UNIVERSITY OF BIRMINGHAM

A protocol study to investigate the acute vascular and metabolic effects of nicotinamide riboside and pterostilbene co-supplementation

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ABSTRACT

Effective mitochondrial and vascular function are key to maintaining general health and optimising endurance exercise performance. This thesis aims to discuss some key players regulating these functionalities, and a promising strategy to upregulate these pathways. Specifically, the co-supplementation of nicotinamide riboside (NR), an NAD+ precursor, and pterostilbene (PT), a polyphenol (together referred to as NRPT). Clinical data regarding the independent and combined metabolic and vascular actions of NR, PT, and NRPT supplementation are reviewed in detail. The possible beneficial effects of co-supplementation on glycaemic control will also be discussed, as well as the role of post-exercise hypoxic exposure on post-exercise glucose uptake. The review discusses the use of two stimuli, acute hyperglycaemia and acute hypoxic exposure, which could potentiate the vascular effects of NRPT. Within this, an in-depth summation of literature regarding the impact of a standardised glucose load on flow-mediated dilation in healthy populations is provided.

Currently, only four clinical trials have investigated NRPT supplementation (chronic and short-term), either in vulnerable or elderly populations with a limited range of metabolic and vascular outcome measures. Consequently, no data exists regarding acute dosages, supplementation in younger cohorts, or its impact during exercise. The original aim of this thesis was to address this gap in the literature; however, due to COVID-19 the research could not take place. Therefore, this thesis contains the rationale for this study and the proposed methodology that planned to utilise a randomised, double-blind, and placebo-controlled design. Briefly, the impact of acute NRPT supplementation on vascular function, substrate utilisation during exercise, and post-exercise glycaemic control were going to be assessed, with the primary outcome measure being brachial artery flow-mediated dilation (FMD). The relatively underexplored area of post-exercise hypoxic exposure on post-exercise glucose uptake was also included in the investigation.

Lastly, this thesis also contains results of an FMD repeatability study conducted prior to COVID-19. Completion of this was required before the start of experimental testing to demonstrate a certain degree of sonography competence to ensure FMD measurements would be reliable. Each participant (n=8) attended three lab visits, where FMD was measured twice, 1 hr apart, totalling six measurements. Analysis showed an inter- and intra-day coefficient of variation of 13.7% and 9.0%, respectively.

CONTENTS

ABBREVIATIONS

- **NR:** Nicotinamide Riboside
- **NAD+:** Nicotinamide adenine dinucleotide
- **PT:** Pterostilbene
- **NO:** Nitric oxide
- **eNOS:** Endothelial nitric oxide synthase
- **OGTT:** Oral glucose tolerance test
- **BH_4:** tetrahydrobiopterin
- **$\dot{V}O_{2max}$:** Rate of maximal oxygen consumption
- **FMD:** Flow-mediated dilation
- **T_{max}:** Time to peak plasma concentration
- **RONS:** Reactive oxygen and nitrogen species
- **RER:** Respiratory exchange ratio
- **NIRS:** Near-infrared spectroscopy
- **PWV:** Pulse wave velocity
- **HOMA-IR:** Homeostatic Model Assessment for Insulin Resistance
- **ATP:** Adenosine triphosphate
- **OXINOS:** oxidative-inflammatory-nitrosative stress
- **SIRT:** Sirtuin
- **ETC:** Electron transport chain
- **VSMC:** Vascular smooth muscle cell
- **NAAD:** nicotinic acid adenine dinucleotide
- **RWP:** Red wine polyphenol
- **HLC:** Hepatic liver content
- **PGC-1α:**
- **OXPHOS:** Oxidative phosphorylation
- **GLUT4:** Glucose transporter 4
- **NMN:** Nicotinamide mononucleotide
- **LDL:** Low-density lipoprotein
- **AMPK**
- **SNA:** Sympathetic nerve activity

1. LITERATURE REVIEW

1.1. Introduction

Mitochondrial and vascular function are integral to general health, with dysfunctions contributing to a multitude of cardiovascular, metabolic and neurodegenerative diseases (Jani and Rajkumar, 2006; Sorrentino, Menzies and Auwerx, 2017). Often, these dysfunctions are age-related and are associated with declines in cellular nicotinamide adenine dinucleotide (NAD+) levels (Mouchiroud, Houtkooper and Auwerx, 2013) and redox status (Liguori *et al.*, 2018), the balance between pro-oxidants and anti-oxidants. Outside of a clinical context, efficient mitochondrial and vascular function are essential for optimising exercise performance. Mitochondrial function determines an athlete's ability to produce energy aerobically and therefore their endurance performance. Meanwhile, the vasculature is required to redistribute blood flow to the active skeletal muscle, providing vital oxygen and nutrients whilst removing detrimental waste products. In sport, athletes not only need to perform well during the event, but also recover quickly to cope with busy training and competitive schedules. It is important to quickly replenish muscle glycogen stores, a process mediated by both insulin-dependent and –independent mechanisms (Alghannam, Gonzalez and Betts, 2018). Unsurprisingly, strategies aimed at enhancing mitochondrial function, vascular function and metabolic control are highly sought after due to their significance for both clinical and athletic populations. This review will discuss the key players regulating these functionalities and evaluate the metabolic and vascular potential of the two components (nicotinamide riboside and pterostilbene, NRPT) that comprise a promising, commercially available nutraceutical supplement (Basis, Elysium Health Inc). Alongside this, literature surrounding the impact of acute hypoxic exposure and post-prandial hyperglycaemia on endothelial function will be summarised. Finally, the potential for post-exercise hypoxic exposure to augment skeletal muscle glucose uptake, and thus improve post-exercise metabolic recovery will be discussed.

1.2. Key Players of Mitochondrial and Vascular Function

Nicotinamide Adenine Dinucleotide (NAD+)

NAD+ and Mitochondrial Function

The mitochondria has roles in heat production, cell apoptosis and calcium storage; however, its primary function is to produce energy in the form of adenosine triphosphate (ATP) (Vakifahmetoglu-Norberg, Ouchida and Norberg, 2017). Cellular NAD+ is compartmentalised within the nucleus, cytoplasm and mitochondria (Cantó, Menzies and Auwerx, 2015); although, examination of mouse skeletal muscle revealed mitochondrial NAD+ levels were 2-fold greater than the rest of the cell

(Pirinen *et al.*, 2014). NAD+ and its reduced counterpart, NADH, form an important redox couple that regulates cellular energy metabolism. NADP+ is the phosphorylated homolog of NAD+. Unlike the NAD+/NADH redox couple, which primarily operate in oxidative, catabolic reactions, NADP+/NADPH drive predominantly reductive, anabolic reactions including the synthesis of fatty acids and cholesterol (Bogan and Brenner, 2008).

A central determinant of ATP production is the bioavailability of NAD+, due to its role as a vital cofactor for many oxioreductases within energy metabolism, thus regulating their enzymatic activity. Specifically, NAD+ acts as a reducing equivalent in glycolysis, the tricarboxylic acid cycle (TCA) and beta-oxidation; the NADH formed shuttles electrons to the electron transport chain (ETC) for ATP production via oxidative phosphorylation on the inner mitochondrial membrane (Cantó, Menzies and Auwerx, 2015), see figure 1 below. Animal studies that manipulated NAD+ biosynthesis have demonstrated lower NAD+ levels are a hallmark of cellular senescence (Cantó, Menzies and Auwerx, 2015), and associated with impaired ATP production (Oyarzún *et al.*, 2015), insulin resistance (Yoshino *et al.*, 2011), and attenuated oxidative phosphorylation (Gomes *et al.*, 2013). There is some evidence to suggest that, in mice fed a high-fat diet, increasing NAD+ bioavailability through chronic supplementation with an NAD+ precursor increased oxidative metabolism and mitochondrial biogenesis (Canto *et al.* 2012; Gariani *et al.*, 2016). However, these findings have not been consistently replicated (Williams *et al.*, 2021), possibly due to substantial differences in the magnitude of baseline NAD+ depletion.

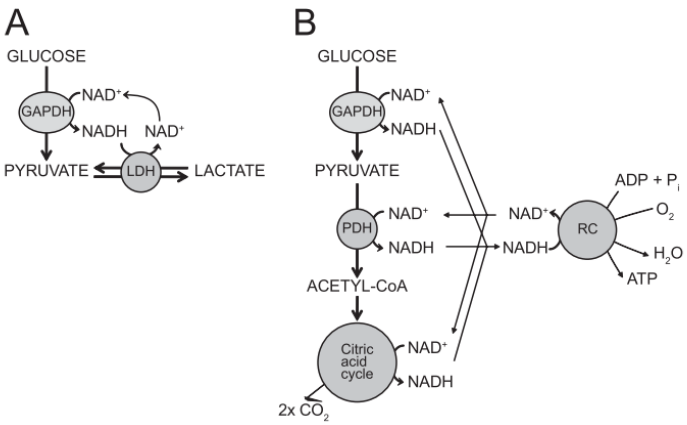

Figure 1: *NADH is produced during glycolysis in the glyceraldehyde-3-phosphate (GADPH) reaction. Panel A shows anaerobic glucose metabolism, whereby, to maintain production of ATP during periods of high glycolytic flux, NADH is recycled to NAD+ by pyruvate dehydrogenase (PDH), at the cost of lactate formation. Panel B shows aerobic glucose metabolism, whereby NADH formed during glycolysis, link reaction, and citric acid/TCA cycle is shuttled to the respiratory chain (RC/ETC), providing electrons for the synthesis of ATP. (From Houtkooper et al. (2010)).*

NAD+ and Vascular Function

As mentioned previously, NAD(H) and homologous NADP(H) redox couples are involved a wide variety of reactions and pathways. Vascular endothelial function is primarily regulated by the enzyme endothelial nitric oxide synthase (eNOS), which produces the vasodilatory molecule, nitric oxide (Davignon and Ganz, 2004). This reaction requires the presence of several cofactors, one of which is NADPH. NADPH provides electrons to the reductase domain of eNOS, which in turn are shuttled to the catalytic centre of the oxygenase domain to form nitric oxide (Davignon and Ganz, 2004). This electron flux is a key determinant of eNOS-mediated nitric oxide production and thus highlights the importance of maintaining homeostasis of the NADP(H) redox couple to prevent endothelial cell dysfunction.

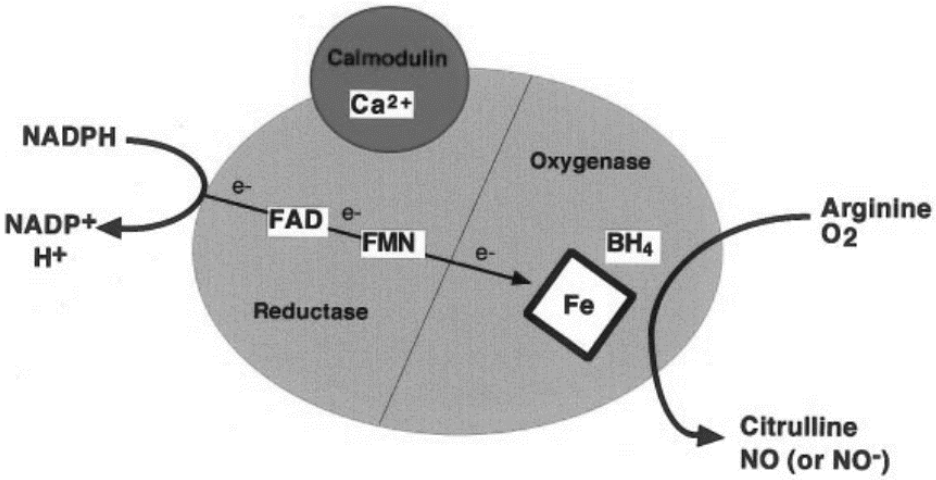

Figure 2: *Electrons (e-) are donated by NADPH to the reductase domain of the enzyme and proceed via flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) redox carriers to the oxygenase domain. There they interact with the haem iron (Fe) and tetrahydrobiopterin (BH_4) at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and nitric oxide (NO) as products. (From Alderton et al. (2001)).*

Maintaining cellular redox status is important to allow proper vasodilatory function of endothelial cells. Reactive oxygen and nitrogen species (RONS), in particular superoxide, have a high affinity for nitric oxide and react with it to form peroxynitrite (Pryor and Squadrito, 1995), reducing nitric oxide bioavailability for vasodilation. These series of reactions have been termed 'OXINOS' stress, known to be implicated in human endothelial dysfunction (Pierce *et al.*, 2009; Bailey *et al.*, 2013; Wadley *et al.*, 2014). Similarly, the age-related reduction in bioavailability of eNOS substrates and cofactors (Bode-Böger *et al.*, 2003; Eskurza *et al.*, 2005) is thought to be mediated by elevated RONS (Jacobson *et al.*, 2007), resulting in eNOS uncoupling and thus impaired nitric oxide production (Yang *et al.*, 2009). NADP(H) is a key regulator redox homeostasis, preventing excessive RONS accumulation. NADPH is a vital cofactor for glutathione- and thioredoxin-reducatases which are essential for glutathione peroxidase- and peroxiredoxin-mediated RONS removal, respectively (Buettner, G,

Wagnera, B, and Rodgers, 2013). Augmenting the biosynthesis of NAD+ has elicited beneficial *in vivo* effects on redox homeostasis in rodents (de Picciotto *et al.*, 2016; de Castro *et al.*, 2020) and humans (Dolopikou *et al.*, 2019). However, a delicate balance must be struck to prevent reductive stress (Handy and Loscalzo, 2017), as high levels of NADPH can provide substrate for RONS-producing enzymes, nicotinamide adenine dinucleotide phosphate oxidases (NOX) (Handy and Loscalzo, 2012).

Sirtuins

Sirtuins are a family of NAD+-dependant enzymes, primarily acting as deacetylases. There are currently seven known mammalian homologs (SIRT 1-7) with diverse tissue and sub-cellular localisation and functionalities (Cantó, Menzies and Auwerx, 2015). Sirtuins first became of interest when a seminal paper demonstrated SIR2 overexpression increased lifespan in budding yeast (Kaeberlein, McVey and Guarente, 1999); since then, it is generally agreed that sirtuins, amongst other factors, are closely involved in mammalian calorie-restriction mediated lifespan extension (Park, Mori and Shimokawa, 2013). Of the seven, SIRT1 is the most extensively studied and SIRT1, 2 and 3 appear to have the strongest deacetylation activity (Cantó, Menzies and Auwerx, 2015). Sirtuins are important regulators for a plethora of cellular processes and functions; including, maintaining metabolic and redox homeostasis, optimising mitochondrial and vascular function as well as modulating our circadian rhythms (Borradaile and Pickering, 2009; Boutant and Cantó, 2014; Cantó, Menzies and Auwerx, 2015; Masri, Bellet and Sassone-corsi, 2016; Singh *et al.*, 2018). Due to their wide spanning influence, sirtuins have been studied as therapeutic targets for cardiovascular, metabolic, and neurodegenerative diseases (Borradaile and Pickering, 2009; Haigis and Sinclair, 2010; Houtkooper, Pirinen and Auwerx, 2012; Kane and Sinclair, 2018).

NAD+ levels represent cellular energy availability, meaning sirtuins could be considered metabolic energy sensors (Canto *et al.*, 2012) based on their response to energy/nutrient stress induced by calorie restriction (Chen *et al.*, 2008) or exercise (Cantó *et al.*, 2010). Given sirtuins NAD+-dependency, it is unsurprising that sirtuin activity is consistently reported to be correlated with NAD+ bioavailability (Borradaile and Pickering, 2009; Houtkooper, Pirinen and Auwerx, 2012; Cantó, Menzies and Auwerx, 2015). Physiological concentrations of NAD+ rarely fluctuate more than 2-fold (Houtkooper *et al.*, 2010); however, *in vivo* animal research has shown that augmenting NAD+ bioavailability through dietary interventions enhances SIRT1 activity (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014; Kiss *et al.*, 2020). It should be remembered that sirtuins are not the only NAD+-dependant enzymes, NAD+ is also a substrate for poly(ADP-ribose)polymerases (PARPs) and cyclic ADP-ribose synthases (cADPRs) (Cantó, Menzies and Auwerx, 2015), creating competition for a finite NAD+ pool. PARPs have instrumental roles in DNA repair, inflammation and cell apoptosis,

whilst cADPRS generate second messengers, implicated in calcium and insulin signalling (Cantó, Menzies and Auwerx, 2015). An extensive review by Canto *et al.* (2013) discusses SIRT-PARP interactions, and how these enzymes can up-, or down-regulate one another's activity. Another key NAD+ consumer is the glycoprotein, CD38. Its expression/activity has been shown to increase with age in mice and humans (Polzonetti *et al.*, 2012; Camacho-Pereira *et al.*, 2016). CD38-mediated NAD+ depletion is thought to be linked with markers of oxidative stress (Hu *et al.*, 2014;. Musolino *et al.*, 2011) and age-related elevations in inflammation (Hogan *et al.*, 2019).

In addition to the NAD+/NADH ratio, sirtuin activity can also be modulated by plant-derived polyphenols, including resveratrol and pterostilbene (Borra, Smith and Denu, 2005; Cheng *et al.*, 2016). Alongside their anti-oxidant properties, sirtuins are thought to be implicated in the beneficial health effects elicited by these molecules (Tsai, Ho and Chen, 2017)

Sirtuins and Mitochondrial Function

Sirtuins are essential for optimising efficiency of the mitochondria's energy producing role. SIRT3, 4, and 5 are primarily localised within the mitochondria itself (Michishita *et al.*, 2005) and interact directly with mitochondrial proteins. Although SIRT1 is primarily localised within the nucleus (Michishita *et al.*, 2005), it is integral for mitochondrial and metabolic homeostasis through regulation of multiple transcription factors such as peroxisome proliferator-activated receptor λ (PPARλ), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), forkhead box protein 1 (FOXO1) and p53 (Feige and Johan, 2008). SIRT1-mediated deacetylation of PGC-1α is associated with increased fatty acid oxidation, mitochondrial biogenesis, and oxidative phosphorylation (Lagouge *et al.*, 2006; Gerhart-Hines *et al.*, 2007; Feige *et al.*, 2008; Rodgers *et al.*, 2008; Bai *et al.*, 2011; Canto *et al.*, 2012; Gomes *et al.*, 2013).

SIRT3 directly interacts with complexes 1 and 2 of the ETC, enhancing their activity and overall ATP production through oxidative phosphorylation (Houtkooper, Pirinen and Auwerx, 2012). SIRT3 is also thought to promote fatty acid oxidation by deacetylating and thus upregulating activity of long-chain acyl coenzyme A dehydrogenase (LCAD) (Hirschey *et al.*, 2010), an enzyme within the beta oxidation pathway. The importance of SIRT3 to mitochondrial function has also been demonstrated *in vivo*, with SIRT3 knockout mice exhibiting decreased oxygen consumption (Jing *et al.*, 2011).

RONS are known to disrupt or damage mitochondrial proteins and membranes, resulting in dysfunction of the organelle (Bhat *et al.*, 2015). The sirtuin family is heavily implicated in the modulation of RONS and anti-oxidant enzymes, reviewed in detail by Singh *et al.* (2018). For example, SIRT1, 2, and 3 upregulate transcription of the mitochondrial anti-oxidant enzyme,

superoxide dismutase 2 (SOD2). Alongside impaired oxygen consumption, SIRT3 knockout mice also experienced elevated RONS (Jing *et al.*, 2011).

However, some studies using skeletal muscle-specific SIRT1 knockout mice have found no effect on glucose tolerance, whole-body and muscle-specific insulin sensitivity (Schenck *et al.*, 2011), or mitochondrial adaptations to exercise training (Menzies *et al.* 2013; Philp *et al.*, 2011). Similar findings exist with SIRT3 knockout mice, liver- and muscle-specific SIRT3 deficiency did not effect global metabolic homeostasis, despite hyperacetylation of mitochondrial proteins (Fernandez-Marcos *et al.*, 2012). These data suggest efficient redundancy mechanism exist *in vivo.*

Taken together, sirtuins potentially regulate mitochondrial function in numerous ways, including direct interaction with ETC proteins, through modulating mitochondrial transcription factors, or indirectly, by reducing radical damage to the ETC and mitochondrial membranes. Strategies to increase sirtuin activity could therefore hold therapeutic potential for diseases in which mitochondrial dysfunction is responsible.

Sirtuins and Vascular Function

Sirtuins are also extensively implicated within vascular function, both directly and indirectly (Borradaile and Pickering, 2009). The importance of eNOS, and subsequent nitric oxide production, to the vasculature is well documented (Sandoo *et al.*, 2010) and discussed further in the section below. There is *in vitro* and *in vivo* evidence that SIRT1 directly interacts with eNOS, increasing its activity through deacetylation (Mattagajasingh *et al.*, 2007). This study also demonstrated that SIRT1 inhibition impaired endothelium-dependant dilation in rat aorta. The importance of SIRT1 to the vasculature is reinforced by research showing SIRT1 inhibition induced premature senescence of human umbilical vein endothelial cells (Ota *et al.*, 2007).

Sirtuins also have roles combatting oxidative stress and, closely coupled, inflammation. As previously discussed, RONS are a major player in the deterioration of vascular function with age (Wadley, Veldhuijzen Van Zanten and Aldred, 2013), decreasing nitric oxide availability and also increasing pro-atherogenic oxidised low density lipoprotein (ox-LDL) (Kita *et al.*, 2001). Similarly, inflammatory cytokines, which are inherently linked to oxidative stress (Wadley, Veldhuijzen Van Zanten and Aldred, 2013), are known to increase pro-atherogenic adhesion molecules (Pasceri, Willerson and Yeh, 2000) and downregulate eNOS expression (Zhang *et al.*, 2009).

Given the modulatory roles of sirtuins on RONS and anti-oxidant enzymes (Singh *et al.*, 2018), upregulating sirtuin activity could be a key mechanism for maintaining vascular health. Several sirtuins have been identified to modulate the expression of anti-oxidant genes and thus anti-oxidant

enzymes, via sirtuin-mediated deacetylation of transcription factors including nuclear factor E2-related factor 2 (NRF2), FOXO3a and PGC-1α (Singh *et al.*, 2018). Nuclear factor kappa B (NF-κB) is a redox-sensitive transcription factor, stimulated by both RONS and inflammatory cytokines to upregulate transcription of pro-inflammatory and pro-oxidative genes (Donato *et al.*, 2009). In humans, NF-κB activity increases with age (Donato *et al.*, 2007) and is correlated with impaired endothelium-dependant dilation (Pierce *et al.*, 2009). SIRT1 is a potent inhibitor of NF-κB (Yeung *et al.*, 2004) and thus could help modulate redox status and limit oxidative stress-induced vascular dysfunction, present in cardiovascular disease and the ageing process.

Nitric Oxide

Although nitric oxide is best known for its role within the vasculature, it is also an important signalling molecule involved in the regulation of metabolic homeostasis (Levine, Punihaole and Levine, 2012; Litvinova *et al.*, 2015); thus explaining why impairments in nitric oxide bioavailability are associated with numerous cardiometabolic diseases (Widlansky *et al.*, 2003). Nitric oxide is produced in tissues by four isoforms of nitric oxide synthase (NOS): endothelial (eNOS), mitochondrial (mtNOS), neuronal (nNOS), and inducible (iNOS) (Levine, Punihaole and Levine, 2012). These enzymes catalyse the production of nitric oxide from L-arginine, requiring oxygen and cofactors including NADPH, tetrahydrobiopterin (BH_4), heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin (Alderton, Cooper and Knowles, 2001).

Nitric Oxide and Vascular Function

Vascular nitric oxide is produced by the endothelial isoform, eNOS, and its vasoactive properties dictate its critical role as a regulator of vascular tone, blood pressure, and blood flow (Sandoo et al., 2010). Physiological stimuli that activate eNOS include insulin, vascular endothelial growth factor (VEGF), acetylcholine, and bradykinin (Förstermann and Sessa, 2012); however, mechanical shear stress is considered to be the most powerful regulator of eNOS activity and is instrumental for the correct pairing of skeletal muscle perfusion with metabolic demand (Niebauer and Cooke, 1996). Shear stress is a force produced in response to increased blood flow through vessels and is detected by endothelial mechanotransducers (Ando and Yamamoto, 2013), stimulation of which triggers the influx of calcium into the endothelial cells and activation of a signal transduction pathway, involving phosphatidylinositide 3-kinases (PI3K) (Papapetropoulos et al., 1997) and Akt (Fulton et al., 1999), that ultimately results in the phosphorylation of eNOS, increasing electron flux and enzymatic

activity. The subsequent nitric oxide formed diffuses in to the vascular smooth muscle cells (VSMC), stimulating guanylate cyclase to increase intracellular cyclic guanosine monophosphate (cGMP), causing VSMC to relax and allowing vessels to dilate (Carvajal et al., 2000).

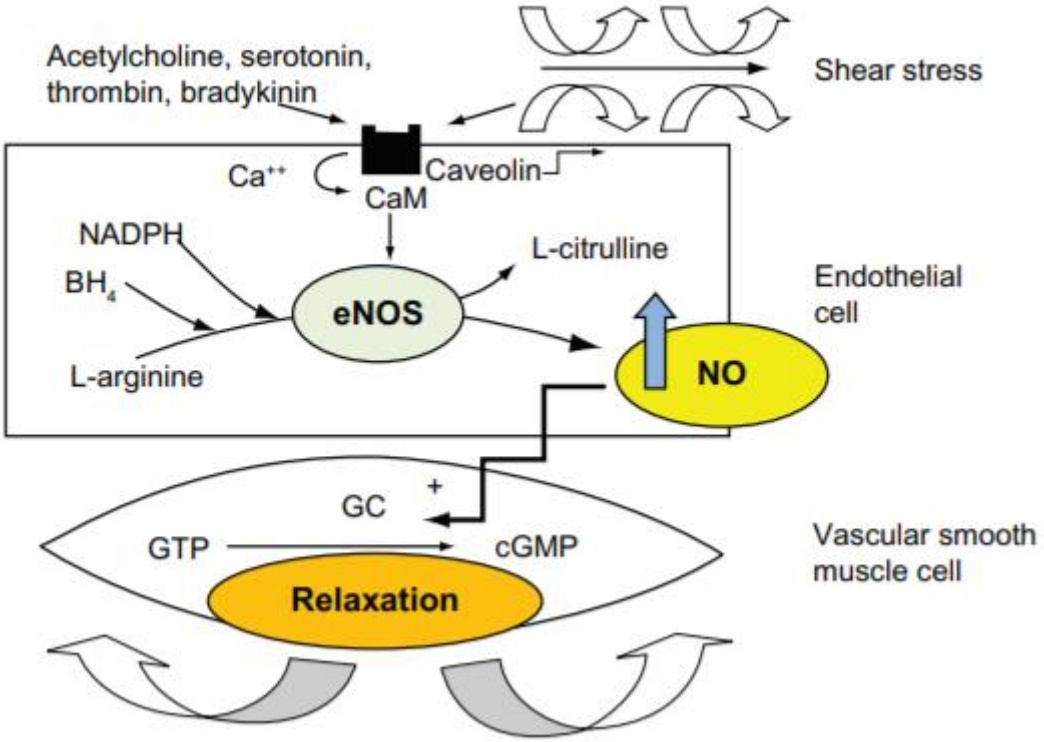

Figure 3: *Shear stress stimulates eNOS-mediated nitric oxide (NO) production which, upon diffusing into vascular smooth muscle cells (VSMC), triggers an increase in cyclic guanosine monophosphate (cGMP) causing VSMC relaxation and thus vessel dilation. (From Toblli et al. (2012)).*

As discussed, the activity of eNOS is regulated by several factors; including sirtuin-mediated deacetylation (Mattagajasingh *et al.*, 2007), bioavailability of the necessary substrate/co-factors (Förstermann and Sessa, 2012) and phosphorlyation status (Fulton, Gratton and Sessa, 2001). Shear-stress increases eNOS phosphorylation, a process initiated by elevations of intracellular calcium; however, phosphorylation of eNOS, independent of calcium, is sufficient to increase activity and production of nitric oxide from the enzyme (Fulton *et al.*, 1999). eNOS can be phosphorylated by various kinases, which target specific residues (Fulton, Gratton and Sessa, 2001), meaning strategies aimed at maximising the phosphorylation status of eNOS could optimise nitric oxide production and thus vasodilatory function.

Apart from its vasoactive role within blood vessels, nitric oxide possesses multiple anti-atherosclerotic properties that help maintain vascular function. These include the inhibition of platelet aggregation and adhesion to vessel walls, preventing the release of platelet-derived growth factors that stimulate VSMC proliferation (Förstermann and Sessa, 2012), and limiting expression of

adhesion molecules responsible for initiating atherosclerotic lesions (Förstermann and Sessa, 2012). Altogether, the importance of nitric oxide's role within the vasculature is abundantly clear and thus it is essential to maintain adequate bioavailability of this molecule.

Nitric Oxide and Mitochondrial Function

Although nitric oxide is primarily a vasoactive molecule, eNOS-derived nitric oxide has also been implicated in mitochondrial biogenesis (Nisoli and Carruba, 2006). In human coronary arterial endothelial cells, resveratrol-induced mitochondrial biogenesis was prevented by the inhibition of eNOS (Csiszar *et al.*, 2009). Furthermore, mice devoid of eNOS exhibit lower mitochondrial content in a variety of tissues, associated with impaired ATP production (Nisoli *et al.*, 2004); similarly, eNOS-knockout mice show impaired cold-induced mitochondrial biogenesis (Nisoli *et al.*, 2003). These effects of nitric oxide are thought to be mediated by a cGMP-dependant increased expression of PGC-1α (Nisoli *et al.*, 2003).

Nitric oxide is also thought to have roles within the mitochondria, interacting directly with mitochondrial proteins. Interestingly, nitric oxide elicits opposing acute and chronic effects on mitochondrial oxidative metabolism. Specifically, nitric oxide acutely inhibits ETC complexes (Brown, 1995; Brown and Borutaite, 2004), partially depolarising the mitochondrial membrane (Dedkova and Blatter, 2005) to reduce oxidative metabolism (Nisoli and Carruba, 2006). Conversely, nitric oxide increases oxidative metabolism chronically (Nisoli and Carruba, 2006), primarily mediated by upregulation of mitochondrial biogenesis, as previously discussed. Paradoxically, the acute nitric oxide-mediated inhibition of oxidative metabolism is associated with increased mitochondrial function. Nitric oxide impairs ETC complex activity; however, the resultant membrane depolarisation reduces RONS production (Gutierrez *et al.*, 2006), in turn limiting mitochondrial disruption and damage. It appears nitric oxide acutely improves mitochondrial efficiency by organising the ETC, allowing sufficient ATP production whilst lowering oxygen consumption and subsequent RONS production. Evidence also indicates that nitric oxide regulates mitochondrial beta-oxidation. Doulias *et al.* (2013) found that nitric oxide-mediated S-nitrosylation improves the catalytic efficiency of a key enzyme within this pathway, very long chain acyl-coA dehydrogenase (VLCAD), suggesting that impaired bioavailability may hinder fatty acid oxidation.

Evidence for the *in vivo* role of nitric oxide on whole-body oxygen consumption is equivocal. NOS inhibition increased basal oxygen consumption (estimated using cardiac output and oxygen extraction) by ~25% in conscious dogs (Shen *et al.*, 1994); whereas in humans, NOS inhibition had no effect on exercising limb oxygen consumption (Bradley, Kingwell and McConell, 1999; Rådegran and Saltin, 1999; Heinonen *et al.*, 2013). In contrast, exogenous nitrate supplementation, considered a

reliable method to augment endogenous nitric oxide (Stanaway *et al.*, 2017), has yielded relatively consistent results regarding oxygen consumption and metabolic efficiency in humans (Jones, 2014). Two studies investigating short-term nitrate supplementation demonstrate its effectiveness at reducing the oxygen cost of low-, moderate- and high-intensity exercise, as well as increasing time-to-exhaustion (S. J. Bailey *et al.*, 2009; Lansley, Winyard, Fulford, *et al.*, 2011). Similarly, acute nitrate supplementation improved cycling economy during a 4- and 16-km time-trial, by 11 and 7% respectively; although only the 4-km time trial met the statistical significance threshold (Lansley, Winyard, Bailey, *et al.*, 2011). However, similar research failed to observe changes in exercising oxygen consumption following acute nitrate supplementation (Garnacho-castaño *et al.*, 2018; Kocoloski and Crecelius, 2018). These whole-body and performance data are reinforced by investigation of skeletal muscle mitochondria harvested from individuals supplemented with nitrate, which show enhanced efficiency (Larsen *et al.*, 2011); as determined using the respiratory control ratio and oxidative phosphorylation efficiency ratio. Nitrate supplementation also appears to alter skeletal muscle bioenergetics during exercise, blunting the decline in phosphocreatine stores and decreasing total ATP turnover (S. J. Bailey *et al.*, 2010). Collectively, this evidence suggests that augmenting nitric oxide bioavailability in humans has the potential to improve metabolic efficiency during exercise by utilising its acute inhibitory effects on the mitochondrial ETC.

The discussed literature shows that nitric oxide is not only a vasoactive molecule, but an important regulator of transcription and signalling pathways that control energy metabolism. Ensuring adequate nitric oxide bioavailability is therefore essential to optimise vascular and mitochondrial function. Importantly, endogenous nitric oxide can be manipulated through exogenous nitrate supplementation or strategies that aim to augment eNOS activity/efficiency.

1.3. Relevance of Mitochondrial and Vascular Function

Ageing and Disease

Mitochondrial function is known to decline with age and is thought to be inherently linked to an age-related decline in NAD+ bioavailability (Mouchiroud, Houtkooper and Auwerx, 2013). Animal work has illustrated clear age-related NAD+ decline in diverse tissues (Braidy *et al.*, 2011; Yoshino *et al.*, 2011; Gomes *et al.*, 2013); however, evidence in humans is not so well documented. Cross-sectional research has indicated a negative correlation between NAD+ and age in human skin cells (Massudi *et al.*, 2012); in line with this, magnetic resonance-based *in vivo* NAD+ assays also show a negative correlation between intracellular NAD+ and age in the human brain (Zhu *et al.*, 2015). More recently, Dolopikou *et al.* (2019) found that compared to young, older individuals (23 vs 72 yrs) have significantly lower levels of erythrocyte NAD(P)H, suggesting impaired NAD+ metabolism. Overall,

the literature would suggest the existence of an age-related decline in NAD+. Individual's lifestyles will also influence NAD+ bioavailability, animal studies demonstrate high-fat diets reduce NAD+ in multiple tissues (Bai *et al.*, 2011; Yoshino *et al.*, 2011; Canto *et al.*, 2012; Okabe *et al.*, 2019), whilst exercise positively affects the NAD+ pool (Cantó *et al.*, 2009, 2010). These lifestyle-mediated alterations in NAD+ are likely linked to the mitochondrial dysfunction observed in obese and insulin resistant populations (Bhatti, Bhatti and Reddy, 2017).

Expression/activity of the NAD+ consumer, CD38 increases with age humans (Polzonetti *et al.*, 2012). CD38-mediated NAD+ depletion is thought to be linked with elevated oxidative stress (Hu *et al.*, 2014; Musolino *et al.*, 2011) and age-related elevations in inflammation (Hogan *et al.*, 2019). Activity of another family of NAD+ consumers, PARPs, also increase with elevated oxidative stress (Martin-Guerrero *et al.*, 2020). Oxidative stress is elevated in elderly and metabolically challenged populations (Cui, Kong and Zhang, 2012; Manna and Jain, 2015).

Mitochondrial dysfunction can impair energy availability and increase RONS production, RONS-induced oxidative damage of mitochondrial DNA and proteins creates a positive-feedback loop for further deterioration (Cui, Kong and Zhang, 2012). Ultimately, mitochondrial dysfunction will disrupt cellular energy homeostasis, resulting in premature cell senescence and apoptosis (Vakifahmetoglu-Norberg, Ouchida and Norberg, 2017). Therefore, the mitochondria are thought to be heavily implicated within the ageing process (Lanza and Nair, 2010) and are considered to be an important factor in the pathology of a multitude of diseases; including, heart failure (Brown *et al.*, 2017), sarcopenia (Hiona and Leeuwenburgh, 2008), neurodegeneration (Lin and Beal, 2006; Zhou *et al.*, 2015) and type-2-diabetes (Petersen *et al.*, 2003; Abdul-Ghani and DeFronzo, 2008).

Similarly to the mitochondria, there is an age-related decline in vascular structure and function (Xu *et al.*, 2017). Endothelial dysfunction has been shown to precede atherosclerosis (Juonala *et al.*, 2004; Halcox *et al.*, 2009), the precursor to more severe cardiovascular diseases (CVD). It was reported that in 2016, CVD was responsible for 31% of global deaths, highlighting the importance for developing novel therapeutic strategies. This age-related decline is partially attributable to an oxidative stress-mediated reduction in nitric oxide bioavailability (Donato *et al.*, 2007). Lifestyle choices will also interfere with vascular function. Generally, acute and chronic exposure to high-fat or high-carbohydrate meals (Lacroix *et al.*, 2012; Mah and Bruno, 2012; Thom *et al.*, 2016), as well as sedentary behaviour (Zheng *et al.*, 2021), are associated with attenuations of vascular function. The development of hypertension and atherosclerosis are two integral components associated with the onset of CVD, usually regulated by the compliance-enhancing and anti-atherogenic effects of nitric oxide within the vasculature. Flow-mediated dilation (FMD) is an increasingly popular method to

non-invasively assess, predominantly, nitric oxide-mediated endothelial function (Green *et al.*, 2014). Meta-analyses have proven a clinical significance of FMD, concluding that a 1% improvement in FMD equates to a significant 8-13% reduction in CVD risk, relevant for both high- and low-risk populations (Green *et al.*, 2011; Ras *et al.*, 2013; Matsuzawa *et al.*, 2015). This evidence highlights the importance of maintaining nitric oxide availability to prevent age-, and lifestyle-related vascular impairments, which inevitably result in disease.

Exercise

Exercise is a stressor that tests the limits of the mitochondria's functional capacity. Endurance exercise performance relies on the continuous production of ATP through predominantly aerobic pathways, pathways reliant on NAD(H) for redox reactions. During periods of higher glycolytic flux, to maintain NAD+ levels and allow continuation of energy production, NADH reduces pyruvate to lactate, rather than shuttling electrons to the ETC for oxidative phosphorylation. This shift towards anaerobic energy systems does not allow for complete oxidation of glucose, limiting ATP yield per glucose molecule. Interventions that boost the NAD+ pool may therefore allow more NADH to be shuttled to the ETC at a given glycolytic flux (which corresponds to exercise intensity), improving energy efficiency and reducing lactate production.

NAD(H) is also essential for fatty acid metabolism, acting as a co-factor for the key beta-oxidation enzyme, 3-hydroxyacyl-CoA dehydrogenase (Houten *et al.*, 2016). This enzyme is commonly used as an *ex vivo* marker of mitochondrial beta oxidation capacity, based on research that found its activity/abundance increases following endurance exercise training (Kiens *et al.*, 1993; Shaw *et al.*, 2020). The NAD+ requirements of complete fatty acid oxidation outweigh that of a glucose molecule. For example, the long chain free fatty acid, palmitate, has a 16 carbon aliphatic chain and thus requires 7 NAD+ (seven rounds of beta oxidation) (Talley and Mohiuddin, 2022). In comparison, glucose requires only 4 NAD+ (glycolysis and link reaction) (Chaudry and Varacallo, 2021). Therefore, under conditions of attenuated NAD+ availability, the bountiful source of energy from beta-oxidation could be compromised.

Mitochondrial efficiency is essential for exercise performance, improving the ATP yield per oxygen molecule and thus reducing the oxygen cost of a given workload. As outlined above, nitric oxide appears to have a key role regulating this efficiency through its interactions with the ETC (Lansley, Winyard, Bailey, *et al.*, 2011; Lansley, Winyard, Fulford, *et al.*, 2011; Larsen *et al.*, 2011).

During exercise, the vasculature is responsible for the large redistribution of blood flow to active skeletal muscle, providing valuable oxygen and nutrients for energy production whilst removing detrimental, efficiency comprising waste products. It is therefore unsurprising that perfusion is well

documented to be tightly coupled with metabolic demand (Joyner and Casey, 2015). During steady-state exercise, blood flow is thought to be primarily regulated by mechanical mechanisms, specifically the muscle pump and elevations in shear-stress, as well as a cocktail of local vasodilatory metabolic factors (Joyner and Casey, 2015). Given that nitric oxide is such an important vasodilator, produced in response to shear-stress, increasing its bioavailability during exercise could enhance perfusion to active skeletal muscle. In normoxic conditions, maximal aerobic capacity is limited by skeletal muscle perfusion (Bassett and Howley, 2000), and thus the ability to augment this during exercise could potentiate performance improvements.

Somewhat surprisingly, eNOS inhibition during exercise appears to have no significant effect on exercising muscle blood flow (Bradley, Kingwell and McConell, 1999; Rådegran and Saltin, 1999; Heinonen *et al.*, 2011, 2013); only during rest and recovery (Rådegran and Saltin, 1999; Heinonen *et al.*, 2011, 2013), or when combined with other inhibitors (Boushel *et al.*, 2002; Kalliokoski *et al.*, 2006; Heinonen *et al.*, 2011), does it detrimentally impact blood flow. However, baseline (Totzeck *et al.*, 2012) and post-exercise (Rassaf *et al.*, 2007) nitrite concentration, indicative of eNOS-mediated nitric oxide synthesis (Lauer *et al.*, 2001), are positively associated with exercise capacity, suggesting nitric oxide is indeed key to optimising exercising blood flow. Both of these studies also assessed baseline endothelial function using FMD, reporting similar positive associations with exercise capacity. However, the relationship between FMD and exercise performance is not clear cut, with some evidence illustrating a negative association between training status and FMD (Schroeder *et al.*, 2019). FMD is inversely related to baseline arterial diameter (Herrington *et al.*, 2001), meaning training-induced vascular remodelling (increased arterial diameter) (Green *et al.*, 2017) could interfere with interpretation of results.

Overall, this evidence highlights the integral roles of mitochondrial and vascular dysfunction within disease, as well as how optimising these functionalities could improve exercise efficiency, and ultimately exercise performance for healthy individuals. In particular, strategies aimed at increasing nitric oxide and NAD+ bioavailability have potential to positively influence both mitochondrial and vascular function *in vivo,* posing an exciting avenue to explore potential health-related and ergogenic effects.

1.4. Dietary Interventions Relating to Mitochondrial and Vascular Function

Dietary interventions targeting mitochondrial and vascular function are becoming increasingly more popular. Many of these have been designed to manipulate the, previously discussed, 'key players', including NAD+ precursors and polyphenols. Nutraceutical supplements aim to compliment the diet

but also provide health benefits. The following sections aim to provide a brief history of these two supplemental strategies as well as an overview of their potential effects.

NAD+ Precursors

NAD+ precursors are generally forms of vitamin B3 that aim to boost the intracellular NAD+ pool (e.g. nicotinic acid, nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR)). The therapeutic potential of NAD+ precursors arise from NAD+'s pivotal role in three separate systems, as described detailed earlier. To summarise, these potential pathways are: i) increasing mitochondrial NAD+ bioavailability to help promote oxidative metabolism, increasing electron shuttling to the ETC and increasing beta-oxidation; ii) improving redox status by increasing NADP(H), a key regulator of redox homeostasis - the disturbance of which is associated with the pathology of numerous cardiometabolic dysfunctions and diseases, and iii) elevating activity of the NAD+-dependant enzymes, sirtuins, known to positively regulate a vast number of pathways related to metabolism, redox status, and the vasculature.

Historically, nicotinic acid has successfully been used as a treatment for pellagra, a disease related to a nicotinamide deficiency (Elvehjem *et al.*, 1937). Nicotinic acid is also widely recognised for its positive effects on dyslipidaemia, favourably altering levels of high- and low-density lipoproteins whilst reducing levels of triacylglycerol in plasma (Altschul, Hoffer and Stephen, 1955). The mediator of these lipid lowering effects remains unclear; however, with contradictory results regarding the involvement of the GRP109A receptor, it is likely that an NAD+-mediated increase of sirtuin activity is responsible (Okabe *et al.*, 2019). In practice, the clinical use of nicotinic acid was limited due to poorly tolerated side-effect of cutaneous flushing (Birjmohun *et al.*, 2005).

In more recent years, the NAD+ precursors NR and NMN have attracted attention after demonstrating therapeutic metabolic and vascular effects in rodent models (Yoshino *et al.*, 2011; Canto *et al.*, 2012; Khan *et al.*, 2014; de Picciotto *et al.*, 2016; Okabe *et al.*, 2019). These findings prompted the start of clinical trials investigating the possible health benefits of NAD+ precursors. Recent data highlight promising effects on blood pressure (Martens *et al.*, 2018) and redox status (Dolopikou *et al.*, 2019; Elhassan *et al.*, 2019). Larger scale clinical studies using a variety of demographics are necessary to confirm these potential health benefits. Despite promising results regarding endurance capacity (Canto *et al.*, 2012; Costford *et al.*, 2018) and energy production (Gomes *et al.*, 2013) in animals, little work has been done to determine the possible exercise-related benefits of NAD+ augmentation, and whether these supplements could be utilised by athletes during training or competition.

Polyphenols

Polyphenols are a family of organic phytochemicals known to exert beneficial cardiometabolic effects in humans (Arts and Hollman, 2005; Liu *et al.*, 2014; Berman *et al.*, 2017; Cory *et al.*, 2018; Weaver *et al.*, 2021). There are over 8,000 identified phenolic compounds, sub-categorised in to four groups: flavonoids, phenolic acids, lignans, and stilbenes (in order of prevalence) (Pandey and Rizvi, 2009). They occur naturally in the diet, primarily in fruits and vegetables, but also in nuts and cocoa-containing foodstuffs (Woodward *et al.*, 2018). The therapeutic potential of these biomolecules was discovered after observation of the 'French Paradox' in the 1980s, whereby the French population exhibited low incidence of coronary heart disease, despite high intakes of saturated fats and tobacco use (Ferrières, 2004). This paradox was subsequently attributed to their moderate red wine consumption (Renaud and De Lorgeril, 1992), shown to contain a complex variety of polyphenols (Dell'Agli, Buscialà and Bosisio, 2004), termed red wine polyphenols (RWP). This discovery prompted further research in to whether the consumption of polyphenol-rich foods offered cardiometabolic protection. It is now understood that the health benefits associated with the 'Mediterranean Diet' are not solely mediated by polyphenols, but also related to higher intakes of nitrates and polyunsaturated fatty acids (Estruch *et al.*, 2018). The therapeutic action of polyphenols has been largely attributed to their anti-oxidant and anti-inflammatory capacities (Zhang and Tsao, 2016), however, it is now apparent that polyphenols can interact (directly or indirectly) with key metabolic and vascular enzymes to elicit beneficial cardiometabolic effects (Kim *et al.*, 2014; Chung *et al.*, 2010; Momtaz *et al.*, 2019).

Resveratrol is arguably the most extensively researched red wine polyphenol; alongside its anti-oxidant capacity (Xia *et al.*, 2017), animal data show resveratrol can stimulate activity of eNOS (Li *et al.*, 2019), 5' adenosine monophosphate-activated protein kinase (AMPK) (Lan *et al.*, 2017) and SIRT1 (Borra, Smith and Denu, 2005), highlighting its potential to reach a diverse range of cardiometabolic process. Resveratrol-mediated upregulation of AMPK and SIRT1 protein levels have also been reported in human trials (Timmers *et al.*, 2011; Goh *et al.*, 2014; Hoseini *et al.*, 2019), improving various metabolic- and redox-related outcome measures. Resveratrol has illustrated largely consistent positive health effects in diseased animal models (Zordoky, Robertson and Dyck, 2014; Weaver *et al.*, 2021), but results from clinical studies are less conclusive (Novelle *et al.*, 2015; Xia *et al.*, 2017; Ramírez-Garza *et al.*, 2018). Generally, more pronounced effects are reported in vulnerable versus healthy populations. Despite irregular findings, it would appear resveratrol has the capacity to improve peripheral (Wong *et al.*, 2011, 2013; Li *et al.*, 2013) and cerebral (Kennedy *et al.*, 2010; Wightman *et al.*, 2014) vascular function, systolic blood pressure (Weaver *et al.*, 2021), glycaemic control (Liu *et al.*, 2014), and oxidative stress (Seyyedebrahimi *et al.*, 2018; Hoseini *et al.*, 2019).

Factors contributing to the inconsistent findings of clinical studies include: an inability to reproduce levels of control used in animal models, a lack of knowledge regarding the optimal dosage for humans, and resveratrol's inherent poor oral bioavailability (Walle, 2011). Although most of the literature focuses on resveratrol, other phenolic compounds have similar potential. Pterostilbene, a dimethyl analog of resveratrol, has demonstrated superior bioavailability (Kapetanovic *et al.*, 2011) whilst eliciting similar actions to resveratrol (Tsai, Ho and Chen, 2017).

The next section of this review will focus on two specific supplements, one from each of the previously discussed nutraceutical groupings, and how co-supplementation has been suggested to optimise their effects within the body. The summarised research will focus primarily on results from randomised clinical trials, in which either a placebo/control arm or a cross-over design was used. Existing knowledge will be reviewed whilst highlighting gaps in the literature that warrant further exploration.

1.5. Nicotinamide Riboside

The direct NAD+ precursor, nicotinamide riboside (NR), is a form of vitamin B3 found naturally, in small amounts, in milk (Trammell, Yu, *et al.*, 2016). In comparison to its predecessors, nicotinamide and nicotinic acid, animal data demonstrates that NR has superior pharmacokinetics (Trammell, Schmidt, *et al.*, 2016). Such data, amongst others (Airhart *et al.*, 2017; Conze, Brenner and Kruger, 2019), also illustrate that oral NR supplementation can increase plasma and peripheral blood mononuclear cell (PBMC) NAD+ bioavailability in healthy humans. Conze *et al.* (2019) and Trammell *et al.* (2016) both identified a dose-dependent effect of NR on NAD+, with Trammell suggesting the peak NAD+ response is observed 8 hours post-supplementation; although, considering measurements were only taken at 1, 2, 4, 8, and 24 hrs post-ingestion, it is plausible that the peak may occur later than this (but before 24 h). Interestingly, Frederick *et al.* (2015) suggests that the sites of action for NAD+ precursors reside outside of skeletal muscle, based on their observation that tissue-specific increases in NAD+ did not augment mitochondrial function in mice. Therefore, the ability of NR to reliably increase blood NAD+ metabolism in humans would imply it does have potential to elicit beneficial metabolic effects. However, contradictory animal research describes an important role of intramuscular NAD+ on muscle degeneration and endurance capacity (Frederick *et al.*, 2016; Costford *et al.*, 2018); even so, supplementation of NR in humans has also proven to increase skeletal muscle nicotinic acid adenine dinucleotide (NAAD) (Elhassan *et al.*, 2019), considered the most sensitive biomarker of NAD+ metabolism (Trammell, Schmidt, *et al.*, 2016). Importantly, while other NAD+ precursors, such as nicotinic acid and nicotinamide, have been limited due to unfavourable effects regarding cutaneous flushing (Birjmohun *et al.*, 2005) or the inhibition of sirtuins (Bitterman *et al.*, 2002), human-based studies to date using NR-based supplements have found no evidence of flushing (Airhart *et al.*, 2017;

Martens *et al.*, 2018; Conze, Brenner and Kruger, 2019), and the majority of animal-based studies indicate a stimulatory effect on sirtuins, specifically SIRT1 (Canto *et al.*, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014).

To date, only nine clinical trials supplementing isolated NR have been conducted. Of the nine, three were dedicated to determining either the safety and bioavailability (Airhart *et al.*, 2017; Conze, Brenner and Kruger, 2019) or the pharmacokinetic profile (Trammell, Schmidt, *et al.*, 2016) of NR, with the remaining six attempting to catalogue the influence of NR on cardiometabolic- or redox-related variables (Dollerup *et al.*, 2018; Martens *et al.*, 2018; Dolopikou *et al.*, 2019; Elhassan *et al.*, 2019; Remie *et al.*, 2020; Stocks *et al.*, 2021). The findings of these six trials (summarised in Table 1) will now be discussed. All six were randomised, double-blind, placebo-controlled trials, with only Dollerup *et al.* (2018) not including a cross-over arm. The majority were chronic in nature (3-12 wk), while Dolopikou *et al.* (2019) and Stocks *et al.* (2021) investigated acute (2 hr) and short-term (1 wk) effects, respectively.

Table 1: *Summary of all published clinical trials (as of May 2021) investigating the effects of nicotinamide riboside (NR) supplementation.*

PWV, pulse wave velocity; SBP/DBP, systolic/diastolic blood pressure; FMD, flow-mediated dilation; VOP, venous occlusion plethysmography; OGTT, oral glucose tolerance test; HEC, hyperinsulinemic euglycemic clamp; HLC, hepatic lipid content; FFM, fat-free mass; NEFA, non-esterified fatty acids; LDH, lactate dehydrogenase; TTE, time-to-exhaustion; MnSOD, mitochondrial superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; CAT, catalase; TNF-α, tumor necrosis factor; IL, interleukin.

	Population				Outcome Measures						
Study	**Health Status**	**Mean Age**	**n (M:F)**	**Supplementation**	**Vascular**	**Metabolic**	**Exercise**	**Mitochondria**	**Sirtuins**	**Redox Status**	**Inflammation**
Martens *et al.* (2018) (α = 0.006)	Healthy	65	24 (11:13)	1 g/d for 6 wks	↓ PWV (-45.1 m s^{-1}) (p = 0.033) ↓ SBP (-3.9 mmHg) (p = 0.048) ↓ DBP (-2 mmHg) (p = 0.030) ↔ FMD	↔ energy expenditure (rest) ↔ substrate utilisation (rest & exercise) ↔ insulin sensitivity (OGTT)	↔ VO_{2max} ↔ TTE ↔ motor function	N/A	N/A	N/A	N/A
Dollerup *et al.* (2018/2020)	Obese, insulin resistant	59	20 per group (40:0)	2 g/d for 12 wks	N/A	↔ energy expenditure (rest) ↔ substrate utilisation (rest) ↔ insulin sensitivity (HEC) ↓HLC (-2% vs 0.2%) (p = 0.13) ↔ body composition	N/A	↔	↔ global skeletal muscle acetylation status	N/A	N/A
Remie *et al.* (2020)	Overweight / Obese	59	13 (6:7)	1 g/d for 6 wks	↔ SBP & DBP (24 hr)	↔ substrate utilisation (rest) ↔ insulin sensitivity (HEC) ↔ HLC **↑ FFM (1.34%)** **↑ sleeping metabolic rate**	N/A	↔	N/A	N/A	↔
Elhassan *et al.* (2019)	Healthy	75	12 (12:0)	1 g/d for 3 wks	↔ SBP & DBP ↔ VOP	↔ substrate utilisation (rest) ↔ insulin sensitivity (OGTT)	N/A	↔	↔ global skeletal muscle acetylation status	N/A	**↓ TNF-α** **↓ IL-2, -5, & -6**
Stocks *et al.* (2021)	Healthy	23	8 (8:0)	1 g/d for 1 wk	N/A	↔ substrate utilisation (rest & exercise) ↔ lactate (rest and exercise) ↔ glycerol (rest and exercise) ↔ NEFA (rest and exercise)	N/A	↔	↔ global skeletal muscle acetylation status ↔ p53 or MnSOD acetyation	N/A	N/A
Dolopikou *et al.* (2019)	Healthy young Healthy old	23 70	12 (12:0) 12 (12:0)	500 mg [measurements 2 hrs post)	N/A	<u>Old</u> **↑ LDH (ΔNR = 8% vs Δplacebo = -13%)** ↑ isokinetic lactate	<u>Old</u> **↑ isometric peak torque** **↑ fatigue resistance** ↔ concentric peak torque, VO_{2max}	N/A	N/A	<u>Old</u> **↑ NAD(P)H** **↓ baseline F_2-isoprostanes (-18%)** ↑ glutathione (ΔNR = 17% vs Δplacebo = -8%) (p = 0.063) **↑ GR** ↔ SOD, CAT, GPx,	N/A
					N/A	<u>Young</u> ↔ LDH	<u>Young</u> ↔ VO_{2max} ↔ isometric/concentric peak torque ↔ fatigue resistance	N/A	N/A	<u>Young</u> **↑ NAD(P)H** **↓ F_2-isoprostanes (ΔNR = -6% vs Δplacebo = 13%)** ↑ baseline SOD (16%) (p = 0.058) **↑ SOD (ΔNR = 16% vs Δplacebo = 13%)** **↓ GPx** ↔ CAT	N/A

Chronic Interventions

In 24 older individuals (aged = 65±7), Martens *et al.* (2018) found 6 week NR supplementation (1 g/d) significantly increased PBMC NAD+ by 60%, and stimulated a near fivefold increase in NAAD, the most sensitive biomarker of NAD+ synthesis (Trammell, Schmidt, *et al.*, 2016). NR had a tendency to reduce both blood pressure (systolic, -3.9 mmHg; diastolic, -2.0 mmHg) and carotid-femoral pulse wave velocity (PWV), considered the clinical "gold-standard" measurement of aortic arterial stiffness. Although these vascular effects are promising, not all variables followed suit, with brachial artery FMD exhibiting no change. In line with this, metabolic variables including, energy expenditure, substrate utilisation, and insulin sensitivity were unchanged post-supplementation. It is therefore unsurprising that maximal and sub-maximal exercise capacity remained unaffected. The authors suggest that the observed reductions in systolic blood pressure were primarily driven by participants with stage 1 hypertension, indicating a greater potential of NR supplementation in vulnerable, versus healthy, populations.

Dollerup and colleagues (2018) used a higher dose (2 g/d) for 12 weeks in 20 obese, insulin resistant men, with a further 20 receiving placebo (Dollerup *et al.*, 2018). Supplementation significantly increased a plethora of urinary NR- and NAD+-derived metabolites; however, similarly to the aforementioned study, NR lacked metabolic action, eliciting no changes in energy expenditure, substrate utilisation, insulin sensitivity or body composition. One encouraging, non-significant, finding was a larger reduction in hepatic liver content (HLC) in the NR treated group (-2.0%), compared with placebo (-0.2%) - especially when considering the NR group's pre-treatment HLC was 2.8% lower than placebo. Hepatic steatosis is associated with a worse risk profile for a number of cardiometabolic diseases (Ndumele *et al.*, 2011), strategies to reduce HLC could therefore have wide-ranging health benefits. Recently, further analysis of this data set has been published, primarily focussing on NR-mediated changes of mitochondrial function (Dollerup *et al.*, 2020). NR had no effect on numerous markers of mitochondrial function; including, maximal oxidative capacity, oxidative phosphorylation (OXPHOS), SIRT3 protein levels, citrate synthase activity, and PGC-1α. Potentially explaining why NR did not induce changes in glycaemic control, levels of proteins relating to glucose metabolism, such as glucose transporter 4 (GLUT4), were unaffected. The therapeutic actions of NR are hypothesised to be partially attributable to increased sirtuin-mediated deacetylation of proteins and transcription factors (Canto *et al.*, 2012; Khan *et al.*, 2014; Wang *et al.*, 2018); however, alongside the absence of metabolic actions, NR did not modify the acetylation status of muscle proteins, despite stimulating NAD+ metabolism. Interestingly, participants given placebo experienced a significant 20% fall in the content of intra-myofibrillar mitochondria in type 1 fibres, whereas no change was seen in the NR

group. This would suggest NR prevents natural attenuations in mitochondrial fractional area within this population; however, without a cross-over arm, the impact of between-group differences is unknown. Only males were included in this study and therefore the possibility of sex differences in the response to NR cannot be excluded, a suggestion that has recently been proposed (Remie *et al.*, 2020).

A recently published study adopted the same supplementary protocol as Martens *et al.* (2018), dosing NR at 1 g/d for 6 weeks (Remie *et al.*, 2020). Thirteen overweight or obese participants (aged 59±5) were recruited. Similar to other research (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020), NR elicited no change in steady-state NAD(H) or NADP(H) concentrations in skeletal muscle; however, levels of NAAD increased by 677%. One novel observation was that NR had a small, but significant, beneficial effect on fat-free mass (+1.33%), the authors suggest this increase was responsible for the concurrent significant elevations in sleeping metabolic rate. These data are in contrast to previous research, employing supplementary periods of 6 (Martens *et al.*, 2018) and 12 (Dollerup *et al.*, 2018) weeks, both of which failed to report NR-induced changes in body composition. Interestingly, the observed improvements in fat-free mass were primarily driven by the females within the cohort, this could possibly relate to the natural higher body fat percentage seen in women (A. S. Jackson *et al.*, 2002). Interestingly, NR significantly altered skeletal muscle carnitine metabolism, increasing baseline and exercise-induced [acetylcarnitine] in skeletal muscle. Given the roles of carnitine in the transport of long chain fatty acids in to the mitochondria, and as an acetyl group buffer during periods of high acetyl-CoA generation (Stephens, Constantin-teodosiu and Greenhaff, 2007), NR-mediated alterations in acetylcarnitine may be indicative of NR-mediated adjustments in oxidative metabolism. Although these results are promising, statistical analysis did not include adjustments for multiple comparisons, and therefore the possibility of type 1 errors cannot be excluded. In line with the aforementioned studies, NR supplementation had no effect on mitochondrial function, insulin sensitivity (whole-body, hepatic, or white adipose tissue), hepatic and skeletal muscle lipid accumulation, or resting substrate oxidation. The present studies results regarding HLC do conflict with those of Dollerup *et al.* (2018), who reported a non-significant declines post-supplementation. This discrepancy is possibly explained by differences in durations of supplementary periods or daily dosage; however, more likely is the fact that, in contrast to the population studied by Dollerup *et al.* (2018), the cohort studied by Remie *et al.* did not have elevated baseline HLC. With regard to NR-mediated vascular effects, unlike Martens *et al.* (2018), NR did not elicit changes in blood pressure, along with a host of other markers of cardiac function; however, the cohort's cardiac status was deemed healthy, with Martens *et al.* (2018) suggesting that NR-mediated vascular effects may be more apparent in those with pre-existing dysfunction.

The final remaining chronic NR intervention was conducted by Elhassan *et al.* (2019); where 12 aged individuals (median age = 75), with no history of metabolic disease, received NR at 1 g/d for 3 weeks. As with the other studies reviewed above, NR increased whole-blood NAD+ levels, whereas in muscle, levels of NAAD, but not NAD+, were elevated. Consistent with previous work, NR did not change insulin sensitivity, resting substrate oxidation, or *ex vivo* mitochondrial bioenergetics, including citrate synthase activity, maximal oxidative capacity, and OXPHOS protein levels. Interestingly, NR downregulated genes related to energy metabolism, this is contrary to animal data suggesting an upregulation of genes relating to oxidative metabolism (Canto *et al.*, 2012; Gong *et al.*, 2013; Khan *et al.*, 2014). However, this downregulation did not affect the expression of glycolytic enzymes or mitochondrial bioenergetics. Surprisingly, given that the cohort's baseline mean systolic and diastolic blood pressure was at least 10 mmHg greater than the population studied by Martens *et al.* (2018), NR did not improve either of these variables. These data would indicate that a 3-week supplementary period is not long enough for NR to have measurable vascular effects. Changes in forearm muscle blood flow were also assessed, using venous occlusion plethysmography (VOP). NR induced no changes in muscle blood flow at baseline or following a glucose load, a stimulus which increases blood flow. Dollerup *et al.* (2020) and Elhassan *et al.* (2019) both found that muscle protein acetylation status was unaffected following NR supplementation, suggesting that sirtuins were not upregulated and may therefore explain why the potential cardiometabolic actions of NR remain absent. Despite this, NR supplementation did appear to beneficially regulate inflammatory markers, significantly suppressing levels of circulating pro-inflammatory cytokines; specifically, tumour necrosis factor alpha (TNF-α) and interleukins (IL) 2, 5 and 6. In contrast, Remie *et al.* (2020) found 6 weeks of NR to have no effect on numerous inflammatory markers, apart from a tendency to decrease IL-α; however, TNF-α and IL-12 were the only shared markers between the two studies.

Recently, Stocks *et al.* (2021) utilised a short-term NR supplementation protocol (1 g/d for 1 week) in healthy, young male adults (n=8). Due to the lack of significant effects reported in previous sedentary clinical trials, as outlined above, this study aimed to potentiate the beneficial effects of NR by using exercise to induce cellular stress. Participants completed 1 hour steady-state cycling exercise (60% W_{max}) to examine changes in a range of metabolic markers; including, carbohydrate and fat oxidation, RER, lactate, glycerol, and non-esterified fatty acids (NEFA). No differences were seen in these variables following NR supplementation during exercise, or at rest. This may relate to the lack of change in skeletal muscle NAD+, despite a successful NR-induced increase in NAD+ metabolism. Muscle protein acetylation status was assessed to infer NR-induced changes in sirtuin activity; similarly to Dollerup *et al.* (2020) and Elhassan *et al.* (2019), no effect was seen. Specific acetylation of respective downstream targets of SIRT1 and 3 were also measured, but did not differ between groups.

Further corroborating previous findings, NR did not alter baseline *ex vivo* mitochondrial function or expression of PGC-1α. Despite the lack of NR-mediated changes in substrate metabolism, the concept of using a cellular stressor, such as exercise or hypoxic exposure (or even in combination), to potentiate effects is still valid and should be further explored. If repeated within a population with pre-existing NAD+ deficiency, this strategy may indeed elicit meaningful effects of NR in humans. Stocks and colleagues do acknowledge that the study was primarily powered to detect changes in exercising fat oxidation, and therefore NR-mediated effects on other variables may have been hidden.

Summary

The literature does show that NR supplementation reliably stimulates NAD+ metabolism in humans, assessed using biomarkers in blood, urine and skeletal muscle. However, data indicate that, in muscle, NR does not elicit changes in steady-state NAD+ levels (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Remie *et al.*, 2020; Stocks *et al.*, 2021). These findings highlight that NAD+ is under tight homeostatic regulation in humans, which may be responsible for the discrepancies in results between animal and clinical studies. Although this may appear damaging for the efficacy of NR, rodent studies have documented beneficial actions of NR in the absence of elevated muscle NAD+ concentrations (Khan *et al.*, 2014; Frederick *et al.*, 2016). It is possible that skeletal muscle NAD+ content is augmented in the acute stages of NR supplementation (first 24 hrs), before regulation by negative feedback loops, which may therefore allow effects to be observed.

An increase in sirtuin-mediated NAD+ degradation could potentially explain why skeletal muscle NAD+ levels seem to be unaffected by NR. However, opposing this theory are data from Dollerup *et al.* (2020), Elhassan *et al.* (2019), and Stocks *et al.* (2021), which show no change in muscle acetylation status following NR supplementation. These results may be linked to pharmacokinetic data from rodents that suggest that the majority of orally consumed NR is extensively metabolised at the liver in to nicotinamide (Liu *et al.*, 2018), a sirtuin inhibitor (Avalos, Bever and Wolberger, 2005). Co-supplementation of NR alongside a small-molecule sirtuin activator may be necessary to offset the potential inhibitory effects of nicotinamide potentiate effects, and the lack of change in muscle NAD+ bioavailability, thought to be a key regulator of sirtuin activity (Houtkooper, Pirinen and Auwerx, 2012).

Taken together, these intervention studies demonstrate a potentially limited effect of NR on metabolic pathways and mitochondrial function, aside from positive trends regarding HLC (Dollerup *et al.*, 2018), maintenance of mitochondrial fractional area in type 1 fibres (Dollerup *et al.*, 2020), and elevations in fat free mass (Remie *et al.*, 2020). The lack of effect in metabolically challenged cohorts is somewhat surprising, particularly when animal models yield consistent metabolic improvements

(Okabe *et al.*, 2019) and *in vitro* human data suggest positive mitochondrial outcomes (Felici *et al.*, 2015; Traba *et al.*, 2015). Dollerup *et al.* (2018) proposed that the inability to differentiate whether the beneficial effects of NR in animals are direct, or secondary to a reduction in weight gain, is partially attributable to the equivocal findings of animal and clinical trials. This relates to the fact that in humans, obesity and its associated metabolic disorders (e.g., insulin resistance) develop over decades, whereas in animals, high-fat diet-induced obesity occurs over a much shorter time frame. Another factor to consider is that any given supplementary period within rodents will represent a much larger percentage of their lifespan. This suggests that to potentiate the beneficial actions of NR in humans, supplementary periods need to be in the range of months-years, rather than weeks. In support of this, 6 month acipixmox supplementation, a nicotinic acid analog, improved metabolic parameters in obese individuals, significantly reducing fasting glucose and tending to reduce insulin resistance, assessed by homeostatic model assessment of insulin resistance (HOMA-IR) (Makimura *et al.*, 2016). In addition, differences in dosages between rodent and human trials may explain why clinical trials observe little significant effects of NR. Stocks *et al.* (2021) highlight how a common 400 mg/kg/d dose in rodents, which elicits significant beneficial metabolic outcomes (Canto *et al.*, 2012), is comparable to 2 g/d in humans. This dosage is double that used in the majority of clinical NR trials, and even when utilised did not significantly improve metabolic outcomes in an obese population (Dollerup *et al.*, 2018).

With regard to vascular function, only one chronic intervention documented favourable changes; specifically, in relation to blood pressure and arterial stiffness, with the largest effects seen in hypertensive individuals (Martens *et al.*, 2018). This replicates results from rodent studies that have used the NAD+ precursor, NMN, to improve endothelial-dependent dilation and arterial stiffness by reducing oxidative stress (de Picciotto *et al.*, 2016), and elevate resting skeletal muscle perfusion through SIRT1-dependent increases in capillary density (Das *et al.*, 2018). On the other hand, Elhassan *et al.* (2019), Remie *et al.* (2020), and a safety trial by Conze *et al.* (2019) (1 g/d NR for 8 weeks) found no effect of NR on blood pressure; however, populations of the latter two trials had blood pressure within the normal healthy range, and the other implemented a relatively short (3 week) supplementary period (Elhassan *et al.*, 2019). These vascular data follow suit with metabolic results, which suggest that NR-mediated improvements in HLC are only apparent in populations with an elevated baseline (Remie *et al.*, 2020). It would appear that a certain degree of pre-existing dysfunction and a supplementary of duration of >3 weeks is necessary in order for NR to exert beneficial vascular effects.

The chronic data surrounding the effect of NR on inflammation is also somewhat conflicting, with Elhassan *et al.* (2019) reporting significant reductions, whilst Remie *et al.* (2020) saw no changes. The prevalence of chronic, systemic inflammation increases with age and is implicated in multiple age-

related diseases (Chung *et al.*, 2009). This age-related increase potentially explains why, compared to the population studied by Elhassan *et al.* (median age = 75), the relatively younger cohort studied by Remie *et al.* (aged 59±5) experienced no anti-inflammatory effects. This would further reinforce the vascular and metabolic data that indicate NR will have the greatest impact in populations with the most severe pre-existing impairment. The anti-inflammatory mechanism of NR is still unclear, with authors suggesting that NR potentially opposes the NAD+-consuming actions of CD38, expression of which increases with age (Polzonetti *et al.*, 2012). Another explanation relates to the inherent coupling of oxidative stress and inflammation by NF-κB, with increases in one upregulating the other (Donato *et al.*, 2009). Recently, NR has improved redox status in humans (Dolopikou *et al.*, 2019) and depressed mitochondrial membrane potential in mice (Vannini *et al.*, 2019), a principal regulator of radical production (Gutierrez *et al.*, 2006). Research also indicates that NR elevates NADPH levels in blood (Martens *et al.*, 2018; Dolopikou *et al.*, 2019), but not skeletal muscle (Dollerup *et al.*, 2020; Remie *et al.*, 2020). NADPH is an essential co-factor for glutathione peroxidase- and peroxiredoxin-mediated RONS removal (Buettner, G, Wagnera, B, and Rodgers, 2013). Therefore, NR-mediated reductions in oxidative stress could suppress NF-κB-mediated increases of inflammatory cytokines. Further research is warranted to determine the therapeutic potential of NR in chronic inflammatory diseases.

Finally, it is important to recognise that studies to date have primarily investigated the effects of NR on mostly males, with only 20 of the summated 97 participants being female. Therefore, the applicability of their findings is therefore inherently limited to one sex and does not allow for the detection of potential sex differences in the response to NR. Interestingly, Remie *et al.* (2020) proposed that the significant elevations in fat-free mass observed following NR supplementation were driven primarily by the female portion of the cohort. NMN, another NAD+ precursor, has recently been shown to improve insulin sensitivity in prediabetic women following 10 week supplementation (250 mg/d) (Yoshino *et al.*, 2021), supporting the suggestion made by Remie and colleagues that NR may have superior metabolic effects within a female population. Clearly, future research needs to endeavour to include female participants within their investigations in order to improve our understanding of possible sex-dependant responses.

Acute Interventions

To date, only one study has investigated the acute potential of NR supplementation, which focussed on its role within redox homeostasis and exercise performance (Dolopikou *et al.*, 2019). Just 500 mg of NR was supplemented to twelve old and twelve young male individuals (aged 71.5±1.0 and 22.9±1.0, respectively), measurements were recorded 2 hours post-supplementation. Significantly increased levels of erythrocyte NADH (59% or 51%) and NADPH (38% or 32%) were observed in both

groups, more so in the older cohort; however, only the older cohort experienced concurrent improvements in resting markers of redox status, with significant reductions in urinary F_2-isoprostanes (-18%), representing lipid peroxidation, and a tendency to increase erythrocyte glutathione (17%), a vital non-enzymatic anti-oxidant. Although resting F_2-isoprostanes did not significantly change in the young group, when the pre- to post-supplement change was compared with placebo, NR did significantly reduce F_2-isoprostanes. These results are likely related to the observed relative baseline impairments of these markers, and NAD(P)H levels, in the older group when compared to the young. Similar to the redox markers, only the older cohort saw improvements in exercise performance, measured using isometric knee extensor peak torque and fatigue resistance; whereas, $\dot{V}O_{2max}$ and concentric peak torque remained unaffected in both groups. In line with data from chronic interventions, this study would suggest a beneficial role of acute NR supplementation - only in populations with prior dysfunction/deficiencies, in this case relating to redox status. However, given that pharmacokinetic insight from Trammell *et al.* (2016) indicates the peak response of the NAD+ metabolome to NR occurs (at least) 8 hours post-supplementation, the 2-hour period used in the present study may restrict the ability of NR to elicit more pronounced effects, and thus contribute to the absence of significant functional change in the younger cohort. The authors also suggest the time frame used did not allow for post-translational modification of enzymes, possibly explaining why the activity of key anti-oxidant enzymes was unaffected. Again, this trial included only male participants meaning the results cannot be generalised to the female population without further investigation.

Future Directions

It is clear that clinical research involving NR supplementation is in its early stages and that new research is needed to consolidate its vascular-, metabolic-, and redox-related effects. Initial findings would suggest a therapeutic potential of chronic NR supplementation in cardiometabolic and inflammatory diseases; however, longer supplementary periods (≥ 6 months) may be necessary confirm longer lasting effects. Supplementation in the elderly (≥ 70 years), who are understood to exhibit NAD+ deficiencies, have demonstrated positive results regarding redox status and inflammation (Dolopikou *et al.*, 2019; Elhassan *et al.*, 2019). This avenue of research should be explored further, especially given the importance of radical- and inflammatory-damage to the ageing process (Chung *et al.*, 2009; Liguori *et al.*, 2018). Finally, future research needs to include females to allow determination of potential sex-dependant responses.

Acute supplementation appears to have more relevance within an exercise setting, as an ergogenic aid. This ergogenic potential was explored by Dolopikou *et al.* (2019); however, with this study's focus primarily on redox homeostasis, it lacked information regarding NR-mediated modifications of

vascular and metabolic variables, which are key to exercise performance. Using maximal aerobic capacity and peak torque to evaluate performance could also be considered to lack relevance, given NR's theoretical potential to boost oxidative metabolism and that the majority of sports/exercise involve varying degrees of continuous sub-maximal exercise and/or are limited by maximal oxidative capacity. Arguably, prior to attempting to augment exercise performance, the physiological effects of NR during exercise should be categorised first, in order to understand which exercise mode NR could most favourably affect. This was attempted by Stocks *et al.* (2021), who found no impact of NR on series of metabolic variables; however, the small sample size, short supplementary period, and lack of analysis of vascular variables mean further research is warranted. Skeletal muscle NAD+ is consistently reported to remain unchanged following short-term or chronic NR trials. In comparison, acute supplementation has the potential to utilise an elevated intra-muscular NAD+ pool before it is negatively regulated back to baseline levels, thus allowing NR to exert meaningful effects on the physiological responses to exercise.

NR is hypothesised to exert its beneficial effects through sirtuins, by increasing bioavailability of their substrate, NAD+. However, several studies reported no change in muscle deacetylation status following NR supplementation, an indicator of sirtuin activity (Elhassan *et al.*, 2019; Dollerup *et al.*, 2020; Stocks *et al.*, 2021). These data indicate that simply increasing substrate bioavailability is not enough to stimulate sirtuin activity in humans. Supplementing NR in combination with a sirtuin activator may be required to augment activity of this pathway, in turn promoting the hypothesised actions.

1.6. Pterostilbene

Pterostilbene (PT) is phenolic compound, a member of the stilbene family, which is found naturally in grapes, almonds, and blueberries. PT is a dimethyl analog of the more extensively studied polyphenol resveratrol, sharing a multitude of beneficial biological actions in animal models of disease (Tsai, Ho and Chen, 2017). The presence of these additional methoxy groups is what gives PT superior bioavailability compared to resveratrol (Liu *et al.*, 2020), due to an extended half-life (Lin, Yue and Ho, 2009) as well as increased oral and lipophilic absorption (McCormack and McFadden, 2013). A study in rats quantified this difference in bioavailability, concluding a substantial fourfold improvement (Kapetanovic *et al.*, 2011). To date, there is no human pharmacokinetic data regarding PT. In rodents, evidence shows the time taken to reach peak plasma concentrations (T_{max}) is longer than resveratrol and is somewhat dose-dependent, with a reported T_{max} of 2-4 hours (Lin, Yue and Ho, 2009; Kapetanovic *et al.*, 2011; Yeo, Ho and Lin, 2013). PT has numerous similarities to resveratrol with regards to the mechanistic pathways it interacts with. For example, PT has anti-oxidant and anti-

inflammatory capacities (Li, Li and Lin, 2018), with initial *in vitro* (Guo *et al.*, 2016; Zhang *et al*., 2021) and *in vivo* animal (Cheng *et al.*, 2016; Liu *et al.*, 2019) research also suggesting PT can activate SIRT1. Furthermore, *in vitro* data found PT to stimulate AMPK (Ren, Rimando and Mathews, 2018) and eNOS (Park *et al.*, 2015), as well as acting as a peroxisome proliferator activated receptor-alpha (PPAR-α) agonist (Rimando *et al.*, 2015). Taken together, these data show the diverse cellular pathways that PT has potential to modulate; thus, there is clear metabolic and vascular therapeutic potential of PT supplementation.

Clinical trials investigating isolated PT supplementation are lacking; to date, only two have been published (Riche *et al.*, 2013, 2014), both of which were randomised, double-blind, placebo-controlled trials, although both utilised the same sample cohort. Due to the lack of clinical data, the next portion of this review will summarise results from *in vivo* animal research using PT, as well as the previously mentioned clinical trials by Riche and colleagues. Given the similarities of resveratrol and PT, and in order to highlight promising avenues for PT supplementation; clinical studies, that are equally well-controlled and well-designed, focussing on the metabolic and vascular effects of resveratrol, and how these relate to exercise, will be discussed.

In Vivo Animal Trials

Although there are currently only a limited number of *in vivo* animal trials that have investigated PT, likewise to resveratrol, the majority report consistent health benefits of chronic supplementation in disease models. With regards to metabolism, PT reliably exhibits anti-obesogenic effects (Gómez-Zorita *et al.*, 2014; Aguirre *et al.*, 2016; La Spina *et al.*, 2019; Trepiana *et al.*, 2019), including reducing adipocyte lipogenesis, increasing hepatic fatty acid oxidation and attenuating weight gain. Similarly, PT supplementation has improved glycaemic control and insulin sensitivity in diabetic rats (Pari and Satheesh, 2006; Gómez-Zorita *et al.*, 2015; Elango *et al.*, 2016; Kosuru and Singh, 2017), with Pari and Satheesh (2006) and Kosuru and Singh (2017) positively comparing PT to metformin, the primary medication for type-2-diabetes. Several studies have also documented a cardioprotective role of PT, favourably altering lipid profiles in hypercholesteraemic hamsters (Rimando *et al.*, 2005) and attenuating the loss of cardiac function post-myocardial ischaemia/reperfusion injury in rats (Wu *et al.*, 2017; Yu *et al.*, 2017); however, both Wu *et al.* (2017) and Yu *et al.* (2017) administered PT intravenously and therefore results may lack relevance in humans. All of the discussed studies, bar one (Yu *et al.*, 2017), supplemented PT chronically, with several attributing beneficial health effects to PT-mediated anti-oxidant and anti-inflammatory actions (Kosuru and Singh, 2017; Wu *et al.*, 2017; Yu *et al.*, 2017). Overall, *in vivo* animal research would suggest a role of chronic PT supplementation in

metabolically challenged populations and in diseases related to oxidative stress; however, consistently translating these effects to humans is yet to be seen.

Pterostilbene Clinical Trials

Expectedly, the first published study investigating PT was primarily evaluating safety (Riche *et al.*, 2013). Here, PT was supplemented for 6-8 weeks at either a high (250 mg/d) or low (100 mg/d) dose in hypercholesteraemic participants, 55% of whom were also hypertensive. Both doses of PT were deemed safe with only a minimal number of low severity adverse reactions. During this study, alterations of various cardiometabolic parameters were also recorded, these data were presented in a separate publication the following year (Riche *et al.*, 2014). Over both doses, PT tended to dose-dependently attenuate blood pressure compared to placebo; although, these reductions only became significant with the high dose, lowering both systolic (-7.8 mmHg) and diastolic (-7.3 mmHg) blood pressure. In contrast to these beneficial vascular effects, both PT doses significantly increased low-density lipoproteins (LDL) to levels of public health concern. Alongside this, a sub-group analysis of participants not taking cholesterol medication revealed high dose PT significantly reduced levels of high-density lipoproteins (HDL). Evidently, with tremendous blood pressure attenuations on the one hand, and highly unfavourable alterations in lipid profiles on the other, preliminary clinical data for the use of chronic PT supplementation is conflicting. Further, as highlighted in the above sections describing NR supplementation, the ability to repeat findings from animal research in humans is by no means guaranteed. This is exemplified by the contradictory results, regarding PT on lipid profiles, documented by Rimando *et al.* (2005) and Riche *et al.* (2014). These promising vascular effects of PT should continue to be explored, particularly in hypertensives (without clinical levels of dyslipidaemia), with larger scale clinical trials.

Resveratrol Clinical Trials

Vascular Effects

A recent meta-analysis from Weaver *et al.* (2021) found that chronic resveratrol supplementation (n=18 studies) significantly reduced systolic blood pressure by 3.7 mmHg, reinforcing the promising actions of PT observed by Riche *et al.* (2014). In contrast, data from Weaver *et al.* (2021) show no effect of chronic RWP supplementation on vascular function in 8 studies (as indexed by measures of FMD), whereas all acute interventions analysed (n=3) did cause a significant improvement in FMD. Of the three studies, two administered resveratrol, either in an obese (Wong *et al.*, 2011) or hypertensive (Marques *et al.*, 2018) population. Wong *et al.* (2011) supplemented resveratrol in 19 individuals at a dose of 30, 90, and 270 mg, all of which significantly increased FMD one hour later by ~2.5-3.0%. Similarly, Marques *et al.* (2018) reported a mean 1.8% increase in FMD 1.5 hrs post-supplementation

(300 mg) in 24 participants. Significant improvements in endothelial function following acute RPW supplementation have also been reported in healthy populations (Li *et al.*, 2013). Given its superior bioavailability, it is highly probable that acute PT supplementation could also augment endothelial function within these vulnerable populations, but potentially in healthy individuals as well. PT-induced transient elevations in endothelial function could have implications within exercise performance or to counteract stimuli, such as post-prandial hyperglycaemia, which temporarily evoke endothelial dysfunction.

Metabolic Effects

As previously mentioned, findings from resveratrol clinical trials are far from consistent (Zordoky, Robertson and Dyck, 2014; Novelle *et al.*, 2015), likely related to the diversity in dosages, durations, and populations investigated. However, there are still an abundant number of well-designed studies that report beneficial metabolic effects. For example, three randomised, double-blind, placebo-controlled trials in type-2-diabetics (combined cohort size n=133), that supplemented resveratrol at 150 (Timmers *et al.*, 2011), 500 (Hoseini *et al.*, 2019), or 1000 (Movahed *et al.*, 2013) mg/d for 30, 28, or 45 days, respectively, all demonstrated favourable effects on blood glucose and insulin sensitivity. Alongside this, a meta-analysis of 11 studies reported that in diabetics, resveratrol significantly reduces fasting glucose, insulin, glycated haemoglobin, and insulin resistance (Liu *et al.*, 2014). Timmers *et al.* (2011) also reported resveratrol-mediated improvements in *ex vivo* mitochondrial function and PGC-1α protein content. These data indicate that PT may have potential to modulate glycaemic control, clinical trials in insulin resistant populations could potentiate these effects. The possibility of PT-induced changes in mitochondrial efficiency, and thus oxidative capacity/substrate utilisation should be explored.

Exercise Effects

Somewhat surprisingly, given resveratrol's vascular and metabolic potential, there is limited research investigating its interaction with exercise. There is some evidence to suggest an inhibitory effect of resveratrol on exercise training-induced adaptations (Gliemann *et al.*, 2013; Olesen *et al.*, 2014; Scribbans *et al.*, 2014); although, conclusions from Gliemann *et al.* (2013) have been criticised for over-interpretation of the data (Smoliga and Blanchard, 2013; Budford and Anton, 2014). Conversely, Alway *et al.* (2017) found that in response to 12 weeks exercise training, concurrent resveratrol supplementation augmented absolute maximal oxygen uptake, mitochondrial density, and knee extensor peak torque in older adults. Despite apparent *in vitro* anti-inflammatory actions of resveratrol, one week supplementation (600 mg/d) prior to a marathon elicited no suppression of post-marathon inflammatory markers or muscle soreness (Laupheimer *et al.*, 2014). However, results

should be interpreted with caution due to a limited sample size (n=7), no cross-over arm, and a lack of temporal consistency surrounding pre- and post-marathon measures. Within the context of exercise performance, Voduc *et al.* (2014) supplemented resveratrol for four weeks in sedentary adults and found no change in maximal aerobic capacity or time to exhaustion. Clearly, further research is warranted to conclusively establish the role of resveratrol on exercise physiology and performance, future work should use outcome measures with greater ecological validity such as time-trials. This summary highlights the lack of knowledge regarding the acute impact of resveratrol on the cardiometabolic responses to exercise, or how it could be used to enhance post-exercise recovery. With respect to PT, future studies investigating PT supplementation should first attempt to document how it modifies vascular and metabolic variables during exercise and recovery, prior to exploring its ergogenic potential for performance.

Future Directions

Evidently, little is known about the clinical applications of PT supplementation. The only clinical trial available would suggest that chronically, PT can beneficially affect the vasculature but potentially detrimentally alter lipid profiles. Future research should develop an understanding of PT's vascular potential, consolidating the effects seen regarding blood pressure and exploring its impacts on other vascular variables. Given resveratrol's ability to improve FMD, particularly in the acute setting, manipulation of this variable should also be targeted with PT supplementation, allowing a clear comparison between the two analogous stilbenoids. Alongside resveratrol's largely consistent positive effects in type-2-diabetics, *in vivo* animal research highlights anti-obesogenic and insulin sensitising actions of PT; together, these data advocate clinical studies investigating chronic PT supplementation in metabolically challenged populations, although lipid profiles should be closely monitored for adverse effects given the findings of Riche *et al.* (2014). Finally, the speculated metabolic and vascular effects of PT deem it a logical supplement to beneficially alter the cardiometabolic responses to exercise. These potential actions would likely favour oxidative metabolism and therefore future research should focus on sub-maximal exercise to potentiate this. The use of supplemental strategies to enhance the post-exercise recovery period, particularly post-exercise glucose uptake, are not well explored. Data from *in vivo* animal PT, and clinical resveratrol, trials both show supplemented-induced improvements of glycaemic control. These actions could potentially be utilised by athletes with busy training or competitive schedules to optimise their recovery of endogenous fuel stores.

1.7. Co-supplementation: Nicotinamide Riboside and Pterostilbene

NR and PT have been combined within a commercially available nutraceutical supplement, called Basis (Elysium Health) – for the remainder of this review this supplement will be referred to as NRPT. The combination of an NAD+ precursor with a polyphenol give this supplement potentially powerful metabolic-, vascular-, and redox-related actions. These actions could be benefit those in disease states, such as type-2-diabetes and atherosclerosis, or within exercise, as an ergogenic aid or to improve recovery. Of these potential actions, some are supplement-specific whereas others share a mechanistic pathway. For example, NR-induced increases of NAD(H)/NADP(H) bioavailability could directly alter mitochondrial bioenergetics (Oyarzún *et al.*, 2015) and improve recycling of non-enzymatic anti-oxidants back to their reduced form (Buettner, G, Wagnera, B, and Rodgers, 2013); likewise, PT can directly phosphorylate eNOS to increase nitric oxide bioavailability (Park *et al.*, 2015), or improve redox status through radical quenching or inhibiting radical production (McCormack and McFadden, 2013).

Importantly, both of these supplements share an ability to interact with the sirtuins family of enzymes, and in particular SIRT1, which may be key due to its regulatory role in copious cellular pathways (Borradaile and Pickering, 2009) and its proposed implications within a multitude of cardiometabolic diseases (Kane and Sinclair, 2018). *In vivo* animal research demonstrates that PT can upregulate SIRT1 activity (Cheng *et al.*, 2016; Liu *et al.*, 2019); alongside this, favourable actions of the PT analogue, resveratrol, in clinical trials have been partially attributed to increased SIRT1 activity (Timmers *et al.*, 2011; Hoseini *et al.*, 2019). As previously discussed, NAD+ acts as a substrate for sirtuins and therefore the bioavailability of this molecule can limit their activity (Borradaile and Pickering, 2009). Given sirtuins NAD+-dependency, the co-supplementation of NR alongside PT could be vital in order to optimise sirtuin-mediated effects. In support of this idea, the majority of NR (only) clinical trials have lacked meaningful changes in a variety of outcome measures (Table 1) including elevating sirtuin activity, which suggests that a sirtuin activator may help to potentiate NR's actions. Figure 4 below visually displays the pathways that NR and PT are hypothesised to regulate, and importantly highlights how the majority of these are shared between the two nutraceuticals.

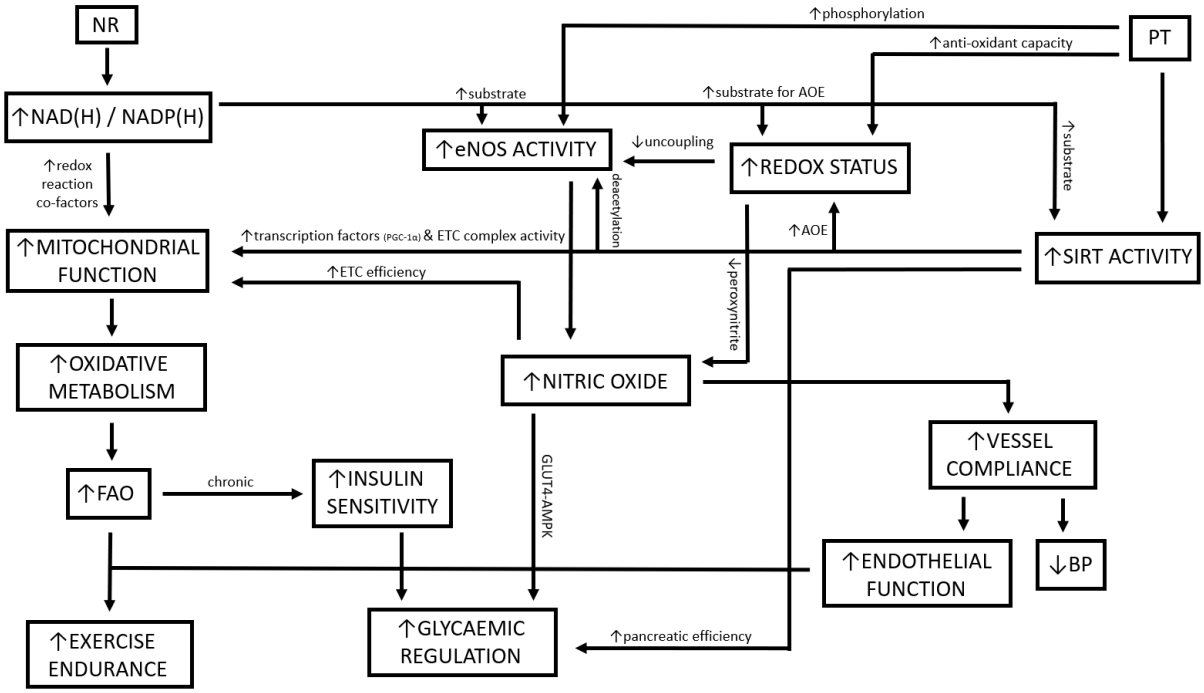

Figure 4: *An overview of the potential pathways, relating to the key players of mitochondrial and vascular function, that nicotinamide riboside (NR) and pterostilbene (PT) could interact with to affect vascular and metabolic outcomes.*

eNOS, endothelial nitric oxide synthase; SIRT, sirtuin; FAO, fatty acid oxidation; BP, blood pressure; ETC, electron transport chain; AOE, anti-oxidant enzymes.

Clinical Trials

To date, only three clinical trials investigating NRPT supplementation have been conducted, all utilising a randomised, double-blind, and placebo-controlled design. The first investigation by Dellinger *et al.* (2017) supplemented NRPT in 120 older adults at either the recommended daily dose (NR, 250 mg/d; PT, 50 mg/d), double the recommended dose (NR, 500 mg/d; PT, 100 mg/d), or placebo for 8 weeks. Overall, NRPT was well tolerated and significantly increased whole-blood NAD+ levels by 40% and 55% in the low and high dose groups, respectively. A promising finding of this work was a considerable decline in both systolic (-3.2 mmHg; non-significant) and diastolic (-3.4 mmHg; $p<0.05$) blood pressure following low, but not high, dose NRPT supplementation. However, despite all three groups experiencing increases in LDL after 8 weeks, both NRPT groups saw significantly higher, dose-dependent, elevations compared to placebo. The authors suggest this might relate to the significant between-group differences in baseline LDL concentrations, with changes primarily driven by those participants that were classified as being overweight (body mass index (BMI) = 25-32). These results from Dellinger *et al.* (2017) are comparable with those from Riche *et al.* (2014), in which chronic PT supplementation attenuated blood pressure but markedly increased LDL in participants with dyslipidaemia (mean BMI = 30). As such, these data consolidate the suggestion that PT supplementation, in overweight individuals, can adversely alter lipid profiles and thus future research investigating this cohort should be aware of this possibility; however, it also consolidates the potential

vascular enhancing actions on PT, which warrants further study and across a wider range of vascular health biomarkers (e.g. FMD, arterial stiffness).

Following this research, de la Rubia *et al.* (2019) supplemented NRPT in individuals with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease hypothesised to involve alterations in SIRT1 activity. Participants received either four times the recommended dose (NR, 1000 mg/d; PT, 200 mg/d) or placebo for 4 months (each group n = 10). NRPT elicited significant improvements in a variety of clinical endpoints relating to ALS and in some cases was considered to slow the progression of the disease. Although these results are promising, a cross-over study design was not possible and significant differences were seen in a number of baseline outcome measures between the placebo and NRPT groups. When taken together, these differences could imply that the NRPT group had a more severe form of the disease, which may confound the group comparisons.

The most recent study supplemented NRPT for just two days in 24 hospitalised participants with acute kidney injury (AKI) (Simic *et al.*, 2020). The rationale of this study was based on findings from animal research that demonstrated AKI impairs NAD+ bioavailability (Ralto, Rhee and Parikh, 2020). The studied cohort was divided in to four groups in order to investigate a range of daily dosages, groups received either a single, double, triple, or quadruple amount of the recommended daily NRPT (NR, 250 mg/d; PT, 50 mg/d), whilst one individual from each group received placebo. The study found that after two days, AKI reduced whole blood NAD+ by 50%, whereas NRPT supplementation significantly increased NAD+ by 37% (combined data from all groups). At a group level the effects of NRPT on NAD+ seemed to be dose-dependent, as indicated by the progressive increases observed between the groups taking single, double, and triple dose NRPT. However, only the double group dosage (NR, 500 mg/d; PT, 100 mg/d) showed a statistically significant increase in NAD+, and NAD+ levels were lower at the highest dose. Nevertheless, all doses of NRPT was deemed safe, with only three reports of minor gastrointestinal side effects. These data consolidate the conclusions made by Dellinger *et al.* (2017), confirming that NRPT is well tolerated and does reliably augment NAD+ metabolism in humans.

Future Directions

As highlighted above, while clinical research of NRPT is still in its early stages, chronic supplementation has demonstrated an ability to reliably stimulate NAD+ metabolism, favourably alter blood pressure, and improve ALS clinical endpoints. Additional research using pair-matched groups, will help develop our understanding of the therapeutic potential of NRPT within ALS. The potential for short-term supplementation to limit the severity of AKI is also promising. Repeated bouts of AKI are thought to contribute to chronic kidney disease (CKD), which is thought to affect 697.5 million people worldwide (Bikbov *et al.*, 2020). AKI is most commonly seen in medical settings; however, heat stress and exercise

are also known to induce AKI; for example, in agricultural workers (Kupferman *et al.*, 2018) and ultra-endurance athletes (Hodgson *et al.*, 2017). Therefore, further research is warranted to determine whether NRPT can attenuate biomarkers of AKI, and potentially reduce risk of CKD, within such populations.

Forthcoming research investigating NRPT should implement it within the settings previously discussed in detail under the '*Future Directions*' sections of NR and PT. Specifically, chronic supplementation should be targeted within disease states where vascular or metabolic dysfunction is present, such as hypertension and type-2-diaebetes. Supplementation in obese populations or those with metabolic syndrome are also promising avenues, although the potential adverse PT-induced lipid alterations mean individuals with hypercholesterolaemia should be excluded. Acute supplementation should be focussed on exploring the vascular actions of NRPT on the vasculature and its role within, predominantly aerobic, exercise, due to its hypothesised effects on oxidative metabolism. Prior to exploring the ergogenic potential for NRPT, its impact on the cardiometabolic responses to exercise should be catalogued, enabling us to understand the duration/intensity of exercise where it could have the greatest effect. Hypothesised NRPT-induced improvements in glycaemic control could also be applied during the post-exercise recovery period, increasing glucose uptake and thus allowing a faster recovery of muscle glycogen – this avenue of research is discussed in detail below in section 1.9: Post-Exercise Recovery.

1.8. Promising Settings to Investigate NRPT Vascular Effects

The vascular potential of NRPT relates to its proposed ability to augment nitric oxide bioavailability, either by increasing eNOS activity (via phosphorylation and SIRT1-mediated deacetylation) or via improved redox status, reducing the interactions of RONS with nitric oxide. As previously mentioned, this vascular potential could be utilised in contexts such as exercise or disease, in which vascular dysfunction can limit performance or promote disease progression. Beneficial vascular effects of nutraceuticals, like NRPT, are most commonly seen when there is a degree of dysfunction and thus more severe deficiencies in the biological targets of supplementation. Within humans, this dysfunction can be pathological in nature (e.g. hypertension) or purposefully induced by a physiological stressor. This section will focus on two stimuli, hypoxia and hyperglycaemia, which have been shown to transiently induce vascular dysfunction in healthy populations and could therefore be used to potentiate vascular effects of NRPT.

Hypoxia

A recent review from Tymko *et al.* (2019) shows that the majority, but not all (Iglesias *et al.*, 2015; Rieger *et al.*, 2017), of the literature reports a detrimental effect of laboratory-induced acute hypoxic exposure on brachial artery endothelial function; assessed as FMD (Lewis *et al.*, 2014, 2017; Tremblay, Howe, *et al.*, 2018), glyceryl trinitrate (GTN)-induced dilation (Lewis *et al.*, 2017), or the FMD/GTN ratio (Frøbert *et al.*, 2008). The findings of Lewis *et al.* (2017) and Tremblay *et al.* (2018) indicated that hypoxia-induced alterations in shear rate patterns (i.e. increased retrograde and oscillatory flow) and overall magnitude, alongside impairments of vascular smooth muscle function, were partially attributable to the observed attenuation of vascular function. Although these investigations were well designed, results from Lewis *et al.* (2017) were in response to non-physiological isocapnic hypoxia; which, relative to hypobaric or normobaric hypoxia, will result in elevated arterial carbon dioxide, known to modify brachial blood flow patterns and sympathetic nerve activity (SNA) (Somers *et al.*, 1989; Vantanajal *et al.*, 2007). The significant FMD attenuation reported by Tremblay *et al.* (2018) was accompanied by an increase in baseline diameter, a variable inversely related to FMD (Thijssen *et al.*, 2008), which was not accounted for in the analysis. However, other studies have shown that when this increase is accounted for (Frøbert *et al.*, 2008; Lewis *et al.*, 2017), or in milder hypoxic exposures which do not alter baseline diameter (Lewis *et al.*, 2014, 2017), reductions in FMD are still apparent. Although not widely investigated, unlike the brachial artery, femoral artery FMD appears to be preserved following a nine day trek to 5050 m (Tremblay, Hoiland, *et al.*, 2018), possibly relating to the localised exercise of the lower limbs (Tymko *et al.*, 2019). Studies investigating acute hypoxic exposure have only focussed on brachial artery FMD (Tymko *et al.*, 2019); therefore, future research should identify whether, independent of exercise, the femoral artery has a greater resistance to hypoxia-induced endothelial dysfunction.

The mechanisms underlying hypoxia-induced endothelial dysfunction are not fully understood; however, hypoxia-mediated alterations in SNA and redox status are thought to be implicated (Tymko *et al.*, 2019). Lewis *et al.* (2017) demonstrated a clear positive association between the magnitude of impairment and the magnitude of hypoxia employed, possibly related to hypoxic severity-dependent increases of sympathoexcitation (Rowell *et al.*, 1989). The literature generally shows that elevations in SNA, induced with lower-body negative pressure, mental stress, or static handgrip exercise (Seals, 1989; Hijmering *et al.*, 2002; Spieker *et al.*, 2002; Padilla *et al.*, 2010), but not cold pressor test (Thijssen *et al.*, 2006; Padilla *et al.*, 2010), will impair vascular function. It has been suggested that SNA indirectly induces vascular dysfunction by increasing retrograde shear (Padilla *et al.*, 2010; Thijssen *et al.*, 2014), known to dose-dependently impair FMD (Thijssen *et al.*, 2009). Hypoxia-induced retrograde shear may result from an SNA-mediated elevation in vasomotor tone, which have been shown to completely and

partially mask hypoxic vasodilation of the forearm (Weisbrod *et al.*, 2001; Moradkhan *et al.*, 2010). The role of heightened SNA within hypoxia-induced endothelial dysfunction is reinforced by reports that α_1-adrenoreceptor blockade administration partially ameliorated the attenuations of FMD observed following 3 hours of severe normobaric hypoxia (Lewis *et al.*, 2014). Taken together, hypoxia-induced vascular dysfunction could therefore be consequential to sympathoexcitation-mediated stimulation of vasomotor tone and elevations of retrograde shear.

Alongside increases in SNA, acute hypoxia is known to increase free radical production (D. M. Bailey *et al.*, 2009; Woodside *et al.*, 2014) and thus markers of oxidative stress (Magalhães *et al.*, 2004; Pialoux *et al.*, 2009). Although the exact mechanism driving hypoxia-induced RONS is unclear, elevated radical release from mitochondrial ETC complexes is thought to contribute (Wang *et al.*, 2007). Indeed, exaggerated radical production can lead to cellular damage and vascular dysfunction (Liguori *et al.*, 2018). For example, RONS can interact with nitric oxide (Pryor and Squadrito, 1995), increasing OXINOS stress and reducing bioavailability of this important vasoactive molecule, an effect documented following acute and short-term hypoxic exposure (D. M. Bailey *et al.*, 2009, 2010). OXINOS stress appears to contribute to oscillatory shear stress-induced endothelial dysfunction (Johnson *et al.*, 2013), a shear stress pattern also induced by hypoxia (Tremblay, Howe, *et al.*, 2018). An indirect link between redox status and endothelial dysfunction is also possible; in hypertensive individuals, oxidative stress has been suggested to promote sympathoexcitation (Bruno *et al.*, 2012), which, as previously mentioned, is thought to stimulate detrimental shear stress patterns (Padilla *et al.*, 2010). Therefore, as well as heightened SNA, OXINOS stress-mediated declines in nitric oxide bioavailability and increased oscillatory shear stress may be contributing to hypoxia-induced endothelial dysfunction.

Interestingly, despite these apparent hypoxia-induced reductions in endothelial function of conduit arteries, acute hypoxic exposure actually augments limb blood flow and vascular conductance, both at rest (Dinenno, Joyner and Halliwill, 2003; Moradkhan *et al.*, 2010) and during sub-maximal exercise (Casey and Joyner, 2012). Local skeletal muscle metabolic vasodilation, stimulated by hypoxaemia (Dinenno, 2016), must override and compensate for hypoxia-mediated sympathetic vasoconstriction and reductions in endothelial function of larger resistance vessels. Although skeletal muscle perfusion is the key determinant of performance at sea level (Bassett and Howley, 2000), the diffusion limitation at pulmonary and peripheral circulations are considered to be more important at altitude (Grocott, Levett and Ward, 2019). Therefore, given the net vasodilatory effect of hypoxaemia (Casey and Joyner, 2012), it is unlikely that hypoxia-induced endothelial dysfunction restricts blood flow to active muscle to a degree that could impair performance.

Hypoxia-induced endothelial dysfunction has been associated with high-altitude pulmonary oedema (HAPE); Berger *et al.* (2005) found that following acute hypoxic exposure, the endothelium-dependent vasodilation response to acetylcholine infusion of the brachial artery, was impaired in HAPE-susceptible patients only. High altitude-induced pulmonary hypertension is an important factor that contributes to HAPE, thought to be primarily mediated by hypoxic pulmonary vasoconstriction (HPV); however, findings from Berger *et al.* (2005) suggest that systemic endothelial dysfunction may also contribute. A hypoxia-induced imbalance of vasodilator and vasoconstrictor molecules is thought to cause HPV, with concurrent elevations in endothelin-1a (Naeije *et al.*, 2010) and reductions in nitric oxide (Duplain *et al.*, 2000; D. M. Bailey *et al.*, 2010) reported. There is some evidence to suggest a radical-mediated reduction of pulmonary nitric oxide bioavailability, following observations that pulmonary artery pressure was significantly, positively, correlated with both radical species output and 3-nitrotyrosine (3-NT), a marker of OXINOS stress (D. M. Bailey *et al.*, 2010). Pulmonary hypertension, and subsequent HAPE, can not only cause serious health complications (Paralikar, 2012), but is also thought limit exercise performance at altitude. Several studies have shown that administration of a systemic vasodilator attenuates pulmonary hypertension and improves hypoxic exercise performance (Ghofrani *et al.*, 2004; Richalet *et al.*, 2005; Hsu *et al.*, 2006; Faoro *et al.*, 2007; Naeije *et al.*, 2010), with Naeije *et al.* (2010) attributing 33% of hypoxia-mediated reductions in $\dot{V}O_{2max}$ to pulmonary hypertension. However, there is still debate between the aforementioned studies as to whether performance improvements resulting from attenuations in pulmonary hypertension are mediated by elevations of cardiac output or arterial oxygen saturation. Taken together, these data suggest that systemic hypoxia-induced endothelial dysfunction may contribute to high-altitude pulmonary hypertension, and thus may impair high-altitude exercise performance by limiting alveolar oxygen diffusion. Reductions in pulmonary nitric oxide bioavailability, potentially resulting from OXINOS stress, appear important for the increase in HPV; therefore, strategies to augment nitric oxide bioavailability and improve redox status could work to prevent HAPE and attenuate hypoxia-mediated declines in performance.

Dietary manipulation has previously been proven to effectively offset hypoxia-induced endothelial dysfunction in a healthy cohort. For example, Bakker *et al.* (2015) found exogenous nitrate supplementation, to increase nitric oxide bioavailability, prevented attenuations in FMD following a five-day trek to 4200 m. Alongside the more obvious vasoactive properties of nitric oxide that make it a primary determinant of endothelial function, nitric oxide is also a sympathoinhibitory substance (Patel, Li and Hirooka, 2001) and could therefore have attenuated hypoxia-induced sympathoexcitation to improve FMD, although SNA was not measured in this study. To the best of our knowledge, no research has investigated the effects of acute nutraceutical supplementation on

hypoxia-induced endothelial dysfunction. With regard to the nutraceutical NRPT, it has potential to augment nitric oxide availability, similar to nitrate supplementation, but also to improve redox status; which, as previously discussed, are two key variables associated with hypoxia-induced endothelial dysfunction. From a methodological perspective, using acute hypoxic exposure to transiently induce endothelial dysfunction could be a useful tool to test the vascular efficacy of NRPT in healthy populations. Positive results could lead to further exploration of NRPT supplementation within high altitude-induced pulmonary hypertension, and how this may help attenuate HAPE or performance declines following hypoxic exposure.

Acute Hyperglycaemia

A glucose load, and subsequent hyperglycaemia, transiently impairs endothelial function. For example, a meta-analysis carried out by Loader *et al.* (2015) concluded that acute hyperglycaemia significantly impairs the endothelial function of healthy, type-2-diabetic, and hypertensive populations by 1.14%, 1.40%, and 2.74%, respectively. Within the analysed studies, hyperglycaemia was either induced through intravenous infusion or oral administration, including a standardised 75 g oral glucose tolerance test (OGTT), various sugar or 'energy' drinks, and various carbohydrate meals. The impaired endothelial function was assessed by multiple methods, predominantly via FMD but also by acetylcholine-mediated dilation and venous occlusion plethysmography. Due to its practicality, non-invasive nature, and clinical significance, FMD is becoming an increasingly popular method to assess endothelial function in humans. Although this meta-analysis gives a useful broad overview, it does not isolate and quantify the effect of hyperglycaemia on FMD. The between-study comparability is also limited by the diverse methodologies used to induce hyperglycaemia, and several of the analysed studies share data from the same group of participants. Another consideration regarding the studies presented by Loader *et al.* (2015), are the between-study inconsistencies of FMD protocol, specifically surrounding the cuff placement, which is known to strongly influence the outcome measure and therefore limit comparability between studies (Green *et al.*, 2014). Therefore, a brief summation of research specifically measuring the effects of a physiological relevant, standardised OGTT on the subsequent brachial artery FMD of healthy participants is provided here (see Table 2).

All studies reviewed here shared the following methodological approaches: recruited healthy participants; induced acute hyperglycaemia using a 75 g OGTT, and assessed endothelial function with FMD following the recommended, standardised guidelines (Thijssen *et al.*, 2019). Furthermore, if multiple studies had shared data from a single group of participants, only one was included within this review. In total, 11 studies met the inclusion criteria, two of which investigated two distinct healthy groups simultaneously; therefore, the overall number of data sets reviewed was 13, with a combined

sample size of 199. Following the OGTT, 7 data sets showed significant attenuations in FMD (Title *et al.*, 2000; Xiang *et al.*, 2008a; Watanabe *et al.*, 2011; L. Wang *et al.*, 2013; Mah *et al.*, 2013; Greyling *et al.*, 2015; Williams *et al.*, 2019), 2 demonstrated significant elevations in FMD (Dengel *et al.*, 2007; Major-Pedersen *et al.*, 2008), and the remaining 4 reported no significant change (Kawano *et al.*, 1999; Xiang *et al.*, 2008a; Xiang *et al.*, 2008b; Williams *et al.*, 2019). Table 2 below summarises the study characteristics and displays the key findings, relating to the magnitude and timing of the FMD response.

Table 2: *Findings from studies investigating the effects of acute hyperglycaemia, induced by an OGTT (75 g), on brachial artery FMD, measured using the recommended protocol (forearm cuff occlusion), in healthy populations. OGTT, oral glucose tolerance test; FMD, flow-mediated dilation; T2D, type-2-diabeties.*

Study	n (M:F)	Sub-groups	Age	Blood glucose (mmol/L)		FMD Timepoints (post-OGTT)	Peak FMD Response (%)	Significance	Time of Peak FMD Response
				Fasting	Peak post-prandial				
Williams *et al.* (2019)	17 (0:17)	Tested during early follicular phase (EF)	21			1, 1.5 & 2 hrs	↓ 2.5	**p<0.05**	1.5 hrs
		Tested during late follicular phase (LF)					↓ 1.6	p=0.052	2 hrs
Greyling *et al. (* 2015)	10 (10:0)	*NA*	57	4.9 ± 0.5	~ 8.0	1, 2 & 2.5 hrs	↓ 1.4	**p<0.05**	1 hr
Mah *et al.* (2013)	15 (15:0)	*NA*	21.8	5.21 ± 0.10	~ 8.8	30, 60, 90, 120, 150 & 180 mins	↓ 2.8	**p<0.05**	1 hr
Wang *et al.* (2013)	33 (12:21)	*NA*	51.36	5.43 ± 0.45	6.49 ± 0.89	2 hrs	↓ 3.72	**p<0.05**	*NA*
Watanbe *et al.* (2011)	14 (8:6)	*NA*	33.4	4.65 ± 0.43	6.07 ± 1.61	1, 2 & 3 hrs	↓ 1.63	**p<0.05**	1 hr
Major-Pederson (2008)	10 (6:4)	*NA*	41.1	4.12 ± 0.12	6.21 ± 0.79	1, 2, 3 & 4 hrs	↑ 4.8	**p<0.05**	3 hrs
Xiang *et al.* (2008a)	32 (18:14)	n=17 with no family history of T2D	40	4.59 ± 0.49	9.31 ± 1.05	1 & 2 hrs	↓ 0.33	p>0.05	1 hr
		n=15 with a family history of T2D	39	5.13 ± 0.40	9.4 ±1.03		↓ 1.12	**p<0.05**	1 hr
Xiang et al. (2008b)	26 (14:12)	*NA*	50	4.89 ± 0.45	9.52 ± 1.19	1 & 2 hours	↓ 0.57	p>0.05	1 hr
Dengel *et al.* (2007)	15 (7:8)	*NA*	11.3	4.78 ± 0.07	~ 8.0	1 & 2 hrs	↑ 1.6	**p<0.05**	2 hrs
Title *et al.* (2000)	10 (6:4)	*NA*	25.5	5.2 ± 0.7	7.9 ± 3.0	1, 2, 3 & 4 hrs	↓ 2.8	**p<0.05**	2 hrs
Kawano *et al.* (1999)	17 (11:6)	*NA*	52.6	5.02 ± 0.07	8.52 ± 0.35	1 & 2 hrs	↓ 3.29	p>0.05	1 hr

In the 7 data sets reporting significant impairment, the average peak attenuation in FMD was 2.28%, ranging between 1.12-3.72%. All, excluding one (L. Wang *et al.*, 2013), measured FMD at least twice, generally every hour post-OGTT, allowing identification of the temporal pattern of FMD impairment. The majority (n=4) of studies found that maximal FMD impairment occurred 1 hour post-OGTT, whilst the other 2 reported this occurred later, at either 1.5 or 2 hours post-OGTT.

In contrast, Dengel *et al.* (2007) and Major-Pedersen *et al.* (2008) observed an increase in FMD post-glucose load; specifically, a peak increase of 1.6% 2 hours post-OGTT and 4.8% 3 hours post-OGTT, respectively. One possible explanation for the observations by Dengel *et al* (2007) was that they examined children rather than adults. Regarding the findings of Major-Pedersen *et al* (2008), the authors suggest the participant's relatively low levels of fasting and post-prandial [glucose] may partially explain the differences observed. If metabolic health is a key factor determining the magnitude and direction of change in FMD it would be expected that type-2-diabetics and populations with impaired glucose tolerance experience the greatest vascular impairments. Although this finding has been proven (Kawano *et al.*, 1999; Xiang *et al.*, 2008b), other research has failed to replicate this

result (L. Wang *et al.*, 2013; Greyling *et al.*, 2015; Loader *et al.*, 2015), suggesting that the marginally superior markers of glucose homeostasis recorded within the cohort studied by Major-Pedersen *et al.* (2008) are unlikely to explain the vast elevations in post-prandial FMD. The authors attributed the rather delayed nature of the vascular response to the slow-onset vasodilatory effect of insulin; however, other research measuring FMD 3 or 4 hours post-OGTT have found it to remain depressed or returned to baseline (Title *et al.*, 2000; Watanabe *et al.*, 2011; Mah *et al.*, 2013).

Four of the included data sets reported no significant effect of an OGTT on post-prandial FMD; however, in line with the majority of the literature, all four found FMD tended to decrease post-OGTT. Early research by Kawano *et al.* (1999) tested 17 participants and saw a substantial mean peak FMD reduction of 3.29%, the lack of significance is potentially attributable to the use of a highly conservative post-hoc analysis (Scheffé's procedure) (Kim, 2015). The non-significant reduction shown by Xiang *et al.* (2008b) was smaller (0.57%); however, like Kawano *et al*, this study also employed Scheffé's post-hoc analysis, possibly masking the vascular impacts of the OGTT. Another shared limitation of these studies is the lack of control for smoking status and dietary supplement usage, which are both known to influence vascular function. The remaining two data sets that showed no significant effect were part of larger studies assessing the impact of another variable on post-prandial hyperglycaemia-induced endothelial dysfunction. Firstly, Xiang *et al.* (2008a) investigated whether a family history of type-2-diabetes affected the FMD response to an OGTT. The authors showed that post-prandial FMD did not significantly change from baseline in participants without a family history of type-2-diabetes; conversely, participants with a family history experienced a significant peak reduction of 1.12%. Although the two groups had similar peak post-prandial blood [glucose], fasting [glucose] was significantly greater in participants with a family history of type-2-diabetes. This finding reinforces suggestions made by Major-Pedersen *et al.* (2008) that an individual's fasting [glucose], and thus metabolic health, will influence their vascular response to a glucose load, with healthier populations experiencing less dysfunction. The other non-significant result was from Williams *et al.* (2019), which investigated whether menstrual cycle phase (early *vs* late follicular) changed the FMD response to an OGTT. Peak FMD was significantly attenuated (-2.5%) during the early follicular phase whereas only non-significant reductions (-1.6%) were seen during late follicular; however, this result borderlines significance (p=0.052) and significant reductions were seen in this group at an earlier time point.

Taken together, the majority of literature indicates a detrimental impact of a glucose load on FMD, the severity of which may be partially influenced by metabolic health and menstrual cycle phase. Significant negative correlations between blood [glucose] and FMD indicate that the degree of hyperglycaemia is a key determinant of the vascular response (Watanabe *et al.*, 2011; Mah *et al.*,

2013); however, unsurprisingly, insulin concentrations rise in a similar manner to glucose and thus are also correlated with FMD. Given the vasodilatory role of insulin (Muniyappa and Quon, 2007) and evidence that hyperglycaemia-induced impairments of endothelial function remain after inhibition of insulin secretion (Williams *et al.*, 1998), it is likely that hyperglycaemia, rather than hyperinsulinemia, is responsible for the observed declines in FMD.

There are numerous hypotheses surrounding the cause of hyperglycaemia-induced endothelial dysfunction; the most common relates to detrimental changes in redox status, whereas others suggest alterations in pathways controlling nitric oxide signalling and vasoconstrictor production may also contribute. Although the exact mechanism(s) is unclear, elevated glucose is known to increase endothelial cell radical production *in vitro* (Tesfamariam and Cohen, 1992; Du, Stockklauser-Färber and Rösen, 1999). These findings are largely paralleled by *in vivo* clinical data, which demonstrate how transient hyperglycaemia increases systemic biomarkers of oxidative stress in healthy populations (Konukoğlu *et al.*, 1997; Ceriello *et al.*, 1998; Xiang *et al.*, 2008a; L. Wang *et al.*, 2013; Mah *et al.*, 2013). However, these increases have not been universally observed, with some investigations not showing this elevated hyperglycaemia-induced oxidative stress in humans (Kawano *et al.*, 1999; Title *et al.*, 2000; Zhu *et al.*, 2007; G. Da Xiang *et al.*, 2008); although, two of these studies observed non-significant increases (Kawano *et al.*, 1999; Xiang *et al.*, 2008b). This discrepancy is possibly due to methodological differences in the analysis of markers; for example, there is a large degree of variation in the sensitivity of different techniques used to measure malondialdehyde (MDA), a frequently used marker (Moselhy *et al.*, 2013; Spirlandeli, Deminice and Jordao, 2014). The role of oxidative stress is reinforced by evidence highlighting strong inverse relationships between FMD responses and markers of lipid peroxidation (Kawano *et al.*, 1999; Xiang *et al.*, 2008b; Mah *et al.*, 2013), as well as research showing anti-oxidant supplementation can partially ameliorate hyperglycaemia-induced endothelial dysfunction (Title *et al.*, 2000; Xiang *et al.*, 2008b; Grassi *et al.*, 2012; Mah *et al.*, 2013).

It is likely that the concept of 'nutrient stress' is involved in hyperglycaemia-induced radical production, whereby an over-supply of respiratory substrates (i.e. glucose) to the mitochondria promotes electron leakage and ultimately radical generation (M. J. Jackson *et al.*, 2002). Research from Nishikawa *et al.* (2000) indicates that complex 2, but not complex 1, of the ETC is influential to glucose-mediated radical production. Radicals are known to impact a number of pathways that regulate the synthesis and degradation of nitric oxide and will thus impact endothelial vasodilatory function. Markers of nitric oxide bioavailability have been shown to decline post-OGTT (Ceriello *et al.*, 2002; L. Wang *et al.*, 2013; Mah *et al.*, 2013), explaining the impaired FMD responses observed. Only a short summation of the mechanisms surrounding hyperglycaemia-induced endothelial dysfunction

will be provided as they have been explored in detail by Rammos *et al.* (2008) and Mah and Bruno (2012).

As previously discussed (section 1.2), due to their high affinity for one-another, radicals have the capacity to directly scavenge nitric oxide, resulting in the formation of peroxynitrite (Pryor and Squadrito, 1995). Alongside this, *in vitro* research implicates hyperglycaemia-induced radicals in the accumulation of asymmetric dimethylarginine (ADMA) (Frombaum *et al.*, 2011) (a competitive inhibitor of eNOS), elevated arginase activity (Romero *et al.*, 2008) (reducing bioavailability of the eNOS substrate, L-arginine) and the increased oxidation (Crabtree *et al.*, 2008), and thus diminished bioavailability (Ihlemann *et al.*, 2003), of BH4 (an eNOS cofactor). There is also clinical evidence suggesting that hyperglycaemia-induced radicals damage the endothelial glycocalyx, a layer of proteoglycans that are thought to have a vasculoprotective role, as well as regulating vascular permeability and facilitating the mechanotransduction of shear stress (Reitsma *et al.*, 2007). Nieuwdorp *et al.* (2006) found normoinsulinemic hyperglycaemia significantly reduced glycocalyx volume in healthy participants, an effect that was blocked by infusion of the exogenous anti-oxidant, N-acetylcysteine (NAC). These data, in combination with animal research showing hyperglycaemia attenuated shear stress-, but not acetylcholine-mediated dilation (Kelly *et al.*, 2006), indicate that hyperglycaemia-induced radicals impair the mechanotransduction of shear stress and ultimately nitric oxide synthesis.

Alongside this, upregulation of inflammatory processes are hypothesised to contribute to hyperglycaemia-induced radical release. Hyperglycaemia can stimulate pro-inflammatory cytokine release directly, via protein kinase C activation (Quagliaro *et al.*, 2003), or indirectly, due to its reciprocal relationship with oxidative stress (Mah and Bruno, 2012; Wadley, Veldhuijzen Van Zanten and Aldred, 2013). Inflammation promotes radical bursts from neutrophils (Gougerot-Pocidalo *et al.,* 2002), expression of NOX4 (Williams *et al.*, 2012) (a radical producing enzyme that is the predominant source of vascular oxidative stress (Ago *et al.*, 2004)) and upregulates iNOS (Kleinert, Art and Pautz, 2003) (a NOS isoform that more readily releases radicals whilst reducing the bioavailability of shared substrate and cofactors, consequently uncoupling eNOS). Inflammatory responses to an OGTT are seen in healthy, middle-aged populations (Festa *et al.*, 2002; Aljada *et al.*, 2006), with an exaggerated response observed in those with impaired glucose tolerance (Ceriello *et al.*, 2004; Konukoglu, Firtina and Serin, 2008; Derosa *et al.*, 2010). However, research indicates that in younger, healthy participants, this pro-inflammatory response is absent (Mah *et al.*, 2013; Wopereis *et al.*, 2013). Despite lacking inflammatory markers, the younger population studied by Mah *et al.* (2013) did exhibit hyperglycaemia-induced endothelial dysfunction, indicating that inflammation is not a key factor explaining the vascular impairment in younger cohorts.

To summarise, *in vitro* evidence indicates that acute hyperglycaemia stimulates radical production, which detrimentally interact with pathways controlling the synthesis and degradation of the vasodilator, nitric oxide. Indeed, markers of oxidative stress and impaired nitric oxide bioavailability have been documented in clinical trials. Given the importance of nitric oxide bioavailability to FMD (Green *et al.*, 2014), this likely explains the acute endothelial dysfunction observed following a glucose load. As previously discussed, NRPT supplementation has the potential to increase nitric oxide bioavailability, either via its anti-oxidant capacity or upregulation of eNOS activity. There is already some clinical evidence that polyphenols can protect endothelial function from OGTT-induced declines. For example, Grassi *et al.* (2012) found that 3-days consumption of flavonoid-rich dark chocolate, compared to white chocolate, significantly attenuated declines in FMD following a glucose load. Improvements in endothelial function are likely explained by the concurrent reductions in markers of hyperglycaemia-induced oxidative stress observed following the dark chocolate protocol. Resveratrol has also been shown to reduce ADMA accumulation, an eNOS inhibitor, in bovine aortic endothelial cells exposed to 24-hour hyperglycaemia (Frombaum *et al.*, 2011). These attributes suggest that NRPT could help attenuate or even abolish hyperglycaemia-induced endothelial dysfunction, which appears related to elevations in oxidative stress. NRPT supplementation may therefore be relevant to those experiencing regular bouts of acute, diet-induced post-prandial vascular dysfunction. Repeated, transient episodes of vascular dysfunction are hypothesised to be instrumental in the pathogenesis of atherosclerosis (Bonetti, Lerman and Lerman, 2003).

The next portion of this review will focus on the post-exercise recovery period and will discuss how acute NRPT supplementation may be used to optimise glucose uptake during this time. Following this, the potential for post-exercise acute hypoxic exposure to augment glucose uptake will be reviewed. Although the mechanistic link between hypoxia and glucose uptake has been known for decades, its role within post-exercise glycaemic control remains relatively unexplored.

1.9. Post-Exercise Recovery

Exercise demands energy that our skeletal muscle produces using intramuscular stores of glycogen and fat, as well as releasing glucose and fatty acids from the liver and adipose tissue, respectively (Hawley, 2001). Dependant on the intensity and duration of the exercise, stores of glycogen and fat will suffer varied degrees of depletion, which will need replenishing in the post-exercise recovery period (Hawley, 2001). Generally, intramuscular glycogen stores are considered the most important fuel source; based on its essential role powering strenuous exercise (Hermansen, Hultman and Saltin, 1967), its inverse relationship with fatigue perception (Noakes, Snow and Febbraio, 2004), and how its baseline levels are directly related to aerobic endurance (Bergström *et al.*, 1967). Therefore, rapid replenishment of this fuel source is vital for high-level athletes who are undertaking busy training or competitive schedules.

Post-exercise muscle glycogen re-synthesis is thought to have a biphasic response (Price *et al.*, 1994), the first being a more rapid, insulin-independent phase, lasting 30-60 mins post-exercise. This is thought to be mediated by residual, exercise-induced, GLUT4 on the cell membrane and a heightened glycogen synthase activity, resultant from low, exercise-induced, glycogen concentrations (Ivy and Kuo, 1998). The second, insulin-dependent, phase is more gradual in nature, with re-synthesis occurring at an ~80% lower rate (Price *et al.*, 1994); here, re-synthesis relies on the sensitivity of GLUT4 and glycogen synthase to insulin stimulation. Although factors regulating glycogen synthase activity are important, it is now believed that substrate availability, and thus glucose transport, is the rate-limiting element of muscle glycogen synthesis - this has been shown under basal and insulin-stimulated conditions (Ivy and Kuo, 1998).

Somewhat unsurprisingly, post-exercise exogenous carbohydrate feeding is known to be a key driver of glycogen re-synthesis (Alghannam, Gonzalez and Betts, 2018); stimulating insulin release (thus triggering GLUT4 translocation) and increasing glycogen synthase activity, whilst providing a large influx of essential substrate. Interestingly, co-ingestion of protein alongside carbohydrate can enhance post-exercise glycogen re-synthesis, thought to be related to their synergistic effects on insulin secretion; however, the literature is far from consistent and lacks definitive conclusions on whether the effects are insulin-mediated or resultant from increased energy intake (Alghannam, Gonzalez and Betts, 2018). Although dietary manipulation is known to be a strong modulator of glycogen re-synthesis, the potential role of micro-nutrient supplements to augment the recovery process has not been widely explored. Similarly, hypoxia has long been documented to augment glucose transport; however, to the best of our knowledge, the impact of post-exercise hypoxic exposure on glucose uptake in humans has not been investigated.

NRPT and Glycaemic Control

This section will discuss the various regulatory pathways that NRPT could potentially interact with to augment glucose uptake, and ultimately glycaemic control; and how acute supplementation offers a possible strategy to manipulate post-exercise glucose recovery.

Firstly, as previously discussed, NRPT has the potential to enhance the activity of the NAD+-dependent deacetylase, SIRT1. SIRT1 has been proposed to regulate glucose-stimulated insulin secretion by reducing expression of uncoupling protein-2 in the pancreas (Moynihan *et al.*, 2005; Bordone *et al.*, 2006), preventing mitochondrial uncoupling and thus allowing a more efficient ATP synthesis, required to trigger the release of insulin. SIRT1 has also been suggested to optimise the action of insulin at the target tissues (Sun *et al.*, 2007); however, SIRT1 ablation in the skeletal muscle of mice does not appear to influence insulin sensitivity (Boutant and Cantó, 2014). Therefore, NRPT-mediated elevations in SIRT1 activity could improve pancreatic efficiency, enhancing the release of insulin in response to post-exercise carbohydrate feeding to promote glucose uptake at the skeletal muscle.

A rather under-appreciated regulator of glucose uptake is nitric oxide, and as mentioned already, NRPT has the capacity to increase the bioavailability of this molecule through SIRT1- and PT-mediated stimulation of eNOS. In skeletal muscle, AMPK is involved in exercise-induced glucose uptake, phosphorylating the protein TBC1D4 (tre-2/USP6, BUB2, cdc16 domain family member 4) to trigger GLUT4 translocation (O'Neill, 2013). Some of the first evidence to highlight a regulatory role of nitric oxide on this pathway used NOS inhibition to abolish exercise-induced elevations of sarcolemmal membrane GLUT4 in rodents (Roberts *et al.*, 1997). Future animal research reinforced these findings, successfully using nitric oxide donors and NOS inhibitors to manipulate AMPK-mediated GLUT4 translocation and glucose uptake (Li *et al.*, 2004). Although not all data agree (Heinonen *et al.*, 2013), an importance of nitric oxide for glycaemic control in humans is also apparent, with two studies demonstrating impaired exercising leg glucose uptake following NOS inhibition, independent of limb blood flow (Bradley, Kingwell and McConell, 1999; Kingwell *et al.*, 2002). Residual AMPK-mediated phosphorylation of TBC1D4 (Treebak *et al.*, 2009) is thought to be partially attributable to post-exercise increases in insulin sensitivity (O'Neill, 2013); therefore, augmenting nitric oxide bioavailability could optimise this pathway and ultimately enhance the glucose uptake response to carbohydrate feeding post-exercise.

In a similar vein, various phenolic compounds have been shown to activate AMPK (Kim *et al.*, 2016), primarily by increasing the ratio of adenosine monophosphate (AMP) to ATP (Kim *et al.*, 2016; Lan *et al.*, 2017). Currently, only *in vitro* (Lin *et al.*, 2012; Ren, Rimando and Mathews, 2018) and *in vivo*

(Gómez-Zorita *et al.*, 2014) animal evidence is available that shows PT-mediated AMPK activation. There is some clinical evidence illustrating that PT analogue, resveratrol, activates AMPK *in vivo* (Timmers *et al.*, 2011; Goh *et al.*, 2014); although, not all findings agree (Yoshino *et al.*, 2012; Poulsen *et al.*, 2013). Animal data has already shown PT supplementation to increase muscle GLUT4 expression (Gómez-Zorita *et al.*, 2015); therefore, it is possible that PT supplementation could enhance glucose uptake through AMPK-mediated GLUT4 translocation. Furthermore, there is evidence from SIRT1-knockout mice (Price *et al.*, 2012) and human cell culture models (Hou *et al.*, 2008) to suggest resveratrol-mediated AMPK activation is, at least partially, SIRT1-dependant. Therefore, co-supplementation of NR to increase NAD+ bioavailability, the SIRT1 substrate, is essential to the optimisation of this pathway.

Given that glucose uptake is the product of the arteriovenous glucose difference and blood flow, post-exercise skeletal muscle perfusion, and thus substrate delivery, is a key factor influencing post-exercise glucose uptake. Following dynamic exercise, skeletal muscle blood flow transiently increases before gradually declining and returning to resting levels in 20-30 mins (Bangsbo and Hellsten, 1998). As previously discussed, nitric oxide is also a potent vasoactive molecule responsible for regulating vascular tone; therefore, NRPT-mediated increases in nitric oxide bioavailability could work to augment post-exercise skeletal muscle blood flow and thus glucose uptake.

Another important factor affecting glucose uptake is redox status; this is clear given that elevations in systemic oxidative stress are common within insulin resistant populations (Tiwari *et al.*, 2013; Bigagli and Lodovici, 2019), with the majority of the literature reporting improvements of insulin sensitivity within this cohort following anti-oxidant supplementation (Dal and Sigrist, 2016). Free radicals are capable of interacting with, and ultimately disrupting, the insulin cascade in a multitude of tissues, including skeletal muscle, thus impairing insulin-stimulated GLUT4 translocation (Garcia-Bailo *et al.*, 2011). NRPT can potentially augment redox status via several mechanisms: i) elevations in NADP(H) ii) sirtuin-mediated upregulation of anti-oxidant genes and inhibition of NF-κB, and iii) PT anti-oxidant capacity. Consequently, NRPT-mediated improvements in redox status could enhance skeletal muscle insulin-sensitivity and thus increase glucose uptake following carbohydrate feeding during the post-exercise recovery period.

Taken together, these data indicate that NRPT is a promising nutraceutical to augment post-exercise glucose uptake in response to carbohydrate feeding. NRPT would primarily act to optimise insulin-dependent mechanisms; possibly enhancing insulin secretion and signalling transduction. On the other hand, it also has potential to increase glucose uptake independent of insulin, through activation and optimisation of the AMPK-GLUT4 pathway, or elevations in blood flow, and thus

substrate delivery to skeletal muscle. Therefore, clinical research investigating the acute effects of NRPT supplementation on post-exercise glycaemic control is warranted.

Hypoxia and Glycaemic Control

The effects of hypoxia on glucose transport within skeletal muscle were first documented over 60 years ago, with Randle and Smith (1958) demonstrating that one hour of hypoxic exposure significantly increased glucose uptake in an isolated rat diaphragm muscle model. It is now understood that this effect is mediated by hypoxia-induced GLUT4 translocation (Cartee *et al.*, 1991; Bashan *et al.*, 1995; Zierath *et al.*, 1998) and that this translocation occurs independent of insulin (Cartee *et al.*, 1991; Azevedo *et al.*, 1995; Lee, Hansen and Holloszy, 1995; Yeh *et al.*, 1995); thus prompting the hypothesis that the intracellular GLUT4 pools utilised by hypoxia and insulin are distinct from one another (Zhang, Behrooz and Ismail-Beigi, 1999). Hypoxia-induced elevations of glucose transport in skeletal muscle are thought to rely primarily on AMPK- (Mu *et al.*, 2001; Fisher *et al.*, 2002) and calcium/calmodulin-dependent protein kinase (CaMK)-dependent (Cartee *et al.*, 1991; Wright *et al.*, 2005) pathways, as shown in rodents. Given that acute exercise stimulates these same pathways (Richter and Hargreaves, 2013), and that combining hypoxia with muscular contractions does not have an additive effect on glucose uptake (Cartee *et al.*, 1991), it is largely believed that hypoxia and exercise increase glucose uptake via the same signalling mechanisms. Conversely, there is evidence to indicate that these two stimuli can target exclusive mechanistic pathways; including, additive effects of exercise and hypoxia on post-exercise insulin sensitivity in humans (Mackenzie *et al.*, 2011), differential *in vitro* effects of enzymatic inhibitors on contractile- and hypoxia-induced glucose uptake (Wojtaszewski *et al.*, 1998), and evidence of a compensatory glucose uptake mechanism in response to contractions, but not hypoxia, in GLUT4-null mice (Zierath *et al.*, 1998; Ryder *et al.*, 1999).

Clinical data investigating the effects of acute hypoxic exposure on glycaemic control are limited and inconsistent, with no research exploring a role of post-exercise hypoxia on glucose uptake. Azevedo *et al.* (1995) was the first to document a stimulatory effect of hypoxia on glucose transport, independent of insulin, in human skeletal muscle; however, these data lack practical relevance as muscle was studied *ex vivo* and incubated under non-physiological conditions. The forthcoming discussed studies relate to *in vivo* human research, all of which utilised a randomised, cross-over design. Kelly *et al.* (2010) demonstrated that following an OGTT conducted in hypoxia (simulated 4300m; Fractional inspired O_2 (FiO_2) = ~12.3%) significantly supressed elevations of plasma glucose in young, healthy adults (compared to normoxia responses). Insulin concentrations did not differ between trials, and thus, although non-significant, hypoxia increased insulin sensitivity, as

determined using HOMA-IR. The authors concluded that elevations in muscle glucose uptake were likely responsible for the lower plasma glucose concentrations observed. However, paralleled increases of blood lactate and epinephrine may suggest that rather than being stored, glucose was being utilised in response to epinephrine-induced glycogenolysis. Furthermore, research conducted in type-2-diabetics illustrated an insulin sensitising role of acute (60 minute) hypoxia (FiO_2 = 14.6%; Oxygen saturation (SpO_2) = ~91%) during a subsequent normoxic intravenous glucose tolerance test (IVGTT) (Mackenzie *et al.*, 2011). Unlike Kelly *et al.* (2010), hypoxia-mediated reductions of insulin, not glucose, during the IVGTT appear to be primarily responsible for improvements in glycaemic regulation observed by Mackenzie *et al.* (2011). Mackenzie *et al.* (2011) also found that compared with normoxic exercise, hypoxic exercise significantly increased post-exercise insulin sensitivity. Although this study utilised a cross-over design, a healthy control group, not taking medication, would allow for more robust conclusions to be made.

In contrast, other research has documented a detrimental effect of acute hypoxia on insulin sensitivity, determined using the hyperinsulinaemic euglycaemic clamp technique, in healthy, young adults (Oltmanns *et al.*, 2004; Peltonen *et al.*, 2012). Oltmanns *et al.* (2004) demonstrated that just 30 minutes of hypoxic gas exposure (SpO_2 = 75%) significantly impaired glucose tolerance for the following two hours, highlighting that hypoxia-induced increases in epinephrine were partially responsible. Epinephrine is known to inhibit whole-body (Deibert and DeFronzo, 1980; Baron, Wallace and Brechtel, 1987) and skeletal muscle (Laakso *et al.*, 1992) insulin-mediated glucose uptake. Similarly, Peltonen *et al.* (2012) had participants breathe hypoxic gas (FiO_2 = 11%; SpO_2 = ~65%) for three hours, during which the glucose infusion rates required to maintain euglycaemia were 50% lower than the normoxic control ($p < 0.05$). Peltonen *et al.* also reported hypoxia-induced elevations of epinephrine, and that inhibiting epinephrine release resulted in partial abrogation (~25%) of hypoxia-induced attenuations of insulin sensitivity. Interestingly, despite reporting similar hypoxia-induced increases in epinephrine as Oltmanns *et al.* and Peltonen *et al.*, Kelly and colleagues saw a stimulatory, rather than inhibitory, effect on glycaemic regulation. Collectively, these data highlight that acute, relatively severe, hypoxia promotes glucose intolerance in men, which is at least partially attributable to heightened sympathetic activation and the resultant release of catecholamines, known to adversely affect glycaemic regulation.

There are clear discrepancies in the literature regarding the role of hypoxia-induced increases of catecholamines on glucose uptake. Early research demonstrated an inhibitory effect of non-physiological levels of epinephrine (~10-fold increase) on insulin release/action (Deibert and DeFronzo, 1980; Lager *et al.*, 1986; Baron, Wallace and Brechtel, 1987). This effect would be expected to be significantly weaker, or even absent, in the aforementioned studies which report

smaller 2-3-fold increases, potentially explaining the inconsistent findings. Data from Peltonen and colleagues show epinephrine was responsible for only ~25% of hypoxia-induced impairments, suggesting involvement of other factors. Physiological concentrations of epinephrine are known to enhance skeletal muscle blood flow (Freyschuss *et al.*, 1986; Linde *et al.*, 1989), a key determinant of muscle glucose uptake. Discrepancies in literature are possibly explained by increases in blood flow, and thus substrate delivery, partially compensating for epinephrine's inhibitory effect on insulin (Laakso *et al.*, 1992).

Despite largely consistent results of a stimulatory effect of acute hypoxia on muscle glucose transport from *in vitro* and animal studies (Cartee *et al.*, 1991; Azevedo *et al.*, 1995; Zierath *et al.*, 1998; Fisher *et al.*, 2002), these findings have not been conclusively replicated in clinical trials. The apparent differences between the aforementioned studies could relate to differences in the populations investigated, the severity and duration of hypoxic exposure, or the method employed to measure the efficiency of glycaemic control. Despite these inconsistencies, the promising beneficial effects of post-exercise hypoxic exposure on muscle glucose uptake remain, particularly given the additive effects of hypoxia and exercise on insulin sensitivity reported by Mackenzie *et al.* (2011). Kelly and colleagues data suggest that hypoxia-induced elevations in muscle glucose uptake were coupled with increases in glucose utilisation rather than storage: However, it is plausible that post-exercise, hypoxia-mediated glucose uptake would work to promote the opposite, due to the overriding impact of depleted glycogen stores and exercise-induced increases of insulin sensitivity. The studies in which a beneficial effect was reported utilised carbohydrate ingestion, rather than infusion, which is arguably a more physiologically relevant stimulus. Exercise-induced elevations in insulin sensitivity diminish with time following cessation of contraction-stimulated signalling pathways, thus reducing the capacity for rapid glucose uptake. Therefore, a post-exercise hypoxic stimulus could work to maintain stimulation of those insulin-independent pathways, increasing GLUT4 translocation, and thus sarcolemmal membrane permeability, to promote glucose uptake and prolong elevations of insulin sensitivity, ultimately ensuring rapid muscle glycogen resynthesis.

2.0. Future Research

Having discussed the importance of optimising mitochondrial and vascular function for both health and exercise, research investigating strategies to achieve this hold great promise. Such strategies should target the aforementioned 'key players' – bioavailability of NAD+ and nitric oxide, sirtuin activity, and redox status. Nutraceuticals and other dietary interventions have shown the capacity to affect these key players, although not all simultaneously. Conversely, NRPT supplementation offers a unique opportunity to accomplish simultaneous activation of all these pathways by co-

supplementing a polyphenol alongside an NAD+ precursor. NRPT will likely have its most meaningful effects in populations with pre-existing dysfunctions relating to the vasculature and metabolism; however, exposure to stimuli that transiently induce dysfunction, such as acute hypoxia or hyperglycaemia, could be used when investigating healthy populations. Using such stimuli should help potentiate beneficial effects of NRPT, but if successful, results could have ramifications for preventing the progression of periodic post-prandial vascular dysfunction in to atherosclerosis or attenuating the decline in exercise performance at high-altitude. Little literature exists that focusses on the acute potential of NR and PT, a setting that lends itself to the manipulation of outcomes relating to exercise, rather than disease. As well as the physiological responses to exercise, this also includes the post-exercise recovery period, in which maximising glucose uptake and thus glycogen synthesis is essential for athletes with busy training or competitive schedules. This review also discussed the potential of post-exercise hypoxic exposure to augment post-exercise glucose uptake, an avenue of research that remains completely unexplored in humans.

In attempt to fill several gaps in the literature, this Masters research project primarily intended to document the acute effects of NRPT supplementation on baseline vascular function, substrate utilisation and vascular function during sub-maximal continuous exercise, and post-exercise glycaemic regulation. Measuring glycaemic regulation using an OGTT provides a strong hyperglycaemic response, this stimulus can therefore also be used to assess if NRPT can prevent, or even abolish, hyperglycaemia-induced vascular dysfunction. Alongside cataloguing the physiological effects of NRPT, the research project also intended to expand our understanding of how hypoxia impacts glycaemic regulation in humans by using post-exercise hypoxic exposure. Similarly to the glucose load, acute hypoxia also induces vascular dysfunction and could therefore give further insights in to the vascular potential of NRPT.

Unfortunately, despite the research project being fully designed and having received ethical approval, the COVID-19 pandemic prevented the execution of this research project. The following chapter outlines the proposed methodology of this intended study that was designed to explore the research questions outlined in the paragraph above.

2. PROPOSED METHODOLOGY

2.1. Ethics

Ethical approval was granted by the University of Birmingham's ethical review committee for this proposed protocol (ERN_19-1912). All work would be carried out in compliance with the General Medical Council's Research Guidelines and run to the principles of Good Clinical Practice.

2.2. Participants

At least 20 moderately trained participants would be recruited for this study. Brachial artery FMD (primary outcome measure) was significantly increased following acute supplementation of pterostilbene's analog, resveratrol, in a placebo-controlled, cross-over trial of 19 participants (Wong *et al.*, 2011). A moderate training status was anticipated to improve consistency of the physiological responses to exercise protocol, and allow comparison of metabolic and vascular variables to existing literature. Participants would be screened using a General Health and Lifestyle Questionnaire (including allergies) and required to meet the following inclusion criteria: 1) aged 18-50; 2) healthy, with no history of cardiovascular, metabolic, or respiratory disease; 3) not taking over-the-counter medication or supplements with potential metabolic/vascular effects; 4) not allergic to nicotinamide riboside or pterostilbene, and 5) females must be on effective oral contraception. In addition, participants needed to achieve a $\dot{V}O_{2max}$ of ≥50 or ≥45 ml/kg/min for males and females, respectively, confirmed at the first visit (see below). Participants received detailed information sheets regarding the experimental protocol and could ask questions before signing an informed consent form.

2.3. Study Design

The proposed study involves an acute dietary supplementation intervention and was to be conducted as a randomised, double-bind, placebo-controlled trial with a full cross-over. Participants would be asked to attend the Human Performance Laboratory in the University of Birmingham's School of Sport, Exercise & Rehabilitation Sciences on five separate occasions (Figure 5). The first visit involves a $\dot{V}O_{2max}$ test and short familiarisation of the brachial artery FMD protocol. The remaining four experimental sessions share a largely identical protocol, briefly, consisting of baseline vascular measures, 1 hr steady-state exercise, and a 4 hr post-exercise recovery period. The only differences between sessions are the supplement taken prior (placebo vs NRPT) and the environmental conditions during the post-exercise recovery period, half were conducted in normal room air (normoxia) and half in hypoxia (simulated altitude of 3500 m). To prevent any bias, both the participant and research team would be blinded to the supplement being taken at each visit. NRPT (Basis, Elysium Health) and placebo supplements would be coded in to two groups (A and B) by

a colleague independent to the study, the code would be broken once all data is collected and preliminary analysis completed. Following visit 1, participants would be randomised into supplemental group A or B - they would receive this supplement for the first arm of the study (visits 2 and 3) and then swap to the other for the second arm (visits 4 and 5). Visit 2 would take place at least two days after visit 1, while for the remaining visits there would be a minimum seven day wash-out period to prevent carry-over effects of NRPT supplementation. During the second arm of the study (visits 3 and 4) participants would receive the alternative supplementation to that they were given during the first arm (visits 2 and 3). Each arm consisted of two experimental sessions, involving either a normoxic- or hypoxic-recovery period – the order of sessions within each arm was randomised (see figure 5). All experimental sessions would begin at the same time of day (7:00-9:00 am).

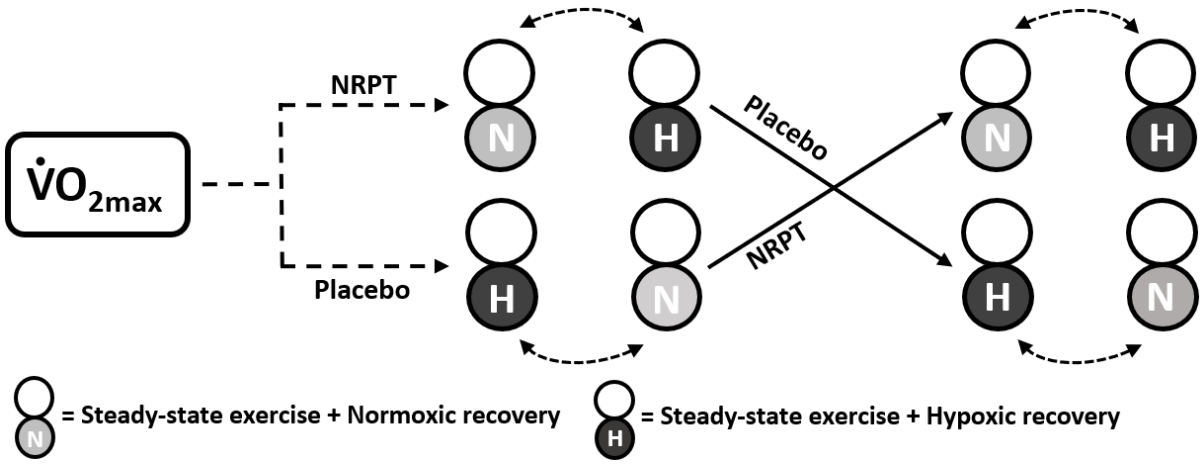

Figure 5: *Schematic demonstrating the cross-over study design, involving one familiarisation session followed by four experimental visits. Dashed lines indicate randomisation (supplement and experimental visit order).*

Visit 1: $\dot{V}O_{2max}$/familiarisation

Participants would be provided an information sheet and asked to complete a General Health and Lifestyle Questionnaire to determine eligibility, before signing the informed consent form. Participants would then be equipped with a heart rate monitor (M430, Polar, Finland) and facemask, connected to a metabolic cart (Vyntus CPX, Carefusion, Germany), for collection of respiratory gas data. Baseline measures of heart rate, oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) would be taken after a 5 min rest period. Participants would undertake a 5 min warm-up, with resistance gradually increased until a Rate of Perceived Exertion (RPE) score of 11 is reached (~60-100 W), at which point the test would begin. Resistance would be increased by 30 W every 2 min until volitional exhaustion or cadence drops below 50 rpm. Upon completion, resistance would be reduced to ~60W for a minimum 5 min cool-down. Following the cool-down period, equipment

would be removed and data assessed to determine whether individuals met the $\dot{V}O_{2max}$ inclusion criteria. If achieved, participants are then familiarised with the FMD protocol used in future experimental sessions, after which they are free to leave. The purpose of this FMD familiarisation should help avoid stress-induced vascular responses during experimental sessions.

Standardisation Procedures

Prior to the four experimental sessions participants would be asked to refrain from vigorous exercise and the consumption of alcohol and foodstuffs containing high levels of phenolic compounds/nitrate (see prohibited foods list: Section 2.9) in the preceding 24-hours, and caffeine consumption in the preceding 12-hours. Prohibited foods are those considered to have a significant effect on pathways which NRPT is hypothesised to act on, primarily surrounding nitric oxide bioavailability, redox status, muscle metabolism, or glycaemic regulation (as outlined in previous chapter). Together, these standardisation measures are intended to prevent influence of these variables on metabolic and vascular outcome measures. For example, FMD, the primary outcome measure, is known to be acutely affected by exercise (Dawson *et al.*, 2013), alcohol (Hijmering *et al.*, 2007), caffeine (Buscemi *et al.*, 2010), polyphenols (Hooper *et al.*, 2012; Li *et al.*, 2013; Rodriguez-mateos *et al.*, 2014), and nitrate (Lara *et al.*, 2016). Dietary factors could also potentially interfere with determination of substrate utilisation. Although not all evidence agrees (Garnacho-castaño *et al.*, 2018; Kocoloski and Crecelius, 2018), short-term nitrate supplementation has been proven to alter oxygen consumption during exercise (Larsen *et al.*, 2007, 2010; S. J. Bailey *et al.*, 2009; Lansley, Winyard, Fulford, *et al.*, 2011). Similarly, acute caffeine consumption has been shown to enhance fat oxidation during sub-maximal (Ruiz-Moreno *et al.*, 2020) and maximal (Ramírez-Maldonado *et al.*, 2021) exercise.

Due to the timing and duration of experimental sessions it is important that participants are fed; therefore, participants would be provided with a commercially available, pre-packaged, protein-based standardised meal and asked to consume this 2.5 hr prior to arrival at each experimental session. Fat oxidation during exercise, another key outcome measure, is supressed and augmented by pre-exercise feeding of carbohydrate (Coyle *et al*., 1997; Horowitz *et al.*, 1997) and fat (Gregory *et al.*, 2011; Murakami *et al.*, 2012), respectively. Conversely, evidence suggests that pre-exercise protein intake has a minimal effect on fat oxidation (Impey *et al.*, 2015). The standardised pre-session meal is also important to reliably determine FMD. A meta-analysis reported that acutely, irrespective of macronutrient composition, meal consumption significantly impairs FMD by ~2% (Thom *et al.*, 2016). High-fat and high-carbohydrate meals negatively affect endothelial function (Koning and Rabelink, 2002; Lacroix *et al.*, 2012; Mah and Bruno, 2012). Protein-specific effects have only been investigated in two studies, which suggest protein has a minimal effect on FMD. One

investigation found that protein intake had a protective effect on fat-induced FMD declines (Smeets, Mensink and Joris, 2020), whilst the other reported that protein significantly reduced FMD (Phillips *et al.*, 2014); however, this was likely due to a significantly higher baseline diameter. The standardised protein-based meal will therefore help ensure consistency of metabolic and vascular responses between-sessions. Furthermore, participants would complete a food diary for the day prior to visit 2, and asked to replicate this for the day before visits 3-5.

Experimental sessions would be scheduled for the same time of day to prevent interference of the natural diurnal variation associated with FMD (ter Avest *et al.*, 2005), glucose tolerance (Poggiogalle, Jamshed and Peterson, 2018), substrate utilisation (Ramírez-Maldonado *et al.*, 2021), and redox status (Blanco *et al.*, 2007). Female participants would be tested during pill-taking weeks, where hormones are most stable. FMD (Williams *et al.*, 2001), arterial stiffness (Spaczyński *et al.*, 2014), and redox status (Cornelli *et al.*, 2013) are influenced by hormonal changes. Ambient temperature affects FMD (Widlanksy *et al.*, 2007), substrate utilisation (Gagnon *et al.*, 2020), and glycaemic regulation (Dumke *et al.*, 2015); therefore, all experimental sessions would be conducted in a climate-controlled laboratory. Additionally, throughout the duration of the study, participants would be asked to refrain from making significant changes to their training schedules and dietary habits.

Experimental Protocol

At 12 and 2.5 hrs prior to visits 2-5, participants would be asked to consume the supplements provided, either: NRPT (250 mg NR, 50 mg PT) or placebo - totalling 500 mg NR and 100 mg PT. These timings were chosen based on pharmacokinetic data that show the peak NR-induced elevations in NAD+ metabolism (Trammell, Schmidt, *et al.*, 2016) and the T_{max} of PT (Kapetanovic *et al.*, 2011) occur at ≥ 8 hrs and ~3 hrs post-supplementation, respectively. Participant's adherence to the supplementation and standardisation procedures would be recorded on arrival. Participants would then complete 20 min supine rest in preparation for resting vascular measures, after which they would be equipped with a cannula (antecubital vein), near-infrared spectroscopy (NIRS) probe (vastus lateralis), heart rate monitor (Polar), and facemask, connected to a metabolic cart. A resting venous blood sample would be taken for determination of baseline redox status.

On a cycle ergometer (Lode), participants would complete a 5 min warm-up (60-100 W) before completing 1 hr at 60% W_{max} in normoxia. This intensity is considered aerobic and promotes fat oxidation, a variable which NRPT is hypothesised to augment. Research shows that fat is the predominant fuel source during sub-maximal exercise (<~65% $\dot{V}O_{2max}$) (Romijn *et al.*, 1993; Van Loon *et al.*, 2001) and that the exercise intensity which elicits maximal fat oxidation rates (Fat_{max}) lies between 55-75% $\dot{V}O_{2max}$ (Van Loon *et al.*, 2001; Achten, Venables and Jeukendrup, 2003; Bircher and

Knechtle, 2004; Lanzi *et al.*, 2014), depending on training status (Fat_{max} increases with cardiorespiratory fitness (Purdom *et al.*, 2018)). A 60% W_{max} intensity was chosen as this should correspond to Fat_{max} for the fitness levels of participants recruited, based on data from studies investigating participants with similar $\dot{V}O_{2max}$ (52-58 ml/kg/min) (Achten, Venables and Jeukendrup, 2003; Robinson *et al.*, 2015). Blood samples would be taken at 15 min intervals throughout steady-state exercise for determination of lactate and glycerol concentrations, markers of fat oxidation. Respective decreases (Achten & Jeukendrup., 2004) and increases (Robinson *et al.*, 2016) in these variables are considered potential markers of increased exercise-induced fat oxidation. Upon completion of exercise, all equipment (except the cannula) would be removed from the participant and they would be seated in either normoxic or hypoxic conditions, depending on randomisation. The University of Birmingham's environmental chamber would be used to induce normobaric hypoxia (F_iO_2 = 13.5%; simulated altitude = ~3500m). This degree of hypoxia was chosen as research involving similar acute exposures have demonstrated significant improvements and impairments in glycaemic regulation (Mackenzie *et al.*, 2011) and endothelial function (Lewis *et al.*, 2014, 2017), respectively. Once seated, participants would be asked to complete an OGTT and consume 75 g glucose dissolved in 250 mL water, after which the 4 hr post-exercise recovery period would begin.

The 4 hr duration was chosen based on the biphasic response of post-exercise muscle glycogen resynthesis (Price *et al.*, 1994), and thus glucose uptake. The initial rapid insulin-independent phase lasting ~1 hr is thought to be attributable to exercise-induced residual membrane-bound GLUT4 and low glycogen concentrations (Ivy and Kuo, 1998). These effects will likely overpower any NRPT- or hypoxia-induced changes in glycaemic control. The second, more gradual, phase is insulin-dependent and can last for many hours, depending on the original degree of glycogen depletion (Alghannam, Gonzalez and Betts, 2018). Therefore, the 4 hr duration should allow identification of the potential effects of NRPT, which are hypothesised to be primarily insulin-dependent, and hypoxic exposure, hypothesised to maintain stimulation of insulin-independent pathways, activity of which are thought to decline after ~1 hr (Goodyear *et al*., 1990). For analysis of NRPT- or hypoxia-induced alterations in glycaemic regulation, blood samples would be taken at 30, 60, 90, 120, 180 and 240 min post-OGTT for determination of glucose and insulin concentrations. Acute hypoxic exposure has been suggested to augment glucose clearance from the blood by increasing glucose utilisation, and thus lactate production (Kelly *et al.*, 2010). Therefore, to help determine whether post-exercise hypoxic exposure affects glucose utilisation or storage, lactate would also be measured at all aforementioned timepoints.

To assess hyperglycaemia- and hypoxia-induced alterations in vascular function, FMD and arterial stiffness would be measured at 60 and 210 min. Acute hypoxia has previously been shown to significantly reduce FMD at these timepoints (Lewis *et al.*, 2014). The first timepoint is also relevant to hyperglycaemia-induced FMD impairment, with the majority of literature concluding that peak attenuation occurs 1 hr post-OGTT (Mah *et al.*, 2013; Greyling *et al.*, 2015). Hyperglycaemia-induced vascular dysfunction is thought to be primarily attributable to elevations in RONS production (Mah and Bruno, 2012). Significantly elevated biomarkers of oxidative stress have been documented following an OGTT in healthy populations (G. D. Xiang *et al.*, 2008; L. Wang *et al.*, 2013), with evidence indicating a peak at 1 hr post-OGTT (Mah *et al.*, 2013). Therefore, the 60 min blood sample would also be used to measure hyperglycaemia-induced declines in redox status, and thus potential NRPT-induced attenuations.

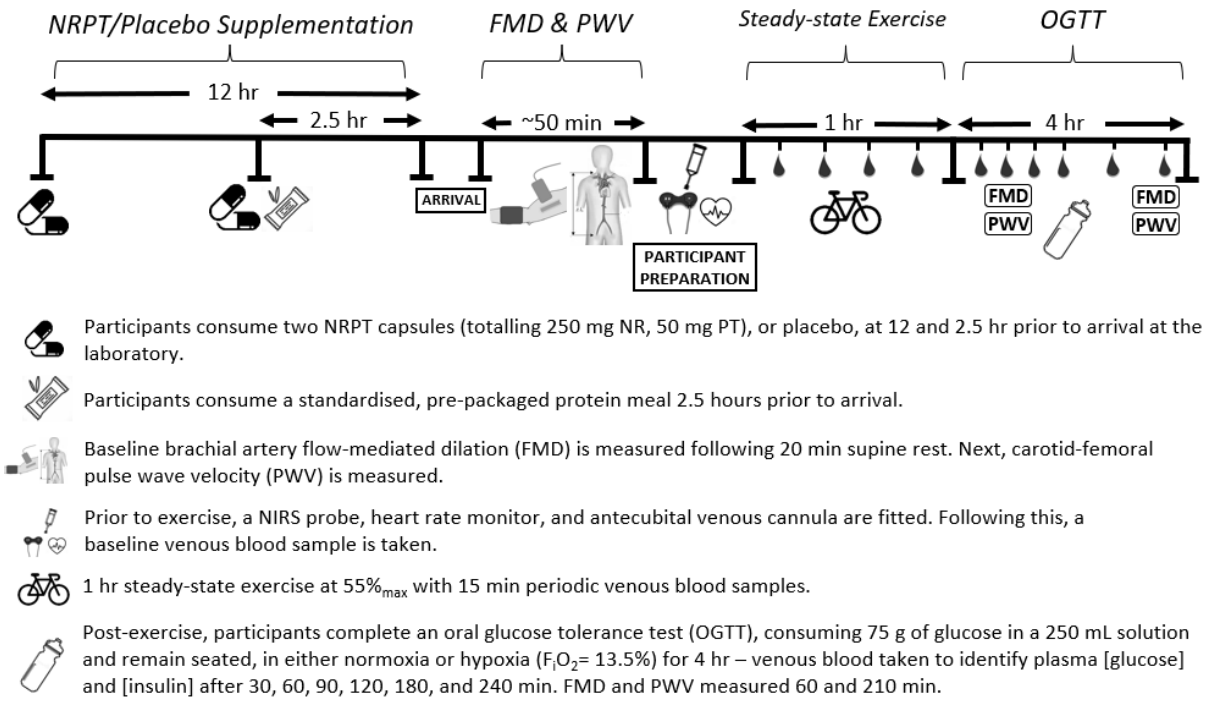

Figure 6: *Timeline and summation of the experimental protocol.*

2.4. Vascular Outcome Measures

FMD

Protocol

The FMD protocol will be conducted in line with recommended, standardised guidelines (Thijssen *et al.*, 2019). Upon arrival to the laboratory, participants would be asked to lay supine with the right arm outstretched with palm up-facing, level with the heart, resting on a table positioned at equal height to the bed. A blood pressure cuff would then be positioned distal to the imaged artery, around the forearm, ~2 cm from the antecubital fossa, the exact position would be recorded and photographed to ensure that the cuff position could remain consistent across all visits. Cuff placement has been shown to influence the degree to which nitric oxide mediates the FMD response. A meta-analysis (Green *et al.*, 2014) found that compared with proximal, the subsequent dilatory response to distal cuff occlusion is primarily nitric oxide-mediated (~70% vs ~30%). Given the proposed study's hypotheses, it is important to utilise the distal cuff placement which will highlight changes in nitric oxide-mediated dilation. Another advantage of utilising distal, over proximal, cuff placement is that it reduces variability in FMD measurements (Peretz *et al.*, 2007), likely due to the superior image quality achieved. To prevent movement of the arm during the imaging process, support should be provided under the wrist, using towels, and the upper arm, using and a mouldable pillow. After ensuring the participant is comfortable, they must complete 20 min supine rest, allowing for the stabilisation of haemodynamic parameters.

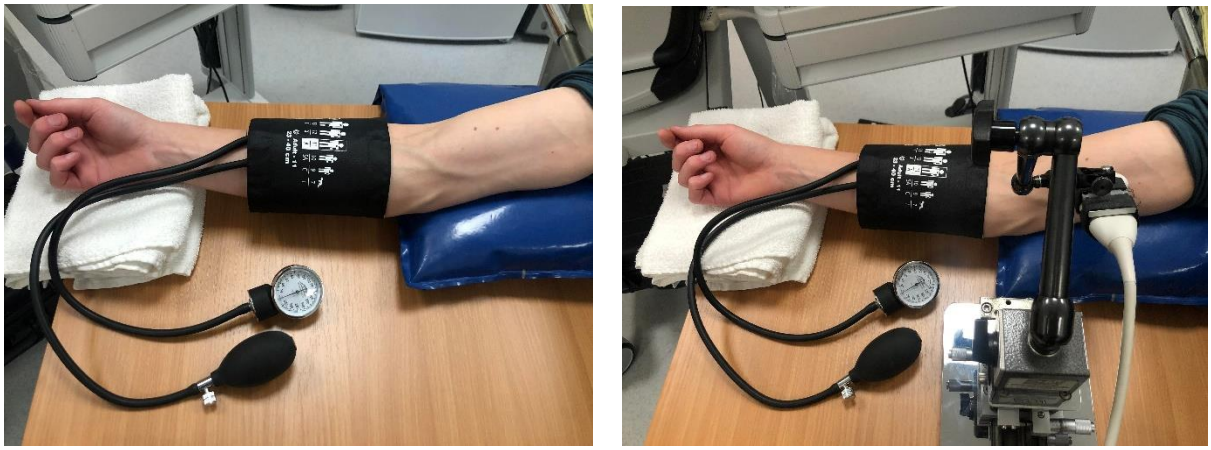

Figure 7: *Arm, support, and distal cuff placement of FMD protocol*

Once an adequate image has been established (see detail below), the eleven-minute protocol can begin. This starts with a 1-min period where baseline diameter and shear rate can be determined. Next, the forearm cuff is inflated and sustained at suprasystolic pressure (~220 mmHg) for 5 min, preventing blood entering the lower limb and instead accumulating in the upper arm. During cuff occlusion the image of the artery can be distorted due to movement of the skin; if necessary, in the final ~10 s of this period, small adjustments to the probe position can be made in order to regain image quality. After 5 min, the cuff is then rapidly deflated (<2 s) and remains so for a further 5 mins, in which artery diameter and shear rate are continuously recorded. Further small adjustments to the probe position can be made post-cuff deflation to improve image clarity, these must be completed within 15 s of cuff deflation. The sudden release of built-up pressure that occurs with cuff deflation enables the rapid flow of blood into the forearm, consequently increasing shear stress in the brachial artery, and ultimately triggering a dilatory response (Pyke and Tschakovsky, 2005).

Upon completion of the protocol, it is important to record an accurate position of the probe in relation to the arm, which will allow for consistency in the arterial segment analysed between visits. Photos displaying the position/angle of the probe can be taken, as well as recording the distance and angle from the elbow's medial epicondyle to the centre of the probe. Recording distinctive anatomical landmarks evident in the ultrasound image can also be used to ensure that the same arterial segment is analysed between visits. Finally, the probe, cuff and excess gel can be removed from the participant.

Ultrasound Probe Positioning

Maintaining a high-quality image of the target artery is essential for accurate and repeatable measurements of FMD, an example is shown in Figure 8. The ideal image should display a longitudinal arterial segment, orientated parallel to the probe. The arterial walls should be bright and clearly defined (double lines of Pignoli), in order to optimise the edge-detection software used in analysis and provide the most precise diameter measurements. In contrast, the lumen should be dark and unobstructed to provide a strong pulsed-wave Doppler velocity signal. Guidelines from Thijssen *et al.* (2019) advise the use of probe-holders, based on evidence that they are associated with greater FMD reproducibility (Greyling *et al.*, 2016). Primarily, probe-holders help to ensure image stability; however, they also allow for fine-tuning of the probe position to enhance

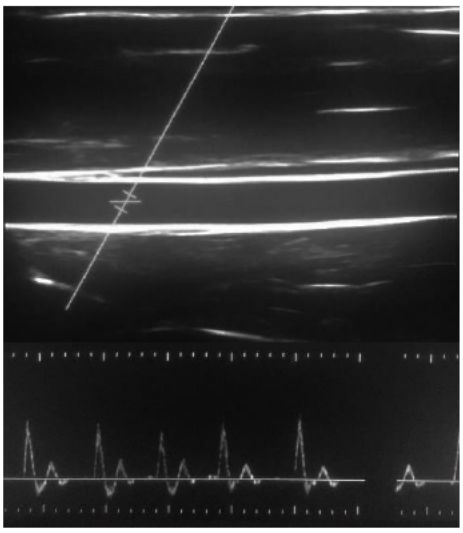

Figure 8: *Example longitudinal image of the brachial artery, with clear arterial walls and strong pulsed waved Doppler signal.*

image clarity. The on-screen contrast can be adjusted to enhance image definition; however, the primary aim should always be to get the perfect image from probe positioning alone.

Ultrasound Set-Up

The ultrasound system available is the Terason Duplex Doppler system (uSmart 3300 NexGen; Terason; USA), in conjunction with a 15-4MHz frequency linear array ultrasound transducer (15L4 Smart MarK™). There are three primary imaging modes that are utilised during data collection: B-mode (to measure arterial diameter), pulsed waved Doppler (to measure blood flow velocity and thus calculate shear rate) and colour Doppler (to help distinguish between arteries and veins). An important consideration when employing duplex ultrasound (the simultaneous recording of B-mode diameter and pulsed waved Doppler velocity) is that the two modes require vastly different orientations of the ultrasound beam in relation to the vessel to function optimally (90^{o} vs 0^{o}, respectively). Evidently, a compromise in beam orientation is necessary which enables accurate measurements of both diameter and velocity. Research has shown that error increases exponentially with angles >60^{o} and therefore guidelines recommend an angle <60-70^{o} to be used (Thijssen *et al.*, 2019). Subsequently, this proposed protocol intended to use a 60^{o} beam-vessel angle. Based on manufacturer recommendation, an image depth of 3 cm should be used to optimise visibility of the brachial artery, whilst reducing that of the vein. The sample volume of the Doppler velocity should be positioned in a dark, unobstructed region of the lumen, outside of the primary region of interest (to prevent interference during analysis), where a strong pulsed wave signal is achieved (as shown in Figure 8).

Shear Rate

Measurements of arterial diameter and blood flow velocity can be used to calculate shear rate ($\dot{\gamma}$). FMD Studio calculates shear rate using the following formula: $\dot{\gamma}$ = (4 x blood velocity) / vessel diameter. Shear stress is the tangential force produced as blood flows against a vessel and is detected by mechanotransducers in the endothelium (Ando and Yamamoto, 2013), triggering the synthesis of vasoactive substances, including nitric oxide (Buga *et al.*, 1991) and prostaglandins (Rubanyi and Vanhoutte, 1986). Adjusting shear rate for blood viscosity allows shear stress to be determined; however, because blood viscosity is generally considered to be consistent within/between groups/individuals (Boot *et al.*, 2002; Padilla *et al.*, 2008), shear rate is an acceptable proxy for shear stress. Interventions that alter blood viscosity (e.g., chronic hypoxic exposure or exercise training (El-Sayed, 1998; Tremblay, Hoiland, *et al.*, 2018)) should report the shear stress, not rate, as this may influence results. Given the stimulatory effect of shear rate on vasodilator synthesis, it is unsurprising that the magnitude of shear rate is proportional to the

magnitude of FMD observed (Carter *et al.*, 2013), with research highlighting that the overall mean shear rate, rather than the peak, is the most important factor (Pyke and Tschakovsky, 2007). Therefore, it is important that studies aiming to manipulate FMD also record and report shear rate, as an intervention may wish to achieve this independent of changes in shear rate, or vice versa. In relation to this proposed protocol, NRPT is hypothesised to elevate FMD by augmenting the bioavailability of nitric oxide, and therefore shear rate should remain constant.

Analysis

FMD Studio, within Cardiovascular Suite (v.2.81, Quipu, Italy), contains the edge-detection and wall-tracking software that would be used to analyse each FMD protocol. Analysis can be conducted offline using recorded real-time footage of the longitudinal artery and pulsed-wave velocity graph (shown in Figure 8). An arterial segment should be selected that allows for accurate and consistent edge-detection during the first minute, the ~3 min post-cuff deflation, and the final minute. Diameter and velocity data from the first minute of the protocol should be recorded to establish baseline values. The next portion of the data to be recorded is from 10 s prior to cuff-deflation, until after the peak diameter has clearly been reached and has begun to return to baseline (generally ~2 mins post-cuff deflation). It is important to start the recording slightly prior to cuff-deflation to capture of the full shear rate response, not just the peak, as the overall mean is a more powerful determinant of the FMD response (Pyke and Tschakovsky, 2007). The final portion of data to record is the last minute (recovery), which should show the arterial diameter back at baseline values, although sometimes it remains slightly higher. This recovery data can be used to calculate FMD in instances where the baseline data recorded is highly fluctuating and therefore not reliable. The most likely cause of unreliable baseline data is due to participant movement during the protocol.

Of the 11 min protocol, ~4-5 min of diameter and velocity data should be recorded. Here, the data should be snipped further to remove clear errors in the data, errors could be resultant from participant movement or inconsistencies with the edge-detection software. Ideally, baseline and recovery data should be completely stable and be of similar absolute values. Immediately post-deflation, diameter should then be seen to progressively rise until the peak is reached, after which it begins to gradually return to baseline. The software calculates the mean diameter and shear rate during baseline and recovery data collection periods, as well as the peak reached post-deflation. Area under the curve (AUC) shear rate is also calculated for the period between cuff deflation and peak diameter attainment. FMD is subsequently calculated using the equation: ((post-occlusion peak diameter – baseline diameter) / baseline diameter) x 100. This process should be repeated at least

twice, ideally 3-4 times, on different arterial segments, to allow determination of mean values for baseline diameter, FMD, and AUC shear rate.

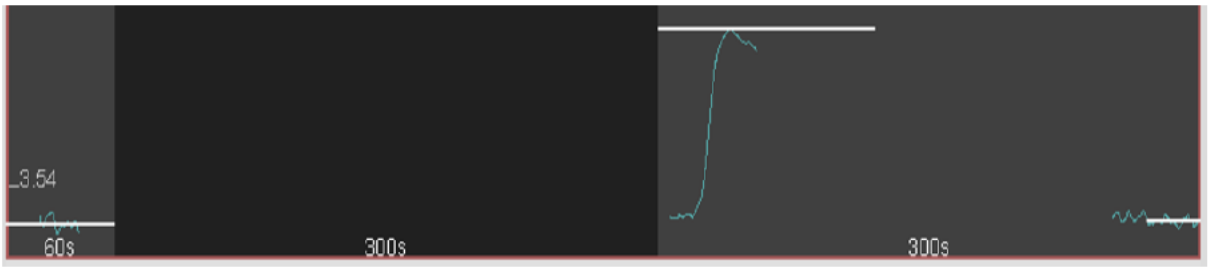

Figure 9: *Example analysis of arterial diameter (mm) during 11 min FMD protocol.*

Arterial Stiffness

Pulse wave velocity (PWV) is widely accepted as an indicator of arterial stiffness and is an independent predictor of cardiovascular risk (Meaume *et al.*, 2001; Cruickshank *et al.*, 2002; Mattace-Raso *et al.*, 2006). PWV is defined as the speed with which a pulse wave travels along a length of an artery (Oliver and Webb, 2003). Arterial stiffness of the aortic and aorto-iliac pathway can be assessed non-invasively using carotid-femoral PWV (cfPWV), deemed the gold-standard due to its clinical relevancy (Laurent *et al.*, 2006). For this proposed study, cfPWV would be measured using the Vicorder (Skidmore Industries, UK), an automated and operator-friendly device that uses an oscillometric technique to create pulse pressure waves at proximal and distal blood pressure cuffs. The Vicorder has been validated against other methods used to assess arterial stiffness and is considered to have a good repeatability in healthy populations (Hickson *et al.*, 2009), and those with peripheral artery disease (Shahin *et al.*, 2013).

Measurements should be taken supine, following ≥5 min rest to allow for the stabilisation of haemodynamic variables. Arterial path length is determined superficially by measuring the distance between the suprasternal notch and the centre of the thigh cuff. A 100 mm wide cuff would be placed around the right upper thigh with another 30 mm cuff around the neck, level with the right carotid artery. Both cuffs would then be inflated to 65 mmHg and for the next 3-5 s, pulse waveforms are simultaneously recorded at each site (carotid and femoral). The trace is frozen when at least three sufficient quality waveforms have been recorded. The Vicorder automatically

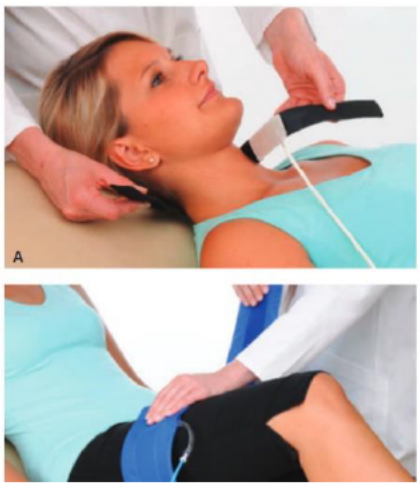

Figure 10: *Example of correct carotid (A) and femoral (B) cuff placement. Taken from Muller et al. (2013)*

calculates foot-to-foot transit time of the proximal and distal waves and uses this information, alongside the inputted path length, to quantify cfPWV . cfPWV is recorded from two sufficient quality waveforms, within ≤ 0.5 m/s of each other, and averaged.

Near-infrared Spectroscopy (NIRS)

NIRS is a validated non-invasive technique to estimate *in vivo* local tissue oxygenation and perfusion (Mancini *et al.*, 1994). Prior to steady-state exercise, the NIRS probe, connected to a monitor (NIRO-200NX; Hamamatsu Photonics K.K., Japan) would be attached to the lower third of the vastus lateralis and secured with tape. The probe would be located ~10 cm proximal to the patella with the precise position recorded for each participant to ensure placement consistency between-sessions. Human tissue is relatively transparent to near-infrared light emitted by the probe, absorption is oxygen-dependant and determined by chromophores, oxyhaemoglobin (O2Hb) or deoxyhaemoglobin (HHb) (Mancini *et al.*, 1994). Absolute values of [O2Hb], [HHb] and [Total Haemoglobin] (tHb) cannot be calculated, but the relative change (micromoles per litre) from baseline is detectable based on an application of the modified Beer-Lambert law (Al-Rawi, Smielewski and Kirkpatrick, 2001). Total Oxygenation Index (TOI) measures mixed arterial-venous oxygen saturation and represents the balance between oxygen delivery and consumption (Mancini *et al.*, 1994), calculated using: ([O2Hb] / [tHb]) x 100. Normalised Total Haemoglobin Index (nTHI) measures perfusion, estimating local blood flow using the ratio of current [tHb] to initial [tHb]. TOI (%) and nTHI are calculated by the NIRO-200NX (Spatially Resolved Spectroscopy method) using the light attenuation slope along the distance from the emitting point as detected by two photodiodes in the detection probe. TOI and nTHI would be continuously recorded throughout the 1 hr steady-state exercise.

Nitric Oxide Bioavailability

NO is a powerful vasodilator and is the primary driver of FMD (when using distal cuff placement) (Green *et al.*, 2014); therefore, FMD can be used as an indirect bioassay for NO bioavailability, whereby elevations in FMD indicate elevations in NO, and vice versa. In addition to this, for this proposed study the bioavailability of NO would be assessed in the blood indirectly by measuring plasma NO metabolites, nitrite and nitrate (NOx). The Griess assay (Miranda, Espey and Wink, 2001) is the most widely used method for NOx determination due to its methodological simplicity and low cost. NO is a highly reactive molecule with a short half-life, making direct quantification difficult; however, the Griess assay is proven to be a valid and reliable method to assess NO bioavailability (Tsikas, 2005; Y. Wang *et al.*, 2013). It has been shown that only plasma nitrite, not nitrate, reflects

regional eNOS activity (Lauer *et al.*, 2001). Methods able to quantify isolated nitrite are available but come with a heightened level of complexity and cost (Tsikas, 2005; Bryan and Grisham, 2007).

2.5. Metabolic Outcome Measures

Exercise Substrate Utilisation

Respiratory gases would be collected continuously during the 1 hr steady-state exercise protocol through a leak-free face mask fitted with a volume transducer and gas analyser, connected to a Vyntus Metabolic Cart (CPX, CareFusion, Germany). $\dot{V}O_2$ and $\dot{V}CO_2$ would be determined on a breath-by-breath basis, exported as 5 s averages for analysis. Analysis of expired gases using indirect calorimetry would be used to quantity substrate utilisation. Prior to all experimental sessions, gas and volume calibrations would be completed using known concentrations of gases (O_2/CO_2) and the in-built, automatic Vyntus system, respectively.

The respiratory exchange ratio (RER) is an indicator of relative substrate utilisation during exercise, assuming that protein oxidation is negligible. Values range from 0.7-1.0, where 0.7 and 1.0 indicate that the primary fuel source is fat or carbohydrate, respectively. RER can exceed 1.0 during intense exercise; however, this reflects changes in non-oxidative CO_2 production rather than changes in oxidative metabolism (Jeukendrup and Wallis, 2005). RER will be continuously recorded during exercise and is automatically calculated by the metabolic cart using the following equation:

$$RER = \dot{V}CO_2 / \dot{V}O_2$$

Estimated absolute rates of carbohydrate and fat oxidation (grams/min) during exercise would be calculated from the raw $\dot{V}O_2$ and $\dot{V}CO_2$ data, using equations proposed by Jeukendrup and Wallis (2005). Like RER, these equations are confounded by non-oxidative CO_2 production; therefore, accurate determination of these variables is only possible at intensities that do not promote lactate accumulation (<75% $\dot{V}O_{2max}$) (Jeukendrup and Wallis, 2005).

$$\text{Carbohydrate Oxidation (g/min)} = (4.210 \times \dot{V}CO_2) – (2.962 \times \dot{V}O_2)$$

$$\text{Fat Oxidation (g/min)} = (1.695 \times \dot{V}CO_2) – (1.701 \times \dot{V}O_2)$$

Exercise Metabolism Blood Markers

Blood samples taken at 15 min intervals during steady-state exercise would be used to quantify plasma glycerol and lactate, markers of exercise-induced fat oxidation and aerobic metabolism, respectively. Whole blood (10 mL) would be collected in to EDTA vacutainers and immediately kept on ice prior to being centrifuged at 5000 g for 15 min at 4 °C. Plasma samples would then be stored at -80°C until analysed.

Post-exercise Glucose Control

This will be assessed using an OGTT protocol, whereby participants consume a 75 g glucose load dissolved in 250 mL water. Plasma concentrations of glucose and insulin would be determined at the aforementioned timepoints. A reduction in the blood glucose response would be inferred as an increased glucose clearance. Insulin sensitivity during the recovery period would also be calculated using the HOMA-IR method (Matthews *et al.*, 1985) and the following equation:

$$\text{HOMA-IR} = ([\text{glucose (mmol/L)}] \times [\text{insulin (mU/L]}) / 22.5$$

2.6. Redox Status Blood Markers

Redox status, the balance between pro-oxidants and anti-oxidants, would be determined prior to exercise at rest, and 1 hr post-OGTT from a blood plasma/serum. Directly measuring free radicals is challenging; therefore, oxidative adducts, which are indicative of their presence, would be measured in the proposed study. Specifically, lipid peroxidation would be assessed using the thiobarbituric acid reactive substances (TBARS) assay and protein oxidation as protein carbonyls. The ratio of reduced to oxidised glutathione (GSH:GSSG) would also be measured.

TBARS

The TBARS assay is a methodologically simple and low-cost technique to assess lipid peroxidation in human serum, proven to produce consistent and reproducible results ( ). This assay is considered to lack specificity and is prone to artefacts, which limit the validity of the absolute values it produces (Lee *et al.*, 2012; Moselhy *et al.*, 2013). However, assessing the relative change can still be a useful indicator of lipid peroxidation. The PT analogue, resveratrol (Zern *et al.*, 2005; Mcanulty *et al.*, 2013; Seyyedebrahimi *et al.*, 2018), and NR (Dolopikou *et al.*, 2019) have both demonstrated positive effects on lipid peroxidation in clinical trials. The TBARS assay has been successfully used to document the significant oxidative insult induced by acute hyperglycaemia (Xiang *et al.*, 2008a; L. Wang *et al.*, 2013). F_2-isoprostanes are considered the gold-standard for *in vivo* lipid peroxidation quantification. This method uses high-performance liquid chromatography and is very reliable, but also costly (Marrocco, Altieri and Peluso, 2017).

Protein Carbonyls

This is the most commonly used marker of oxidative protein damage (Chevion and Berenshtein, 2000), primarily due to advantages relating to the speed of formation and stability (Marrocco, Altieri and Peluso, 2017). Protein carbonyls can be assessed by ELISA, meaning a simple, relatively low-cost procedure with proven validity and reproducibility (Buss *et al.*, 1997).

GSH:GSSG

Glutathione (GSH) is a tripeptide that functions as a non-enzymatic endogenous anti-oxidant, it is the most abundant anti-oxidant in aerobic cells (Wu *et al.*, 2004). GSH accepts an electron from the free radical hydrogen peroxide, forming GSSG (its oxidised state) and water (Owen and Butterfield, 2010). GSSG is then recycled back to its reduced state using the cofactor NADP(H). Therefore, GSH:GSSG reflects an overall oxidation status at a given time and when measured in the blood, is considered a useful indicator of whole-organism redox status and a marker of pathological conditions in humans (Pastore *et al.*, 2003). There are numerous techniques available to quantify GSG and GSSG in biological samples, including enzymatic assays, HPLC, mass spectrometry, and capillary electrophoresis (Pastore *et al.*, 2003). Spectrophotometric (Owen and Butterfield, 2010) and fluorometric (Hissin and Hilf, 1976) assays represent the easiest and most cost-effective methods for quantifying GSH and GSSG; however, they are suggested to lack specificity due to the impact of sample acidification on GSSG and interference of other proteins/thiols (Rossi *et al.*, 2002). Similarly, to TBARS, these methods may lack accuracy in relation to the true values of GSH/GSSG; although, they still provide a valuable gross overview of redox status which can be used to assess potential changes.

2.7. Data Analysis

Steady-state Exercise

Overall mean $\dot{V}O_2$, $\dot{V}CO_2$, RER, nTHI, and TOI would be calculated for the entire 1 hr steady-state exercise. Means would also be calculated for periodic 10 min intervals (0-10 min, 10-20 min…). $\dot{V}O_2$ and $\dot{V}CO_2$ values would be used to calculate corresponding mean rates of carbohydrate and fat oxidation during exercise. AUC values for oxidation rates would be calculated using the trapezoidal rule. Mean exercising glycerol and lactate concentrations would be determined from the four blood samples.

Post-exercise Recovery

AUC values for plasma glucose, insulin, and lactate concentrations would be calculated at each timepoint (30, 60, 90, 120, 180, and 240 min post-OGTT).

2.8. Statistical Analysis

Three repeated measures ANOVAs would be used for analyses.

One-way ANOVA (supplement [placebo vs NRPT]) to compare means regarding fat and carbohydrate oxidation (overall and AUC), RER, nTHI, TOI, lactate, and glycerol during exercise.

Two-way ANOVA (supplement [placebo vs NRPT] x exercise time [0-10 vs 10-20 vs 20-30 vs 30-40 vs 40-50 vs 50-60 min]) to compare means regarding fat and carbohydrate oxidation, RER, nTHI, and TOI during exercise.

Two-way ANOVA (supplement [placebo vs NRPT] x environment [normoxia vs hypoxia]) to compare means regarding plasma glucose, insulin, and lactate AUC.

Three-way ANOVA (supplement [placebo vs NRPT] x environment [normoxia vs hypoxia]) x time [baseline vs post-$OGTT_{60\ min}$ vs post-$OGTT_{210\ min}$] to compare means regarding FMD, PWV, and redox markers.

2.9. Prohibited Foods List

Polyphenols (based on Pérez-Jiménez et al., 2010)

Fruit

- apples
- apricots
- black chokeberries
- black and red currants
- black elderberries
- black grapes
- blackberries
- blueberries
- cherries
- grapes
- grapefruit
- lemon
- nectarines
- peaches
- pears
- pomegranate
- plums
- raspberries
- strawberries

Vegetables

- artichokes
- asparagus
- broccoli
- carrots
- endives
- potatoes
- red chicory
- red lettuce
- red and yellow onions
- spinach
- shallots

Legumes

- black beans
- tempeh
- tofu
- soybean sprouts
- soy meat
- soy milk
- soy yogurt
- white beans

Nuts and Seeds

- almonds
- chestnuts
- hazelnuts
- flax seeds
- pecans
- walnuts

Other

- black tea
- capers
- cocoa powder
- coffee
- dark chocolate
- ginger
- green tea
- olives and olive oil
- rapeseed oil
- red wine
- vinegar

Nitrate

- All foods from middle, high, and very high groups in Table 3 below.

Table 3: *Classification of vegetables according to nitrate content. Taken from Hord et al., 2009.*

Nitrate content (mg/100 g fresh weight)	Vegetable varieties
Very low, <20	Artichoke, asparagus, broad bean, eggplant, garlic, onion, green bean, mushroom, pea, pepper, potato, summer squash, sweet potato, tomato, watermelon
Low, 20 to <50	Broccoli, carrot, cauliflower, cucumber, pumpkin, chicory
Middle, 50 to <100	Cabbage, dill, turnip, savoy cabbage
High, 100 to <250	Celeriac, Chinese cabbage, endive, fennel, kohlrabi, leek, parsley
Very high, >250	Celery, cress, chervil, lettuce, red beetroot, spinach, rocket (rucola)

3. FMD REPEATABILITY STUDY

3.1. FMD Background

FMD is becoming an increasingly popular vascular outcome measure, used within sport, nutrition, and clinical healthcare. The growing popularity likely relates to its non-invasive nature, relatively low cost, short and simple protocol (~30 min), and clinical relevance. FMD describes the percentage increase in arterial diameter from baseline to its peak, following exposure to a single bout of elevated blood flow (reactive hyperemia). It is a measure of conduit artery vascular responsiveness and can be used as a non-invasive assay for bioavailability of the vasoactive molecule, nitric oxide. FMD can be indicative of endothelial function, particularly in clinically vulnerable groups. Evidence has shown that endothelial dysfunction, assessed using brachial artery FMD, is a precursor to atherosclerosis, diagnosed using carotid intima-media thickness (Juonala *et al.*, 2004; Halcox *et al.*, 2009). Atherosclerosis is a systemic circulatory disease which is implicated in the manifestation of many cardiovascular diseases, including ischemic heart disease (IHD), ischemic stroke, and peripheral artery disease (Herrington *et al.*, 2016). To reinforce the clinical importance of FMD, meta-analyses have found that an absolute increase of brachial artery FMD by 1% reduces the risk of cardiovascular events by 8-13% (Thijssen *et al.*, 2019), emphasising its potential within cardiovascular health assessment. In 2016, it was reported that CVD accounted for 31% of global deaths (Gregory A Roth *et al.*, 2018) and that between 1990 and 2013, IHD was the leading cause of premature adult mortality worldwide (Naghavi *et al.*, 2015); therefore, practical, non-invasive assessment of endothelial function is an important tool for early identification of cardiovascular disease risk.

The FMD response of the brachial artery is the most extensively researched, primarily due to accessibility and the ease of image acquisition; however, analysis of lower limb FMD via the femoral artery is becoming more common. The normal range of FMD lies between 5-9% (Bots *et al.*, 2005); however, this varies significantly between populations. For example, FMD is known to be influenced by age (decreasing with age) (Skaug *et al.*, 2012), sex (greater in women) (Skaug *et al.*, 2012), and disease states (lower in hypertensives (Simova *et al.*, 2008), CVD (Onkelinx *et al.*, 2012) and those with dyslipidaemia (Donald *et al.*, 2008)). There is conflicting data surrounding the impact of cardiorespiratory fitness on FMD, with research showing positive (Kasikcioglu *et al.*, 2005; Buscemi *et al.*, 2013), negative (Schroeder *et al.*, 2019), or no association (Wang *et al.*, 2014) between the two variables. Discrepancies in findings may relate to variations in FMD protocol or the fitness levels of the participants studied. The results from Schroeder *et al.* (2019) demonstrate a potential paradox, that fitter people have a lower FMD and by extension poorer endothelial function, which conflicts with the well-established positive impact that regular exercise has on cardiovascular health

(Nystoriak and Bhatnagar, 2018).These data highlight an important factor to remember when comparing FMD and endothelial function between populations, baseline arterial diameter – which has a strong, inverse relationship with FMD (Herrington *et al.*, 2001). Obviously, fitter people do not experience a larger degree of endothelial dysfunction; instead, training-induced vascular remodelling means they may have a larger baseline diameter (Green *et al.*, 2017) and thus a lower percentage increase to their peak.

3.2. FMD Variability

Given that only a small absolute increase in FMD of 1% is deemed clinically significant, reliability of this measure is vital. Indeed, it is generally accepted that to consider an intervention successful at having a meaningful impact on FMD, it must cause an absolute increase/decrease of 1%. For an individual with an FMD of 7%, an absolute increase of 1% equates to a relative increase of only 14.3%. Therefore, for an intervention to reliably document its effect on FMD, it is essential that the sonographer is able to consistently reproduce intra- and inter-day FMD results of individuals/groups with a variation, ideally, of less than the meaningful relative change. In healthy populations and when following the recommended expert-consensus guidelines, a meta-analysis concludes that brachial artery FMD has an excellent-to-good reproducibility (coefficient of variation = 9.3%) (van Mil *et al.*, 2016). This is in line with previous research, which also notes a higher inter-day, than intra-day, variation (9.9% vs 12.9%) (Ghiadoni *et al.*, 2012). Sources of variation when measuring FMD are attributable to either the participant or the methodology. This variability increases in populations which are older, have a lower baseline FMD, or experience a degree of clinical hypertension/dyslipidaemia (van Mil *et al.*, 2016). Regarding methodology, both prolonging time between scans and a laboratory with less experience of measuring FMD have been shown to increase variability (van Mil *et al.*, 2016).

These sources of variability reinforces the importance of adhering to recommended guidelines and ensuring that the sonographer has an appropriate skill level. It has been proposed that for a sonographer to be considered qualified they must complete adequate training (Thijssen *et al.*, 2019); following which, their coefficient of variation (CV) between repeated scans for arterial diameter and FMD should be <2% and <15%, respectively (Ghiadoni *et al.*, 2012; Charakida *et al.*, 2013). Given that the presence of cardiovascular risk factors is known to increase FMD variability (Craiem *et al.*, 2007), sonographers intending to measure FMD in clinical populations should undergo more rigorous training, including experience of scanning the population in question. Since 1992, when FMD was originally introduced (Celermajer *et al.*, 1992), there has been an abundance of inconsistencies within the experimental protocol utilised, meaning that between-study results lack comparability. Ultimately, this is why FMD has not been formally adopted as a tool for clinical cardiovascular health

assessment. In attempt to promote standardisation of FMD protocol, Thijssen and colleagues (2019) proposed a series of guidelines that would allow for optimal reliability and reproducibility of FMD measurements. Unsurprisingly, stricter adherence to these guidelines has proven to improve FMD reproducibility (Greyling *et al.*, 2016).

As previously discussed, the primary outcome measure of the proposed intervention is FMD, with individuals undergoing multiple scans at multiple visits. Therefore, based on the aforementioned guidelines, it was necessary that appropriate sonographer training was conducted prior to data collection. This aimed to ensure a skill level of sufficient quality to reliably reproduce FMD results, and thus allow detection of NRPT's vascular potential. As part of this training, an FMD repeatability study was conducted so that sonographer intra- and inter-day variability could be assessed, the methodology and results of which are described below.

3.3. Methodology

Participants

8 students (1 female), aged 20-25, from the University of Birmingham volunteered to participate in this study. Prior to the first visit, all participants signed consent forms and completed a general health and lifestyle questionnaire. The purpose of the questionnaire was to determine whether it was safe for an individual to participate in the study, as well as identify any lifestyle factors which may influence the results or meet the exclusion criteria (e.g. smoking).

Study Design

There were three laboratory visits in total for each participant, each occurring at the same time of day and separated by at least 24 hours. Each visit shared an identical experimental protocol, including two brachial artery FMD measurements taken one hour apart. FMD was measured in the right brachial artery and was conducted in line with recommended guidelines (Thijssen *et al.*, 2019). The protocol used has been described in detail previously (Section 2.4). In brief, this involved an initial 20 min supine rest period, after which the ultrasound probe was used to obtain a clear longitudinal image of the brachial artery. The image was then continuously recorded for the length of the FMD protocol (11 min). This included 1 min of rest to determine baseline values, followed by 5 min of forearm ischaemia (induced by a distal blood pressure cuff inflated to ~220 mmHg), and concluded with a 5 min post-cuff deflation recording. Between measurements, participants rested in a seated posture. Upon completion of the second measurement, participants were free to leave.

Standardisation Procedures

FMD is known to be acutely manipulated by a plethora of factors, including diet, alcohol, physical activity, time of day, stress, temperature, and supplements/medication (Thijssen *et al.*, 2019). Therefore, due to the nature of this study, it was important to understand the effects of these factors so that they could be controlled for. Controlling participant-related variables allows for a more accurate representation of sonographer FMD variability. To minimise the potential impact of some of the more easily controlled confounding variables, participants were asked to refrain from drinking alcohol and performing vigorous physical activity in the 24 hours prior to each visit, and caffeine consumption in the 12 hours prior. Caffeine (Papamichael *et al.*, 2005; Buscemi *et al.*, 2010) and alcohol (Hijmering *et al.*, 2007) acutely depress FMD whereas exercise elicits a biphasic response, an immediate decrease (< 1 hr) followed by a recovery to normal, or even supranormal, levels (Dawson *et al.*, 2013). Also, to prevent influence of the natural diurnal variation in FMD (ter Avest *et al.*, 2005), all of a participant's visits were scheduled for the same time. Hormonal fluctuations associated with the menstrual cycle have also been demonstrated to influence an individual's FMD (Williams *et al.*, 2001); therefore, only females taking oral contraception were included in the study, with all of their visits completed during pill-taking weeks. FMD measurements were conducted in climate controlled vascular laboratories to prevent potential interference of ambient temperature changes (Widlanksy *et al.*, 2007; Donald *et al.*, 2010). During the time taken to complete all three visits, participants were asked not to make any substantial changes to their dietary and exercise training routines.

3.4. Analyses

In total, 48 scans from 8 participants were analysed (6 each). Given the recommended guidelines advice regarding attainment of specific CV values for arterial diameter and FMD before a sonographer is deemed qualified to measure FMD reliably, this was deemed the primary outcome measure. Intra- and inter-day CV for both variables were calculated using the following equation:

CV = standard deviation / mean.

Further analysis of FMD variability included Bland Altman plots and Pearson's correlation coefficient. Bland Altman analysis allows quantification of the degree of agreement between two quantitative measurements (Giavarina, 2015); in this case, two FMD measurements from the same visit, or two FMD measurements from different visits. Pearson's correlation coefficient was used to quantify the magnitude and direction of a relationship between two variables (Schober, Boer, & Schwarte., (2018). Thresholds proposed by Hopkins *et al.* (2009) were used to assess the magnitude of each

relationship. Correlations were deemed trivial, small, moderate, strong, and very strong – corresponding to r values of <0.1, 0.1-0.5, 0.5-0.7, and 0.7-0.9, respectively.

3.5. Results

Table 4: *Mean and standard deviation (SD) of baseline diameter (top) and FMD (bottom) of each participant throughout the protocol, including individual and overall inter- and intra-day coefficient of variations for both variables.*

		Visit 1		Visit 2		Visit 3		Combined Visits		Coefficient of Variation (%)	
	Participant	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Intra-day	Inter-day
Baseline Diameter (mm)	1	4.18	0.09	4.24	0.03	4.14	0.04	4.19	0.07	1.34	1.75
	2	3.71	0.03	3.84	0.03	3.90	0.04	3.82	0.09	0.96	2.27
	3	3.47	0.05	3.64	0.05	3.64	0.10	3.58	0.11	1.76	2.93
	4	3.56	0.08	3.46	0.08	3.58	0.04	3.54	0.08	1.89	2.36
	5	4.06	0.07	4.08	0.09	4.07	0.06	4.07	0.07	1.83	1.77
	6	3.87	0.12	3.87	0.06	3.86	0.13	3.87	0.10	2.64	2.51
	7	4.39	0.03	4.22	0.08	4.16	0.01	4.26	0.11	0.96	2.49
	8	4.51	0.08	4.40	0.01	4.52	0.05	4.48	0.07	1.09	1.63
Overall										**1.56**	**2.21**

		Visit 1		Visit 2		Visit 3		Combined Visits		Coefficient of Variation (%)	
	Participant	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Intra-day	Inter-day
FMD (%)	1	5.81	0.15	5.71	0.48	4.12	0.27	5.21	0.83	5.81	15.95
	2	4.80	0.31	4.46	0.42	5.50	0.33	4.92	0.57	7.28	11.51
	3	6.60	0.29	5.06	0.74	5.92	0.48	5.86	0.80	9.04	13.73
	4	7.21	0.65	8.12	0.86	5.63	0.77	6.99	1.29	11.09	18.50
	5	6.66	0.98	7.02	0.47	7.40	1.54	7.03	1.13	14.11	16.05
	6	4.00	0.43	4.48	0.31	5.41	0.42	4.63	0.67	8.51	14.40
	7	6.43	0.81	6.04	0.77	6.68	0.29	6.38	0.70	9.88	10.96
	8	3.80	0.19	3.76	0.16	4.14	0.38	3.90	0.32	6.19	8.26
Overall										**8.99**	**13.67**

Inter-day

Table 4 shows that mean inter-day CV for brachial artery baseline diameter and FMD was 2.21% and 13.67%, respectively. Bland Altman graph analysis (Figure 11) shows that the mean inter-day difference in FMD was 0.04%, with values ranging between -0.02-0.08. Upper and lower limits of agreement were 2.08% and -2.00%, respectively. Pearson's correlation coefficients were calculated for mean FMD between visits 1-2, 1-3, and 2-3, producing moderate-to-strong correlations of values of 0.864, 0.572, and 0.507, respectively. Figure 12 illustrates the Pearson's correlation coefficients between inter-day baseline diameter from the different visits, showing a very strong correlation (r > 0.95).

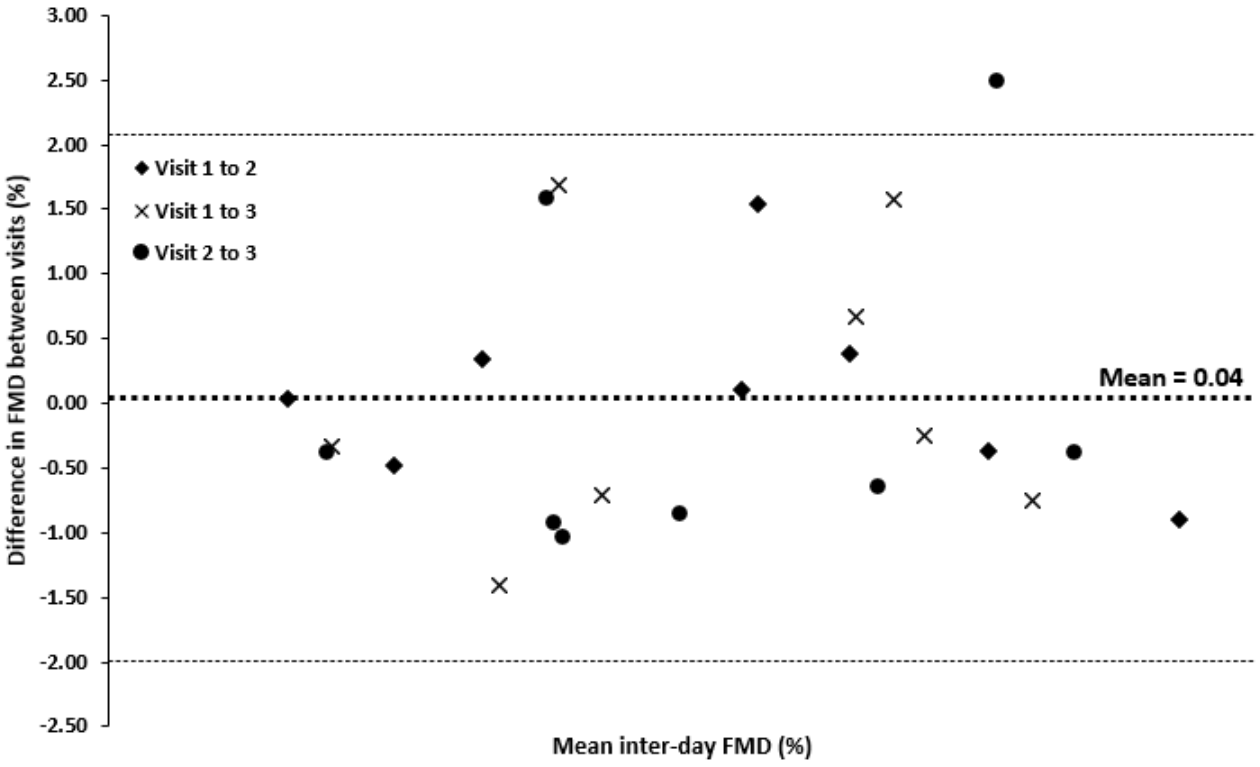

Figure 11: *Bland Altman Plot of differences between inter-day FMD (%) against the mean of the two measures. Dashed lines represent mean difference (thick) and upper/lower limits of agreement (thin).*

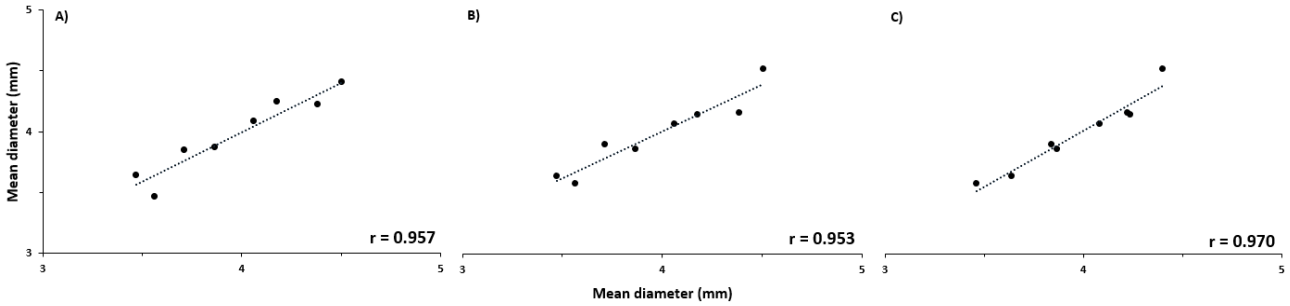

Figure 12: *Relationship between inter-day mean baseline diameter (mm) measured between visits 1-2 (A), 1-3 (B), and 2-3 (C).*

Intra-day

Mean intra-day CV for brachial artery baseline diameter and FMD was 1.56% and 8.99%, respectively (Table 4). Bland Altman graph analysis (Figure 13) shows that the mean inter-day difference in FMD was -0.02%, with values ranging between -0.22-0.13%. Upper and lower limits of agreement were 0.76% and -0.79%, respectively. Figure 14 shows the individual Pearson's correlation coefficients between each set of visits, showing a very strong correlation ($r > 0.95$).

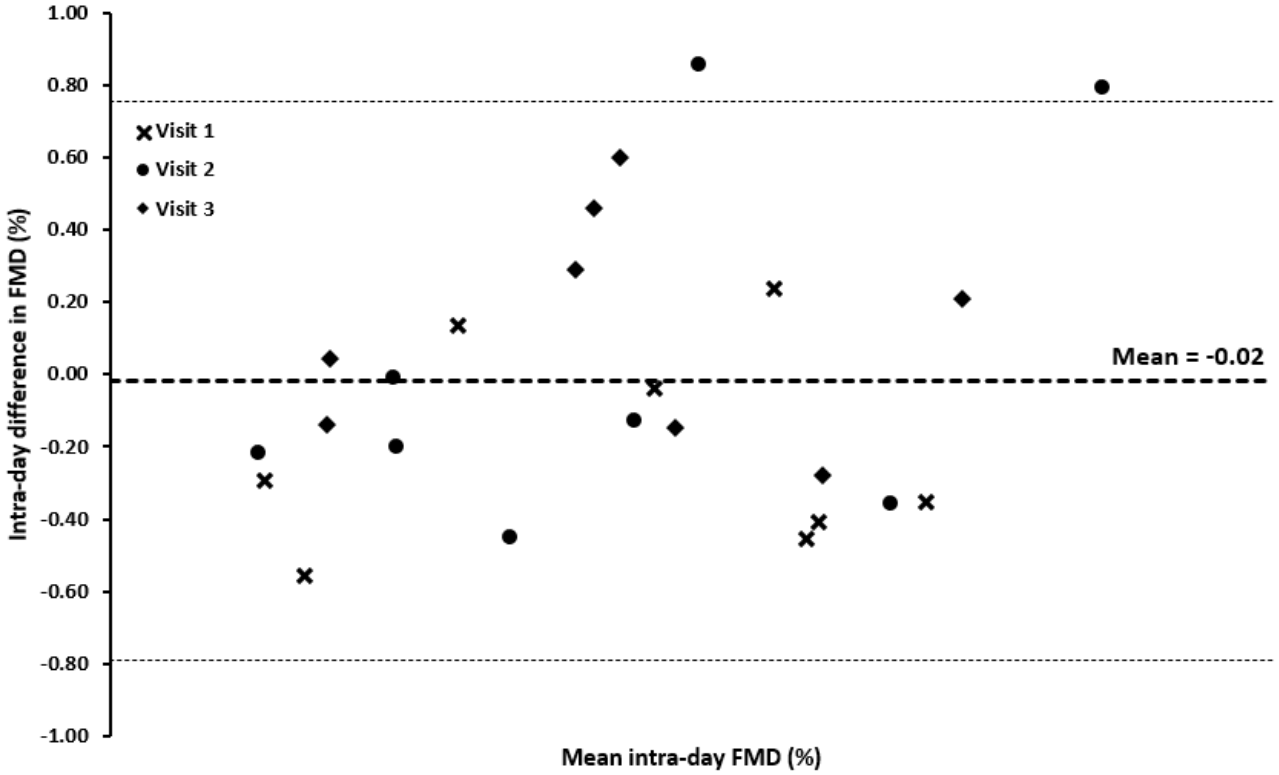

Figure 13: *Bland Altman Plot of differences between inter-day FMD (%) against the mean of the two measures. Dashed lines represent mean difference (thick) and upper/lower limits of agreement (thin).*

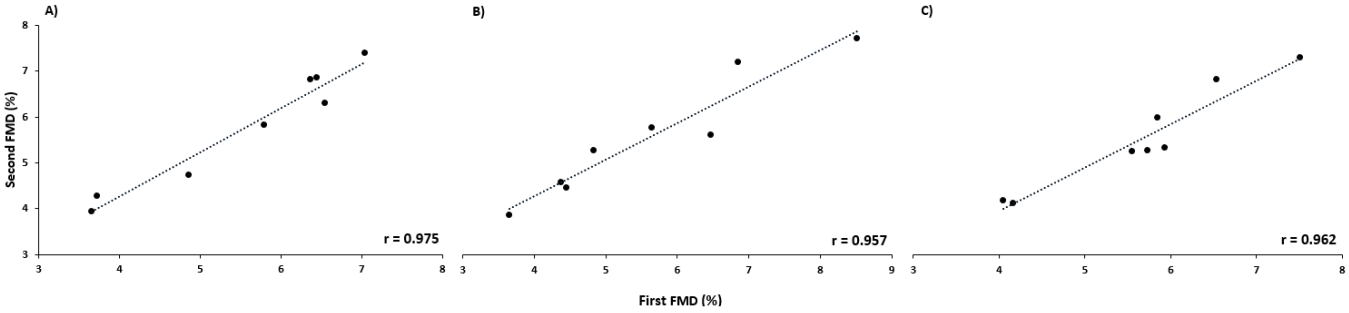

Figure 14: *Relationship between two intra-day FMD measurements, separated by 1 hr, during visit 1 (A), 2 (B), and 3 (C).*

3.6. Discussion

Thijssen *et al.* (2019) proposed that prior to data collection, a sonographer must achieve inter-day CV for baseline arterial diameter and FMD of <2% and <15%, respectively. The results of this repeatability study show that the requirement of inter-day FMD CV was met (13.67%), but not baseline arterial diameter (2.21%). In contrast, intra-day CV for both variables were less and met recommended requirements. This lower intra-day CV was consistent with previously reported repeatability data (Ghiadoni *et al.*, 2012), which is likely due to the short time between measures, ease to replicate the exact same set up (marks of skin etc) and lower biological variation related to standardisation procedures (discussed below). Despite inter-day baseline diameter CV not meeting the proposed standards, analysis of Pearson's correlation coefficient showed a very strong relationship between measurements ($r > 0.95$). Bland Altman analysis demonstrated that both inter- and intra-day FMD measurements showed good levels of agreement, with only 1 and 2 values, respectively, falling outside two standard deviations from the mean difference.

Although these results are promising of near-adequate sonographer ability, there are a few factors that need to be considered. Firstly, this repeatability study only included eight participants, Charakida *et al.* (2013) suggests that a minimum of ten repeat scans should be conducted before determination of FMD CV. This was the studies initial aim; however, the COVID-19 pandemic interrupted this study and its continuation was not possible. Secondly, only one of the eight participants was female and although the methodological process is the same, due to their often-smaller build and thus arterial diameter (Pham *et al.*, 2016), obtaining a clear image of the artery can sometimes be challenging. Therefore, gaining further experience of measuring FMD in females prior to data collection should be considered. It should also be remembered that all participants were young and healthy, and that given how FMD variability increases with age and clinical vulnerability (van Mil *et al.*, 2016), a separate repeatability study should be conducted within such populations prior to their inclusion.

These results show that inter-day FMD CV met the recommended guidelines and was comparable to existing research (Ghiadoni *et al.*, 2012); however, this value did fall relatively close to the unacceptable range of ≥15%. Moreover, only a moderate overall association was demonstrated between inter-day FMD ($r = 0.65$), suggesting that this variability could be improved further. Given that intra-day FMD showed far lower variability, it is likely that the somewhat basic levels of standardisation procedures may have contributed to the augmented inter-day variability observed, rather than sonographer-related factors. For example, acutely, diet is known to have a profound effect on brachial artery FMD, with evidence that the intake of carbohydrate (Mah *et al.*,

2011; Mah and Bruno, 2012), fat (Lin, Tsai and Chen, 2008; Lacroix *et al.*, 2012), nitrate (Lara *et al.*, 2016), and phenolic compound-containing foodstuffs (Schroeter *et al.*, 2006; Faridi *et al.*, 2008; Li *et al.*, 2013; Rodriguez-mateos *et al.*, 2014) significantly influence FMD. Similarly, hydration status (Arnaoutis *et al.*, 2017), mental stress (Ghiadoni *et al.*, 2000), and sleep quality (Grassi *et al.*, 2016) have all been found to influence FMD. For simplicity, no control measures were put in place to limit the impact of these confounding variables; however, it is likely that with more rigorous control measures, inter-day FMD CV would be lower.

To conclude, the results of this FMD repeatability study demonstrate near-adequate sonographer ability to reliably measure FMD. The inter-day CV criteria was met for FMD, but not for baseline diameter, which was only 0.21% above the proposed threshold. However, due to the COVID-19 pandemic, clinical testing has not been possible and therefore no FMD scans have been performed for 1 year. In this time, it is likely that sonographer skill level will have regressed due to lack of practice; consequently, it will be necessary to conduct a second repeatability study prior to further data collection to regain and demonstrate proficiency.

SUMMARY

This thesis provides an overview of the key players regulating mitochondrial and vascular function, and the importance of these functionalities to general health, ageing, and exercise performance. Clearly, strategies aimed at enhancing or preserving these functionalities are highly sought after within these contexts. Nutraceutical supplementation appears a promising strategy to achieve this, with a review of the literature highlighting the potential beneficial impact of NR and PT on some of the key players of mitochondrial and vasculature function – sirtuin activity, eNOS activity, redox status, and NAD+ bioavailability.

Clinical trials investigating these supplements are in their early stages and have achieved variable results thus far. Chronic NR supplementation has demonstrated some positive effects on blood pressure and arterial stiffness (Martens *et al.*, 2018), body composition (Remie *et al.*, 2020), and inflammation (Elhassan *et al.*, 2019). Better outcomes will possibly be seen when using supplementary periods of ≥ 6 months, in elderly (≥ 70 years) and metabolically challenged populations who experience the most mitochondrial and vascular dysfunction (Mouchiroud, Houtkooper and Auwerx, 2013; Bhatti, Bhatti and Reddy, 2017; Xu *et al.*, 2017), and severe NAD+ deficiencies (Massudi *et al.*, 2012; Okabe *et al.*, 2019). Even less data exists on chronic PT supplementation, the only clinical trial available found PT reduced blood pressure, but induced unfavourable changes in lipid profiles (Riche *et al.*, 2014).

NR and PT share a number of important mechanistic pathways, particularly upregulating sirtuin activity. It is therefore hypothesised that co-supplementation may help potentiate beneficial cardiometabolic outcomes, which are currently lacking in isolated NR and PT trials. Chronic NRPT clinical trials have shown positive effects on blood pressure (Dellinger *et al.*, 2017) and ALS outcomes (Rubia *et al.*, 2019).

There are evidently large gaps in the literature, particularly surrounding the effects of acute NRPT supplementation, which this thesis aimed to address. Acute NRPT supplementations offers the potential to utilise an elevated NAD+ pool before it normalises via a negative feedback loop, this may be especially relevant within the context of exercise. This thesis contains a detailed study design intended to robustly investigate the impact of acute NRPT supplementation on vascular function (at rest and during exercise), substrate utilisation during exercise, and post-exercise glycaemic control. Furthermore, the role of post-exercise hypoxic exposure on post-exercise glucose uptake was also planned to be explored, an area of research that is not well studied.

COVID-19 prevented the completion of this study; however, endeavour should still be made to conduct similar interventions. The full extent of NRPT's cardiometabolic effects are still not understood, particularly how they relate to exercise and whether they differ between acute and chronic supplementation strategies. Similarly, post-exercise hypoxic exposure is a promising strategy to augment post-exercise glucose uptake, which remains unexplored. Positive findings within this area could have meaningful implications for athletes' recovery process.

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