MASTER OF PHILOSOPHY

Heterogeneity of Endothelial Dysfunction and the Role of Nutritional Therapy

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Award date:
2021

Awarding institution:
Bangor University

PRIFYSGOL
BANGOR
UNIVERSITY

Heterogeneity of Endothelial Dysfunction and the Role of Nutritional Therapy

By

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A thesis submitted to Bangor University for the degree of Master of Philosophy

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September 2020

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ACKNOWLEDGMENTS

I wish to thank my supervisors Jamie and Gabs for your dedicated supervisions. Furthermore, I would like to extend my gratitude to Aamer, who supported and supervised me during the early stages of my MPhil. Despite the many challenges that 2020 presented us, your support and guidance has been outstanding throughout. I am extremely grateful for the opportunity to work with you both, and I can't express how thankful I am for your support and guidance.

In addition to the support from my supervisors, my research would not have been possible if it wasn't for the dedicated work of Kevin and Jason. Both technicians have been outstanding in their roles, and have always been happy to go beyond their responsibilities to help others. Other members of the School of Sport, Health and Exercise Sciences have also been a big part of my MPhil. Hans-Peter Kubis and Jonathan Moore are two members who supported me through my studies, both academically and emotionally. I also want to extent my appreciation to all the undergraduate and postgraduate students who were played a role in my research studies.

To the friends I have made in the department, you have all been a blessing. Working in an office filled with such amazing people has been a joy. Your support and friendship are things that I will always miss the most, and no research environment will ever be the same without you. Someday I wish to be in a position where I can employ all of you to work beside me and change the world, but until then I can only thank you sincerely.

Finally, I want to thank my one of a kind family. My 'nain' has always supported me in everything that I do, and I am honoured to make her proud. For my resilience and robustness, I would like to thank my mother, who was the person responsible for making me the determined person I am today. Lastly, but most importantly, I want to thank my wonderful girlfriend Jess. For the highs and lows, she has always been there for me, and I can never repay her for her support over the years.

STATEMENTS

Statement of Originality

The work presented in this thesis is entirely from the studies of the individual student, except where otherwise stated. Where derivations are presented and the origin of the work is either wholly or in part from other sources, then full reference is given to the original author. This work has not been presented previously for any degree, nor is it at present under consideration by any other degree awarding body.

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THESIS FORMAT

The present MPhil thesis consists of 4 Chapters that demonstrate the research skills and expertise I have developed over the course of the degree. Chapter 1 introduces the vascular research that forms the foundation of this thesis. Two independent experimental studies (Chapter 2 & Chapter 3) follow the introduction.

The first study (Chapter 2) was an experimental study in a simulated hypoxic environment. This study aimed to investigate the deleterious effect that acute hypoxia on vascular endothelial function, in micro and large blood vessels. Chapter 2 is being prepared for submission to the *Journal of Experimental Physiology,* has yet to be formatted to the requirements of the journal.

The second study (Chapter 3) was a placebo-controlled interventional study that investigated the effect of dietary nitrate on cardiovascular outcomes, including micro and large vascular endothelial function. Chapter 3 is a peer-reviewed article in the *Nutrients* journal and has been included as published.

Lastly, Chapter 5 of the thesis is a general discussion of the main findings of the empirical research. The critical discussion highlights the importance and implications of the findings and provides guidance for future vascular research.

ABSTRACT

Previous work has demonstrated that endothelial dysfunction (ED) plays a pivotal role in the development and progression of atherosclerosis. Several risk factor that increase the risk of ED have been identified; including hypoxia and ageing; however, most of the research only asses the degree of impairment in a single vascular bed (micro or large blood vessels). Similarly, studies investigating the potential influence of various therapies for ED, fail to account for the heterogeneity of the endothelium. The overall objective of the present thesis was to measure systemic endothelial responses (microvascular and large vascular beds) to acute hypoxia, and to assess the potential benefit of dietary nitrate (NO_3^-) therapy on systemic endothelial function (EF).

The specific aims of the first study (Chapter 2) were to determine the effects of acute hypoxia on microvascular and large-vessel EF [via non-invasive laser Doppler imaging (LDI) with acetylcholine (ACh) iontophoresis, and flow-mediated dilatation (FMD)], respectively]. Compared to normoxia, endothelium-dependent microvascular dilation was reduced after 30 minutes of exposure ($\tilde{x}\Delta$ = -109%, {IQR: 542.7}, p = 0.05). Alternatively, hypoxia did not affect endothelium-independent microvascular function after 30 minutes of exposure. Compared to normoxia, hypoxia significantly reduced allometrically scaled FMD responses after 60 min ($\bar{x}\Delta$-1.21%, $p < 0.001$). The magnitude of the decline in microvascular EF was correlated with cardiorespiratory fitness (r = 0.45, p = 0.02), but was not associated with large-vessel EF (r = -0.09 = p = 0.68). This study concluded that acute exposure to hypoxia reduced endothelium-dependent vascular function, in micro and large vascular beds. The decline in microvascular EF was approximately twice as large as that observed in the large blood vessel, demonstrating the heterogenous responses of the endothelium.

Study 2 (Chapter 3) examined the effects of a 4-week dietary NO_3^- intervention (beetroot juice (BRJ)) *vs*. placebo (PLA) on microvascular and large-vessel EF [via non-invasive LDI with ACh iontophoresis, and FMD] in healthy older adults. Plasma NO_3^- increased following 2-weeks of dietary NO_3^- supplementation ($p < 0.05$) along with a concomitant improvement in systolic and diastolic blood BP (−6mmHg and −4mmHg, respectively) ($p < 0.05$). However, there were no significant differences in endothelium-dependent or endothelium-independent microvascular function between groups. FMD increased by 1.5% following 2-weeks of dietary NO_3^- and were sustained until week 4 ($p < 0.05$), with only a minimal (0.1%) change for the PLA group. This study concluded that chronic dietary NO_3^- ingestion significantly improves BP and large-vessel EF in healthy older adults. However, 4-weeks of BRJ did not alter microvascular function.

To conclude, the present work highlights the importance of assessing EF in multiple vascular beds when investigating endothelial response to harmful stimuli or therapeutics. Acute hypoxia significantly reduces microvascular and large-vessel EF in healthy young adults. The decrease in microvascular EF was two-fold greater than the observed reduction in large-vessel EF. Secondly, the present work demonstrated that dietary NO_3^- supplementation significantly improved large-vessel EF in healthy older adults. However, no microvascular EF improvements were detected. Collectively, these findings emphasise the importance of assessing EF in multiple vascular beds.

LIST OF FIGURES, TABLES AND ABBREVIATIONS

FIGURES

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ABBREVIATIONS

ACh	acetylcholine
BP	blood pressure
BRJ	beetroot juice
cIMT	carotid intima-media thickness
CVD	cardiovascular disease
DBP	diastolic blood pressure
ED	endothelial dysfunction
EF	endothelial function
eNOS	endothelial nitric oxide synthase
FMD	flow-mediated dilatation
HIF-1	hypoxia-inducible factor-1
ICAM-1	intercellular adhesion molecule-1
LDI	laser Doppler imaging
LDL-C	low-density lipoprotein cholesterol
NADPH	dihydronicotinamide-adenine dinucleotise phosphate
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
oxLDL	oxidised low-density lipoprotein
PLA	placebo
RNS	reactive nitrogen species
ROS	reactive oxygen species
SBP	systolic blood pressure
SNP	sodium nitroprusside

SVR	systemic vascular resistance
VCAM-1	vascular cell adhesion molecule-1
WK2	week 2
WK4	week 4

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE BURDEN OF ENDOTHELIAL DYSFUNCTION

The endothelium forms a semi-permeable membrane between the blood and vascular matrix [1], and is also a dynamic organ that plays a pivotal role in vascular homeostasis [2]. To maintain vascular homeostasis, endothelial cells are responsible for releasing multiple vasoactive factors to fulfil the physiological duties of the endothelium. [2,3]. Endothelium-dependent vasoactive factors help regulate thrombosis, inflammation and angiogenesis [4]. Furthermore, the endothelium produces various vasodilating and vasoconstricting factors which allows the endothelium to regulate vascular tone to ensure adequate blood flow and blood pressure (BP) [5].

Similar to any other organ, the endothelium can become damaged and dysfunctional. Traditionally, endothelial dysfunction (ED) is characterised by an imbalance between the bioavailability of vasodilators and vasoconstrictors, favouring vasoconstriction [6]. This imbalance consequently leads to impaired endothelium-dependent vasodilation. In particular, the reduction in the bioavailability of the vasodilator, nitric oxide (NO), is the primary characteristic that defines ED [7]. In addition to its vasodilatory role, NO also inhibits the expression on endothelin-1, a potent endothelium-dependent vasoconstrictor [8]. Therefore, the reduction in NO bioavailability indirectly promotes a further imbalance by upregulating the production of vasoconstrictors. Endothelial dysfunction plays a critical role in the early stages of atherosclerosis and thus it is considered a significant marker for cardiovascular disease (CVD) (see "From Endothelial Dysfunction to Atherosclerosis").

The existing relationship between ED and atherosclerosis has detrimental impact on the heart and the vasculature. A comprehensive systematic review and meta-analysis, proposed that a 1 standard deviation (SD) reduction in a NO-meditated assessment of EF (flow-mediated dilatation (FMD)) is associated with doubled cardiovascular risk [9]. Due to the reduction in endothelium-dependent vasodilation, excessive shear stress is forced upon the vascular wall, which leads to atherosclerotic lesions and plaque development [10]. Consequently, the vascular lumen becomes narrower and stiffer, blood flow is restricted, and the risk of ischaemia is significantly increased. Collectively, these series of events increases the prevalence of coronary heart disease and ischemic heart disease [11], which according to the World Health Organisation, are the primary causes of global deaths [12]. Furthermore, Elbaz et al. reported that 81% of patients with coronary artery disease demonstrated signs of ED [13]. The association between ED and CVD is significant, and the prognostic value of assessing EF has been highlighted by several studies [14–17].

1.2 Heterogeneity of the Endothelium: Microvascular and Large Blood Vessels

Despite the promising findings to support the prognostic value of EF testing for CVD, the heterogenous nature of the endothelium makes it difficult to compare the findings. The endothelium forms the inner lining of the entire vasculature and it's function and structure differs between microvascular and large-blood vessels [1]. Furthermore, there is evidence to suggest that the endothelial phenotype can also be organ-specific [18]. Endothelial heterogeneity is predominantly driven by the surrounding stimulus and the interaction endothelial cells have with other cells [19,20].

In clinical populations considered at risk of ED, it has been reported that microvascular EF does not correlate with large blood vessel EF within individuals [21]. From their findings, Sandoo *et al*. concluded that signs of ED in the microvasculature does not necessarily mean that ED is present in large vascular beds. Microvascular blood vessels, defined as arterioles, capillaries and venules, are proposed to be at a greater risk of ED [22–24]. Furthermore, microvascular ED is believed to precede ED in larger blood vessels, particularly in individuals with metabolic syndrome [25]. The elevated risk of accelerated microvascular ED is believed to be associated with the prevalence of vascular adhesion molecules that makes the microvasculature more susceptible to inflammation [26,27]. Jekell *et al*. suggested that microvascular ED has greater prognostic value, particularly in hypertensive patients. However, Jekell and colleagues also proposed that large-vessel ED can be considered as better marker of existing atherosclerotic manifestations [28].

1.2.1 Methods of Assessing Microvascular and Large-vessel Endothelial Function

Laser Doppler technology is frequently used to measure microvascular reactivity. Briefly, the interaction between the laser light and the erythrocytes results in the backscattering of light. Static cells scatter light on the same wavelength as emitted, however the light reflected from moving cells undergoes small changes in wavelength. The light that is reflected from both static and mobile cells represents the velocity and concentration of red blood cells, currently described as the laser Doppler effect [29]. This method does not provide measurements of actual blood flow (ml/min), but rather represented as blood flux (perfusion). However, linear

relationship between blood flux and blood flow has been reported in previous research, suggesting that blood flux is a valid indirect measure of blood flow [30].

Since the discovery of the laser Doppler technology, microvascular reactivity has been primarily assessed using laser Doppler flowmetry and Laser Doppler imaging (LDI) techniques [31]. Primarily, laser Doppler technology is most reliable and valid when assessing cutaneous microcirculation in human trials [31]. However, significant correlations between peripheral vascular function and coronary vessel function has been reported by multiple studies[32–36], Laser Doppler flowmetry uses a single-point probe that emits low-level laser light through an optical fibre and detects the backscatter of light. The single-point probe provides a continuous measure of blood flux, but it does not account for the heterogeneity of the microcirculation [37,38]. In contrast, LDI uses the laser Doppler principle over a larger area than laser Doppler flowmetry thereby accounting for spatial heterogeneity of the cutaneous circulation, but does not provide a continuous measure of blood flux.

Various stimuli can be used to induce microvascular reactivity, including thermal, shear stress and pharmaceutical interventions/manipulations. However, when assessing microvascular EF, pharmacological stimulation is most commonly used. Acetylcholine is frequently administered to trigger NO synthesis and consequently stimulating endothelium-dependent vasodilatation. By binding to muscarinic ACh receptor M_3 on the endothelium, intracellular Ca^{++} levels are elevated which initiates a cascade of events leading to the relaxation of vascular smooth muscle cells [5]. However, some evidence does suggest that ACh mediated vasodilation is not entirely dependent on NO release and may be the result of activation of prostacyclin [39]. Acetylcholine can be administered using iontophoresis, which

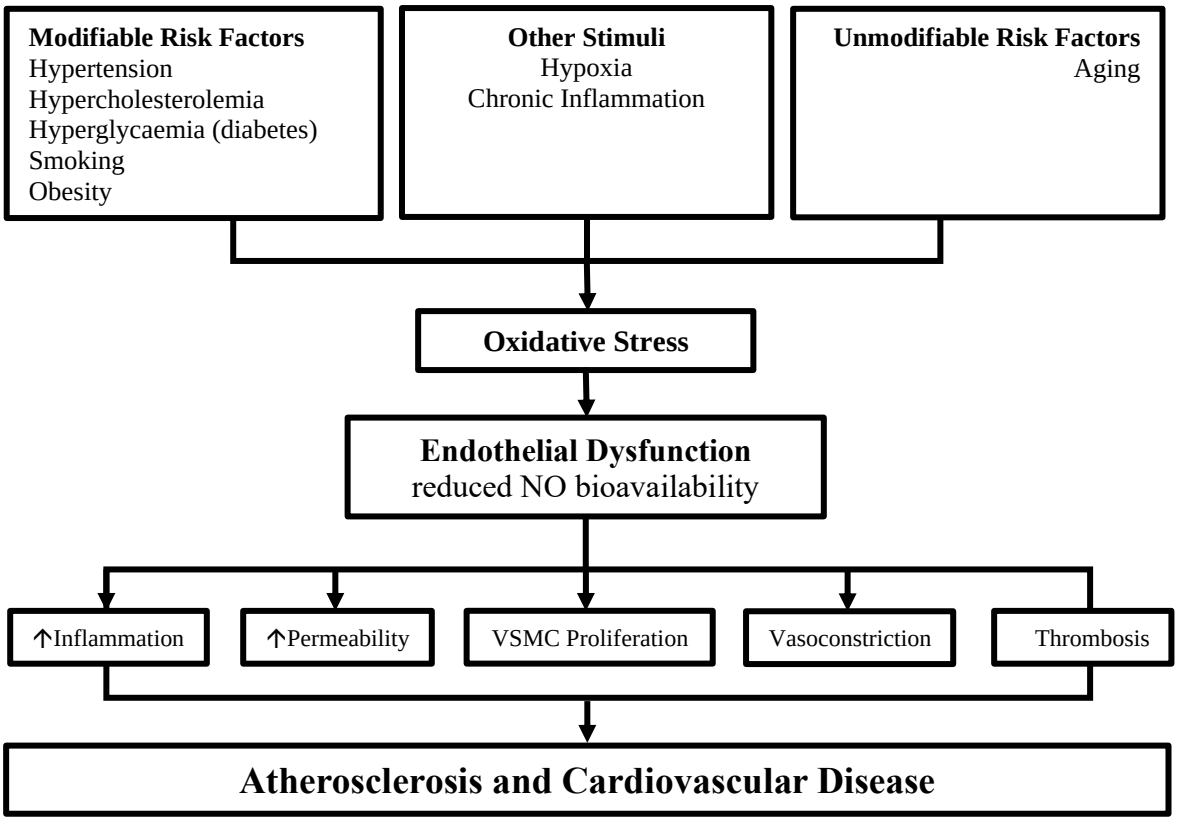
uses direct electrical current to deliver the vasoactive drug into the micro-circulation under the skin [40]. Using laser Doppler technology to assess the changes in blood flux (perfusion) in response to the delivery of ACh provides a measure of microvascular EF.

Large-vessel EF is commonly determined by FMD, whereby changes in arterial diameter are measured in response to reactive hyperaemia. This technique is a non-invasive technique that uses vascular ultrasound to scan vascular structures for offline diameter analysis. A brief period of arterial occlusion stimulates a reactive hyperaemia, which directly causes an increase in shear stress. Shear stress is a stimulus that activates mechanoreceptors located on the endothelial membrane, which directly increases intracellular NO synthesis [41,42]. The NO-mediated vasodilatory response to shear stress causes lumen diameter to increase in comparison to resting diameter [43]. A large change in diameter indicates that the endothelium is functioning well, and is able to produce an ample supply of NO. This non-invasive technique of assessing large-vessel EF is considered to be a valid and reliable method [44,45]. However, it is a technique that is highly user-dependent and should always be performed by a singular competent sonographer. Moreover, it is common practice in studies that use FMD to report the coefficient of variation value for their assigned sonographer.

1.3 Factors that Increase the Risk of Endothelial Dysfunction

Hypertension, hypercholesterolemia, hyperglycaemia, smoking and obesity are all modifiable risk factors that are implicated in the pathophysiology of ED. Additionally, there are unmodifiable risk factors that increase the risk of ED, such as ageing. A noteworthy characteristic that the aforementioned risks share is their capacity to upregulate the production

of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Moreover, Park et al. concluded that the progression from risk factor to ED is primarily mediated by the overexpression of ROS/RNS, described as oxidative stress (Figure 1. 1) [46]. In addition to the aforementioned risk factors for ED, other stimuli are also implied to cause oxidative stress-induced ED, including chronic inflammation and hypoxia [47–50]. Despite the sources of



ROS/RNS being different, their role in the pathophysiology of ED is similar.

Figure 1. 1. The sources of oxidative stress that triggers a reduction in nitric oxide (NO) bioavailability that leads to endothelial dysfunction (ED). NO: nitric oxide; VSMC: vascular smooth muscle cell.

1.4 Endothelial Dysfunction: the Role of Oxidative Stress

The production of ROS/RNS and a defective antioxidant response are the primary causes of reduced NO bioactivity [51,52]. Experimental models have observed that nicotinamide

adenine dinucleotide phosphate (NADPH) oxidase is the primary source of vascular ROS/RNS, however, other enzymes including xanthine oxidase also contribute to the upregulation [53]. Additionally, the enzyme responsible for producing NO, endothelial nitric oxide synthase (eNOS), is also a considered to be a source of oxidative stress [54]. The presence of ROS/RNS alters the function of eNOS, referred to as eNOS uncoupling, whereby enzyme activity no longer results in the production of NO [55]. Consequently, eNOS produces superoxide, which reacts rapidly with NO to generate peroxynitrite and thus limiting the protective effects of NO (Figure 4. 1) [56]. There is some evidence to suggest antioxidative therapy, such as Vitamin C and E supplementation can reduce NADPH oxidase dependent superoxide, and improve EF [57,58].

Oxidative stress also impairs the production of NO by limiting the endothelial nitric oxide synthase eNOS substrate availability [59]. In healthy endothelial cells, eNOS converts L-arginine and oxygen to L-citrulline and NO. However, other enzymes compete for L-arginine such as arginase. Arginase competes for L-arginine and produces urea and $_{L}$-ornithine. Cardiovascular disease risk factors and ED are associated with an increase in arginase activity, consequently leaving less substrate (L-arginine) for eNOS [60,61]. Interestingly, Reule *et al*. were able to demonstrate significant improvements in EF following four week of L-arginine supplementation in patients with an elevated risk of ED [62].

1.5 The Relationship between Hypoxia and Endothelial Dysfunction

Hypoxia can be defined as a condition whereby the body is deprived of an adequate oxygen supply. Severe hypoxia can impair several physiological functions including vascular

homeostasis and is believed to be implicated in numerous cardiovascular conditions [63–65]. Furthermore, hypoxia is proposed to be implicated in numerous stages of atherosclerosis development and progression [63,66,67]. However, the effects of hypoxia on EF remain unclear. Some studies have reported that hypoxia significantly diminishes endothelium-dependent NO bioavailability and the capacity to regulate vascular tone [48–50], and others report no change [68,69]. The discrepancies between findings may partly be explained by methodological considerations such as, type of exposure (simulated *vs*. high altitude), length of exposure and severity of hypoxic exposure. Nonetheless, there are several proposed mechanisms that may trigger hypoxia-induced ED, such as reduced expression of essential proteins for NO production [70,71], heightened sympathetic nerve activity [72], oxidative and inflammatory stress [73–75].

Briefly, hypoxia stimulates the activation of a transcriptional complexes, termed hypoxia-inducible factors (HIF). The upregulation of active HIF, primarily hypoxia-inducible factor-1 (HIF-1), stimulates metabolic changes within endothelial cells [76]. Changes in endothelial metabolism have been associated with an increase in the production of ROS/RNS that are generated from a number of sources, such as the mitochondrial electron transport system, xanthine oxidase, cytochrome p450, uncoupled eNOS [74]. However, the primary source of ROS in endothelial cells is derived from NADPH-oxidase. Hypoxia exposure may cause NADPH-oxidase to convert molecular oxygen to superoxide [73]. Once superoxide is synthesised in ample amounts, it reacts rapidly with NO to produce peroxynitrite. This reaction prevents the transportation of NO to vascular smooth muscle cells, and thus inhibits the vasodilatory effect of NO [77]. In addition to the changes in endothelial metabolism, the interaction between HIF-1 and endothelial cells evokes proinflammatory reactions, which can trigger the expression of endothelial adhesion molecules [75]. Overexpression of adhesion

molecules leads to endothelial activation that make cells more susceptible to the infiltration of inflammatory molecules that leads to further ROS/RNS production [27].

1.6 From Endothelial Dysfunction to Atherosclerosis

The progression of ED will eventually result in an increase in vascular permeability, vascular smooth muscle cell proliferation, upregulation of adhesion molecules and reduced ability to regulate vascular tone [78]. These series of events that occur during ED are considered to be pivotal in the initial stages of atherosclerosis [79]. By definition atherosclerosis is a disease that is characterised by vascular stenosis resulting from the formation of intimal plaques made of fat, cholesterol and other circulating substances [80]. Formation of atheromatous plaques can lead to further damage to the vascular walls that then stimulates thrombus formation that can cause obstruction to blood flow. Although the early stages of atherosclerosis are triggered by ED, the progression of the disease is driven by inflammation and lipid oxidation.

In addition to ED, endothelium activation also triggers the atherosclerotic process [81]. Activation of endothelial cells is a term used to describe the expression of cell-surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin [82]. Expression of the aforementioned adhesion molecules is believed to be stimulated by proinflammatory cytokines and ROS/RNS [82–84], LDL-C and oxLDL [85]. The anti-inflammatory properties of NO is believed to reduce the expression of cell-surface adhesion molecules, by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$_k$B) pathway [86]. Administration of various NO donors has been reported to supress cell-surface adhesion molecules gene

transcription by suppressing NF-$_k$B activity [87,88]. However, in the presence of ED, endothelium-derived NO is diminished and therefore the NF-$_k$B pathway is no longer suppressed, and thus endothelial activation occurs. Endothelial dysfunction and endothelial activation are closely associated and both events are integral in the early stages of atherosclerosis.

Both VCAM-1 and ICAM-1 are considered as strong indicators of both endothelial activation and ED [89]. These adhesion molecules are proteins that belong to the immunoglobulin superfamily group. They are expressed on endothelial cells and in the circulation in their soluble form. Moreover, circulating levels of VCAM-1 and ICAM-1 correlate with the levels which are expressed on the endothelial cell surface [90]. Once expressed on endothelial cells, the adhesion molecules trigger the leukocyte extravasation cascade [91]. Briefly, the leukocyte extravasation cascade is a series of events that lead to the migration of leukocytes into the subendothelial space. As the endothelium becomes more dysfunctional, other harmful substances, such as lipids are able migrate into the subendothelial space [92]. According to previous experiments, the transmigration of low-density lipoprotein cholesterol (LDL-C) occurs *via* two pathways [93–95]. In the 1980s, it was discovered that some of that LDL-C were transported across the endothelium in vesicles [93,94], however it was later suggested that this pathway only accounted for <10% of LDL-C flux [95]. The additional 90% of LDL-C flux was implied to be associated with leaky endothelial junctions [95]. Endothelial apoptosis can lead to larger junctions between endothelial that allows for macromolecules to permeate through [96].

After endothelial transmigration of LDL-C, it is oxidised as it interacts with various ROS/RNS, which are produced predominantly by endothelial cells [97,98]. Once oxidised, oxidized low-density lipoprotein (oxLDL) stimulates further endothelial cell activation [99,100]. Oxidised low-density lipoprotein are cytotoxic and pro-apoptotic lipids that are central in human atherosclerosis [101]. Due to the modifications in protein structure of LDL-C once oxidised, they are no longer recognised by LDL-C receptors and are subsequently scavenged by macrophages. Macrophages scavenge oxLDL in order to protect the vasculature from their harmful actions. Once a macrophage engulfs oxLDL, macrophages are then classed as foam cells [102]. The interaction between oxLDL and scavenger receptors located on the macrophages induces the release of proinflammatory cytokines and other immune cells, which subsequently leads to chronic state of inflammation within the vascular walls [103,104]. Proinflammatory conditions further stimulates the expression of cell adhesion molecule and thus prompts the progression of ED and atherosclerosis.

Subendothelial accumulation of foams cells and oxLDL form fatty streaks on the vascular walls, clinically defined as atheroma or atheromatous plaque, will eventually lead to early signs of vascular stenosis. The abundance of proatherosclerotic substances, including foam cells and oxLDL are pivotal in the development and progression of vascular lesions [105]. However, during the later stage of atherosclerosis, the formation of a fibrous cap comprised extracellular matrix components, isolate vascular lesions and atheromas from the lumen [106]. The factors situated in the extracellular matrix, such as thrombin, fibroblast growth factor and matrix metallo proteinases also stimulate the migration of vascular smooth muscle cells, which promotes further plaque progression [107]. Within atheromatous plaques, foam cell apoptosis triggers the release of its contents and give the plaque its necrotic core [108]. Furthermore, the apoptosis of other cells including vascular smooth muscle cells and endothelial cells is also

associated with the upregulation of proinflammatory cytokines, which can lead to plaque instability [109].

In the advanced stages of atherosclerosis, proliferation of intra-atheroma vascular smooth muscle cells triggers proinflammatory signals and thereby increasing the levels of inflammation in atheromatous plaques [110]. In addition to the upregulation of vascular smooth muscle cells, large quantities of angiogenic factors have been reported to be present within atheromas [111]. These factors stimulates the formation of fragile blood vessel (neovessles) to provide a blood supply to the atheroma. However, these neovessles are prone to rupture and can lead to the formation of intraplaque haemorrhages. The release of haemoglobin from erythrocytes within the haemorrhage can stimulate the production of ROS and other proinflammatory cytokines [112]. There is an associated risk with developed plaques that they will rupture and therefore will no longer be isolated from the lumen. As the plaque consists of several thrombogenic factors, platelets will aggregate to protect the vascular lesion resulting in a thrombus and will lead to further stenosis.

1.7 THERAPIES FOR ENDOTHELIAL DYSFUNCTION

Several mechanisms are involved in the pathology of ED, such as oxidative stress, inflammation, increased synthesis of vasoconstrictors and decreased production of vasodilators. Therefore, treating ED poses many challenges, as the source of dysfunction can be difficult to identify, and may not limited to a singular source. Although, a reduction in NO bioavailability is the primary marker of ED, the endothelium also regulates the production of numerous other vasoactive substances, including endothelium-derived hyperpolarising factors

(EDHF), prostacyclin, angiotensin II, endothelin-1, thrombomodulin and adhesion molecules. Disturbances in the regulation of the aforementioned endothelium-derived vasoactive factors may also contribute to the ED process and thus, an effective treatment for ED must be targeted towards maintaining an adequate balance to achieve homeostasis.

Pharmaceutical drugs such as eNOS enhancers, angiotensin-converting enzyme inhibitors and antioxidant agents have been shown to exert therapeutic effects on the endothelium [113]. In spite of this, pharmacotherapy that targets ED has not yet been translated into the clinic setting to date [114]. However, there is a large body of research that has demonstrated the effectiveness of non-pharmacological therapeutics for ED treatment [115–117]. For the purpose of this chapter, non-pharmacological therapy is considered as lifestyle changes including, exercise and dietary interventions. Most of the research that has investigated the relationship between non-pharmacological therapeutics and EF have focused on the eNOS vasodilatory system. However, due to the heterogenous nature of the endothelium, many previous studies have reported inconsistent findings, and thus it's important to evaluate the available evidence.

1.7.1 Physical Exercise for Endothelial Function

The relationship between cardiovascular health and physical activity was first described in 1958, by Morris *et al.* [118]. It was reported that increased levels of physical activity reduced the risk of CVD and cardiovascular mortality. Moreover, frequent exercise significantly lowers the risk of developing traditional CVD risk factors such as hypertension, obesity and hypercholesterolemia [119]. Reductions in traditional CVD risk factors considerably reduces the risk of ED; however, independent of the effect of exercise on risk factors, physical activity

is directly associated with improvements in EF [120]. Maintaining a high fitness status with frequent exercise training, prevents and restores the natural decline in EF that occurs during advanced ageing, particularly in the microcirculation [121,122]. In contrast, cross-sectional observations in young men and women, demonstrated that fitness status is not associated with EF in the absence of any risk factors of ED [123,124]. However, these cross-sectional studies reported that resting arterial diameter were larger in fit individuals, and thus may limit the detection of any improvements in endothelium-dependent vasodilation.

In addition to the preventative benefits of physical exercise, a comprehensive meta-analysis that included sixteen studies, concluded that ≥8-weeks of exercise training restored EF in diabetic patients with signs of ED [125]. Although the mentioned meta-analysis reported promising findings to support the use of physical exercise for ED treatment, Qiu and colleagues only included studies that used FMD as a method of EF assessment. The FMD assessment is a measure of endothelium-dependent NO-mediated vasodilation, specifically for large conduit arteries. Considering that diabetes is CVD risk factor that is most commonly associated with microvascular complication [126], the inclusion of studies that examined the effect of exercise training on microvascular EF would have strengthened the findings of Qiu *et al*. Nonetheless, it has been reported elsewhere that continuous or interval training for 12-weeks is associated with improvements in microvascular and large-vessel reactivity in individuals with diabetes [127]. In contrast, Middlebrooke *et al*. reported no microvascular improvements following a longer exercise intervention (6-months) [128]. However, Middlebrooke and colleagues also failed to observe any significant improvements in aerobic fitness during the study, which possibly suggests that the exercise programme intensity was insufficient to stimulate any meaningful benefits.

Other disease populations, such as rheumatoid arthritis patients have also got an increased risk of CVD, and the magnitude of CVD morbidity in rheumatoid arthritis is proposed to be equivalent to that observed in individuals with diabetes [129]. In both diabetes and rheumatoid arthritis, ED is a prevalent condition that increases the risk of CVD, and occurs in the microcirculation prior to the larger vascular beds [130]. A cross-sectional study by Metsios *et al*. reported that rheumatoid arthritis patients who are less physically active have significantly worse CVD risk profile compared with physically active patients [131]. Additionally, Cooney *et al*. demonstrated that 8-weeks of supervised exercise improved markers of CVD risk, including BP and body composition; however, there was no assessment of EF [132]. Nonetheless, more recent work by Metsios and colleagues, suggested that a 6-month tailored aerobic and resistance exercise intervention improve microvascular and large-vessel EF by approximately 60 and 78%, respectively in rheumatoid arthritis patients [133].

According to a 2018 meta-analysis including five randomised control trials, aerobic exercise improves FMD responses by 1.45%, in individuals with hypertension [134]. Moreover, a small increase of 1% in FMD may reduce CVD outcomes by approximately 13% for hypertensives [135]. Although aerobic exercise is most associated with improvements in cardiovascular health [136], a recent randomised clinical trial proposed that different modalities of exercise, including resistance and combined training significantly improves EF and lowers BP in hypertensive patients [137]. Moreover, 8-weeks of aerobic or resistance training resulted in similar improvements in FMD (+3.2% and +4.0%, respectively) whilst a combined training method produced greater improvements (+6.8%).

There is compelling evidence to suggest that physical exercise in any form can reduce the risk of CVD [138]. Exercise interventions also significantly augments NO bioavailability, which has been shown to improve for large-vessel EF, more so in individuals with risk factors for ED or CVD [139]. Currently, there are a limited amount of studies that have studied the effects of exercise on microvascular EF [127,133,140], which have reported paradoxical results. Therefore, more research to examine the relationship between physical exercise and microvascular EF is warranted, particularly in at risk populations. In spite of all the evidence to support the use of exercise to treat or prevent ED, one must consider that physical activity might not be possible for some frail individuals, and thus it is important to discuss alternative therapies for ED, such as diet therapy.

1.7.2 Healthy Diets for Endothelial Function

Numerous nutritional therapies have been shown improve cardiovascular health, including the Mediterranean and Japanese diet that are rich in antioxidant rich foods and dietary NO_3^- [141,142]. The Mediterranean diet is often recognised as a diet rich in antioxidants due to the large presence of bioactive nutrients [143]. Most commonly, the diet includes a moderate amount of fish, dairy and meat, olive oil, unrefined cereals, fruit, vegetables and wine [144]. Similarly, the Japanese diet, traditionally considered as a rice dish complimented with fish and a variety of vegetables, is also a diet that is considered to have antioxidative properties [145]. Vegetables that are prevalent in both the Mediterranean and Japanese diet are green leafy vegetables. Green leafy vegetables are the primary source of dietary NO_3^- for humans and are strongly associated with endothelial and cardiovascular benefits [146]. In animal models, it has been shown that long-term deficiency in dietary NO_3^- intake increases the prevalence of ED and cardiovascular mortality [147]. The benefits of dietary NO_3^- are achieved through serial

reductions of NO_3^- until the formation NO is achieved. This series of reaction are recognised as the Nitrate-Nitrite-Nitric Oxide Pathway (NNN Pathway) (Figure 1. 2) [148].

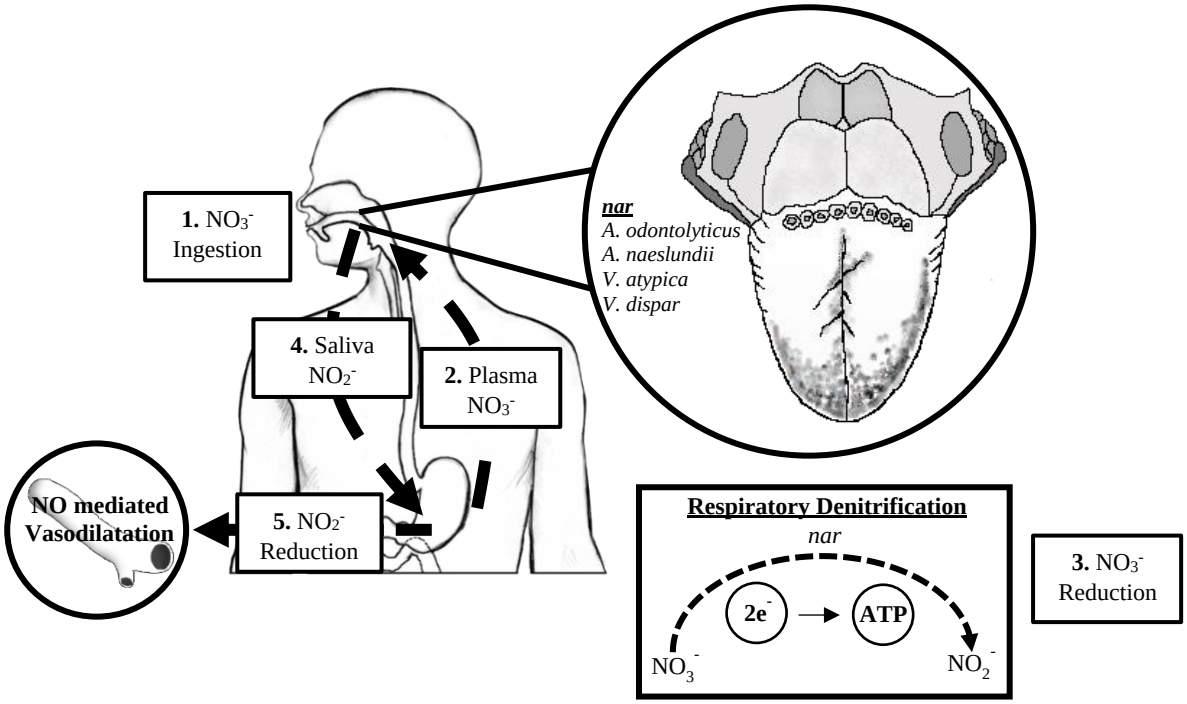


Figure 1. 2. The Nitrate-Nitrite-Nitric Oxide Pathway [149]. A: Actinomyces; NO: nitric oxide; NO_2^-: nitrite; NO_3^-: nitrate; V: Veillonella.

1.8 Dietary Nitrate: the Exogenous Source of Nitric Oxide

Approximately 80% of the dietary NO_3^- that humans consume come from green leafy and root vegetables, including, spinach, rocket and red beetroot [150]. Over the years, the favourable and unfavourable effects of dietary NO_3^- and nitrite (NO_2^-) have been a topic of debate in scientific literature. Early research, in animals, discovered that nitrosamines that are secondary to NO_2^- consumption are highly carcinogenic [151]. The nitrosation of NO_2^- occurs in the acidic environment of the stomach and was once believed to significantly increase ones risk of colorectal and gastric cancer [152]. Since this discovery was made, comprehensive reviews and meta-analyses of epidemiological studies have deemed the evidence to be inconclusive, with some studies showing high NO_3^- intake can reduce the risk of gastric cancer [153–155].

However, more recently, dietary NO_3^- is better recognised as an exogenous source of NO, that is independent of the endothelium.

Once the dietary NO_3^- is ingested it is absorbed across the gut wall and into the blood plasma [156]. Twenty five percent of the circulating NO_3^- enters the enterosalivary circuit and the rest is excreted by the kidneys [157]. Primarily, NO_3^- enters the enterosalivary circuit from saliva secretion. Circulating NO_3^- is actively transported to the acinar cells of the salivary glands *via* sialin (SLC17A5) proteins located in the plasma membrane, and is then secreted into the oral cavity at a 10 to 20-fold higher concentration [158,159] . Facultative anaerobes located deep in the crypts of the posterior area on the dorsal surface of the tongue, such as *Actinomyces* and *Veilonella* species, possess NO_3^- reductase enzymes that are capable to reduce salivary NO_3^- to NO_2^- [160,161]. Most mammalian cells do not have this enzyme, and thus the reduction of dietary NO_3^- is dependent on NO_3^- reducing anaerobic bacteria. Individuals with a higher abundance of oral NO_3^- reducing bacteria have significantly higher salivary NO_2^- concentration [162]. Furthermore, lower salivary NO_2^- has been reported during the administration of antibacterial mouthwash [163] or during swallowing prevention [164].

Following the oral reduction of NO_3^- to NO_2^-, the saliva is subsequently swallowed, whereby most of the NO_2^- is reduced to NO in the gastric acidic milieu [164]. However, a small percentage of salivary NO_2^- is absorbed across the gut wall and transported into circulation. During dietary NO_3^- supplementation interventions, plasma NO_3^- and NO_2^- both increase concomitantly. Following the transport of NO_2^- into the circulation, it is reduced further to NO, under certain conditions and by specific enzymes [148]. Currently there are five proposed pathways that describe how elevated plasma NO_3^- and NO_2^- augment NO bioavailability:

deoxyhaemoglobin [165], deoxymyoglobin [166], xanthine oxidase [167], acidification [168] and mitochondrial enzymes/coenzymes [169] (Figure 1. 3).

Deoxyhaemoglobin/ myoglobin: $NO_2^- + Fe^{2+} + H^+ \rightarrow \mathbf{NO} + Fe^{3+} + OH^-$

Xanthine oxidoreductase: $NO_2^- + Mo^{4+} + H^+ \rightarrow \mathbf{NO} + Mo^{5+} + OH^-$

Acidification:

$$NO_2^- + H^+ \rightarrow HNO_2$$
$$2\ HNO_2 \rightarrow 2\ N_2O_3 + H_2O$$
$$N_2O_3 \rightarrow \mathbf{NO} + NO_2$$

Mitochondrial Enzymes/Coenzymes: $NO_2^- \rightarrow$ [Cytochrome *c* oxidase / Ubiquinone] $\rightarrow \mathbf{NO}$

Figure 1. 3. Five possible physiological pathways that convert nitrite into NO. Fe^{2+}: ferrous iron $^{2+}$; Fe^{3+}: ferrous iron $^{3+}$; H^+: hydrogen; H_2O: dihydrogen oxide; HNO_2: nitrous acid; Mo^{4+}: molybdenum $^{4+}$; Mo^{5+}: molybdenum $^{5+}$; N_2O_3: dinitrogen trioxide; NO: nitric oxide; NO_2^-: nitrite; OH^-: hydroxide.

1.8.1 Dietary Nitrate: its Cardioprotective Role

Acute and chronic consumption of dietary NO_3^- has been shown to offer numerous cardioprotective benefits for healthy individuals and people at risk of CVD [170] (Table 1. 1). In the presence of an intact enterosalivary circuit, the NNN pathway increases the bioavailability of NO independently of the endothelium, and thus providing an alternative source of NO for individuals with ED. Moreover, having an alternative source of NO can prevent development and progression of ED, and subsequently atherosclerosis. Most studies report improvements in EF, arterial stiffness and BP in response to dietary NO_3^-, all factors which predispose an individual to CVD. However, there is a degree of inconsistency amongst study findings which may be explained by variables including; NO_3^- content, source of NO_3^-, population and study length.

The relationship between dietary NO_3^- and cardioprotective benefits exists in a dose dependent manner [171,172]. It is possible to achieve a diet sufficiently rich in NO_3^-, by eating vegetables including, red beetroot, spinach, celery and lettuce. However, the content of NO_3^- within these vegetables may vary according to where they were grown [150,173]. Therefore, manufactured NO_3^- supplements, such as Beet It Sport by James White Drinks Ltd, which provides 6.2 mmol of dietary NO_3^- are most effective for a specific beneficial dose. Most studies demonstrate that supplements containing 4 to 20mg of NO_3^-, per kilogram body weight, are sufficient for lowering BP, improving EF and reducing platelet aggregation and arterial stiffness [174]. However, higher levels of NO_3^- in supplements corresponds to higher levels of circulating NO_3^-, but similar levels of circulating NO_2^- [171,172], due to the rapid reduction of NO_2^- with a half-life of ~20–45 min *in vivo* [175,176].

Table 1. 1. A summary of the recent studies that have investigated the effect of inorganic nitrate supplementation in populations at risk of endothelial dysfunction (ED) and cardiovascular disease (CVD).

Author	Subjects	Primary Outcome	Secondary Outcomes	NO_3^- Dosage	Placebo	Findings	Intervention Length	Study Design
Litwin et al., 2019 [177]	15 overweight/obese	Post High Fat Meal Reactive Hyperaemia (plethysmo-graphic method)	BP AI Carotid-Femoral PWV Endothelial Cell Protein Expression Plasma NO_2^- & NO_3^- Saliva NO_2^- & NO_3^-	70 ml (NO_3^-: 6.2 mmol) of BRJ	**Placebo 1**: 70ml of NO_3^- depleted BRJ **Placebo 2**: 70ml of Powder drink devoid of polyphenols and NO_3^- **Placebo 3**: 70ml of Powder drink devoid of polyphenols and NO_3^-, plus potassium NO_3	Reactive Hyperaemia (*Acute & Chronic*) ↔ SBP (*Acute & Chronic*) ↔ DBP (*Acute & Chronic*) ↔ AI (*Acute & Chronic*) ↔ Carotid-Femoral PWV (*Acute & Chronic*) ↔ Endothelial Cell Protein Expression (*Acute & Chronic*) ↔ Plasma NO_2^-/NO_3^- (*Acute & Chronic*) ↑ Saliva NO_2^-/NO_3^- (*Acute & Chronic*) ↑	Acute (4-hours) [Assessed at Baseline, 1-hour *(only biological samples)*, 2-hours *(only biological samples)* and 4-hours]. Chronic (4-weeks) [Assessed at Baseline and Week 4]	Randomized, Double-blind, Placebo-controlled, Crossover Study
Broxterman et al., 2019 (Study 1) [178]	13 with Hypertension (taking	BP	PLM Induced Leg Blood Flow (Microvascular Function)	70 ml (NO_3^-: 6.2	70ml of NO_3^- depleted BRJ	SBP ↔ DBP ↔	Chronic (3-days) [Assessed at Day 3]	Double-blind, Placebo-controlled, Crossover Study

	antihypertensive medication)		FMD Plasma NO_2^- & NO_3^-	mmol) of BRJ		MAP ↔ PLM Induced Leg Blood Flow (Microvascular Function) ↔ FMD ↔ Plasma NO_2^- & NO_3^- ↑		
Broxterman et al., 2019 (Study 2) [178]	14 with Hypertension (not taking antihypertensive medication)	BP and antihypertensive medication status in mediating the efficacy of NO_3^- supplementation	PLM Induced Leg Blood Flow (Microvascular Function) FMD Plasma NO_2^- & NO_3^-	70 ml (NO_3^-: 6.2 mmol) of BRJ	70ml of NO_3^- depleted BRJ	SBP ↓ DBP ↓ MAP ↓ PLM Induced Leg Blood Flow (Microvascular Function) ↑ FMD ↑ Plasma NO_2^- & NO_3^- ↑	Chronic (3-days) [Assessed at Day 3]	Double-blind, Placebo-controlled, Crossover Study
Asgary et al., 2016 [179]	24 with Hypertension	BP	FMD Systemic inflammation (hs-CRP, TNF-α & IL-6) Total antioxidant capacity Adhesion molecules (ICAM-1, VCAM-1 & E-selectin)	**Treatment 1:** 250 ml of Raw BRJ **Treatment 2:** 250 ml of Cooked BRJ	No placebo	SBP ↓ (*Raw & Cooked*) DBP ↓ (*Raw & Cooked*) FMD ↑ (*Raw & Cooked*) hs-CRP, TNF-α & IL-6 ↓ (*Raw & Cooked*) Total antioxidant capacity ↑ (*Raw only)*	Chronic (2-weeks) [Assessed at Baseline and Week 2]	Randomized Single-blind, Crossover Pilot Study

			Lipid profile (TG, LDL & HDL)			ICAM-1, VCAM-1 & E-selectin ↓ (*Raw & Cooked*) TG & LDL ↓ (*Raw only)* HDL ↔ (*Raw & Cooked*)		
Velmurugan et al., 2016 [180]	69 with Hypercholesterolemia	FMD	Aortic PWV AI Saliva/Plasma/Urine NO_2^- & NO_3^- BP Oral microbiome profiling Platelet flow cytometry Platelet-monocyte aggregate expression Plasma oxLDL Systemic inflammation (hs-CRP)	250 ml (NO_3^-: 6.0 mmol) of BRJ	250 ml (NO_3^-: 0.001 mmol) of NO_3^- Depleted BRJ	FMD ↑ Aortic PWV ↑ AI ↑ NO_2^- & NO_3^- in all Biological Samples ↑ SBP ↓ DBP ↔ *Neisseria flavescens* ↑ *Rothia mucilaginosa* ↔ Platelet P-selectin ↓ Platelet-monocyte aggregate levels ↓ Plasma Oxidised LDL/Uric Acid/CXCL1/hs-CRP ↔	Chronic (6-weeks) [Assessed at Baseline and Week 6]	Randomized, Double-blind, Placebo-controlled, Parallel Study
Kapil et al., 2015 [181]	64 with Hypertension (Drug Naïve vs Treated)	BP	AI PWV FMD	250 ml (NO_3^-: 6.4	250 ml (NO_3^-: 0.007 mmol) of NO_3^-	*All Findings Reported for Drug Naïve and Treated Because no*	Chronic (4-weeks)	Prospective Single-center, Double-blind, Randomized, Placebo-controlled

			Saliva/Plasma/Urine NO_2^-& NO_3^- Plasma cGMP	mmol) of BRJ	Depleted BRJ	*Difference was Found Between Them* NO_2^-& NO_3^- in all Biological Samples ↑ Plasma cGMP ↑ SBP ↓ & DBP ↓ (*Returned Towards Baseline After 2-week Washout*) AI ↓ PWV ↓ FMD ↑	[Assessed Baseline & Week 4]	
Bondonno et al., 2015 [182]	27 with Hypertension	BP	Saliva/Plasma/Urine NO_2^-& NO_3^-	70 ml (NO_3^-: 5.5 mmol) of BRJ	70 ml of NO_3^- Depleted BRJ	SBP ↔ DBP ↔ NO_2^-& NO_3^- in all Biological Samples ↑	Chronic (1-week) [Assessed at Baseline and Day 7]	Randomized, Double-blind, Placebo-controlled, Crossover
Bondonno et al., 2014 [183]	38 with Pre-hypertension	BP	PWV AI Plasma and Salivary NO_2^-& NO_3^-	High NO_3^- Vegetables (NO_3^-: 6.45 mmol)	Low NO_3^- Vegetables	SBP ↔ DBP ↔ PWV ↔ AI ↔ NO_2^-& NO_3^- in all Biological Samples ↑	Chronic (1-week) [Assessed at Baseline and Day 7]	Randomized, Placebo controlled, Crossover
Rammos et al., 2014 [184]	21 Older Adults at Moderate Risk of CVD	FMD	CIMT AI	Sodium NO_3^- (0.15 mmol per	Sodium Chloride (0.1 mmol per	FMD ↑ CIMT ↔	Acute (24-hours)	Randomized, Placebo-controlled, Double-blinded

			PWV BP Plasma NO_2^-& NO_3^-	kilogram of body weight)	kilogram of body weight	AI ↓ PWV ↓ SBP ↓ DBP ↔ Plasma NO_2^-& NO_3^- ↑	[Assessed at Baseline and 24-hours]	
Jajja et al., 2014 [185]	21 Elderly and Obese Patients	BP	Salivary and Urinary NO_2^-& NO_3^-	70 ml ((NO_3^-: 4.84 – 6.45 mmol) of BRJ	200 ml of NO_3^- Depleted Blackcurrant Juice	SBP ↓ (*During Final Week*) DBP ↔ Salivary and Urine NO_2^-& NO_3^- ↑	Chronic (4-weeks) [*last week without supplementation*] [Assessed at Baseline, Week 3 and Week 4]	2-arm, Parallel, Randomized Clinical Trial
Ghosh et al., 2013 [186]	15 with Grade 1 Hypertension	BP	PWV Plasma NO_2^-& NO_3 Plasma cGMP	250 ml (NO_3^-: 3.3 mmol) of BRJ	250 ml of Water	SBP ↓ DBP ↓ PWV ↓ Plasma NO_2^-& NO_3 ↑ Plasma cGMP ↑	Acute (24-hours) [Assessed at Baseline and 24-hours]	Randomized, Open-label Crossover
Gilchrist et al., 2013 [187]	27 with Type II Diabetes	BP	FMD LDI Insulin Sensitivity Plasma NO_2^-& NO_3^-	250 ml (NO_3^-: 7.5 mmol) of BRJ	250 ml (NO_3^-: 0.002 mmol) of NO_3^- Depleted BRJ	SBP ↔ DBP ↔ MAP↔ FMD ↔ LDI (ACh) ↔ LDI (SNP) ↔	Chronic (2-weeks) [Assessed at Baseline and Week 2]	Randomized Double-blind, Placebo Controlled Crossover

						Whole-body glucose disposal ↔ Plasma NO_2^-& NO_3^- ↑		
Kelly et al., 2013 [188]	12 Older Adults	BP	O_2 Uptake Kinetics, Muscle (PCr) and Cognitve Function	500 ml (NO_3^-: 9.6 mmol) of BRJ	500 ml of NO_3^- Depleted Orange Juice	SBP ↓ DBP ↓ VO_2 ↔ Functional Capacity ↔ Low-intensity Knee Extension Exercise ↔ Recovery Kinetics ↔ Cognitive Performance↔ Brain Metabolic Concentrations ↔	Chronic (3-days) [Assessed at Baseline and Day 3]	Randomized, Double-blind, Placebo-controlled, Crossover
Kenjale et al., 2011 [189]	8 with Peripheral Artery Disease	Exercise Performance (VO_2 and PCr)	BP Plasma NO_2^-& NO_3^- FMD	500 ml (NO_3^-: 9.1 mmol)	500 ml of NO_3^- Depleted Orange Juice	Exercise Tolerance ↑ SBP ↓ (*2-min Post Exercise*) DBP↓ (*At Rest and During Exercise*) Plasma NO_2^-& NO_3^- ↑ FMD ↔	Acute (4-hours) [Assessed Hourly]	Randomized, Double-blind, Placebo-controlled, Crossover

↑: Significantly Increased; ↓: Significantly Decreased; ↔: No Significant Difference; AI: Augmentation Index; BP: Blood Pressure; cGMP: Cyclic Guanosine Monophosphate; CIMT: Carotid Intima-Media Thickness; CVD: Cardiovascular Disease; DBP: Diastolic BP; Diff: Difference; FMD: Flow-mediated Dilatation; HDL: high-density lipoprotein; hs-CRP: high sensitivity C-reactive protein; ICAM-1: intercellular adhesion molecule 1; LDL: low-density lipoprotein; MAP: Mean Arterial Pressure; NO_2^-: Nitrite; NO_3^-: Nitrate; oxLDL: oxidised low-density lipoprotein; PLM: passive leg movement; PWV: Pulse Wave Velocity; SBP: Systolic BP; Sig: Significant; TG: triglyceride; VCAM-1: vascular cell adhesion molecule 1.

In addition to the dose-response relationship between dietary NO_3^- and cardioprotective benefits, there also exists a time-response relationship. Hobbs *et al*. performed hourly blood collections following a beetroot juice (BRJ) supplement, in order to determine the length of time it took for circulating NO_3^- to peak [167]. In conclusion, they reported that peak plasma NO_3^- occurs 3-hours following BRJ intake, along with concomitant peaking of BP reductions. Furthermore, Hobbs and colleagues also reported levels of circulating NO_2^- to be at their highest post 3-hours. Many studies have investigated the acute (≤ 24-hours) responses to dietary NO_3^- (Table 1. 1), with most treatments showing BP reductions in healthy and clinical populations [186,190]. In spite of this, concomitant improvements in EF are less common in acute studies [189,191]. Dietary NO_3^- is proposed to lower BP through numerous mechanisms, and therefore acute improvements in BP may not be entirely endothelium-dependent [192]. However, in older individuals with moderate risk of CVD, relative body mass NO_3^- supplementation (0.15mmol per kg of body weight) resulted in improved large-vessel EF, only 24-hours post ingestion, which may be explained by the intervention being tailored to individuals, thus making the treatment highly effective. [184].

Currently, there are only a limited amount of studies that have investigated ≥2-weeks NO_3^- interventions on EF, and the findings are inconclusive. In patients with hypertension or hypercholesterolaemia, 4-weeks and 6-weeks of NO_3^- supplementation significantly improved large-vessel EF compared to placebo, respectively [180,181]. Conversely, Gilchrist *et al*. did not observe any improvements in microvascular or large-vessel EF following a 2-week intervention in patients with type II diabetes. Moreover, Gilchrist and colleagues also failed to report any reductions in BP. Gilchrist and colleagues argued that the patient sample they recruited had several other comorbidities which possibly prevented any NO_3^--induced

cardioprotective benefits. Additionally, patients whom were already taking vasoactive medication for their retrospective conditions, were not excluded from the study.

It is clear that dietary NO_3^- supplementation is effective for some populations more than others, with improvements in EF greater in individuals with moderate risk of ED [180,181,184]. However, as discussed previously, factors including NO_3^- dosage, treatment duration and the oral microflora can also affect how well one responds to dietary NO_3^-. In addition to these factors, the form by which NO_3^- is ingested also dictates how an individual may respond. Most commonly, green leafy vegetables are used as supplementation due to their rich NO_3^- content. Bondonno *et al*. demonstrated that by simply introducing a moderate amount of vegetables high in NO_3^- into the diets of prehypertensive individuals significantly elevated their circulating NO_3^-, however they reported no concomitant improvement in any vascular parameters [183]. As it is difficult to quantify the NO_3^- concentration of organic vegetables, it is possible that the investigators had no control of NO_3^- dosage, and thus failed to detect any vascular improvements.

Commercially available NO_3^- rich BRJ drinks are a popular supplement that are often used in dietary NO_3^- trials, due to the regulated NO_3^- concentration. However, currently there is no evidence to support the use of beetroot based products in solid food form for ED [193]. Juicing NO_3^- rich vegetables allows for easier ingestion, increases digestibility and is associated with better outcomes [179]. Additionally, the treatment of beetroot (cooked *vs*. raw) prior to juicing can attenuate the potency of the associated cardioprotective benefits [179]. Following 2-weeks of raw or cooked BRJ supplementation, large-vessel EF improved significantly in hypertensive subjects [179]. However, the improvements in endothelium-dependent vasodilatation were

significantly higher after treatment with raw BRJ compared with cooked BRJ. The failure to measure plasma NO_3^- or NO_2^- is a limitation of this study, which makes it difficult to explain why raw BRJ is more effective. Elsewhere, it has been suggested that boiling leafy vegetables lowers the NO_3^- levels [194], therefore it is possible that raw BRJ elevates NO bioavailability more so than cooked BRJ.

Asgary *et al.* also investigated the effect of BRJ on vascular inflammation and oxidative biomarkers. As discussed previously, both vascular inflammation and oxidative stress largely contribute towards ED. Furthermore, it is also implied that the progression from ED risk factors to the development of ED is primarily mediated by oxidative stress [46]. Although red beetroot is known to improve EF by increasing NO bioavailability, Asgary and colleagues also reported that raw BRJ significantly improved total antioxidant capacity. Beetroot is a vegetable that contains anti-oxidants, including, betalains (betacyanins and betaxanthins), flavonoid and polyphenols, and therefore improvements in EF is response to BRJ may partly be attributed to the anti-oxidative properties of beetroot [195]. Additionally, it has been proposed in animal models, that other exogenous NO donors similar to beetroot directly scavenges and suppress' the production of ROS/RNS such as superoxide in diabetic vasculature [196,197]. This highlights the importance of selecting an appropriate placebo treatment in comparative studies, as NO_3^- depleted BRJ remains to have antioxidants and thus can potentially improve EF. Nitrate-mediated improvements in EF is primarily the result of increased NO bioavailability, however, the ability of dietary NO_3^- to reduce oxidative stress may also decrease the risk of endothelial damage.

1.9 SUMMARY

Physical activity and diet are modifiable risk factors that have been shown to affect oxidative stress and consequently EF. However, majority of this research is performed in large blood vessels due to the accessible non-invasive techniques that are available including; FMD, pulse wave analysis and pulse wave velocity [5]. Non-invasive assessment of microvascular EF, such as LDI with iontophoresis of ACh are considered to be more technically demanding, thus less *in vivo* research is conducted in the microvasculature. Therefore, the impact of ED risk factor on microvascular EF are unclear. Moreover, little is known about the potential benefits of endothelial therapies of microvascular EF. Gaining a better understanding of microvascular ED and potential therapeutic treatments is of great importance as microvascular ED is believed to precede the development of large-vessel ED [25].

Oxidative stress is a common characteristic that all risk factors for ED share, and is proposed to be the primary cause of dysfunction. Acute and chronic hypoxia are stimuli that promote oxidative stress and has been shown to cause a decline in EF. Traditionally, hypoxia is caused by disease or extreme environmental conditions. However, simulated hypoxia can also elicit similar physiological responses, which allows researcher to investigate the effect of hypoxia on specific mechanisms, such as microvascular and large-vessel EF. Being able to assess EF in both microvascular and large-vessel bed is an opportunity to gain a greater understanding of the endothelium's heterogenous responses oxidative stress.

To understand the benefits of nutritional endothelial therapy, specifically dietary NO_3^-, for the microvasculature and large blood vessels, one must consider studying individuals at an elevated risk of ED. As aging is an unmodifiable risk factor for ED, the potential benefits of dietary

NO_3^- for systemic EF is relevant for the human population. Although hypoxia and aging are both different risk factors for ED, they share similar pathophysiology. Both risk factors are associated with the upregulation of ROS/RNS and also inflammatory responses that trigger ED.

1.10 THESIS AIMS

The overall aim of this thesis is to better understand how the endothelium responds to stress and treatment in different vascular beds using non-invasive assessments of microvascular and large-vessel EF. Currently, there is a limited amount of studies that assess EF in multiple vascular beds in response to hypoxia or dietary NO3- therapy. To address this gap in the literature, this thesis will examine the sensitivity of the endothelium to hypoxia in microvascular and large vascular beds, and to trial a dietary NO3- intervention that could potentially reduce the risk of ED in individuals who are susceptible to oxidative stress-mediated ED. For these investigations, two studies were completed and are summarised below

The specific aims of the first study (Chapter 2) were to determine to what degree did acute hypoxia impair the eNOS vasodilatory system in microvasculature and large blood vessels of healthy adults. Secondly, the study aimed to determine if cardiorespiratory fitness protects against hypoxia-induced endothelial decline.

Lastly, the second study (Chapter 3) aimed to determine if four weeks of dietary NO_3^- supplementation could improve EF in a population susceptible to age-associated endothelial

impairments, but otherwise healthy. Again, EF was assessed in microvessels and large blood vessels to determine the sensitivity of the heterogenous endothelium to dietary NO_3^- treatment.

CHAPTER 2: THE DELETERIOUS EFFECTS OF ACUTE HYPOXIA ON MICROVASCULAR AND LARGE-VESSEL ENDOTHELIAL FUNCTION

2. 1 ABSTRACT

Hypoxia is associated with diminished bioavailability of the endothelium-derived vasodilator, NO. Diminished NO bioavailability can have deleterious effects on EF. The endothelium is a heterogeneous organ; therefore, a comprehensive assessment of EF is critical to understand the significance of hypoxia-induced ED. We hypothesized that acute hypoxia would have deleterious effects on microvascular and large-vessel EF. Twenty-nine healthy adults (age: 24 (4 SD) years) completed normoxic and hypoxic [inspired O_2 fraction (FiO_2) = 0.209] trials in this double-blinded, counterbalanced crossover study. After 30 min, we assessed LDI-determined perfusion response to iontophoresis of ACh as a measure of endothelium-dependent microvascular function, and iontophoresis of sodium nitroprusside (SNP) as a measure of endothelium-independent microvascular function. After 60 min, we assessed brachial FMD as a measure of large-vessel EF. Thirty minutes of hypoxia reduced endothelium-dependent microvascular function determined by perfusion response to ACh ($\tilde{x}\Delta$ = -109%, {IQR: 542.7}, $p = 0.05$), but not endothelium-independent determined by perfusion response to SNP ($\tilde{x}\Delta$ 69%, {IQR: 453.7}, $p = 0.6$). In addition, 60 minutes of hypoxia reduced allometrically-scaled FMD compared to normoxia ($\bar{x}\Delta$-1.19 [-1.80, -0.58] %, $p < 0.001$). The decline in microvascular EF was associated with cardiorespiratory fitness ($r = 0.45$, $p = 0.02$). In conclusion, acute exposure to normobaric hypoxia significantly reduced endothelium-dependent vasodilatory capacity in small and large vascular beds. Furthermore, these findings suggest that cardiorespiratory fitness may provide some protection against hypoxia-induced microvascular ED.

2. 2 INTRODUCTION

Hypoxia can cause disturbances to vascular homeostasis, and is believed to be implicated in numerous stages of atherosclerosis development and progression, including ED [63,66,67]. A healthy endothelium maintains homeostasis by regulating vascular tone, coagulation and inflammation. Chronic and acute hypoxic exposure has been shown to trigger endothelial damage and vascular inflammation [198], increasing an individuals risk of vascular injury that can lead to adverse outcomes, such as CVD [65]. Moreover, the progressive nature of CVD is also proposed to exacerbate vascular hypoxia [199], resulting in a reciprocal cycle. The endothelium plays a pivotal role in this cycle, and thus it is important to understand the deleterious effects of hypoxia on EF.

Nitric oxide is recognised as an endothelium-derived vasodilator that plays a central role in maintaining vascular homeostasis [5]. Endogenously, NO is produced by an oxygen-dependent enzyme, eNOS [200]. In endothelial cells, L-arginine and molecular oxygen bind to eNOS which stimulates NO synthesis, thus when oxygen availability is reduced, eNOS activity decreases [201]. Once synthesised, NO is transported to the vascular smooth muscle cells, whereby it reduces intracellular Ca^{2+} concentrations and prevents the contraction of smooth muscle cells [202]. Previous studies have demonstrated that hypoxia triggers pathways that diminish the bioavailability and action of NO [70,71,73,74]. The production of NO is limited during hypoxia due to prevalence of oxidative stress. Overexpression of hypoxia-induced ROS is proposed to upregulate the scavenging of NO [73,74] and downregulate the expression of endothelium eNOS [70,71]. A reduction in the expression of

NO can result in an imbalance between endothelium-derived vasoactive factors, contributing towards ED.

Flow-mediated dilatation is a well-established technique that uses reactive hyperaemia to assess the endothelial NO vasodilatory system in large blood vessels [43]. Previous research has shown that FMD responses decline by as much as 45% during acute hypobaric hypoxia exposure [48–50]. However, the authors also reported a decline in endothelium-independent vasodilation, suggesting that ED did not fully account for the reduction in vasodilatory capacity. To better understand the underlying reason for these vascular impairments, it is important to also examine the microvascular responses to hypoxia, as evidence suggests that microvascular dysfunction precedes large-vessel dysfunction [25]. Peripheral microvascular ED is an indicator of systemic ED and atherosclerotic risk, and is considered a major cause of cardiovascular mortality [22–24,203]. Furthermore, the microcirculation comprises a much larger surface area of the circulatory system which leads to greater ROS production, therefore the risk of injury is significantly elevated in the microcirculation [204].

Recent research by Treml *et al*. suggests that the cutaneous microvascular response to reactive hyperaemia is significantly lower following 6 and 12 hours of normobaric hypoxia [205]. However, it is unclear whether cutaneous reactive hyperaemia is mediated by the NO vasodilatory system, similar to the FMD procedure [206]. Thus, it cannot be concluded that the reported decline in microvascular function is associated with ED. Alternatively, iontophoretic application of ACh on human skin increases microvascular endothelium-dependent vasodilation [207]. Laser Doppler imaging can be used concomitantly to assess changes in cutaneous perfusion in response to the delivery of ACh over a large surface area,

accounting for spatial differences. During this assessment, co-administration of an eNOS inhibitor has been demonstrated to reduce microvascular vasodilatory response significantly, suggesting that ACh induced vasodilation is partly dependent on NO bioavailability [208].

In spite of the overwhelming literature to suggest that acute hypoxia attenuates EF, there is some evidence which implies that microvascular reactivity is heightened during hypoxia [209]. However, the use of laser Doppler flowmetry limited their findings, as it only measures perfusion changes at a single point. This means it is difficult to ensure repeated measurements between trials are taken from the same location, and it does not account for the spatial heterogeneity of the cutaneous vasculature. Therefore, these findings should be interpreted with caution. Furthermore, the primary increase in microvascular perfusion in response to the thermal stimulus is not believed to be endothelium-dependent [210].

Not only is it crucial to identify stimuli that may trigger the development or progression of ED, it is also important to understand how humans may be able to protect the endothelium against damage. Over the years, it has been established that lifestyle modifications including diets high in green leafy vegetables and increasing physical activity can prevent and reverse ED [211–213]. However, despite the strong evidence to suggest that hypoxia can have a deleterious effect on EF, there has yet to be a study that examines how these affects might be mitigated. To populations that suffer long-term hypoxia, such as obstructive sleep apnoea patients [214], it is important to consider factors that can possibly protect the endothelium from excessive hypoxia-induced damage. As exercise intervention studies have already been shown to cause improvements in EF [211]; prospective studies should consider examining the relationship between the fitness status and endothelial changes in response to hypoxia. Collectively, these

studies might be able to highlight the importance of physical activity and fitness for individuals who have a higher risk of hypoxia-induced ED.

To understand the systemic effect of hypoxia on the endothelium, it is important to assess EF in different vascular beds. The present double-blind, counterbalanced crossover study sought to determine the effect of hypoxia on microvascular and large-vessel function. Our aims were to i) replicate the previous FMD findings reported by Lewis *et al*. (significant decreases in FMD following acute hypoxia) [49], and to assess and compare the effects of acute hypoxia on ii) endothelium-dependent microvascular function determined by perfusion response to iontophoresis of ACh, iii) endothelium-independent microvascular function determined by perfusion response to iontophoresis of SNP. Furthermore, we aimed to assess the relationship between cardiorespiratory fitness and the changes in EF. We hypothesised that a degree of ED was present in both vascular beds, but cardiorespiratory fitness would partly protect against the magnitude of dysfunction. However, as the risk of injury is increased for microvascular endothelial cells, we hypothesise that the extent of dysfunction will be greater in the microcirculation.

2. 3 METHODS

2. 3. 1 PARTICIPANTS

Twenty-nine healthy adults (17 men) were recruited into the study (age: 24 (4) years). Participants had not travelled to altitude (≥ 1500 m) in the preceding six months, and had no medical contraindications to maximal exercise testing. Female participants were studied during the early follicular phase of their cycle, or the placebo phase of oral contraceptives. All

participants were briefed on the nature and the purpose of the investigation before written consent was taken along with a short demographic questionnaire to ensure that they satisfied the study criteria. Ethical approval was granted by the Ethics Committee of the School of Sport, Health, and Exercise Sciences at Bangor University (Ethics ID: P19-16/17) and the study was performed in accordance with the guidelines of the WMA Declaration of Helsinki (2013).

2. 3. 2 STUDY DESIGN

The study followed a double-blind, repeated-measures, counterbalanced crossover design. Participants completed three separate laboratory visitations. During the first visit, individuals completed baseline health and fitness assessments, including, a carotid intima-media thickness (cIMT) assessment and a maximal exercise test. Participants then completed normoxic [inspired O_2 fraction (FiO_2) = 0.209] and hypoxic (FiO_2 = 0.120) experimental trials in a regulated altitude chamber, separated by at least five days. Participants were randomly allocated to conditions in a counterbalanced order, using a computer-generated randomized list [215]. In experimental trials, participants rested in a recumbent position for 20 min before manual BP, heart rate and blood saturation were recorded. These vital signs were measured every 30 min for the duration of the experimental trial. Whilst remaining in a recumbent position, vascular function of the small and large blood vessels was assessed after 30 and 60 min, respectively (separated by a minimum of 15 min).

2. 3. 3 BASELINE PROCEDURES

2. 3. 3. 1 CAROTID INTIMA-MEDIA THICKNESS

Assessment of advanced but subclinical atherosclerosis was completed using cIMT. The right and left carotid arteries were imaged 1–2 cm proximal to the carotid bulb [216], using a high-resolution ultrasound machine (Acuson X300, Siemens Healthcare GmbH; Erlangen: Germany) attached to a high frequency linear array transducer. Participants lay supine with a 45° tilt of the neck to align the carotid artery for scanning. Images were acquired at end-diastole, determined by the ECG R-peak. Three images were acquired for each side (left and right), with the cIMT measured in each and averaged across the three images for each side, and across both sides. All analysis was completed off-line using artery measurement software. All images were acquired and analysed by GMKR (the between-day reliability of this technique is equal to coefficient of variation of 4.1%). Increased atherosclerotic risk was defined as having cIMT measurements greater than 1.0mm in accordance with Simon *et al*.[217].

2. 3. 3. 2 MAXIMAL EXERCISE TEST

To determine cardiorespiratory fitness levels (VO_{2max}), participants completed a running test to exhaustion on a motorized treadmill (H-P-Cosmos, Sports & Medical GmbH; Nussdorf-Traunstein: Germany) with simultaneous online gas analysis (Cortex Metalyzer, Biophysik GmbH; Leipzig: Germany).

The test protocol was designed so that participants reached maximum between 10–15 min regardless of fitness level, using a similar method to da Silva and colleagues [218]. VO_{2max} was estimated using the Matthews equation [219], and work rates were calculated using the ACSM

metabolic equations for treadmill running. The test protocol began with an 8 min warm up at 50% estimated maximum and subsequent 2 min rest, followed by a ramped increase in work rate from 50% estimated maximum to 100% estimate maximum over 10 min. The ramp of the slope continued until exhaustion to obtain VO_{2peak}. After a 10 min rest, participants completed a validation stage at 110% of the work rate at exhaustion to obtain VO_{2max}. VO_{2max} was identified if the validation VO_2 had a greater than 3% negative discrepancy of the modelled 110% VO_{2peak} [220]. Heart rate and Rating of Perceived Exertion (RPE assessed by the Borg CR100) [221] was recorded each minute of the test.

2. 3. 4 Experimental Procedures

2. 3. 4. 1 Microvascular function: Laser Doppler Imaging

Both endothelium-dependent (ACh) and endothelium-independent (SNP) microvascular function were assessed in normoxia and hypoxia after 30 min using LDI (moorLDI2, Moor Instruments, Devon, UK) with iontophoresis. All LDI assessments were completed under temperature-controlled conditions (25 (2) °C) and measured according to previously established methodology [45]. Simultaneous delivery of ACh (Miochol, Bausch & Lomb Inc. Berlin, Germany) and SNP (Rottapharm S.L., Barcelona, Spain) was performed using an iontophoresis controller (MIC2, Moor Instruments, Devon, UK) to assess endothelium-dependent and endothelium-independent cutaneous vasodilation, respectively. Perfusion changes in response to the delivery of both vasoactive drugs were assessed on the participant's volar aspect of the right forearm. The full protocol that was used for this study has been described in detail previously [45]. In summary, a baseline scan was performed before a series of ten scans with an iontophoresis charge of 30µA was delivered to administer 2.5ml of 1%

ACh and 1% SNP. ACh and SNP drugs were diluted with 0.9% saline and delivered into the skin *via* an anode and cathode internal electrode Perspex chamber (⌀22mm) (ION 6, Moor Instruments, Devon, UK), respectively. Following ten scans with iontophoresis, two further recovery scans were performed without the delivery of the vasoactive drugs.

The exposure-time-response protocol took approximately 15–20 min and all scans were performed in natural lighting conditions, with most of the ambient lighting restricted. Additionally, the settings of the laser Doppler imager (moorLDI2-IR, Moor Instruments, Axminster, Devon, UK) were kept consistent for all scans and acetate sheets labelled with anatomical markers were used to ensure delivery site was consistent across trials. Measurements of perfusion were carried out offline using the moorLDI Review V6.1 software. Perfusion values were quantified for ACh and SNP calculating the median for each region of interest since LDI data will rarely have Gaussian distribution [222]. Results are presented as a percentage change in perfusion from baseline:

$$\left(\frac{Peak\ Perfusion - Baseline\ Perfusion}{Baseline\ Perfusion}\ x\ 100 = \text{ACh\% or SNP\%}\right).$$

2. 3. 4. 2 LARGE-VESSEL ENDOTHELIAL FUNCTION: FLOW-MEDIATED DILATATION

Large-vessel EF was assessed using FMD under temperature-controlled conditions (25 (2) °C) in normoxia and hypoxia after 60 minutes. The FMD procedure was performed as previously described in detail [45]. Briefly, a 2 min baseline ultrasound scan of the brachial artery was followed by 5 min of occlusion, achieved by inflating a BP cuff placed around the wrist to suprasystolic pressures (220mmHg). After 5 min, the cuff was deflated rapidly to induce reactive hyperaemia. To capture maximal dilation, a 3 min scan was performed following cuff deflation.

A Siemens Acuson X300 Ultrasound scanner was used with a multifrequency linear-array vascular probe set at 7.3MHz (Acuson X300, Siemens Healthcare GmbH; Erlangen: Germany) to perform the FMD procedure. B-mode images were captured at 15 frames per second to record a 120 s baseline and a 210 s clip following 5 min of occlusion. To capture the initial reactive hyperaemic response to cuff deflation, the recording was initiated 30 s before cuff release; therefore, only 180 s was used for the analysis. Images were analysed offline using an automated edge detection software (Brachial Analyser, Medical Imaging Applications, USA). The Brachial Analyser software is capable to detect the peak of the R-wave; therefore, this inbuilt feature was used to include only the images at the peak of the R-wave. The recommended image quality standard was set at a confidence threshold ≥70%. From the frames which were accepted the change in diameter from baseline to peak was calculated as follows; $\frac{Peak\ Diameter - Baseline\ Diameter}{Baseline\ Diameter} \times 100$ = FMD%. To account for the differences in baseline diameter, all the data was allometrically scaled as per the Atkinson and Batterham guideline [223]. All FMD scans were completed by DTJ (the between day reliability of this technique is equal to coefficient of variation of 8.5%).

2. 3. 5 Statistical Analyses

The assumption of normality was examined with the Shapiro-Wilk test. For primary analysis (to determine the effect of hypoxia on vascular function), paired t-tests were applied on normally distributed data and Wilcoxon signed rank test was used for non-parametric data. P values < 0.05 were considered to indicate statistical significance. Also, effect sizes for paired t-tests (by Cohen's *d*) are presented as the mean difference divided by the pooled SD between both normoxic and hypoxic time points and can be interpreted as small (> 0.2), medium (>

0.5), large (> 0.5), very large (0.7) and extremely large (0.9). Alternatively, effect sizes for Wilcoxon signed rank test (by Rosenthal's *r*) are presented as the Z scores divided by the square root of the sample size between both normoxic and hypoxic time points and can be interpreted as small (> 0.2), medium (> 0.3) and large (> 0.5).

A-priori sample size estimation for the primary analysis indicated that 10 participants were needed to produce an 80% chance of obtaining statistical significance at the 0.05 level for a 2-tailed design, based on a minimum important difference of 3.1 %, a SD of the difference of 1.7 %, and an estimated average correlation of 0.5 (data from Lewis *et al*. [49]). Results for all normally distributed data are presented as mean differences ($\Delta\bar{x}$) with 95% confidence intervals [95% CI]. The results of non-parametric analysis are presented as the median differences ($\tilde{x}\Delta$) and interquartile range (IQR). Due to poor image quality, three participants' scans were removed from the FMD analysis, and three different participants' scans were removed from the microvascular analysis. The removal of this data was performed before statistical analysis.

The effect of hypoxia on FMD was determined by a paired t-test comparing normoxia and hypoxia in the first instance. Additionally, the allometric scaling approach was used to adjust for baseline diameter in the calculation of FMD (Atkinson & Batterham, 2013). Briefly, baseline diameters and peak diameters were logarithmically transformed, and then a linear mixed model with repeated measures was performed in SPSS, where the baseline diameter were selected as covariates. Covariate adjusted means for diameter change were obtained from this SPSS model and then back-transformed.

To determine the relationships between the decline in EF with cardiorespiratory fitness (VO_{2max}), Pearson's correlations were used for parametric data and Spearmen's correlations for non-parametric data. For all correlational analyses, the strength of a relationship was determined by the correlation coefficient value, and P values < 0.05 were considered to indicate statistical significance.

2. 4 RESULTS

2. 4 .1 VASCULAR DEMOGRAPHIC (CAROTID INTIMA-MEDIA THICKNESS)

Baseline cIMT measurements were recorded to screen for any subclinical signs of atherosclerosis. For measurements of the right common carotid artery, the mean value was reported to be 0.46mm (SD = 0.07), and the left common carotid artery was measured to be 0.45mm (SD = 0.07) (Table 2. 1). Carotid intima-media thickness measurements of <1.0mm are considered to be normal [217].

	Minimum (mm)	Maximum (mm)	SD (mm)
Right CCA IMT	0.36	0.71	0.07
Left CCA IMT	0.35	0.58	0.07
Mean CCA IMT	0.37	0.56	0.06

Table 2. 1. Measurement values of the common carotid artery intima-media thickness (cIMT). CCA: common carotid artery; IMT: intima-media thickness.

2. 4. 2 PHYSIOLOGICAL RESPONSES TO 30 AND 60-MIN HYPOXIA

Resting physiological responses were recorded at 30 and 60 min during the trial. Hypoxia decreased SpO_2 compared to normoxia after 30 min ($\bar{x}\Delta$−19 [−20, −17] %) and 60 min ($\bar{x}\Delta$−18 [−20, −15] %; $p < 0.001$) exposure. Hypoxia significantly increased heart rate compared to normoxia after 30 min exposure ($\bar{x}\Delta$12 [8, 6] beats/min; $p < 0.001$) and remained elevated after 60 minutes ($\bar{x}\Delta$11 [6, 16] beats/min; $p < 0.001$). Hypoxia increased mean arterial BP compared to normoxia after 30 min ($\bar{x}\Delta$4 [1, 7] mmHg; $p = 0.02$; but had no effect on mean arterial BP after 60 min ($\bar{x}\Delta$0 [−4, 4] mmHg; $p = 1.0$).

2. 4. 3 Effect of Hypoxia on Microvascular Function

Mean baseline perfusion was calculated by calculating the average perfusion values for both regions of interest during the baseline scan. Compared to normoxia, hypoxia did not affect baseline perfusion after 30 min ($\tilde{x}\Delta = 0.5$; {IQR: 13.8}, $p = 0.6$). As expected, perfusion values increased in response to the iontophoresis of ACh and SNP during both trials. Compared to normoxia, endothelium-dependent (ACh) microvascular dilation was reduced after 30 minutes of exposure for 73% of participants ($\tilde{x}\Delta$ = -109%, {IQR: 542.7}, $p = 0.05$) (Figure 2. 1). Compared to normoxia, hypoxia did not affect endothelium-independent (SNP) microvascular function after 30 minutes of exposure, however, 42% of participants has lower responses during hypoxic trial ($\tilde{x}\Delta$ 69%, {IQR: 453.7}, $p = 0.6$).

2. 4. 4 Effect of Hypoxia on Flow-mediated Dilatation

In comparison to normoxia, hypoxia significantly increased baseline brachial diameter by 2.9% after 60 min ($\bar{x}\Delta$0.11 [0.03, 0.19] mm; $p = 0.01$). As baseline diameters were different between conditions, FMD results are presented as unscaled and allometrically scaled responses (Figure

2. 2). Compared to normoxia, hypoxia significantly reduced unscaled FMD responses in 85% of participants after 60 min ($\bar{x}\Delta$-1.19 [-1.80, -0.58] %, $p < 0.001$). Compared to normoxia, hypoxia significantly reduced allometrically scaled FMD responses in 85% of participants after 60 min ($\bar{x}\Delta$-1.21%, $p < 0.001$; relative −18.2%).

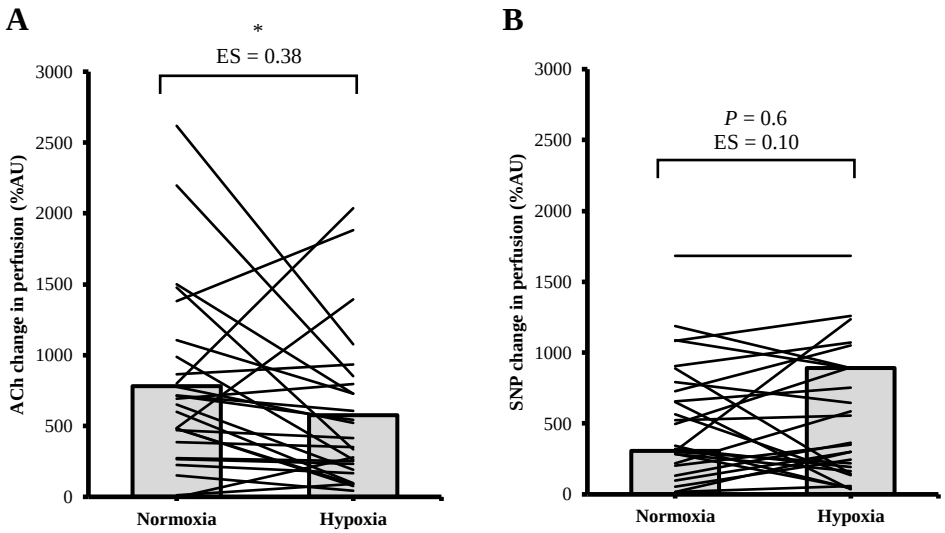


Figure 2. 1. The effect of normoxia and hypoxia on microvascular function (n = 26)
Date presented as median as well as individual responses. (**A**) Microvascular response to acetylcholine (ACh) was significantly impaired during hypoxia. (**B**) Microvascular response to sodium nitroprusside (SNP) remained unchanged. *Significant difference between conditions, $p < 0.001$. Effects sizes (ES; by Cohen's d) can be interpreted as small (>0.2), medium (>0.5), large (>0.5), very large (0.7) and extremely large (0.9). ACh: acetylcholine; AU: arbitrary units; SNP: sodium nitroprusside.

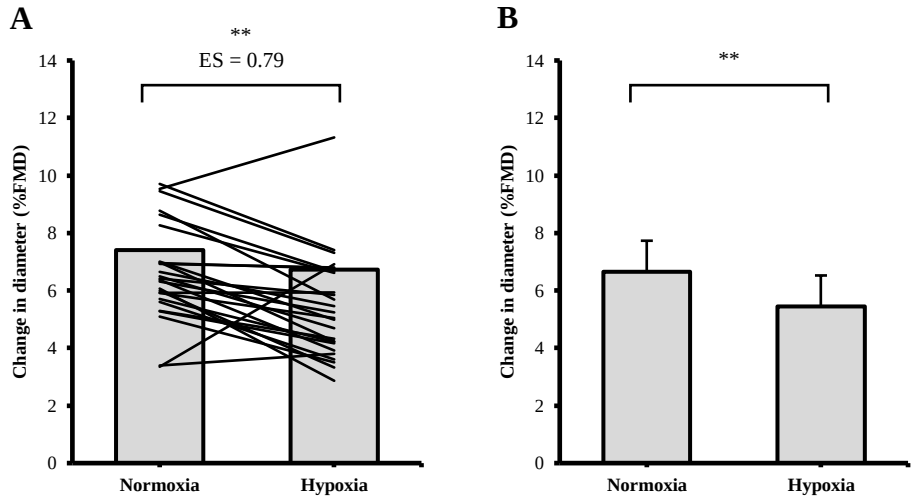


Figure 2. 2. The effect of normoxia and hypoxia on flow-mediated dilatation (FMD) (n = 26)
Uncorrected data (**A**; paired t-test; presented as mean as well as individual responses) and allometrically scaled data for differences in baseline diameter (**B**; linear mix model; presented as mean (SD). Flow-mediated dilatation (FMD) response were significantly lower during hypoxia. **Significant difference between conditions, $p < 0.001$. Effects sizes (ES; by Cohen's d) can be interpreted as small (>0.2), medium (>0.5), large (>0.5), very large (0.7) and extremely large (0.9). FMD: flow-mediated dilatation.

2. 4. 5 THE ASSOCIATION BETWEEN CARDIORESPIRATORY FITNESS WITH ENDOTHELIAL FUNCTION

Cardiorespiratory fitness was not associated with microvascular EF (r = -0.47; p = 0.07) or large-vessel EF (r = 0.06; p = 0.76) at normal conditions (Figure 2. 3). The magnitude of the decline in microvascular EF was correlated with cardiorespiratory fitness (r = 0.45, p = 0.02), but was not associated with large-vessel EF (r = -0.09; p = 0.68).

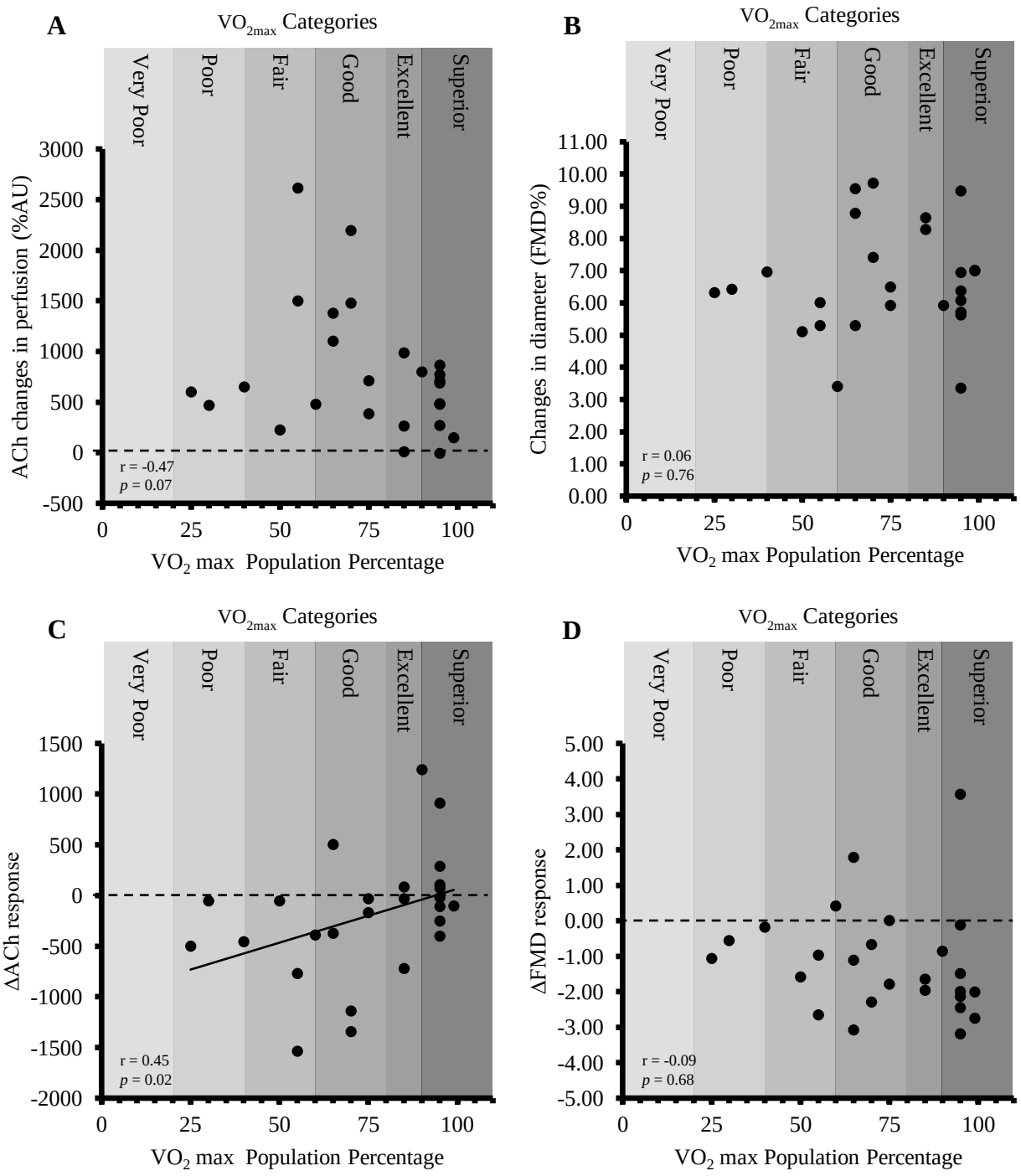


Figure 2. 3. The association between cardiorespiratory fitness and endothelial function (EF). During normoxia, cardiorespiratory fitness was not associated with (**A**) microvascular EF (r = -0.47; *p* = 0.07) or (**B**) large-vessel EF (r = 0.06; *p* = 0.76). (**C**) Higher cardiorespiratory fitness levels were associated with the decline in microvascular EF between normoxia and hypoxia (r = 0.45; *p* = 0.02), but was (**D**) not associated with large-vessel EF (r = -0.09; *p* = 0.68). Cardiorespiratory data is presented as the VO_2 max score as a population percentage, according to the American College of Sports Medicine guidelines [224]. ACh: acetylcholine; AU: arbitrary units; FMD: flow-mediated dilatation.

2. 5 DISCUSSION

The principle findings of this study are that 30 minutes of hypoxia reduced endothelium-dependent microvascular function (43% reduction in perfusion response to ACh), but did not affect endothelium-independent microvascular function (no change in perfusion response to SNP). Moreover, 60 min hypoxia reduced endothelium-dependent large-vessel vasodilatation (18% reduction in FMD). Notably, the extent of dysfunction was approximately two-fold higher in the microcirculation compared to the large-vessels. Additionally, we are the first to demonstrate individuals with greater cardiorespiratory fitness preserve microvascular EF during hypoxic exposure.

The present study is the first to our knowledge to examine the effect of hypoxia on microvascular and large-vessel EF in the same study. The difference in the degree of dysfunction between vascular beds suggests that hypoxia may activate specific mechanisms, which effect EF differently. Assessed separately, microvascular and large-vessel function have been reported to decline following acute hypoxia [48,49,205]. However, some studies have also reported increased vascular reactivity following hypoxic exposure [209]. Differences in vascular stimulation methods and the length and type of hypoxic exposure makes it difficult to compare these published findings. Therefore, when investigating the effects of acute hypoxia

on EF, it is important to consider assessing EF in multiple vascular beds for a comprehensive understanding of the underlying mechanisms.

Using isocapnic hypoxia, Lewis *et al*. concluded that normobaric hypoxia-induced FMD reductions were more pronounced after 30 minutes of severe hypoxia ($P_{ET}O_2$ 50 mmHg) compared to mild hypoxia ($P_{ET}O_2$ 75 mmHg) [48]. This finding suggests that hypoxaemia severity is associated with ED. However, the small range of SpO_2 that were recorded during hypoxia in the present study (range = 70–86%, SD = 5%) suggests that the hypoxic stimulus was relatively homogenous across participants, with most participants at a similar $P_{ET}O_2$ of ~42 mmHg. Thus, the minimal range makes it difficult to evaluate the relationship between hypoxaemia severity and ED. Nonetheless, our results do suggest that hypoxia has a greater deleterious effect of microvascular EF than that of the large-vessels, suggesting that the microvasculature endothelium may be more sensitive to hypoxia than larger blood vessels, highlighting the importance of assessing both microvascular and large-vessel EF in hypoxia studies.

Most of the literature implies that hypoxia-induced ED is linked to NO deficiency [225,226]. The synthesis of NO is an oxygen-dependent reaction, and therefore lower oxygen availability would imply a reduction in NO synthesis. In animal and human *in vitro* models, chronic hypoxia (> 24 h) exposure has been proposed to downregulate the expression of eNOS, thus, blocking the synthesis of NO synthesis [70,71]. However, Prieto *et al*. suggested that acute hypoxic exposure (< 24 h) does not decrease eNOS protein expression, but rather, eNOS' capacity to produce NO is affected [227]. L-arginine oxidation *via* eNOS is the primary source of NO in endothelial cells, but other enzymes including arginase-I and arginase-II also compete for the same substrate. Krotova *et al*. reported that the activation of hypoxia-inducible factor 1

(HIF-1) elevates the expression and activity of arginase-II in the human lung microvasculature, thus limiting the bioavailability of NO [228]. To our knowledge, this finding has not been replicated in large blood vessels. Thus, it is possible that the upregulation of arginase-II in the microvasculature could explain the greater degree of ED in the microvasculature that we observed.

Hypoxia stimulates the activation and expression of HIF-1 and other transcriptional complex, which prompts metabolic changes within endothelial cells of small and large blood vessels. The changes in endothelial metabolism have been associated with nicotinamide adenine dinucleotide phosphate (NADH) oxidase-dependent increases in ROS, primarily, superoxide [73,74]. When an ample amount of superoxide is synthesised, it reacts rapidly with NO to produce peroxynitrite and thereby prevents NO's vasodilatory effect on vascular smooth muscle cells [77]. In addition to the changes in endothelial metabolism, the interaction between HIF-1 and endothelial cells evokes proinflammatory reactions [75]. The prevalence of adhesion molecules are proposed to be higher in microvascular endothelial cells compared to large-vessel endothelial cells [26]. The overexpression of adhesion molecules makes the microvasculature more susceptible to the infiltration of inflammatory molecules [27], which can activate endothelial cells and diminish NO bioavailability. Finally, acute hypoxia directly increases sympathetic outflow and in turn, attenuates NO-dependent vasodilation [72]. Sympathetic excitation does not only stimulate vasoconstriction, but also increases retrograde shear rate, thus limiting FMD response [229,230]. In summary, the available evidence suggests that acute hypoxia diminishes NO bioavailability by reducing eNOS activity, upregulating reactive species and inflammation, and increasing sympathetic activity, and thus directly impairs the endothelial NO vasodilatory system. Further research is warranted to investigate

the relative contribution of the aforementioned mechanisms of ED between different vascular beds.

Cardiorespiratory fitness is positively associated with cardiovascular health [231]. Exercise interventions have been reported to significantly improve EF [211,212] and prevent and restore age-related endothelial decline [212]. Moreover, exercise-induced improvements in EF have been directly associated with increases in NO bioavailability [211]. However, independent of training interventions, resting FMD responses are not associated with fitness status in young adults. In the present study, while cardiorespiratory fitness was not associated with microvascular or large-vessel EF, the hypoxia-induced decline in microvascular function was negatively correlated with cardiorespiratory fitness. Those with the lowest cardiorespiratory fitness had the greatest hypoxia-induced reduction in microvascular function. This significant relationship provides some evidence to suggest that cardiorespiratory fitness may provide some protection against hypoxia-induced microvascular ED. In contrast, we did not observe a similar relationship between FMD decline and cardiorespiratory fitness, possibly because the microvasculature is more sensitive to hypoxia-induced dysfunction. Here, we demonstrate the importance of physical fitness for microvascular function in hypoxia, which warrants future research in populations that suffer long-term hypoxia and vascular dysfunction.

LIMITATIONS

The vascular techniques that were used in the current study are sensitive to changes in measurement location. Despite our best efforts to ensure that the same measurement location was used between trials (i.e. using anatomical landmarks and labelled acetate sheets), it is

possible that there were small variations in LDI and ultrasound measurement location. However, it has been previously reported that different measurement locations would increase variance, thus making it more difficult to identify mean changes and provide statistical significance [232,233]. In addition to using baseline diameter for covariate-adjusted means, some researchers propose that FMD data should also be normalised for variation in the shear rate [234,235]. For the present study, shear rate was not recorded. However, Atkinson *et al*. suggested that normalising one variable (i.e. baseline diameter), by another variable (i.e. shear rate), is not good practice when analysing FMD data [236]. Furthermore, Atkinson *et al*. implied that scaling FMD to baseline diameter differences should outweigh the variation in shear rate.

CONCLUSION

To conclude, acute exposure to normobaric hypoxia reduced endothelium-dependent vascular function, in small and large vascular beds. The decline in microvascular EF was approximately twice as large as that observed in the large blood vessel, demonstrating the sensitivity of the microvascular endothelium to hypoxia. Furthermore, our data suggests that a greater cardiorespiratory fitness was protective against the hypoxia-induced reduction in microvascular EF but this warrants further investigation. Collectively, these findings highlight the sensitivity of the microvascular circulation to hypoxic insult, particularly in those with poor cardiorespiratory fitness.

CHAPTER 3: THE EFFECTS OF BEETROOT JUICE ON MICROVASCULAR AND LARGE-VESSEL ENDOTHELIAL FUNCTION: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY IN HEALTHY OLDER ADULTS

3. 1 ABSTRACT

Dietary NO_3^- has been reported to improve EF and BP. However, most studies only assess large-vessel EF with little research on the microvasculature. Thus, the aim of the present study is to examine NO_3^- supplementation on microvascular and large-vessel EF and BP.

Twenty older adults (63 ± 6 years) were randomised to a BRJ or placebo (PLA) group for 28 (±7) days and attended three laboratory visitations. Across visitations, microvascular and large-vessel EF were assessed by LDI with iontophoresis of vasoactive substances and FMD, respectively. Plasma NO_3^- concentrations, BP and the presence of NO_3^- reducing bacteria were also assessed.

Plasma NO_3^- increased following 2-weeks of BRJ supplementation ($p < 0.05$) along with concomitant improvements in systolic and diastolic BP (−6mmHg and −4mmHg, respectively) ($p < 0.05$). BP remained unchanged in the PLA group. There were no significant differences in endothelium-dependent or endothelium-independent microvascular responses between groups. FMD increased by 1.5% following 2-weeks of BRJ ($p < 0.05$), with only a minimal (0.1%) change for the PLA group.

Two weeks of BRJ supplementation improved large-vessel EF and BP in older adults. The improvements observed in the present study are likely to be greater in populations presenting with ED. Thus, further prospective studies are warranted in individuals at greater risk for CVD.

3.2 INTRODUCTION

The endothelium is the inner most lining of the vasculature and is a dynamic organ that plays a pivotal role in regulating vascular tone [5]. Various vasoactive factors are released by endothelial cells, which cause the vascular smooth muscle cells to contract or relax, resulting in vasoconstriction or vasodilation, respectively. One such factor is NO which is a potent vasodilator that is important for maintaining vascular homeostasis [237]. Endothelial dysfunction is characterised by a reduction in NO bioavailability and can increase CVD risk, particularly in older individuals [238,239]. Reductions in NO bioavailability in older individuals could be due to alternations in eNOS expression and activity [240]. Additionally, it has been suggested that a considerable amount of NO is consumed by superoxide in later years of life [241,242]. Thus, an alternative source of NO, which is independent of the endothelium, could help to maintain vascular homeostasis.

Vegetables such as beetroot and spinach contain high levels of NO_3^- and when consumed, they can increase NO bioavailability independently of the endothelium [243]. When ingested, dietary NO_3^- is partially absorbed into the circulation and is later taken up by the salivary glands. Within the oral cavity NO_3^- becomes 10 to 20 fold more concentrated in the saliva [164]. Further to this, salivary NO_3^- is reduced to NO_2^- by commensal facultative anaerobic bacteria, located on the posterior region of the tongue. After swallowing, most of the salivary NO_2^- is converted to NO in the acidic conditions of the stomach; however, a small percentage is transported into the circulation. Once in the circulation, NO_2^- is converted to NO through enzymatic and non-enzymatic reactions [165–169]. This endothelium-independent production of NO is commonly described as the entero-salivary NNN pathway [148]. A growing body of

literature identifies dietary NO_3^- as an exogenous source of NO which has the capacity to improve EF in individuals at risk of CVD [244].

A prospective study (over 14 years) in a cohort of older adults, reported that a diet low in NO_3^- rich vegetables was correlated with the prevalence of CVD mortality [245]; this conclusion is also in line with previous findings from the same research group [246]. Furthermore, the high NO_3^- content of a Mediterranean and Japanese diet has been suggested to be associated with significant blood BP improvements [142,247]. As well as diets which are naturally rich in NO_3^-, supplements such as BRJ have been demonstrated to have beneficial effects on BP [167,180,181,185,186,248–251]. Although these discoveries support the cardioprotective features of dietary NO_3^-, little is known about the underlying mechanisms. However, some authors conclude that the improvements in BP are the result of reductions in systemic vascular resistance (SVR) [252].

Systemic vascular resistance is partly regulated by the endothelium. Previous research has shown that impairments in EF can lead to greater SVR [253]. In humans, EF is commonly assessed in conduit arteries using FMD, which measures the endothelium-dependent dilatory response to reactive hyperaemia. In a large cohort of 68 hypertensive patients, FMD responses were significantly improved following four weeks of daily BRJ consumption [181]. Furthermore, both systolic BP (SBP) and diastolic BP (DBP) were reduced following one week of the intervention and were sustained throughout the four week supplementation period [181]. Interestingly, there is limited research available, that has investigated the effect of NO_3^- supplementation on microvascular EF, despite the microvasculature being the primary regulator of SVR [254]. Moreover, microvascular ED is believed to precede ED in the larger blood vessels [255]. However, due to the heterogeneity of the endothelium, no clear

associations can be made between vascular beds [21,256]. Therefore, it is important to understand the underlying principles of microvascular EF as it has a much larger surface area and is at a greater risk of becoming dysfunctional.

Gilchrist *et al*. examined the effects of a two week BRJ intervention on microvascular function, large-vessel function and BP in individuals diagnosed with type II diabetes (T2D) [187]. Large-vessel function was assessed using nitroglycerine-mediated dilatation (endothelium-independent) and FMD (endothelium-dependent), whilst microvascular function was measured using LDI with iontophoresis of endothelium-dependent (ACh) and endothelium-independent (SNP) agonists [39]. Contrary to Gilchrist and colleagues' hypothesis, the intervention did not result in any significant improvements in vascular function or BP. The authors concluded that subjects had irreversibly diminished vascular reactivity, due to having multiple risk factors for CVD. Nonetheless, a longer intervention of six weeks has been shown to improve FMD and reduce SBP in hypercholesterolemic patients, although no microvascular assessment was performed in this study [180].

It appears that longer interventions are necessary to examine the chronic effect of dietary NO_3^- supplementation in individuals at risk of CVD. Additionally, further examination about the effect of NO_3^- supplementation on microvascular function is warranted in order to improve understanding of the underlying mechanisms that lead to BP improvements. Therefore, the present study aims to examine the effect of BRJ supplementation on microvascular function, large-vessel EF and BP in older adults over a four week period. We hypothesise that four weeks of daily supplementation with NO_3^- rich BRJ will increase NO bioavailability, along with concomitant improvements in vascular function and BP.

3.3 MATERIALS AND METHODS

3. 3. 1 PARTICIPANTS

Thirty-seven healthy older adults were recruited and assessed for eligibility by e-mail, flyers and advertisements (Figure 3. 1). Participants were allocated to each group using stratified randomisation based on age (± 3 years) and gender to ensure similarities between both arms. From the sample, 16 people did not reply to the invitation, 1 person was excluded on medical grounds. Therefore, 20 healthy older adults (age: 63 ± 6 years) participated in the present study. Due to personal reasons, 2 individuals withdrew from the study following a week of their intervention. Participants were briefed on the nature and the purpose of the investigation before written consent was taken along with a short demographic questionnaire to ensure that they satisfied the study criteria.

To avoid the effects of the menstrual cycle, only men and post-menopausal women aged 55 or older were recruited for the study. Exclusion criteria included any history of acute coronary syndrome or established CVD, use of antihypertensive medication, chronic diseases or acute illnesses, highly trained/active individuals, diets rich in green leafy vegetables and habitual use of antibacterial mouthwash. All experimental procedures were performed in accordance with the ethical standards of Declaration of Helsinki and approved by the institutional ethics committee (S02-16/17).

3. 3. 2 EXPERIMENTAL DESIGN

A randomized placebo-controlled trial utilising a parallel study design was used to test the hypothesis. Those assessing the outcomes were blinded to the conditions and participants were never told which treatment they were allocated to, only that both drinks had health benefits. For the duration of the study, all participants were required to attend three laboratory visits, separated by 14 (± 7) days. All visits were performed in a thermoregulated laboratory (21-22^{o}C) which started between the hours of 07:30-09:00am and lasted approximately 2-hours. Participants arrived following a 12-hour fast and were asked to refrain from caffeine. They were also instructed to avoid strenuous exercise 24 hours prior to testing. Lastly, participants were asked to complete a three day food diary and the seven day International Physical Activity Questionnaire [257] before attending the laboratory sessions. Over the period of the study, all participants were asked to maintain their usual diet and physical activity levels.

During each visit, anthropometric data was collected prior to any other assessments, which included; height, body mass, body mass index (BMI) and body fat percentage (TANITA C-300; Tanita Ltd, Japan). Venous blood samples were collected into EDTA collection tubes and were centrifuged immediately. The plasma was separated into 500µl aliquots before being transferred to a -80^{o}C freezer for later analysis. Following the blood sample, a tongue scrape sample, containing potential NO_3^- reducing bacteria, was collected from the posterior region of the tongue. Samples were transferred into a NO_3^- medium and immediately incubated. Subsequently, participants were asked to lie down for 10-minutes before three separate automated BP measurements were taken (separated by 2-minutes) and the mean value was recorded. Whilst remaining in a recumbent position, vascular function of the small and large blood vessels were assessed (separated by 15-minutes).

3. 3. 3 DRINK INTERVENTION

Daily consumption of the active or placebo (PLA) treatment was instructed for the duration of their enrolment. The active treatment was a 70ml NO_3^- rich BRJ drink (Beet-It Sport Shot 400; James White Ltd, Ashbocking, UK), providing approximately 400mg of NO_3^- each day. For the PLA group, prune juice (Sunsweet Growers Inc, Kingston upon Hull, UK) was selected because of the negligible NO_3^- levels (<0.01mM) [258] and similar consistency and colour to BRJ. Additionally, carbohydrate and fibre content of both treatments have been reported to be similar [258]. All participants were asked to consume their allocated drink in the morning and to keep it consistent for the duration of the study, with exception of testing days.

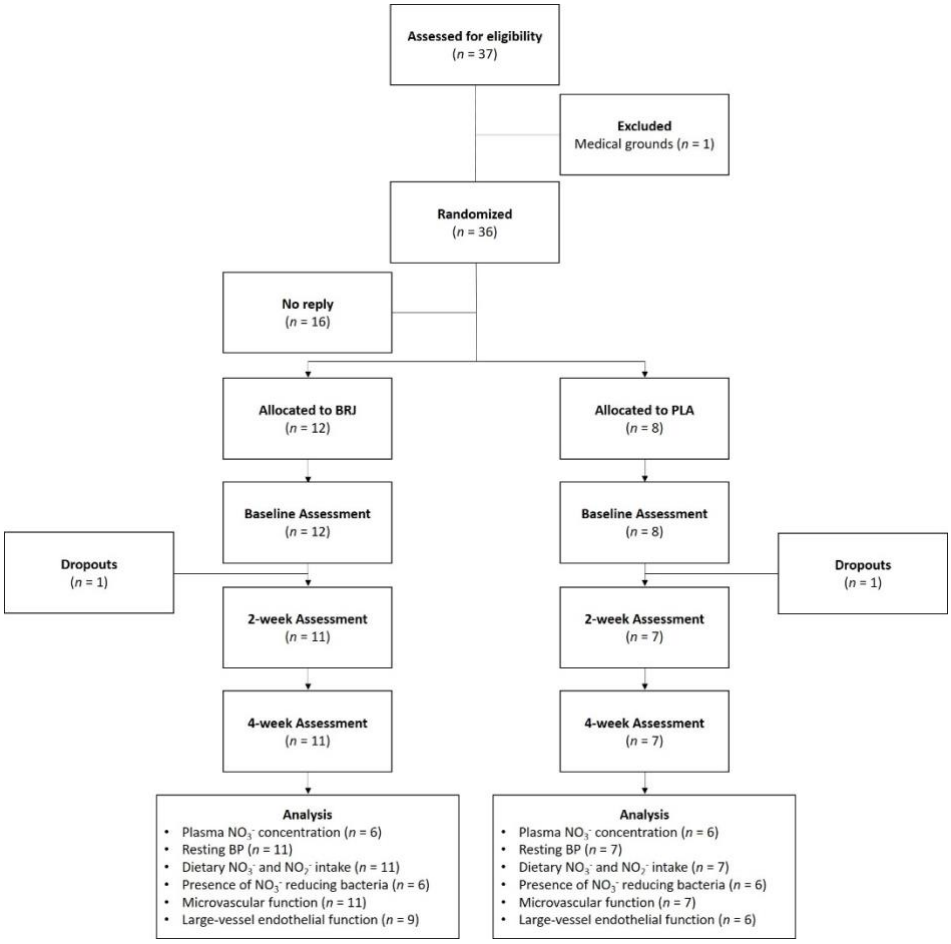


Figure 3. 1. Consolidated Standards of Reporting Trials (CONSORT) flowchart of study.

3. 3. 4 DIETARY NITRATE AND NITRITE INTAKE

The NO_3^- and NO_2^- content was determined by a database containing NO_3^- and NO_2^- values from 7,703 foods and beverage records from 432 publications [259]. To develop the most accurate and appropriate values in terms of the study population location, a similar method previously described [260] was used with priority given to: 1) the UK/European countries from 1990 to present; 2) the UK/European countries from 1960 – 1989; 3) countries with predominately Western diets (Australia, U.S, and Canada) from 1990 to present; 4) countries with predominately Western diets from 1960 – 1989; 5) countries with predominately non-Western diets. The cut-off dates were chosen to account for changes in laboratory methods, food preservation techniques and manufacturing technologies (the addition of ascorbate during meat processing (added to reduce the formation of nitrosamines), and legislation regulating the amounts of NO_3^- and NO_2^- used in the curing process created significant reductions in food and beverages [261,262].

Nitrate and NO_2^- intake was calculated by multiplying the food or beverage item consumed (grams per day) as the weighted mean value of that item identified using the multistep process mentioned above. Weighted mean was selected as some mean values were adversely affected by extreme values. If a NO_3^- or NO_2^- value was unattainable a value of 0 mg/g was assigned to the food or beverage item. Cooking methods such as baked, blanched, boiled, broiled, cooked, fried, microwaved, raw, and steamed were reported when relevant and available.

If the serving amount for a food or beverage item was missing from the recall data, the recommended serving size amount was calculated using summary estimates (in grams) as defined by standard serving sizes in nutritional analysis software [263,264]. In the case of

multicomponent foods (e.g., pizza, juices, salad mix, soup, and curries), nutrient values were determined by calculating the NO_3^- and NO_2^- content of all ingredients contained in the recipe list, or in the case of commercial products, looking at the ingredients list or using the recipe reproduction [265]. Recipes were selected from reliable and common online kitchen resource materials. Total NO_3^- and NO_2^- (mg/d) were determined by calculating the sum of daily NO_3^- and NO_2^- values.

3. 3. 5 PRESENCE OF NITRATE REDUCING BACTERIA

A tongue scraping was collected from each participant during their first visit following an oral examination to ensure there were no signs of any mouth infections. The scrapings were performed using a disposable acrylic spatula from the posterior region of the tongue and were collected into sterile disposable pipette (Fisher Scientific, Cat. No. 13489108). Samples were transferred into 6mL of NO_3^- rich medium (Ingredients per litre of deionized water: Pancreatic Digest of Gelatin 5.0gm; Beef Extract: 3.0gm; Potassium Nitrate: 1.00gm) (Hardy Diagnostics, Cat. No. K42) and incubated for 54 hours at 37 ˚C, 95% humidity and 5% CO_2. To ensure a similar inoculum size, the weight gain of the broth was kept constant at approximately 10µg.

Following incubation, 1mL of the NO_3^- broth was extracted through a Puradisc 25 Polyethersulfone 0.2µm sterilizing grade filter (Fisher Scientific, Cat. No. 6780-2502) to ensure that any bacteria were eliminated. Content of the syringe was then emptied into separate 500µL aliquots and immediately frozen at -80˚ for later analysis of NO_3^-. The levels of NO_3^- in the medium were analysed before and after incubation. Reductions in NO_3^- levels are expressed as a percentage change (ΔNO_3^-%), in order to determine the presence of NO_3^- reducing bacteria. All the NO_3^- analysis is described in more detail in Section 3. 3. 8.

3. 3. 6 MICROVASCULAR FUNCTION

Iontophoresis administration of ACh (Miochol-E, Novartis, UK) and SNP (Nitroprussiat Fides, Rottapharm SL, Spain) was performed using an iontophoresis controller (MIC-Ie, Moor Instruments Ltd, UK) in order to assess cutaneous endothelium-dependent and endothelium-independent vasodilation, respectively. Perfusion changes on the anterior surface of the forearm in response to the delivery of both vasoactive drugs were assessed using LDI, which measures blood flux (AU). The full protocol that was used for this study has been described in detail previously [45]. In summary, one baseline scan was performed before a series of ten scans with an iontophoresis charge of 30μA was delivered to administer 1% ACh and 1% SNP. ACh and SNP drugs were diluted with 0.9% saline and delivered into the skin *via* an anode and cathode internal electrode Perspex chamber (⌀22mm) (ION 6, Moor Instruments Ltd, UK), respectively. Following ten scans with iontophoresis, two further recovery scans were performed without the delivery of the vasoactive drugs.

The exposure-time-response protocol took approximately 15-20 minutes and all of the scans were performed in natural lighting conditions, with most of the ambient lighting restricted. Additionally, the settings of the laser Doppler imager (moorLDI2-IR, Moor Instruments, Axminster, Devon, UK) were kept consistent for all scans. Measurements of perfusion were carried out offline using the moorLDI Review V6.1 software and results are presented as a percentage change in perfusion from baseline ($\frac{Peak\ Flux - Baseline\ Flux}{Baseline\ Flux}\ x\ 100 =$ ACh% or SNP%).

3. 3. 7 LARGE-VESSEL ENDOTHELIAL FUNCTION

The FMD procedure is a non-invasive technique that is commonly used on the brachial artery as an assessment of global large-vessel EF. Several published guidelines are available for this technique [266–269], however the present study used Sandoo and Kitas' protocol [45]. Briefly, a 2-minute baseline ultrasound scan of the brachial artery was followed by 5-minutes of arterial occlusion, achieved by inflating a BP cuff that was placed around the wrist to 220mmHg. After a 5-minute period of ischemia, the BP cuff was deflated rapidly, and a further 3-minute scan of the artery was performed.

A Siemens Acuson X300 Ultrasound scanner was used with a multifrequency linear-array vascular probe set at 7.3MHz (Siemens PLC, Camberley, UK) to perform the FMD procedure. B-mode images were captured at 15 frames per second to record a 120s baseline and a 210s clip following 5 minutes of occlusion. To capture the initial reactive hyperaemic response to the deflation of the BP cuff, the recording was initiated 30s before cuff release; therefore, only 180s was used for the analysis. Images were analysed offline using an automated edge detection software (Brachial Analyser, Medical Imaging Applications, USA). The Brachial Analyser software is capable to detect the peak of the R-wave; therefore, this inbuilt feature was used to include only the images at the peak of the R-wave. Frames which did not meet the recommended quality standard (confidence threshold <70%) were rejected. From the frames which were accepted the change in diameter from baseline to peak was calculated as follows; $\frac{Peak\ Diameter - Baseline\ Diameter}{Baseline\ Diameter}\ x\ 100$ = FMD%. To account for the differences in baseline diameter, all the data was allometrically scaled as per the Atkinson and Batterham guideline [223]. The coefficient of variation for the sonographer (DTJ) was reported as 8.5%.

3. 3. 8 Analysis of Nitrate concentration

Whole venous blood samples were collected into EDTA collection tubes following a 12-hour fast. Samples were immediately stored on ice or placed in a pre-chilled (4°C) centrifuge. Once samples were centrifuged, 500µl plasma aliquots were instantly stored at -80ºC. Prior to analysis, plasma samples were thawed and centrifuged again through a Amicon 30kDa molecular weight filter to reduce protein content [270]. Similarly, the sample of NO_3^- medium, for determining the presence of bacteria (Section 2.5), were also filtered in the same manner. Analysis of NO_3^- concentrations was performed using the Griess Reagent system as previously described by Miranda *et al.* [271]. Blood samples were analysed at baseline, week two (WK2) and week four (WK4), whilst medium samples were only analysed at baseline. Medium samples were diluted accordingly to ensure the NO_3^- values would fall within the standard range. Sodium nitrate was used for standard quantification and the results were expressed in micromoles (µM). The coefficients of variation (n = 35, in duplicate) of the methods were 2.9% to intra-assay and 4.0% to inter-assay.

3. 3. 9 Statistical Analysis

Per-protocol analysis was utilised for the present study. The assumption of normality, homogeneity of variances and sphericity were examined with the Shapiro-Wilk, Levene and Mauchly tests, respectively. For primary analysis on all dependent variables, a 2 x 3 (treatment x time) repeated-measures analysis of variance (RM ANOVA) was used ($p < 0.05$). When statistical significance was found, additional post-hoc tests with Bonferroni adjustments were conducted. If groups were different at baseline for any of the dependent variables, baseline

values were used as covariates. Results for all normally distributed data are presented as mean ± SD. Data that were skewed were log transformed, however the median and interquartile range (IQR) of the raw data are presented. All analyses were performed using a commercially available statistical package (IBM SPSS Statistics version 22 for Windows, Chicago, IL).

3.4 RESULTS

A total of 18 participants completed all three study visits between January 2017 to December 2018. According to verbal confirmation, both interventions were well tolerated for the duration of the study. No serious adverse events were experienced, however common side effect such as beeturia and faecal discolouration were reported. Baseline demographic characteristics were similar for both treatment allocations (Table 3. 1). None of the participants had any uncontrolled conditions and were considered to be healthy individuals.

Treatment Allocation	BRJ	PLA
Demographics		
n	11	7
age (y)	65 ± 8	61 ± 5
Height (cm)	167.4 ± 9.5	165.3 ± 6.2
Mass (kg)	73.2 ± 16.6	73.3 ± 8.0
BMI (kg/m^2)	26.2 ± 6.3	26.9 ± 2.1
Body Fat (%)	30.7 ± 10.5	33.8 ± 7.7
Dietary Intake		
NO_3^-	151.8 ± 77.8	185.3 ± 75.7
NO_2^-	7.4 ± 3.8	5.8 ± 1.9
Clinical BP		
SBP	129 ± 12	124 ± 14
DBP	75 ± 8	79 ± 3

Table 3. 1. Baseline characteristics stratified by treatment allocation. Data are presented as mean ± SD. (BMI = body mass index; DBP = diastolic blood pressure; NO_3^- = nitrate; NO_2^- = nitrite; SBP = systolic blood pressure).

3.4.1 PLASMA NITRATE CONCENTRATIONS

Plasma samples were analysed for 12 individuals (6 BRJ; 6 PLA), due to insufficient volumes from the remaining 6 participants (Figure 3. 2). Across the duration of the study, NO_3^- levels were highest in the BRJ group following two weeks of supplementation but declined slightly from WK2 to WK4. Mean plasma NO_3^- concentrations of the BRJ group increased from 34 ± 8µM to 132 ± 83µM following two weeks ($p < 0.05$), with concentrations measuring at 78 ± 41µM at four weeks ($p = 0.60$). Furthermore, NO_3^- levels were significantly greater compared to PLA treatment at WK2 and WK4 ($p < 0.05$). There were no significant changes in plasma NO_3^- concentration within the PLA group.

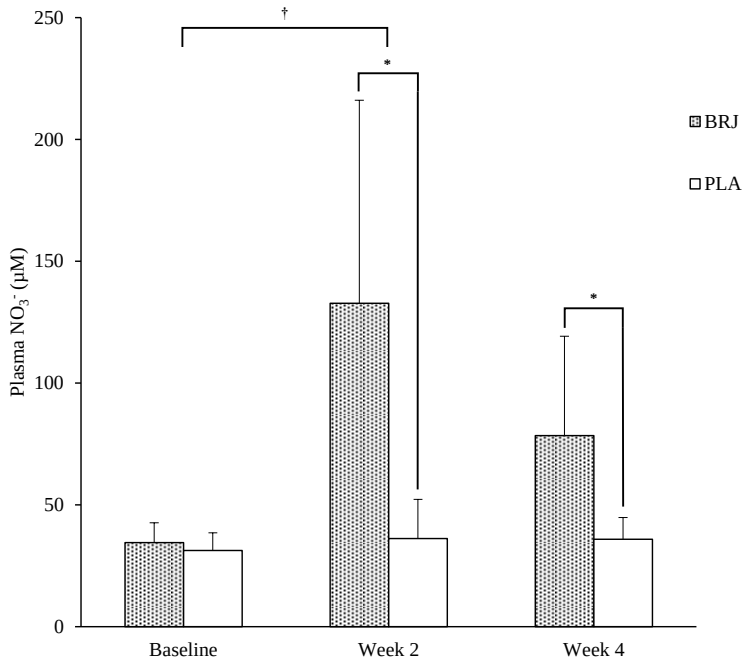


Figure 3. 2. The effect of treatments on the plasma nitrate (NO_3^-) levels over four weeks (n = 12). Data expressed as mean ± SD. *Significant group differences by treatment (BRJ versus PLA), $p < 0.05$. † Significant differences across time by post-hoc analysis (Baseline versus 2 weeks versus 4 weeks), $p < 0.05$ for Bonferroni post hoc test. (NO_3^- = nitrate).

3.4.2 RESTING BLOOD PRESSURE

Following two weeks of BRJ ingestion, SBP reduced by −6 ± 7mmHg and DBP by −4 ± 3mmHg ($p < 0.05$). However, from WK2 to WK4, SBP increased by 2 ± 7mmHg ($p = 1.00$) and DBP by 2 ± 4mmHg ($p = 0.49$). Within the PLA treatment group, SBP reduced by −1 ± 10mmHg following 2 weeks and DBP by −0 ± 5 ($p = 1.00$). From WK2 to WK4, SBP was further reduced by −4 ± 7mmHg ($p = 0.70$) and DBP by −0 ± 3mmHg ($p = 1.00$). None of the BP changes for PLA treatment reached statistical significance. Additionally, no significant differences were observed between the groups at any time point (Figure 3. 3).

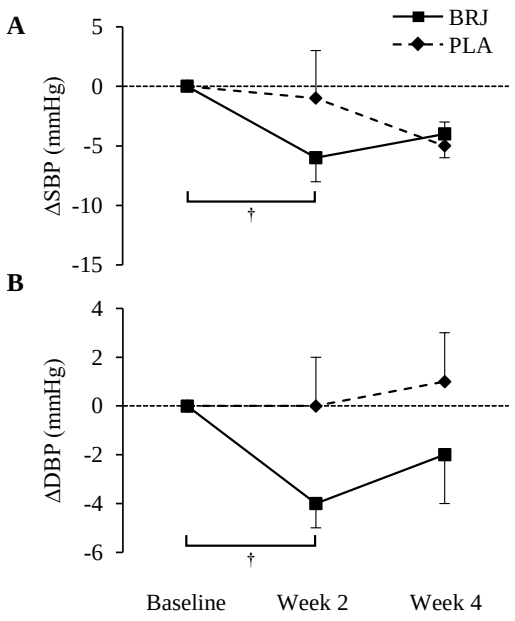


Figure 3. 3. The effect of treatments on the changes in resting blood pressure (BP) over four weeks (n = 18)

Data expressed as mean ± SD changes from the values measured during the baseline visit (A; SBP) (B; DBP). † Significant main effect for time (Baseline versus 2 weeks versus 4 weeks), $p < 0.05$ for Bonferroni post hoc test. (DBP = diastolic blood pressure; SBP = systolic blood pressure).

3.4.3 Dietary Nitrate and Nitrite intake

Analysis of the three-day self-report food diary was performed in order to quantify the NO_3^- and NO_2^- intake at baseline, before their second visitation and before their final visitation (Table 3. 2). At each time-point, the intake of NO_3^- and NO_2^- did not significantly differ between groups ($p = 0.78$; $p = 0.49$, respectively) Furthermore, no difference were observed over the four weeks within the BRJ and PLA groups ($p = 0.56$; $p = 0.67$, respectively).

Treatment Allocation	BRJ	PLA
NO_3^- (mg)		
Baseline	151.8 ± 77.8	185.3 ± 75.7
WK2	182.5 ± 72.4	200.2 ± 63.2
WK4	195.4 ± 66.9	190.4 ± 113.6
NO_2^- (mg)		
Baseline	7.4 ± 3.8	5.8 ± 1.9
WK2	6.2 ± 4.0	8.2 ± 2.9
WK4	8.1 ± 4.4	5.1 ± 2.3

Table 3. 2. Quantification of the amount of NO_3^- and NO_2^-, measured with a three-day self-report food diary. Data are presented as mean ± SD. (NO3- = nitrate; NO2- = nitrite; WK2 = week two; WK4 = week four).

3.4.4 PRESENCE OF NITRATE REDUCING BACTERIA

Twelve participants were included for the oral microbiome assessment (6 BRJ; 6 PLA). This assessment was used to determine the presence of NO_3^- reducing bacteria. Every participant who was included for this assessment demonstrated the capacity to reduce NO_3^-. Following 54-hours in incubation, the tongue scrape samples from each participant successfully reduced all of the NO_3^- in the broth medium, meaning a 100% reduction of the available NO_3^- substrate. These results confirmed the presence of NO_3^- reducing bacteria in the oral cavity of the 12 participants included for this analysis.

3.4.5 MICROVASCULAR FUNCTION

Blood flux was quantified by calculating the median rather than the mean for each region of interest. This is due to the fact the LDI data will rarely have Gaussian distribution [222]. The flux responses to ACh and SNP were not normally distributed for any of the laboratory visits, therefore log-transformed data was used to test for differences. However, the results are presented using raw data as percentage changes in flux with IQR. (Figure 3. 4). Contrary to our hypothesis, no significant differences in endothelium-dependent (ACh) or endothelium-independent (SNP) microvascular responses were observed over the three visitations.

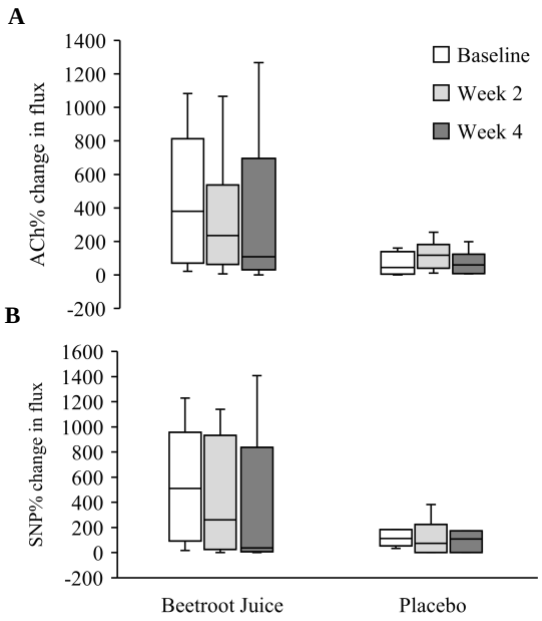


Figure 3. 4. The effect of treatments on laser Doppler imaging (LDI) with iontophoresis over four weeks (n = 18). Log-transformed data was used for all analyses, as the untransformed data was not normally distributed. Untransformed data expressed as median (IQR). (ACh = acetylcholine; LDI = laser Doppler imaging; IQR = interquartile range; SNP = sodium nitroprusside).

As expected, flux values increased in response to the iontophoresis of ACh for both groups. During the baseline visit, the median responses were measured at 380.0% (IQR: 743.6%) for the BRJ group and 44.3% (IQR: 133.3) for the PLA ($p < 0.05$). Significant differences between groups were reported at baseline; therefore, these values were used as a covariate to test for difference between groups at WK2 and WK4. Median responses at WK2 were 234.9% (IQR:

473.8%) for BRJ group and 117.8% (IQR: 141.8%) for the PLA group ($p = 0.75$). Furthermore, no significant differences between groups was observed at WK4, with the BRJ response measured at 108.1% (IQR: 665.45%) and PLA response measured at 59.1% (IQR: 130.94%) ($p = 0.42$).

As expected, flux values increased in response to the iontophoresis of SNP for both groups. During the baseline visit, the median responses were measured at 510.0% (IQR: 864.5%) for the BRJ group and 112.3% (IQR: 131.0) for the PLA ($p = 0.15$). Median responses at WK2 were 260% (IQR: 906.4%) for BRJ group and 73.1% (IQR: 223.3%) for the PLA group ($p = 0.43$). Furthermore, no significant differences between groups was observed at WK4, with the BRJ response measured at 33.4% (IQR: 830.8%) and PLA response measured at 107.0% (IQR: 171.9%) ($p = 0.80$).

3.4.6 Large-vessel Endothelial Function

Data loss occurred for 3 participants, therefore only 15 participants (9 BRJ; 6 PLA) were included for the main FMD analysis. FMD responses increased from baseline to WK2 for the BRJ treatment and the improvements were sustained until WK4. At baseline, endothelium-dependent vasodilation was 5.6 ± 1.8% in the BRJ group and for the PLA group it was 6.4 ± 2.5% ($p = 0.49$). Within the BRJ group, FMD values increased by 1.5 ± 1.8% at WK2 ($p < 0.05$), however values only increased by 0.1 ± 0.8% in the PLA groups ($p = 0.84$). After four weeks, FMD values increased by 1.5 ± 1.5% for the BRJ treatment ($p < 0.05$). The PLA group failed to reach any significance after 4 weeks, although a small improvement of 0.5 ± 1.0% was reported ($p = 0.23$). Further to this, no main effect between groups were found at WK2 (p

= 0.58) or WK4 (p = 0.85) (Figure 3. 5A). Using appropriate allometric scaling methods on the data did not change any of the outcomes; however, corrected data is presented in Figure 3. 5B.

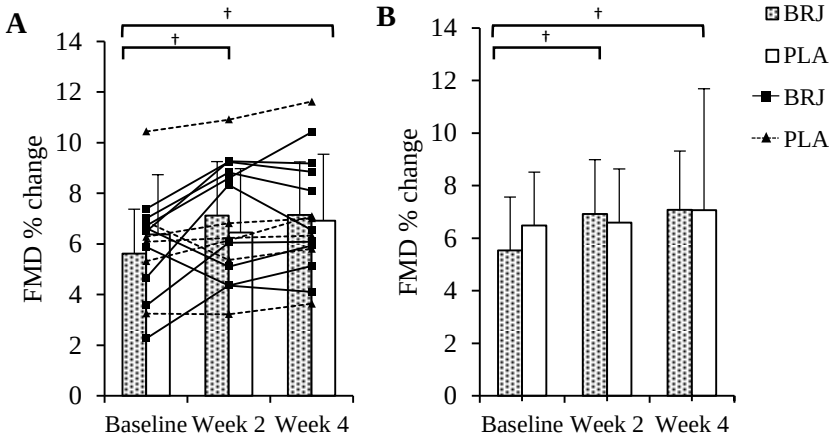


Figure 3. 5. The effect of treatments on flow-mediated dilatation (FMD) over four weeks (n = 15). Uncorrected data (A; mixed model ANOVA; presented as means ± SD, as well as individual responses) and allometrically scaled data (B; linear mix model; presented as means ± SD). † Significant main effect for time (Baseline versus 2 weeks versus 4 weeks), $p < 0.05$ for Bonferroni post hoc test. (FMD = flow-mediated dilatation).

3. 5 DISCUSSION

To our knowledge, this is the first study to investigate the effect of a dietary NO_3^- intervention on vascular function in small and large vascular beds of older adults. The findings indicate that BRJ improves large-vessel EF and reduces BP after two weeks. Improvements in large-vessel EF were sustained until the fourth week, however SBP values increased slightly from WK2 to WK4, corresponding with the pattern in plasma NO_3^- levels.

Analysis of the food diaries confirmed that the potential influence of dietary NO_3^- and NO_2^- intake is negligible, as levels were similar between groups at all time-points. These findings extend on previous acute studies performed in both healthy [167,191,193,251,272] and at risk populations [184,187,189]. Aside from the present study, only a few have tested long-term

interventions on EF and BP in humans (≥4 weeks) [180,181]. Despite the majority of the literature reporting significant improvements in large-vessel EF, the present study along with Gilchrist *et al*. observed no changes in microvascular function in response to BRJ supplementation [187]. Furthermore, the present study demonstrated that most of the participants' oral microbiome had the capacity to reduce NO_3^-, avoiding potential non-responders.

Siervo *et al*. implied that the ageing process disrupts the entero-salivary NNN pathway and reduces the potential benefits of dietary NO_3^- [273]. The argument made by Siervo *et al*. was based on the presumption that modifications which occur to the aging oral microbiome might diminish the effectiveness of dietary NO_3^- [274]. However, many studies, including the present one, have reported vascular improvements in response to NO_3^- supplementation in elderly individuals [184,185,249] and evidence from the present study suggests that the ageing oral microbiome is able to convert NO_3^- into NO_2^-. Furthermore, six weeks of BRJ supplementation has been associated with the proliferation of bacterial species which possess the NO_3^- reductase enzyme [180,275]. Therefore, long-term interventions could potentially transform non-responders into responders.

Dietary NO_3^- can reduce BP by several mechanisms (average reductions: SBP; −4.4mmHg and DBP; −1.1mmHg [276]) according to a comprehensive review by Carlström *et al.* [192]. The evidence implies that improvements in EF is the primary explanation for these reductions, even when endogenous NO bioavailability is reduced. Indeed, exogenous sources of NO has been shown to induce vasodilation, which consequently reduces SVR [277]. Interestingly, these

vascular responses to dietary NO_3^- consumption occur shortly after ingestion and coincides with BP reductions. In support of this statement, Bondonno *et al*. demonstrated significant improvements in FMD responses two hours post NO_3^- ingestion, which were also accompanied by reductions in SBP [272]. However, futures studies are required to determine the benefits from long-term NO_3^- supplementation on the burden of hypertension in individuals at risk of CVD.

To date, the longest intervention performed in humans was a 6-week study, in hypercholesterolemic individuals [180]. The authors reported that over the duration of the study, FMD responses improved by 24%, SBP and DBP were reduced (−4.1mmHg and −1.5mmHg, respectively) and the abundance of NO_3^- reducing bacteria was significantly greater. Further support for long-term supplementation was published by Kapil *et al*. in 2015. They reported a 20% improvement in FMD responses following four weeks of BRJ supplementation in a hypertensive cohort, which was accompanied by reductions in SBP and DBP (−7.7mmHg and −2.4mmHg, respectively). Similarly, the present study found improvements of 27% in FMD responses and reductions of −4.1mmHg and −2.1mmHg in SBP and DBP, respectively. It should be noted that many of the aforementioned studies fail to analyse their FMD data in the correct manner according to the guidelines of Atkinson *et al*. [236], therefore caution must be taken when interpreting these results. However, the present study analysed raw and corrected FMD data. The outcome of both analyses resulted in the same statistical finding. Collectively, these positive findings imply that long-term use of NO_3^- supplements can reduce BP and improve EF of the large blood vessels. However, despite the microvasculature being the primary regulator of SVR [254], few studies have investigated the long-term use of NO_3^- on microvascular function.

Contrary to our hypothesis, four weeks of NO_3^- supplementation did not lead to significant changes in endothelium-dependent or endothelium-independent microvascular reactivity. A potential reason for this finding is the fact that the pharmacologically vasoactive factors that were administered do not stimulate the conversion of NO_2^- into NO that happens under the ischemic conditions of the FMD procedure. However, Wong *et al*. stimulated microvascular vasodilation using reactive hyperaemia and found that three days of BRJ supplementation did not alter cutaneous vascular reactivity [278]. It should be noted that they only tested seven healthy individuals, three of whom were females, tested at no specific phase of their menstrual cycle. The authors proposed that cutaneous reactive hyperaemia is not NO mediated and is rather to be largely dependent on cutaneous sensory-nerve and/or calcium activated potassium channels [279]. In support of this argument, the same study reported no changes in cutaneous reactive hyperaemia after NOS inhibition. Laser Doppler flowmetry was used as a method of measuring cutaneous blood flow by this group, which only measures perfusion at a single point. However, LDI scans a larger region of interest and thereby accounts for the spatial heterogeneity of skin blood flow [31]. Further research is warranted in order to investigate the effects of NO_3^- supplements on the microcirculation.

The participants for the present study were recruited from a healthy aging population and most of them showed no signs of hypertension. Although significant improvements in large-vessel EF and BP were observed after two weeks of BRJ, it was not expected for improvements to continue indefinitely, due to the good health of the participants. The available evidence suggests that inorganic NO_3^- is most effective for individuals with some degree of ED [180,181,184,280]. However, the changes in plasma NO_3^- levels corresponded with the changes in BP, thus when plasma NO_3^- levels increased from two weeks to four weeks, so did

BP. Therefore, an alternative reason why improvements were not sustained for the duration of the study could be explained by the reductions in circulating NO_3^-. The exact cause of the diminished NO_3^- availability is not known. Some authors believe that NO_3^- and NO_2^- clearance is upregulated with prolonged exposure [281], however compliance to this intervention was only confirmed verbally, therefore we are unsure if every participant adhered to their allocated drinks.

LIMITATIONS

A limitation of the present study was the small sample size, due to difficulty with recruitment. As previously mentioned, compliance to the intervention was only confirmed verbally at the end of the trial; however, biological markers did support this declaration to an extent. Although plasma NO_3^- levels were measured, we did not have the facilities to analyse NO_2^- concentrations. However, previous finding suggests that significant increases in plasma NO_2^- occur alongside increases in plasma NO_3^- in elderly people with T2D [187].

CONCLUSION

In conclusion, this study was the first to demonstrate that chronic BRJ ingestion significantly improves SBP, DBP and large-vessel EF in healthy older adults. These changes corresponded to the changes in plasma NO_3^- levels throughout the follow-up. Although no changes in microvascular function were observed, further prospective studies examining the long-term impact of inorganic NO_3^- supplementation on this vascular bed in patient populations at risk of CVD are warranted.

CHAPTER 4: GENERAL DISCUSSION

4.1 OVERVIEW OF MAIN FINDINGS

The primary objectives of the present thesis were to understand the effect of hypoxia on EF in multiple different vascular beds and to determine whether cardioprotective factors including physical exercise and dietary NO_3^- protects and restores EF. Two independent research studies were conducted to satisfy the objectives of this current thesis. In the first study (Chapter 2), was we reported that acute hypoxia reduced microvascular and large-vessel EF. The findings from this study also established that in comparison to large blood vessels, the endothelium of the microcirculation is more sensitive to hypoxia-induced ED. Finally, it was discovered that individuals with greater cardiorespiratory fitness levels, were less susceptible to the deleterious effects of acute hypoxia on microvascular EF.

The second study of the present thesis demonstrated that chronic supplementation of BRJ, rich in NO_3^-, improves large-vessel EF in older, but otherwise healthy adults. The concomitant increases in circulating NO_3^- concentration, suggests that the vascular improvements were associated with an augmentation in NO bioavailability. With increased NO bioavailability, the endothelium had a greater capacity to regulate vascular tone, therefore significant reductions in BP were also observed. However, there were no significant improvements in microvascular EF, in spite of the microvasculature being the primary regulator of systemic BP.

4.2 HYPOXIA-INDUCED ENDOTHELIAL DYSFUNCTION

It has been reported previously that hypoxia diminishes the bioavailability and action of endothelium derived NO [70,71,73,74]. Although the primary cause of NO attenuation is unclear, most of the research suggests that the prevalence of oxidative and inflammatory stress during hypoxia, triggers a cascade of events that consequently leads to diminished NO. Independent of the endothelium, hypoxia is also proposed to moderate vascular function due to the corresponding increases in sympathetic outflow [72,229,230]. However, a pro-oxidative and pro-inflammatory environment have a greater impact on EF as majority of the literature suggests [282]. Hypoxia-induced inflammation and oxidative stress impairs the capacity of the endothelium to produce NO by downregulating the expression of eNOS [70,71], scavenging NO to produce peroxynitrite [73,74] and stimulating arginase synthesis [228] (Figure 4. 1). Collectively, these events can disrupt the balance between endothelium-derived vasoactive factors. Consequently, the imbalance between vasoactive factors favours vasoconstrictors and thus impairs the endothelium's vasodilatory capacity.

In addition to eNOS dependent NO, NO is also synthesised by inducible NO synthase. Unlike, eNOS dependent NO, iNOS is activated by immunostimulating cytokines. The overproduction of iNOS dependent NO has a greater tendency to react with superoxide than eNOS dependent NO [283]. Inevitably, the reaction between superoxide and NO will result in the formation of peroxynitrite, which triggers the uncoupling of eNOS, therefore preventing the beneficial vasodilatory effects of NO. Thompson *et al*. reported that the pro-inflammatory and pro-oxidative environment associated with hypoxia [284], upregulates the activation of iNOS which may partly explain the deleterious effect of hypoxia on endothelium-dependent vasodilatation. In comparison to larger vascular beds, microvascular endothelial cells express more adhesion molecules and therefore making them more susceptible to the infiltration of

inflammatory cytokines [27], which can stimulate the activation of iNOS and reduce eNOS dependent NO.

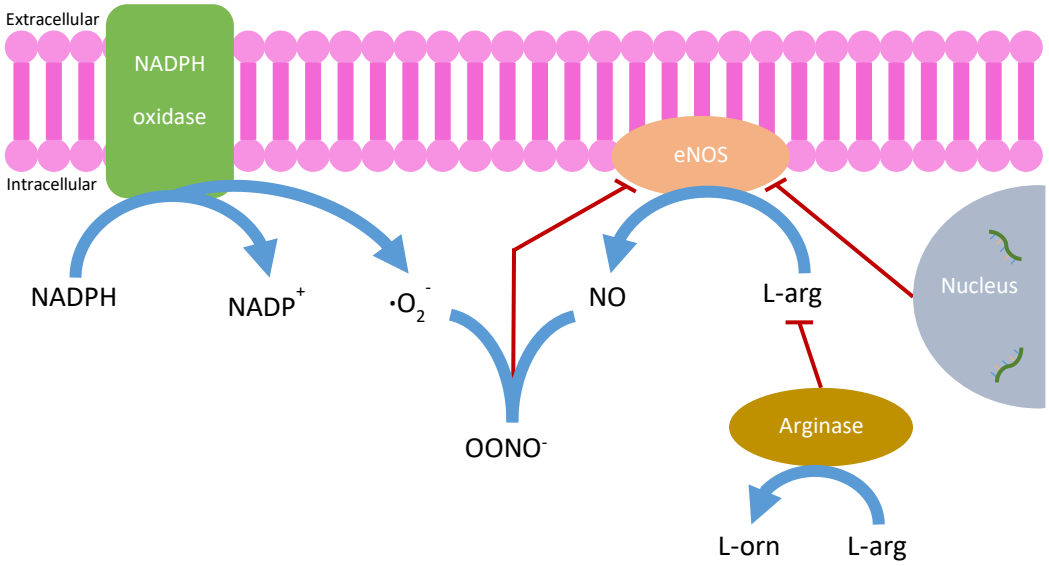


Figure 4. 1. Hypoxia-induced molecular changes that reduce endothelial function.
Hypoxia activates dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and the expression of superoxide ($\cdot O_2^-$) is upregulated. Superoxide rapidly scavenges nitric oxide (NO), and reacts to produce peroxynitrite ($OONO^-$), which diminished endothelial nitric oxide synthase (eNOS) dependent NO. eNOS protein expression is reduced. Hypoxia activates arginase enzymes that compete with eNOS for L-arganine (L-arg), to produce L-ornithine (L-orn). eNOS: endothelial nitric oxide synthase; L-arg: L-arganine; L-orn: L-ornithine; NADPH: dihydronicotinamide-adenine dinucleotide phosphate; NO: nitric oxide; $\cdot O_2^-$: superoxide; $OONO^-$: peroxynitrite.

The results of the first study of the present thesis, supports the arguments that the microvascular is more susceptible to ED [25,27,204]. We demonstrated that ACh stimulated changes in microvascular perfusion was reduced by 43% following 30-minutes of hypoxia, whereby large-vessel FMD changes were only reduced by 18%, but remined significant. Nonetheless, some authors have reported reductions of 45% in large-vessel FMD [49] and others reporting lower reductions in microvascular reactivity of 31% [205]. However, it remains difficult to make direct comparisons between studies as there are several methodological considerations such as differences in assessment techniques (laser Doppler *vs*. ultrasound) and endothelial stimuli

(pharmacological *vs*. shear stress). To our knowledge, this is the first study to assess microvascular and large-vessel EF side by side, in response to acute hypoxic exposure. Our findings demonstrate that the heterogenous endothelium responds differently to hypoxia and highlights the importance of conducting a comprehensive assessment of EF in experimental studies.

4.2.1 THE PROTECTIVE BENEFITS OF CARDIORESPIRATORY FITNESS AGAINST HYPOXIA-INDUCED ENDOTHELIAL DYSFUNCTION

Physical exercise and cardiorespiratory fitness status have been shown to be associated with superior EF [120,211,212]. However, in the absence of any exercise intervention, the relationship between cardiorespiratory fitness and EF does not exist in healthy individuals with no developed risk factors for ED [123,124]. These findings suggest that greater fitness levels possibly protect the endothelium from the cytotoxicity that's associated with the risk factors of ED. Having a high fitness status is the result of long-term exercise training. Moreover, long-term exercise training has been proposed to reduce the production of ROS/RNS [285] and also enhance antioxidant defences [286]. Additionally, physical exercise stimulates increases in shear stress that is believed to increase NO bioavailability that occurs due to upregulation of eNOS expression [287]. A comprehensive review by Maiorana *et al*. provide a detailed description of the specific event that occur in response to exercise training in populations at risk of ED [288].

Cardiorespiratory fitness can be considered as a barrier of defence against ED for populations with an elevated risk. This is true for both small and large blood vessels [122,289]. In the present thesis, it was demonstrated that cardiorespiratory fitness may also protect against

hypoxia-induced reductions in EF in individuals who presented no risk factors for CVD or ED. However, this relationship only existed between the decline in microvascular EF and cardiorespiratory fitness. The microcirculation is proposed to be more sensitive to inflammatory and oxidative stress as they exhibit more adhesion molecules than larger blood vessels [26,27]. Therefore, the anti-oxidative benefits of long-term exercise may provide greater protection for microvascular endothelial cells against hypoxia, as they are more suspectable to oxidative stress-induced dysfunction compared to larger vessel.

4.3 Dietary Nitrate and Endothelial Function

Endothelial dysfunction is recognised as a significant marker for CVD and is considered to be a vascular disorder that is closely associated with the development and progression of atherosclerosis. Although a healthy endothelium maintains vascular homeostasis by regulating coagulation and inflammation, it is primarily responsible for regulating vascular tone. When the endothelium is unable to maintain a balance between endothelium-derived vasodilators and vasoconstrictors, regulation of vascular tone is impaired, and the endothelium is considered to be dysfunctional. Although there are several endothelium-derived vasoactive factors, ED is primarily characterised by a significant reduction in NO bioavailability.

The vasodilatory properties of NO was first discovered in 1980 by Furchgott and Zawadzki [237] and was initially recognised as the endothelium-derived relaxing factor before being identified as NO in 1987 [290]. In addition to the role of vascular tone regulation, the free radical also has several other physiological functions including reducing oxidative stress and inflammation status [148]. Endogenously generated NO is produced through the oxidation of

L-arginine in endothelial cells, this reaction is catalysed by eNOS and is termed 'The L-arginine Pathway' [200]. However, more recent research has discovered that under certain physiological conditions, circulating NO_2^- is also able to be converted into NO. Consumption of dietary NO_3^- or NO_2^- is known to be a significant method of upregulating circulating NO_2^- levels. There is an overwhelming amount of evidence to suggest that dietary NO_3^- is an exogenous NO source that can counter the decline in endothelium-derived NO and has also been shown to improve EF in the absence of any signs ED.

In the second study of this present thesis (Chapter 3), we were able to demonstrate that 4-weeks of dietary NO_3^- supplementation increased circulating levels of NO_3^-, accompanied by concomitant improvements in large-vessel EF and systemic BP. However, in spite of these promising findings, we failed to detect any significant improvements in microvascular function (endothelium-dependent or endothelium-independent). This null finding can be explained by many reasons. The NNN pathway is dependent on numerous enzymatic reactions, with the first conversion occurring in the enterosalivary circuit. Although the present study confirmed the presence of NO_3^- reducing bacteria, Siervo *et al*. proposed that modifications which occur to the aging oral microbiome might diminish the effectiveness of dietary NO_3^- [61]. Therefore, the changes in the ageing oral microbiome could suggest that increases in circulating NO_2^-, might be lower than that observes in younger populations. The failure to measure circulating levels of NO_2^- is a limitation of second study (Chapter 3). Consequently, it is difficult to support the argument that the modifications to the ageing oral microbiome attenuated increase in NO bioavailability, and thus prevent microvascular improvements.

Another enzymatic reaction that occurs in the NNN pathway, is the reaction that converts circulating NO_2^- into NO. Nitrite is converted into NO *via* five possible pathways; deoxyhaemoglobin [165], deoxymyoglobin [166], xanthine oxidase [167], acidification [168] and mitochondrial enzymes/coenzymes [169] (Figure 1. 3). Four of these pathways are enzyme-dependent, however acidification of NO_2^- occurs independent of enzymatic reactions. During the FMD procedure, the brief period of occlusion causes tissues to become ischemic. The ischemic environment triggers the release of hydrogen ions, and produces a decrease in cell pH [168,291,292]. In contrast, the technique used to assess microvascular function assessment in study 2 (Chapter 3) did not stimulate the release of hydrogen ions and thus it is possible that the conversion on NO_2^- to NO was not stimulated in the microvasculature. Furthermore, there is evidence to suggest that the cutaneous microcirculation is largely NO independent and is rather to be mediated by cutaneous sensory-nerve and calcium activated potassium channels [279]. Despite no improvements in microvascular EF in study 2, we did observe significant reductions in BP, and thus it is possible that improvements in microvascular EF did occur on some level. The microvasculature is considered to be the primary regulator of systemic as vascular resistance, and therefore is pivotal in maintaining normal systemic BP [254].

4.4 Discrepancies Between Microvascular and Large Blood Vessel Endothelial Function

The majority of the research demonstrating dietary NO_3^--dependent increases in NO bioavailability are associated with concomitant improvements in EF [178–181,184,293]. However, in spite of the favourable outcomes that many authors report, most studies have only assessed large-vessel EF, with the microvasculature being somewhat neglected. Interestingly,

the development of microvasculature ED precedes large-vessel ED [25], thus microvascular endothelial research should be the priority for therapeutic studies. Moreover, the microcirculation comprises a large surface area of the circulatory system and is more susceptible to endothelial damage due to the high abundance of vascular adhesion molecules [26,27].

Significant correlations between peripheral microvascular function and coronary vessel function has been reported by multiple studies [32–36], however this relationship is not replicated between large-vessels and the coronary circulation [294]. Collectively, these findings indicate that microvascular ED is stronger predictor of future cardiac events [22,23]. Interestingly, some authors make the argument that it is not necessary to assess microvascular and large-vessel EF independently due to the significant associations between small and large blood vessel EF in healthy individuals [295,296]. Nonetheless, this relationship is not evident in clinical populations with an elevated risk of ED [21], an thus a comprehensive assessment of EF should always be considered, especially in individuals at risk of CVD.

Other than the second study of the present thesis, only two other studies have assessed microvascular and large-vessel EF in response to NO_3^- supplementation [178,187]. However, the findings of these studies are inconclusive, with some reporting improvement in both vascular beds and other reporting no improvements in neither. The studies which reported no improvements in EF recruited individuals with diabetes and hypertension; however, Gilchrist *et al*. did not account for any medication that patients were taking for their conditions [187]. It is possible that patients may be taking pharmaceutical vasoactive substances during the dietary NO_3^- intervention, and therefore making any additional improvements difficult to detect. In

contrast, Broxterman *et al*. recruited hypertensive patients who were refrained from taking any vasoactive substances, responded well to three days of dietary NO_3^- supplementation [178]. They reported that the microvascular and large-vessel EF was significantly better following three days of dietary NO_3^- supplements in comparison to a placebo group. Furthermore, they reported that the change in large-vessel EF during the trials were correlated with the change in circulating NO_3^- levels, suggesting that the improvements were directly associated with dietary NO_3^--dependent increases in NO bioavailability.

In conclusion, it appears that the current evidence suggests a strong correlation between dietary NO_3^- and large-vessel EF, primarily when assessed using the FMD procedure. The extent of the improvements is highly dependent on numerous factors such as the studied population, source of NO_3^-, NO_3^- concentration and intervention duration. In addition to improvements in EF, studies frequently report concomitant reductions in BP, specifically in individuals who display signs of hypertension [178,179,181]. By increasing NO bioavailability and restoring the ability of blood vessels to vasodilate, the vascular resistance within the vessels decreases, which ultimately leads to reductions in BP [297]. Despite the small number of studies that have examined the direct effect of dietary NO_3^- on microvascular EF, the favourable reductions in BP can be considered as an indirect marker of microvascular EF improvements, due to the microcirculation being the primary regulator of vascular resistance [254]. Nonetheless the lack of consistency between methods of assessing microvascular EF and the discrepancies between the findings warrants further research in this field.

4.4 Overall Interpretation and Implications

As stated in the general introduction, the endothelium is a heterogenous organ that differs in structure and in function between vascular beds [1]. Both experimental chapters of this present thesis highlights the importance of performing a comprehensive assessment of EF. The findings suggest that the microvascular and large-vessel endothelium does not respond to harmful stimuli or therapeutic treatment in a similar manner. Firstly, we identified that hypoxia can reduce microvascular and large-vessel EF in healthy young adults. The diminished ability to vasodilate was approximately two-folds greater in microvascular beds in comparison to the larger-vessels. Due to the higher abundance of adhesion molecules on the surface of microvascular endothelial cells, it is possible that hypoxia-induced inflammation and oxidative stress is more damaging for these cells in comparison to large blood vessels [26,27].

Although the hypoxic exposure was simulated in the first study, systemic hypoxia is a concern for certain clinical population whom are also at risk of ED. Patients with CVD, diabetes and respiratory complication are all conditions that are associated with hypoxia [64,298,299]. The existing risk of ED for the aforementioned patient groups may be significantly elevated as a result of heightened inflammation and oxidative stress caused by hypoxia. In addition to clinical populations, ageing is also characterised increase risk of hypoxia and ED [300] [239]. With some evidence suggesting that older adults are at risk of age- and hypoxia-induced ED, it was in the interest of our second study to trial a dietary intervention to minimise this risk. Previous studies have already demonstrated that acute dietary NO_3^- improves age or hypoxia-induced ED, independently [184,301]. The findings of our second study, also suggests that chronic dietary NO_3^- also improves large-vessel endothelium-dependent vasodilation for older adults. However, dietary NO_3^- dependent increases in NO bioavailability did not significantly change microvascular EF.

Overall, the present thesis has demonstrated that a hypoxic stimulus can have catastrophic effects on EF, which are exacerbated in the microvasculature. For individuals who present risk factors of ED, and are at risk of being hypoxic, microvascular endothelial complications could precede and be greater than impairments large-vessel complications [255]. Therefore, it is important to perform a comprehensive assessment of EF when evaluating potential therapeutic treatments to prevent or restore ED. Although the discoveries of our second study contradicted our hypothesis, the findings may prompt further research to examine the effect of long-term dietary NO_3^- supplementation on microvascular and large-vessel EF in individual at risk of ED, whilst addressing the limitations and considerations that were acknowledged.

4.5 Limitations and Methodological Considerations

In the present thesis, both study 1 and 2 used different stimuli to assess microvascular and large-vessel EF, independently. To assess microvascular endothelium-dependent and endothelium-independent function, LDI was used to measure cutaneous perfusion changes in response to pharmacological stimuli (ACh and SNP, respectively). However, there is some research to suggest that cutaneous vascular tone is not predominantly regulated by the NO vasodilatory system [279]. Coadministration of a NO inhibitor has been shown to reduce ACh mediated vasodilatation by only 30-40% [302]. Alternatively, the FMD assessment uses a physiological stimulus (shear stress) to stimulate NO production, and during coadministration of a NO inhibitor, the vasodilatory response is completely eradicated [303]. Interestingly, Takase *et al*. reported a significant correlation between microvascular and large-vessels EF when ACh was used to stimulate vasodilation [304]. However, Anderson *et al*. also reported a significant relationship between microvascular and large-vessel EF whilst using different

stimuli (pharmacological and physiological, respectively) [203]. The different stimuli used for the assessment of microvascular and large-vessel EF in study 1 and study 2 makes it difficult to compare the extent of decline or improvement following hypoxic exposure or a dietary NO_3^- intervention.

4.6 Future Directions

In Chapter 2, it was identified that hypoxia significantly impaired the ability of the endothelium to regulate vascular tone. According to the available evidence, it was suggested that the decline in EF was primarily caused by the upregulation of inflammatory cells and ROS/RNS. However, in study 1 we failed to measure any biomarkers of inflammation or oxidative stress. To better understand the physiological mechanisms that causes the endothelium to become dysfunctional during hypoxia, analysis of the aforementioned biomarkers is warranted. A laboratory-based study with healthy participants would allow researchers to examine how hypoxia-induced ED is mediated by the prevalence of inflammation and oxidative stress, such as C-reactive protein, tumor necrosis factor-α, interleukin 6 and ROS. Additionally, future research should examine the relationship between the duration and/or intensity of hypoxia and inflammatory and oxidative stress mediated ED. Previous research by Lewis *et al*. indicates that severe hypoxia impairs EF greater the milder intensities, however, this group also failed to measure any biomarkers of inflammation or oxidative stress [49].

The relationship between cardiorespiratory fitness and hypoxia-induced microvascular endothelial decline reported in study 1, provides only some evidence to suggest that fitness protects the endothelium during hypoxia. To better understand how physical exercise may

prevent such substantial reductions in NO bioavailability during hypoxic exposure, an intervention study is a better method to evaluate the direct impact of the preventative potential of physical exercise [305]. In order to evaluate if regular physical exercise can minimise the hypoxia-induced decline in EF, future research should monitor cardiorespiratory fitness changes in response to and exercise intervention and whether this relates to changes in EF.

Although there is an overwhelming amount of research demonstrating the favourable effects of dietary NO_3^- on EF, the long-term effects are yet to be discovered. Once considered to be carcinogenic [151,152], dietary NO_3^- is no longer regarded to be a health risk and thus it is safe and feasible to investigate the potential benefits of chronic supplementation. To date, the longest dietary NO_3^- supplementation intervention is 6-weeks [180]. The findings of this study revealed that long-term NO_3^- supplementation can improve EF, reduce arterial stiffness and lower BP for individuals with hypercholesterolaemia. Further research is warranted to investigate the potential of longer intervention trials, especially for population groups considered to have an elevated risk of ED. Additionally, future studies should acknowledge the methodological consideration that have been reported previously in the present thesis.

4.7 Conclusion

The present work highlights the importance of assessing EF in multiple vascular beds when investigating endothelial response to harmful stimuli or therapeutics. The major conclusion that can be drawn from Chapter 2 is that acute hypoxia can significantly diminish NO bioavailability in healthy young adults. Consequently, the ability of endothelium to regulate vascular tone is reduce and therefore endothelium-dependent vasodilation is impaired. The two-fold decrease in microvascular EF was substantially greater than the observed reduction

in large-vessel EF, emphasising the importance of tailoring therapeutic interventions towards preserving or restoring microvascular EF.

The major conclusion from Chapter 3 is that dietary NO_3^- supplementation, in the form of BRJ, can significantly improve large-vessel EF in healthy older adults with potential age-mediated ED. As with many previous studies, the improvements were associated with concomitant increases in circulating NO_3^- and reduction in BP. The failure to detect improvement is microvascular EF, warrants further research to investigate alternative techniques that more accurately reflects the endothelial NO vasodilatory system. Furthermore, future studies are needed to confirm whether the benefit translate to long-term benefits for CV outcomes.

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