into spheroechinocytes. The term ‘echinocyte’ describes an RBC with regular finger-like projections of cytoplasm around its circumference. The tips of these echinocytic spines can release haemoglobin-containing microvesicles of lipid bilayer through exovesiculation (Hess 2014). While most extracellular Hb in packed red blood cell units is contained within these microparticles, cell-free Hb also increases as a function of storage duration (Liu, Zhao et al. 2013). When Hb is encapsulated within a RBC, it scavenges ˙NO up to 1000 times slower than when the Hb is not within a RBC, possibly through the effect intravascular flow and shear stress has on particles, and the subsequent formation of a cell-free zone (Liao, Hein et al. 1999). This RBC-free zone exists near the blood vessel wall, can be as much as 20-25% of the vessel’s diameter, and allows the outflow of ˙NO into the endovascular smooth muscle layer, while reducing interaction between endothelium-released ˙NO and the circulating RBCs (Butler, Megson et al. 1998, Liao, Hein et al. 1999). As older stored blood contains more microparticles and free haemoglobin than normal blood *in vivo*, it would therefore have a far greater ˙NO scavenging capacity. Free Hb exists as a homogenous solution within the blood vessels and is free to enter the cell-free zone where it readily scavenges ˙NO given its very high affinity. Hence, stored blood has a far greater propensity for ˙NO scavenging. The extracellular Hb increases the transfused blood’s immunogenicity, thrombogenicity, its pro-inflammatory and pro-coagulant nature, and its ˙NO scavenging activity (Liao, Hein et al. 1999, Bennett-Guerrero, Veldman et al. 2007, Stamler, Singel et al. 2008, D'Alessandro, Kriebardis et al. 2014). The reduction in ˙NO bioavailability after blood transfusions may therefore have a further detrimental effect on the transfusion

recipient's ability to vasodilate in conditions of hypoxia. This may influence the burden of symptoms in people with chronic anaemia requiring transfusion support.

In an animal model of bacterial pneumonia with septic shock, massive transfusion of 42 day old blood was found to profoundly increase mortality, lung injury and degree of haemodynamic shock compared to 7 day old blood (Solomon, Wang et al. 2013). Stored RBCs were found to haemolyse *in vivo* resulting in persistent increased intravascular cell-free haemoglobin over days, and more ˙NO scavenging. As a result, the older blood was found to be more vasoactive; during transfusion, systemic blood pressures and pulmonary artery pressures were higher. The pulmonary hypertension remained elevated for 10 hours after transfusion and was severe enough to adversely affect left ventricular filling and cause right ventricular dilatation. Increased free oxyhaemoglobin, a vasoconstrictive substance in the plasma, was found to worsen ischaemic vascular damage in the lung at the site of the pneumonia. Hence, older blood had a detrimental effect on outcomes in a canine model of septic shock and massive transfusion through reduction in ˙NO bioavailability.

In contrast to the paper described above (Solomon, Wang et al. 2013), transfusion with older blood had no detrimental effect on outcome in a different animal model, this time mimicking haemorrhagic shock (Solomon, Cortés-Puch et al. 2015). They noted that the physiological response to this insult was altered, with an association between transfusion of 42 day old blood and a reduction in norepinephrine requirements and cardiac outputs. This also demonstrated a trend towards improved survival in this cohort of animals compared to those given 7 day old blood transfusion. Hence this study suggests that the vasoconstrictive effect of reduced

˙NO bioavailability associated with older blood was perhaps beneficial in this population of patients at high risk of hypoxic ischaemia/reperfusion injury.

Sickle cell disorders including sickle cell disease represent some of the commonest inherited genetic disorders worldwide, affecting 2.28 of conceptions per 1000, causing 3.4% of mortality in children under the age of 5 (Modell and Darlison 2008). This condition is caused by a single nucleotide polymorphism (glutamine to valine substitution) at the 6^{th} residue of the beta globin gene found on the short arm of chromosome 11 (Stuart and Nagel). HbS, the variant beta haemoglobin product of this mutation, polymerises readily when deoxygenated, distorting the red cell morphology into sickle or crescent forms, causing haemolysis and vaso-occlusion. This increased haemolysis explains the association between sickle cell disease and an increase in haemoglobin-containing red blood cell microparticles (Liu, Zhao et al. 2013). This extracellular haemoglobin readily scavenges ˙NO, contributing to the hypertensive and thrombotic tendency seen in sickle cell disease and other disorders of intravascular haemolysis, along with the potentially life-threatening sequelae of acute haemolytic transfusion reactions (Wallis 2005). It also promotes painful episodes of vaso-occlusion, which are the leading cause of hospitalisation and emergency department visits in sickle cell disease, and are associated with increased mortality (Morris, Kuypers et al. 2013). In an attempt to overcome the reduced bioavailability of ˙NO, this group gave children with sickle cell disease dietary arginine supplementation, hence providing increased substrate for the L-arginine NOS pathway of ˙NO production amongst other effects, counteracting the vasoconstrictive effect of intravascular haemolysis, increased free haemoglobin and increased ˙NO scavenging. This intervention brought about an improvement in pain and a 54% reduction in opioid use in hospitalised children with painful episodes of

vaso-occlusion indicating improved oxygenation of affected tissues (Morris, Kuypers et al. 2013). Supplementation of dietary NO_3^- could have a beneficial effect in patients with sickle cell disease via similar mechanisms; this intervention has been demonstrated to decrease middle cerebral artery blood flow velocity and hence cerebrovascular resistance (Bond, Curry et al. 2013). This simple intervention could potentially reduce the risk of stroke in patients with sickle cell disease, although this effect is yet to be studied in this population.

No previous studies have investigated the effect of dietary NO_3^- supplementation on patients with chronic anaemia or those on transfusion programmes.

1.6 THE NEUROCOGNITIVE EFFECTS OF CHEMOTHERAPY

Chemotherapy-related cognitive impairment sometimes referred to by patients as 'Chemobrain', was first described in the 1970s (Weiss, Walker et al. 1974), affecting between 17 and 75% of cancer patients with a plethora of malignancies undergoing a range of treatment modalities (Myers 2009). Patients with Non-Hodgkin's Lymphoma treated with RCHOP or R Bendamustine chemotherapy were found to have an objective cognitive impairment compared to control during the 3 months following chemotherapy (Zimmer, Mierau et al. 2014). Women with breast cancer reported a deterioration in memory and distraction, while an objective worsening of attention, concentration, memory and processing speed was reported when compared to controls (Ganz 2012). While many short-term studies have revealed this effect, a more durable cognitive impairment has also been revealed in patients with breast cancer (Wefel, Lenzi et al. 2004). In this study, prospective neuropsychiatric testing was undertaken in people newly diagnosed with this condition, and at short-term (3 weeks) and long-term (6 months) intervals after chemotherapy. They noted that 33% had cognitive impairment before treatment started, and 61% displayed a deterioration in their baseline function, particularly in attention, learning and processing speed. At the long-term follow-up, 50% of patients had not recovered to their baseline cognitive function.

The pathophysiology of chemobrain is likely to be multifactorial but is poorly understood. Potential contributors include social factors such as age, educational levels, IQ and social support, and psychological factors such as anxiety and depression. Physical factors including fatigue, disease site, stage, co-morbidities,

hormone levels, cytokines, damage to neural progenitors and presence of the apolipoprotein E4 allele can all influence cognitive function. Finally, cancer treatment factors affecting cognition include treatment type, timing, duration and any concomitant management (Myers 2009).

Central nervous system (CNS) function is affected by both chemotherapy and radiotherapy, via variable mechanisms depending on the agent involved. The drugs or their metabolites can cause direct injury to the grey or white matter, microvascular injury or immune-mediated CNS inflammatory responses (Wefel, Lenzi et al. 2004). Interleukin-6, a pro-inflammatory cytokine able to penetrate the blood-brain barrier, was found in higher levels in post-chemotherapy blood samples from patients with breast cancer complaining of fatigue than those who were not (Zimmer, Mierau et al. 2014). Doxorubicin chemotherapy was reported to induce an increase in tissue necrosis factor α (TNFα) in a murine study. This was associated with a decline in CNS mitochondrial respiration and mitochondrial protein nitration in wild type mice, but not in iNOS knock-out mice. This single study suggests ˙NO might mediate Doxorubicin-induced CNS injury (Tangpong, Cole et al. 2007), although this has not been studied in the human population. Conversely, a further murine study demonstrated a reduction in doxorubicin-induced ventricular dysfunction, cell death, oxidative stress and mitochondrial respiratory chain damage in mice given seven days of sodium nitrate supplementation (Zhu, Kukreja et al. 2011). This group described a significant decrease in tissue peroxidation as a result of preservation of mitochondrial complex I activity and oxidative phosphorylation. Hence, dietary nitrate supplementation may provide a cardioprotective effect in humans undergoing chemotherapy with anthracyclines, although further work is required to fully establish the nature of this relationship.

˙NO may also have a protective role in the aetiology of chemotherapy-related cognitive impairment. Magnetic resonance diffusion tensor imaging of patients with breast cancer post-chemotherapy showed decreased white matter integrity in tracts involved in cognition in the fronto-parietal and occipital lobes. This correlated with deterioration in objective measures of cognitive function, and suggests some uncoupling of blood flow and neuronal activity (Deprez, Amant et al. 2011)

Hence, although this has not been studied in this population previously, dietary supplementation with inorganic NO_3^- might have some benefit in countering the detrimental effects cancers and their treatments have on cognitive function, through modification of cerebral blood flow, subsequent improvement in cerebrovascular neuronal coupling, and hence matching of oxygen supply with neuronal activity (Bond, Curry et al. 2013).

1.7 LITERATURE REVIEW SUMMARY

˙NO is a simple yet extremely active gas molecule which plays a fundamental role in human homeostatic mechanisms. Produced through the L-arginine – NOS pathway or through sequential reduction of dietary NO_3^- via the enterosalivary circulation, it has a profound influence on mitochondrial respiration, vasodilation, muscle contractile efficiency and force, exercise efficiency, platelet aggregation, cerebral blood flow and neurotransmission. Three decades of research have demonstrated potential clinical efficacy of increasing the bioavailability of ˙NO in both health and disease through manipulation of its production through either of the pathways above.

This includes treatment of pathological conditions as diverse as sickle cell disease (Morris, Kuypers et al. 2013), chronic obstructive pulmonary disease (Berry, Justus et al. 2015), peripheral vascular disease (Allen, Giordano et al. 2012), type 2 diabetes mellitus (Gilchrist, Winyard et al. 2014) and cognitive impairment (Piknova, Kocharyan et al. 2011) amongst others. It has not yet been studied in the anaemic population but, given its wide-ranging effects, particularly those on the circulation, the matching of oxygen demand and supply, and efficiency of muscle metabolism and respiration, dietary NO_3^- may provide a viable complement to existing treatments. Of further interest is the storage deficit associated with donated blood; the biochemical and morphological changes which occur between donation and transfusion may explain why patients requiring blood transfusions still experience symptoms related to anaemia despite improvement in haemoglobin. Dietary supplementation of NO_3^- could potentially ameliorate or overcome these symptoms and improve the quality of life, exercise function and cognitive function of patients with anaemia and cancer.

$^{\bullet}NO$ inhibits platelet aggregation (Alheid, Frolich et al. 1987, Apostoli, Solomon et al. 2014). As having a cancer diagnosis is associated with increased risk of thrombosis (Lee and Levine 2003), dietary supplementation with NO_3^- might overcome this effect through inhibition of aggregation and improvement in endothelial function.

1.8 AIMS AND OBJECTIVES

The aim of this research was to perform a pilot study examining the feasibility of recruiting patients with cancer related anaemia to a randomized controlled trial studying nitrate supplementation. Patients with malignancies usually develop anaemia as a consequence of infiltration of their bone marrow by their disease, or as a consequence of therapy. This dynamic patient population can undergo intensive treatment or develop unpredicted complications of their cancer or its therapies, and are often subjected to psychological stresses related to their diagnosis and prognosis. This pilot study aimed to provide estimates of differences in potential outcome measures and guide the likely requirement for resources to run a full scale study. Following this pilot study, a larger study will be planned, investigating whether nitrate supplementation is beneficial for patients with anaemia with respect to symptoms of anaemia, cognitive function, muscle strength and efficiency, and platelet aggregation.

The key objectives were to assess:

- Percentage of patients who are eligible for inclusion in the study
- Of patients who are eligible, percentage who consent to study entry
- Of patients consenting to study entry, percentage who complete the study
- Percentage of enrolled participants who complete all aspects of the study
- Percentage of those enrolled who complete all aspects of the study except exercise testing

- Refinement of nitrate and nitrite measurements and other data collection methods
- Estimation of standard deviations of the main outcome measures:
    - Quality of life
    - Exercise tolerance and related measures
    - Platelet function

1.9 HYPOTHESES

Relative to a nitrate-depleted placebo beetroot juice, dietary supplementation of anaemic patients with nitrate-rich beetroot juice will have no effect on:

- Thrombogenicity
- Muscle phosphocreatine recovery rate
- Exercise tolerance
- Cognitive function
- Quality of life

CHAPTER 2: METHODS

2.1 SUBJECTS

Prior to initiation of patient recruitment and data collection, this trial was registered as a clinical trial at the Integrated Research Application System (IRAS, reference number 123901). Ethical approval was also obtained from the south west regional ethics committee (REC, reference 14/SW/0081, Appendix 6). The Royal Devon and Exeter Foundation NHS Trust gave its approval for this study to proceed (Research and Development reference number 1404914, Appendix 1), while the University of Exeter also approved its initiation for the purposes of a Doctor of Medicine Degree (candidate number 032816).

Adult patients with anaemia attending the Haematology and Oncology Departments of the Royal Devon and Exeter Hospital were offered recruitment into the trial. Patients in these departments are anaemic for a variety of different reasons, including chemotherapy side effects, aplastic anaemia, acute and chronic leukaemias, lymphoma, myeloma, chronic blood loss and bone marrow infiltration with solid tumour.

Advice was sought from the Royal Devon and Exeter Foundation NHS Trust Consultant Statistician regarding participant recruitment and power calculation. As this was a pilot study, no power calculation was performed as no previous data was available on which to base it. However, the aim was to recruit 30-40 patients. Data generated by this study would therefore allow power calculations to be performed to

inform future similar studies of the number of participants required to give results statistical significance.

Inclusion Criteria

- Age >18 years
- Haemoglobin level of <120 g/l
- Blood transfusion requirement of less than 3 units per fortnight
- Ability to give informed consent
- Written consent
- Understanding of the procedures to be undertaken as part of the study
- Willingness to participate in exercise testing and follow intervention guidelines and other instructions provided by the experimenters.
- ECOG (Eastern Co-operative Oncology Group) performance status of 2 or less

Exclusion Criteria

- Allergy to beetroot.
- Significant cardiac, respiratory, renal or liver comorbidities

- Aspirin, clopidogrel, dipyridamole, NSAID or other anticoagulant use (e.g. warfarin, low molecular weight heparin or other oral anticoagulants) will be excluded from the platelet aggregometry arm of the study.
- Platelet count of <50 will be excluded from platelet aggregometry
- Musculoskeletal disorders that would make exercise testing impractical (inability to walk one mile)
- Inability to lie flat for the conduction of exercise testing
- Anyone unable to have an MRI scan was offered alternative exercise testing (as assessed by MR 'Participant Safety Checklist')

Of note, while the use of antibacterial mouthwashes is commonplace amongst the population of patients within the oncology and haematology departments who were approached for recruitment to the current study, this was not restricted amongst participants. Many used Difflam® 0.15% (methylparahydroxybenzoate and ethanol) or Corsodyl® (chlorhexidine digluconate) in order to reduce both chemotherapy-induced oropharyngeal inflammation and bacterial growth. Given the chemotherapy and immunosuppression of this population of patients, their inherent vulnerability to infection meant that antibacterial mouthwash use was not used as an exclusion criterion despite its effect on the oral microbiome and the potential for an effect on the individual's ability to reduce salivary nitrate to nitrite in the oral cavity.

Informed consent

All trial participants were given a Patient Information Sheet (Appendix 2) giving detailed information about the trial objectives, structure, interventions, investigations,

risks and benefits in layman's terms. After a period of time sufficient to digest this information, they met with a member of the research team on a one-to-one basis. The trial was explained to them verbally, explaining each of the above points and checking the subject's understanding throughout. The researcher made clear that enrolment in the trial had no effect on the normal treatment of their underlying health condition, and that their usual healthcare team would not be informed of the results of their tests. Participants were also reassured that they were free to end their involvement in the trial at any point and that this would not influence their usual care. They were given the opportunity to ask any questions throughout the discussions, which were answered fully and in layman's terms. Once satisfied with all aspects of the trial, if they were still keen to be enrolled, written consent was obtained by the researcher.

2.2 GENERAL RESEARCH DESIGN

This was a prospective, balanced randomised cross-over pilot study. Once recruited into the trial, participants underwent baseline assessment of quality of life, muscle performance and exercise capability along with blood tests. They were randomised to receive either concentrated beetroot juice rich in nitrates (BR) (Beet It Sport Pro-Elite Shot, James White Drinks Ltd, Ashbocking, Suffolk, UK), or nitrate-deplete placebo beetroot juice (PL) (Nitrate-Depleted Beet It Sport Pro-Elite Shot, James White Drinks Ltd, Ashbocking, Suffolk). The latter juice is produced purely for research purposes by passing beetroot juice through a column containing the anion exchange resin Purolite a520e which exchanges nitrate for chloride (Gilchrist, Winyard et al. 2014). Normally used in the treatment of water for human

consumption, Purolite a320e produces a safe dietary product which is identical in its appearance, taste, texture and smell to BR.

After their initial baseline appointment, participants drank 5 x 70ml bottles of their allocated juice at 48, 36, 24, 12 and 2.5 hours prior to their second point of assessment. All of the above assessments were repeated following this period of supplementation at experimental visit 2. Once completed, participants were asked to return 3-6 weeks after their second appointment for a repeat of all of the above assessments to update their baseline results. They were then given 5 x 70 ml bottles of either nitrate-rich or nitrate-deplete beetroot juice (whatever they did not receive at randomisation) which they again drank in the days leading up to their final assessment at 48, 36, 24, 12 and 2.5 hours prior to their appointment (Figure 5). To avoid any 'order effect', in which a patient received BR first, but the effects were carried over to the period when they received PL, there was a minimum three week 'washout' period before testing was repeated. Data from healthy subjects suggested that the effects of nitrate are lost within 24 hours (Wylie, Kelly et al. 2013) so it was postulated that three weeks would be ample to prevent carry-over of the active compound.

One disadvantage of this 'cross-over' design was the inadvertent introduction of bias, whereby patients who remained in the study long enough to complete the second half of the study may not have been comparable to the group as a whole. Patients receiving chemotherapy have regular fluctuations in their blood counts and general well-being. One of the advantages of the cross-over design is that each participant acts as their own control; this can be helpful in unstable participant populations. For example, patients are likely to suffer from the effects of their cancer early on in

treatment, but over time, will typically be more affected by the side effects of treatment. The cross-over design aimed to help correct for these fluctuations over time, ensuring that data collection would inform the researchers about the effects of nitrate supplementation, rather than measuring the changes in physiology caused by active malignancy or chemotherapy. However, participants who remained in the study for all four visits may have performed better in all of their tests than those who were forced to withdraw as a result of ill health, hence giving a potential source of confounding. The other reason a cross-over design was utilised in this study was that it is much more acceptable to patients to know that they will at some point receive the 'active' ingredient.

Prior to initiation of recruitment to the study, both beetroot juice and nitrate-depleted beetroot juice were code labelled by a scientist in the University of Exeter Department of Sport and Health Sciences not otherwise involved in this research. The person labelling the supplements ensured that their order was counter-balanced; meaning equal numbers of participants received each intervention at the first point of supplementation. Therefore, no-one involved in the study knew the order of supplementation until after data collection was complete. Hence participants, clinicians, research nurses and scientists were all blinded to the order of supplementation for each participant until the coding was revealed upon data analysis once data collection was complete.

Through this study design, each participant was their own internal control, reducing confounding and selection bias. In order to reduce recruitment bias, consecutive patients attending haematology/oncology outpatient clinic who were eligible to enter the trial were approached to consider participation in the study. Blinding ensured

there was no recall, reporting or observer bias. Trained research staff used the functional assessment of cancer therapy – anaemia scale (FACT-An) validated questionnaire (Appendix 3) (Cella 1997, Cella, Eton et al. 2002) to measure participant quality of life and the ‘functional assessment of cancer therapy - cognition (FACT-cog) questionnaire (appendix 4) (Jacobs, Jacobsen et al. 2007, Bell, Dhillon et al. 2018) to assess their cognitive function. These validated questionnaires were devised in order to obtain reproducible, validated methods of obtaining objective qualitative feedback of the symptomatic burden participants experienced as a result of anaemia, and the effect that cancer and its therapy has on a participant’s cognitive function. Each questionnaire uses a standard set of questions, allowing participants to select one of five possible responses to each one. The responses are scored according to the guidelines accompanying the questionnaires and were allocated a total score of between 0 and 188 (FACT-An) or 0 and 132 (FACT-Cog). The higher the score obtained, the better the quality of life and lower the symptomatic burden experienced by that patient. The participants completed these questionnaires unaided reducing observer bias. Questionnaires were used with the kind permission of the Functional Assessment of Chronic Illness Therapy (FACIT) group.

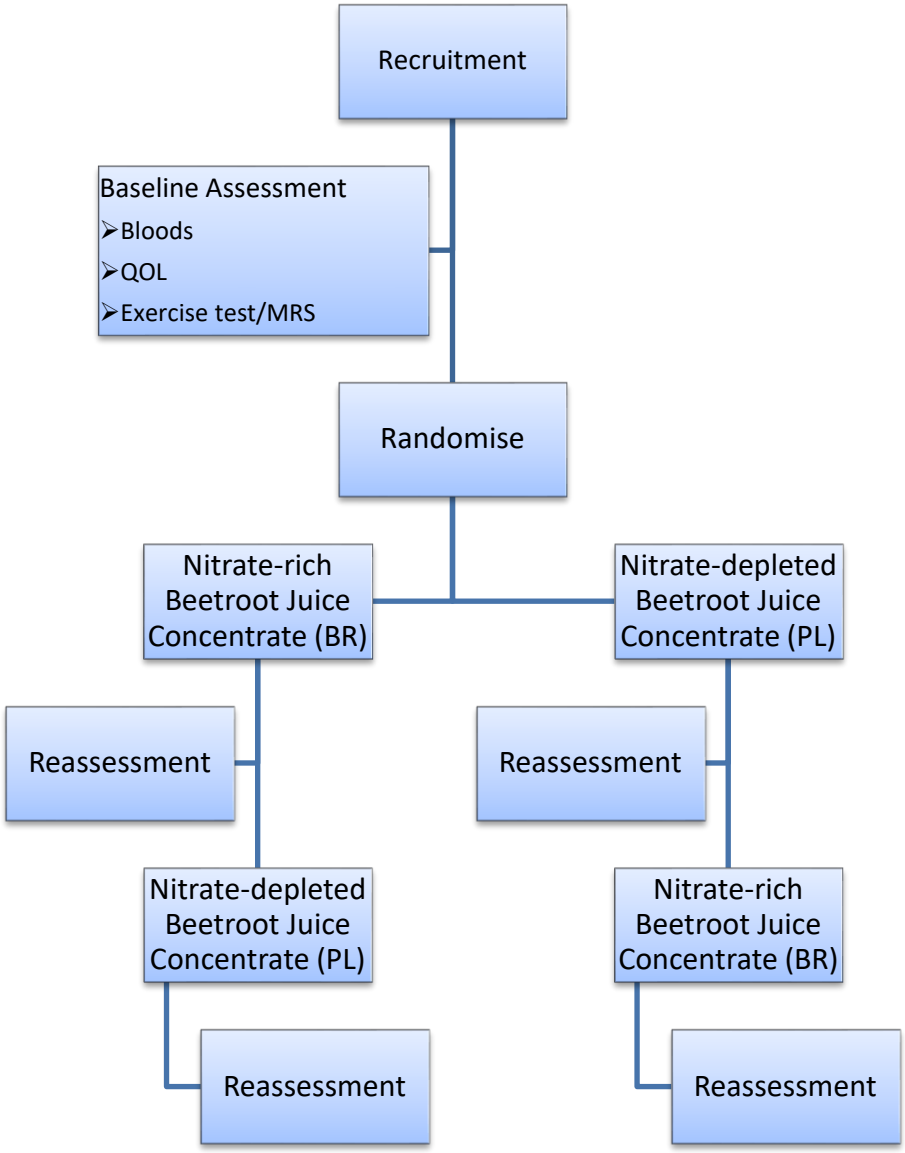


Figure 5: Experimental Design

2.3 HEALTH AND SAFETY

Researchers underwent appropriate health and safety training prior to obtaining access to the biochemistry laboratories, and ensured these guidelines were adhered to. A Control of Substances Hazardous to Health (COSHH) health and safety assessment was performed before any investigations or sample analyses were undertaken. All researchers wore disposable latex gloves and laboratory coats when handling blood products, while any sharps and biohazards used were disposed of in a sharps bin.

All exercise physiology testing procedures were compliant with the University of Exeter health and safety policies. This included strict maintenance of hygiene and cleanliness through regular cleaning of work surfaces, ergometers and other equipment in the exercise physiology laboratory. Each participant was afforded an appropriate period of 'warm-down' after the cycle ergometry test.

The hygiene and cleanliness of the magnetic resonance spectroscopy room was also maintained through regular cleaning. Access to this area was strictly controlled given the extremely powerful magnetic field; only staff specifically trained in the operation of the MRI scanner were allowed to perform this part of the experimental procedure, and completed an MRI safety checklist before initiation of data collection. All tests were performed under the direct supervision of a Post-Doctoral Research Fellow in MRI. Participants also completed an MRI safety checklist prior to each scan to ensure they were suitable candidates to complete that arm of the investigations, and were reminded to remove all metallic objects before entering the scanner room.

All investigations were performed under close supervision by a member of the research team who was also a qualified medical doctor. Participant symptoms, oxygen saturations, heart rate and age-predicted heart rate reserve were constantly monitored during exercise. The participants were given instructions about when to terminate the ergometry according to their symptoms and heart rate, ensuring they did not exercise excessively. Basic resuscitation equipment was available in the laboratory.

2.4 ADVERSE EVENTS

This study of dietary supplementation had some potential risks. The above exclusion criteria prevented patients with significant pre-existing co-morbidities from entering the trial, reducing the risk from gentle exercise testing. However, there was a significant risk associated with asking unhealthy and potentially unstable patients to exercise as this could have precipitated cardiovascular or respiratory compromise. Magnetic resonance spectroscopy was chosen as a means of assessing muscle strength and metabolic activity because it is non-invasive and safe, as it does not expose participants to ionising radiation.

Beetroot juice itself was taken in small quantities repeatedly, but does not taste particularly pleasant. Study participants were reassured that, should they feel unable to drink the supplements, they could withdraw from the study with no effect on their ongoing medical care.

An adverse event was defined as the appearance or worsening of any undesirable sign, symptom, or medical condition occurring after starting the study even if the event was not considered to relate to the study intervention. Medical conditions and diseases present before starting the study were only considered adverse events if they worsened after starting the study. Abnormal laboratory values or test results constituted adverse events only if they induced clinical signs or symptoms that were considered clinically significant or required therapy.

The occurrence of adverse events was sought by non-directive questioning of the participant at each visit during the study. Adverse events also could have been detected when they were volunteered by the participant during and between visits, or through physical examination, laboratory test or other assessments. Any adverse events were to be recorded within the participant's clinical research file with the following information:

- The severity grade (mild, moderate, severe)
- Its relationship to the study (suspected/not suspected)
- Its duration (start and end dates or if continuing at final exam)
- Whether it constituted a serious adverse event (SAE)

Any adverse events would have been treated with one or more of the following: No action taken (i.e. further observation only); Study permanently discontinued due to this adverse event; Concomitant medication given; Non-drug therapy given; Patient

hospitalised / patient's hospitalisation prolonged. The action taken to treat the adverse event was recorded in the participant's clinical research file.

Adverse events which affected participants would have been followed until resolution or until they were judged to be permanent, and an assessment would have occurred at each visit (or more frequently, if necessary) of any changes in severity, the suspected relationship to the study intervention (beetroot juice), the interventions required to treat it, and the outcome.

To ensure patient safety, every serious adverse event (SAE), regardless of suspected causality, occurring after the patient had provided informed consent and until 90 days after the patient had stopped study participation was reported to the chief Investigator. A log of all adverse events was recorded in the patient's CRF, which was shared with the Research and Development department.

2.5 DATA COLLECTION AND QUALITY CONTROL

Case report forms (CRFs) were used to collect the data. At the end of each participant's involvement in the study, the research nurse was responsible for ensuring the accuracy, completeness, legibility and timelines of the data reported in the CRFs. The CRFs were completed in black or blue ink. Only the chief investigator and those personnel authorised by him were allowed to enter or change data in the CRFs. All laboratory data and observations were transcribed into the CRFs. ^{31}P-MRS scans were reported in summary in the CRFs. The original reports, traces and films were retained by the principal investigators for future reference.

Corrections were made only by striking out any errors, with a single stroke, and not by using correction fluid. The correct entry was entered by the side. The incorrect figure remained visible and the correction was initialled and dated by the person authorised by the chief investigator to make the correction.

After all the queries were resolved at the end of the study, the chief investigator confirmed this by signing off the CRFs. The original CRFs were subsequently archived by the research nurse.

2.6 DATA MANAGEMENT

Participants were given a Unique Identifying Number (UIN) upon recruitment to the study. All blood samples, questionnaire responses and ergometry performance data were anonymised using this UIN. These results were not accessible to the clinician caring for the patient. Research nurses gathered anonymised information using the Functional Assessment of Cancer Therapy - Anaemia (FACT-An) and Functional Assessment of Cancer Therapy - Cognition (FACT-Cog) questionnaires and a trial data collection sheet at each clinic appointment. Exercise testing, blood tests and ^{31}P-MRS results were documented using the same participant UIN.

The only personal data stored was in the written CRFs, and comprised solely of the participant's initials, date of birth, and hospital number. CRFs were stored according to hospital trust policy, in the locked room retained specifically for the purpose of all haematology trials.

In order to ensure participant confidentiality, the NHS Code of Confidentiality was followed, along with the GMC's 'Confidentiality' guideline (2009). Patient identifiers

were kept to a minimum through use of the UIN on results from blood tests, exercise testing and MRS along with FACT-An and FACT-Cog questionnaires.

2.7 MEASUREMENT PROCEDURES

This thesis reports the results of 104 exercise tests. These were conducted in an air-conditioned exercise physiology laboratory and an air-conditioned ^{31}P-magnetic resonance spectroscopy room, each at sea level, with an ambient temperature of 19-21°C. Also included are the results of 104 Functional Assessment of Chronic Illness Therapy (FACIT) questionnaires, investigating participants' cognitive function and quality of life. These questionnaires were completed in the presence of a trained researcher in a formal interview setting with minimal distraction and interruption. All procedures were in accordance with the standards set out by the *Declaration of Helsinki* and *Good Clinical Practice*. They were approved by the National Regional Ethics Service Committee South West (ref: 14/SW/0081), the University of Exeter and the Royal Devon and Exeter Hospital Research and Development department prior to initiation of data collection.

2.7.1 DESCRIPTIVE DATA

Prior to study entry, the participant's height, weight, gender and underlying diagnosis were recorded on the CRF, along with their age. Each time they attended for exercise physiology testing and questionnaires, their haemoglobin was also

recorded, along with the number of days since their last transfusion of packed red cells and the number of days since their last course of chemotherapy. They then went on to undergo the specific testing as detailed below.

2.7.2 BLOOD COLLECTION AND PROCESSING

Upon arrival at each appointment, participants underwent venepuncture using a 21G butterfly needle. Blood was drawn into room temperature vacutainers appropriate to the experimental arms that individual was enrolled in. All participants gave 8 ml of blood into a clotted vacutainer (Sarstedt S-Monovette, Nümbrecht, Germany) which was later used for quantitation of ferritin, serum iron, transferrin and transferrin saturation. They also gave 8ml of blood into Lithium-Heparin vacutainers (later used for nitrate and nitrite quantitation) and 8ml into EDTA vacutainers (later used for quantitation of cGMP and haemoglobin.. All lithium heparin samples and all but one EDTA sample were immediately centrifuged at 4000 rpm for 10 minutes which equates to a g-force of 1729 g (Heraeus Labofuge 200 centrifuge, Thermo Fisher Scientific Inc, Waltham, MA, USA). Following centrifugation, the supernatant plasma was immediately removed and placed in 1.5 ml reaction tubes before transferring on ice to -80 °C storage freezers pending analysis (Kapil, Rathod et al. 2018).

During the same venepuncture process, those participants who satisfied the inclusion and exclusion criteria for platelet aggregometry gave 18ml of blood in Citrate vacutainers (Sarstedt, Germany) which were stored vertically for 30 minutes at room temperature, prior to centrifugation and ongoing processing as detailed in

the section on platelet aggregometry. Further laboratory analysis of each blood sample is outlined below (section 2.7.7).

2.7.3 FACT-AN AND FACT-COG QUESTIONNAIRES

Upon completion of venepuncture, trained researchers explained how to complete the Functional Assessment of Cancer Therapy - Anaemia (FACT-An) and Functional Assessment of Cancer Therapy – Cognitive Function (FACT-Cog) questionnaires (Yellen, Cella et al. 1997, Wagner, Sweet et al. 2009) before participants were asked to complete the forms themselves. Researchers were available throughout should the participant have any queries. Investigators assisted those patients who were unable to self-complete by reading the questions for them, and asking the participant to choose which response from a card of options they were given for the duration of the questionnaire. Responses were scored according to the scoring template and a score of between 0 and 188 was allocated to each completed FACT-An questionnaire and between 0 and 132 for each completed FACT-Cog questionnaire. Higher scores indicated better quality of life and cognitive function respectively.

2.7.4 BASELINE OBSERVATIONS

After the quality of life questionnaires were completed, following 10 minutes of seated rest in a quiet, darkened room, baseline blood pressure of the brachial artery (systolic and diastolic) and heart rate was recorded using a DINAMAP® ProCare

Monitor (General Electric Healthcare, Little Chalfont, United Kingdom) and the mean of three readings was recorded. This widely used monitor contains a sensitive transducer which measures cuff pressure, and attributes tiny oscillations in pressure to the appearance and disappearance of the arterial pulse once it overcomes the occlusive pressure exerted by the cuff as the cuff deflates. These points correspond to the systolic and diastolic blood pressure while pulse pressure is calculated as the difference between the two. Blood pressure and pulse was checked four times over four consecutive minutes; the first values were discarded while the mean of the readings from the second, third and fourth minutes was recorded as the patient's pre-exercise values in order to maximise accuracy.

2.7.5 CYCLE ERGOMETRY

After completion of venepuncture, questionnaires and baseline observations, all participants underwent a symptom-limited incremental cycle ergometer test at each experimental visit to the exercise physiology laboratory. These were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) which was regularly recalibrated by a laboratory technician in order to ensure maintenance of data quality throughout the study. Pulmonary oxygen uptake and carbon dioxide exhalation was measured throughout the test on a breath-by-breath basis in order to determine the participant's gas exchange threshold (GET, see relevant section). Each test started with a 2 minute seated rest, followed by 3 minutes of unloaded pedalling, before commencing a linear work rate increase to intolerance. Participants were asked to maintain a cadence which they found

comfortable during the unloaded pedalling. As each participant maintained the same cadence throughout their tests, the ergometer was able to impose a fixed work rate increase through electronic braking via flywheel resistance. Hence participants pedalled with a known power output ('ramp rate') during this section of the test. The power output of 10 watts per minute was selected by the researcher aiming to achieve the limit of exercise tolerance in around 8-12 minutes. Participants were instructed to exercise until they were breathing heavily, the exercise became challenging or when the pedal rate began to feel uncomfortably fast. Alternatively, if their heart rate reached 80% of age-predicted maximum, the exercise test was terminated. This maximum was determined by subtracting the patient's age from 220; for example, a 60 year old patient will have a maximum heart rate of 160, meaning they were allowed to exercise until they reached a heart rate of 128 beats per minute (160 x 0.8). Time to exhaustion was documented.

Pulmonary gas exchange

Dynamic breath-by-breath pulmonary gas exchange and ventilation was analysed throughout all cycle ergometry tests using an open circuit system. Prior to every tests, a 3-litre calibration syringe was used to calibrate flow volume, while a pressurised gas cylinder containing known concentrations of oxygen and carbon dioxide (close to 15% and 5% respectively with 80% nitrogen) was used to calibrate the analysis of participants' respiratory gases. An appropriate low dead space, tight-fitting face mask was selected according to the size and shape of each participant's face. This mask was connected to a metabolic cart analyser (Jaeger Oxycon Pro, Hoechberg, Germany) containing a transducer.

The widely used metabolic analyser quantified the carbon dioxide component of exhaled air using differential infrared absorption, and the oxygen component via paramagnetic absorption. The first technique relies on the fact that molecules such as CO_2 containing dissimilar atoms will absorb infrared radiation and convert this energy into molecular vibration. The frequency of this vibration depends on molecular mass and atomic bonding within each molecule within the gas being analysed. Different molecules therefore absorb infrared at specific wavelengths and can be quantified (Langton and Hutton 2009). Oxygen quantification is enabled by the somewhat unusual characteristic of molecular oxygen; it is magnetic as a consequence of the two unpaired electrons in its outer electron ring, and is attracted into an electromagnetic field. Using a switched electromagnetic field generated at approximately 110Hz and a pressure transducer, a pressure difference is created between the reference sample (clean air) and the patient's sample. A sensitive transducer then detects pressure variations and converts them to a DC voltage which is directly proportional to the concentration of oxygen (Langton and Hutton 2009).

The metabolic cart simultaneously quantified ventilation through a bidirectional digital volume sensor ('TripleV'). Breath-by-breath raw data on minute ventilation (VE), oxygen uptake (VO_2) and carbon dioxide output (VCO_2) was displayed digitally during cycle ergometry on a real-time basis after automated correction of concentration and volume signals of each breath. This was then exported for later analysis on completion of the study. This analysis included assessment of the VO_{2peak}, maximum power output, time to exercise limit, and calculation of gas exchange or anaerobic threshold. Gas exchange threshold (GET) is defined as the

work rate at which anaerobic metabolism supplements aerobic mechanisms during progressive exercise.

Oxygen Saturations

Participants' oxygen saturations were continuously analysed peripherally with a finger probe using a Masimo SET Rainbow Pulse Co-Oximetry monitoring platform (Masimo Europe Ltd, Matrix House, Basing View, Basingstoke, Hampshire, RG21 4DZ). Conventional pulse oximetry cannot distinguish between arterial and venous blood in the exercising patient as it assumes that the only blood moving in the patient's finger is arterial, whereas in exercise, venous blood moves more rapidly than at rest. As a result, these analysers under-read the arterial oxygen content as they cannot distinguish between the two blood pools, whereas the Masimo SET technology overcomes this difficulty. This widely used technology utilises adaptive filters in parallel processing mode along with conventional red over infrared algorithms to identify the venous blood signal and extract the arterial signal, giving an accurate measure of arterial oxygen saturation (Barker 2002). It also assessed methaemoglobin as part of its standard capability.

Heart rate

During cycle ergometry, participants' heart rate was recorded every 5 seconds using short range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). This raw data was displayed to the researcher digitally, and was used to ensure no participant exceeded 80% of their age-predicted maximum heart rate during exercise. It was exported for further analysis on completion of the test.

2.7.6 ^{31}PHOSPHOROUS - MAGNETIC RESONANCE SPECTROSCOPY (^{31}P-MRS)

At each experimental visit, those participants who were not precluded from entering the magnetic resonance imaging (MRI) scanner underwent an exercise protocol involving single-legged knee extensions while the quadriceps muscles of their right leg were analysed.

During the ^{31}P magnetic resonance spectroscopy scan, participants underwent a gentle exercise protocol using a single-leg knee extension ergometer. Prior to their first such scan they underwent familiarisation in order to ensure they were comfortable with the exercise that was required of them, and to find an appropriate load for the ergometer. This was determined as the mass which caused their quadriceps muscles to begin to ache at the end of 24 seconds of exercise, and at which the participant judged the exercise severity as 7/10 if 1/10 is very light exercise and 10/10 is extremely difficult.

Participants lay prone in the bore of the MRS scanner and Velcro straps were used to immobilise their lumbar back, buttocks and thighs. Their right foot was attached securely to a padded foot brace again using a Velcro strap. The brace was in turn attached to the custom-built, non-ferromagnetic ergometer by a rope and pulley system housed in a nylon frame. The rope was attached to a load basket into which brass plates of known weight were placed (Figure 6).

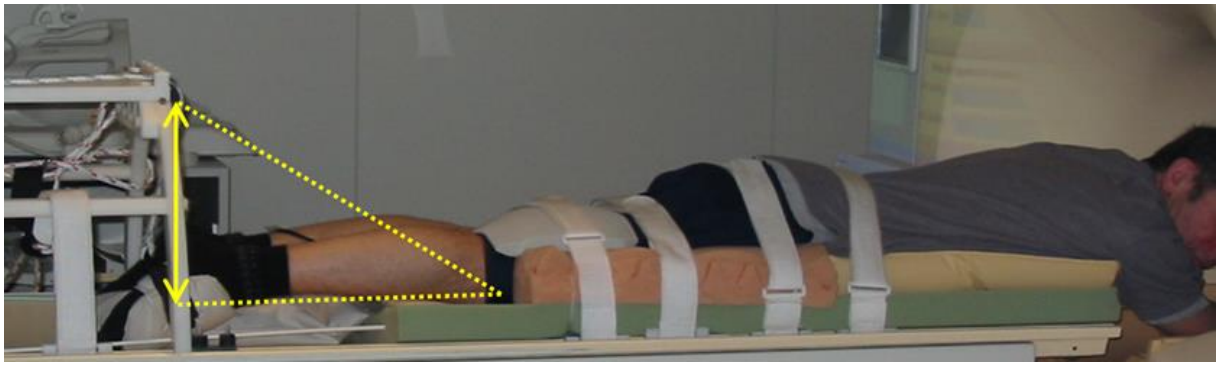


Figure 6. Knee extension ergometry during ^{31}P-magnetic resonance spectroscopy. This figure shows a volunteer ready to perform knee-extension exercise in the MR scanner. A weight is attached to the foot via a rope and a padded foot strap. The exercise is performed by extending the knee, so that the foot will move up and down as indicated by the yellow arrow. The grey pad strapped to the quadriceps of the right leg is the ^{31}P transmit / receive surface coil (see below).

Participants were asked to undergo 24 seconds of right leg knee extensions over a distance of 22cm, followed by a 3.5 minute rest. During these 24 second bursts, participants extended their leg 40 times per minute, timing each exercise downstroke with an auditory stimulus from the MRI scanner. This stimulus was emitted by noisy expansion and contraction of the magnetic coils within the scanner as pulses of electricity passed through them causing predictable changes in the magnetic field they created. All power levels were later estimated according to the masses used, as rate of PCr recovery was the measure of interest in this study and exercise was merely used as a means to reduce intracellular PCr. To ensure a constant work rate and muscle position relative to the magnetic coil, participants were visually queued and given verbal feedback by the researcher.

^{31}P magnetic resonance spectroscopy (^{31}P-MRS) offers a unique means of performing non-invasive, longitudinal analysis of energy metabolism in skeletal

muscle during exercise. The human body comprises predominantly of water. Magnetic fields generated by the powerful electromagnets in the MRI scanner cause excitation and hence, alignment of all protons within the body. Additional, smaller specific magnetic fields are applied during the scan to allow orientation of the data generated, and also to create an individual label for each proton relative to its velocity. Simultaneously, specific radio frequencies (RFs) are applied to the tissue of interest in the subject, such as the quadriceps muscle in the current study. These alter the alignment of the protons within that tissue, before the RF signal ceases, causing the protons to move back into alignment. This movement of protons generates a nucleic signal which is amplified and used to create detailed images or calculate volumes within the body. In this study, it was used to assess the kinetics of phosphorous metabolites within the right quadriceps muscle group.

Participants in this trial performed the exercise protocol whilst lying prone in a 1.5T superconducting magnetic resonance scanner (Gyroscan Clinical Intera, Philips Medical Systems, Best, The Netherlands). A 6cm ^{31}P transmit / receive surface coil was placed within the subject bed, centred over the quadriceps muscle. This was matched and tuned before an automatic shimming protocol was performed. The term shimming refers to the process of removing small inhomogeneities which are present in the magnetic field. This process is both passive, whereby sheet metal is placed at specific locations on the external surface of the MRI machine, and active. In active shimming, an electric current is passed through specifically designed coils and is adjusted and optimised to fine tune the homogeneity of the magnetic field.

Once shimming was complete, an unsaturated ^{31}P spectrum was acquired to allow for T1 correction of those spectra obtained during exercise. Throughout the entire

exercise protocol, phosphorous spectra were acquired every 1.5 seconds at a spectral width of 1500Hz, generating 1000 data points. The multi-pulse experiment was repeated four times and for each repetition the phases of the radiofrequency pulses were varied through a carefully designed sequence (phase cycling). Through combination of the free induction decays from each repetition, this reinforced desired phosphorous signals through addition and allowed unwanted signals to cancel each other out, providing an accurate ^{31}P magnetic resonance spectrum of the participant's quadriceps muscle group every 6 seconds (Jones, Wilkerson et al. 2008).

Analysis of each ^{31}P spectrum was performed by the jMRUI (version 3) software package (Java-based Magnetic Resonance User Interface) which used the AMARES (Advanced Method for Accurate, Robust and Efficient Spectral) fitting algorithm. Spectra were fitted assuming the presence of the following peaks; P_i (inorganic phosphorous), PCr (phosphocreatine), phosphodiester, α-ATP (two peaks, amplitude ratio 1:1), β-ATP (three peaks, amplitude ratio 1:2:1), and γ-ATP (two peaks, amplitude ratio 1:1). A baseline spectrum was obtained for each subject prior to exercise initiation with long repetition time (20 s). This allowed quantification of the unsaturated peak amplitudes and, hence, allowed the relative amplitudes during exercise to be corrected for partial saturation. Intracellular pH was calculated using the chemical shift in the position of the P_i peak relative to the PCr peak.

The rate of PCr recovery was of particular interest in this study. PCr is a high-energy phosphate reserve found in vertebrates and some invertebrates. It is used to maintain ATP levels in the activation of cells in excitable tissues such as muscles and the brain, overcoming the very limited supply of ATP within cells in those

tissues. Without PCr, excitable cells would only maintain a muscle contraction for a few seconds before their intracellular ATP reservoir is exhausted (Wallimann, Wyss et al. 1992). ATP is continuously replenished from large intracellular pools, catalysed by the enzyme creatine kinase through the following reaction:

$$MgADP^{-} + PCr^{2+} + H^{+} \leftrightarrow MgATP^{2-} + Cr$$

Recovery of the intramuscular PCr reservoir following exercise is inversely proportional to the rate of oxygen consumption (Mahler 1985, Haseler, Hogan et al. 1999); therefore the rate constant of the exponential PCr recovery curve could be used as an index of oxidative ATP production (Roussel, Bendahan et al. 2000). This provides an *in vivo* assessment of skeletal muscle mitochondrial function.

2.7.7 BLOOD ANALYSIS

Platelet Aggregometry

Those participants who were not excluded from this arm of the study were advised to avoid aspirin-containing medications and non-steroidal anti-inflammatory drugs (NSAIDs) for 14 days prior to their appointment, and caffeine and alcohol for twenty-four hours. 6 x 3ml citrated vacutainers (Sarstedt S-Monovette, Nümbrecht, Germany) of venous blood were taken from both the participant and from one of the researchers acting as a control. The person acting as control also avoided aspirin, NSAIDs, caffeine and alcohol. Venepuncture was as atraumatic as possible, using a 21g needle and minimal use of the tourniquet to avoid artefactual platelet activation. Blood samples were left to stand for a minimum of 30 minutes before centrifugation

using a Heraeus Labofuge 200 centrifuge (Thermo Fisher Scientific Inc, Waltham, MA, USA) at 1600rpm for 5 minutes. Samples were visually inspected for clarity before the supernatant platelet rich plasma (PRP) was carefully pipetted using a plastic Pasteur pipette into a separate plastic tube for the patient and control specimens, which were capped. The previously centrifuged specimen tubes were then returned to the centrifuge for a further 10 minutes at 4000rpm before the supernatant was pipetted off into plastic tubes marked platelet poor plasma (PPP) for both the participant and the control.

Portions (0.5 ml) of each of the participant's and control's PPP and PRP were pipetted into aliquot tubes and a platelet count was obtained using a Coulter DXH analyser (Beckman Coulter Ltd, Miami, FL, USA). Aggregation should be performed on PRP with platelet counts of at least $100x10^9$/L, White Cell Count (WCC) of $<0.5x10^9$/L and Red Cell Count (RBC) of $<0.5x10^9$/L. If the RBC or WCC were higher than 0.5, the sample was re-centrifuged. PPP was analysed to ensure platelet count was $<10x10^9$/L, WCC of $<0.5x10^9$/L and RBC of $<0.5x10^9$/L. Light transmission platelet aggregometry was then performed on both the control and the study samples simultaneously using a PAP-8 platelet aggregation profiler (BioData Corporation, Horsham, PA, USA), with the reagents ADP (10 μM), arachidonic acid (5 mg/ml), collagen (1.9 mg/ml) and epinephrine ($1x10^{-3}$ M). Prior to the addition of reagents, light aggregometry was performed on the PRP to ensure no spontaneous aggregation occurred. All aggregation testing was completed within 3 hours of venepuncture.

Nitrate and Nitrite Quantification

At each participant appointment, 8 ml of venous blood was taken for nitrate (NO_3^-) and nitrite (NO_2^-) quantification in Lithium Heparin vacutainers (Sarstedt S-Monovette, Nümbrecht, Germany) before immediate centrifugation at 4000rpm for 10 minutes (Heraeus Labofuge 200 centrifuge, Thermo Fisher Scientific Inc, Waltham, MA, USA). Using a plastic pipette, the supernatant plasma was then immediately removed to prevent reduction of ˙NO by haemoglobin to methaemoglobin. The plasma was placed in 1.5ml reaction tubes before transferring on ice to -80°C storage freezers pending analysis.

The samples were later tested using a nitric oxide analyser based on gas phase chemiluminescence (Sievers NOA 280i analyser, General Electric Healthcare, Little Chalfont, UK). This technique involves chemical reduction of NO_3^- and NO_2^- to ˙NO which is then quantified, hence giving an indirect measure of NO_3^- or NO_2^- contained within the sample. ˙NO quantification is performed through its gas phase reaction with ozone in the chemiluminescence chamber, producing an excited state of nitrogen dioxide (NO_2) which emits light in the infrared region of the electromagnetic spectrum. The quantity of photons produced in this reaction is directly proportionate to the concentration of ˙NO within the chemiluminescence chamber. The intensity of this light is amplified and then quantified by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in the nitric oxide analyser, producing an analogue millivolt output signal. Nitrate and nitrite concentration of patient samples is derived from the integral of the ˙NO-generated millivolt signal over time via comparison with those obtained for standard concentrations of nitrate and nitrite (Bateman, Ellis et al. 2002).

A standard curve was prepared daily using sodium nitrate at known concentrations ranging between 100nM and 10μM. Resultant luminescence was plotted via signal area in mV and used as a reference to quantify participant NO_2^- and NO_3^- concentrations.

In order to quantify NO_3^-, all participant samples were deproteinised using zinc sulphate precipitation prior to analysis. 100 μl of patient plasma was added to 500 μl of 0.18 M sodium hydroxide (NaOH), vortexed and incubated at room temperature for 10 minutes. 300 μl of 5% zinc sulphate ($ZnSO_4$) was added before the resultant solution was vortexed again and incubated at room temperature for a further 10 minutes. This was centrifuged at 14000 rpm for 10 minutes before storage in a refrigerator pending analysis. 50 μL of sample plasma was injected into a sealed system at 95°C and reduced to ˙NO using a 5 ml solution of vanadium (III) chloride (VCl) in 1 M hydrochloric acid (0.8% w/v). All ˙NO produced then bubbled up a feed line through a filter into the chemiluminescence chamber where it was quantified through the analysis outlined above (Bateman, Ellis et al. 2002). Each participant plasma sample was analysed in duplicate in order to improve reliability.

When quantifying NO_2^-, samples did not require deproteinisation as less bubbling and foaming occurred during quantitation of NO_2^- than NO_3^-. 50 μL of sample plasma was injected into a sealed system at 30°C and reduced using 5ml glacial acetic acid and 1 ml sodium iodide (NaI) solution. ˙NO produced by these reductions was again bubbled up a feed line into the chemiluminescence chamber where it reacted with ozone, produced an excited state of nitrogen dioxide and photons which were then quantified as outlined above; thus NO_2^- concentration was deduced (Bateman, Ellis et al. 2002).

Guanosine 3', 5'-cyclic Monophosphate (cGMP) Quantification

cGMP is formed by the action of the enzyme guanylate cyclase on glycerine triphosphate. Presence of ˙NO stimulates the action of guanylate cyclase, hence stimulating cGMP levels. Blood was taken in a vacutainer containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) before centrifugation at 4000 rpm for 10 minutes. The supernatant plasma was pipetted into reaction tubes which were then placed on ice before storage in a -80°C freezer pending analysis at a later date. cGMP quantification used a colourimetric competitive immunoassay kit from Enzo Life Sciences (Enzo Life Sciences Inc, Farmingdale, New York, USA). Participant samples were treated with 0.1 M hydrochloric acid to stop endogenous phosphodiesterase activity and stabilise any released cGMP. They were then acetylated through the addition of acetic anhydride and triethylamine. The kit uses a polyclonal antibody to cGMP which competitively binds either the cGMP in the standards or samples, or an alkaline phosphatase molecule which has cGMP covalently attached to it. After a simultaneous incubation the excess reagents are washed off and a substrate is added. After another shorter incubation period, the enzyme reaction is stopped and the yellow colour generated is read on a microplate reader at 405 nm wavelength. The intensity of the bound yellow colour is inversely proportional to the concentration of cGMP in either standards or participant samples. The measured optical density is used to calculate the concentration of cGMP.

Haemoglobin Quantification

Haemoglobin was quantified using a Coulter DXH analyser (Beckman Coulter Ltd, Miami, FL, USA) courtesy of the Royal Devon and Exeter Hospital Department of

Haematology. This lyses whole blood before quantification of haemoglobin photometrically at 525 nm.

Ferritin, Serum Iron, Transferrin and Transferrin Saturation Quantification

Ferritin, serum iron, transferrin and transferrin saturation were determined using Roche E170 automated immunoassay platforms (Roche Diagnostics Ltd, West Sussex, UK) courtesy of the Royal Devon and Exeter Hospital Department of Biochemistry.

2.8 STATISTICAL ANALYSIS

The statistical analysis plan was incorporated within the study protocol. It dictated the exclusion and exclusion criteria and general study population, the target number of participants to include in the trial, and the randomisation and blinding methodology as outlined above. No interim analysis was intended given the small size of the study population. All data obtained from participants who completed all study visits were analysed fully, meaning those participants who withdrew from further involvement in the trial after the second experimental visit were not included in the analysis. This was intended to ensure that the cross-over design was effective at providing its own control. There was very little missing data except for the platelet aggregometry data; very few participants had no missing data in this aspect of the trial, but only those with aggregometry results from all four experimental visits were included in the analysis given the expected marked inter-subject variation.

All data were assessed for normality and are presented in the results section as means +/- standard deviation (SD) +/- 95% confidence intervals (CI). Observed differences in the parameter of interest were tested using students' two tailed paired t-tests when comparing two groups of data and one way analysis of variant (ANOVA) when comparing three groups. Pair-wise post-hoc analysis of statistically significant ANOVA results was performed using the Fisher's Least Significant Difference (LSD) test as appropriate. Non-parametric data was analysed for significance using the Wilcoxon matched-pairs signed rank test. Further analysis was performed calculating the pre- versus post-supplementation delta for each group of data, then an unrelated sample test was used to compare these values.

Statistical significance was accepted at $p = < 0.05$, while a trend towards significant results was accepted as $p = < 0.1$ as recommended by the statistician during initial consultations. Results which were statistically significant following post hoc analysis are denoted with an asterisk (*) in figures and their legends. Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA), GraphPad Prism 5.04 (GraphPad software incorporated, San Diego, California, USA) and SPSS software version 26.0 (IBM, Chicago, Illinois, USA)

CHAPTER 3: RESULTS

Participant recruitment was easily achievable at a rate which matched the availability of MRI appointments; when approached, many of the patient population were motivated and keen to enrol. While the reasons for this enthusiasm were not recorded formally, a number of participants volunteered that they felt significant goodwill towards the recruiting departments in the local hospital, as many pointed out that they had received excellent care there. Altruism was a recurring theme within participants' comments, as many wanted to 'give something back.' Others hoped that their enrolment in the study would afford them some empowerment in their control of symptoms and treatment of their disease. However, a number of participants were living with terminal diagnoses and, while neither the Patient Information Sheet (Appendix 2) nor researchers suggested it may have any effect on their underlying cancer, one could speculate that they felt this trial may have enabled them access to a 'treatment' that offered them hope of improved symptoms and survival. This reflects formally gathered reasons from qualitative research investigating the incentives reported by those patients volunteering to participate in health research. The main reasons identified were that the trial allowed patients access to medications that offered hope of relief, to better care and to technologies for monitoring illness and health (Townsend and Cox 2013).

Thirty-three participants were recruited into the study out of 36 who were approached (92.5%) (Figure 7). Of those who were recruited, twenty-six people attended all 4 experimental visits (79%). Three participants withdrew from the study prior to their first visit due to ill health; two had progression of malignancy while one

had severe pneumonia and rib fracture. One participant withdrew after changing his mind about involvement as he had a poor prognosis and did not want to commit time to travelling the significant distance to Exeter from his home. A further participant was withdrawn by the investigator after another trial which the patient had been actively involved in declined to give their consent for her involvement in this trial. Two participants withdrew after attending for their first two experimental visits for personal reasons and as a result of chemotherapy side effects respectively.

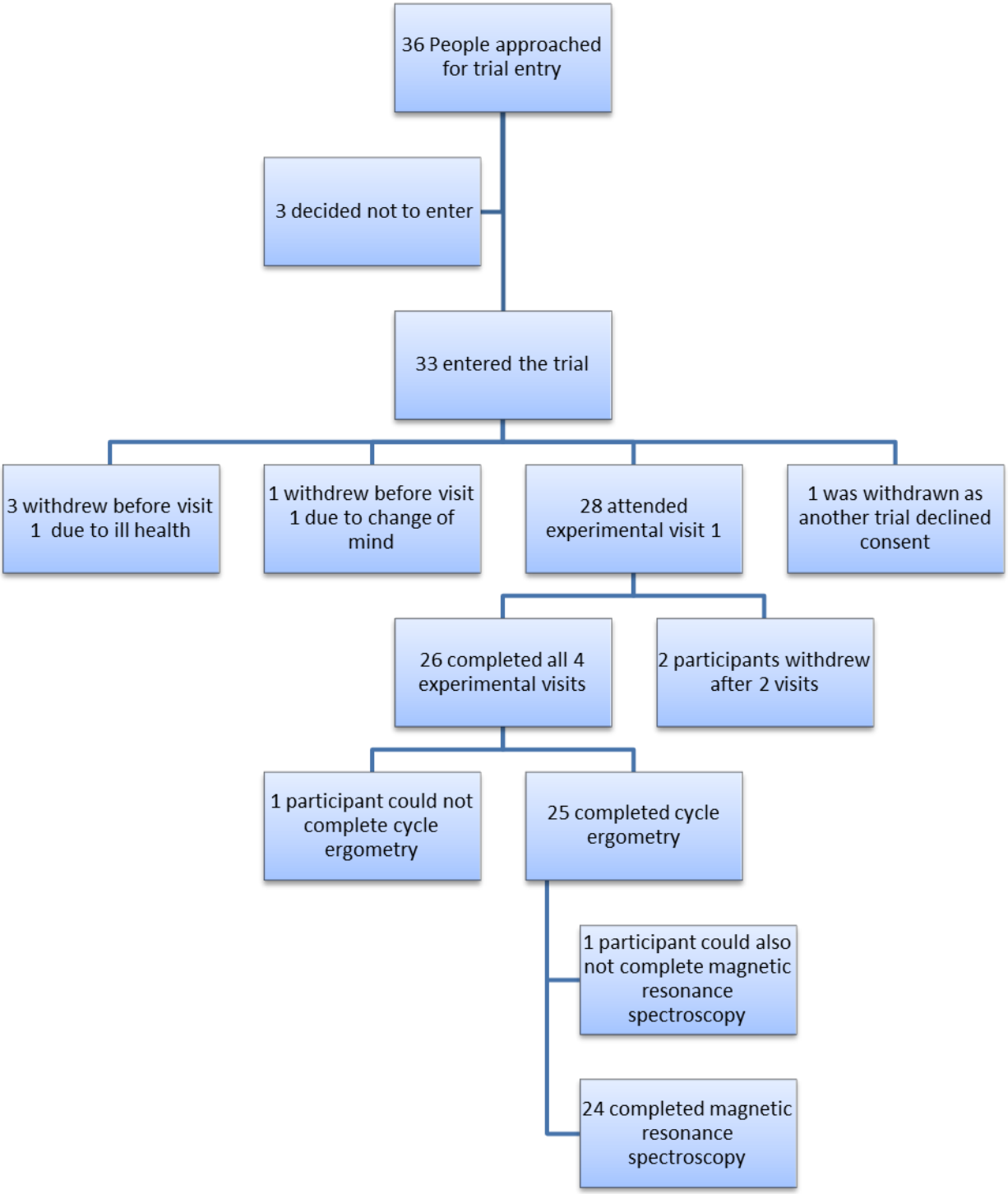


Figure 7: Consort diagram. This demonstrates that 33 of 36 people approached to enter the trial decided to do so (92.5%). 5 people withdrew or were withdrawn from the trial before their first experimental visit, either as a result of ill health due to progressive malignancy or pneumonia, and one because a trial she was already enrolled in declined her permission to enter the current study. 2 participants withdrew after 2 of their experimental visits due to personal reasons or ill health, while 26

completed all experimental visits. One person was unable to tolerate cycle ergometry while one further participant was also unable to complete magnetic resonance spectroscopy.

Of the 26 participants who proceeded with all four experimental visits, one was precluded from involvement in the ^{31}P-MRS arm of the study having previously had a spinal decompression / stabilisation procedure during which titanium rods were inserted into her spine. This participant was excluded as her titanium implants were large and the theoretical risks associated with MRI scanning were slightly increased compared to those without these rods. These risks include heating of the metal through induction of electrical currents in the rods, which may have been capable of causing localised tissue heating (Davis, Crooks et al. 1981). In reality, as titanium implants are paramagnetic and therefore not affected by magnetic fields, this risk was negligible but the need to ensure no patient was harmed by their trial entry was reason enough for her to be excluded (Kim, Choi et al. 2019). A further patient withdrew from involvement in the MRI arm as his marked thoracic kyphosis made lying in the prone position in the bore of the MRI scanner intolerable. The same patient also experienced dizziness and light-headedness after just over 2 minutes of cycling without any resistance during cycle ergometry so, although he recovered within seconds of stopping exercise, he was also withdrawn from that arm for safety reasons.

Hence, 24 participants completed all aspects of the study, one person completed all but the MRI arm, and one person completed all but the MRI and cycle ergometry arms.

3.1 POPULATION DEMOGRAPHICS

Results of the 26 patients who completed all 4 experimental visits in this pilot study are included in this analysis (Table 2). Their age ranged from 40 to 93 years old with a mean of 66 and standard deviation of 12 years. There was a slight preponderance for male sex (15 of 26 participants, 57.7%). The study group were anaemic for a variety of reasons, representing a spectrum of haematological and oncological disorders. 50% of participants were undergoing active chemotherapy of varying intensities during their trial involvement.

Age (years)	Mean	61
	Range	40 – 93
	Standard Deviation	12
Gender: n (%)	Male	15 (57.7%)
	Female	11 (42.3%
Diagnosis: n (%)	Low-Grade Non-Hodgkin's Lymphoma	5 (19.2%)
	Myeloma	4 (15.4%)
	Myelodysplasia	5 (19.2%)
	Gastric Carcinoma	2 (7.7%)
	Chronic Lymphocytic Leukaemia	1 (3.8%)
	Myelofibrosis	2 (7.7%)
	Myeloproliferative Neoplasm	3 (11.5%)
	Aplastic Anaemia	1 (3.8%)
	Colorectal Carcinoma	1 (3.8%)
	Diagnosis unclear	2 (7.7%)
Ongoing Chemotherapy	Rutuximab & Bendamustine	1 (3.8%)
	Irinotecan & Capecitabine	1 (3.8%)
	Lenalidomide	2 (7.7%)
	Pomalidomide	1 (3.8%)
	Rituximab, Cyclophosphamide, Vincristine & Prednisolone	2 (7.7%)
	Hydroxycarbamide	3 (11.5%)
	Rituximab & Chlorambucil	1 (3.8%)
	Etoposide, Oxaliplatin & Capecitabine	1 (3.8%)
	Etoposide, Carboplatin & Capecitabine	1 (3.8%)
	Azacitidine	1 (3.8%)
	Nil	13 (50%)

Table 2: Population Demographics. This table demonstrates the breadth of demographic characterising those participants who enrolled in this study. There was a broad range in the age of the 26 people, from the young middle-aged to the very elderly. There was a slight preponderance for male sex, while their health was very heterogeneous. They had a wide variety of medical problems causing their anaemia ranging from anaemia of

uncertain benign aetiology which was behaving in a benign manner, to anaemia as a result of terminal malignancy. They were also on a variety of different treatments; while half of the study participants were on no chemotherapy, the remainder were on treatments ranging from relatively intensive chemotherapy such as the etoposide, oxaliplatin and capecitabine combination, to the very gentle such as hydroxycarbamide.

3.2 BLOOD ANALYSIS: HAEMOGLOBIN

Three participants were given a blood transfusion of 2 units of packed red cells within 2 months of any of their experimental visits. One received this blood 60 days prior to their first visit and hence 71 days before their second visit which was post-BR supplementation visit. This participant's Hb was stable thereafter, measuring 99 g/l, 93 g/l, 98 g/l and 95 g/l at each of their experimental visits. One participant was transfused 5 days prior to visit three, an unsupplemented visit, which was 12 days prior to the post-PL supplementation visit. Despite this change, this participant's Hb was similar at each of the experimental visits (92 g/l, 99 g/l at the visits before transfusion, then 99 g/l and 102 g/l after transfusion). This participant was transfused as his Hb had fallen significantly between the second and third visits, but the Hb on each of the visit days was similar. A further participant received a blood transfusion 5 days prior to visit 2 (i.e. 5 days before their post-BR supplementation visit). As a result, this participant's Hb rose from 77 g/l to 96 g/l between baseline 1 and the post-BR supplementation visit. However, despite receiving no further transfusions, this participant's Hb actually continued to rise prior to their next experimental visit as

a result of response to chemotherapy, and their next Hb at the second baseline visit was 123 g/l.

While the mean lifespan of human RBCs *in vivo* is 110-118 days (Mock, Matthews et al. 2011), transfused red blood cells survival is similar, with a maximum reported lifespan of 135 days after transfusion. A proportion of these cells are removed from the circulation within 24 hours of transfusion; this varies according to the duration of storage of the cells, and can be between 9% and 23% (Luten, Roerdinkholder-Stoelwinder et al. 2008). Hence, some of these transfused cells were still present in the first participant's circulation at the time of testing despite the amount of time which had elapsed between transfusion and that visit. The other two participants' transfusions are potentially more significant given the proximity to their experimental visits, but are balanced in terms of experimental arms; one potentially affected the participant's performance on their post-PL supplementation visit and the other potentially affected that participant's post-BR supplementation visit. However, those participants receiving transfusions did not display a significant improvement in their haemoglobin level following receipt of blood.

Blood samples from participants were photometrically analysed at the Royal Devon and Exeter Hospital haematology laboratory using Coulter DXH analysers (Beckman Coulter Ltd, Miami, FL, USA). The interbatch coefficient of variation was up to 1.8%.

The haemoglobin concentrations of all participants at all study visits demonstrated a normal distribution, while the mean concentration was 111.7 g/l, indicating relatively mild anaemia; the normal range of Hb concentration is 120 – 160 g/l in females and 130 – 180 g/l in males. The Hb of the participant population was expected to be dynamic as it was subject to external factors such as progression or relapse of

malignancies, initiation or cessation of chemotherapy or radiotherapy, bleeding, transfusion etc. Hence, the crossover study design was chosen in an attempt to counter any fluctuation in the level of anaemia during the participants' involvement in this trial. However, there was a trend towards a difference in the haemoglobin between the experimental arms. Of those participants who received BR at their first supplemented visit, the mean haemoglobin increased from 105.0 g/l (standard deviation 10.6 g/l, 95% confidence interval [CI] 6.0) at their post-BR visit, to 110.7 g/l (SD 12.7 g/l, 95% CI 7.2) at their post-PL supplementation visit, an increase of 16% (Figure 8).

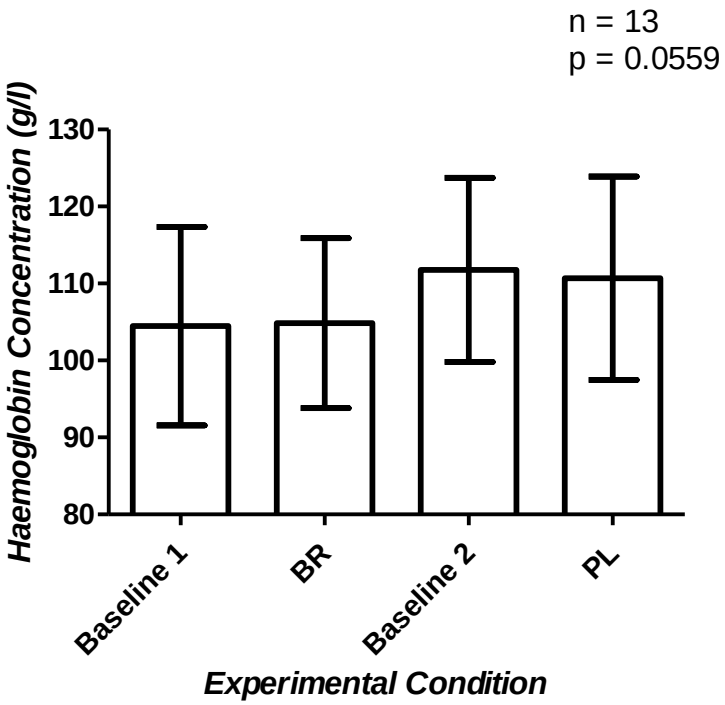


Figure 8: Mean haemoglobin concentration (g/l) in the cohort of participants who received BR at first post-supplementation experimental visit. This bar graph demonstrates the mean and standard deviations of the haemoglobin concentrations of those 13 participants in the current study who received five 70 ml bottles of nitrate-rich beetroot juice (BR) in the 48 hours before their second experimental visit, and five 70 ml bottles of nitrate-deplete beetroot juice (placebo

[PL]) in the 48 hours prior to their fourth experimental visit. This figure demonstrates that the participants' haemoglobin concentration increased during the 3 to 6 weeks washout period between the BR supplemented visit and their return to the next baseline visit. Hence the mean haemoglobin concentration was significantly higher at the experimental visit investigating the effects of supplementation with PL (Hb 110.7 g/l) than following BR supplementation (Hb 105.0 g/l) (paired one tail t-test p = 0.04). Students' t test comparison of the two baseline (non-supplemented) visits, demonstrated no statistically significant difference (p = 0.15). However, an ANOVA analysis demonstrated that there was a trend towards statistical significance amongst all group differences (p = 0.0559). See Appendix 5: Raw Data Graphs for the raw data for each participant's haemoglobin change with time.

The Haemoglobin results of 2 of the participants who received BR prior to their first supplemented visit had a significant effect on the change in the group mean through the experimental timepoints. The first such participant's Hb increased from 104 g/l at the post-BR visit to 137 g/l at the post-PL visit, while the second such participant's Hb improved from 96 g/l to 126 g/l over the same time period (See raw data graph in appendix 5). Each of these participants demonstrated a response to chemotherapy, with improvement in anaemia as a result of reduced bone marrow infiltration with lymphoma following its treatment. One participant received no transfusions of blood, while the second received two units of blood after their initial baseline visit as outlined above, but their haemoglobin continued to rise despite needing no further blood.

The remaining 13 participants received PL at first supplementation and BR at the second. Their mean Hb in the first post-supplementation visit was 118.5 g/l (SD 9.3 g/l, CI 5.3) while it fell to 113.3 g/l (SD 9.0 g/l, CI 5.1) post-BR (Figure 9).

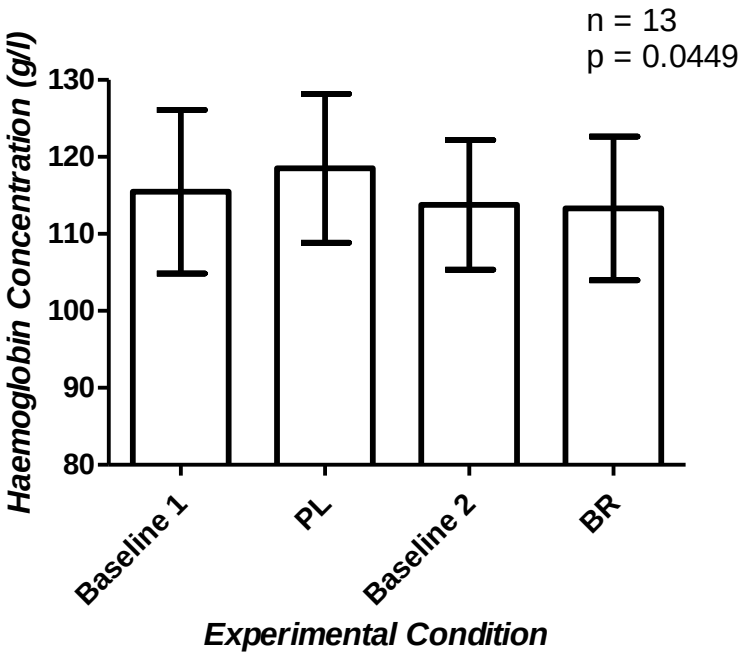


Figure 9: Mean haemoglobin concentration (g/l) of the cohort of 13 participants who received PL at first supplementation visit. This bar graph demonstrates the mean and standard deviations of the haemoglobin concentrations of those 13 participants in the current study who received nitrate-deplete beetroot juice (placebo [PL]) in 48 hours before their second experimental visit, and nitrate-rich beetroot juice (BR) in the 48 hours prior to their fourth experimental visit. This figure demonstrates that the participants' haemoglobin concentration fell during the 3 to 6 weeks washout period between the PL supplemented visit and their return to the next baseline visit. Hence the mean haemoglobin concentration was significantly lower at the experimental visit investigating the effects of supplementation with BR (Hb 113.3 g/l) than following PL supplementation (Hb 118.5 g/l) (paired two tail t-test p = 0.01). There was no difference between the two baseline (non-supplemented) visits (p = 0.47), although, a one way ANOVA of all data sets demonstrated statistical significance (p = 0.0449). Further analysis using the Fisher's Least Significant Difference (LSD) post hoc test demonstrated no signficant difference however.

Of this group of participants who received PL then BR prior to their post-supplementation visits, there were less marked changes in the Haemoglobin.

However, one participant's Hb fell from 126 g/l to 108 g/l and a second fell from 120 g/l to 108 g/l, while no participants in this group had an improvement in their Hb of greater than 10 g/l during their involvement in the trial (See appendix 5 for raw data).

Hence, whether they received BR at their first or their second supplementation, the mean Hb for participants at their post-PL supplementation visit was 114.7 g/l (standard deviation 10.9 g/l, CI 4.6) while the mean Hb of all participants at the time of their post-BR supplementation visit was 109.1 g/l (standard deviation 10.9 g/l, CI 4.2, p-value 0.0028) (**Figure 10**). The mean Hb at each of the supplementation visits was 111.4 g/l (SD 11.6, CI 3.1), meaning no two experimental conditions were performed when the group mean Hb was at the same level.

Two participants had a swing in their Hb between their post-BR and post-PL visits in excess of 30 g/l while another 3 had a swing in excess of 12 g/l. Whatever the order of actual supplementation, all of these larger fluctuations in Hb were between a lower value at the post-BR visit and a higher value post-PL. The crossover study design was selected in an attempt to overcome such bias, but chance appears to have skewed the level of anaemia between the two post-supplementation visits. This pilot study has therefore highlighted that fluctuation in clinical variables such as haemoglobin concentration would affect power calculations when designing a full study. All haemoglobin fluctuations demonstrated were due to progression of underlying cancers, the effect of chemotherapy itself on the process of haematopoiesis within the bone marrow, or due to response of malignancy to chemotherapy.

Blood transfusions did not significantly impact on haemoglobin through the study. If Hb data for those participants who received transfusions were excluded from this

analysis, the mean Hb of participants at baseline visits was 113.1 g/l (SD 8.98), 115.5 g/l post-PL supplementation (SD 11.5) and 110.8 g/l post BR (SD 10.4). Compared to the mean of the entire cohort of all participants, these mean values were 1.7g/l greater at baseline, 0.8g/l higher post-PL and 0.9 g/l higher post-BR. Hence data for those participants who received transfusions was included in the remaining analysis of data obtained in the current study.

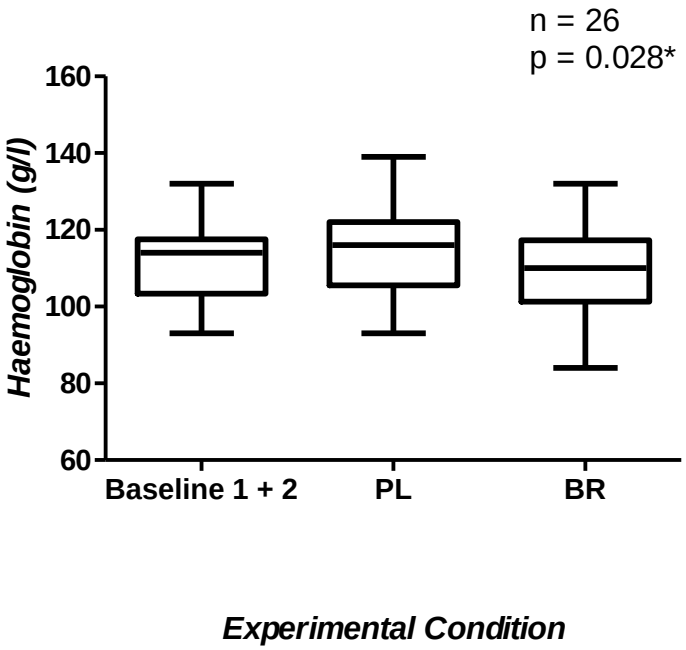


Figure 10: Haemoglobin concentration (g/l) of all participants at non-supplemented (Baseline 1 + 2), post-PL and post-BR study visits. This bar chart compares the mean haemoglobin concentration of participants at their baseline (non-supplemented) visits with both the haemoglobin concentrations at their experimental visits after a 48 hour period of supplementation with nitrate-rich beetroot juice (BR) and after a 48 hour period of supplementation with nitrate-depleted beetroot juice (PL). The error bars display the standard deviations. This graph demonstrates that, irrespective of the order in which the 26 participants received the different beetroot juice supplements, their mean haemoglobin concentration was higher at the 'PL' visit (114.6 g/l, SD 12 g/l, CI 109.8 – 119.5 g/l) than the 'BR' visit (109.7 g/l, SD 10.9 g/l, CI 104.7 – 113.5 g/l). This data reaches statistical significance (two tailed t-test p =

0.008). The mean haemoglobin concentration at all baseline experimental visits (i.e. participants' first and third experimental visits before which they received no beetroot juice supplementation) was 111.4 g/l (SD 9.8 g/l, CI 107.4 – 115.3 g/l), lower than PL ($p = 0.047$) and higher than BR ($p = 0.03$). An ANOVA analysis of all data demonstrates statisitical significance ($p = 0.028$) while LSD post hoc tests demonstrate a signficant difference between all experimental conditions (LSD tests comparing each condition range from 0.007 to 0.47).

As this was a pilot study, only relatively small numbers of participants were enrolled. It was not powered to overcome chance changes in variables such as Hb despite the fact such variables can have a profound effect on the results the study obtained. A larger study involving more participants could potentially have enrolled people whose Hb was higher during the post-BR visit than the PL. This could have negated the skew exerted by the small number of participants whose Hb changed markedly between the two supplemented visits.

3.3 BLOOD ANALYSIS: NITRATE AND NITRITE

One of the key objectives of this study was to ascertain whether the nitrate-nitrite-˙NO enterosalivary pathway is intact in people with anaemia related to haematological and oncological conditions and their treatments.

3.3.1 NITRATE

The nitrate (NO_3^-) concentration of stored participant plasma samples was quantified using gas-phase chemiluminescence (see section '2.7.7 Blood Analysis'). Prior to this, nitrate in plasma was sequentially chemically reduced to nitrite (NO_2^-) then nitric oxide (˙NO) which was then quantified, hence giving an indirect measure of the NO_3^- concentration of the sample. ˙NO quantitation was performed through its reaction with ozone in the chemiluminescence chamber, yielding an excited state of nitrogen dioxide. This emits light in the infra-red region of the electromagnetic spectrum, which is amplified and then quantified by a red-sensitive photomultiplier tube. This produced an analogue millivolt output signal. The NO_3^- concentration within the plasma sample was derived from an integral of the ˙NO-generated millivolt signal over time in comparison with those previously obtained for standard concentrations of NO_3^-.

Standard curves were created daily using known concentrations of sodium nitrate ranging from 250 nM to 10 µM over at least five points. The resultant luminescence

was plotted via signal area in mV and a standard curve was produced (**Figure 11: Standard curve derived from area under the curve in millivolts compared to known concentrations of nitrate**. A range of concentrations of sodium nitrate were reduced sequentially to nitrite then nitric oxide which, when exposed to ozone in the chemiluminescence reaction chamber produced nitrogen dioxide which emitted photons of light in the infra red spectrum. This was amplified and quantified producing an area under the curve measured in millivolts. The R2 value of 0.998 indicates very good fit.Figure 11).

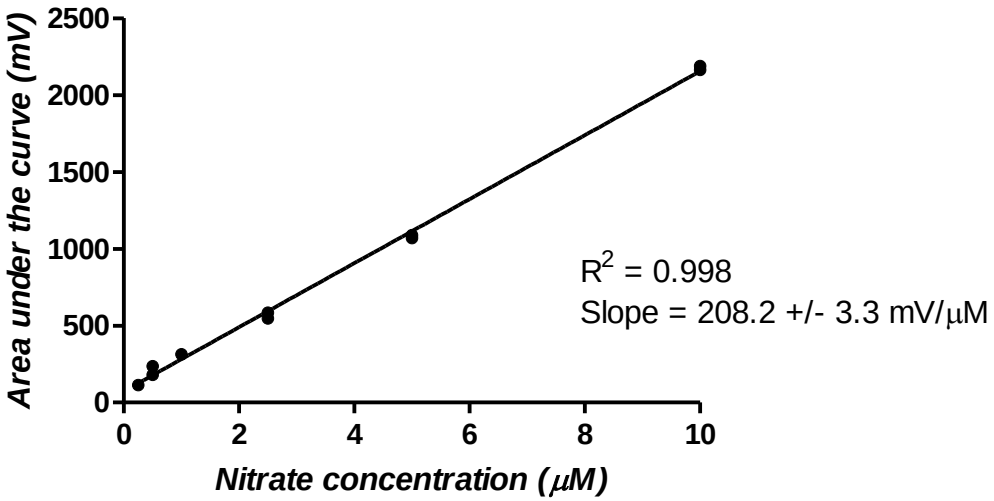


Figure 11: Standard curve derived from area under the curve in millivolts compared to known concentrations of nitrate. A range of concentrations of sodium nitrate were reduced sequentially to nitrite then nitric oxide which, when exposed to ozone in the chemiluminescence reaction chamber produced nitrogen dioxide which emitted photons of light in the infra red spectrum. This was amplified and quantified producing an area under the curve measured in millivolts. The R^2 value of 0.998 indicates very good fit.

Once satisfied with the quality of the daily standard curve, analysis of plasma samples from all four experimental visits of each of the 26 participants was performed using the same technique of gas-phase chemiluminescence which was

used to make the standard curves. A screenshot of one such batch is displayed below (Figure 12)

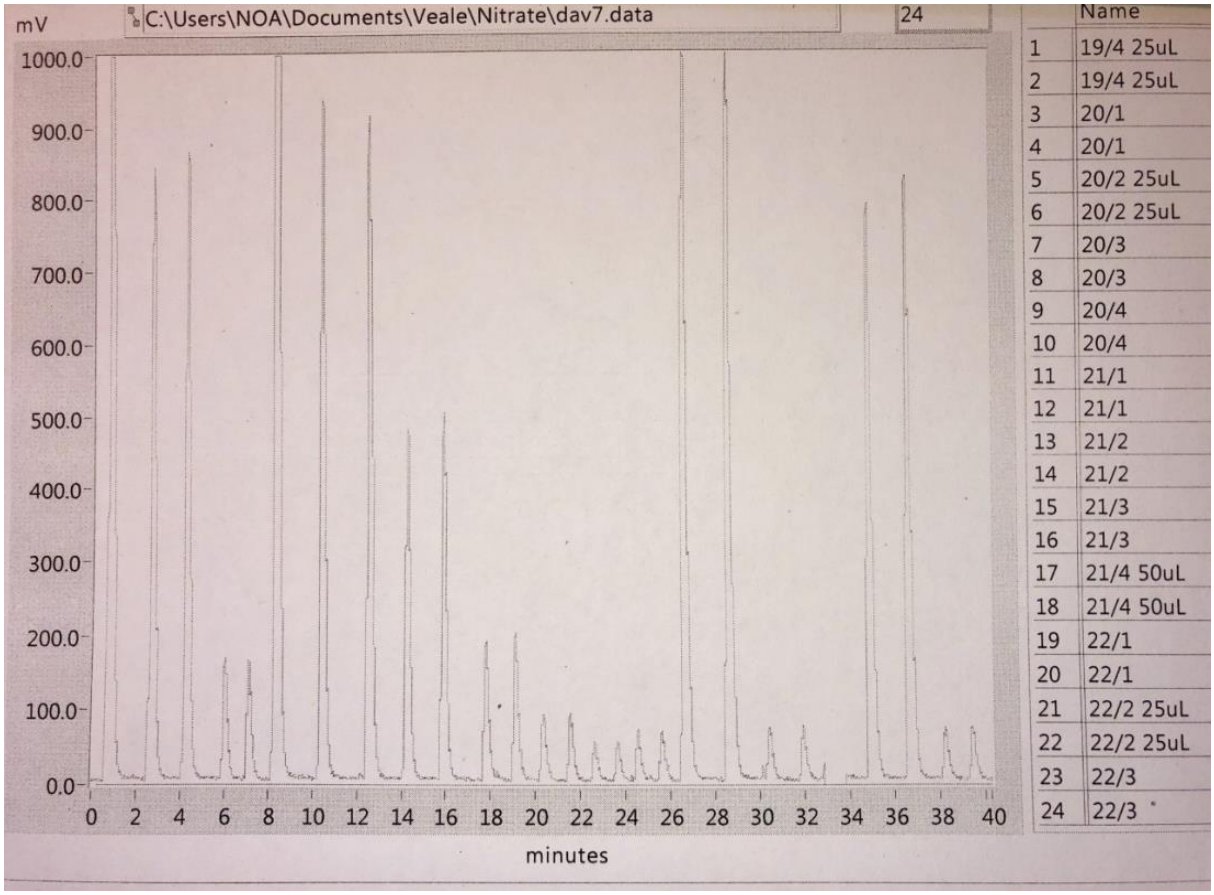


Figure 12: Nitrate quantification through chemiluminescence. This figure is a screenshot of the analysis of plasma samples from 4 participants (unique identification numbers 19, 20, 21 and 22), showing the electrical output from chemiluminescence. Each curve corresponds to a separate sample analysis. Quantification of the area under each curve was used to calculate the nitrate concentration of that patient's plasma sample. Every sample was analysed in duplicate in order to improve accuracy of the results. While 50 µl of participant samples was analysed in most cases, only 25 µl of Participant 19's samples from their fourth experimental visit (name '19/4') was analysed as this visit followed supplementation with BR and therefore their millivolt output was anticipated to rise above the scale used in this batch. This decision was made as in response to problems with samples from earlier batches; those samples from BR-supplemented participants repeatedly generated outputs in millivolts which rose above this scale.

Their areas under the curve and therefore nitrate concentrations could not be quantified as some of the data was not captured, so the samples had to be reanalysed using 25 µl aliquots of participant plasma rather than 50 µl. No serial dilutions were performed on these high-nitrate concentration samples. If the millivolt output of samples from participants at their BR supplementation visit was lower than anticipated, analysis was repeated without reducing the volume analysed.

Analysis of all 26 participants' plasma samples from each experimental visit was performed in duplicate to improve accuracy. There was significant variability in participants' plasma nitrate concentration results as described below. This affected the standard deviations and therefore coefficient of variation (CV) for the assay, with the latter varying from 15.6% in all participants at the post-BR supplementation visit, to 12.5% at the post-PL supplementation visit.

While there was significant variability amongst the trial population, 25 of the 26 participants in this study (96%) demonstrated an increase in plasma nitrate concentration following supplementation with BR compared to the mean of their baseline visits and after PL supplementation (Figure 13). This demonstrates that those participants recruited into this pilot study were compliant with their supplementation, and despite their various medical complaints and treatments (chemotherapy and otherwise), they absorbed it in good quantity. The only participant who did not develop an increase in nitrate concentration took over 10 different herbal remedies and lived on a diet which only consisted of juiced fruit and vegetables. While the precise content of the herbal remedies is unclear, her diet was extremely rich in nitrate prior to and during her involvement with this trial. The mean of this participant's two unsupplemented baseline visits and her post-PL visit was 18.1 µM, higher than all other participants, although this was only 0.1 µM greater than the second highest patient. This information is an important guide for the design

of future trials following this initial pilot study, as such participants would probably have been excluded from any future trial as a result of the high risk of confounding.

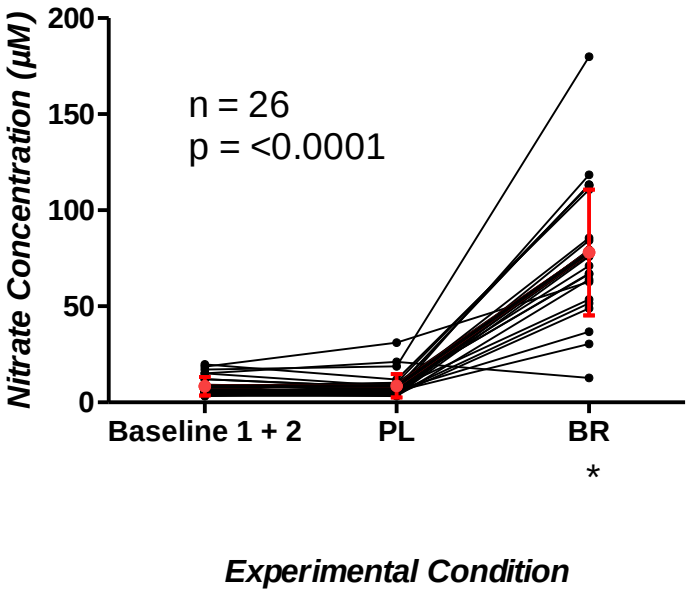


Figure 13: Plasma nitrate concentration at non-supplemented, post-PL and post-BR experimental conditions. Black points and lines indicate each of the 26 individual participants' plasma nitrate concentration at all of their experimental conditions, while the red data points and red line displays the mean of the entire cohort of participants within this study. The red error bars demonstrate the standard deviation of the means. The mean was used as these data are parametric. The experimental condition 'Baseline 1 + 2' indicates all nitrate concentrations of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visit following a period of supplementaion. Participants ingested a 70 ml bottle of nitrate-depleted beetroot juice (PL) at 2.5, 12, 24, 36 and 48 hours before study visit 'PL', while they ingested a 70 ml bottle of nitrate-rich beetroot juice (BR) at the same time points prior to visit 'BR'. Each data point represents the mean of duplicate nitrate concentrations quantified by gas-phase chemiluminescence analysis of each participant plasma sample from every experimental visit (see Methods section 2.8.7). This graph demonstrates that, while there was some variation in the individual response to BR supplementation, the mean plasma nitrate concentration was significantly higher at experimental condition BR (78.0 µM, SD 33.5, CV 15.6%) than at either baseline (8.4 µM, SD 58.8, CV 10.3%) or PL (8.6 µM, SD 72.3, CV 12.5%)

(one way ANOVA p = < 0.0001, LSD test confirms statistical significant difference [*] in baseline 1 + 2 vs BR and in baseline 1 + 2 vs BR).

The mean plasma nitrate for participants at baseline was 8.4 µM (standard deviation 4.9 µM) while the mean concentration at the post-PL visit was 8.6 µM (SD 6.1 µM), giving a mean of all of these experimental timepoints of 8.5 µM. In comparison, the mean plasma nitrate for participants in this study at the post-BR supplementation visit was 78.0 µM. Hence, despite some variability in the degree of this response between individuals, this cohort of anaemic participants absorbed a significant quantity of the ingested nitrate within their BR supplementation, with an increase in the mean plasma concentration of 664%.

3.3.1 NITRITE

The nitrite (NO_2^-) concentration of stored participant plasma samples was quantified using gas-phase chemiluminescence (see '2.7.7 Blood Analysis'). Prior to this, 50 µL of sample plasma was injected into a sealed system at 30°C and reduced using 5ml glacial acetic acid and 1 ml sodium iodide (NaI) solution. Nitric oxide ($^{\cdot}NO$) produced by these reductions was bubbled up a feed line into the chemiluminescence chamber for quantification which was then quantified as outlined above, hence giving an indirect measure of the NO_2^- concentration of the sample. The NO_2^- concentration within the plasma sample was derived from an integral of the $^{\cdot}NO$-generated millivolt signal over time in comparison with those previously obtained for standard concentrations of NO_2^-.

Standard curves were created daily using known concentrations of sodium nitrate ranging from 250 nM to 10 µM over at least five points. The resultant luminescence was plotted via signal area in mV and a standard curve was produced. Analysis of all 26 participants' plasma samples from each experimental visit was performed in duplicate to improve accuracy. Similar to the results obtained from plasma NO_3^- concentration quantification, there was significant variability in participants' plasma nitrite concentration results as described below. This affected the standard deviations and therefore coefficient of variation (CV) for the assay. The CV ranged from 5.8% and 13.8% in the baseline and post-PL supplementation visits respectively, to 9.5% at all of the post-BR supplementation visits.

A significant increase in plasma nitrite (NO_2^-) after supplementation with BR was observed in the majority of the participants in this trial (Figure 14). The median NO_2^- concentration of this cohort at their baseline (non-supplemented) visits was 135 nM (SD 79 nM, IQR 88 - 165 nM) while the median plasma NO_2^- following PL supplementation was 112 nM (SD 69 nM, IQR 92 - 147 nM). This median concentration rose markedly to 485 nM (SD 1006 nM, IQR 365 – 1104 nM) following supplementation with BR. This was statistically significant according to a 2 way ANOVA ($p = <0.0001$) and LSD post hoc test (LSD = 0.000).

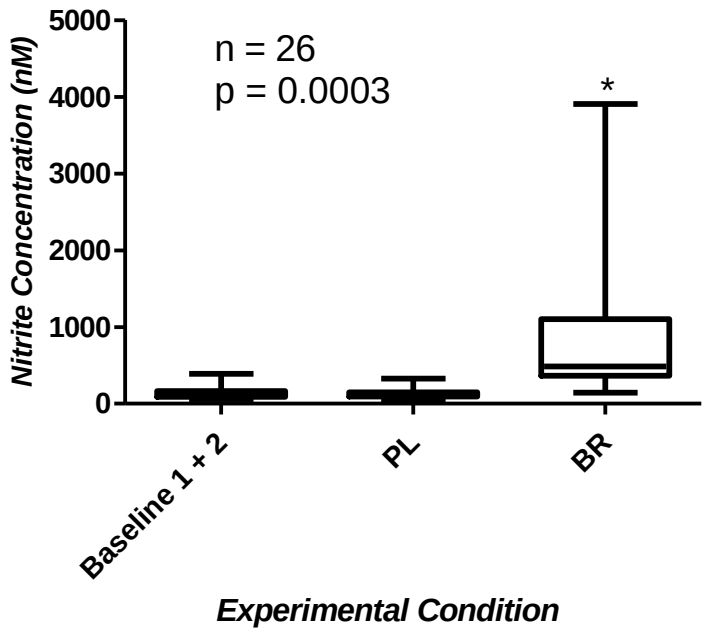


Figure 14: Plasma nitrite concentrations of participants at each experimental visit. Unlike most results in the current study, these data were non-parametric in distribution according to visual inspection and Shapiro-Wilk test exhibiting a positive skew in each experimental condition. Hence a box and whisker plot was used to display the median, interquartile range and extreme nitrite concentrations of 26 participants' plasma samples from each of their 4 visits. The experimental condition 'Baseline 1 + 2' indicates all nitrate concentrations of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visit following a period of supplementation. This graph demonstrates that the median plasma nitrite concentration was significantly higher at experimental condition BR (485 nM, SD 1006 nM, interquartile range 365 – 1104 nM) than at either baseline (135 nM, SD 79 nM, IQR 88 – 165 nM) or PL (112 nM, SD 69 nM, IQR 92 – 147 nM) (2 way ANOVA $p = < 0.0001$, statistically significant according to Fisher's LSD post hoc analysis [LSD = 0.000]). The data was positively skewed and therefore made non-parametric by two participants' nitrite concentration at condition BR which rose far more than the remainder of the participants. See Appendix 5: Raw Data Graphs for the individual nitrite concentration results.

While the group median NO_2^- concentration rose significantly following BR supplementation compared to their non-supplemented and PL-supplemented visits, there was a large variation in the degree of this response with a standard deviation of the BR experimental state of 1006 nM. NO_2^- concentration rose by a median of 1235% between the post-PL visit and the post-BR condition. However, 2 participants' plasma NO_2^- concentration increased to over 3750 nM, in excess of 2000%, while at the other end of the spectrum, one participant's plasma NO_2^- concentration only rose by 155% to 142 nM. Interestingly, the latter person with a minimal response in plasma NO_2^- was the same participant in whom there was observed only a minor increase in plasma NO_3^- concentration following supplementation. Hence it would seem likely that, as there was only a modest rise in plasma NO_3^-, there was only a small increase in available substrate for the facultative anaerobic bacteria located on the base of her tongue, and therefore less plasma NO_2^- was produced. As outlined above, this participant routinely ingested large quantities of nitrate daily as a result of a 'juicing' diet, so this may have ameliorated her response to oral nitrate and therefore her plasma nitrite concentration. She also had previously undergone significant surgery to her upper gastro-intestinal tract in order to treat oesophageal carcinoma, so anatomic and gastric pH changes may have altered the enterosalivary circulation of her dietary nitrate. One must also consider the possibility that she was non-compliant with the supplementation regimen, although the participant appeared highly motivated in each of her experimental visits.

In summary, almost all participants involved in this pilot study derived a good response to BR supplementation both in terms of an increase in their plasma NO_3^- and NO_2^- concentration. This indicates that they were compliant with

supplementation and absorbed it despite their medical complaints, previous surgeries in some cases, co-morbidities and treatments.

3.4 QUALITY OF LIFE

The quality of life of participants enrolled in the trial was recorded through sequential monitoring of their responses to the FACT-An questionnaire at each of their four trial visits (Yellen, Cella et al. 1997, Wagner, Sweet et al. 2009). This subjective score of the severity of symptoms associated with anaemia has been validated to assess the quality of life of people experiencing anaemia. Participants in this trial were asked to self-complete this questionnaire which included 57 questions about a range of factors which contribute to their quality of life. They were asked about their emotional wellbeing, functional wellbeing, physical wellbeing and social / family wellbeing over the previous seven days. Each answer was allocated a score out of 4, giving a total which was scored out of 188. The higher this total score was, the fewer and milder the symptoms the participant was experiencing at the time they completed the questionnaire.

Following the initial explanation about how to complete this questionnaire, participants involved in the trial required very little help or instruction and found it straightforward to fill in. The only problem encountered was that occasional patients missed out a question by mistake, although these were spotted by the research team at that appointment and the patient was prompted to complete all they were happy to answer. As a result, all questionnaires were fully completed. Responses were scored

according to the scoring template and a score of between 0 and 188 was allocated to each completed FACT-An questionnaire.

The coefficient of variation observed amongst the total scores of all completed questionnaires was 15.3 %, demonstrating some breadth of the range of scores obtained.

This study demonstrated that supplementation with both PL and BR brought about an improvement in the median values recorded compared to the median of all baseline visits of this cohort (Figure 15). At their baseline visits, the participants' median FACT-An score was 147 (standard deviation 20) out of a possible 188; this rose by 8 to 155 (SD 24) after supplementation with PL (t test p = 0.009, CI -10.1 to -1.6). This median score also increased from baseline by 4 points after supplementation with BR to 151 (SD 25, t test p = 0.025, CI -8.2 to -0.61). These results indicate that supplementation with either BR or PL brought about a small subjective improvement in the quality of life of the trial participants.

The above results were derived from a comparison of the FACT-An scores on each of the post-supplementation visits with the mean of the participant's baseline FACT-An scores. However, further analysis was performed analysing the change in FACT-An score between each baseline visit and the following post-supplementation visit in an attempt to limit the confounding effect of any change in each participant's wellbeing through the study. This demonstrated no significant difference in the FACT-An score between baseline and post-PL supplementation (Δ4 points, SD 13, CI 4.9, p = 0.44). It also showed no significant difference between the baseline pre-BR and the post-BR supplementation visit's FACT-An score (Δ7 points, SD 8, CI 3.1, p = 0.44).

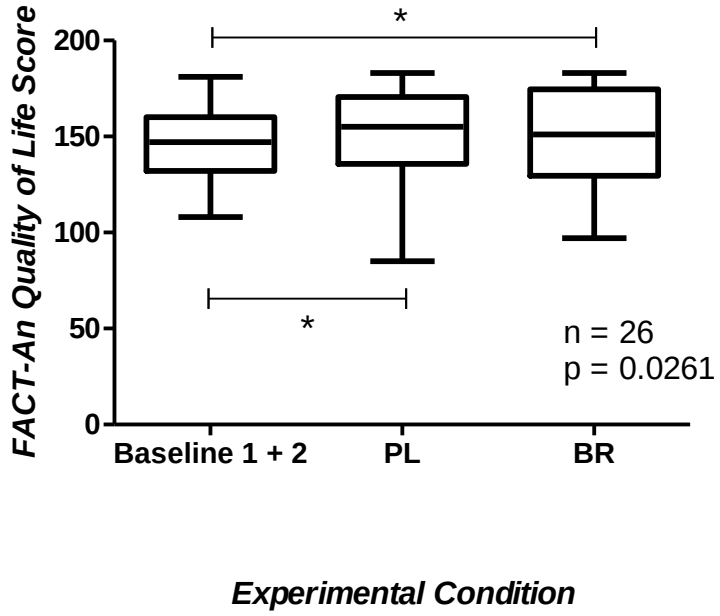


Figure 15: FACT-An quality of life scores of participants at each experimental visit. All 26 participants were asked to self-complete a Functional Assessment of Cancer Therapy - Anaemia (FACT-An) questionnaire to objectively quantify their quality of life over the seven days leading up to each of their 4 experimental visits, with a higher score indicating a better quality of life. The experimental condition 'Baseline 1 + 2' indicates all FACT-An scores of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visits following a period of supplementation with either nitrate-depleted beetroot juice (PL) or nitrate-rich beetroot juice (BR). This box whisker plot shows the median, interquartile range and the extreme total FACT-An scores recorded at each experimental visit. This graph demonstrates statistically significant difference in the FACT-An scores of each experimental visit, with improvement following both PL and BR supplementation when compared to baseline 1 + 2 (one way ANOVA p = 0.0261). A Fisher's LSD demonstrates post-hoc statistical significance between Baseline 1 + 2 and PL (0.009), and between Baseline 1 + 2 and BR (0.025). The graph demonstrates a broad range of responses particularly at the 'PL' experimental visit.

The degree of improvement of FACT-An scoring after supplementation was relatively small. Significant work has been performed recently in order to assist interpretation of these scores; the Functional Assessment of Chronic Illness Therapy

(FACIT) group has issued guidance regarding the minimal important differences (MIDs) for scores and scales of several similar qualitative assessments of health-related quality of life, including FACT-An. They define MID as the "smallest difference in score in the domain of interest that patients perceive as important, either beneficial or harmful, and that would lead the clinician to consider a change in the patient's management" (Cella, Eton et al. 2002). According to their ‘tentative’ guidance, the MID for FACT-An is 7 points, meaning the observed difference in patients’ symptoms is statistically significant but may not be clinically significant across this population of participants. However, some individual participants derived a larger improvement in the FACT-An score results (Figure 16). Participants 5 and 15 both recorded an improvement in the FACT-An score of 16 points between baseline and post-BR supplementation, although participant 15 also derived a FACT-An benefit of 16 points from the PL supplementation compared to the baseline. Participant 5 did not report any benefit in their FACT-An score from PL supplementation, however.

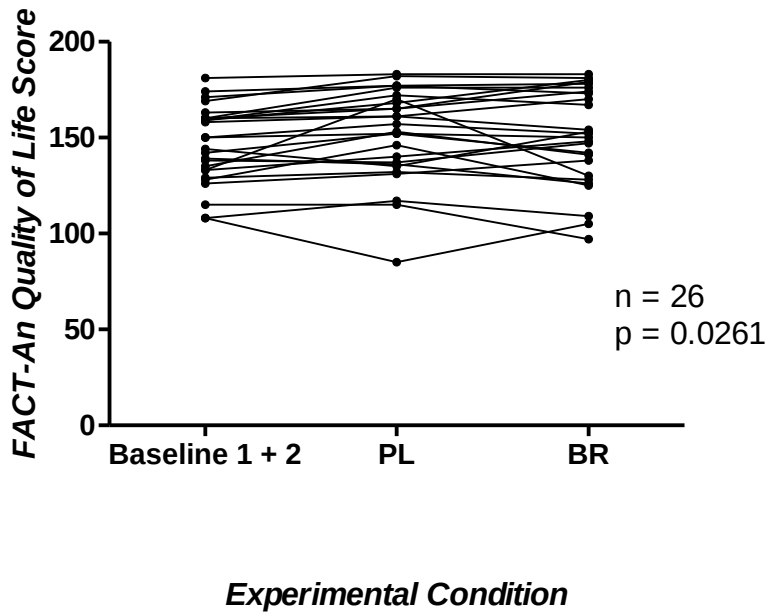


Figure 16: FACT-An quality of life scores of each individual participant at each experimental condition. All 26 participants were asked to complete a Functional Assessment of Cancer Therapy - Anaemia (FACT-An) questionnaire to objectively quantify their quality of life over the seven days leading up to each of their 4 experimental visits, with a higher score indicating a better quality of life. The experimental condition 'Baseline 1 + 2' indicates all FACT-An scores of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visits following a period of supplementaion.. The total FACT-An Quality of Life score recorded for each individual participant at their baseline and post-supplementation visits, demonstrated a marked range in scores and individual variability through the study (SD of all scores at all experimental visits was 22.8).

This subjective, qualitative aspect of the study of this cohort of anaemic participants demonstrated significant variability in all FACT-An quality of life scores with a coefficient of variation (CV) of 15.3%. The highest scoring individual rated their quality of life as 184 out of 188 while the lowest score recorded by any participant was only 85. The mean score of all participants at all study visits was 149 out of 188, while the standard deviation of all scores was 23. There was no correlation between

extent of change of FACT-An score post-supplementation and level of baseline rating.

3.5 COGNITIVE FUNCTION

The cognitive function of all participants within this study was objectively assessed at each trial visit through sequential monitoring of their responses to the FACT-Cog questionnaire. This 37-item questionnaire is divided into six cognitive domains: memory, concentration, mental acuity, verbal fluency, functional interference, and multitasking ability. This questionnaire also includes two other subscales, “noticeability” (comments from others) and “effect of perceived cognitive impairment on quality of life.” Participants record their answers on a 5-point scale ranging from 0, “never,” to 4, “several times a day,” the frequency of each occurrence over the 7 days leading up to the test. In the sections on perceived cognitive abilities and the effect of cognitive impairment on quality of life, responses were rated on a 5-point severity scale ranging from 0, “not at all,” to 4, “very much.” The FACT-Cog total score was derived from the summation of all of the individual subscale scores, ranging from 0 to 132, with higher scores indicating better cognitive functioning.

Within the 26 study participants, there was a marked variability in FACT-Cog responses, ranging from 37 to 132 out of 132 (SD 24), with a mean score of 107 and a CV of 22.2%. There was a minor improvement in the recorded score between baseline and both post-supplementation visits, irrespective of the type of supplementation consumed. The mean of all participants’ FACT-Cog total score was 105 (SD 23) at baseline and rose by 4 to 109 (SD 24) after supplementation with PL

(p = 0.021, CI -7.1 to -0.6) and by 3 to 108 after BR (p = 0.013, CI -6.0 to -0.8) (Figure 17). Each of these values represents only ~3% of the total FACT-Cog score respectively which makes the clinical significance of this improvement uncertain. Indeed, Cheung et al (Cheung, Foo et al. 2014) reported that the minimum clinically important difference in FACT-Cog scored amongst a population of Chinese patients with breast cancer ranged from 6.9 – 10.6 depending on the statistical method used for interpretation.

The above results were derived from a comparison of the FACT-Cog scores on each of the post-supplementation visits with the mean of the participant's baseline FACT-Cog scores. However, further analysis was performed analysing the change in FACT-Cog score between each baseline visit and the following post-supplementation visit in an attempt to limit the confounding effect of any change in each participant's cognitive function through the study. This demonstrated no significant difference in the FACT-Cog score between baseline and post-PL supplementation (Δ5 points, SD 9.5, CI 3.7, p = 0.41). It also showed no significant difference between the baseline pre-BR and the post-BR supplementation visits' FACT-Cog score (Δ3 points, SD 7.3, CI 2.8, p = 0.41).

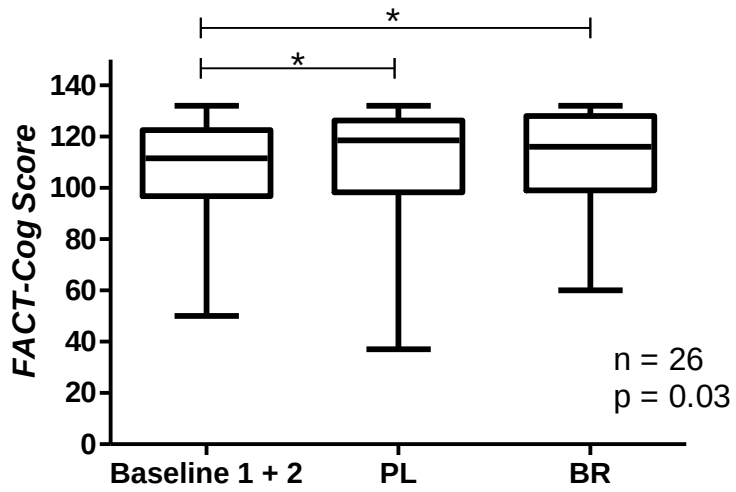


Figure 17: FACT-Cog cognitive function scores at each experimental condition. All 26 participants were asked to complete a Functional Assessment of Cancer Therapy - Cognition (FACT-Cog) questionnaire to objectively quantify their cognitive function over the seven days leading up to each of their 4 experimental visits. The experimental condition 'Baseline 1 + 2' indicates all FACT-Cog scores of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visits following a period of supplementaion with either nitrate-depleted beetroot juice (PL) or nitrate-rich beetroot juice (BR). This box and whisker plot shows the median, interquartile ranges and extreme values of the FACT-Cog scores recorded by the trial participants. This demonstrates the presence of some extreme outliers whose self-reported cognitive function was markedly impaired by their health problem, although this was consistent for those participants across each of the experimental visits. It showed no clinically significant difference in any of the experimental conditions, although statistical significance was reached (1 way ANOVA p value = 0.03). Fisher's LSD test confirmed statistically significant differences between the

conditions Baseline 1 + 2 and PL (0.021), and between Baseline 1 + 2 and BR (0.013).

3.6 CYCLE ERGOMETRY

Individuals enrolled in this study had a striking variability in physical characteristics, ranging from relatively frail nonagenarians to quite athletic individuals in their early 40s. While anyone with significant cardiac or respiratory co-morbidities were excluded from the study, all participants were carefully observed during their cycle ergometry testing by a trained physician. All participants were strongly advised to cease exercise if they had any adverse symptoms such as dizziness or chest discomfort, if they reached 80% of their age-predicted maximum heart rate, if their oxygen saturations fell below 92% and if they experienced the following: If they felt they were breathing heavily; If they felt the exercise was challenging or; If they felt that maintaining a steady pedalling rate was uncomfortably fast.

Most participants managed this well. However, the eldest person began to experience slight dizziness with no associated symptoms or deterioration in heart rate or pulse oximetry during the 3 minutes warm-up phase during which he pedalled without resistance. He was immediately advised to cease and his symptoms resolved within a few seconds, but this episode precluded him from further participation in the cycle ergometry aspect of this study. Hence, 25 of the 26 study

participants (96%) who attended all 4 investigational visits completed the cycle ergometry testing on each visit.

Twenty-four of the 25 participants (96%) who were able to complete the cycle ergometry stopped the test themselves because they were experiencing one of the above symptoms. Only one participant (4%) was asked to cease the protocol on each of his experimental visits by the researcher as he had reached 80% of his age-predicted maximum heart rate.

3.6.1 GAS EXCHANGE THRESHOLD

Oxygen saturations and methaemoglobin quantitation were observed during each cycle ergometry test. No participants developed methaemoglobinaemia.

Pulmonary oxygen uptake and carbon dioxide output were recorded breath-by-breath throughout a ramp incremental exercise test in order to determine the gas exchange threshold (GET).

Data obtained was normal in distribution. The mean work rate at which GET was reached at baseline study visits was 51 Watts (W) (SD 12 W), while this work rate was 2 W higher (53 W, SD 11 W) after PL supplementation ($p = 0.042$, CI -3.933 to -0.077). Those same participants reached their GET at a lower work rate by 1 W after BR supplementation (50 W, SD 12 W) compared to the mean of their baseline visits (p value not statistically significant at 0.34) while a comparison of the two supplemented experimental conditions reveals that GET after PL was 3 W higher than following BR supplementation ($p = 0.014$, CI 0.71 to 5.73) (Figure 18). There

was marked variation between the GET amongst participants within the current study, ranging from 27 W to 78 W.

Further analysis was performed comparing the individual change in GET between each participant's pre- and post-supplementation visits rather than comparing BR and PL with the mean of the two baseline visits. This demonstrated no significant change in GET at either PL; the cohort mean GET at this post-supplementation visit was 1 W higher than the baseline immediately prior to it (SD 6.6, CI 2.6, p = 0.96). Similarly, there was no significant change between the post-BR supplementation visit and the baseline visit beforehand; the cohort mean GET at BR was 1W higher than the baseline it followed (SD 6.1, CI 2.4, p = 0.96).

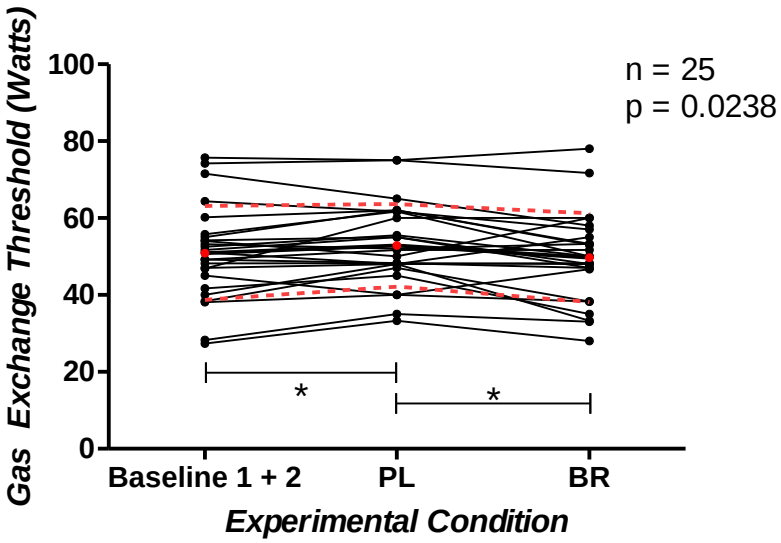


Figure 18: Work rate (Watts) at which gas exchange threshold is reached at each experimental condition. 25 participants underwent a sub-maximal incremental exercise physiology test on an electronically braked cycle ergometer at each experimental visit. Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This graph demonstrates each participant's individual GET (black symbols and lines), the mean GET at each experimental condition (red

symbols and solid line) and the standard deviation at each condition (red dotted line). GET was reached at 51 W work rate in the baseline 1 + 2 experimental condition, but rose to 53 W after PL supplementation (t test p = 0.042). There was no statistical difference between GET work rate at baseline and BR. One way ANOVA analysis of all groups p = 0.0238, while post-hoc LSD test shows statistically significant difference between conditions Baseline 1 + 2 and PL (0.042) and between PL and BR (0.014) but not between Baseline 1 + 2 and BR.

3.6.2 $VO_{2\ PEAK}$ AND PEAK POWER DURING A SYMPTOMS-LIMITED INCREMENTAL EXERCISE TEST

Breath by breath oxygen uptake (VO_2) was displayed digitally during cycle ergometry on a real-time basis after automated correction of concentration and volume signals of each breath. On completion of the study, analysis of this raw data was performed to determine the participants' $VO_{2\ peak}$, the maximum rate of oxygen uptake measured during this incremental exercise. As the participants were given restrictions preventing maximal exercise to ensure none came to harm, the maximum measured VO_2 was technically not VO_{2max}. Twenty-five of the 26 patients who completed all four experimental visits were included in this aspect of the analysis. The only exclusion was the elderly gentleman who was precluded from cycle ergometry testing as outlined above.

The mean observed $VO_{2\ peak}$ across the entire cohort of participants at all experimental visits was 1209 ml/kg/min (SD 459 ml/min/kg). This standard deviation demonstrates the large range in exercise capability amongst this cohort of people, and represents the marked variation in individuals' age, co-morbidities, haemoglobin and concurrent treatment. The mean $VO_{2\ peak}$ was 1170 ml/kg/min at the baseline

experimental visits (SD 375 ml/kg/min) (Figure 19). There was a trend for this to be higher at the post-PL experimental visit, with an observed increase to 1230 ml/kg/min which was not quite statistically significant (p = 0.062, CI -123 ml/kg/min to 3.16 ml/kg/min). There was no difference in the mean $VO_{2\ peak}$ between baselines and at the post-BR supplementation visit (1153 ml/kg/min, p = 0.43). When comparing the post-supplementation visits, the $VO_{2\ peak}$ following PL supplementation tended to be higher than that recorded following BR supplementation (mean 78 ml/kg/min, p = 0.09, CI -13 ml/kg/min to 168 ml/kg/min).

Further analysis of this data assessing the change in $VO_{2\ peak}$ between each of the individual baselines with the post-supplementation visits showed no significant difference between baseline and PL (Δ8 ml/kg/min, SD 139, CI 55, p 0.54) or between baseline and BR (Δ27 ml/kg/min, SD 71, CI 28, p 0.54).

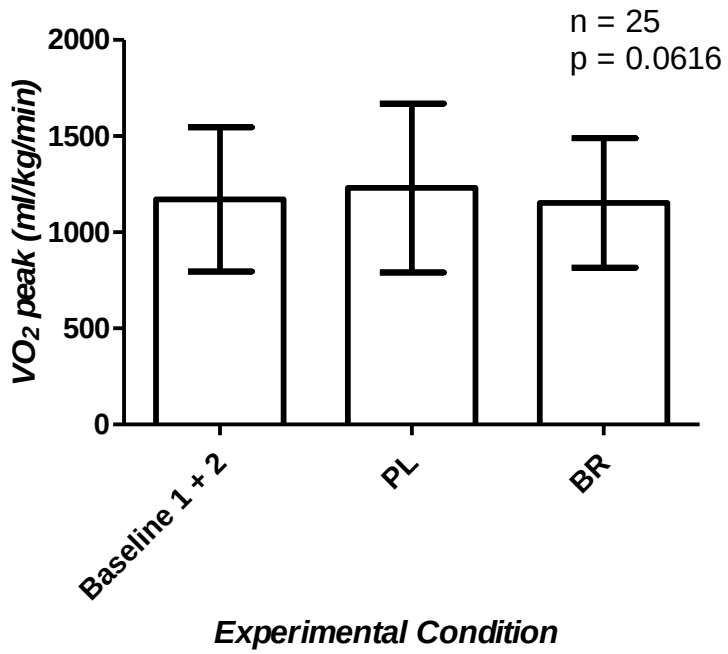


Figure 19: Change in $VO_{2\ peak}$ (ml/kg/min) observed with each experimental condition. This bar graph shows the means and standard deviations of maximal

oxygen consumption ($VO_{2\ peak}$) of 25 participants whilst performing a sub-maximal incremental electronically braked cycle ergometry test at each of their experimental visits. Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent acute supplementation prior to the 'PL' and 'BR' experimental conditions. This bar graph demonstrates no statistically significant difference in observed $VO_{2\ peak}$ at any of the experimental visits. There was a trend for increased $VO_{2\ peak}$ between baseline (1170 ml/kg/min) and PL experimental visits (1230 ml/kg/min) although this was not significant ($p = 0.06$). Similarly, $VO_{2\ peak}$ following BR supplementation tended to be slightly lower (1153 ml/kg/min) than PL but this too was did not reach statistical significance ($p = 0.09$). This trend towards differences between all of the means is confirmed through a 1 way ANOVA analysis ($p = 0.0616$).

The 25 participants who completed cycle ergometry exercise testing at each of their 4 experimental visits maintained the same pedalling cadence while performing a linear work rate increase of 10 W / minute which continued to intolerance. The ergometer imposed a fixed work rate increase through electronic flywheel braking, enabling the peak power generated to be calculated following completion of the exercise test.

The mean peak power recorded amongst all participants at all experimental visits was 112 W (standard deviation 44 W). The variability in physical fitness of participants was again demonstrated by this measure of maximal exercise capacity, with peak powers of those able to perform the test ranging between 59 W and 236 W (Figure 20).

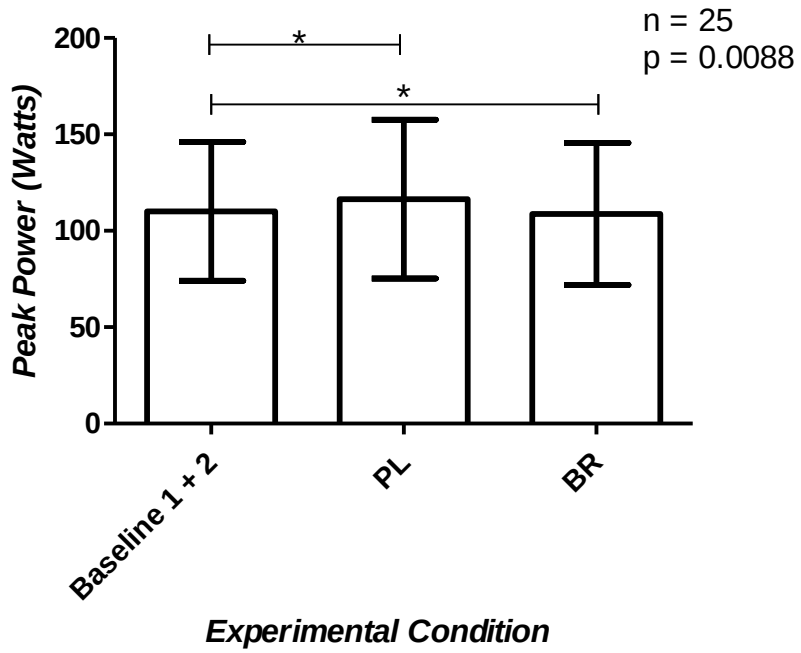


Figure 20: Peak power observed at each experimental condition. This bar chart shows the mean and standard deviation of maximal power output in Watts achieved by 25 participants whilst performing a symptoms-limited incremental electronically braked cycle ergometry test at each of their experimental visits. Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent 48 hours of beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This graph demonstrates a statistically significant increase in peak power at the experimental visits following PL supplementation (116 W) compared to both baseline (peak power = 110 W, t test p = 0.014) and 'BR' (peak power = 109 W, t test p = 0.027). A one way ANOVA calculation showed the difference between data sets was statistically significant (p = 0.0088) while LSD test concluded that comparison of PL with both baseline and BR was significant (*). The error bars signify SD, demonstrating a broad range between peak power particularly in the post-PL experimental visit.

The mean peak power recorded across the baseline visits was 110 W (SD 36 W). This increased to 116 W (SD 41 W) at the post-PL supplementation experimental visit (t test p = 0.014, CI -11.2 W to 1.41 W). There was no statistically significant

difference when comparing the peak power after BR supplementation (109 W, SD 37 W) with the mean of the peak power at baseline (p = 0.449). However, comparison of peak power between the post-intervention trial visits showed a statistically significant improvement in performance at the post-PL visit over the post-BR supplementation of 8 W (p = 0.027, CI 0.96 W to 14.29 W). Hence, there was a small but statistically significant improvement in peak power following supplementation with nitrate-depleted beetroot juice (PL) over both baseline and post-BR study visits.

There was no significant change in the peak power between participants' pre-PL baseline and post-PL supplementation visits (Δ4 W, SD 10.5, CI 4.1, p = 0.27) or between their pre-BR baseline and post-BR supplemented visits (Δ1 W, SD 8.2, CI 3.2, p = 0.27).

Time elapsed prior to the intolerance of exercise during a cycle ergometry linear work rate increase was recorded as the time limit of exercise tolerance (T_{lim}). As demonstrated by all of the other physiological measures in the cycle ergometry testing, there was marked variation of the T_{lim} between participants and also between each individual's study visits. The population mean T_{lim} was 560 seconds (s) (SD 258 s) with a maximum of 1532 s and minimum of 160 s (Figure 21).

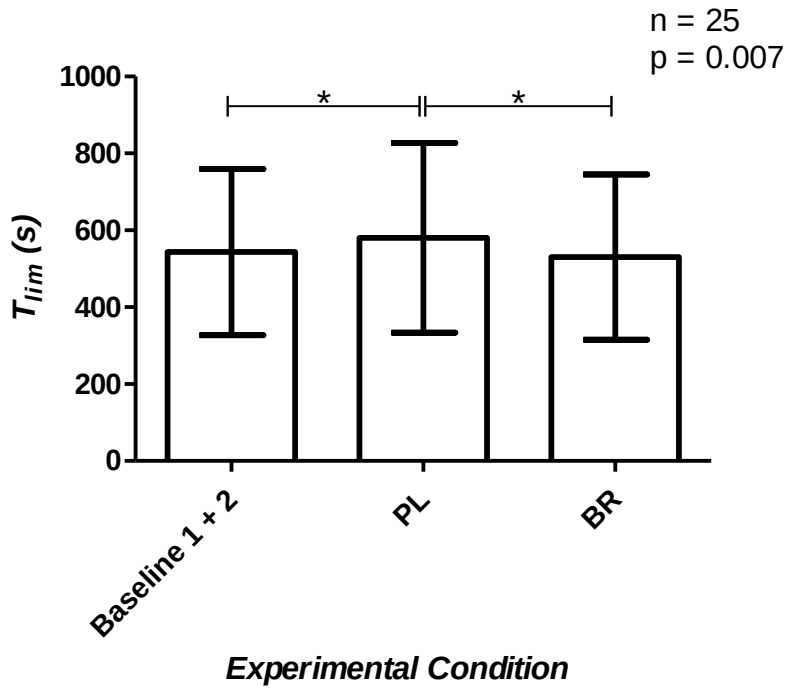


Figure 21: Time to limit of exercise tolerance (T_{lim}) of cycle ergometry testing. This bar chart shows the mean and standard deviation of the T_{lim} of 25 participants who were performing a symptoms-limited incremental electronically braked cycle ergometry test. Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent 48 hours of beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This graph demonstrates that the mean T_{lim} of participants at the 'PL' visit (580 seconds) was higher than the same participants' mean T_{lim} at both the baseline (T_{lim} = 543 s, p = 0.013) and 'BR' experimental conditions (T_{lim} = 530 s, p = 0.023). One way ANOVA calculation shows statistical significance (p = 0.007) while LSD test confirms statistically significant differences between the baseline 1 + 2 and PL (0.013), and between PL and BR supplemented visits (0.023), but not between baseline 1 + 2 and BR.

Supplementation with PL was associated with a statistically significant improvement in T_{lim} over the mean of the baseline visits of 37 s (p = 0.013*, CI = -65.99 to -8.42). There was no difference in T_{lim} following BR supplementation and baseline visits (p = 0.252). Comparison of the two post-supplementation visits demonstrates a 50 s

difference in T_{lim} (580 s compared to 530 s) following PL supplementation compared to BR (p = 0.023*, CI = 7.666 to 92.494).

When looking at the data in a different manner by comparing the change in T_{lim} between the pre-PL baseline and the post-PL visits, this apparent change becomes statistically not significant (Δ 24 s, SD 62 s, CI 24 s, p = 0.14). Similarly, there is no significant change in T_{lim} between pre-BR baseline and post-BR supplementation (Δ 1 s, SD 41 s, CI 16 s, p = 0.14).

Hence there was a physiologically and statistically significant improvement in T_{lim} following PL supplementation compared to both the mean of the non-supplemented visits and with the post-BR supplemented visits. This difference was not demonstrated when comparing the change in T_{lim} between individual baselines and supplemented visits. However, the observed difference in haemoglobin concentration between PL and BR experimental conditions (see section 3.2) was positively correlated with the difference in T_{lim} between the same conditions (r = 0.64, p – 0.0006).

3.7 ^{31}PHOSPHOROUS (^{31}P) MAGNETIC RESONANCE SPECTROSCOPY

^{31}Phosphorous magnetic resonance spectroscopy (^{31}P-MRS) was performed at each experimental visit by 24 of the 26 participants (92%). The two people who did not complete this aspect of the testing were excluded by the investigator. One participant had previously undergone spinal decompression / reconstruction surgery and a significant length of her spine was supported by a titanium framework of rods and

screws. She was therefore excluded as the quantity of titanium in her body was felt to preclude her from this aspect of the study, despite the fact the risk was probably negligible given titanium's non-ferrous nature. The second participant was unable to perform this aspect of testing as he had an age-related marked increase in thoracic kyphosis. As a result, he was unable to lie supine in the scanner without significant discomfort regarding his head position, and a sensation of breathlessness. Hence, he was advised to not perform this part of the testing. All other participants tolerated the procedure without adverse effect.

Participants repeatedly completed 24 seconds of exercise with a single leg knee extension ergometer whilst lying in the bore of the 1.5T superconducting magnet, followed by 3.5 minutes of recovery. Recorded ^{31}P-MRS results were used to determine the rate of phosophocreatine (PCr) recovery, while PCr concentration and pH of the quadriceps muscle was calculated at completion of exercise.

3.7.1 RATE OF PHOSPHOCREATINE RECOVERY

There was no difference in the rate of phosphocreatine (PCr) recovery amongst any of the trial conditions. The time constant (τ) rate of PCr recovery amongst all 24 participants who completed the scans was 42 seconds (s) (SD 9) (Figure 22).

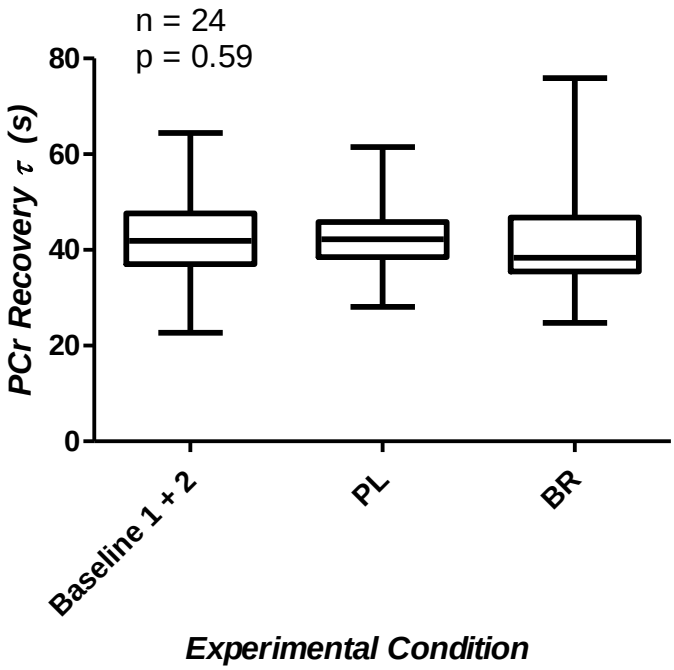


Figure 22: The time constant of phosphocreatine (PCr) recovery in each experimental condition. 24 participants completed an exercise protocol involving single-legged knee extensions while the quadriceps muscle of their right leg was analysed during ^{31}Phosphorous magnetic resonance spectroscopy (^{31}P-MRS) inside the bore of a 1.5T superconducting magnetic resonance scanner (see methods section 2.8.6). Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent 48 hours of beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This box and whisker plot displays the medians, interquartile range and extremes of observed PCr recovery rate amongst all participants at each experimental visit. It shows that there was no statistically significant difference amongst the medians of any of the experimental conditions (one way ANOVA p = 0.59).

At baseline visits, the median PCr time constant was 42 seconds (SD 10, CI 38.05 – 46.33). At the post-BR experimental visit, the median PCr time constant was 38 seconds (SD 10, CI 36.5 – 45.24). Following supplementation with PL, median PCr recovery was 42 s (SD 7, CI 39.27 – 45.55), which was not different from baseline (p

= 0.43, CI -3.04 – 2.60). Comparing the two post-supplementation experimental visits, there was no significant difference between PCr recovery τ following BR supplementation (38 s) and PL supplementation (42 s). A one way ANOVA analysis of all experimental visits confirmed no statistically significant difference between experimental conditions (p = 0.59).

Further interrogation of the data demonstrated no significant change in the PCr time constant between participants' pre-PL baseline and post-PL supplementation visits (Δ0 s, SD 6, CI 2.42, p = 0.21) or between their pre-BR baseline and post-BR supplemented visits (Δ-3 s, SD 9, CI 3.78, p = 0.21).

3.7.2 END-EXERCISE PH

There was no significant difference in calculated pH of the quadriceps muscle at termination of the knee-extension exercise protocol between any of the experimental conditions (Figure 23). One participant had an extremely outlying pH of 6.70 at one of their baseline visits, with pH between 7.12 and 7.16 at each of their other visits. This much more acidotic pH was observed in the absence of any symptoms of cramp or marked fatigue during the single leg ergometry suggesting this is a spurious result. Hence, it has been excluded from all analysis bar the graph in Figure 23**Figure 23**.

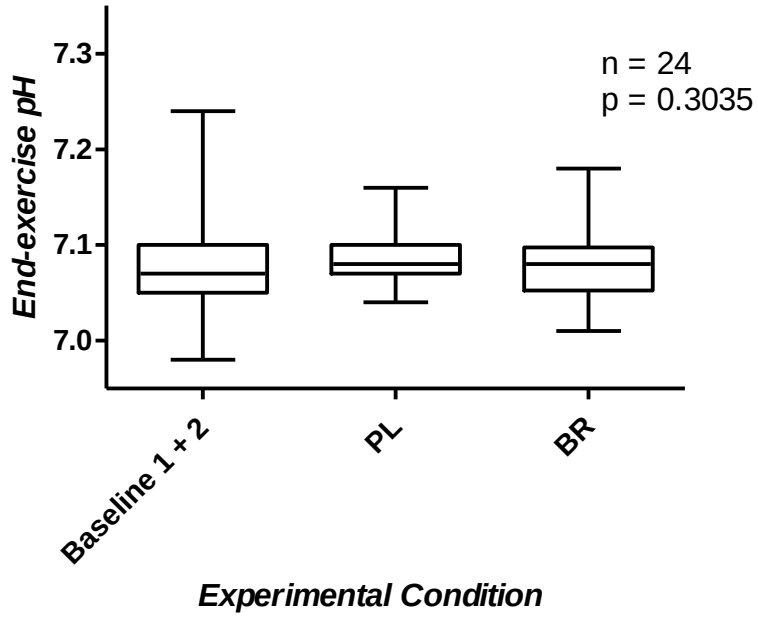


Figure 23: Quadriceps muscle pH at the termination of exercise regime during ^{31}phosphorous magnetic resonance spectroscopy (^{31}P-MRS). Magnetic resonance spectroscopy was performed while 24 participants underwent a low intensity exercise protocol involving single leg knee extension against resistance inside the bore of a 1.5T superconducting magnetic resonance scanner (see '2.7.6 ^{31}Phosphorous - Magnetic Resonance Spectroscopy (^{31}P-MRS)'). Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent 48 hours of beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This box and whisker plot demonstrates the medians, interquartile ranges and extremes of end-exercise pH amongst the 24 participants at each experimental visit. It demonstrates that there was no statistically significant difference in end-exercise pH between any of the experimental conditions (one way ANOVA p = 0.3035), and that two extreme outliers were recorded amongst baseline visits.

The median pH of all the other baseline experimental visits was pH 7.08 (SD 0.041, 95% CI 7.07 – 7.09). Following supplementation with PL, end-exercise pH was 7.09 (SD 0.03, 95% CI 7.07 – 7.10). Following supplementation with BR, end-exercise pH was lower than each of the other experimental conditions at pH 7.08 (SD 0.04, 95% CI 7.06 – 7.09). Further analysis of the data demonstrated no significant change in

the pH between participants' pre-PL baseline and post-PL supplementation visits (Δ0 pH, SD 0.05, CI 0.052, p = 0.01) or between their pre-BR baseline and post-BR supplemented visits (Δ0 pH, SD 0.03, CI 0.01, p = 0.01).

Hence supplementation with beetroot juice made no difference to pH (one way ANOVA p = 0.3035).

3.8 PLATELET AGGREGOMETRY

Fourteen of the 26 participants (54%) were excluded from the platelet aggregometry aspect of this study at their screening visit as they were taking aspirin, clopidogrel, non-steroidal anti-inflammatory drugs, warfarin or another anticoagulant. Only three participants (11.5%) were able to undergo platelet aggregometry testing on each of their four experimental visits. The remainder were unable to proceed with this test on one or more of their visits for a number of reasons. Some participants were excluded from this aspect of the study as they ingested an antiplatelet, anticoagulant or non-steroidal anti-inflammatory drug within a week before the experimental visit. Most of these took over the counter aspirin or ibuprofen for pain such as tension headache, having forgotten that such an act would preclude them from this aspect of the study. Others were excluded because their platelet rich plasma (PRP) failed quality control because of insufficient platelet count (fewer than 50 x 10^9/l). In all such cases, this was always associated with a marked reduction in numbers of other blood cells as a result of the effect their underlying disease or chemotherapy was exerting on their bone marrow function at that time. Thirdly, a number of participants were excluded

on at least one of their experimental visits due to a lack of reagent tubes for use in the platelet aggregometer as a result of supply problems from the manufacturer.

Platelet aggregation testing measures the ability of various agonists to platelets to induce *in vitro* activation and platelet-to-platelet activation. Classically Born aggregometry uses platelet rich plasma [PRP] but whole blood aggregometry can be also used. In the Born aggregometer, PRP is rapidly stirred in a cuvette at 37°C and the cuvette is positioned in a well between a light source and a photocell (Born and Cross 1963). When an agonist is added the platelets initially change shape from discs to rounded forms with extended filopodia, resulting in a small, transient decrease in light transmission that is followed by an increase as they aggregate or stick together in a fibrinogen-dependent manner. Platelet aggregation reduces the degree of side scatter of light, increasing light transmission through the cuvette. This increase is detected by the photocell. A curve comparing light transmission with time elapsed is generated as seen in the sample from the second experimental visit of participant number 26 below (Figure 24).

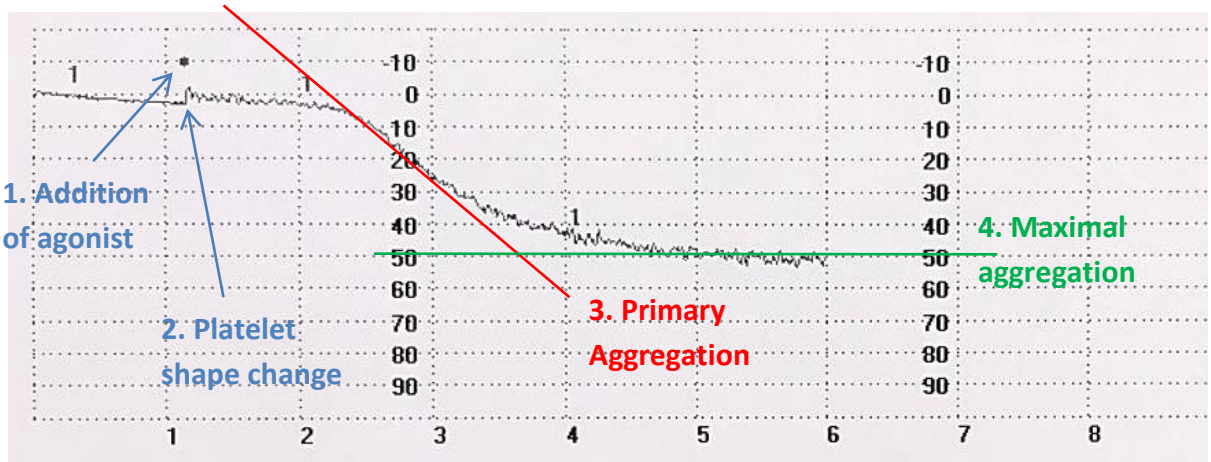


Figure 24: Platelet aggregometry trace from participant number 26 in response to collagen. Venepuncture and the preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP) was performed as described in section 2.7.7. This figure

displays monophasic platelet aggregation of PRP from the second experimental visit of participant 26 in response to the addition of the platelet aggregation agonist, collagen. The x axis of this figure depicts time elapsed (minutes) while the y axis displays percentage of light transmission through the cuvette containing PRP and agonist within the platelet aggregometer. Collagen (agonist) is added to the PRP at blue arrow 1, which binds to the glycoprotein VI and glycoprotein Ia/IIa receptors on platelets inducing granule release. This results in a shape change in platelets within the sample resulting in a transient slight reduction in light transmission (blue arrow 2). After a lag phase of just less than a minute which is typical in platelet aggregometry agonised by collagen, platelets within the sample soon begin to aggregate, the rate of which is measured as the primary aggregation of the sample (red line), a measure of the platelets' ability to aggregate. The point at which maximal light transmission is reached is the maximal aggregation of the sample. This example demonstrates maximal aggregation at only 51% (green line). It is a monophasic curve, indicating that no secondary aggregation occurred, whereby platelets often release nucleotides such as adenosine diphosphate (ADP) and irreversibly change in shape producing a secondary phase of aggregation. The reason no secondary aggregation was observed in this sample could be that either there was an insufficient initial stimulus, or that the activated platelets were unable to make sufficient thromboxane A2 and thus could not sustain glycoprotein IIb/IIIa activation.

The majority of participants' samples were analysed simultaneously in 8 separate reaction chambers within the platelet aggregometer, assessing the platelet aggregation response of both participant and control platelet rich plasma to the addition of 4 different agonists, adenosine diphosphate, arachidonic acid, collagen, and epinephrine. The platelet aggregometer analysed the curve of optical density change over time, calculating the primary aggregation, primary slope, secondary aggregation, area under the curve, lag phase, final aggregation and disaggregation (Figure 25).

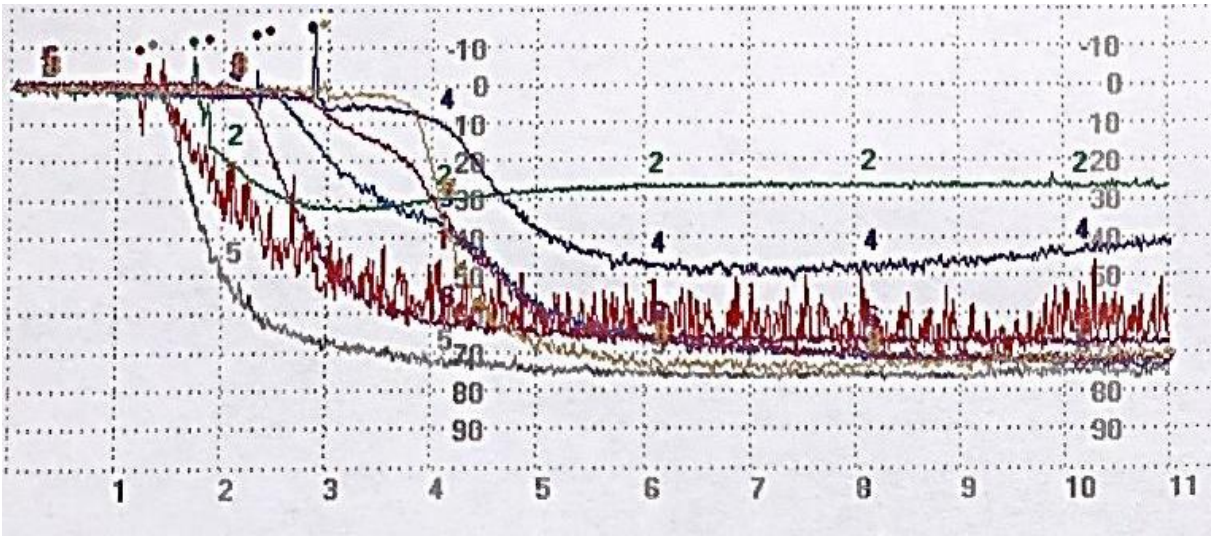


Figure 25: Platelet aggregometry trace for participant 12 experimental visit 2 and control in response to agonists. Platelet rich plasma was produced as outlined above from both participant and healthy volunteer control (methods section 2.7.7). Platelet aggregation was analysed in response to the addition of 4 agonists, adenosine diphosphate (ADP), arachidonic acid (AA), epinephrine and collagen. The resultant platelet shape change, aggregation and secondary aggregation produced an increase in light transmission as detected by the photo cell. Channels 1 to 4 are samples from participant 12 demonstrating aggregation upon the addition of each of the above agonists, while channels 5 – 8 display aggregation of control PRP with the same agonists.

In all but one of the participant and control sample analyses, platelets within the PRP aggregated successfully on the addition of agonists. However, samples from participant 12's first experimental visit did not aggregate in response to any agonists despite passing the PRP quality control check (Figure 26). The sample contained 68 x 10^9/l platelets (accepted if greater than 50 x 10^9/l, normal range 150 x 10^9/l – 400 x 10^9/l), a white cell count of 0.1 x 10^9/l (accepted if less than 0.5 x 10^9/l, normal range 3.8 – 10.6 x 10^9/l), and a red cell count of 0.01 x 10^{12}/l (accepted if less than 0.5 x 10^{12}/l, normal range 4.5 – 5.9 x 10^{12}/l). On visual inspection, it was evident that the magnetic stirring rods had failed to function, explaining the lack of aggregation;

platelets will only aggregate if they are activated by an agonist and they are in contact with one another (Born and Cross 1963). A repeat of the analysis was not complicated by the same equipment failure and aggregometry occurred as expected.

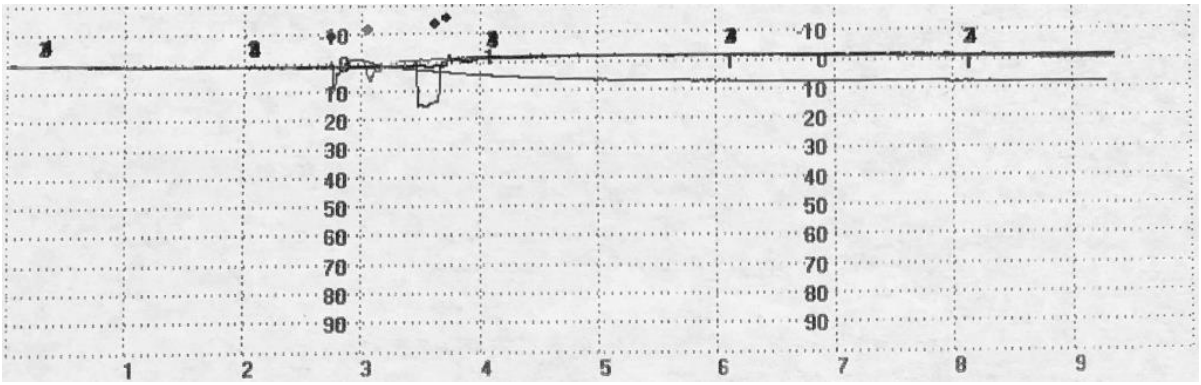


Figure 26: Failed platelet aggregometry of platelet rich plasma (PRP) samples from participant 12's first experimental visit. This participant's PRP was produced as described above (methods section 2.7.7) and passed quality control quantification of platelets (68 x 10^9/l), white cells (0.1 x 10^9/l) and red cells (0.01 x 10^{12}/l). However, the addition of the platelet aggregation agonists ADP, arachidonic acid, collagen and epinephrine induced no platelet aggregation. On visual inspection of the well containing PRP and agonist, it was apparent that the magnetic stirring rod had failed to function and the sample was therefore unable to aggregate. A repeat analysis brought about successful aggregation in all wells.

3.8.1 PLATELET AGGREGATION INDUCED BY ADP

Of those three participants in whom a complete set of platelet aggregometry data was available, there was no discernible difference between their aggregometry results at any of the experimental conditions (ANOVA p = 0.77). The mean primary slope of aggregation following the addition of ADP at the baseline experimental stage was 37.7 (SD 20.5, 95% CI 16.2 – 59.2). After supplementation with BR, this

mean primary slope of aggregation rose to 45.3 (SD 25.5, 95% CI -18.1 – 108.8) although this was not statistically significant (t test p = 0.70). Following PL supplementation, the primary slope of aggregation was 41.3 (SD 4.0, 95% CI 31.3 – 51.4), with no significant difference compared to baseline 1 + 2 (t test p = 0.34). Each of these mean values for the primary slope of aggregation following ADP addition was within the normal range quoted by the manufacturer of the PAP8 analyser in the analyser's handbook.

The median final platelet aggregation at 5 minutes after the addition of ADP was 65% at baseline, 73% following PL and 77% following BR. The comparison of each of these non-parametric data through Wilcoxon matched-pairs signed rank test showed no statistically significant difference between experimental conditions (p = 0.50 comparing baseline and PL, p = 1.0 comparing baseline and BR, and p = 0.75 comparing BR and PL), while ANOVA analysis gave p = 0.66 for all of the observations regarding final platelet aggregation (Figure 27).

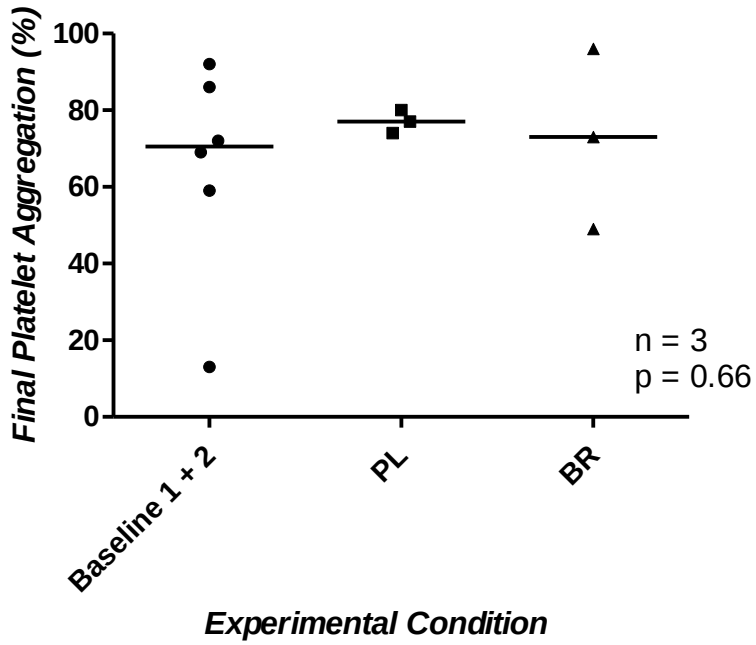


Figure 27: Final platelet aggregation following the addition of ADP. This scatter graph depicts the total percentage of platelet aggregation for platelet rich plasma samples from the three participants in this study who successfully completed aggregometry testing after each of their four experimental visits. Participant samples were analysed through light aggregometry of platelet rich plasma in response to the addition of the platelet agonist ADP (see section 2.7.7 for methods). This figure demonstrates that one participant's baseline experimental visit blood test did not successfully aggregate, while all other specimens aggregated to an acceptable degree (manufacturer's reference range 63 – 89%). The median final percentage aggregation (depicted by the line on the graph) was not statistically different between each experimental condition and was thus not affected by supplementation with nitrate-rich beetroot juice (BR) or nitrate-depleted placebo (PL) (ANOVA p = 0.66)

The manufacturer's quoted reference range for this total platelet aggregation was 63% – 89%, indicating that all median values were within the normal range, although three individual samples aggregated less fully than this level. The platelets of one participant did not aggregate well with any agonist at experimental visit 3 (non-

supplemented, baseline) without any obvious reason; they had taken no medications as listed above and the platelet rich plasma passed quality control standards.

Further analysis of all available data including those from participants who did not have results from each of their four experimental visits still demonstrated no significant difference in the primary aggregation or final aggregation of platelets in response to the addition of ADP. The primary aggregation at baseline was 73.4% (SD16, CI 8.4), PL was 83.7% (SD17.7, CI 10.9), and BR was 72.5 (SD 16.8, CI 9.9) with p values of 0.17 and 0.35 derived from T tests comparing each of the conditions, and an ANOVA comparing all supplementation states of $p = 0.34$. The final aggregation at baseline was 76.2% (SD 14.4, CI 7.5), PL was 83.5 % (SD 14.2, CI 8.8) and BR was 72.7 (SD 14.8, CI 8.7) with t-test p values of 0.36 and 0.11 and an ANOVA of $p = 0.27$. Hence no difference was observed between experimental conditions in platelet aggregation in response to ADP.

3.8.2 PLATELET AGGREGATION INDUCED BY ARACHIDONIC ACID

Following the addition of arachidonic acid to platelet rich plasma, platelets rapidly release alpha granules which initiate primary aggregation. While one participant's platelets did not aggregate well at one of their baseline (non-supplemented) experimental visits as described following ADP, the remaining participants' platelet function appeared to be in the normal range. The median rate of primary aggregation at baseline was 59, while it was 52 following BR supplementation although this did not represent a statistically significant difference ($p = 0.75$). Following PL

supplementation, the median rate of primary aggregation measured 71 but again, this was not statistically significant (baseline vs. BR t-test p = 0.45, BR vs. PL t-test p = 1.0) (Figure 28).

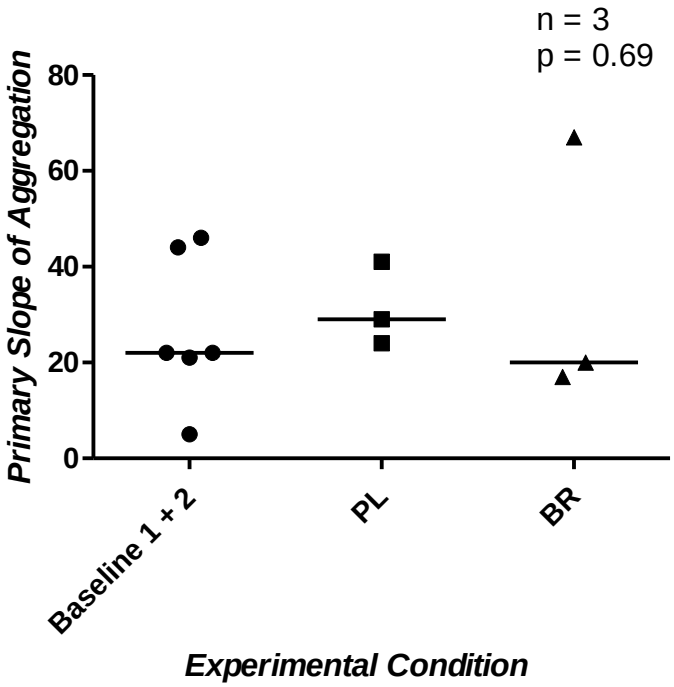


Figure 28: Primary slope of platelet aggregation following the addition of arachidonic acid to platelet rich plasma (PRP). This scatter graph depicts the primary slope of platelet aggregation for platelet rich plasma samples from the three participants in this study who successfully completed aggregometry testing after each of their four experimental visits. Participant samples were analysed through light aggregometry of platelet rich plasma in response to the addition of the platelet agonist arachidonic acid (see section 2.7.7). This figure demonstrates that PRP taken at one participant's experimental visit following supplementation with nitrate-rich beetroot juice (BR) was very rapidly, while the remainder of PRP aggregated more slowly in each experimental condition. One participant's PRP sample did not aggregate well at their baseline visit with a slow rate of aggregation, as also demonstrated in response to ADP (see Figure 27). The median primary slope of aggregation (depicted by the line on the graph) was not statistically different between

each experimental condition and was thus not affected by supplementation with nitrate-rich beetroot juice (BR) or nitrate-depleted placebo (PL) (ANOVA p = 0.69).

Total aggregation (not displayed in graph form) following the addition of arachidonic acid was within the normal range apart from one participant at one of their baseline visits. The mean total aggregation at baseline was 53.8% (SD 22.6), was 53.0% (SD 43.6) following supplementation with BR and 52.3% following PL supplementation (SD 24.2). There were no statistically significant differences between the baseline, PL or BR experimental conditions (p = 0.88).

Further analysis of all available data including those from participants who did not have results from each of their four experimental visits still demonstrated no significant difference in the primary aggregation or final aggregation of platelets in response to the addition of arachidonic acid. The primary aggregation at baseline was 63.79% (SD 10.93, CI 5.72), PL was 67.7% (SD 17.77, CI 11.01), and BR was 61.0% (SD 24.07, CI 14.23) with p values of 0.62 and 0.5 derived from T tests comparing each of the conditions, and an ANOVA comparing all supplementation states giving a p value of p = .075. The final aggregation at baseline was 61.71% (SD 11.83, CI 6.2), PL was 65.50 % (SD 16.69, CI 10.34) and BR was 58.67 (SD 25.72, CI 15.20) with t-test p values of 0.55 and 0.47 and an ANOVA of p = 0.74. Hence no difference was observed between experimental conditions in platelet aggregation in response to arachidonic acid.

3.9 BLOOD ANALYSIS: CYCLIC GUANOSINE MONOPHOSPHATE

Plasma cyclic guanosine monophosphate (cGMP) concentration was quantified in blood samples taken from all 26 participants who completed the entire study at each of their experimental visits. After centrifugation, plasma samples were stored at -80 °C pending analysis. During this analysis as outlined in methods section 2.8.7, cGMP was quantified in two large batches of all participant samples along with 5 standard samples of known cGMP concentrations per batch. All of the standard and participant samples were analysed in duplicate using a colourimetric competitive immunoassay kit. This resulted in a change in the optical density of the reactants within a plastic plate, which in turn was used to quantify the cGMP concentration of the sample. The results from standard samples were used to plot a standard curve (Figure 29).

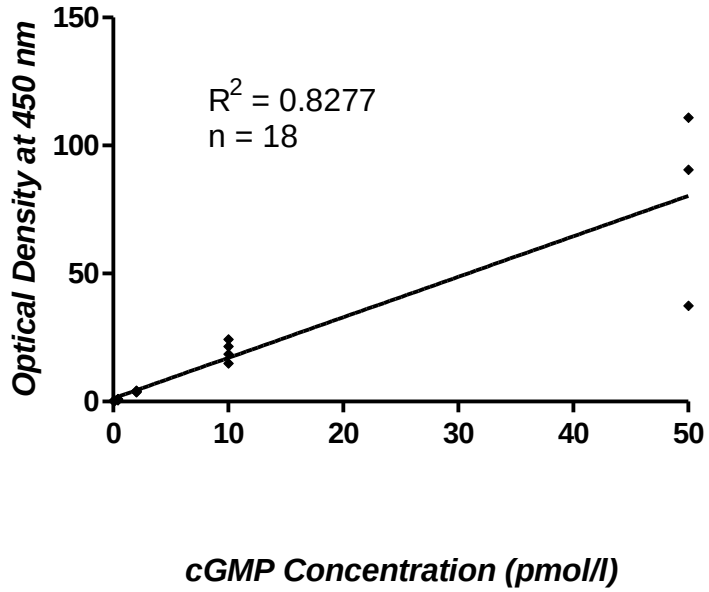


Figure 29: cGMP quantitation standard curve. This demonstrates the results from enzyme linked immunospecific assay of standard samples of known concentrations

of cGMP. Included are 5 standard samples each analysed in duplicate alongside two batches of participant plasma samples. The standards contained cGMP concentrations of 50 pmol/l, 10pmol/l, 2 pmol/l, 0.4 pmol/l and 0.08v pmol/l. The mean concentrations of all four analyses of the same standard samples were 50.38 pmol/l (SD 7.23 pmol/l), 9.92 pmol/l (SD 2.03 pmol/l), 2.01 pmol/l (SD 0.12 pmol/l), 0.41 pmol/l (SD 0.1 pmol/l) and 0.15 pmol/l (SD 0.12 pmol/l) respectively. The coefficient of variation for the assay was 29.1 % if all standard concentrations were included in the analysis, although if the least accurate higher concentration (50 pmol/l) sample results were excluded from this analysis, the CV of this assay was 16.7%. While the accuracy of the assay was thus demonstrated to be poor at the highest standard concentration of cGMP, none of the participants' cGMP concentrations reached 10 pmol/l and therefore the loss of accuracy at far higher concentrations was not felt to have a detrimental effect on the validity of data generated from participant samples.

The mean plasma cGMP amongst all samples was 2.15 µmol/l (SD 1.41 µmol /l). There was no difference in cGMP between baseline or after supplementation with either BR or PL (Figure 30) (ANOVA p = 0.36). The mean cGMP concentration at baseline visit was 2.24 µmol/l (SD 1.17 µmol/l, 95% CI 1.77 – 2.71). There was a trend towards a greater cGMP concentration after BR (2.51 µmol/l, SD 1.17 µmol/l) compared to baseline (p = 0.079). Mean cGMP after supplementation with PL measured 2.48 µmol/l (SD 1.65 µmol/l, 95% CI 1.82 – 3.15) but the difference between PL and baseline was not statistically insignificant (p = 0.32). There was no difference in mean cGMP between BR and PL supplementation (p = 0.93).

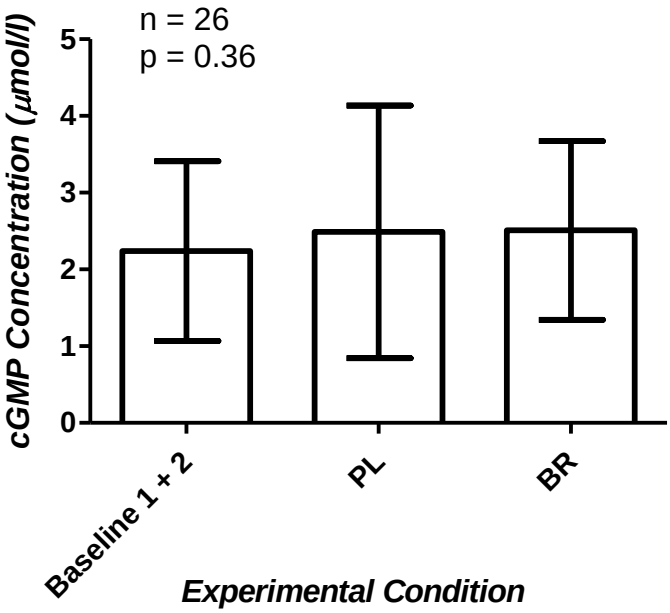


Figure 30: Plasma cGMP concentration at each experimental visit. This bar chart demonstrates the mean and standard deviations of measured concentrations of cGMP amongst 26 participants' plasma samples. Baseline 1 + 2 refers to the two non-supplemented visits, while participants underwent 48 hours of beetroot juice supplementation prior to the 'PL' and 'BR' experimental conditions. This demonstrates that there was no statistically significant difference between the mean cGMP plasma concentration amongst any of the experimental conditions, although there was a trend to suggest an increase from baseline (mean cGMP = 2.15 µmol/l) to BR (mean cGMP = 2.51 µmol/l, p = 0.079) and PL (mean cGMP = 2.48 µmol/l, p = 0.32). ANOVA p = 0.36.

3.10: BLOOD PRESSURE

At each experimental visit, after all 26 participants had undergone blood tests and completed quality of life questionnaires, following 10 minutes of seated rest in a

quiet, darkened room, baseline blood pressure of the brachial artery (systolic and diastolic) was measured three times and the mean of the readings was recorded.

3.10.1: SYSTOLIC BLOOD PRESSURE

The mean systolic blood pressure of all 26 participants at their baseline (non-supplemented) experimental visits was 122 mmHg (SD 15 mmHg, CI 116 – 128 mmHg). After acute supplementation with nitrate-depleted beetroot juice (PL), the mean systolic blood pressure was 116 mmHg (SD 15 mmHg, CI 110 – 124 mmHg), while systolic blood pressure was 117 mmHg following 48 hours of supplementation with nitrate-rich beetroot juice (BR) (SD 15, CI 112 – 123 mmHg) (Figure 31). A one way repeated measures ANOVA of these data demonstrates statistical significance ($p = 0.0081$), while post hoc Fisher's LSD test confirms a statistically significant difference between baseline and PL ($p = 0.005$) and between baseline and BR ($p = 0.011$) but no difference between PL and BR.

Further interrogation of this data analysing the change in systolic blood pressure between the baseline visit prior to PL and the post-PL supplemented visits demonstrates no statistically significant difference (Δ -6 mmHg, SD 11, CI 4) while comparison of pre and post BR supplementation visits similarly showed no statistically significant difference in systolic blood pressure (Δ -4 mmHg, SD 9, CI 4) with a t-test p value of 0.44 (this data is not depicted in the figure below).

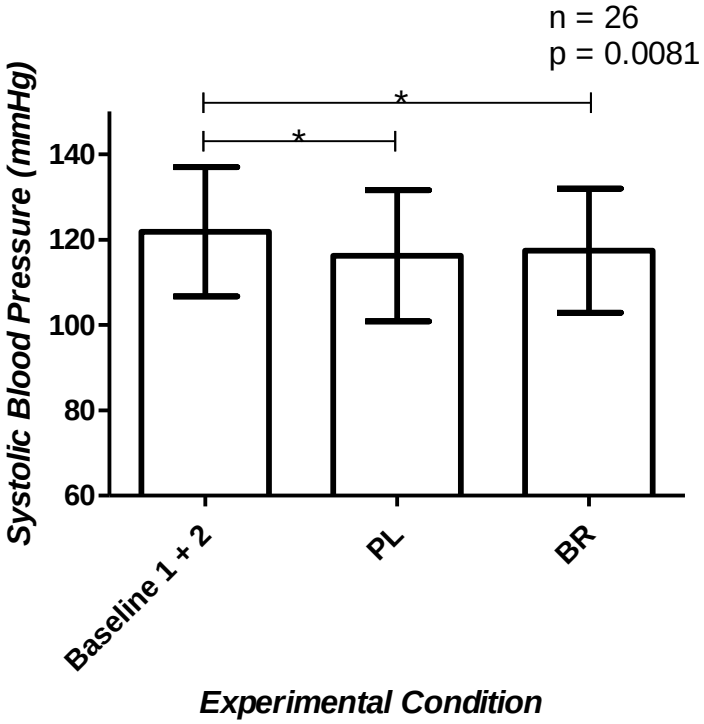


Figure 31: Systolic blood pressure at each experimental condition. This bar chart demonstrates the mean and standard deviations of the observed systolic blood pressures of 26 participants at each of their experimental conditions. The experimental condition 'Baseline 1 + 2' indicates all systolic blood pressures of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visits following a period of supplementation. This graph demonstrates that the mean systolic blood pressure was higher at the baseline visits (122 mmHg, SD 15 mmHg, CI 116 – 128 mmHg) than at each of the supplemented visits (PL = 116 mmHg, SD 15 mmHg, CI 110 – 124 mmHg; BR = 117 mmHg, SD 15 mmHg, CI 112 – 123 mmHg) (p = 0.0081). LSD shows statistical significant difference between Baseline and PL (p = 0.005) and between baseline and BR (p = 0.011).

3.10.2 DIASTOLIC BLOOD PRESSURE

The mean diastolic blood pressure of all 26 participants at their baseline (non-supplemented) experimental visits was 68 mmHg (SD 5 mmHg, CI 66 - 70 mmHg).

After acute supplementation with nitrate-depleted beetroot juice (PL), the mean systolic blood pressure was 66 mmHg (SD 6 mmHg, CI 64 – 69 mmHg), while systolic blood pressure was 65 mmHg following 48 hours of supplementation with nitrate-rich beetroot juice (BR) (SD 5 mmHg, CI 63 - 67 mmHg) (Figure 32). A one way repeated measures ANOVA of these data demonstrates a trend towards statistical significance ($p = 0.0535$). Comparison of diastolic blood pressures using a students' repeated measures t test between baseline and PL also showed a trend towards significance ($p = 0.0897$), while there was a statistically significant difference between baseline and BR ($p = 0.0067$). There was no difference between BR and PL.

Further interrogation of this data analysing the change in diastolic blood pressure between the baseline visit prior to PL and the post-PL supplemented visits demonstrates no statistically significant difference (Δ -1 mmHg, SD 5, CI 2) while comparison of pre and post BR supplementation visits similarly showed no statistically significant difference in diastolic blood pressure (Δ -1 mmHg, SD 5, CI 2) with a t-test p value of 0.86.

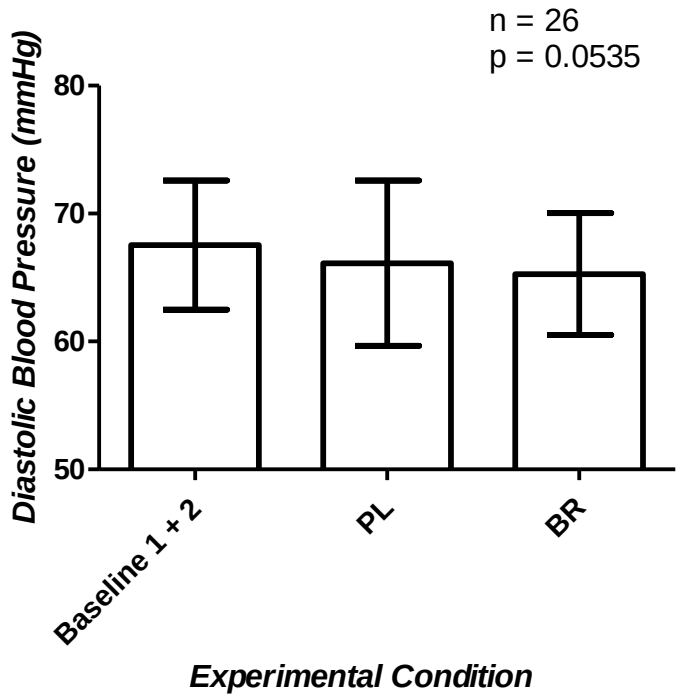


Figure 32: Diastolic blood pressure at each experimental condition. This bar chart demonstrates the mean and standard deviations of the observed diastolic blood pressures of 26 participants at each of their experimental conditions. The experimental conditions are described in **Figure 31** above. This graph demonstrates a trend towards statistically significant differences in the mean diastolic blood pressure between each experimental visit; this was 68 mmHg at the baseline visits (SD 5 mmHg, CI 66 – 70 mmHg), 66 mmHg at PL (SD 6 mmHg, CI 64 - 69 mmHg) and 65 mmHg at BR (SD 5 mmHg, CI 63 - 67 mmHg) (ANOVA p = 0.0535). Paired students' t test comparisons showed a statistically significant difference between baseline and BR (p = 0.0067) and a trend towards statistically significant difference between baseline and PL (p = 0.0897) but no change between BR and PL.

3.11 BLOOD ANALYSIS: FERRITIN AND IRON STUDIES

Serum ferritin, iron, transferrin and transferrin saturation were quantified in all 26 participants who completed each of the experimental visits, although 7 of the 104

samples (6.7%) were automatically not processed by the Royal Devon and Exeter Hospital biochemistry laboratory as they breached the hospital policy that this test could not be repeated on the same person before 28 days had elapsed since the previous sample.

The mean ferritin concentration across all participants' visits was 358 µg/l (normal range 30 – 200 µg/l). The batch to batch CV was up to 2.8%. No participants were iron deficient with a ferritin beneath this range, while a number of participants had a ferritin far in excess of this range (Figure 33). Serum transferrin and transferrin saturation on those patients with elevated ferritin concentration demonstrated no evidence of iron overload, indicating that the elevation in ferritin was probably caused by an acute phase response or liver disease, both of which were most likely to have been caused by their underlying malignancy. Of note, any patients exhibiting symptoms of acute illness such as infection, or whose baseline observations such as temperature were indicative of such an illness at the beginning of their experimental visit would have been excluded from that day of experiments. No such events occurred for any of the participants during any of their visits.

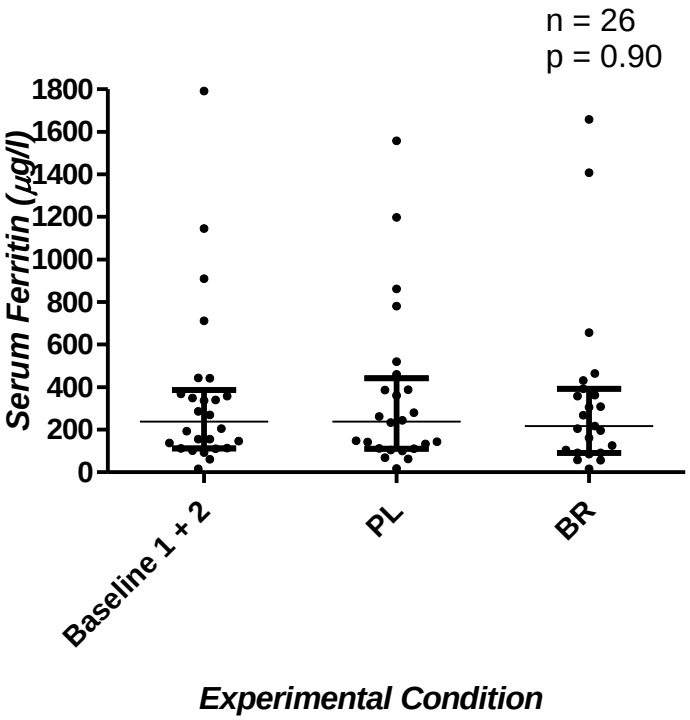


Figure 33: Serum ferritin of participant blood samples from each experimental visit. This scatter graph demonstrates serum ferritin concentrations from all 26 participants' plasma samples from venepuncture at each of their four experimental visits, along with the median and the interquartile range of results obtained after each experimental condition. The experimental conditions and ferritin analysis are described in **Figure 30** and in methods section 2.7.7 respectively. This graph demonstrates that the median of all participants' ferritin at each experimental condition was above the normal range (i.e. greater than 200 µg/l), and a small number of participants had vastly elevated ferritin concentrations. There was no significant difference between the experimental conditions (ANOVA p = 0.90)

Supplementation with either BR or PL had no significant effect on serum ferritin compared to either baseline or the other supplemented state (p = 0.68, 0.82 and 0.73 respectively), while ANOVA analysis of the entire data set showed no significant difference (p = 0.90).

The mean of participants' serum iron concentration was within the normal range at each experimental condition, measuring 16.0 µmol/l (95% CI 12.2 – 19.8 µmol/l, SD 7.85 µmol/l) at their baseline visits, 19.1 µmol/l (95% CI 14.4 µmol/l to 23.7 µmol/l , SD 9.6 µmol/l) at the visit following 48 hours of oral supplementation with nitrate-rich beetroot juice (BR) and 21.2 µmol/l (95% CI 16.6 – 25.8 µmol/l , SD 9.6 µmol/l) at the experimental visit which followed 48 hours of ingestion of oral nitrate-depleted beetroot juice (PL) (Figure 34). The laboratory quoted a normal reference range of 10.0 – 30.0 µmol/l according to the 95% confidence intervals of those members of the local population of approximately 500000 patients who had undergone this test, while the batch to batch CV was up to 2.6%. There was no statistically significant difference when comparing the serum iron of participants at baseline and post-BR supplementation visits ($p = 0.22$) or when comparing PL with BR ($p = 0.43$), making an ANOVA analysis not statistically significant ($p = 0.11$), although the increase between the mean serum iron at baseline and PL was significant ($p = 0.03$).

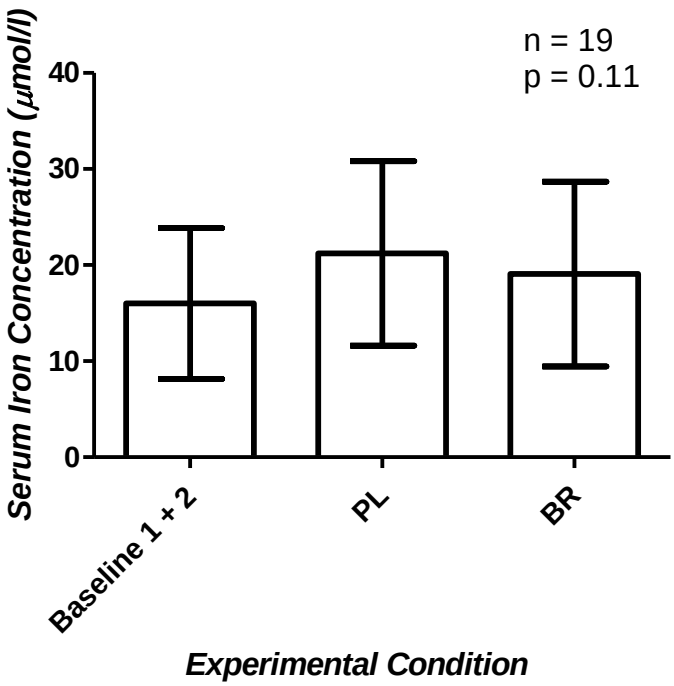


Figure 34: Serum iron concentration (µmol/l) of participant plasma at each experimental condition. This bar graph demonstrates the mean and standard deviations of serum iron concentration of the plasma samples from 19 participants. The experimental conditions and serum iron analysis are described in figure 29 and methods section 2.7.7 above. This demonstrates that there was a statistically significant increase in serum iron from 16.0 µmol/l (95% CI 12.2 – 19.8 µmol/l, SD 7.85 µmol/l) at their baseline visits to 21.2 µmol/l (95% CI 16.6 – 25.8 µmol/l, SD 9.6 µmol/l) following a 48 hour period of supplementation with nitrate-depleted beetroot juice (PL) (p = 0.03), although all means were within the normal range for serum iron. There was no difference between baseline and BR serum iron (p = 0.22). ANOVA analysis of the entire data set gave p = 0.11.

The mean transferrin concentration for all 18 participants from whom results were available from plasma collected at each of their four experimental visits was 30.5 µmol/l (95% CI 28.7 – 32.3 µmol/l, SD 6.5 µmol/l). The laboratory quoted a normal reference range of 25.2 – 45.4 µmol/l according to the 95% confidence intervals of those members of the local population of approximately 500000 patients who had

undergone this test. The batch to batch CV of this assay was up to 1.8%. There was no significant difference between any of the experimental conditions indicating that a 48 hour period of supplementation with either nitrate-rich or nitrate-depleted beetroot juice did not affect this variable (p values of 0.84, 0.28 and 0.48 respectively).

The mean transferrin saturation concentration for all 19 participants from whom results were available from plasma collected at each of their four experimental visits was 33.8% (95% CI 28.6 – 39.0%, SD 19.7%). There was also no significant difference between any of the experimental conditions (p values of 0.54, 0.12 and 0.36 respectively).

Those participants with the highest serum ferritin levels were also found to have the highest transferrin concentration and transferrin saturation. One participant was found to have a plasma ferritin concentration of between 1558 µg/l and 1791 µg/l, transferrin concentration of between 40.7 µmol/l and 47.2 µmol/l, transferrin saturation consistently measuring 84%, and a serum iron concentration of between 34 and 38 µmol/l. All of the above indicate that, while this participant had metastatic oesophageal carcinoma with liver metastases which probably exaggerated the increase in ferritin and transferrin through their role as acute phase proteins which rise in cases of liver disease, he was also overloaded with iron presumably as a result of recurrent blood transfusions during chemotherapy.

3.12 RESULTS SUMMARY

The results of this pilot study investigating the effect of oral supplementation with inorganic nitrate in people with anaemia demonstrated that the majority of patients with anaemia who were approached for trial entry were keen to enrol. Of those, only a small minority did not satisfy the inclusion and exclusion criteria of this study, although one participant was excluded from the cycle ergometry testing and two were excluded from the magnetic resonance spectroscopy analysis. Retention of participants was very good, with 93% completing all four experimental visits.

The study was not powered to prove or disprove the null hypotheses stated. As expected, it demonstrated that clinical factors within this unstable and clinically varied population of patients can occur such as fluctuations in haemoglobin concentration. Thus information obtained will be used to inform power calculations when planning a larger study in the future.

The current study also found that the enterosalivary circulation of nitrate was intact even in this population of participants with medical conditions such as gastrointestinal cancers, on chemotherapy, using mouthwashes and antibiotics, with previous histories of significant gastrointestinal surgery.

CHAPTER 4: DISCUSSION

This pilot study is the first to successfully investigate the effects of supplementation with dietary inorganic nitrate in people with anaemia. While there is a wealth of research regarding the effect of supplementation in both health (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Lansley, Winyard et al. 2011, Kelly, Fulford et al. 2013, Larsen, Schiffer et al. 2014) and disease (Bryan, Calvert et al. 2007, Allen, Giordano et al. 2012, Baliga, Milsom et al. 2012, Bond, Curry et al. 2013, Gilchrist, Winyard et al. 2014), no previous research has investigated the effect in this an anaemic population. This pilot study investigated the following experimental hypotheses which were drawn through extrapolation of the existing literature in this field of research. Relative to a nitrate-depleted placebo beetroot juice, this study hypothesised that dietary supplementation of anaemic patients with nitrate-rich beetroot juice would affect:

- Thrombogenicity
- Muscle phosphocreatine recovery rate
- Exercise tolerance
- Cognitive function
- Quality of life

Thus, this pilot study intended to explore whether people with anaemia could derive significant clinical and physiological benefit from such an intervention.

4.1 ACHIEVEMENT OF KEY OBJECTIVES

This study was easy to recruit for, successfully achieving its key objectives and hence, reflecting excellent patient motivation. When approached to offer recruitment, most of the eligible population were motivated and keen for some empowerment in their control of symptoms and treatment of their disease. Part of their enthusiasm to enter the study was borne out of the goodwill they felt towards the recruiting departments, as many pointed out that they had received excellent care there and, therefore, wanted to 'give something back.' The exact percentage of those patients approached to consider trial entry who consented to screening is not known, as people were told of the trial in a number of different departments in the hospital by several different healthcare professionals, not all of whom kept a clear record of this activity. The small number of patients screened for this study who then withdrew their consent prior to any experimental visits did so with very good reasons. Two participants were diagnosed with progressive malignancy with poor prognosis between their consent for trial entry and their first experimental visit, and they quickly became too unwell to attend. One patient knew his life expectancy was sadly only a few months at the time of enrolment but having consented for trial entry, changed his mind as he did not want to commit his time to this study. A further participant withdrew his consent after developing pneumonia with rib fractures, and felt too unwell to proceed. One participant was excluded by the principal investigator of a national trial which she was already enrolled in, who declined to give consent for her enrolment. Hence 28 of the 33 patients screened for study entry were enrolled to participate (84.8%).

Two participants decided to withdraw from the trial after two of their four experimental visits; one did so because he was on intensive chemotherapy and felt too physically and psychologically exhausted to continue with this study, and another did so because his wife had become unwell and he was her main carer.

Thus, the study recruited very well amongst those people who were eligible for enrolment. Retention of participants who began the trial was excellent, with a low drop-out rate (7% of those enrolled) which was beyond the participants' control as a result of ill health or external factors unrelated to this study.

4.2 THE BIOCHEMICAL EFFECT OF NITRATE SUPPLEMENTATION

This was a pilot study intended to assess feasibility of a future, larger investigation into the efficacy of inorganic nitrate supplementation on the physiology and symptomatology of people with anaemia. Hence, a relatively small population was recruited containing individuals who were variably anaemic, suffering from a wide range of medical conditions contributing to their anaemia, had a broad range of co-morbid medical conditions, and who were undergoing vastly differing treatments for their medical complaints.

One of the key objectives of this study was to ascertain whether the nitrate-nitrite-$^{\cdot}$NO enterosalivary pathway is intact in people with anaemia related to haematological and oncological conditions and their treatments. Through this pathway, oral nitrate supplementation in healthy individuals can increase the bioavailability of $^{\cdot}$NO, hence influencing mitochondrial respiration, vasodilation,

muscle contractile efficiency and force, exercise efficiency, platelet aggregation, cerebral blood flow and neurotransmission.

4.2.1 NITRATE

This study demonstrated that the mean plasma nitrate of all individuals rose from 8.4 µM at the non-supplemented baseline visit and 8.5 µM at the experimental visit following supplementation with PL, to 78.2 µM following BR supplementation. Hence, this study demonstrated that inorganic dietary nitrate supplementation was sufficiently bioavailable to overcome some significant potential hurdles such as previous major upper gastrointestinal surgery to treat oesophageal and gastric malignancies, concurrent use of a variety of chemotherapies and the use of other medications such as isosorbide mononitrate.

The mean of the observed plasma nitrate at baseline visits and following supplementation with PL was 8.5 µM, significantly lower than that reported in healthy individuals by previous studies (19.7 µM, (Moshage, Kok et al. 1995), 35.9 µM (Suzuki, Yagi et al. 2001), 32.7 µM (Dykhuizen, Masson et al. 1996)). One further study (Vanhatalo, Blackwell et al. 2018) found that the mean plasma nitrate concentration in young (18 – 22 year old) and older (70 – 80) healthy adults was 28 µM in each group. This research was conducted in the same laboratory at the University of Exeter, using the same methodology for nitrate quantification as the present study. Hence, this confirms that the low nitrate concentration found in this

cohort of participants was not due to the difference of methodology between laboratories.

A number of possible factors may have contributed to this difference. Firstly, while Moshage *et al* did not stipulate the demographics of the study population who provided their blood samples for analysis, one would assume that participants were working or studying in the Department of Medicine at the University of Groningen, the Netherlands, and therefore, that they were younger than the participants in the present study who ranged from 40 – 93 years old. Similarly, the control group in the study by Suzuki et al. were aged 33.1 years old on average, while Dykhuizen did not report the age of their control volunteers. Increasing age is associated with greater likelihood of gastrointestinal (Newton 2004) and vascular disease (Savji, Rockman et al. 2013), increased use of concurrent medications (Kaufman, Kelly et al. 2002), and therefore absorption of dietary nitrate and the function of the nitrate-nitrite-˙NO enterosalivary pathway could be affected. For example, gastrointestinal disease such as gastro-oesophageal reflux or peptic ulcer disease is very commonly treated with proton pump inhibitors which increase the pH of the stomach. This may in turn affect the generation of reactive nitrogen species from swallowed nitrite, which is normally potentiated in the acidic environment of the stomach. Vascular disease may include atherosclerosis, the accumulation of plasma-derived lipoproteins within the arterial intima (Stary, Chandler et al. 1994). ˙NO metabolism is affected by this disease, as the atherosclerotic plaques are associated with reduced endothelial nitric oxide synthase activity (Khambata, Ghosh et al. 2017), thus reducing the bioavailability of ˙NO and potentially affecting plasma nitrate concentrations. Ischaemic heart disease may be treated with organic nitrates which are used to vasodilate the coronary arteries, thus reducing myocardial ischaemia and angina

symptoms. However, the haemodynamic and anti-anginal effects of organic nitrates are rapidly lost upon long-term low-dose administration of this medication due to the rapid development of tolerance and of endothelial dysfunction, which in most cases is linked with oxidative stress (Daiber and Münzel 2015). Hence, use of this drug may have ameliorated the response of that participant to inorganic nitrate supplementation.

Secondly, all but one of the anaemic participants in the present study were of white British ethnic origin, many of whom had origins in the South West of England, while those enrolled in previous studies were presumably Dutch (Moshage, Kok et al. 1995), Japanese (Suzuki, Yagi et al. 2001), and Glaswegian (Dykhuizen, Masson et al. 1996). There is no published evidence comparing the normal plasma nitrate concentration between ethnic groups but differing population habits regarding tobacco consumption, alcohol and diet should be considered as possible contributory factors.

Thirdly, concurrent use of medications in this study could have affected the absorption and metabolism of participants' habitually ingested dietary inorganic nitrate. For example, one patient was taking isosorbide mononitrate in order to control angina; this may have had a bearing on his response to dietary inorganic supplementation as outlined at the top of this page (Daiber and Münzel 2015). Fourthly, three participants in this study (11.5%) had active malignancy in their gastrointestinal tract. The risk of developing gastric or colorectal carcinoma is increased by some lifestyle factors such as tobacco consumption and a diet lacking in vegetables (Danaei, Vander Hoorn et al. 2005), meaning that those participants' lifelong dietary habits may have contained few vegetables and could have

contributed to the development of their malignancies. Vegetables are the source of 60 – 80% of dietary nitrate in a typical Western diet (Lundberg, Weitzberg et al. 2004) and therefore, the presence of gastrointestinal malignancy may explain their reduced baseline plasma nitrate concentration compared to that of healthy individuals, and could reflect a diet poor in vegetables. However, it is important to note that the above points are speculative causes for the low concentration of plasma nitrate observed at baseline and post-supplementation with nitrate-depleted beetroot juice in the current study, and that this thesis presents no definite evidence to prove or disprove them.

Despite the above factors and the relatively low plasma nitrate concentration at baseline and post-PL supplementation in comparison to healthy cohorts, 96% of the participants in this study derived a marked increase in their plasma nitrate following supplementation with nitrate-rich BR; the mean plasma nitrate for participants in this study at the post-BR supplementation visit was 78.0 µM. Hence, despite some variability in the degree of this response between individuals, this cohort of anaemic people absorbed a significant quantity of the ingested nitrate within their BR supplementation, with an increase in the mean plasma concentration by 664%. While their baseline nitrate concentration was lower than that of healthy individuals (Moshage, Kok et al. 1995, Dykhuizen, Masson et al. 1996, Suzuki, Yagi et al. 2001, Vanhatalo, Blackwell et al. 2018), the change from pre- to post-supplementation was comparable to that of healthy people. This would suggest that the absorption of ingested nitrate amongst participants in this trial was efficient, meaning that their low baseline plasma nitrate concentration was not due to impaired absorption but to some other factor as outlined above, including dietary habits, malignancy, co-morbidities, chemotherapy and concurrent medications.

The only participant who did not develop an increase in nitrate concentration took over 10 different herbal remedies and lived on an extremely strict diet which only consisted of juiced fruit and vegetables. While the precise content of the herbal remedies is unclear, her diet was extremely rich in nitrate prior to and during her involvement with this trial. The mean of this participant's two unsupplemented baseline visits and her post-PL visit was 18.1 μM, higher than all other participants, although this was only 0.1 μM greater than the second highest patient. This nitrate-rich diet may have influenced absorption of nitrate in the beetroot juice she received in the trial intervention, although this seems unlikely given its ability to rapidly penetrate the gastric wall. Distribution of the nitrate was have been altered; if potential molecules such as DNICs or thiols were already saturated with NO, the impact of ingestion of more nitrate may have been affected in this patient. In this case, her constant exposure to high levels of nitrate may have caused downregulation of the nitrate-nitrite-˙NO enterosalivary pathway and of absorption of ingested nitrate in the small bowel, attenuating her response in terms of resultant increase in plasma nitrate concentration after further doses of nitrate. Interestingly, tachyphylaxis, an acute decrease in response to a drug after its administration, has been demonstrated in human responses to organic nitrates such as nitroglycerine and other nitrovasodilators (Cohen and Kirk 1973), which require a drug-free interval to maintain their efficacy when applied transdermally. The same loss of effect has also been observed in chronic ingestion of organic nitrate via decreased bioactivation, increased ROS production and endothelial dysfunction (Omar, Artime et al. 2012). However, there is no evidence to suggest that inorganic nitrate induces tolerance build-up in the same way as organic nitrate, with studies examining

efficacy of such supplementation over a period of up to six months (Kapil, Khambata et al. 2015, Velmurugan, Gan et al. 2016, Faconti, Mills et al. 2019).

Another possible reason for this participant's lack of response in plasma nitrate concentration is her medical history; she had oesophageal carcinoma and had undergone a total oesophagectomy. While in healthy individuals, nitrate is rapidly and completely absorbed in the proximal jejunum, this lady's previous surgery may have affected this process through a number of possible mechanisms. Firstly, the total oesophagectomy may have reduced the transit time of orally ingested substances, meaning the dietary nitrate supplementation may have reached and passed through the jejunum sooner and faster than other participants, limiting her ability to absorb it. An alternative explanation is that her known oesophageal carcinoma could have metastasised to involve her jejunum or ileum unbeknown to her and the research team. If so, this may have also brought about a partial bowel obstruction and an alteration in her ability to absorb ingested nitrate.

Another variable which may have influenced this participant's plasma nitrate concentration is that she was also undergoing chemotherapy treatment with Epirubicin, Carboplatin and Capecitabine chemotherapy. While other participants were being treated with these drugs as single agents or in combination with different chemotherapeutic agents, no one else was taking this combination. This chemotherapy regime has been associated with mucositis, or chemotherapy-induced damage of the mucosal membranes (Rothermundt, Hubner et al. 2006), this participant did not complain of diarrhoea and therefore one must assume her small bowel function was not significantly hindered by this chemotherapy regimen and was able, therefore, to absorb nitrate.

Another possible explanation for this participant's attenuated response to BR supplementation is poor compliance. However, it was felt that this is a very unlikely explanation; this lady had been informed by her medical team that she had a terminal malignancy and was willing to try anything which might help, including the 'juicing' diet consisting only of fruit and vegetables and her numerous herbal remedies. She was extremely motivated, actively sought me out to request trial recruitment, and gave no indication that she was finding the supplement unpalatable or not consuming it as directed.

4.2.2 NITRITE

This study has shown that oral dietary supplementation with beetroot juice containing inorganic nitrate brings about a marked rise in plasma nitrite, proving that the nitrate-nitrite-˙NO enterosalivary circulation was functioning in the majority of individuals included in this trial. It also, therefore, suggests that the oral microbiome involved in nitrate metabolism was similar to that of a normal healthy person, and included facultative anaerobic bacterial genera such as *Actinomyces* and *Veillonella* that used NO_3^- from the BR supplementation as a terminal electron acceptor and produced NO_2^- as a by-product. This was somewhat unexpected as many of the participants in this study were using bactericidal mouthwash such as chlorhexidine digluconate (Corsodyl®), while others were treated with antibiotics either prophylactically or in response to infection. Mouthwash and antibiotics may have attenuated the quantity of oral bacteria capable of involvement in this crucial reaction in the enterosalivary

circulation as previously reported (McDonagh, Wylie et al. 2015, Pinheiro, Amaral et al. 2015).

Similarly, 3 participants had active infections between their experimental visits. While they were treated with antibiotics which may have reduced the number of commensal oral bacteria and impaired the function of the nitrate-nitrite-˙NO enterosalivary circulation, such infections may have increased the bioavailability of ˙NO within the vasculature through an increase in inducible nitric oxide synthase (iNOS) (Neilly, Copland et al. 1995). This response is demonstrated in patients with sepsis and neutrophilia, as iNOS within neutrophils produces ˙NO through the arginine-NO system. Hence, those participants in this trial with infections may have had increased ˙NO and NO_2^-, potentially affecting their response to dietary supplementation with inorganic nitrate or placebo.

Significant variation was noted in the degree of rise in plasma nitrite concentration within this cohort, with a standard deviation of the BR experimental condition of 1006 nM. For example, two participants' plasma NO_2^- concentration increased to over 3750 nM, in excess of 2000%, while at the other end of the spectrum, one participant's plasma NO_2^- concentration only increased by 155% to 142 nM. Interestingly, the latter person with a very modest response in plasma NO_2^- was the same participant in whom there was observed only a minor increase in plasma NO_3^- concentration following BR supplementation. Hence it would seem likely that, as there was only a minor rise in plasma NO_3^-, there was a minor increase in available substrate (NO_3^-) for the facultative anaerobic bacteria located on the base of her tongue, and therefore less NO_2^- was produced and absorbed into the blood.

Of the two participants in whom a very large response to BR supplementation was observed, one participant had a history of gastric carcinoma and, having previously undergone a total gastrectomy, was being treated with Epirubicin, Oxaliplatin and Capecitabine chemotherapy. Unlike the patient with a poor response to BR supplementation however, his diet consisted of relatively few vegetables and other foods rich in nitrate. One could speculate that the contrast between these two participants' responses despite similar chemotherapy regimens and cancers (both upper gastro-intestinal) could suggest that the biggest contributory factor in determining the depth of their response to BR supplementation was their usual dietary intake of NO_3^-. This may reflect variability in the individuals' pharmacokinetics around ingested inorganic nitrate. The lack of response observed in the patient who likely consumed huge quantities of dietary inorganic nitrate under normal circumstances may have been due reduced ability to absorb ingested nitrate, although this seems unlikely given its ability to rapidly penetrate the gastric wall. Distribution of the nitrate and nitrite may have been altered; if potential molecules such as DNICs or thiols were already saturated with NO, the impact of ingestion of more nitrate may have been affected in this patient. Similarly, an as-yet unknown homeostatic mechanism may exist providing negative feedback to limit the efficacy of the nitrate-nitrite-˙NO reduction pathway. Another possible explanation is the oral microbiome as outlined above; each of these participants was on active chemotherapy and was therefore advised to maintain good oral hygiene. They were prescribed mouthwash, some of which was antibacterial, by their oncology and haematology teams and, while this is known to attenuate the rise in NO_2^- following ingestion of NO_3^- (Govoni, Jansson et al. 2008), it was felt that, from an ethical perspective, it was not justified to not ask participants to omit such treatment given

the resultant increased risk of potentially life-threatening infections at this time when they were at significant risk of developing neutropaenic sepsis. In the author's personal experience, significant numbers of patients on chemotherapy do not heed advice to use mouthwashes; hence a potential difference in their mouthwash compliance might explain the variation in the response of these two participants' NO_2^- and NO_3^- plasma concentrations following dietary NO_3^- supplementation. However, this is made unlikely by the fact that both NO_2^- and NO_3^- were attenuated while only NO_2^- production through the nitrate-nitrite-$^{\bullet}NO$ enterosalivary circulation relies on the presence of the oral microbiome.

Another participant was demonstrated to have a high baseline plasma NO_2^- concentration of 375 nM at his post-PL supplementation visit. This gentleman had a history of ischaemic heart disease and had been prescribed oral isosorbide mononitrate to dilate his coronary arteries and reduce symptoms of angina pectoris. Despite this chronic supplementation, he still derived a good response to oral NO_3^- supplementation, with an increase of plasma NO_2^- concentration to 1733 nM at his post-BR experimental visit.

The mean plasma NO_2^- concentration of this cohort of 26 participants was 136 nmol/l when at their experimental visits which were either non-supplemented or had followed a 48 hour period of nitrate-depleted beetroot juice supplementation. Normal values of plasma NO_2^- reported amongst healthy individuals are comparable to this; a recent publication producing data from the same laboratory as the current study reported a median NO_2^- concentration of 232nmol/l. Other laboratories elsewhere in the world give similar results, with Bondonno et al. (Bondonno, Liu et al. 2014) describing unsupplemented plasma nitrite of 262 nmol/l in their study based in Perth,

Australia. This possible slight reduction in baseline and un-supplemented NO_2^- concentration amongst participants may well reflect the use of medications such as antibiotics and antimicrobial mouthwashes as outlined above. 50% of the participants in the present study were on chemotherapy during their period of trial activity. A common side effect of chemotherapy, particularly regimens which include corticosteroids, is gastritis and peptic ulceration. Hence, many of the participants in the current study were taking omeprazole or alternative proton pump inhibitor medications which reduce the risk of such side effects. However, omeprazole has been found to cause a large increase in the NO_2^- concentration of gastric secretions (Mowat, Carswell et al. 1998). Therefore one could speculate that this NO_2^-rich fluid may not be entirely re-absorbed during their gastrointestinal transit and could lead to a reduction in their plasma NO_2^- concentration. Similarly, esomeprazole, a similar proton pump inhibitor to omeprazole, has been demonstrated to blunt the blood pressure lowering effect of intravenous or oral sodium nitrite in healthy individuals despite an expected rise in those individuals' plasma nitrite concentration (Montenegro, Sundqvist et al. 2017). The mechanism for this change is not clear, but may demonstrate that the physiological effect of blood pressure lowering is dependent on bioactivation in the acidic stomach environment. S-nitroso groups (RSNOs) may be the link between the effect proton pump inhibitors exert on the bioactivity of ingested nitrates and nitrites. The formation and degradation of RSNOs is a dynamic process largely determined by the prevailing redox environment which would be significantly altered by the effect of proton pump inhibitors on gastric pH. RSNOs have bioactivities similar to those of ˙NO but with half-lives in the order of hours (Stamler, Jaraki et al. 1992).

An alternative explanation for this observed reduction in plasma NO_2^- compared to healthy individuals is that it may have been caused by some of the participants' underlying medical conditions; 2 participants had gastric cancer which had previously been treated with gastrectomy operations in which their stomachs had been removed in their entirety. This would potentially affect the absorption of swallowed NO_3^- and NO_2^- and could reduce baseline plasma concentrations as a result. A further participant had a history of colorectal carcinoma which had been treated with surgical removal of the entire length of their ascending and transverse colon, hence requiring an iliostomy. This external bag bypasses the patient's colon and / or small bowel and can potentially reduce reabsorption of substances such as NO_2^- from the intestinal lumen. However, as 98% of nitrate and nitrite reabsorption occurs in the small intestine, this is less likely to have exerted a significant effect on plasma nitrite.

In summary, almost all participants involved in this pilot study derived a good response to BR supplementation both in terms of an increase in their plasma NO_3^- and NO_2^- concentration, although there was marked variation in the degree of this response. This indicates that they were compliant with supplementation and absorbed NO_3^- effectively despite their active medical complaints, co-morbidities and treatments which in some cases included previous major gastrointestinal surgery. These factors which were expected to limit NO_3^- uptake and interfere with the nitrate-nitrite-$^{\bullet}NO$ reduction pathway, probably explain the large range in responses to this supplementation.

4.2.3 CYCLIC GUANOSINE MONOPHOSPHATE

The plasma cGMP concentration of participants in this trial was unaffected by supplementation with dietary inorganic nitrate. cGMP is formed by the action of guanylate cyclase on glycerine triphosphate. Presence of ˙NO stimulates the action of guanylate cyclase, hence increasing cGMP levels. While the nitrate and nitrite concentrations increased following BR supplementation, plasma cGMP did not alter to a statistically significant level. However, a student's paired t test of the observed increase in plasma cGMP concentration suggested a trend for an increase in cGMP following BR supplementation ($p = 0.079$). The small numbers of participants and between subject variability in responses to supplementation in this study are likely reasons that this level did not reach significance. An expanded investigation enrolling more participants is recommended to explore the cGMP response to nitrate supplementation in anaemic patients.

4.3 THE PHYSIOLOGICAL EFFECT OF NITRATE SUPPLEMENTATION

The mean systolic blood pressure of this cohort of participants was relatively low measuring 122 mmHg. While formal body mass index was not recorded during the current study, no participants appeared to be significantly overweight and a number had lost weight as a result of their malignancy itself, treatments they were undergoing such as surgery or chemotherapy, and lifestyle changes in order to improve their general health such as the 'juicing' diet as outlined above. The

observed physiological responses to nitrate supplementation during the current study gave unexpected results according to existing evidence. Firstly, blood pressure was anticipated to be lowest in participants at their experimental visit which had been preceded by acute supplementation with nitrate-rich beetroot juice, consistent with previous research in healthy individuals which reports this to be due to the nitrate component of the supplementation (Kelly, Fulford et al. 2013, Berry, Justus et al. 2015). The participants in this study did indeed have a statistically significant reduction in their systolic blood pressure following a 48 hour period of supplementation with nitrate-rich beetroot juice (BR) compared to baseline. However, this same systolic blood pressure reduction was also observed following ingestion of nitrate-depleted beetroot juice (PL) compared to the baseline visit.

There are a number of potential explanations for this observed effect. Firstly, each of the supplemented visits occurred 7 – 10 days after each of the baseline visits, whereas the baseline visits were either 4 – 6 weeks following their initial supplementation visit, or they were the first ever experimental visit. One could speculate that participants may have felt more anxious or excited prior to their baseline study visits, and may have been more relaxed a few days later in the supplemented experimental state. In susceptible people, emotional stress results in immediate stimulation of the sympathetic nervous system, with a vasomotor response that results in a high-output state and elevated blood pressure (Mustacchi 1990). Hence, the potentially heightened sympathetic activity at the baseline visits may have brought about an increase in systolic blood pressure in some individuals.

A further speculative explanation for the observed reduction in systolic blood pressure following supplementation with both PL and BR is that, rather than nitrate,

another component of the beetroot juice may have caused a reduction in blood pressure. Epidemiological evidence suggests that antioxidants and vitamin C found in beetroot juice may be therapeutic in lowering blood pressure, although interventional trials have had mixed results with these substances (Schiffrin 2010). These antioxidants include carotenoids, phenolic acids and flavonoids. Beetroot is also one of few vegetables which contain a group of highly bioactive pigments known as betalains, which appear to have anti-oxidant and anti-inflammatory capabilities in vitro and in animal models (Clifford, Howatson et al. 2015). Hence this component of beetroot may have influenced participants' blood pressure following acute juice supplementation. Folate deficient hypertensive rats have been found to have a raised blood pressure and increased oxidative stress compared to folate replete controls (Pravenec, Kožich et al. 2012). A number of the individuals in the current study had risk factors for folate deficiency such as previous gastrointestinal surgery, chemotherapy, and high cell-turnover malignancies. Hence those participants may have had a higher systolic blood pressure at baseline compared to placebo if they underwent supplementation with folate-rich beetroot juice. Similar epidemiological studies have demonstrated an inverse relationship between dietary iron uptake and blood pressure (Tzoulaki, Brown et al. 2008) so, as beetroot juice is rich in iron, this component may have led to a reduction in systolic blood pressure. However, given the short duration of supplementation in this current study (48 hours), this explanation is made unlikely.

Similar to the unexpected results findings regarding blood pressure response to supplementation, the hypothesised beneficial effect of inorganic nitrate supplementation (BR) on rate of muscle phosphocreatine (PCr) recovery and exercise tolerance was also not supported by results obtained. Contrary to

expectation, participants' cycle ergometry and magnetic resonance spectroscopy results were superior following supplementation with nitrate-deplete placebo (PL) rather than BR. A major confounding factor which may explain this unexpected finding is the significant variation in haemoglobin (Hb) levels between experimental conditions. The mean Hb concentration in participants attending for their post-PL experimental visit was 114.7 g/l while the same participant population at their post-BR appointment had a mean Hb of 109.1 g/l. Two participants had a change in their Hb between these visits by more than 30 g/l while another 3 had a change in excess of 12 g/l. Whatever the order of actual supplementation, all of these larger fluctuations in Hb were between a lower value at the post-BR visit and a higher value post-PL. Therefore, while the crossover study design was selected in an attempt to overcome such variables, chance appears to have skewed the level of anaemia between each of the post-supplementation visits. Given the plethora of symptoms and physiological disadvantages conveyed by anaemia, such an alteration in the level of Hb could have a significant effect on quality of life, exercise capacity, and muscular physiological function (Wright, Pearce et al. 2014). Hence, the change in haemoglobin is very likely to underlie practically all of the physiological differences between PL and BR.

4.3.1 CYCLE ERGOMETRY

The gas exchange threshold (GET) is a surrogate of the lactate threshold, which is defined as the first loss of linearity in the relationship between oxygen uptake (VO_2) and carbon dioxide output (VCO_2) during incremental exercise (Beaver, Wasserman

et al. 1986). The GET provides a key landmark of aerobic fitness, which is correlated with exercise tolerance in healthy and patient populations (Nishijima, Kondo et al. 2017). Participants who performed the cycle ergometry aspect of this study demonstrated a greater GET following supplementation with PL compared to BR. Similarly, VO_{2peak}, the peak rate of oxygen consumption, was expected to improve following nitrate supplementation whereas there was a statistically insignificant trend towards a deterioration in this measure following BR supplementation compared to PL. The third cycle ergometry parameter which was measured was peak power, which was also hypothesised to improve following BR supplementation compared to both PL and baseline visits. However, there was a statistically significant improvement in peak power following PL supplementation compared to BR, and a trend towards an improvement compared to the baseline (unsupplemented) experimental condition. The fourth and final measure of exercise physiology which was recorded during the cycle ergometry was time to exercise limit (T_{lim}). There was a significant improvement in T_{lim} following PL supplementation compared to both the non-supplemented and post-BR supplemented visits. Hence, across all exercise physiology variables, participants performed consistently better in the PL condition than in BR. As highlighted above, this is likely due to greater Hb concentration in PL than in BR, which would have enabled a greater O_2 delivery to active muscle during incremental exercise that would in turn result in elevated GET, VO_{2peak} and exercise tolerance. The critical effect of haemoglobin fluctuation in dictating all cycle ergometry variables is supported by the finding that the difference in Hb between PL and BR conditions was positively correlated with the difference in Tlim ($r = 0.64$, $P = 0.0006$). Previous literature would support this conclusion drawn from the current study; it is widely accepted that the delivery of oxygen to mitochondria is the principal

limiting factor to the VO_{2peak} measured during large muscle group exercise in humans (Wagner 1995). For example, voluntary donation of 450 ml of whole blood causing a ~5% reduction in the haemoglobin of healthy individuals leads to a significant reduction in the time to exhaustion and VO_{2peak} during severe intensity exercise (Burnley, Roberts et al. 2006). Achieving a similar degree of enhancement in haemoglobin concentration either through reinfusion of packed red blood cells or through exogenous administration of recombinant human erythropoietin leads to an improvement in VO_{2peak} and time to exhaustion during maximal intensity exercise (Spriet, Gledhill et al. 1986, Wilkerson, Rittweger et al. 2005). Reduction in haemoglobin leads to reduced oxygen carrying capacity of the circulating blood and thus the potential for muscle oxygen delivery.

˙NO plays an essential role in maintenance of endothelial function and vascular tone, hence controlling vasodilation, blood pressure and oxygen delivery to hypoxic tissue beds, whilst affording some protection against hypoxic ischaemia/reperfusion injury (Bryan, Calvert et al. 2007, Dezfulian, Raat et al. 2007, Granger and Kvietys 2015). It affects mitochondrial respiration, myocyte calcium handling and ATP turnover, hence contributing to muscle metabolic efficiency in the setting of resting metabolic rate, and in conditions of normoxia and hypoxia (Vanhatalo, Fulford et al. 2011, Larsen, Schiffer et al. 2014). It was therefore hypothesised and expected that dietary inorganic nitrate supplementation would improve GET amongst trial participants through the nitrate-nitrite-˙NO enterosalivary circulation and increased ˙NO bioavailability. Nitrate supplementation was also expected to reflect previously reported beneficial effect on peak power (Lansley, Winyard et al. 2011), T_{lim} and $VO_{2\,max}$ (Bailey, Winyard et al. 2009) through improvement in the delivery of oxygen to

muscle beds during exercise and in the efficiency of ATP generation within myocytes (Bailey, Fulford et al. 2010, Larsen, Schiffer et al. 2011).

There are a number of possible explanations for the discrepancy between data observed during this trial and previously published evidence suggesting that this intervention would convey a physiological advantage in these participants (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Lansley, Winyard et al. 2011). Nitrate supplementation in healthy individuals can modify mitochondrial respiration, vasodilation, muscle contractile efficiency and rate of force generation, platelet aggregation, cerebral blood flow and neurotransmission. However, any potential beneficial effect participants in this study might have derived from nitrate-rich beetroot juice supplementation over placebo could have been obscured by such a change in Hb between the two supplementation conditions. An alteration in Hb between experimental conditions may well have had a greater effect on cycle ergometry performance than any effect nitrate supplementation could have exerted. This likely explanation is supported by the fact that nitrate supplementation has no effect on VO_{2max} during incremental exercise in healthy individuals (Bailey, Winyard et al. 2009), yet there was a trend towards improvement in this measure following PL supplementation compared to the BR and baseline experimental conditions. An increase in plasma Hb concentration would contribute to an improvement in VO_{2max} or VO_{2peak} as a direct consequence of improved oxygen delivery to skeletal muscle, while it would also lead to an increase in peak power output through improved muscular efficiency and / or enhanced blood flow distribution in active muscle.

When considering the cause of this alteration in haemoglobin between experimental conditions, another potential explanation regarding this discrepancy between

subjects' exercise performance reported in previous literature and this study becomes clear. Many of the participants in this trial had cancer-related anaemia either because of bone marrow infiltration with malignancy, or because of chemotherapy side effects. When at their most anaemic, participants had a greater burden of disease, were recovering from more recent chemotherapy or had received a greater cumulative dose of chemotherapy. Active cancer causes fatigue through a variety of mechanisms including direct effect of the tumour, treatment side effects, co-morbid conditions such as malnutrition, thyroid dysfunction and infection. It can also cause fatigue by exacerbating comorbid symptoms like chronic pain, sleep disturbance, and through psychosocial factors (Wagner and Cella 2004). During more intensive chemotherapy, participants may have been more sedentary and experienced deconditioning and skeletal muscle-mass atrophy, resulting in deterioration in their performance during cycle ergometry following BR supplementation.

Some chemotherapy agents themselves can exert a direct cardiotoxic effect. These include anthracyclines such as Epirubicin which 4% of the participants were receiving, and cyclophosphamide which 8% of the study population were treated with. Following these drugs, cardiac output can be diminished and respiratory function can be compromised as a result (Keefe 2002).

Skeletal muscle function can be affected by the use of chemotherapy agents and by tumours themselves. A common side effect of malignancy is weight loss as a result of the release by tumours of inflammatory chemokines and cytokines such as prostaglandin E_2. This factor induces inflammation in muscle tissue and results in muscle wasting (Ardies 2002), and consequentially would have a detrimental effect

on exercise physiology performance when the tumour is more active. Furthermore, metabolically active molecules such as tumour necrosis factor (TNFα) released from tumour or mast cells can necrotise muscle membranes and thus impair excitation – contraction (E-C) coupling (Lucía, Earnest et al. 2003). E-C coupling is the mechanism by which the electrical discharge on the muscle fibre membrane brought about by nerve impulses initiates chemical events inside the fibre—release of intracellular calcium from the sarcoplasmic reticulum. This in turn signals for immediate contractile activity which is followed by calcium reuptake to initiate the relaxation process. Coupling (contraction) and uncoupling (relaxation) continuously operate during any type of exercise or physical activity. Despite common belief, lactate accumulation and lactic acidosis do not alter E-C coupling and thus are not major determinants of muscle fatigue. Other factors independent of lactic-acid build-up are more likely to alter E-C coupling and thus to cause fatigue in healthy people, such as accumulation inside working muscle fibres of inorganic phosphate due to an insufficient rate of ATP resynthesis or accumulation of reactive oxygen species derived from aerobic metabolism (Lucía, Earnest et al. 2003). This effect could explain, at least partly, the increase in muscle fatigue induced by TNFα, and could explain the reduction in T_{lim}, GET and peak power observed in cycle ergometry testing at the post-PL supplementation experimental visit if those patients' cancers were more active at that time.

Nineteen percent of the participants in this study were being treated with high doses of corticosteroids while a further 8% of people were receiving cyclophosphamide. Each of these agents can have adverse effects on the ultrastructure and function of skeletal muscles. These effects include a decline of myofibrillar mass, altered

aerobic metabolism (due to decreased mitochondrial volume or mitochondrial myopathy), or reduced capillarisation (Lucía, Earnest et al. 2003).

Hence those participants receiving chemotherapy at the time of increased anaemia, which coincided with BR supplementation, had a number of physiological changes which could account for this unexpected improvement in exercise physiology performance in the post-PL study visit rather than following inorganic nitrate supplementation.

One further potential source of anaemia-related confounding which was observed in participants in this trial was blood transfusions. Three participants received a blood transfusion within 2 months of any of their experimental visits. One received this blood 40 days prior to their first visit and hence 51 days before their second visit which was post-BR supplementation visit. One participant was transfused within two weeks of their post-PL supplementation visit while a further participant received a blood transfusion 5 days before their post-BR supplementation visit.

While the mean lifespan of human RBCs *in vivo* is 110-118 days (Mock, Matthews et al. 2011), transfused red blood cells survival is similar, with a maximum reported lifespan of 135 days after transfusion. A proportion of these cells are removed from the circulation within 24 hours of transfusion; this varies according to the duration of storage of the cells, and can be between 9% and 23% (Luten, Roerdinkholder-Stoelwinder et al. 2008). Hence, some of these transfused cells were still present in the first participant's circulation at the time of testing despite the amount of time which had elapsed between transfusion and that visit. The other two participants' transfusions were potentially more significant given the proximity to their experimental visits, but were balanced in terms of experimental arms; one potentially

affected the participant's performance on their post-PL supplementation visit and the other potentially affected that participant's post-BR supplementation visit. Therefore, transfusions may have influenced cycle ergometry performance in two participants' post-BR supplementation, and one participant's post-PL visit. Stored red cells have a greater affinity for oxygen and therefore reduced oxygen dissociation in hypoxic tissue beds such as contracting muscles. They also contain less bioavailable ˙NO through deregulation of S-nitrosation of haemoglobin. The latter consequence of storage may limit the red blood cell's ability to induce hypoxic vasodilation and hence, reduces its ability to match oxygen delivery with metabolic demand (Reynolds, Bennett et al. 2013). This uncoupling of delivery and demand of oxygen is also an effect of the increased concentration of haemoglobin-containing microparticles and free haemoglobin in stored blood. This consequence of morphological change in red blood cells as a result of ATP-depletion and lipid loss in stored blood, in turn causes a reduction in bioavailability of ˙NO. Free haemoglobin and microparticles scavenge ˙NO 1000 times faster than haemoglobin within red blood cells (Liu, Zhao et al. 2013). They are also free to enter the cell-free zone at the periphery of blood vessels, where scavenging of ˙NO will limit the ability of ˙NO released by red blood cells in conditions of hypoxia to cause vasodilation (Butler, Megson et al. 1998, Liao, Hein et al. 1999). Hence coupling of oxygen delivery and demand is impaired in the presence of stored blood, and could potentially limit exercise tolerance.

While only 11.5% of participants received blood transfusions within 40 days of their study visits, those people may have demonstrated a detrimental effect of this blood on their physiological responses during cycle ergometry. As 2 participants were transfused prior to BR supplementation, and only one received blood prior to their PL

supplementation, this could have contributed to the observed and unexpected difference in their performance during cycle ergometry which demonstrated improved results following PL supplementation.

The improved exercise performance observed in PL compared to BR may have also been influenced by the placebo effect. . A placebo is defined as;

> '*Any therapeutic procedure (or part of a therapeutic procedure) which is given either deliberately to have an effect, or unknowingly and has an effect on a symptom, syndrome, disease or patient without specific activity for the condition being treated. The placebo is also used as an adequate control in research. The placebo effect is defined as the changes produced by placebo'* (Shapiro 1964).

The subjective nature of the symptoms-limited cycle ergometry test may have enabled a placebo effect to be exerted. Time to exhaustion (T_{lim}) and VO_{2peak} results could have been affected by the participants' knowledge they had previously undergone a period of supplementation before both PL and BR experimental visits, as these variables are both subjective measures of symptoms. As a result, these variables of exercise performance may have improved following beetroot juice, although this potential effect would not have preferentially improved performance for PL rather than BR. The placebo effect is less likely to have altered the GET as this is an objective, submaximal index.

One further potential explanation for the improved performance in cycle ergometry which was observed following supplementation with PL rather than BR, was the contents of the beetroot juice supplements themselves. The nitrate-depleted beetroot

juice (PL) was produced by passing beetroot juice through a column containing the anion exchange resin Purolite a520e which exchanges nitrate for chloride (Lansley, Winyard et al. 2011, Gilchrist, Winyard et al. 2014). The resultant products are very similar bar their nitrate component, with no substantial change in the concentrations of sodium, potassium or magnesium ions, a modest reduction in calcium ion concentration within the PL product and an increase in chloride concentration (Gilchrist, Winyard et al. 2014). However, these minor differences in calcium and chloride content would not be expected to influence exercise performance, and indeed, in healthy individuals who do not experience such fluctuations in Hb concentration, nitrate-rich beetroot juice has been shown to enhance exercise tolerance (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Jones 2014).

While the relatively small numbers in this pilot study were sufficient to inform the researchers regarding recruitment, tolerability and numbers required to power future trials, they were insufficient to overcome the potentially dramatic effect the marked change in Hb of a small number of individual participants had on this cohort's overall physiological responses during exercise. Similarly, the small numbers were insufficient to overcome the progression of malignancy or initiation of chemotherapy during the course of some individuals' experimental involvement. The present study recruited an extremely diverse group of participants, with very different ongoing and past health problems and medications, surgical histories, smoking histories, baseline athletic performances and nutritional states. Any observed differences in performance during cycle ergometry testing could have been caused by the heterogeneity of this group of volunteers; their individual and varying clinical factors were expected to dwarf any observed difference nitrate supplementation may have made in this small, deliberately inclusive pilot study. The potential effect this

heterogeneity of participants may have was countered through the use of a before and after crossover study, so that participants acted as their own controls. However, while this design accounted for the inter-participant variability, it did not overcome the intra-participant variability which was quite marked in a number of volunteers.

4.3.2 ^{31}PHOSPHOROUS MAGNETIC RESONANCE SPECTROSCOPY (^{31}P-MRS)

The muscle pH during a gentle exercise protocol using a single-leg knee extension ergometer was unaffected by experimental condition. It had been hypothesised that dietary inorganic nitrate supplementation with BR would have brought about a decrease in muscle phosphocreatine (PCr) recovery time constant (τ). However no statistically significant difference was observed. Muscle O_2 delivery is a key determinant of the rate at which muscle PCr concentration recovers following exercise (Vanhatalo, Fulford et al. 2011, Vanhatalo, Jones et al. 2014), such that the severity of anaemia would have been a major factor determining the PCr recover τ in this study.. Being less anaemic at the post-PL experimental visit may have caused an improvement in PCr recovery compared to their post-BR and baseline visits, overcoming and reversing any potential benefit nitrate supplementation could have had on the measure of oxidative capacity.

Another possible cause for the observed lack of efficacy of BR supplementation in PCr recovery rate is that malignant muscle cells contain 90% less PCr than healthy controls (Patra, Bera et al. 2008). While none of the participants in this trial were known to have muscular metastases, 3 of the people enrolled in this trial were known

to have disseminated malignancy affecting more than one organ system and it is therefore not inconceivable that their quadriceps could have contained malignant cells.

A potential difference in the degree of muscular metabolic perturbation following supplementation with inorganic nitrate and placebo may have become apparent with further testing involving larger numbers of participants, under conditions where large fluctuations in Hb during the study period could be avoided. However this pilot study was not sufficiently powered to demonstrate such an effect.

4.4 THE EFFECT OF NITRATE SUPPLEMENTATION ON QUALITY OF LIFE

All participants in this study underwent sequential monitoring of their subjective responses to the FACT-An questionnaire at each of their four trial visits, giving validated insight into their quality of life, exploring their emotional wellbeing, functional wellbeing, physical wellbeing and social / family wellbeing. Supplementation with both PL and BR brought about a small but statistically significant improvement in this measure of quality of life compared to the non-supplemented state, with no difference between the two supplemented experimental conditions. Significant work has been performed recently in order to assist interpretation of these scores; the Functional Assessment of Chronic Illness Therapy (FACIT) group has issued guidance regarding the minimal important differences (MIDs) for scores and scales of several similar qualitative assessments of health-related quality of life, including FACT-An. They define MID as the "smallest difference in score in the domain of interest that patients perceive as important,

either beneficial or harmful, and that would lead the clinician to consider a change in the patient's management" (Cella, Eton et al. 2002). According to their ‘tentative’ guidance, the MID for FACT-An is 7 points, meaning the observed difference in the participants involved in the current study is statistically significant but may not be clinically significant across this population.

This small subjective improvement in the quality of life of the trial participants was therefore independent of the nitrate content of their supplements, and has two possible explanations. Firstly, there might have been a placebo effect, as defined above (section 4.3.1). Hence, as participants may have expected that drinking supplements would make them feel physically and emotionally better and answered the quality of life questionnaire accordingly to reflect that. Twenty-three of the 26 participants in this trial had active malignancy during their study involvement, while 50% of all participants were undergoing treatment with chemotherapy at that time. They were a highly motivated group of people, many of whom were striving to try and improve their prognosis by any means possible, including this study supplementation. Hence, there is a strong possibility that this beetroot juice would have made them feel subjectively better in a number of different areas of their daily lives through a purely placebo effect mediated by their psychosocial circumstances. Alternatively, if participants were desperate to derive benefit from this supplementation, reporter bias could be expected as a reflection of their hope that this supplementation would make them feel better, or in order to try and please the investigators.

A further possible explanation for the observed improvement in quality of life following supplementation is the other constituent elements of beetroot juice itself.

Irrespective of its nitrate component, some of the other components of beetroot juice supplements may have brought about this improvement in participants' FACT-An quality of life scores through some unanticipated physiological effect. Beetroot juice is rich in antioxidants, folate, vitamin C, manganese, iron and potassium; possible explanations for the observed beneficial effect include the following. Antioxidant therapy improves quality of life measures in patients with chronic pancreatitis (Kirk, White et al. 2006), while folate deficiency is associated with worse quality of life in patients with heart failure (van der Wal, Comin-Colet et al. 2015). High-dose intravenous and oral vitamin C supplementation has been associated with an improvement in the quality of life of patients with terminal cancer (Yeom, Jung et al. 2007). Beetroot juice is also rich in manganese, a heavy metal which is usually excreted via the biliary tree. Accumulation of manganese is associated with greater fatigue scores in patients with primary biliary sclerosis (Forton, Patel et al. 2004), possibly in part because of accumulation in the central nervous system of the cohort of patient under investigation. Intravenous iron supplementation is of benefit in quality of life and fatigue scores for patients with mild anaemia and iron deficiency (Strauss and Auerbach 2018). There is no evidence potassium supplementation improves quality of life measures. While all of the above components within beetroot juice are potential confounding variables masking or augmenting nitrate-induced effects, this was still chosen as the intervention of choice in the present study over potassium nitrate or sodium nitrate for three reasons. Firstly, in the author's experience, patients often ask in clinic about supplements which they can take to try and improve their symptoms when anaemic, so it was felt likely that such a trial would be more attractive than a tablet-based intervention. Secondly, such a supplement would have been classified as an investigational medicinal product

unlike beetroot juice, and therefore approval would have been required from the medicines and healthcare regulatory authority before the trial could have been opened, a potential source of significant delay. Thirdly, nitrate-rich beetroot juice has been proven to demonstrate significantly different physiological effects when compared to nitrate-depleted placebo in a number of previous studies (Kelly, Vanhatalo et al. 2013, Gilchrist, Winyard et al. 2014, Vanhatalo, Blackwell et al. 2018) and therefore, while the other components of the juice may have altered the physiological effect of this supplementation, this did not appear to mask results in the above studies and others. Hence beetroot juice was selected as the best method of providing reproducible dietary supplementation of inorganic nitrate in the present study.

While there is a lack of published evidence to support this, one final possible explanation for the observed improvement in the FACT-An scores at the participants' second and fourth experimental visits which occurred shortly after their first and third visits, was that there may have been an element of learning and they may have scored more highly on their quality of life and cognitive function questionnaires at the post-supplementation appointments.

The marked range in quality of life scoring is very likely to be merely a reflection of the huge diversity of this group of participants. All subjects in this trial had very different diseases, treatments and prognoses along with marked differences in their physical and psychological co-morbidities. Some participants were faced with a terminal diagnosis and as a result, had a visible heavy psychological burden which must have had an impact on all of the areas assessed within this quality of life questionnaire. At the other end of the spectrum, some participants had longstanding

mild anaemia with no need for treatment, no associated cancer diagnosis and hence, their FACT-An score was less likely to be affected by their minor blood abnormality.

4.5 THE EFFECT OF NITRATE SUPPLEMENTATION ON COGNITION

This study intended to investigate the hypothesis that, relative to a nitrate-depleted placebo beetroot juice, dietary supplementation of anaemic patients with nitrate-rich beetroot juice would beneficially alter cognitive function. Cognitive function was assessed subjectively rather than objectively, by participants self-completing a 37 part questionnaire enquiring about their performance over six cognitive domains; memory, concentration, mental acuity, verbal fluency, functional interference, and multitasking ability. There was a marked difference in the results obtained, with some participants scoring very poorly indeed. However, there was an observed small but statistically significant improvement in cognitive function between the baseline visit and each of the supplemented visits, irrespective of the nitrate component of the supplementation. This observed improvement represented only ~3% of the mean FACT-Cog score at each experimental visit respectively, which makes the clinical significance of this improvement uncertain. Indeed, Cheung et al (Cheung, Foo et al. 2014) reported that the minimum clinically important difference in FACT-Cog scored amongst a population of Chinese patients with breast cancer ranged from 6.9 – 10.6 depending on the statistical method used for interpretation. The participants in the present study were predominately Caucasian and had a number of different conditions leading to their anaemia, so the previously reported guide to interpretation of FACT-Cog scoring may not be applicable to results observed in the current study.

However, the above publication provides further suggestion that the observed differences in the current study in FACT-Cog scores after both BR and PL supplementation were statistically but not clinically significant.

The increased FACT-Cog scores observed after both PL and BR supplementation compared to baseline are likely to have a similar aetiology to the subjective improvement in the FACT-An quality of life scores as discussed above. The improvement in subjective assessment of participants' cognitive function may be a placebo effect; participants enrolled in this study and drank beetroot juice supplements in the hope that they would make them feel better, both in terms of their physical symptoms and their cognitive function. The results of this subjective area of this investigation could therefore have been influenced by the participants' expectations regarding the efficacy of this juice, contributing to a cognitive improvement stemming from their feeling of psychosocial wellbeing, or from reporter bias amongst participants. However, such an improvement might reflect the natural course of the participants' disease, fluctuations in symptoms, regression to the mean, or other concurrent treatments.

Similar to the postulated explanations for improved FACT-An scores above, an alternative explanation for improved FACT-cog scores following acute ingestion of beetroot juice is that another component of the supplement may improve cognition. High dietary intake of antioxidants has been postulated to protect individuals from dementia, although there is conflicting evidence in this area (Crichton, Bryan et al. 2013). Folate supplementation in healthy older adults in the Netherlands significantly improved domains of cognitive function that tend to decline with age (Durga, van Boxtel et al. 2007), while supplementation with potassium of a murine model of

Alzheimer's disease can improve cognition (Cisternas, Lindsay et al. 2015). There is a large body of evidence that maintaining healthy vitamin C levels in humans can have a protective effect against age-related cognitive decline and Alzheimer's disease, although supplementation above a normal healthy diet is of less benefit (Harrison 2012). Those participants in the current study who did not ingest healthy levels of vitamin c may have therefore derived cognitive benefit from this component of the beetroot juice. Manganese is critical for neurodevelopment but has also been implicated in the pathophysiology of several diseases. However, correction of deficiency is associated with an improvement in cognition (Pfalzer and Bowman 2017) and could possibly explain an improvement in the FACT-cog score of participants in the current study. Beetroot juice is rich in iron, supplementation of which has previously been demonstrated to improve verbal learning and memory in adolescent girls who were iron deficient and not anaemic (Bruner, Joffe et al. 1996).

Hence, one can speculate that the minor improvement in reported cognitive function in the current study following supplementation with both nitrate-rich and nitrate-depleted beetroot juice could be to any of a number of other components of the juice.

4.6 THE EFFECT OF NITRATE SUPPLEMENTATION ON THROMBOGENICITY

This pilot study intended to investigate the hypothesis that, relative to a nitrate-depleted placebo beetroot juice, dietary supplementation of anaemic patients with nitrate-rich beetroot juice would beneficially alter thrombogenicity. Unfortunately a

large proportion of the study population were excluded from this part of the trial as a result of concurrent use of antiplatelet, anticoagulant or non-steroidal anti-inflammatory drugs. A number of the remaining participants were also unable to complete this aspect of the study at individual experimental visits, most of whom as a result of a reduction in their platelet count. Like anaemia, this reduction in platelets below normal levels is a very common side effect of all of the chemotherapy regimens the participants were taking, and also of some of the diseases the participants had been diagnosed with. Of those participants who were able to complete this testing, a number of logistical problems were encountered. Firstly, there were periodic supply issues in the delivery of the required reaction tubes which had to be a specific size to fit into the PAP8 platelet aggregometer and without which, the machine was useless. Secondly, the PAP8 aggregometer was sited in the Royal Devon and Exeter Hospital, 0.6 miles away from the exercise physiology and magnetic resonance spectrometry laboratories at the University of Exeter. Analysing the samples before 4 hours had elapsed since venepuncture was difficult; patients underwent their blood tests at the beginning of each experimental visit following observations, underwent cycle ergometry and then magnetic resonance spectrometry testing, before the investigator was able to process the samples which had been centrifuged during cycle ergometry. However, getting samples to the hospital laboratory and completing platelet aggregometry within the recommended 4 hour time window was difficult, and usually the investigator travelled between the two institutions on foot at quite high speed whilst carrying the samples. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared prior to this journey but, as PRP should not be agitated prior to platelet aggregometry, this geographic location may have affected the results obtained amongst the few samples that could

be processed. Unfortunately the location of the analyser could not be changed as it was required on the hospital site for its intended clinical utilisation in the investigation of patients with bleeding disorders.

Platelet aggregometry results were only available for 3 participants out of 26 enrolled in the trial (11.5%). Hence, no statistically significant difference was observed between baseline, post-PL supplementation and post-BR supplementation visits and this hypothesis was not addressed effectively by this study.

4.7 FUTURE WORK

4.7.1 CONSIDERATIONS FOR STUDY DESIGN

While designing this pilot study, advice was sought from a medical statistician regarding the sample size required to provide adequate sufficient power to test the proposed hypotheses. Their recommendation was that no power calculation was possible without any initial results upon which to base it, and hence they suggested that a pilot study was the most appropriate design at this stage. Therefore, in future, a larger investigation should be performed recruiting more participants, the precise number of which should be dictated by power calculations as guided by the results obtained in this study. This future research should be adequately powered to overcome the effect confounding variables might have on the observed results, and could potentially negate the likely effect factors such as variable levels of anaemia in

each experimental condition have on the overall study results. Such research would therefore be better able to conclusively prove or disprove the hypotheses and could guide recommendations regarding nitrate supplementation in people experiencing anaemia. Given the relative ease with which participants in this trial were recruited, the high rates of eligibility and of retention in those approached to enter, it is very likely such a larger investigation in future would also be successful in recruiting enough participants and obtaining sufficient data to satisfy the requirements of the power calculations.

The design of future studies should be considered; they may not use the same cross-over design as the current trial. The cross-over was chosen with the intention that all participants acted as their own internal controls for each experimental condition; it was expected that randomisation of the order of supplementation might overcome any minor fluctuations in the physiological status of the participants. The cross-over design was also utilised because statistically, it is more sensitive than parallel groups, and was therefore hoped that it would detect potentially small effects of dietary supplementation. However, the degree of fluctuation in the haemoglobin of individual participants during their trial involvement was unanticipated and would almost certainly have hidden any potential effect nitrate supplementation has on the variables under investigation. Future work may therefore consider using the parallel groups study design, where one cohort of participants is randomised to receive placebo while another is given nitrate-rich beetroot juice.

Data handling and analysis should be carefully planned prior to any future studies in order to optimise the use of data obtained, without necessitating a much larger study.

4.7.2 MAPPING THE ORAL MICROBIOME

The oral microbiome plays a crucial role in the nitrate-nitrite-˙NO pathway by enabling people to reduce nitrate, the first step in this vital pathway which ultimately increases bioavailability of ˙NO, a biologically highly reactive substance which has a large number of roles in maintenance of homeostasis. This study led to further questions while answering another; the nitrate-nitrite-˙NO enterosalivary circulation functions well to allow a similar increase of plasma nitrate and nitrite following oral supplementation with inorganic nitrate as healthy individuals. However, the participants in this trial had a low plasma nitrate concentration at their non-supplemented or placebo experimental visits compared to those same healthy individuals. Many of the participants in this trial were taking antibiotics or using bactericidal mouthwash; did this influence the species or the quantity of bacteria within their oral microbiome and in turn, affect the baseline activity of the participants' nitrate-nitrite-˙NO enterosalivary circulation and ultimately, their plasma nitric oxide bioavailability? Are some bacterial species which colonise the dorsum of the tongue more effective at nitrate reduction than others, or did the anaemia, chemotherapy drugs or concomitant medications have a greater impact on this activity than the oral microbiome? A future study could provide further insight into this area of interest by collecting oral swabs and saliva samples from two cohorts; people with anaemia and medical complaints, and healthy age matched controls. These samples could be utilised to map the oral microbiome and investigate the dynamics of oral nitrate reduction within health and disease.

4.7.3 S-NITROSOTHIOLS

Following completion of this study, frozen plasma samples obtained have undergone further analysis by a PhD student, Mohammed Abu Alghayth, within the University of Exeter, ensuring that the original patient consent and ethics approvals were not conflicted. Because of the low levels of total RSNOs in human plasma samples, the student modified the size of the standard purge vessel to allow analysis of larger volumes of participant plasma using a tri-iodide chemiluminescent assay. This modification increased the signal peak to noise ratios on the chemiluminescent traces and reduced problems with foaming in the purge vessel. This analysis has proven that, contrary to results reported within previous publications in which pure nitrate salt was orally administered, an approximately ten-fold increase in plasma S-nitrosothiols was observed following inorganic nitrate ingestion in the form of beetroot juice (104 nM) compared to the baseline (12 nM) and nitrate-depleted supplementation (11 nM) experimental conditions. Previous research has indicated that oral supplementation with sodium nitrite or sodium nitrate brought about a far more modest rise in plasma S-nitrosothiol concentration from 5 to 13 nM in hypertensive rats (Pinheiro, Amaral et al. 2015), while sodium nitrate supplementation brought about no increase in S-nitrosothiol concentration in humans (Lundberg and Govoni 2004). This much more marked rise in the current study may be a result of other components within beetroot juice influencing the uptake of nitrate, as these would not be present in pure organic nitrate such as sodium nitrate.

In summary, further analysis of samples obtained from this investigation's participants has demonstrated that oral nitrate ingestion did not just alter plasma nitrite levels, but also other potential reservoirs of ˙NO, including S-nitrosothiols. The physiological importance of such an increase in S-nitrosothiols is yet to be determined, however, and further work is required to fully characterise the significance of these findings.

4.8 CONCLUSION

This pilot study successfully fulfilled its aims, demonstrating that patients for a larger scale study could be recruited from this population of motivated individuals keen to 'give something back' to their healthcare providers. This investigation was not powered to provide any conclusive outcomes regarding the efficacy of dietary supplementation with inorganic nitrate on any of the areas discussed. However, it revealed that almost all patients with anaemia, many of whom in this cohort had active cancers and a host of other medical co-morbidities, were undergoing chemotherapy, and were of vastly differing ages, displayed increases in their plasma nitrate and nitrite concentrations following this supplementation. This indicated that the nitrate-nitrite-˙NO pathway was intact despite the frequent use of antibiotics and bactericidal mouthwashes which may have affected the oral microbiome. This study also demonstrated that supplementation with both nitrate-depleted and nitrate-rich beetroot juice brought about an improvement in the quality of life and cognitive function of participants with anaemia. Finally and contrary to expectations, athletic performance during strenuous cycle ergometry testing was improved following

supplementation with nitrate-depleted placebo juice (PL) compared to both nitrate-rich beetroot juice (BR) and the non-supplemented experimental condition. While other components of the placebo juice such as sucrose or polyphenols may explain this observed improvement of performance at PL over baseline, they would not explain the difference between PL and BR. It seems likely that this area of the study was affected by the confounding effect exerted by an improved haemoglobin concentration observed in participants at the time of the post-PL supplementation experimental visit compared to the post-BR visit. However, given the relatively small numbers of participants recruited into this pilot study, such potential confounding related to chance was unavoidable despite best intentions with the design of this crossover study in which participants acted as their own internal controls.

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APPENDIX 1: ROYAL DEVON AND EXETER FOUNDATION NHS TRUST APPROVAL

Royal Devon and Exeter NHS
NHS Foundation Trust

Dr Paul Kerr
Consultant Haematologist
Royal Devon and Exeter NHS Foundation Trust
Barrack Road
Exeter
EX2 5DW

Royal Devon and Exeter Hospital (Wonford)
Barrack Road
Exeter
EX2 5DW

Tel: 01392 411611
RESEARCH AND DEVELOPMENT DIRECTORATE
Direct Dial: 01392 406933
Direct Fax: 01392 403012
Email: rde-tr.Research@nhs.net
Ref: CB/R&D/CG

10 September 2014

Dear Paul

Study Title: **A pilot study of dietary nitrate supplementation in anaemic patients**
R&D No: **1404914**
MREC Ref: **14/SW/0081**

I have reviewed the Trust R&D file for the above named study, which has received approval from the appropriate regulatory bodies, and I am happy to give approval on behalf of the Royal Devon & Exeter NHS Foundation Trust (RD&E).

The documents approved for use in this study are those approved by ethics, these are detailed on a separate sheet.

As named Investigator for this research that is being undertaken at the RD&E, it is your responsibility to manage and conduct this study in accordance with;

- The requirements of the **Research Governance Framework for Health and Social Care (2005)** and **Medicines for Human Use (Clinical Trials) Regulations 2004** (if applicable).
- **ICH-GCP** (Good Clinical Practice) – It is mandatory for those staff who will be consenting participants into this study to have undertaken GCP and to ensure it is updated every 2 years.
- The **Human Tissue Act 2004** and the **EU Tissue and Cells Directive (2006)** for research involving human tissue.
- The **Data Protection Act 1998** which details the eight principles of 'good information handling'.
- **R&D Standard Operating Procedures** (SOPs) and **Trust policies** which are available on the Trust intranet site

As Lead Investigator for this research, you are required to ensure study specific duties are appropriately delegated and clearly documented on the study Delegation Log. This guarantees clarity of roles and must be signed and dated by each individual on the study and yourself as Lead Investigator.

Safety Reporting
Guidance on the classification of Adverse Events/Reactions (AEs/ARs) / Serious Adverse Events/Reactions (SAEs/SARs) and Suspected Unexpected Serious Adverse Reactions (SUSARs) and the requirements for reporting to the sponsor can be found in the study protocol. For RD&E sponsored studies this is also detailed in the sponsorship letter. All safety events that involve RD&E

patients, that require reporting to the Sponsor, must also be reported by fax to the R&D Office within 24 hours of becoming aware of the event, using the appropriate Trust R&D fax template which can be found on the Adverse Event Reporting pages of the R&D intranet site (http://ian.exe.nhs.uk/welcome/directorates/research-and-development/rd-administration/adverse-event-reporting/).

Progress Reporting
You are required to submit regular recruitment updates to the R&D Office, as well as annual progress reports to Ethics, MHRA (where applicable) and R&D. Please note that new government and Trust targets require you to have recruited your **first patient within 30 days of the date of Trust Approval** and to have recruited your target number of participants within the time frame stipulated on your SSI form (Time to Target).

Monitoring and Audit
Your study may be monitored by the Sponsor and selected for audit by the R&D Office (where RD&E is not the Sponsor) and Regulatory Authorities at any time. The team involved in conducting this research must ensure full co-operation with any requests from any of these bodies. Action may be taken to suspend research if it is found to not be conducted in accordance with the protocol and all applicable regulations.

Archiving
Upon completion of this Research an **End of Study Report** must be submitted to the Regulatory Authorities (this will be done by the CI) and a copy submitted to the R&D Office. All studies must be archived appropriately and in accordance with the applicable Law. Where RD&E is the Sponsor or where the Sponsor has delegated archiving to the Investigator team, it is your responsibility to contact the R&D Office to discuss appropriate archiving arrangements.

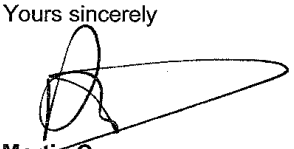
Any publications arising from the Research conducted at this site must be sent to the R&D Office as part of the on-going Research Governance Process.

You should be aware that the Trust accepts no responsibility for the provision of any study drug outside of Clinical Trials and specifically would not fund the continuing prescription of any therapy once the trial has concluded unless there is a written agreement.

Trust Approval is for the duration of the study, as specified in your SSI form. If you have received an Honorary Contract or Letter of Access in order to conduct the above research at this Trust, it is important that you check the termination date on these documents and if applicable contact the R&D Office to extend the document end date.

We wish you every success with your study.

Yours sincerely



Martin Cooper
Medical Director

CC:

Enc: Approved Documents

Study No.:1404914
Study Short Title:Dietary nitrate supplementation in anaemic patients

Patient Related Approved Documents

Doc Name	Version	Date	Date of letter from ethics	Notes
NRES approval letter			27/08/2014	
Protocol	3	29/07/2014	27/08/2014	
Participant Information Sheet	3	27/08/2014	27/08/2014	
Participant Consent Form	1	01/04/2014	27/08/2014	
Poster	1	23/03/2014	27/08/2014	
GP/consultant Information sheet	1	01/04/2014	27/08/2014	
Other: FACT-COG questionnaire)			27/08/2014	
Validated questionnaire - FACT-AN			27/08/2014	

APPENDIX 2: PATIENT INFORMATION SHEET (VERSION 4)

Royal Devon and Exeter NHS
NHS Foundation Trust

A PILOT STUDY OF DIETARY NITRATE SUPPLEMENTATION IN ANAEMIC PATIENTS

WE INVITE YOU TO TAKE PART IN A RESEARCH STUDY

- Before you decide if you would like to take part it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish.
- Ask us if there is anything that is not clear or if you would like more information.
- Take time to decide whether or not you wish to take part.

WHAT IS THE PURPOSE OF THE STUDY

- Being anaemic can make you feel tired and weak
- This is due to reduced oxygen going to your brain and muscles
- Nitric oxide is a naturally occurring chemical that can potentially increase the amount of oxygen travelling to your brain and muscles
- Beetroot juice can boost your levels of nitric oxide naturally
- We want to find out whether concentrated beetroot juice can improve the symptoms of patients who are anaemic
- We will give you concentrated beetroot juice on two occasions;
    - On one occasion normal beetroot juice will be given
    - On the other the beetroot juice will be inactivated to stop it affecting your levels of nitric oxide
- The study involves four visits to our laboratory at the University of Exeter Medical School
- Up to 40 patients will be enrolled onto this study
- This is a pilot study; the information from the study will help plan a larger study.
- The study will not affect your treatment
- You can stop taking part in the study at any time

WHY HAVE I BEEN INVITED TO PARTICIPATE?

We are inviting you to take part in this study because you are anaemic. 'Anaemia' means a lack of haemoglobin; haemoglobin is the chemical that transports oxygen from your lungs to the rest of your body. There are many causes of anaemia – many patients receiving chemotherapy are anaemic; patients who have diseases of the bone marrow can be anaemic; a lack of iron or vitamins can also cause anaemia. If you are anaemic you may feel tired, weak and may find it difficult to concentrate. This is because your body is less able to supply oxygen to your brain and muscles. We want to study whether beetroot juice can improve the quality of life for patients with anaemia.

DO I HAVE TO TAKE PART?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect your normal medical care in any way.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you chose to take part we will meet with you to answer any questions you have; we will take your consent for entry into the trial. We will then book four appointments for you at Exeter University. The research laboratory is about 500 metres from the main hospital (Figure 3). At the first appointment we will undertake the following:

1. A questionnaire measuring your quality of life
2. A test of your ability to think (cognition)
3. An exercise bike test to see how fast you can cycle comfortably
4. A strength test to see how well your muscles are working
5. A blood test looking at the levels of nitric oxide and related chemicals in your blood

The exercise test during the MR scan consists of single-leg knee extensions while lying on your belly inside the MR scanner. We will ask you to do four short bouts of exercise lasting only 24 s each, where you lift and lower a

weight attached to your ankle (see Figure 1). We will monitor your heart rate and the oxygen content of your blood continuously throughout the MR scan using a little light sensor wrapped around your finger by a Velcro strap. After the MR scan you will be asked to perform a cycling test on a stationary bike in the exercise physiology laboratory. The work rate will increase very gradually during this test and we will ask you to continue cycling until you feel that you cannot comfortably keep up with the pedal rate any longer. We will monitor your heart rate using a heart rate monitor wrapped around your chest. We will also use a gas analyser to measure the amount of oxygen your body is using during the cycling and for this purpose we will ask you to wear a facemask during the cycling.

MR spectroscopy uses a powerful magnetic field, radio frequency pulses and a computer to generate detailed functional information of any part of the body. It is a non-invasive procedure and no ionising radiation is used. The MR unit is a large cylinder shaped tube surrounded by a circular magnet. You lie on a moveable couch which slides into the tube putting the area to be scanned in the centre. Devices called coils, which send and receive radio waves, are placed on and around the area of the body to be scanned and, combined with others in the machine, produce signals which are detected by the coils. A computer processes the signals and generates a data output. Not everyone is able to have an MRI scan because of the strength of the magnetic field used to generate images of the body. This is completely harmless to normal body tissues but can be unsafe if you have some implanted metallic devices like a pacemaker or a cochlear implant. Your doctor will go through an MRI safety checklist when obtaining your consent for participation in the study to make sure this test can go ahead.

Blood tests for the study will be sent to the University of Exeter researchers directly involved in this project and to the Royal Devon and Exeter coagulation laboratory. These researchers will perform the tests on how sticky your platelets are and how much of the chemical nitric oxide is present in your blood. Your blood samples will be anonymised and stored in a designated freezer at the University of Exeter until they are tested, before being discarded. The results of these tests will not be available to your doctors and your treatment will carry on exactly as normal. At the end of your involvement in the study the research team will examine your responses to the quality of life questionnaire. This information will then be compared with the results of your MRI exercise test, and your blood tests looking at platelet stickiness and nitric oxide levels.

The quality of life questionnaire is one that has been used in many studies, and takes about ten minutes to complete.

After taking these tests we will give you one of two things:

1. Concentrated beetroot juice (the active supplement)

2. Concentrated juice that has been treated to stop it affecting the levels of nitric oxide in your blood (the inactive supplement)

After taking the beetroot juice for two days we will bring you back to the laboratory and repeat the tests to see if there have been any changes. Six weeks later we will repeat the process; if you received the active supplement of beetroot juice the first time, we will give you the inactivated supplement the second time. If you received the inactive supplement the first time, we will give you the active supplement the second time. Thus all patients will receive both active and inactive supplements. The timings of all the visits are shown in the picture on the following page, and it is anticipated that you will be enrolled on the study over a six to twelve week period (Figure 2 Timeline of investigations). Car parking is available on the campus for all participants in this trial. However, if you have difficulty getting transport to the University for these tests, the Exeter Leukaemia Fund will be able to drive you to and from your appointments. We will not be able to offer dietary supplements to participants after the study has completed.

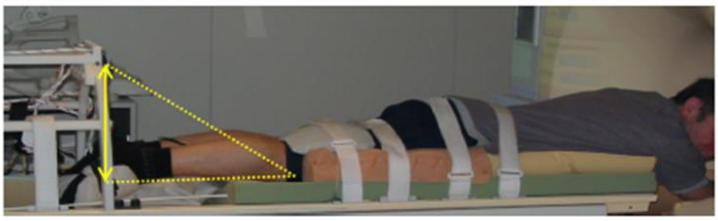


Figure 1 This figure shows a study participant ready to perform knee-extension exercise in the MR scanner. A weight is attached to the participant's foot via a rope and a padded foot strap. The exercise is performed by extending the knee, so that the foot will move up and down as indicated by the yellow arrow.

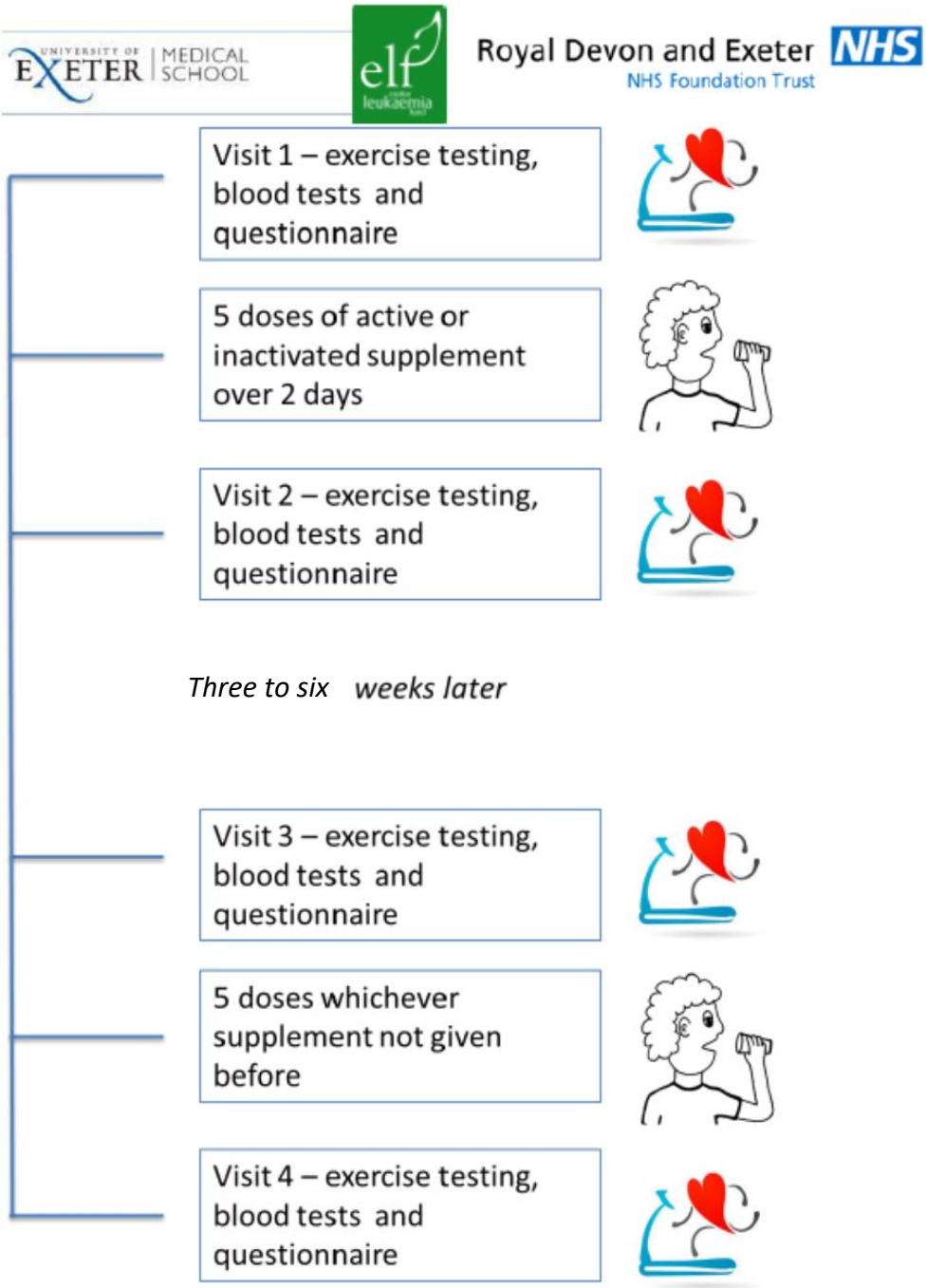


Figure 2 Timeline of investigations

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

We already know that concentrated beetroot juice can have some positive effects. Studies have shown benefits in cognition (ability to think) and ability to exercise. It is possible that you will notice some benefits in how you feel when you are taking beetroot juice. It is not thought that beetroot juice will have any effect on any underlying illness such as cancer, and the study is not specifically looking at this. We do not yet know what the effects of beetroot juice are in patients who are anaemic, and we do not advise you taking beetroot based supplements outside of this study.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

Beetroot juice can change the colour of your bodily fluids; your urine or your faeces may be pink or red during, and for a few days after, you take beetroot juice. It can be difficult to tell this red colouring from blood loss; during the study the doctors looking after you will advise you if you detect such changes.

Although in previous studies people have tolerated the beetroot juice well, you may find the taste unpleasant, and you may experience nausea. If you experience such problems during the study you should tell the doctors running the study; your general practitioner will be informed that you are on the study, as well as your hospital consultant; if you find that you cannot tolerate drinking the beetroot juice for any reason there will be no pressure on you to continue the study – you will have already provided helpful information to the research team since we are interested in how well people tolerate beetroot juice.

Although allergies due to beetroot are rare, some people might be allergic to it. Allergic reactions include rashes, itchiness, chills and fevers and very rarely constriction of the vocal cords and shortness of breath. If you notice any of these symptoms please alert a member of the research team immediately.

If you are already in another trial it is likely that you can also enter this study, but the research team will confirm this when they discuss the study with you.

If you suffer any side effects, or have any anxieties or concerns whilst on the study, please inform a member of the research team, who will be glad to help and advise you accordingly.

WILL WHAT I SAY IN THIS STUDY BE KEPT CONFIDENTIAL?

Everything you tell us will be kept confidential. The data collected on you during the study will not have your name on it, and will be stored securely. Any publications that result from this study will not contain any information from which you could be identified

WHAT SHOULD I DO IF I WANT TO TAKE PART?

If, having read this information sheet, you are happy to take part, please contact Dr Veale on 01392 402850 or by email on dveale@nhs.net and he will arrange to meet you to take consent and check that you are eligible for the study.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

Once 40 patients have completed the study, the data will be collected and published in a scientific journal. Study participants will be informed if this happens and can request a copy by contacting Dr Kerr or Dr Veale (details below). The data will then be used to justify and inform a larger study.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

This study is a collaboration between the doctors at the Royal Devon and Exeter NHS Trust and the scientists at the University of Exeter Medical School and Sport and Health Sciences departments. The chief investigator is Dr Paul Kerr, consultant haematologist who will be working with Dr Dave Veale (haematology doctor), Dr Anni Vanhatalo (senior lecturer), Professor Andy Jones and Professor Paul Winyard.

The research is joint funded by grants from the Exeter Leukaemia Fund and the Research and Development department at the Royal Devon and Exeter NHS Trust. The money will be used to employ Dr Veale to run the study as part of a doctorate. The facilities and dietary supplements will be supplied through Professor Jones' Sport and Heath Sciences departments. None of the researchers will receive any additional money for recruiting patients to the study.

WHO HAS REVIEWED THE STUDY

The study has been reviewed by the regional ethics committee and by the Royal Devon and Exeter Research and Development department.

WHAT IF SOMETHING GOES WRONG OR IF I HAVE A COMPLAINT

If something goes wrong with the trial you will be informed by the chief investigator, Dr Kerr. If you have any concerns that something has gone wrong you should let Dr Kerr or another member of the research team know immediately. If you are not comfortable doing this then you should speak to the Patient Advice and Liaison Service (PALS) whose details are shown below

PATIENT ADVICE AND LIAISON SERVICE

We're here to help

As a patient, relative or carer you might want to be able to turn to someone here at the RD&E for help, advice or support. This is where our patient advice and liaison service – PALS for short – comes in.

PALS is based here at the RD&E to:

Offer you confidential advice, support and information.

Help you sort out your concerns about our services.

Guide you through the range of different NHS services that are available.

Our PALS team offers practical advice and support about the RD&E and can point you in the right direction for the information or services you need.
We can liaise with other hospital staff and with relevant organisations where appropriate to arrange an immediate or prompt solution.
We believe that by responding quickly and sensitively we can often deal with a situation to the satisfaction of all concerned.
PALS will also feed your views back into the system, helping the RD&E to see things from your perspective and improve services in response to your feedback.

Our PALS service is available to anyone using the RD&E's services, including our hospitals at Wonford, Exeter Mobility Centre and the Mardon Centre.
PALS staff are happy to discuss your concerns over the phone or see patients at any of the RD&E sites. Arrangements can be made for this by phoning 01392 40 2093
PALS is available Monday to Friday from 9:30am to 4.30pm.
To contact PALS you can:

Phone: **01392 402093**

Fax: 01392 40 3908

Email: rde-tr.PALS@nhs.net

RESEARCHERS:

Dr Paul Kerr (Haematologist)

Consultant Haematologist, Royal Devon and Exeter NHS Foundation Trust,

EX2 5DW

01392 402850

Paul.kerr1@nhs.net

Dr David Veale

Registrar Haematologist, Royal Devon and Exeter NHS Foundation Trust,

EX2 5DW

01392 402850

dveale@nhs.net

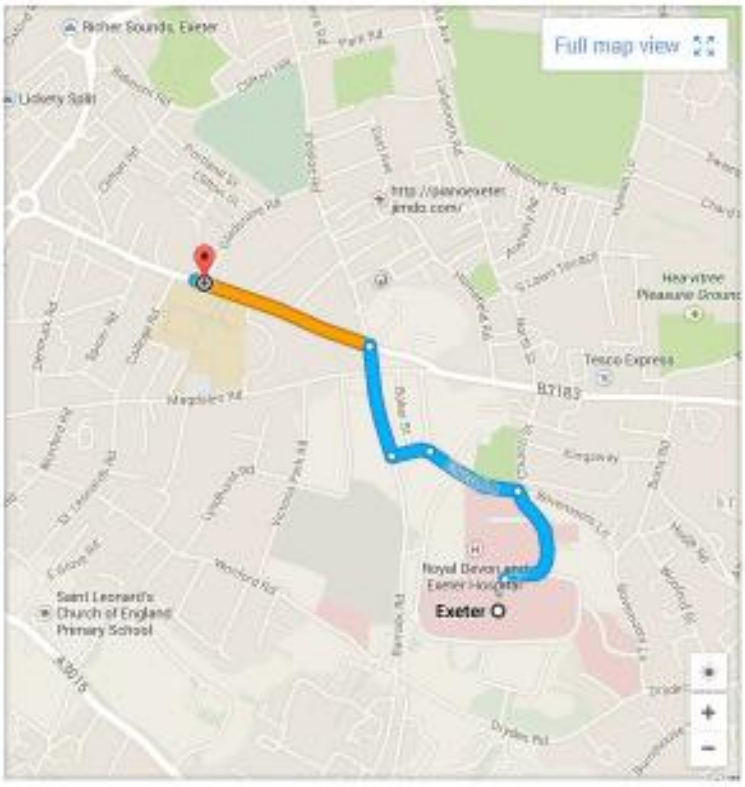


Figure 3 Map showing route between the hospital and the St Luke's campus.

APPENDIX 3: FACT-AN QUESTIONNAIRE (VERSION 4)

FACT-An (Version 4)

Below is a list of statements that other people with your illness have said are important. **Please circle or mark one number per line to indicate your response as it applies to the past 7 days.**

	PHYSICAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4

	SOCIAL/FAMILY WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GS1	I feel close to my friends	0	1	2	3	4
GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1	*Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please mark this box* ☐ *and go to the next section.*					
GS7	I am satisfied with my sex life	0	1	2	3	4

FACT-An (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

	EMOTIONAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GE1	I feel sad	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4

	FUNCTIONAL WELL-BEING	Not at all	A little bit	Some-what	Quite a bit	Very much
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now	0	1	2	3	4

FACT-An (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

	ADDITIONAL CONCERNS	Not at all	A little bit	Some-what	Quite a bit	Very much
HI7	I feel fatigued	0	1	2	3	4
HI12	I feel weak all over	0	1	2	3	4
An1	I feel listless ("washed out")	0	1	2	3	4
An2	I feel tired	0	1	2	3	4
An3	I have trouble starting things because I am tired	0	1	2	3	4
An4	I have trouble finishing things because I am tired	0	1	2	3	4
An5	I have energy	0	1	2	3	4
An6	I have trouble walking	0	1	2	3	4
An7	I am able to do my usual activities	0	1	2	3	4
An8	I need to sleep during the day	0	1	2	3	4
An9	I feel lightheaded (dizzy)	0	1	2	3	4
An10	I get headaches	0	1	2	3	4
B1	I have been short of breath	0	1	2	3	4
An11	I have pain in my chest	0	1	2	3	4
An12	I am too tired to eat	0	1	2	3	4
BL4	I am interested in sex	0	1	2	3	4
An13	I am motivated to do my usual activities	0	1	2	3	4
An14	I need help doing my usual activities	0	1	2	3	4
An15	I am frustrated by being too tired to do the things I want to do	0	1	2	3	4
An16	I have to limit my social activity because I am tired	0	1	2	3	4

APPENDIX 4: FACT-COG QUESTIONNAIRE (VERSION 3)

FACT-Cognitive Function (Version 3)

Below is a list of statements that other people with your condition have said are important. **Please circle or mark one number per line to indicate your response as it applies to the past 7 days.**

	PERCEIVED COGNITIVE IMPAIRMENTS	Never	About once a week	Two to three times a week	Nearly every day	Several times a day
CogA1	I have had trouble forming thoughts	0	1	2	3	4
CogA3	My thinking has been slow	0	1	2	3	4
CogC7	I have had trouble concentrating	0	1	2	3	4
CogM9	I have had trouble finding my way to a familiar place	0	1	2	3	4
CogM10	I have had trouble remembering where I put things, like my keys or my wallet	0	1	2	3	4
CogM12	I have had trouble remembering new information, like phone numbers or simple instructions	0	1	2	3	4
CogV13	I have had trouble recalling the name of an object while talking to someone	0	1	2	3	4
CogV15	I have had trouble finding the right word(s) to express myself	0	1	2	3	4
CogV16	I have used the wrong word when I referred to an object	0	1	2	3	4
CogV17b	I have had trouble saying what I mean in conversations with others	0	1	2	3	4
CogF19	I have walked into a room and forgotten what I meant to get or do there	0	1	2	3	4
CogF23	I have had to work really hard to pay attention or I would make a mistake	0	1	2	3	4
CogF24	I have forgotten names of people soon after being introduced	0	1	2	3	4

FACT-Cog (Version 3)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

		Never	About once a week	Two to three times a week	Nearly every day	Several times a day
CogP25	My reactions in everyday situations have been slow	0	1	2	3	4
CogC31	I have had to work harder than usual to keep track of what I was doing	0	1	2	3	4
CogC32	My thinking has been slower than usual	0	1	2	3	4
CogC33a	I have had to work harder than usual to express myself clearly	0	1	2	3	4
CogC33c	I have had to use written lists more often than usual so I would not forget things	0	1	2	3	4
CogMT1	I have trouble keeping track of what I am doing if I am interrupted	0	1	2	3	4
CogMT2	I have trouble shifting back and forth between different activities that require thinking	0	1	2	3	4

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

	COMMENTS FROM OTHERS	Never	About once a week	Two to three times a week	Nearly every day	Several times a day
CogO1	Other people have told me I seemed to have trouble remembering information	0	1	2	3	4
CogO2	Other people have told me I seemed to have trouble speaking clearly	0	1	2	3	4
CogO3	Other people have told me I seemed to have trouble thinking clearly	0	1	2	3	4
CogO4	Other people have told me I seemed confused	0	1	2	3	4

FACT-Cog (Version 3)

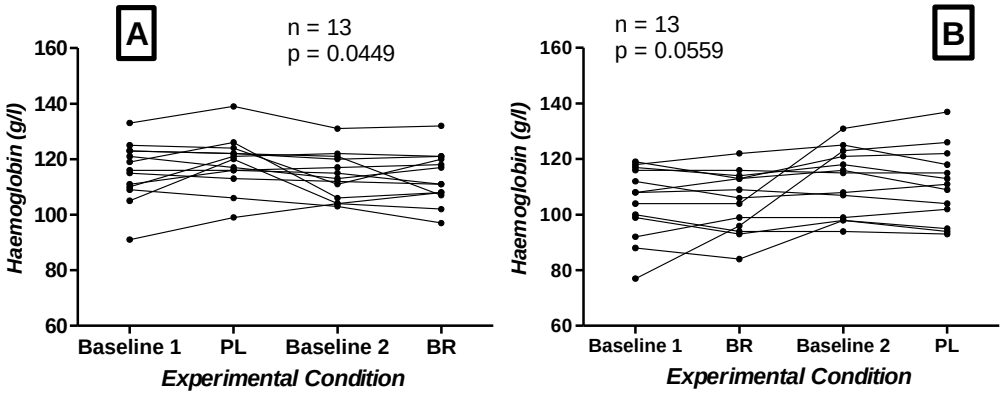
Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	PERCEIVED COGNITIVE ABILITIES	Not at all	A little bit	Some-what	Quite a bit	Very much
Cog PC1	I have been able to concentrate	0	1	2	3	4
Cog PV1	I have been able to bring to mind words that I wanted to use while talking to someone	0	1	2	3	4
Cog PM1	I have been able to remember things, like where I left my keys or wallet	0	1	2	3	4
Cog PM2	I have been able to remember to do things, like take medicine or buy something I needed	0	1	2	3	4
Cog PF1	I am able to pay attention and keep track of what I am doing without extra effort	0	1	2	3	4
Cog PCH 1	My mind is as sharp as it has always been	0	1	2	3	4
Cog PCH 2	My memory is as good as it has always been	0	1	2	3	4
Cog PMT 1	I am able to shift back and forth between two activities that require thinking	0	1	2	3	4
Cog PMT 2	I am able to keep track of what I am doing, even if I am interrupted	0	1	2	3	4

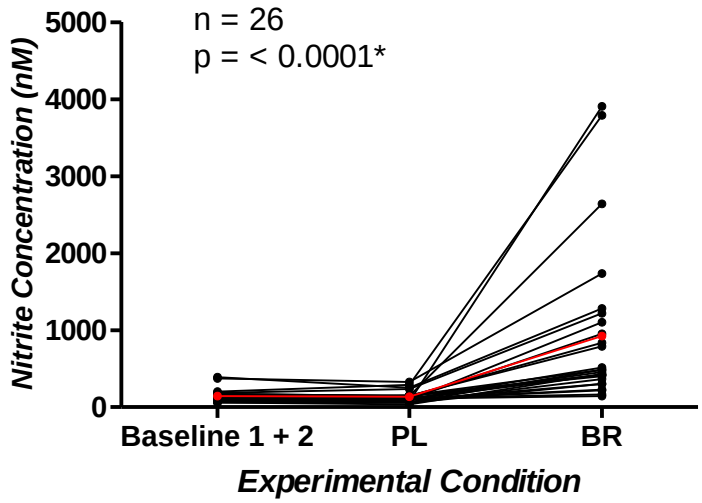
Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	IMPACT ON QUALITY OF LIFE	Not at all	A little bit	Some-what	Quite a bit	Very much
CogQ35	I have been upset about these problems	0	1	2	3	4
CogQ37	These problems have interfered with my ability to work	0	1	2	3	4
CogQ38	These problems have interfered with my ability to do things I enjoy	0	1	2	3	4
CogQ41	These problems have interfered with the quality of my life	0	1	2	3	4

APPENDIX 5: RAW DATA GRAPHS



Raw Data Figure 1. Individual haemoglobin concentrations of 26 participants throughout their experimental visits. These scatter graphs demonstrate the change in haemoglobin concentrations of those 13 participants in the current study who received five 70 ml bottles of nitrate-deplete beetroot juice (placebo [PL]) at 2.5, 12, 24, 36 and 48 hours before their second experimental visit, and five 70 ml bottles of nitrate-rich beetroot juice (BR) at the same time points in the 48 hours prior to their fourth experimental visit (panel A). The experimental timepoints 'Baseline 1' (no beetroot supplementation) and 'PL' were within 7 to 10 days of each other, while 3 to 6 weeks elapsed between 'PL' and 'Baseline 2' (no beetroot supplementation), before only 7 to 10 days passed before they returned for the 'BR' visit. Panel B displays the remaining 13 participants in this study whose order of supplementation was reversed, receiving a 48 hour period of supplementation of BR before visit 2 and of PL before visit 4. This figure demonstrates that, while some individuals displayed little change in haemoglobin with time, a number of participants' Hb was significantly better at their PL experimental condition than their BR timepoint. One way ANOVA of all data sets demonstrated statistical significance ($p = 0.0449$) between the means of the experimental conditions, although this was not significant according to Fisher's Least Significant Difference (LSD) post hoc analysis.



Raw Data Figure 2. Plasma nitrite concentrations of participants at each experimental visit. This scatter plot displays the plasma nitrite concentrations of each of the 26 participants (black data points and lines) and the means values (red data points and lines) at each of the four experimental. The experimental condition 'Baseline 1 + 2' indicates all nitrate concentrations of participants at their non-supplemented visits, while 'PL' and 'BR' indicates the study visit following a period of supplementation. Participants ingested a 70 ml bottle of nitrate-depleted beetroot juice (PL) at 2.5, 12, 24, 36 and 48 hours before study visit 'PL', while they ingested a 70 ml bottle of nitrate-rich beetroot juice (BR) at the same time points prior to visit 'BR'. The nitrite concentration of each stored plasma sample was quantified by gas-phase chemiluminescence analysis of each participant plasma sample from every experimental visit (see Methods section 2.8.7). This graph demonstrates that the mean plasma nitrite concentration was significantly higher at experimental condition BR (923 nM, SD 1006 nM) than at either baseline (142 nM, SD 79 nM) or PL (134 nM, SD 69 nM) (1 way ANOVA p = < 0.0001, statistically significant according to Fisher's LSD post hoc analysis [LSD = 0.000]). The data was skewed and therefore made non-parametric by two participants' nitrite concentration at condition BR which rose far more than the remainder of the participants

APPENDIX 6: REGIONAL ETHICS COMMITTEE FAVOURABLE OPINION

NHS
Health Research Authority
NRES Committee South West - Exeter
Whitefriars
Level 3
Block B
Lewins Mead
Bristol
BS1 2NT

Telephone: 01173421390
Fax:01173420445

27 August 2014

Dr Jonathan P Kerr
Haematology Consultant
Royal Devon and Exeter Foundation NHS Trust
Department of Haematology
Royal Devon and Exeter Hospital
Barrack Road, Exeter
EX2 5DW]

Dear Dr Kerr

Study title:	**A PILOT STUDY OF DIETARY NITRATE SUPPLEMENTATION IN ANAEMIC PATIENTS**
REC reference:	**14/SW/0081**
IRAS project ID:	**123901**

Thank you for your letter of 29th July 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair and Vice-Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mrs Kirsten Peck, nrescommittee.southwest-exeter@nhs.net

Confirmation of ethical opinion

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

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

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

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

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

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

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

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

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

Ethical review of research sites

NHS sites

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

Approved documents

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

Document	*Version*	*Date*
Copies of advertisement materials for research participants	1	23 March 2014
GP/consultant information sheets or letters	1	01 April 2014
Letter from sponsor		01 April 2014
Other [CV paul kerr]	1	23 July 2014
Other [FACT-COG questionnaire]	1	28 July 2014
Other [Letter from funder]		14 November 2013
Other [covering letter]	1	23 July 2014
Other [Email from researcher]		27 August 2014
Other [protocol version 3]	3	23 July 2014
Other [CV A Jones]	1	24 July 2014
Other [cv Prof Winyard]	1	23 July 2014
Other [protocol]	3	29 July 2014
Other [CV Dr Vanhatalo]	1	23 July 2014
Other [cv David Veale]	1	23 July 2014
Participant consent form	1	01 April 2014
Participant information sheet (PIS)	3	27 August 2014
REC Application Form		01 April 2014
Referee's report or other scientific critique report		01 April 2014
Summary CV for Chief Investigator (CI)		04 April 2014
Validated questionnaire [FACT-AN]		

Statement of compliance

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

After ethical review

Reporting requirements

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

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

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

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/SW/0081	**Please quote this number on all correspondence**

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

Yours sincerely

pp.

Dr Denise Sheehan
Chair

Email: nrescommittee.southwest-exeter@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Mr Chris Gardner*
Miss Lynda Garcia, Royal Devon and Exeter NHS Foundation Trust