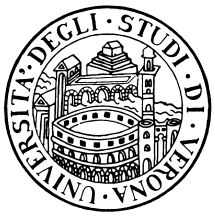


UNIVERSITY OF VERONA

DEPARTMENT OF

Neurosciences Biomedicine and Movement Sciences

PhD

Life and Health Sciences

PhD in

Neuroscience, Psychology and Psychiatry

XXX cycle - 2014 -2017

PhD Thesis title

Effects of Nitrate supplementation during cycling and walking in old and young healthy men

S.S.D. BIO/09 - Physiology

Coordinator: Prof. Leonardo Chelazzi

Supervisor: Prof. Enrico Tam

PhD Candidate: Dott. Luca Dal Sacco

ESEMPIO:

Effects of Nitrate supplementation during cycling and walking in old and young healthy men

Luca Dal Sacco
Tesi di Dottorato
Verona, 15 gennaio 2018

INDEX

ABSTRACT

Effects of Nitrate supplementation during cycling and walking in old and young healthy men

The general objective of the thesis is to investigate the effects induced by nitrates (NO_3^-) supplementation during muscular exercise at moderate and severe intensity, in elderly (60-75 years) and young (20-35 years) people. The NO_3^- contribution, equal to 8.0 mM dissolved in 0.25 L of solution, was provided by means of beetroot juice and continued for a period of 8 days. We evaluated the responses of oxidative metabolism, taking into consideration both central factors as well as peripheral factors. The peculiar and most original aspect of the study is aimed at understanding the effects of NO_3^- supplementation in the elderly. The interest was also that of understanding the mechanisms involved in oxidative metabolic regulation NO3-mediated, in the elderly.

The aging process is associated with functional and structural changes, especially in the cardiovascular and muscular systems, leading to alterations in oxidative metabolism. The marked impairment of the transport of O_2 and its peripheral use, together with the progressive loss of strength and muscle mass, are responsible for the significant and progressive worsening of $\dot{V}O_2$ kinetics that characterizes the elderly.

Also, cardiovascular responses following a nitrate supplementation have been investigated. Indeed, literature remarks that, nitrates are involved both in the circulatory (endothelial) and metabolic (mitochondrial) levels. The interest also was to analyze whether nitrates in the two populations (old and young) have a significant effect on the main hemodynamic components. We intent also to investigate the effects at metabolic level induced by nitrate supplementation during exercise.

Moreover, the metabolic cost of walking per unit of distance travelled is greater in older people than in young adults even when they are healthy and free from gait impairment. Other data indicate that the increase in the cost of the metabolic cost of the walk occurs around the 7th decade of life that coincides approximately with the time in which the changes occur in different biomechanical and bioenergetical factors of walking.

The study was developed in order to describe the trend of energy cost of locomotion on a treadmill at different intensities administered by varying the speed and slope of the instrument.

The main end point is the verification of the efficacy of BR supplementation in improving muscle efficiency in various type of locomotion. In particular, we intend to check if NO_3^-:

i) are able to reduce the energy cost of exercise in elderly subjects in the moderate and severe intensity domain;

ii) are able to improve muscle efficiency in relation to a greater availability of oxygen and its use at the peripheral level.

In short, the aim of the studies was to evaluate, through an integrative approach, the effects of nitrate supplementation on skeletal muscle oxidative metabolism during exercise.

Conclusions:

Main results of these studies suggest that supplementation on nitrate leads to an improvement in the efficiency in the elderly during low-intensity exercise and confirms the improvement in the young during high-intensity exercise.

However, these improvements are probably not due to peripheral improvements in oxygen transport and extraction, so they must be sought elsewhere, for example at the cellular level.

SECTION ONE

Nitric Oxide, Nitrates and Nitrites.

Summary of the section

In this section the NO and its numerous functions are introduced. Moreover, are presented pathway for NO production inside the body and the effects of aging. We expect that NO supplementation has more effects on elderly people due to the more vascular impairment than the young

Finally, general view of the thesis is reported.

1. Introduction

1.1 *Nitric Oxide (NO), Nitrates (NO_3^-) and Nitrites (NO_2^-)*

Nitric oxide (NO) is one of the simplest biological molecule in nature, in terms of cellular signaling device in the modulation of multiple physiological and pathological processes. The discovery of the nitric oxide (NO) pathway in the 1980s represented a critical advance in the understanding of cell signaling and subsequently into major new advancements in many clinical areas including, but not limited to cardiovascular medicine. This seminal finding was viewed as so fundamentally important that the Nobel Prize in Physiology or Medicine was awarded to its discoverers, Drs. Louis J. Ignarro, Robert Furchgott, and Ferid Murad in 1998, 11 years after NO was identified(Bryan and Loscalzo 2011). The Swedish Nobel Assembly sagely noted, "The signal transmission by a gas that is produced by one cell, penetrates through membranes and regulates the function of another cell, represents an entirely new principle for signaling in biological systems." More than a decade after the Nobel Prize was awarded for the discovery of NO (Bryan and Loscalzo 2011).

NO actions are mainly expressed in the vasodilation processes as an endogenous regulator of blood flow, protection from ischemic damage, inhibition of platelet aggregation, nerve transmission, regulation of glucose and calcium homeostasis, muscle contractility (excitation-contraction coupling), breathing mitochondrial and immune and inflammatory response (Clerc et al. 2007; Stamler and Meissner 2001). The half-life of NO in blood is thought to be very short mainly due to rapid inactivation after reaction with hemoglobin (Jon O. Lundberg and Govoni 2004) and the continuous generation of NO is essential for the integrity of the cardiovascular system, and decreased production and/or bioavailability of NO is central to the development of many cardio- vascular disorders (Bryan and Loscalzo 2011), then is necessary to supply produce NO and there are two pathways to produce NO, one of these is NO synthase (NOS) dependent, (L-arginine – NOS pathway), while the other one is NOS independent, (NO_3^- - NO_2^- - NO pathway).

1.2 *L-arginine pathway*

The main source of endogenous NO in mammals is the L-arginine-NO pathway, which is constitutively active in numerous cell types throughout the body (Lundberg,

Weitzberg, and Gladwin 2008). NO is produced endogenously by oxidation of the nitrogen guanidine of L-arginine, by a reaction catalyzed by the NOS similar to cytochrome P450, which require molecular oxygen (O_2) as an electron acceptor, and NADPH (Nicotinamide Adenin Dinucleotide Reduced) and FAD (Flavin Adenosine Dinucleotide) as cofactors (Moncada and Higgs 1993).

There are three distinct NOS isoforms in humans, expressing themselves as latent enzyme, respectively neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), of which the first depend on high concentrations of calcium ions (Ca_2^+). The fact that NOS involve multiple tissue are indicative of the pluripotency of the effects of NO in the physiological field. The gas in question is a free radical whose half-life in the order of milliseconds. and whose diffusion gradient are limited by subsequent oxidation reactions in NO_2^- and NO_3^-, which involve hemoglobin, myoglobin and other superoxide radicals. The stabilization of NO in blood and tissues depends on the L-arginine-NOS pathway as the predominant source of such nitrogenous anions that can be considered as endocrine molecules that can be converted into NO under certain physiological and pathological conditions (Lundberg et al. 2011). Nitrate is the predominant product of final oxidation of NO, whose levels in the blood exceed those of nitrite of at least two orders of magnitude (20-40 μM vs 50-300 nM) (Moncada and Higgs 1993), possessing a half-life in the circle of 5-8h compared to 110s of the other which is rapidly oxidized. It is formed by the reaction of NO with oxyhemoglobin (HbO) which gives NO_3^- and methemoglobin (Doherty et al. 1998; Gladwin et al. 2000). Nitrite, indeed, derives directly from the reaction of two NO molecules with O_2, catalyzed by the plasma enzyme ceruplasmain (Shiva et al. 2006).

1.3 *NO_3^- – NO_2^- – NO pathway*

Nitrates (NO_3^-) and nitrites (NO_2^-) are known predominantly as unwanted residues of the food chain as to potential toxic and carcinogenic effects, or as inert terminal oxidative products resulting from endogenous NO synthesis (Tannenbaum and Correa 1985; Mensinga, Speijers, and Meulenbelt 2003; Lundberg, Weitzberg, and Gladwin 2008). However, more recent studies show that such inorganic molecules are physiologically recycled into the blood and tissues to produce NO and other bioactive nitrogen oxides, by successive reductions (Lundberg et al. 1994; Zweier et al. 1995; Cosby et al. 2003; Lundberg, Weitzberg, and Gladwin 2008). Nitrates and nitrite are,

indeed, substrates of an alternative biochemical pathway of NO production complementary to that of L-arginine-NOS, the NO_3^- – NO_2^- – NO pathway, which improves considerably in physiological or pathological conditions of hypoxia and acidosis, when the action of NOS, oxygen-dependent, is compromised (Giraldez et al. 1997; Østergaard et al. 2007).

The exogenous nitrates deriving from the diet are rapidly absorbed in the bloodstream of the upper gastro-intestinal tract, where they mix with the endogenous nitrates produced by oxidation of the NOS dependent NO, reaching peaks of NO_3^- and of NO_2^- plasma respectively after 1-2h and 2-3h from ingestion, and then return to baseline values after 24h (Webb et al. 2008). After a meal rich in foods containing nitrates (Figure 1 – (Lidder and Webb 2013) the plasma concentration of such anions increases significantly and remains elevated for a prolonged period of time (half-life of NO_3^- in the plasma of 5-6 hours), distributing uniformly in all tissues (Lundberg et al. 2004).

Vegetables	Nitrate content Mean [range] (mg kg^{-1})	Nitrate content mean [range] [mmol per UK portion (80 g)]	Approximate nitrate content per UK portion (80 g) 1 nitrate unit = 1 mmol (62 mg)
High	**1890** [1213–2650]	**2.44** [1.57–3.42]	
Rocket	2597 [2597]	3.35 [3.35–3.35]	
Spinach	2137 [965–4259]	2.76 [1.24–5.50]	
Lettuce	1893 [970–2782]	2.44 [1.26–3.60]	**2**
Radish	1868 [1060–2600]	2.41 [1.37–3.35]	
Beetroot	1459 [644–1800]	1.88 [0.84–2.32]	
Chinese cabbage	1388 [1040–1859]	1.79 [1.34–2.40]	
Medium	**316** [168–518]	**0.41** [0.22–0.67]	
Turnip	624 [307–908]	0.80 [0.40–1.18]	
Cabbage	513 [333–725]	0.66 [0.44–0.94]	
Green beans	496 [449–585]	0.64 [0.58–0.76]	
Leek	398 [56–841]	0.51 [0.06–1.08]	
Spring onion	353 [145–477]	0.46 [0.19–0.61]	**1/2**
Cucumber	240 [151–384]	0.31 [0.19–0.50]	
Carrot	222 [121–316]	0.29 [0.16–0.40]	
Potato	220 [81–713]	0.28 [0.10–0.92]	
Garlic	183 [34–455]	0.24 [0.05–0.58]	
Sweet pepper	117 [93–140]	0.15 [0.11–0.18]	
Green pepper	111 [76–159]	0.14 [0.10–0.21]	
Low	**78** [25–203]	**0.10** [0.03–0.27]	
Onion	87 [23–235]	0.11 [0.03–0.31]	**1/10**
Tomato	69 [27–170]	0.09 [0.03–0.23]	
Water	(mg l^{-1})	(250 ml glass)	(250 ml glass)
Tap	**26** [22.8–30.3]	**0.10** [0.09–0.12]	**1/10**
Mineral	**2.6** [<0.1–6.3]	**0.01** [<0.0004–0.025]	**1/100**

Figure 1
The Nitrate 'Veg-Table': vegetables, ranked from highest to lowest according to mean nitrate content [range] expressed in mg kg-1, mmol per UK portion (80 g) and as a guide as the approximate number of nitrate units per portion (1 nitrate unit = 1 mmol) to facilitate estimation of nitrate intake or to modify intake as desired. Also included is tap water and bottled water for comparison. (Lidder and Webb 2012)

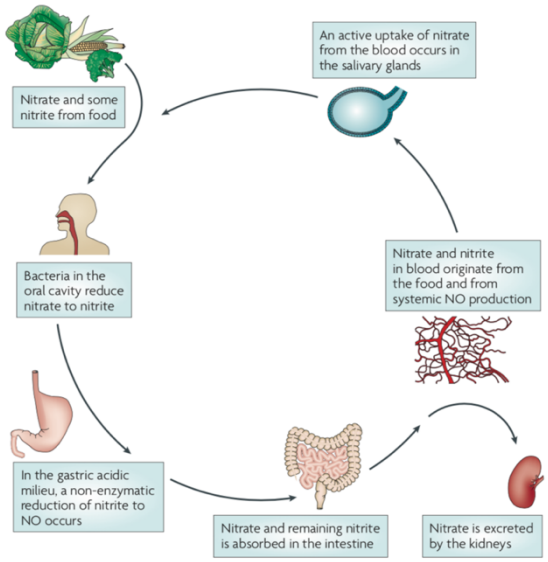


Figure 2
The entero-salivary circulation of nitrate in humans. Ingested inorganic nitrate from dietary sources is rapidly absorbed in the small intestine. Although much of the circulating nitrate is eventually excreted in the urine, up to 25% is actively extracted by the salivary glands and concentrated in saliva. In the mouth, commensal facultative anaerobic bacteria effectively reduce nitrate to nitrite by the action of nitrate reductase enzymes. Nitrate reduction to nitrite requires the presence of these bacteria, as mammalian cells cannot effectively metabolize this anion. In the acidic stomach, nitrite is spontaneously decomposed to form nitric oxide (NO) and other bioactive nitrogen oxides, which regulate important physiological functions. Nitrate and remaining nitrite is absorbed from the intestine into the circulation and can convert to bioactive NO in blood and tissues under physiological hypoxia. (Lundberg et al. 2008)

Although most of the molecules in the bloodstream are excreted in the urine, more than 25% enter the salivary glands, through the entero-salivary circle, from which it is concentrated in the saliva 10-20 times higher than the plasma values.

Once the oral cavity has been reached, the optional anaerobic commensal bacteria, present mainly in the crypts of the tongue, use the nitrates as acceptors of electrons alternative to the oxygen they need for breathing, thus reducing them to nitrites, following the action of enzymes nitrate reductase (Duncan et al. 1995; Lundberg et al. 2004).

When the NO_2^- salivary come into contact with the acidity of the gastric environment (pH = 1.5-3) and with abundant reducing agents in the diet (vitamin C, thiocyanate, polyphenols), they are rapidly deprotonated to nitric acid (HNO_2; pKa ~ 3.3) [1] which spontaneously decomposes into NO and other reactive nitrogen oxides (Lundberg et al. 1994)[2] (Figure 2):

$$NO_3^- + H + \leftrightarrow HNO_2 \qquad [1]$$

$$2HNO_2 \rightarrow N_2O_3 + H_2O$$
$$N_2O_3 \rightarrow NO_2 + NO \qquad [2]$$

The antibacterial effects of NO suggest a role of gas in the protection against pathogens, but also in the regulation of gastric mucosa perfusion and mucus production (Lundberg et al., 1994).

At systemic level, the reduction of NO_2 to NO can occur in various pathways (Figure 3 – Lundberg et al. 2011) that involve hemoglobin (Cosby et al. 2003; Nagababu et al. 2003) myoglobin(Shiva et al. 2007; Rassaf et al. 2007), xanthine oxidoreductase (Zhang et al. 1997; Godber et al. 2000)., ascorbate (Carlsson et al. 2001), polyphenols (Peri et al. 2005; Gago et al. 2007), and proton (Benjamin et al. 1994; Lundberg et al. 1994).

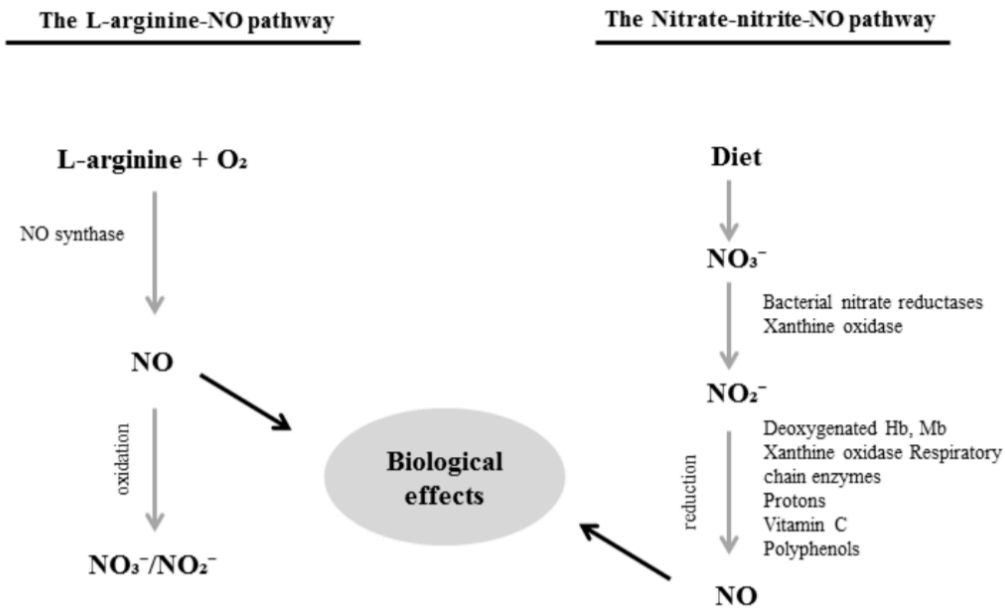


Figure 3:
Two parallel pathways for NO formation in mammals. NO synthases (NOS) catalyse the formation of NO from the substrates L-arginine and molecular oxygen. NO is rapidly oxidized to form nitrite (NO_2^-) and nitrate (NO_3^-), but a recycling of these anions may occur by which NO is formed again. Nitrate reduction to nitrite is mainly carried out by commensal bacteria in the oral cavity and to a lesser extent also by mammalian enzymes in tissues (xanthine oxidase). Once nitrite is formed, several pathways exist with the capacity to further metabolize nitrite to NO and other bio- logically active nitrogen oxides. Most of these pathways are greatly accelerated under hypoxic conditions. Thus, nitrite reduction represents an alternative to the classical NOS pathway for the generation of NO. Our diet (mainly green leafy vegetables) is a major contributor to the body pool of nitrate and ingestion of nitrate may fuel the nitrate–nitrite–NO pathway. Dietary nitrate supplementation is associated with robust NO-like effects including a reduction in blood pressure and inhibition of oxygen consumption in humans. In addition, nitrate or nitrite administration is protective in numerous animal models of cardiovascular disease. (Lundberg et al 2011)

1.4 *NO effects*

NO oversees various roles inside the body. NO is involved in the vasodilation processes as an endogenous regulator of blood flow, protection from ischemic damage, inhibition of platelet aggregation, nerve transmission, regulation of glucose and calcium homeostasis, muscle contractility (excitation-contraction coupling), breathing mitochondrial and immune and inflammatory response (Clerc et al. 2007; Stamler and Meissner 2001).

1.4.1 Vasodilation and blood flow regulation

The vasodilatory effects of NO_2^- supplementation have known since the 30s (Weiss, Wilkins, and Haynes 1937; Furchgott and Bhadrakom 1953; Ignarro et al. 1981) and nowadays numerous studies confirm the vasodilatory effects NO dependent, produced by low doses of NO_2^- following their reduction to NO. Vasodilation inducted by NO is dose dependent (Dejam, Hunter, and Gladwin 2007; Filip J. Larsen et al. 2006) and determines favorable effects on cardiovascular diseases associated with endothelial dysfunction and the reduction of NO bioactivity (arterial hypertension, atherosclerosis, stroke) (Giansante and Fiotti 2006; Plavnik et al. 2007)

Ferguson and colleagues (Ferguson et al. 2013) demonstrated, on a sample of rats, significant reductions in blood pressure and blood lactate concentration during exercise, following dietary supplementation of NO_3^-, as effects dependent on increase in blood flow (~ 38%) to the muscles in exercise, characterized by a high fraction of intermediate fibers (type IIa). The consequent greater contribution of O_2, more adequately distributed within the active tissues, involves a potential reduction of phosphorylation at the substrate level, a better metabolic control and an increase in efficiency, all of which are advantageous conditions for performance.

In a subsequent study, the same authors reported that the PO_2 at the microcirculatory level decreases less rapidly as a result of electrically evoked muscle contractions on rats fed by BR compared to those fed by water (Ferguson et al. 2015). This is consistent with a greater contribution of O_2 during the metabolic transition to exercise, then to the saving of intramuscular phosphocreatine (PCr) and to the attenuation of the concentrations of adenosine diphosphate (ADP) and inorganic phosphate (Pi). These last aspects, in association with the absence of significant variations in muscle

pH between the two types of treatment, indicate that a greater "compensatory" contribution by anaerobic glycolysis to energy production has not been established, as might be the case if establishing a transitory oxygen debt dependent on an ineffective adaptation to local conditions by peripheral perfusion systems (Jones 2014).

The vasodilatory capacity of nitrites, closely coupled to the deoxygenation of hemoglobin (Hb), is associated with the synthesis of NO, whose concentrations increase with decreasing Hb saturation, therefore it depends on bioactivation mechanisms regulated by hypoxia (PO_2 = 20- 40mmHg, which corresponds to the P50 of the hemoglobin, saturated to 40-60%) (Lundberg et al., 2008).

NO_2^- react with deoxyhemoglobin ($HbFe^{+2}$) and a proton (H^+) generating NO and methemoglobin ($HbFe^{+3}$) [3]. NO can subsequently bind to a second deoxyhemoglobin molecule forming an iron-nitrosyl-hemoglobin ($HbFe^{+2}$-NO) [4]:

$$NO_2^- + HbFe^{+2} + H^+ \rightarrow NO + HbFe^{+3} + OH^- \quad [3]$$

$$NO + HbFe^{+2} \rightarrow HbFe^{+2}\text{-}NO \quad [4]$$

This simple reaction has important physiological implications as it has properties necessary for vasodilating effectively under hypoxia and tissue acidosis. In fact, it uses spontaneously nitrates as substrates, deoxyhemoglobin and protons to produce NO, the most powerful known vasodilator.

The maximum reduction rate of NO_2^- to NO occurs at PO_2 of 30 mmHg, at 50% of Hb saturation, due to its greater reactivity with oxyhemoglobin (R-state), even if the reaction takes place only with deoxygenated tetramer (T-state)

It should also be emphasized that the vasodilatory action of NO_3^- and NO_2^- inorganics must be distinguished from that exercised by organic anions (nitroglycerin and amyl-nitrite), since the latter undoubtedly have a greater potency in terms of vasodilatory and anti-anginal effects, but they depend, in the bioactivation of the NO, from the mitochondrial aldehyde dehydrogenase (mtALDH) and from other enzymes subject to inducible tolerance (Li et al. 2005) then to the reduction of the activity of the biological nitroglycerin following the chronic exposure to drugs. The NO_2^- may represent the active metabolite of nitroglycerine capable of passing the enzymatic metabolism subject to tolerance.

1.4.2 Blood pressure and cardiocirculatory factors

The effects of BR supplementation on cardiocirculatory factors have been studied by many researchers. The focus was mainly on the data of: systolic blood pressure (SYS), diastolic blood pressure (DIA), mean arterial pressure (MAP) total peripheral resistance (TPR) and cardiac output (CO).

A study (Bond et al. 2014)with acute supplementation of NO_3^-, by a single dose of 500 ml of beetroot juice (BR) containing ~750 mg of NO_3^- showed effects on cardiovascular response to exercise in the graduated cycle ergometer in overweight young women. BR reduced during all workloads the SYS, the Rate Pressure product (RPp = HR x SYS) index of myocardial oxygen demand and the TPR at rest. However, no effects on DIA, MAP, CO and HR were observed after BR.

Similar effects were found in another study (Lee et al. 2015) that showed a decrease of SYS, DIA, MAP, TRP both in the resting and in the exercise phase after BR supplementation (6.4 mmol/day). The authors highlight that NO_3^- helps to increase the release of oxygen and reduce cardiac work, allowing you to perform the exercises longer before the fatigue begins.

In a study by Liu and colleagues (Liu et al. 2013) arterial stiffness was analyzed following spinach ingestion. The researchers observed a greater arterial elasticity, lower pulse pressure and lower SYS. The spinach diet compared to placebo showed a reduction in ejection time, cardiac output, stroke volume and vascular impedance, while no changes were observed in DIA. The effects on DIA, however, are different in another study by Sobko in 2010 (Sobko et al. 2010) where DIA decreased of 4.5 mmHg.

Another study showed no significant differences before and after 7 days of rich NO_3^- vegetable diet (100/300 mg NO_3^-/day), in blood pressure, heart rate and arterial stiffness (Bondonno et al. 2015)

Therefore, the effects of NO_3^- are various and sometimes in contradiction between them, but it appears a common modification on vascular function.

1.4.3 Protection of tissues from ischaemia-reperfusion injury

Systemic production of NOS-independent NO was first demonstrated in cardiac ischaemia (Duncan et al. 1995), revealing the centrality of these substances in hypoxic signaling.

NO_2^- exert a powerful cytoprotective function from lesions resulting from prolonged ischemia and reperfusion in different organs, as their reduction to NO provides an alternative source of endogenous vasodilator in an environment in which conventional synthesis is compromised (Baker et al. 2007; Tripatara et al. 2007). These findings suggest NO_2^- as a therapeutic opportunity for diseases associated with hypoxic conditions, in particular myocardial infarction, stroke, organ transplantation, cardiopulmonary arrest and disturbances related to sickle cell red blood cells.

Mitochondrial generation of reactive oxygen species (ROS) is a component necessary for mitochondrial cytoprotection (Xu, Ji, and Boysen 2004), but contributes, at high concentrations, to damage, necrosis and cell apoptosis following reperfusion events following ischemia. The NO_2^- are able to nitrosilate the complex I of the electron transport chain in ischemia-reperfusion (Dahm, Moore, and Murphy 2006,Shiva et al. 2007) by inhibiting its activity and limiting the mitochondrial production of ROS, the activation of the permeable mitochondrial pores and the release of cytochrome c, with effects on mitochondria and detectable cytoprotective both in acute (immediately before reperfusion) and remotely (if taken 24 hours before reperfusion.

1.4.4 Mitochondrial efficiency

The reduction of $\dot{V}O_2$ during submaximal exercise, reported by several studies, following dietary supplementation of NO_3^- (Bailey et al. 2009; F. J. Larsen et al. 2011), is due to a greater efficiency of energy metabolism in the oxidation processes of the substrates connected to the synthesis of ATP.

Different possibilities of interaction between NO_3^-, NO_2^-, NO and mitochondria have been identified. The most documented effect of NO is its link with cytochrome C oxidase (COX), an electron acceptor of the transport chain, which, at physiological concentrations, partially inhibits mitochondrial respiration by competing with oxygen (Larsen et al., 2011). This bond, reversible and regulated by oxygen, induces a kinetic constraint that affects the reduction of $\dot{V}O_2$ for the synthesis of ATP with consequent

increase in phosphorylation efficiency, as well as being functional also to control ROS signaling and regulation of tissue oxygen gradients (Thomas 2001).

The effect of NO_2^- seems to be similar to that of NO because the electron transport chain proteins are able to reduce it to NO. Furthermore, nitrogen anions acting independently of the formation of NO in the regulation and expression of tissue proteins inhibit cytochrome C oxygenase, determining a condition that could be perceived by the cell as a mild hypoxia. It follows the triggering of signaling mechanisms that involve a down regulation of the nucleotide adenine translocase (ANT), a protein involved in mitochondrial proton conductance (Larsen et al., 2011) able to reduce the loss of H^+ through the mitochondrial membrane.

The increase in the P/O ratio (~ 19%), classically used to measure the amount of oxygen consumed per ATP molecule produced (Hinkle 2005), is indicative of the fact that a higher proportion of the electrochemical transmembrane potential is destined to at the synthesis of ATP thanks to the lower dissipation of it usually associated with the decoupling of the proton transients through the internal membrane (Larsen et al., 2011).

1.4.5 Contractile muscle properties

Much of the research on the physiology of the effects of BR supplementation has focused on changes in vascular function or energy metabolism (Jones 2014). A recent study has shown important effects also on the intrinsic contractile properties of skeletal muscle (Hernández et al. 2012) highlighting a major increase in contractile force at ≤50 Hz electrical stimulation. At 100 Hz stimulation, the rate of force development was ~35% faster in the NO_3^- treated mice. This effect is attributed to the improvement in the management of intracellular calcium transients, mediated by the NO which, synthesized by the action of nNOS, acts on the calcium channel receptors of the sarcoplasmic reticulum resulting in an increase in the expression of calsequestrin 1 and the receptor of the dihydropyridine. This conduct to an increase in the release of Ca^{2+} and to the ionic sensitivity of the acto-myosin bridges, in particular in the fast fibers (type II). This increase in intracellular [Ca^{2+}], in response to excitation, is probably more useful in situations where saturation is incomplete, for example, in the explosive/growing phase of contraction or at low stimulation frequencies, allowing an improvement in the production of force in these circumstances. The advantages of the

excitation-contraction coupling become more evident in the endurance exercise , characterized by the repeated activation of the muscle fibers (Jones 2014).

The reduction of total ATP expenditure, necessary to support the production of contractile strength, following the supplementation of NO_3^-, with the same muscle tension expressed despite a lower excitation rate, is also linked to the improvement of the Ca^{2+} re-uptake (Hernandez et al., 2012). The impact of this process on the total expenditure, normally equal to about 30-50% (depending on the type of fibers involved) of the ATP consumed by the muscle, is reduced in conditions of higher NO levels as the action of the gas on the ionic channels, after oxidation, protects from the release of excess Ca^{2+} (Haider et al., 2014). In this way, the best excitation-contraction coupling could contribute to reductions in oxygen consumption during sub-maximal exercise with supplementation (Bailey et al. 2010; Vanhatalo et al. 2010; Lansley, Winyard, Fulford, et al. 2011).

1.4.6 Reduction O_2 consumption and exercise tolerance

There are several scientific evidences that attest to the positive effects of NO_3 supplementation on physiological responses to exercise (Bailey et al., 2009; Bailey et al., 2010; Larsen et al. 2010; Vanhatalo et al., 2010; Lansley Winyard, Fulford et al., 2011 and several others).

The first of this group of study was published by Larsen et al in 2007 (Larsen et al. 2007). In this study after sodium nitrate ($NaNO_3$) supplementation (0.1 mmol/kg of body mass per day – 3 day of supplementation) was observed a reduction of $\dot{V}O_2$ (~ 5%) at sub-maximal intensity (45-80% $\dot{V}O_{2max}$), without any change in the concentration of blood lactate (BL), heart rate (HR), ventilation ($\dot{V}E$) and respiratory quotient (RR). Moreover, the study shows a significant increase in the plasma concentration of NO_2^- at rest (~82%) and a reduction in resting blood arterial pressure (BP – 8-6 mmHg). These significant improvements in gross efficiency (output per unit of consumed energy) and delta-efficiency (variation of mechanical output due to unitary variation of energy consumed) suggest a real effect on the efficiency of muscular oxidative metabolism due to an improvement in energy supply or a slower energy cost of the cardiopulmonary processes or a change in substrate use following supplementation (Larsen et al., 2011).

It is well established that the endurance performances depend on the $\dot{V}O_{2max}$ function, on the use of the $\dot{V}O_{2max}$ ($\%\dot{V}O_{2max}$) and on the efficiency of the exercise (Coyle 1995) and considering other factors remain unchanged, an improvement in muscle efficiency should allow greater mechanical output at the same energy cost and therefore result in improved performance. Recognizing the importance of such evidence, Bailey and colleagues (Bailey et al., 2009) examined the influence of NO_3 supplementation (5.6 mmol of NO_3^-/day in 0.5 l of beetroot juice for 6 days) on the kinetics of the $\dot{V}O_2$ during a running exercise on treadmill at moderate intensity (80% of GET) and at high intensity (70%Δ - 70% of the difference between GET and $\dot{V}O_{2max}$). Results of this study report a significant increase in plasma levels of NO_2^- (~ 95%), a reduction in systolic blood pressure (~ 8 mmHg), a reduction in $\dot{V}O_2$ at a steady state (~ 5%) in the exercise moderate intensity and a reduction in the amplitude of the slow component (~ 23%) during high intensity exercise. This last effect was considered as a reflection of the progressive loss of muscular efficiency (Jones, 2014). As for Larsen et al. (Larsen et al., 2007), there are no variations in the concentration of BL, in HR, in $\dot{V}E$ and in RR at the different intensities of exercise or in the $\dot{V}O_2$ peak reached in high-intensity exercise, even if this it is reached later showing a significant increase in the time of exhaustion (~ 16%). A subsequent study of Bailey et al., (Bailey et al., 2010) on knee extension exercise confirms that, compared to placebo, supplementation with beet juice leads to reduced $\dot{V}O_2$ at moderate intensity, but also reduces the amplitude of the slow component and the increase in the time of exhaustion (~25%) at high intensity. A second study by Larsen and colleagues (Larsen et al., 2011) reports, following dietary supplementation with $NaNO_3$ (0.1 mmol/kg body mass per day for 2 days), a significant reduction of $\dot{V}O_2$ (~2.7%) and a significant increase in the time of exhaustion (~7%), in incremental maximal exercise with use of both arms and legs. A similar result of $\dot{V}O_{2max}$ reduction following NO_3 supplementation is also reported by Bescos and colleagues (Bescos et al. 2011) although not a universally confirmed effect.

However, as the influence of the length of the supplementation period adopted by the various studies on the effectiveness of improving the efficiency of the exercise and on performance is not clear, Vanhatalo et al., (Vanhatalo et al., 2010) address the issue, highlighting a significant reduction of steady-state $\dot{V}O_2$ in exercise at moderate intensity (~4%) 2.5 h after the intake of NO_3^- (5.2 mmol per day in 0.5 L of juice for

15 days), that is the same after 5 and 15 days, and an efficiency improvement maintained for at least 2 weeks. There are no changes in the peak $\dot{V}O_2$, while the peak mechanical output is significantly increased at the GET within 15 days of supplementation. The study by Lansley, (Lansley et al. 2011) reports the effect of supplementation of NO_3^- (6.2 mmol in 0.5 L of juice per day for 6 days) in the reduction of $\dot{V}O_2$ in the moderate-intensity treadmill race, the increase in high-intensity exercise tolerance (~15%) and the increase in time of exhaustion in incremental knee extension exercise.

1.4.7 NO_3^- and effects on O_2 delivery

Near Infrared Spectroscopy (NIRS) in vivo has been used since 70s (Jöbsis et al. 1977) as a non-invasive method to assess the oxygen concentrations in tissues, mainly through monitoring the oxygenation and deoxygenation of hemoglobin, although this method cannot measure myoglobin and hemoglobin separately (Miura et al. 1998). In animal and human skeletal muscles during exercise, the changes in oxygenated hemoglobin content measured by NIRS bears a high correlation with the changes in venous hemoglobin oxygen saturation, and could be used to determine the oxygen kinetics in working muscles during exercise (Miura et al. 1998).

Three studies analyzed the effects of BR supplementation (Bailey et al 2009, Breese et al. 2013, 2017)on the O_2 extraction at muscle-level in different intensity domain. All of these studies have been performed on a sample of young subjects and the results are not all in agreement.

Bailey et al. (Bailey et al., 2009) found an improvement of O_2 extraction in the vastus lateralis muscle during exercise at moderate intensity with a lower amplitude of deoxygenated hemoglobin concentration ([HHb]) after BR, but were not observed any changes in kinetics parameters of deoxygenated hemoglobin, so no change in the time constant and the delay time. In high-intensity exercise, they did not observe any significant change.

In the first study by Breese and colleagues (Breese et al., 2013), unlike what Bailey founded, no improvement in extraction was observed, but a change in the [HHb] kinetics, which becomes more rapid after the BR supplementation, during a transition from an exercise to moderate intensity to a more severe one, suggested faster O_2 extraction.

In the most recent study by Breese and colleagues (Breese et al., 2017) the O_2 extraction was investigated in three different muscles: vastus lateralis, vastus medialis and rectus femoris. No changes in the parameters of [HHb] were observed at any muscle site, and in the amplitude at each site. The overall mean, combining three muscle sites, however, reported a [HHb] at the end of the exercise significantly higher after BR, suggesting a greater O_2 extraction spread in all the muscular districts.

1.4.8 NO_3^- effects on exercise

It is important to recognize that, although scientifically important, the time of exhaustion and incremental tests, on which the effects of nitrates were observed from the first studies (Bailey et al., 2009; Bailey et al., 2010; Larsen et al., 2010; Vanhatalo et al., 2010; Lansley et al., 2011a), are evidence related to exercise capacity rather than performance testing. Competitive sport typically requires athletes to complete a certain distance in the shortest possible time and, inherently to this aspect, the considerable size of the effect, in terms of time, in exhaustion tests, are not as extensive in trials over time. However, an improvement of 15% in the time of exhaustion resulting from a given intervention results in an improvement of 1% in the duration performance for which, even if apparently small, such an effect would be highly significant in terms of performance for elite athlete.

Recognizing the importance of assessing the influence of the use of food nitrates on sports performance, Lansley et al. (Lansley et al 2011) has tested acute supplementation of NO_3^- (6.2 mmol in 0.5 L of beet juice) 2.5 h before the tests, in high level cyclists (average $\dot{V}O_{2max}$ = 56 ml/kg/min) obtaining significant increases in the average mechanical power, with the same $\dot{V}O_2$, on the tests of the 4 and 16.1 km with performance improvements of 2.8% and 2.7% respectively. Cermak and colleagues (Cermak, Gibala, and Loon 2012) examined the influence of nitrates (8 mmol of a day for 6 days) on cycle performance tests repeated for 6 days. This shows, in trained cyclists (average $\dot{V}O_{2max}$ = 58 ml/kg/min) a reduction (~5%) of the $\dot{V}O_2$ at submaximal exercise intensity (2 x 30 min at 45 and 65% of peak power) and of the peak power, therefore of the performance over 10 km, without variations of RR, of the blood concentration of lactate, glucose and insulin.

Overall, studies suggest improvements in pedaling efficiency and performance time for events lasting at least 5-30 min, given by dietary supplementation, both acute

(Lansley et al., 2011) and chronic (Cermak et al., 2012), in trained cyclists (53-63 ml/kg/min). Regarding treadmill run performance in active adults, Murphy and colleagues (Murphy et al. 2012) reports the increase in speed (5%) of the last kilometers on the 5 km and the reduction of effort perception in 75 min following the ingestion of 200g of beets cooked (≥ 500 mg or ≥8 mmol of NO_3^-). Similar benefits are also identified by Bond et al, (Bond et al., 2012) on repeated rowing performance on the 500m with ergometer in well trained rowers, after supplementation with beet juice (0.5L per day for 6 days). More recently, the absence of ergogenic effects induced by the use of NO_3^- both in acute and short-term (Jones, 2014, Porcelli et al. 2015)in elite athletes has been demonstrated ($\dot{V}O_{2max}$ > 60 ml/kg/min). Wilkerson et al. (Wilkerson et al., 2012) does not observe any difference on 50 miles time trial in trained cyclists 2.5 hours after supplementation with beet juice (0.5 L) with a tendency to increase the ratio of power to $\dot{V}O_2$. An interesting aspect is given by the relatively low average increase in the plasma concentration of NO_2^- compared to what is found in the less trained subjects suggesting a different responsiveness to treatment with an inverse correlation between the increase in NO_2^- levels and best time in the test. In line with these results, Cermak and colleagues (Cermak et al., 2012) reported no effects of NO_3^- (8.7 mmol in beet juice) in increasing distance traveled in one hour by trained cyclists ($\dot{V}O_{2max}$ = 60 ml/kg/min), as well as Peacock and colleagues, (Peacock et al., 2012) observed no differences with supplementation (10 mmol of NO_3^-) 2.5 h before exercise, on $\dot{V}O_2$ reduction in low-intensity warm up. The absence of differences in the performance of trained cyclists and triathletes ($\dot{V}O_{2max}$ = 60 ml/min/kg) over 40 min, following supplementation (10 mg/kg or 0.16 mmol/kg of $NaNO_3$ per day for 3 days) are also reported in a study by Bescos and colleagues (Bescòs et al., 2012). As noted by Wilkerson., (Wilkerson et al. 2012), there are several reasons why both, fitness level of the subjects and the intensity of the exercise, can influence the effects of NO_3^- supplementation. It was hypothesized a greater activity of NOS (Jones, 2014) in the more trained subjects that would reduce the conversion of NO_3^- to NO by NO_3^- - NO_2^- - NO pathway, but also the presence of higher [NO_2^-] in blood compared to sedentary subjects, therefore the response to a NO_3^- standard dose of may be reduced. The increased capillarization of skeletal muscle minimizes the hypoperfusion of the metabolically active tissue, therefore the hypoxia and acidosis conditions reducing the NO_3^- requirement for the production of NO. For the same reason, low intensity aerobic

exercise, in which the skeletal muscle remains well oxygenated and pH no decreases significantly, no requires the synthesis of NO by NOS-independent pathway. Finally, recent evidences show that the integration of NO_3^- preferentially alters the contractile function of the type II fibers that are typically present in reduced percentages in endurance athletes for which the physiological response to the supplementation is modified (Jones, 2014). Until now, however, studies that showing negligible effects of NO_3^- in elite athletes have used acute supplements (2-3 pre-performance hours) (Cermak et al., 2012; Wilkerson et al., 2012) and in the short term (3 days) (Bescòs et al., 2012), while the researches that indicated the alteration of muscular contractility and mitochondrial enzymes refer to supplementations for longer periods (3-7 days) (Jones, 2014). This aspect increases the possibility that the use of long-term exogenous NO_3^- and/or higher doses has favorable effects on the performance of elite athletes, as suggested by the results of Cermak and colleagues (Cermak et al., 2012) and Vanhatalo and colleagues, (Vanhatalo et al., 2010).

1.4.9 NO_3^- effects on elderly subjects

Currently there is substantial evidence to support the positive effects of increased plasma NO_3^- levels following dietary supplementation, using both $NaNO_3$ and beet juice, can affect physiological responses to exercise (Bailey et al., 2010; Larsen et al., 2007). Most of the studies conducted so far refer to the adult active male population (more or less trained), but substantially excluding the elderly.

It should be considered that the bioavailability of L-arginine is limited in the elderly, as well as the plasma concentrations NO_2^-, marker sensitive to the activity of NOS. This suggests that the synthesis of NO dependent on the L-arginine-NOS pathway could be compromised with the aging process. Furthermore, an increase in the production of superoxides (O_2^-) occurs, predictably in relation to the reduction of the bioavailability of NO, due to the rapidity of reaction between (O_2^-) and NO (Kelly et al. 2013). Given the positive association between NO and vascular health (Ignarro et al. 1999), the perturbations of NO metabolism could contribute to endothelial dysfunction as well as to arterial hypertension that are established with age (Oelze et al. 2014). It is therefore plausible that the dietary supplementation of NO_3^- can improve the bioavailability of NO and with it the vascular function in elderly subjects.

The aging process is linked to a number of functional and structural modifications to the cardiovascular and muscular systems with alterations in the transport and use of O_2. There is evidence that the kinetics of $\dot{V}O_2$ related to the metabolic transition from a resting state to exercise is slowed compared to that of young adults and this may be related to a limited supply of muscle O_2 (DeLorey, Kowalchuk, and Paterson 2005). The reduction of the maximum capacity of oxidative phosphorylation of the elderly further affects the slowing down of the kinetic (Gouspillou et al. 2010). Since dietary supplementation of NO_3^- has shown increases in muscle flow and in the maximum speed of synthesis of ATP, it can be hypothesized that its use in the elderly can accelerate the oxidative metabolism response. This would reduce the metabolic perturbation and the onset of fatigue, thus increasing exercise tolerance (Larsen et al., 2011).

The increased bioavailability of NO could favor an improvement in cerebral blood flow and cognitive functions in old age. There is also a reduction in the brain synthesis capacity of ATP due to oxidative phosphorylation which, together with the chronic ischemia of white matter, leads to the decline of cognitive functions (Kelly et al., 2013).

2. Aging

Aging is an irreversible physiological process that leads to biological changes, that leading to degeneration of tissues, defined by the sum of all the physiological, genetic and molecular changes that accumulate during the years (Sieck 2017). The aging process, still under study, is an event characterized by stochasticity (Vijg and Suh 2013) and by malleability, because the response of cells and tissues is very variable between individuals (Gems, 2013). There are several theories that describe the potential activating mechanisms of this process (Free radical theory, Harman 1953-2003, DNA alteration theory, Vilenchik 1970, Oxidative stress theory, Sohal & Allen 1990), and despite the considerable uncertainties regarding to it, the main hypothesis that aging is characterized as a multifactorial and complex process is widely supported.

The modifications and the impairments produced in the organism lead to a reduction of exercise capacity and adaptation, as they affect the progressive decrease of the functional reserve, enhanced or accelerated by the concomitance with a sedentary or insufficiently active lifestyle (Hardman and Stensel 2013).

These considerations constitute one of the conditions that underlie the present study, aimed at verifying the hypothesis of any positive effects induced by NO_3^- dietary supplementation in elderly subjects. In particular, reference is made to the potential attenuation or modification, even if limited, of the typical aging impairments, shown below, on the practice of physical exercise, the main way of maintaining the efficiency and health of the organism despite the advance of age.

2.1 *Effects of aging*

With age, there is a structural and functional deterioration in most of the physiological systems, even in the absence of perceivable disturbances or overt pathologies. These physiological changes affect a vast range of tissues, organs and functions, the accumulation of which affects the activities of daily life and the maintenance of autonomy, and consequently the quality of life (ACSM 2009).

It is important to present an overview of these processes in order to clarify the causes that influence metabolic responses to exercise in the elderly population.

2.1.1 Muscle function

Aging is characterized by changes in the skeletal muscle that determine the decline of its ability to generate strength, power and resistance, as effects mainly dependent on the progressive and generalized loss of muscle mass in terms of reduction of the volume of individual fibers and their number (Granacher, Zahner, and Gollhofer 2008). This condition, named sarcopenia, is considered by the scientific community as a "geriatric syndrome", which may occur early even towards the age of 50, but which reaches more than 50% of the population over the age of 80 (Alfonso J Cruz-Jentoft et al. 2010; Buffa et al. 2011). It has been shown that from the age of 60 the loss of muscle mass varies between 1.4% and 2.5% annually (Frontera et al. 2000) to achieve losses of force of about 50% and more around at 70-80 years (Taaffe 2006).

There is an alteration of the muscular fiber arrangement ("muscular architecture"), a decrease in protein turnover (Paillard, 2013), a decrease in the number of satellite cells that compromises the regenerative potential and the ability to respond to certain training stimuli, and a faster regression rate of the fast fibers (type II) than the slow ones (type I) (Granacher, Zahner, and Gollhofer 2008). The loss of muscle mass is associated with an increased risk of unfavorable outcomes such as motor disorders, balance deficit, disability and decreased quality of life, as a compromise of the performance of the usual daily activities (A. J. Cruz-Jentoft et al. 2010). The causes of sarcopenia can be multiple; in addition to the aging process, in fact, we can add genetic predisposition, lifestyle and changes in living conditions, but also pathological conditions.

It should also be considered that the functionality of the skeletal muscle in the generation of force and in the production of the movement can be compromised by an incomplete activation of the motor units in central nervous system, by dysfunctions of the peripheral nerve, by the reduction of hormonal factors and changes in the excitation-contraction coupling mechanisms (Frontera et al., 2000; Buffa, 2011).

2.1.2 Cardiovascular functions

The physiological aging of the cardiovascular system manifests itself as a set of structural and functional changes that make it differently responsive to anything that

can change its balance. Main structural modifications should be considered the vascular remodeling in terms of stiffening and thickening of the arterial wall and the consequent reduction of the lumen of the blood vessel, which determines the increase of the peripheral resistances, of the differential pressure and of the propagation speed of the sphygmic wave. (Biagi 2009).

The increase in the parietal thickness of the heart, given by the ratio between the thickness of the wall and the radius of the ventricular chamber, with consequent imbalance of the microcirculation, and volumetric increase of the atria, leads to loss of elasticity of the left ventricle, so is necessary the increase of left atrium contribution for ventricular filling (Biagi, 2009).

Another characteristic feature of the heart of the elderly are the alterations in energy metabolism linked to the reduction of the number of mitochondria, the intracellular levels of ATP and of phosphocreatine, the lower use of fatty acids, the variations in calcium homeostasis and the proteins involved in electromechanical coupling. There are also changes in the diastolic phase due to reduction of the ventricular compliance, potentially associated with alterations in calcium re-uptake, with progressive decrease of the slow filling and increase of the rapid filling due to the compensatory atrial contraction (Biagi, 2009; ACSM, 2009).

Lastly, changes in the systolic phase are highlighted, maintained by a more vigorous atrial contribution, particularly under stress, due to the increase in the afterload, the reduction of aortic compliance and the wall stress of the left ventricle with associated decrease in the sympathetic regulation (reduced tachycardia response to stress). In fact, in the elderly there is a decrease in the maximum heart rate and the ability to increase the ejection fraction during exercise, attributable to a lower sensitivity of the heart to β-adrenergic stimulation (Biagi, 2009; ACSM, 2009).

Overall, therefore, the cardiac changes dependent on the aging process indicate that in most "healthy" elderly the heart at rest is adequate to satisfy the hemodynamic and metabolic demands of the organism. However, when the homeostatic reserve is overcome, it is predisposed to the development of diastolic heart failure, for the impairment of left ventricular filling (reduction of relaxation and compliance), as a condition that is evident in over 50% of subjects after 75 years (Biagi, 2009).

2.1.3 Lung function

The effects of aging on the respiratory system are similar to those occurring in other organs, with a gradual decrease in efficiency dependent on structural and functional changes in the lungs related to age. The main findings include decreasing the volume of the thoracic cavity and lung volumes, and the alteration of the respiratory muscles. Increased stiffness of the rib joints, depending on bone and cartilage changes, leads to a progressive increase in diaphragmatic respiration (Lowery et al. 2013).

Aging is also associated with the modification of the composition of the connective support elements of the alveoli with thickening of the alveolar-capillary membrane which, together with the reduction of the total alveolar surface (from 80 cm at 20 years old to 60 cm at 80 years old) and to the consequent loss of elasticity of the walls of the pulmonary circle, leads to the loss of efficiency of the gaseous exchanges and of the diffusion capacity (Shephard 1993).

There are also structural changes and functional modifications intrinsic to the respiratory muscles. Forced breathing is limited, due to reductions in mitochondrial ATP reserves necessary to sustain a sudden increase in metabolic demand, to atrophy of accessory muscles, to decrease in fiber resistance that can reach 20% around 70 years, to their disorganization and to the transitions related to age (Lowery et al., 2013).

Among the consequent modifications of lung volumes, the increase in the residual volume (VR) is particularly evident which, subtracting from the total lung capacity, reduces the vital capacity (CV) and, despite having little effect on gaseous exchanges, slows the increase in oxygen consumption recorded at the mouth at the beginning of the exercise because the extra air introduced with hyperventilation is mixed with a greater volume of air already present in the alveoli. The decrease in CV follows a slow decrease stared at 20 years old to middle age, and faster in the last years of life, for an average decrease of 24.4 ml/year. In any case, the loss of it is less than the decline of the $\dot{V}O_{2max}$ (Shephard, 1993; ACSM, 2009).

Compared to pulmonary dynamics, the aging process is associated with the narrowing of the small part of respiratory tract, progressively compromising the maximum expiratory speed, but also in this case remains below the decline of the $\dot{V}O_{2max}$. The normal non-uniformity of the current volume distribution is accentuated with limiting repercussions during the exercise (Shephard, 1993).

Finally, the respiratory function in the elderly is affected by the decreased ability to remove mucus from the lungs as a result of the decline of the mechanisms that support it. First there is a reduction in the ability to generate the force necessary for an effective cough in relation to the weakening of the respiratory muscles, but also the ciliary dysfunction (Shephard, 1993; Lowery et al., 2013).

Overall, therefore, the values at rest (current volume and respiratory rate) have small changes, but increases the ventilation for each level of exercise (3-5%/year), that conduct an increased energy cost ($\dot{V}O_2$) for the same exercise. In addition to reducing efficiency (from 23% in young people to 21.5% to 65 years, to the cycle ergometer), there is also an increasing in the energy cost of breathing (from 6% in young to 13% of the total at 70 years old) (Shephard, 1993; ACSM, 2009).

2.1.4 Body composition and metabolism

Aging involves physiological changes that result not only in reduced functional capacity, but also in alterations in body composition.

Studies carried out on the elderly population of industrialized countries indicate a tendency to increase body weight and body mass index (BMI) according to age, in particular from 40 to 70, with an annual average rate of 0.30 kg/year, 0.11 kg/m/year in men and 0.55 kg/year, 0.22 Kg/m/year in women (Buffa, 2011). Fat mass increases progressively in adulthood as a consequence of the reduction in overall energy expenditure, with a preferential increase of the visceral adipose component (intra-abdominal) and redistribution of adipose tissue starting from 45-54 years (ACSM, 2009; Buffa, 2011). This is associated with the reduction of lean mass (FFM) in percentages of 2-3% per decade from 30 to 70 years, due to the skeletal and muscular components (sarcopenia, see Section 2.1.1) and then of metabolically active tissue and important physiological regulator, with loss of total proteins and potassium (ACSM, 2009).

The progressive decrease in bone mass, called osteopenia, depends on the loss of bone mineral content from 40 years (0.7-1% per year). One of the main causes of osteopenia is estrogen deficiency, although calcium deficiency, vitamin D and hyperparathyroidism may contribute to the pathogenesis, and the risk is that turning into osteoporosis, a disease that can lead to bone fragility and an increased susceptibility to fractures (ACSM, 2009; Buffa, 2011).

Parallel to the reduction of the lean mass there are various changes at the metabolic level represented by the decrease in the rate of absolute basal metabolic rate (RMR) and normalized per kg of body mass, the decrease of protein synthesis at the muscular level and of the oxidation capacity of fatty acids during sub-maximal exercise (ACSM, 2009).

3. Overall view of thesis

The general objective of the thesis is to investigate the effects on exercise induced by supplementation of nitrates (NO_3^-), in elderly (60-75 years) and young (20-35 years), during exercise of moderate and severe intensity. The NO_3^- contribution, equal to 8.0 mM dissolved in 0.25 L of solution, was provided by means of beetroot juice and continued for a period of 8 days. We evaluated oxidative metabolism, taking into consideration both central factors as well as peripheral factors.

The aging process is associated with functional and structural changes, especially in the cardiovascular and muscular systems, lead to alterations in oxidative metabolism. The marked impairment of the transport of O_2 and its peripheral use, together with the progressive loss of strength and muscle mass, are responsible for the significant and progressive worsening of $\dot{V}O_2$ kinetics that characterizes the elderly.

Also, cardiovascular responses following a nitrate supplementation have been investigated. Indeed, literature remarks that, nitrates are involved both in the circulatory (endothelial) and metabolic (mitochondrial) levels. The interest is therefore to analyze whether nitrates in the two populations (old and young) have a significant effect on the main hemodynamic components.

Moreover the metabolic cost of walking per unit of distance travelled is greater in older people than in young adults even when they are healthy and free from gait impairment. Other data indicate that the increase in the cost of the metabolic cost of the walk occurs around the 7th decade of life that coincides approximately with the time in which the changes occur in different biomechanical and bioenergetical factors of walking.

We intent also to investigate the effects at metabolic level induced by nitrate supplementation during exercise. The study was developed in order to describe the trend of energy cost of locomotion on a treadmill at different intensities administered by varying the speed and slope of the instrument.

The main end point is the verification of the efficacy of BR supplementation in improving muscle efficiency in various type of locomotion. In particular, we intend to check if NO_3^-:

i) are able to reduce the energy cost of exercise in elderly subjects in the moderate and severe intensity domain;

ii) are able to improve muscle efficiency in relation to a greater availability of oxygen and its use at the peripheral level.

In short, the aim of the studies was to evaluate, through an integrative approach, the effects of nitrate supplementation on skeletal muscle oxidative metabolism during exercise.

SECTION TWO

STUDY ONE

The effects of nitrate supplementation

on different intensities of exercise

Summary of the section

In this section are analyzed the effects of NO_3^-, through supplementation with beetroot juice, on different intensities of exercise. Experimental protocol includes two different transitions from rest to moderate and severe intensities of exercise.

After an introduction on $\dot{V}O_2$ kinetics and NO_3^- effects on them, data of oxygen consumption, muscular oxygen extraction at the muscular level and blood pressure parameters are reported and analyzed.

List of abbreviations

NO_3^-	Nitrates
NO_2^-	Nitrites
NO	Nitric oxide
BR	Beetroot
PL	Placebo
MOD	Moderate intensity of exercise transition
SEV	Severe intensity of exercise transition
R	Rest phase
UP	Unloaded pedalling phase
EXE	Exercise phase
NIRS	Non-invasive near-infrared spectroscopy
ES	Effect size
$\dot{V}O_2$	Oxygen consumption
$\dot{V}CO_2$	Carbon dioxide production
A	Amplitude of $\dot{V}O_2$ kinetics
TD	Time delay of $\dot{V}O_2$ kinetics
τ	Time constant of $\dot{V}O_2$ kinetics
[La]	Lactate concentration
SAT	Saturation of hemoglobin
[HHb]	Concentration of deoxygenated hemoglobin
[HbO_2]	Concentration of oxygenated hemoglobin
[THb]	Total concentration of hemoglobin
SYS	Systolic pressure
DIA	Diastolic pressure
MAP	Mean arterial pressure
TPR	Total peripheral resistance
HR	Heart rate

4. Introduction

4.1 *$\dot{V}O_2$ kinetics*

The study of the kinetics of oxygen consumption ($\dot{V}O_2$) and of the physiological mechanisms that regulate the dynamic response of $\dot{V}O_2$ to constant-load exercise assumes a certain importance if we consider that oxidative metabolism is the main process by which the organism produces energy (David C. Poole and Jones 2012).

The consumption of oxygen by the tissues is described by the Fick equation [1]:

$$\dot{V}O_2 = \dot{Q}x\ \Delta\ (a\text{-}v) \qquad [1]$$

Where: $\dot{Q}$ is the cardiac output, that is the quantity of blood expelled from the left ventricle in the time unit (L / min), corresponding to the product of the heart rate (HR) for the systolic range (SV) ⊿(a-v) represents the arterio-venous difference of the content of O2 (CaO2 - CvO2), therefore the amount of oxygen that the cells can extract and use from the bloodstream during the passage of blood into the capillaries.

The $\dot{V}O_2$ therefore depends on the amount of blood circulated by the heart pump and on the capacity of O_2 utilization by the cells.

The instantaneous increase of muscular work that occurs with the beginning of the exercise at constant load (square wave), starting from a rest condition or a reduced metabolic activity, causes an immediate increase in the speed of synthesis of ATP due to increasing on muscle contraction, until reaching a level of power to that required. The $\dot{V}O_2$, however, follows the mechanical and biochemical events of the contraction with a certain latency, showing insufficient, in the initial phase of the exercise, to satisfy the metabolic demands. The compensation of the delay related to the production of ATP by oxidative pathway determines an increase in the contribution of the anaerobic mechanisms (phosphocreatine and glycolysis) which progressively decreases as a function of time until it is canceled when the coupling between ATP resynthesis speed is achieved by oxidative pathway during moderate exercise(David C. Poole et al. 2008).

The observation of respiratory dynamics in the first few minutes of the exercise can provide important information on the regulation of oxidative metabolism in skeletal muscles, as the $\dot{V}O_2$ measured at the mouth reflects the muscular one (Poole and Jones, 2012). A rapid adjustment of the flow of O_2, dependent on the coordinated

response of the respiratory, cardiac and muscular systems, reduces the need to "perturb" the anaerobic metabolism, thereby depleting the reserves of energy intramuscular substrates and unbalancing the metabolites associated with glycolytic stimulation (ADP, Pi, H^+), with consequent positive effects on exercise tolerance and on the onset of muscular fatigue (Burnley and Jones 2007). By simply considering these dynamics, it can be assumed that the speed with which the aerobic mechanism is able to adapt to a variation in the energy requirements of the muscle in exercise can be a factor that is not negligible for performance (Bassett and Howley 2000). Indeed, an excessive inertia of the aerobic metabolism linked to the latency in the activation time of the enzymes of the Cycle of Krebs and the electron transport chain, or a no optimal distribution of the peripheral flow, can result in a consequent increase in the amount of energy produced anaerobically and as consequence of the O_2 debt (Tschakovsky and Hughson 1999).

The $\dot{V}O_2$ shows a characteristic exponential response to the increase in metabolic demands in constant-load exercise, until a new state of equilibrium is reached. In reality, the phenomenon is not linear, but is made up of the sum of several phases. The increase of the parameter, in the initial phase of the transition from the resting state to the exercise, presents a first rapid adjustment (Phase I: Cardiodynamics), followed by a second phase (Phase II: main or primary component) which represents the real onset. The achievement of steady state is replaced, for loads exceeding the moderate intensity domain (> GET: gaseous exchange threshold), by a third phase (Phase III: Slow component) which progressively tends to increase, reaching (intensity <CP: Critical Power) or not the steady state (intensity> CP) (Whipp and Wasserman 1972). The adjustment of response of the aerobic metabolism to the load follows a trend that can be modelled by the following equation [2], given by the sum of single-exponential functions, each corresponding to a specific phase of kinetics:

$$\dot{V}O_2\,(t) = \dot{V}O_{2BAS} + [A_1\,(1 - e^{(t-TD_1)/\tau_1}\,] + [A_2\,(1 - e^{(t-TD_2)/\tau_2}] + [A_3\,(1 - e^{(t-TD_3)/\tau_3}] \qquad [2]$$

Where: $\dot{V}O_2$ (t) is the $\dot{V}O_2$ at time t – $\dot{V}O_{2BAS}$ is the $\dot{V}O_2$ at rest – A is the amplitude of the variation of the $\dot{V}O_2$ of each phase (1, 2, 3) – TD is the time delay or delay time, which identifies the time instant in which the exponential response begins – τ is the time constant, which defines the time necessary for the $\dot{V}O_2$ to reach 63.2% of the $\dot{V}O_2$ at the steady state.

4.1.1 Phase I

At the beginning of the aerobic exercise at constant load, a sudden and rapid increase in the consumption of oxygen is observed, represented by an exponential function that usually exhausts within 10-25s. This phase, called "cardiodynamics", seems to reflect the early increase in blood flow to the lungs, which depends from the increase in heart rate and myocardial contraction, which are mediated on a neurological level and an increase in venous return (Casaburi et al. 1989). In this period, the blood coming from the muscles in exercise and modified by the cellular metabolism, has not yet reached the lungs, and its composition is determined by rest conditions, without an increasing of muscle extraction of the O_2, and then without appreciable changes of the respiratory quotient, of the partial pressure of oxygen in the venous blood (PO_2) and of the partial pressure of carbon dioxide (PCO_2) (Burnley and Jones, 2007). This phase often shows artifacts, and being considered devoid of metabolic implications, it is normally excluded from analysis (Maione et al. 2015).

4.1.2 Phase II

Phase II follows phase I with a variable delay time, only occasionally higher than 30 s, from the start of exercise. It is characterized by a slow increase in oxygen consumption, with a value of τ in most cases of about 30-40 s, according to a trend represented by an exponential function, attributed to the combination of the continuous increase of venous return from the muscles in exercise and significant reduction of the O_2 content in venous blood compared to the concentration of the same in the lungs. It therefore reflects the consumption of oxygen at the muscular level linked to the increase in mitochondrial respiration and therefore the metabolic changes at this level.

Because the ability to generate energy through substrate phosphorylation is considered finite (both due to a limited capacity and to the accumulation of fatigue-related metabolites), exercise tolerance in subjects with very slow Phase II, such as those with pulmonary, cardiovascular or metabolic diseases or disorders could also be compromised at reduced levels of power. Elite endurance athletes have a remarkably rapid Phase II in order to minimize the extent of O_2 deficiency and therefore the perturbation of homeostasis in the transition from a low to a higher metabolic level (David C Poole et al. 2005).

Moderate intensity of exercise at constant load can be described by Phase I and Phase II of $\dot{V}O_2$ with following equation:

$$\mathbb{V}O_2\,(t) = \mathbb{V}O_{2BAS} + [A_1\,(1 - e^{(t-TD_1)/\tau_1)}\,] + [A_2\,(1 - e^{(t-TD_2)/\tau_2)}] \qquad [3]$$

4.1.3 Phase III

In the exercise performed at an intensity higher than GET, the kinetics of the $\dot{V}O_2$ show a third phase that begins, by definition, around the third minute. This phenomenon, called "slow component", indicates the rate of increase of $\dot{V}O_2$, often in relation to the increase in blood lactate, whose significance has been associated with several possible explanations: physical phenomena such as the increase in body temperature, metabolic changes (glucose resynthesis starting from the lactate produced), but above all the greater recruitment of fast fibers (type II) (Gaesser and Poole 1996).

Phase III is quantified as the increase of $\dot{V}O_2$ in comparison to the steady state value reached up during phase II of the kinetic.

On the causes of this phenomenon, hypotheses have been proposed that take into consideration factors such as catecholamines, lactic acid, hydrogen ions that could act as metabolic stimulators with effects at the peripheral or central level. It seems that it is very linked to the concentration of blood lactate as it is found only in exercises in which a sustained acidosis is established (Whipp and Wasserman 1972), with a width quantitatively linked to level of it. According to others (Poole et al. 1991) 86% of the slow component would be associated with intramuscular factors that refer to the recruitment of type II fibers because of their low oxidative capacity. In any case it is fundamental to recognize that it expresses the reduction of metabolic efficiency that brings an additional consumption of O_2 and increase of the "Gain" (mL O_2/min/ W) (Grassi, Rossiter, and Zoladz 2015), and a delayed achievement of the state stationary, (Poole and Jones, 2012).

4.2 *Kinetics and intensity of exercise*

The profile of the $\dot{V}O_2$ response to the metabolic transition, from the resting state to the constant-load exercise, is characterized by a certain specificity for the intensity of work.

There are usually three main intensity domains determined based on the evoked metabolic adaptations Moderate, Heavy and Severe.

4.2.1 High intensity (Heavy Exercise)

Heavy exercise defines labor intensity higher than GET, but lower than CP, with elevated, but stable blood lactate concentrations during exercise (Burnley and Jones, 2007). The CP represents the asymptote of the Power-duration curve for high-intensity exercise, namely the sub-maximal $\dot{V}O_2$ that can be sustained for a prolonged period of time. These parameters usually coincide with ~ 50% of the delta between GET and $\dot{V}O_{2max}$ (Δ50%).

To this intensity domain, a third phase of the kinetics of the $\dot{V}O_2$ or "slow component" appears, which describes a continuous increase of the parameter, according to a very slow time constant and an amplitude greater than the steady state value. The onset of this phenomenon, which represents 10-20% of the total response, typically occurs 90-180 s after the beginning of the effort (Burnley and Jones, 2007) and tends to stabilize after about 10-20 min (Pringle et al. 2003).

4.2.2 Very high Intensity (Severe Exercise)

The domain of severe intensity is delimited by mechanical outputs between CP and $\dot{V}O_{2max}$ and is associated with high concentrations of blood lactate that continue to increase over time (Scheuermann et al. 2011, Poole and Jones, 2007), but also to the decrease in intramuscular phosphocreatine concentration (Grassi, Rossiter, and Zoladz 2015). Also in this case appears the slow component of the $\dot{V}O_2$ which, however, does not reach equilibrium therefore, in conditions in which the exercise is sustained for a long period of time, it tends to reach its maximum value ($\dot{V}O_{2max}$) bringing the subject to exhaustion (Grassi, Rossiter, and Zoladz 2015) in a time that is faster the more the phase time constant is fast.

4.3 *Kinetics of oxygen consumption: central and peripheral mechanisms*

The kinetics of oxygen consumption are generally considered an index of the general conditions of integrity of the pulmonary, cardiovascular and muscular systems, resulting from the interaction between the mechanisms of regulation of the release of

O_2 and its use by the muscles. The question concerning the factors responsible for the limitation of oxidative metabolism, of a central or peripheral nature, has been widely debated in the literature (Murias, Kowalchuk, and Paterson 2011; De Roia et al. 2012).

The data do not exclude that the immediate response of the $\dot{V}O_2$ to the metabolic transition, in the first ~ 20 s of exercise, can be regulated mainly by intracellular factors, with particular reference to the ability to provide substrates other than O_2 to mitochondria. In particular, since mitochondrial respiration is intimately linked to the rate of hydrolysis of ATP, one or more of the reagents of this process ([ADP] and [Pi], phosphorylation potential and/or [PCr] and [Cr]) are considered potentially responsible for the major limitation in the regulation of oxidative phosphorylation (Meyer, 1988).

Several groups (Poole and Jones, 2012) provided evidence consistent with this theory demonstrating a close coupling between the kinetics of the muscular [PCr] and that of the $\dot{V}O_2$ at different intensities of exercise. Kindig and colleagues (Kindig 2004) reports that the acute inhibition of creatine kinase (CK) in isolated myocytes greatly accelerates the kinetics of intracellular PO_2, in analogy with the transient of $\dot{V}O_2$, suggesting that changes in ADP concentration, following the splitting of CK-catalyzed phosphocreatine at the beginning of the exercise, attenuates the activation of oxidative phosphorylation. In addition, competition for the mitochondrial cytochrome c oxidase link site between the NO from the vascular endothelium and O_2 shows the contribution to regulating the speed of adjustment of the kinetics of the $\dot{V}O_2$ (Jones et al. 2003), as well as the reserve of substrates (acetyl groups and NADH).

Despite the intracellular factors altering the phosphorylation and/or redox potential and influencing the oxidative phosphorylation processes, it is possible that after the initial delay in activation and the increase of oxidative phosphorylation (~ 20 s, potentially due to the intracellular control), all the substrates necessary to drive oxidative metabolism, except O_2, are present in saturating concentrations.

4.3.1 Training effects

Several studies have shown that changes in the oxidative metabolism response occur rapidly in terms of phase II acceleration of 20% and 40% respectively after only two and eight sessions of ET or HIT (McKay, Paterson, and Kowalchuk 2009), but also slow attenuation of the slow component, at severe exercise intensity, after 2 weeks

of RSA (Bailey et al., 2009) or a combination of ET and HIT (Da Boit et al. 2014). In 2013 Williams and colleagues (Williams, Paterson, and Kowalchuk 2013) showed that 2-4 weeks of HIT with intervals at 110% of peak $\dot{V}O_2$ leading to acceleration of the kinetics of $\dot{V}O_2$, in response to light load (20 W → 45% GET) and moderate (45% GET → 90% GET), of similar magnitude. Based on the principle of Henneman and colleagues (Henneman, Somjen, and Carpenter 1965) of the hierarchical recruitment of motor units, greater reductions in muscle glycogen and [PCr] were reported in type I fibers than in type II during ET at 30-80% $\dot{V}O_{2max}$ (Da Boit et al., 2014). The same parameters decrease significantly in type I and II fibers after HIT at intensities higher than $\dot{V}O_{2max}$, with glycogen depletion mainly in fast fibers (Da Boit et al., 2014). Since HIT, above maximal intensities, induces more adaptations in oxidative enzymes in type II fibers than in ET and more important increases in the oxidative capacity of type IIb fibers, oxidative metabolism improves more markedly in these types of fibers after RSA. The study by De Boit and colleagues (Da Boit et al., 2014) shows that two weeks of RSA and ET induce the acceleration of $\dot{V}O_2$ kinetics in the transition from light to moderate intensity (-26% and –22%) and the increase in exercise tolerance in the transition from moderate to severe intensity (+33% and +37%).

4.3.2 VO_2 Kinetics and the elderly

Aging is associated with the decline of cardiorespiratory functions (Biagi, 2009) and of the oxidative capacity of the muscles (Granacher, Zahner, and Gollhofer 2008) that influence the adaptive response of oxidative metabolism to exercise, in relation to the progressive limitations in transport and use of O_2 (Murias, Kowalchuk, and Paterson 2011). Typically, a slowing of the kinetics of the $\dot{V}O_2$ in phase II emerges (Murias et al. 2011), as a factor associated with higher O_2 debt due to less efficient phosphorylation at substrate level and diminished of muscular [PCr] for the synthesis of ATP necessary to sustain a given activity (DeLorey 2004). This leads early to fatigue alerting and reduced exercise tolerance (DeLorey 2004).

In general, there is a compromise of the ability to increase cardiac output with a reduction of O_2 contribution to the limb involved in the exercise (Bell et al. 2001), but what seems to limit the speed of adaptation of the kinetics of the $\dot{V}O_2$ at the increase in the energy requirement is the distribution of the blood flow within the active muscle (duManoir et al. 2010). Beyond the structural changes of the microcirculation (Bradley

J. Behnke and Delp 2010), the reduction of age-related peripheral perfusion, depending on the impairment of the local flow regulation capacity (Maione et al., 2015), is the reflection of a high rate of change in deoxygenated hemoglobin (HHb) in relation to the modification of $\dot{V}O_2$ (Δ[HHb]/ $\Delta\dot{V}O_2$ Ratio) in elderly subjects compared to other younger ones (DeLorey 2004), in addition to a greater transient reduction of PO_2 in the microcirculation (Brad J. Behnke et al. 2005).

Older adults therefore rely on a greater extraction of O_2 during the start of the exercise, probably due to a lower matching between the local blood flow (contribution of O_2 through the microcirculation) and its consumption by the muscle (Murias et al., 2011).

4.3.3 Metabolic transitions and NO_3^-

Dietary supplementation of NO_3^-, reduced to NO_2^- and then to NO and other reactive nitrogen species, has been shown to affect the intensity of $\dot{V}O_2$ reduction of submaximal exercise (Bailey et al. 2010, 2009; Lansley, Winyard, Fulford, et al. 2011; F. J. Larsen et al. 2007; Vanhatalo et al. 2011), in association with improving the intrinsic contractile properties of skeletal muscle active (Vanhatalo et al., 2010), the increase in mitochondrial efficiency in the oxidation processes of substrates related to the synthesis of ATP (Filip J Larsen et al. 2011) and the increase in the contribution of O_2 peripheral in relation to metabolic demands (Ferguson et al. 2013). From this emerges the potential role of NO_3^- exogenous in contributing to the positive modification of the $\dot{V}O_2$ kinetics in metabolic transients (Breese et al., 2013).

Considering that dietary supplementation of NO_3^- promotes improvements in absolute and relative flow distribution to type II muscle fibers (Ferguson et al. 2013), this could improve the coupling between the local contribution of O_2 and its consumption by the muscle, thus accelerating phase II in the metabolic transition from moderate to severe intensity exercise (Breese et al., 2013).

5. Materials and Methods

5.1 *Aim of the study*

The aim of this study is to investigate the effects of nitrate supplementation on muscle oxidative metabolism during moderate and severe intensity exercise on cicloergometer.

During moderate intensity exercise, nitrate supplementation effects on $V'O_2$ kinetic parameters is expected to lead to the acceleration of the oxidative metabolism response, thus to improvements of the $V'O_2$ kinetic parameters with feedback on muscle efficiency. With respect to severe exercise, in which the partial impairment of the availability of O_2 is known, we hypothesize that if nitrates have a predominantly vascular role, they can significantly improve the delivery of O_2. While if the efficiency increases due to treatment, we hypothesize that this is due to mechanisms located at the cellular level (mitochondria), justifying a slight increase in the time constant of Phase II.

5.2 *Subjects*

The study participants were 20 volunteered, healthy, subjects divided in two groups: 10 old (67 ± 4.3 years) and 10 young (25 ± 3.9 years). During subjects' selection phase were recruited 28 men, but 4 refused to participate, 3 were excluded after preliminary medical examination and 1 drop out during first supplementation phase. The remaining 20 non smoking subjects voluntary participated in the study after given their informed and written consensus.

Inclusion criteria to participate at the study were: a normal clinical exam, absence of orthopedic, muscle-skeletal, metabolic, cardiovascular, respiratory or oral cavity pathology,

Exclusion criteria were: abnormal clinical exam, presence of orthopedic, muscle-skeletal, metabolic, cardiovascular or respiratory pathology, obesity (BMI ≥ 30 kg/m^2),.

All procedures were approved by the Department of Neurological and Movement Sciences' ethical committee for research on human subjects.

OLD	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
O1	75	182	88	2718	31	230	148	55	163
O2	63	180	72	2796	39	210	167	45	138
O3	65	182	91	2211	24	194	155	53	140
O4	65	173	77	2153	28	188	183	69	130
O5	67	161	63	2697	43	206	141	60	141
O6	72	172	71	2549	36	221	147	90	172
O7	71	166	66	2620	40	198	155	64	147
O8	67	164	58	1959	34	166	157	62	125
O9	61	163	58	2718	47	230	145	59	161
O10	65	176	78	3161	41	258	150	131	216
Mean	67	172	72	2558	36	210	155	69	153
St. Dev.	4.3	8.0	11.5	355.6	7.0	26.0	12.3	24.9	26.6

YOUNG	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
Y1	26	175	65	4082	62.8	404	183	181	285
Y2	27	183	85.5	4247	49.7	411	188	154	301
Y3	29	182	80	4501	56.3	414	170	161	324
Y4	29	173	70	4291	61.3	357	189	130	279
Y5	30	169	76.5	3256	42.6	270	186	100	207
Y6	25	169	66	3267	49.5	304	189	102	216
Y7	21	166	60	2673	44.6	242	190	80	172
Y8	20	178	66	3598	54.5	296	187	92	222
Y9	21	184	74	3551	48.0	327	190	132	238
Y10	22	173	69	3308	47.9	304	174	89	220
Mean	25	175	71	3677	52	333	185	122	246
St. Dev.	3.9	6.4	7.8	582.8	6.8	61.1	7.0	34.7	48.2

Table 1,2: *The table shows the individual data of subjects examined, Old (up) and Young (down). The values of age (Age, years) of the anthropometric parameters have been reported: height (Height, cm) and body mass (Weight, Kg), of the maximum metabolic power, absolute ($\dot{V}O_{2max}$, mL/min) and relative ($\dot{V}O_{2max}$, mL/min/Kg, , of the maximum mechanical power (Power max, W) and of the maximum heart rate (Hrmax, bpm) detected in the preliminary test, and of the Workloads (W) of the two intensity domains (Moderate: 80 % GET, and Severe: ⊿ 50%, W)*

5.3 *Study design and protocol*

The study is a double-blind crossover design with Nitrate (BR) or Placebo (PL) supplementation. The protocol consisted of a preliminary day of test (D0) in which subjects performed a ramp incremental test (EXP1); followed by 3 alternate testing days (K1, K2, K3) in which $\dot{V}O_2$ kinetics tests were performed (EXP2). This plan was repeated in 4 experimental phases (BDC1, PS1, BDC2, PS2).

In the first phase (BDC1) (basal data collection) basal conditions were measured. In the second phase (PS1) (post supplementation) the conditions after first period of supplementation (randomly selected between NO_3^- or PL) were recorded.

After at least 10 days of washout, the third phase was performed (BDC2) where basal conditions were measured again. In the fourth and last phase (PS2) the conditions after second period of supplementation (opposite of the first period) were determined.

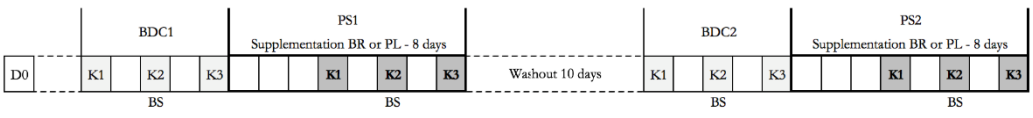


Figure 1: *The representation schematically summarizes order of test. After the preliminary evaluations (D0) follow the four experimental phases (BDC1, PS1, BDC2 and PS2) in each of which the kinetic evaluation protocol is repeated in non-consecutive days (K1, K2 and K3). All subjects underwent 8 days of supplementation with NO_3^- and PL, according to a balanced randomization. In PS1 and PS2 the kinetic evaluation protocol is repeated again. PS1 and BDC2 are separated by 10 days of washout. BS indicated blood sample, that is taken for the determination of the blood concentrations of nitrates and nitrites.*

5.3.1 Supplementation

The BR supplementation was made by beetroot juice (BR) (250 ml/day – Azienda agricola "Aureli" – Ortucchio (AQ) - Italy). The juice was provided in two different formulations: one with high concentration (~8.0 mmol) of NO_3^- and one with low concentration (~0.8 mmol) of NO_3^- (used as a placebo (PL)). The PL was identical in color, taste, smell and texture to the NO_3^- rich BR juice. Supplementation was distributed by an experimenter not involved in laboratory tests and/or in data analysis and the subjects and all the experimenters involved didn't know what supplementation was provided (if BR or PL). The matching of assumptions was known only at the end of data analysis.

This is considered a medium-term supplementation design that lasts for 8 days (Porcelli et al. 2015, Wylie et al. 2013). with ingestion of a single daily dose of 250 ml of juice before breakfast. The measurements of the kinetics started on the third day of treatment. The kinetics protocol took place on average 2.5/3 h after the supplementation. In each phase the same cadence of supplementation/test was repeated.

The subjects independently provided the supplementation following a sheet of instructions delivered to them. They were also warnings on foods to avoid rich in nitrates (spinach, beetroot, salad, rocket and Chinese cabbage) and to avoid the use of antibacterial mouthwash.

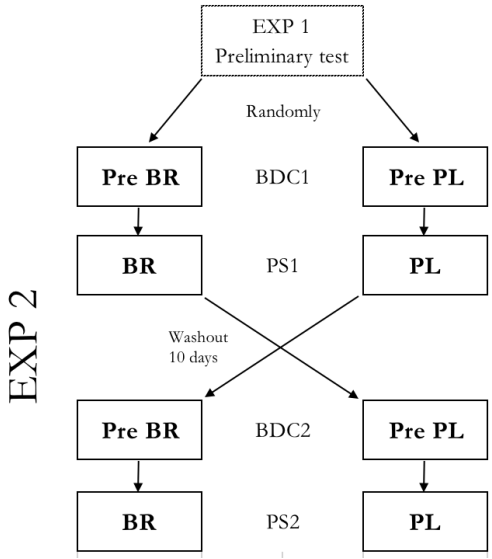


Figure 2: *The representation schematically summarizes the experimental design that structures the presented study, of a longitudinal type in a double-blind crossover. After the preliminary evaluations (EXP1) subjects randomly divided in two groups (BR or PL) and perform first two experimental phases (BDC1 and PS1).*
After 10 days of washout they crossed their condition and change supplementation and perform last two phases (BDC2 and PS2)

5.4 *EXP1 – Preliminary ramp incremental test*

To determine peak of oxygen consumption $\dot{V}O_{2max}$. gas exchange threshold (GET), power output (PO), power output peak (PO_{peak}) and maximal heart rate (HR_{max}) a ramp incremental (RI) test was performed.

RI protocol included 3 min of measurement of baseline condition, where subject remained sit on bike without moving. After that, the subject start cycling at 30 W(warm-up), for 3 min, with self-selected cadence. This cadence was recorded and was maintained during all subsequent tests using visual feedback and verbal encouragement from the experimenters. Warm-up was followed by RI protocol with different workload increments every minute (15, 20, 25, 30 W/min – 2W/8s, 2W/6s. 5W/12s. 3W/6s) in order to maintain entire test duration between 16 and 18 min. Test ended with exhaustion of the subject, and howeevr when the criteria for maximal test were reached ($\dot{V}O_2$ plateau, HR ~ HRmax, [la] >10mM). Failure to maintain the indicated cadence to within 5 rpm (for longer than 5s) during testing despite strong verbal encouragement was considered as the criterion for exhaustion.

In order to obtain a more reliable measure of $\dot{V}O_{2max}$ a verification trial test (VER) was also executed: after 2 min of recovery subjects start pedaling again at 20 W, after 5 min the workload was augmented to constant-work rate equal to 105% of the mechanical power achieved at the end of the ramp test until exhaustion. (David C. Poole, Wilkerson, and Jones 2008)

5.5 *EXP2 – $\dot{V}O_2$ Kinetics test*

To measure physiological adaptations at the onset of exercise a $\dot{V}O_2$ kinetics (EXP2) test was assessed.

EXP2 was performed on cycle ergometer (Excalibur Sport – Lode B.V. – Groningen, The Netherlands) and the protocol provided two square wave transitions of 6 minutes duration, at 2 different intensities: moderate intensity (MOD – 80%GET) and severe intensity (SEV – 50%Δ ;50% of the difference between GET and $\dot{V}O_{2max}$) and was performed in three days (D1, D3, D5).

After 3 minutes of basal condition measurement, subjects start pedaling at 30 rpm (round per minute) for another 3 minutes to warm-up. At the 6^{th} minute the moderate intensity transition started: the workload became equivalent to 80% GET (80% GET

represents the imposed mechanical load in order to reach a metabolic intensity of 80% of GET - gas exchange threshold-). The subject kept his a fix pedaling cadence corresponding to that determined during the RI. The transition lasted 6 minutes and at the end the entire procedure was repeated at severe intensity. At the 18th minute the severe intensity transition started: the workload became equivalent to 50%Δ (50%Δ represents the imposed mechanical load in order to reach a metabolic intensity of 50% of the difference between GET and $\dot{V}O_{2max}$.

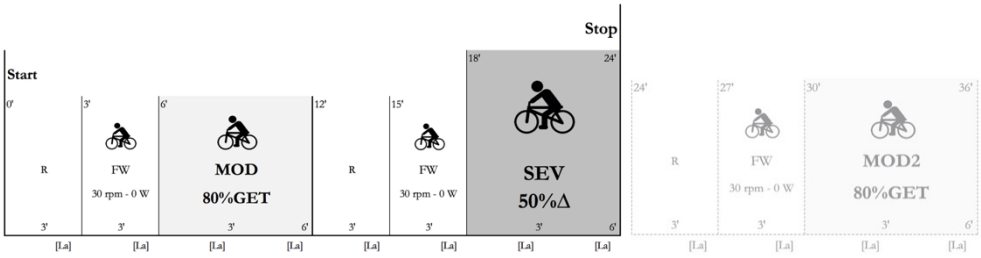


Figure 3: *The representation schematically summarizes the experimental protocol of $\dot{V}O_2$ kinetics. [La] indicates the measurement of lactate concentrations in the last minute of each phase*

5.6 *Measures and instruments*

In all the tests the followingmeasurs were done:

- Pulmonary gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) (Quark CPET – Cosmed srl – Rome. Italy).
- Oxygenated [HbO] and deoxygenated [HHb] hemoglobin concentration on vastus lateralis muscle (VL) by Near InfraRed Spectroscopy (NIRS – OxiplexTS™ – ISS Inc. – Champaign. IL. USA)
- Blood pressure (Portapres® – Finapres medical system B.V. – Enschede. The Netherlands).
- Lactate [La] and Glucose [Glu] concentration (Biosen C-line – EKF Diagnostic – Barleben. Germany), by capillary blood collection (10 μL) from the earlobe performed every 3 minutes, 30s before changing phase.
- Blood samples were collected by venous sampling to (5 + 5 mL) glass EDTA tubes to determine [NO_3^-] and [NO_2^-].

To perform the tests was used

- Cycle ergometer (Excalibur Sport cycle, Lode – Groningen, The Netherlands)

5.6.1 Quark CPET- Cosmed, Rome, Italy

Gas exchanges ($\dot{V}O_2$, $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) were measured breath-by-breath using the metabolimeter with a facial mask.

The concentrations of inhaled and exhaled gases were sampled at a frequency of 100 Hz via a capillary line connected to the mask and quantified by respectively paramagnetic analyzers for O_2 with response time of 120 ms and infrared rays (NDIR technology) for CO_2 with a response time of 100 ms. The measurement of the volume of the respiratory flows was carried out by a flowmeter consisting of a bidirectional digital turbine inside which a movable vanity unit, free to rotate around its axis, rotates at speed and in a direction proportional to the flow of air from which it is invested. The number of rotations was transduced into the parameters of interest by an opto-electronic system with infrared LED diodes based on the frequency of detection of the passage of the blades, integrated and processed by a microcomputer.

Prior to each test, the gas concentration and volume transducer analyzers of the turbine were calibrated using a mixture of a gas with known concentrations, according to the manufacturer's instructions, (FO_2: 0.16; FCO_2: 0.05) and a 3.0 L syringe. Concentration data e volume were aligned temporally, breath-by-breath, taking into account the delay in the passage of the gas to the capillary then the discrepancy between the time of acquisition of the signal by the analyzer and the flow meter, through the calibration of delays.

5.6.2 Portapres® – FMS, Amsterdam, The Netherlands

Non-invasive monitoring of the pressure profile was performed by continuous recording of the pressure pulse with cuff placed at the level of the phalanx distal of the middle or ring finger of the right hand using the photoplethysmographic method.

The mean arterial pressure values (MAP) were calculated as the mean of the integral of any data detected by the Beatscope software (FMS), making the correction for the height difference between the heart and the fingertips and the individual factors of the subject (anthropometric data, age, sex), as indicated by the manufacturer.

5.6.3 NIRS – OxiplexTS™ – ISS Inc. – Champaign. IL. USA

The changes in the oxygenation state at the level of the microcirculation of the muscular tissue of the lateral vastus were measured using a non-invasive method using NIRS (Near Infrared Spectroscopy) spectroscopy. This instrument detects in real time, at a sampling rate of 100Hz, the absolute (micromolar) concentrations of oxyhemoglobin [HbO], deoxyhemoglobin [HHb], total hemoglobin [THb] and tissue oxygenation index (SAT) whose values are expressed and analyzed, second by second, as average data. NIRS light is emitted in the muscle at wavelengths between 690 and 830 nm using light sources and receivers placed at distances of 1.50 - 3.04 cm, with the intake of cellular water at a constant concentration of 70%.

The NIRS probe was positioned after the treatment of the skin surface (degreased, slightly abraded and depilated), at the lower third of the vastus lateralis, calculated as the midpoint of the distance between large trochanter and lateral epicondyle of the femur of the right leg, secured with adhesive tape. Velcro and elastic straps were used to ensure no microspacing of the device and its isolation from external light, minimizing interference during acquisition.

The NIRS probe was calibrated before each test session using a calibration block with known absorption and dispersion coefficients of the known NIRS electromagnetic wave, a procedure performed according to the manufacturer's recommendations.

5.6.4 Lactacidometer

Blood lactate concentrations ([La], mM) and glucose ([Gly], mM) were detected on arterialized capillary blood samples (10 µL) taken from the earlobe. Values were obtained using an electrochemical system (Biosen C_line, EKF Diagnostic, Barleben, Germany).

5.6.5 Kit for blood samples

The evaluation of the plasma concentration of nitrates (NO_3^-) and nitrite (NO_2^-) was carried out on blood samples obtained by venous sampling (5 + 5 mL), for each of the experimental phases (BDC1, PS1, BDC2 and PS2). The intervention was conducted, before the experimental session, by medical staff.

The analysis of the samples, collected in glass tubes containing EDTA anticoagulant, was performed by Borgo Roma hospital chemical laboratory.

5.6.6 Excalibur Sport cycle, Lode – Groningen, The Netherlands

All the tests were performed on an electromagnetic brake cycle ergometer, connected and managed by the metabolimeter (Quark CPET - Cosmed, Rome, Italy).

The electromechanical characteristics of the ergometer allow the application of the workload in 50 ms. The signals of the pedaling frequency (rpm) and of the load (W) were digitized into parallel to a 16-channel analog-to-digital converter (MP100, Biopac Systems, Goleta, CA) and stored on a computer at a frequency of 100Hz.

6. Data analysis

6.1 *Nitrate and Nitrite concentrations*

The blood concentration of nitrates and nitrites was evaluated on plasma with a colorimeter kit (Nitrate/Nitrite Colorimetric Assay Kit - Cayman). The plasma fraction was prepared by ultra-filtration using filters with a 10 KDa cutoff (Amicon). For the test, 10 ul of filtrate were used and the supplier's indications were followed. The reading was done with a reader for 96-well plates at a wavelength of 540 nm (Gralis - Buoty Diagnostics)

6.2 *Maximal oxygen consumption ($\dot{V}O_{2max}$)*

During RI in D0 $\dot{V}O_{2max}$ was determined and it was calculated as the average of the $\dot{V}O_2$ recorded in the last 30 seconds before exhaustion. As maximal power output (PO_{peak}) was considered the last completed load before the end of test.

The results $\dot{V}O_{2max}$ was compared with one recorded during VER. $\dot{V}O_{2max}$ of VER was calculated as the average the $\dot{V}O_2$ recorded in the final 10 seconds before exhaustion. If the difference between two $\dot{V}O_{2max}$ was more of 100 ml$\dot{V}O_2$/min it was calculated average between them, otherwise $\dot{V}O_{2max}$ determined after RI was used.

6.3 *Thresholds*

In order to determine the aerobic threshold (GET), data was individually edited to remove outlier data (more than 4 SD from the local mean) and aligned to the onset of RI. After that Wasserman method was applied. GET has been identified by visual inspection, by three independent expert reviewers and averaging their results as the $\dot{V}O_2$ at which CO_2 output ($\dot{V}CO_2$) began to increase out of proportion in relation to $\dot{V}O_2$ with a systematic rise in the minute ventilation ($\dot{V}E$)-to-$\dot{V}O_2$ relation and end-tidal PO_2 whereas the ventilatory equivalent of $\dot{V}CO_2$ ($\dot{V}E$ /$\dot{V}CO_2$) and end-tidal PCO_2 is stable (Beaver, Wasserman, and Whipp 1986)

On the basis of $\dot{V}O_{2max}$ and GET the PO used in the EXP2 (80%GET - 50%Δ) were defined. To define the PO, it was used the relation between PO and $\dot{V}O_2$ during

RI. The linear regression between the two parameters was applied and with the equation of the regression line the PO corresponding to 80%GET and to 50%Δ has been calculated.

6.4 *Kinetics parameters*

$\dot{V}O_2$ during EXP2 was measured breath by breath. Single data set was individually edited to obtain every second data from breath by breath data. Then linear interpolation second by second was made, through the Spline function (Hughson, Sherrill, and Swanson 1988) which allows to calculate the value of the parameters in the instants of time in which no breaths have been registered. Data were then examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1, PS1, BDC2 and PS2) and intensity of exercise.

On the single data set were calculated $\dot{V}O_2$ values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) averaging the last 30 seconds of each corresponding phase. It was also calculated the amplitude of unloaded pedalling (A_{UP}) as difference between UP_{SS} and R_{SS}. Moreover, the single data set was used for the analysis of $\dot{V}O_2$ kinetics at the onset of exercise. It was calculated net $\dot{V}O_2$ relating to the 360 seconds of exercise subtracting to each value of $\dot{V}O_2$ during exercise the value of UP_{SS}.

Next step was visual data fitting using the algorithm of Levenberg Marquardt (LM) specially implemented in Labview 8.2 (National Instrument. Austin. TX). LM is an interactive regression technique considered standard for solving multivariable non-linear problems, based on an exponential mathematical model with two (phase I and phase II) or three components (phase I, phase II and phase III). according to the intensity of exercise analyzed (MOD. two components – SEV. three components) [9] (Lador 2005; Whipp and Wasserman 1972). In this way have been obtained values of

the amplitude (A). time constant (τ) and time delay (TD) that corresponding to the best fit of the values of the data collected.

Equation used by LM are the subsequent:

$$Y(t) = H(t - TD_1)[A_1 (1 - e^{(t-TD_1)/\tau_1)}] + H(t - TD_2)[A_2 (1 - e^{(t-TD_2)/\tau_2)}] + H(t - TD_3)[A_3 (1 - e^{(t-TD_3)/\tau_3)}] \quad [1]$$

Where: Y(t) is $\dot{V}O_2$ during exercise. $A_1 - A_2 - A_3$ are amplitudes of first – second – third (if present) component. $\tau_1 - \tau_2 - \tau_3$ are time constants of first – second – third (if present) component, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). $TD_1 - TD_2 - TD_3$ are time delays of first – second – third (if present) component.

Referring to equation [1]. $H(t - TD_{1.2.3})$ is related to Heaviside function. defined as:

$$H(t - TD) = \begin{cases} 0 \; if \; t < TD \\ 1 \; if \; t \geq TD \end{cases} \quad [2]$$

It was calculated also mean response time (MRT), a parameter that returns an index of the speed of adjustment of the $\dot{V}O_2$. This index is useful in order to obtain indications regarding the time necessary to the oxidative metabolism to adapt at the variation of energy demands.

$$MRT = [(\tau_1 + TD_1 * A_1) + (\tau_2 + TD_2 * A_2) + (\tau_3 + TD_3 * A_3)]/(A_1 + A_2 + A_3) \quad [3]$$

Where: $A_1 - A_2 - A_3$ are amplitudes of first – second – third (if present) component. $\tau_1 - \tau_2 - \tau_3$ are time constants of first – second – third (if present) component. that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). $TD_1 - TD_2 - TD_3$ are time delays of first – second – third (if present) component.

Finally, the Gain, defined as the ratio between $\dot{V}O_2$ necessary to sustain a given mechanical output and the respective power (W) was calculated. As $\dot{V}O_2$ it was considered the difference between total amplitude (A_{TOT}) and of O_2 consumed at rest (R_{SS}). Gain was calculated as follows, distinguishing the two intensities of exercise:

$$Gain_{MOD} \; (mL/min/W) = A_{TOT}/Workload \; @ \; 80\%GET \quad [4]$$

$$Gain_{SEV} \; (mL/min/W) = A_{TOT}/Workload \; @ \; \Delta 50\% \quad [5]$$

6.5 *NIRS Parameters*

After collecting data second by second with NIRS (OxiplexTS™ – ISS Inc. – Champaign. IL, USA), data were exported with OxiTS™ software (OxiplexTS™ – ISS Inc. – Champaign. IL, USA). Data were then examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1, PS1, BDC2 and PS2) and intensity of exercise.

On the single data set were calculated concentration of deoxygenated hemoglobin [HHb], oxygenated hemoglobin [HbO], total hemoglobin ([THb]) and saturation (SAT) values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) and during exercise moderate (MOD_{SS}) or severe (SEV_{SS}), averaging the last 30 seconds of each corresponding phase.

Next step was visual data fitting of [HHb] data, using the algorithm of Levenberg Marquardt (LM) specially implemented in Labview 8.2 (National Instrument. Austin. TX), LM is an interactive regression technique considered standard for solving multi-variable nonlinear problems, based on an exponential mathematical model with two (phase I and phase II). according to the intensity of exercise analyzed (MOD, two components – SEV, three components). In this way have been obtained values of the amplitude (A). time constant (τ) and time delay (TD) that corresponding to the best fit of the values of the data collected.

Equation used by LM are the subsequent:

$$Y(t) = H(t - TD_1)[A_1 (1 - e^{(t-TD_1)/\tau_1}] + H(t - TD_2)[A_2 \left(1 - e^{(t-TD_2)/\tau_2}\right] \qquad [4]$$

Where: Y(t) is HHb during exercise. $A_1 – A_2$ are amplitudes of first – second (if present) component. $\tau_1 – \tau_2$ are time constants of first – second (if present) component, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). $TD_1 – TD_2$ are time delays of first – second (if present) component.

Referring to equation [4]. $H(t – TD_{1.2})$ is related to Heaviside function. defined as:

$$H(t - TD) = \begin{cases} 0 \; if \; t < TD \\ 1 \; if \; t \geq TD \end{cases} \quad [2]$$

It was calculated also mean response time (MRT_{HHb}) for MOD, a parameter that returns an index of the speed of adjustment of the HHb based on TD and τ.

$$MRT_{HHb} = \tau_1 + TD_1 \quad [5]$$

Where: τ_1 is time constants of first component, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). TD_1 is time delays of first component.

After that the randomization of the subjects was unveil to perform the appropriate matching (BR or PL) and the average and standard deviation were obtained.

6.6 *Δ[HHb]/Δ$\dot{V}O_2$ Ratio*

In order to get an index of matching of microvascular blood flow and O_2 distribution and muscle O2 utilization, Δ[HHb]/Δ$\dot{V}O_2$ Ratio was calculated. This ratio is characterized by an overshoot in the first seconds of exercise, during the on-transient phase. A reduction of overshoot A suggests a better matching of microvascular blood flow and O_2 distribution and muscle O_2 utilization (Murias et al. 2011).

To calculate the ratio the second-by-second amplitude of [HHb] (A_{HHb}) and amplitude of phase II (A_2) of $\dot{V}O_2$ kinetic data were normalized for each subject (0–100% of the response). Normalized A_2 was left shifted by TD_2 for each subject, to remove cardiodynamic phase so the onset of exercise coincided with the beginning of phase II of $\dot{V}O_2$ kinetic and is aligned with the beginning of [HHb] data signal. Data were further averaged into 5-s bins for statistical comparison of the rate of adjustment for [HHb] and $\dot{V}O_2$ kinetic. After that was calculated area under curve (AUC), from the beginning of the signal to 150s to ensure that both signal, [HHb] and $\dot{V}O_2$, had already reached 100% of their amplitude in MOD exercise (Murias et al. 2011), and that the signals are before the beginning of slow component phase in SEV exercise.

6.7 *Blood pressure (BP)*

After collecting data beat by beat with Portapres® (FMS, Amsterdam, The Netherlands) the data was exported using BeatScope® (FMS, Amsterdam, The Netherlands). Then linear interpolation second by second was made, through the Spline function (Hughson et al. 1988) which allows to calculate the value of the parameters in the instants of time in which no breaths have been registered. Data were then examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1, PS1, BDC2 and PS2) and intensity of exercise.

On the single data set were calculated systolic pressure (SYS), diastolic pressure (DIA), mean arterial pressure (MAP) and total peripheral resistance (TPR) values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) and during exercise moderate (MOD_{SS}) or severe (SEV_{SS}), averaging the last 30 seconds of each corresponding phase. After that the randomization of the subjects was unveil to perform the appropriate matching (BR or PL) and the average and standard deviation were obtained.

Due to signal troubles (artifacts, low quality - signal/noise ratio), basal data are averaged in order to obtain a unique more reliable value

6.7.1 Limits

In the acquisition of data with the Portapres® (PP) for the elderly, some signal problems have been found. The data were corrected by means of data collected by a parallel measurement carried out with both PhysioFlow® (PF) and Tango®. this in order to be sure that the estimate of the cardiac output with the PP was reliable, and for parallel measurement of arterial pressure at the brachial level. The correction coefficient was obtained and applied to the data obtained by PP. The pressure signal obtained from the PP beat by beat was calibrated through a factor obtained during R by a measure of independent brachial pressure (Tango monitor).

At R, a correction factor was calculated for cardiac output:

$$F_{COR} = Q_{PF}/Q_{PP} \quad [5]$$

Then the cardiac output signal was multiplied by the factor of correction (5)

$$CO_{Pp} = Q_{PP}*F_{COR} \quad [6]$$

Consequently, TPR were recalculated starting from the correct CO_{PP} signal (Tam et al. 2004)

6.8 *Statistics*

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, USA). After verifying the type of data distribution, using the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test, a two-way ANOVA test was applied, considering Age (Old and Young) and treatment (Pre and Post BR, Pre and PL), for repeated measurements.

Multiple comparison in the post-hoc analysis was performed using the Fisher Test LSD and, when appropriate, the recommended corrections for parametric data (Tukey, Bonferroni and Sidak).

Statistical significance was accepted for $P < 0.05$. The results are expressed as mean ± standard deviation (Mean ± SD). On main relevant data significantly different Cohen's d effect size was calculated. (Sawilowsky 2009)

7. Results – Nitrite and Nitrite concentrations

The first results that are reported are those related to the plasma concentration of nitrates [NO_3^-] and nitrites [NO_2^-].

7.1 *Old*

In elderly subjects supplementation with BR resulted in a significant increase ($P < 0.0001$) in [NO_3^-] compared to the concentrations found in the other conditions. Values of increasing are approximately 93.5% between Pre BR and Post BR (39.87 ± 22.55 µM vs 615.06 ± 317.38 µM), 92.8% between Post BR and Pre PL (615.06 ± 317.38 µM vs 44.03 ± 43.33 µM) and 86.5% between Post BR and PL (615.06 ± 317.38 µM vs 82.94 ± 35.52µM). As for [NO_3^-] also [NO_2^-] significantly increasing after BR compared to the other conditions. The increasing corresponds to 46.1% between Pre BR and Post BR (0.244 ± 0.01 µM vs 0.453 ± 0, 22 µM; $p = 0.0003$), 47% between Post BR and Pre PL (0.453 ± 0.22 µM vs 0.240 ± 0.21 µM, $p = 0.0131$) and 62.3% between Post BR and PL (0.453 ± 0.22 µM vs 0.171 ± 0.12 µM; $p = 0.0017$)

7.2 *Young*

BR supplementation in young has also resulted in a significant increase in plasma levels of both [NO_3^-] and [NO_2^-] in comparison to concentrations without BR.

In [NO_3^-] the improvements given by supplementation were: 92.4% between Pre BR and Post BR (24.32 ± 15.34 µM vs 321.56 ± 246.73 µM), 91.3% between Post BR and Pre PL (321.56 ± 246.73 µM vs 27.85 ± 27.35 µM) and 85.1% between Post BR and PL (321.56 ± 246.73 µM vs 47.73 ± 18.69 µM).

In [NO_2^-], the increases were 44.4% between Pre BR and Post BR (0.301 ± 0.09 µM vs 0.542 ± 0.24 µM, $p = 0.0099$), of 42, 9% between Post BR and Pre PL (0.542 ± 0.24 µM vs 0.309 ± 0.17 µM, $p = 0.0131$) and 52.7% between Post BR and PL (0.542 ± 0.24 µM vs 0.256 ± 0.19 µM; $p = 0.0017$).

OLD	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	39.87 ± 22.55	651.06 ± 317.38*	44.03 ± 43.33	82.94 ± 35.52
[NO_2^-] (µM)	0.244 ± 0.10	0.453 ± 0.22*	0.240 ± 0.21	0.171 ± 0.12

YOUNG	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	24.32 ± 15.34	321.56 ± 246.74*	27.85 ± 27.35	47.73 ± 18.69
[NO_2^-] (µM)	0.301 ± 0.09	0.542 ± 0.23*	0.309 ± 0.17	0.256 ± 0.19

***Table 3,4**: The table shows [NO_3^-] and [NO_2^-] in Old (up) and Young (down). * indicated differences from other condition, $p<0.05$.*

8. Results – $\dot{V}O_2$ kinetics

8.1 *Old*

Here are reported the result related to group old. Results are divided in MOD and SEV.

8.1.1 Moderate intensity

The effect of BR supplementation, in relation MOD exercise (80% GET; Workload @ 80% GET: 69 ± 24.9 W), is significant on the $\dot{V}O_2$ at steady-state (MOD_{SS} – Average of the last 30 seconds) with a statistically significant difference between the Pre BR and BR conditions (1395.5 ± 41.02 mL/min vs. 1324.8 ± 73.81 mL/min, p = 0.0420, ES = 1.184) equal to 70.7 mL/min (5.3%) (Figure 5).

The same occurs on $\dot{V}O_2$ of A_{UP} between Pre BR and BR (142.8 ± 52.65 mL/min vs. 91.9 ± 48.68 mL/min, p = 0.0081) with a variation of 50.9 mL/min (35.6%). Also $\dot{V}O_2$ of A_{TOT} show significant differences between Pre BR and BR (1030.5 ± 276.62 mL/min vs. 948.5 ± 240.55 mL/min, p = 0.0139) with a decrease of 82 mL/min (~ 8%) after treatment.

BR supplementation has positive effects on Gain (mL/min/W) showing reductions of 1.29 mL/min/W (9.1%) between Pre BR and BR values (15.5 ± 2.43 mL/min/W vs 14.2 ± 1.54 mL/min/W, p = 0.0022), and 1.01 mL/min/W (6.5%) between BR and Pre PL (14.2 ± 1.54 mL/min/W vs 15.2 ± 2.04 mL/min/W; p = 0.0265) (Figure 6).

There are no significant effects depending on the treatment of the kinetic parameters related to the cardiodynamic phase (A_1, τ_1, TD_1), and to the main phase (A_2, τ_2, TD_2).

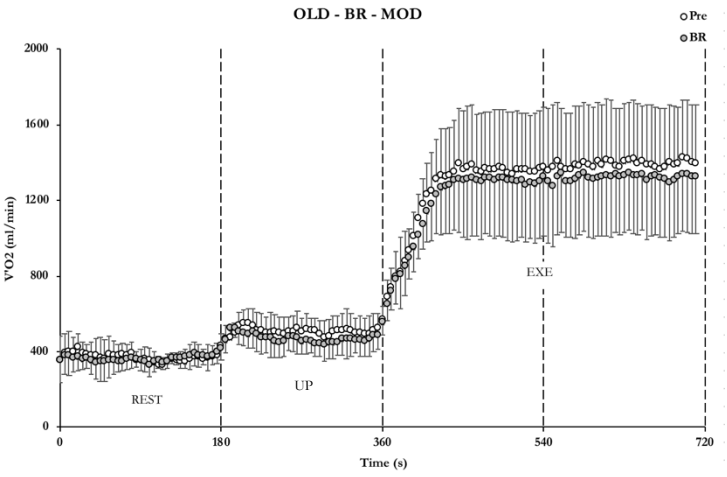
8.1.2 Severe Intensity

In old group during SEV (50%Δ; Workload @ 50%Δ: 152.7 ± 25.08 W), there are no particular differences in metabolic parameters in response to the step following BR supplementation.

Gain and net mechanical efficiency show no significant difference between the different experimental conditions.

OLD	MOD						SEV					
	BR			PL			BR			PL		
	Pre		Post	Pre		Post	Pre		Post	Pre		Post
EXE_{SS} (mL/min)	1395,53 ± 302,28	*°	1324,84 ± 296,84 °	1372,37 ± 249,42	*°	1369,42 ± 280,16 *°	2338,0 ± 283,38 °		2299,4 ± 308,03 °	2340,4 ± 301,48 °		2297,9 ± 284,84 °
A_2 (mL/min)	622,78 ± 270,09		562,00 ± 243,42	602,10 ± 196,94		651,19 ± 241,01	1344,92 ± 316,13		1326,38 ± 343,22	1332,56 ± 275,15		1326,66 ± 267,87
τ_2 (s)	21,89 ± 5,96		23,82 ± 6,93	23,70 ± 4,37		25,12 ± 4,79	31,64 ± 6,80		29,80 ± 4,68	33,31 ± 6,88		32,44 ± 6,60
TD_2 (s)	19,97 ± 6,29		18,79 ± 4,46	19,01 ± 6,71		17,31 ± 5,78	16,67 ± 4,63		16,33 ± 4,05	15,76 ± 6,32		15,29 ± 7,16
A_3 (mL/min)							165,8 ± 57,86 °		206,3 ± 58,48	180,6 ± 80,86 °		167,0 ± 72,44 °
τ_3 (s)							79,3 ± 23,11 °		83,6 ± 20,04	90,5 ± 17,40 °		93,9 ± 24,14 °
TD_3 (s)							192,1 ± 15,19		186,8 ± 13,83	184,6 ± 20,74		185,5 ± 10,06
MRT (s)	29,7 ± 6,33		29,8 ± 9,74	29,6 ± 7,39		31,6 ± 5,81	60,3 ± 8,59		64,3 ± 10,49 °	63,0 ± 11,61		60,9 ± 8,95
Gain (mL/min/W)	15,5 ± 2,43	*°	14,2 ± 1,54	15,2 ± 2,04	*°	15,1 ± 2,37 *°	12,7 ± 0,75		12,6 ± 0,80	12,7 ± 0,89		12,5 ± 0,72
[La] @ R (mM)	1,12 ± 0,37		1,14 ± 0,40	1,14 ± 0,36		1,07 ± 0,24	1,64 ± 0,97		1,49 ± 0,47	1,54 ± 0,70		1,47 ± 0,65

***Table 5**: $\dot{V}O_2$ kinetics in Old group.*
*Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). EXE_{SS} corresponding to the $\dot{V}O_2$ (mL/min) in the last 30 s of exercise. A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of the main phase, (2) and slow component (3), estimated through fitting analysis. A_{TOT} (mL/min) is the value of $\dot{V}O_2$ total amplitude at net of baseline, while A_{UP} (mL/min) is the portion of O_2 consumed during warm up phase at net of the $\dot{V}O_2$ detected at the state of ($A_{UP} = UP_{SS} - R_{SS}$). Gain (mL/min/W) is the net gain calculated as the ratio between A_{TOT} ($EXE_{SS} - R_{SS}$) (mL/min). [La] is lactate concentration at rest. Significance legend (P <0.05): * difference to BR condition, ° to Young*



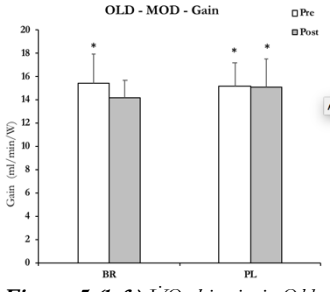


***Figure 5 (left)** $\dot{V}O_2$ kinetics in Old group, during MOD transition from rest to exercise.*
***Figure 6 (up)**: Gain (mL/min/W) in O during MOD.*
*Significance legend (P <0.05): * difference to BR condition*

8.2 *Young*

8.2.1 Moderate intensity

The effect of BR supplementation, in MOD (80% GET; Workload @ 80% GET: 122.1 ± 34.68 W), reduce significantly only TD_2 (Pre BR 16.6 ± 4.19 s vs BR 12.9 ± 4.37 s; p = 0.0498), that results anticipated by 3.7 s. There are no significant differences on the values of $\dot{V}O_2$ at MOD_{SS}, on kinetic parameters, and on Gain.

8.2.2 Severe Intensity

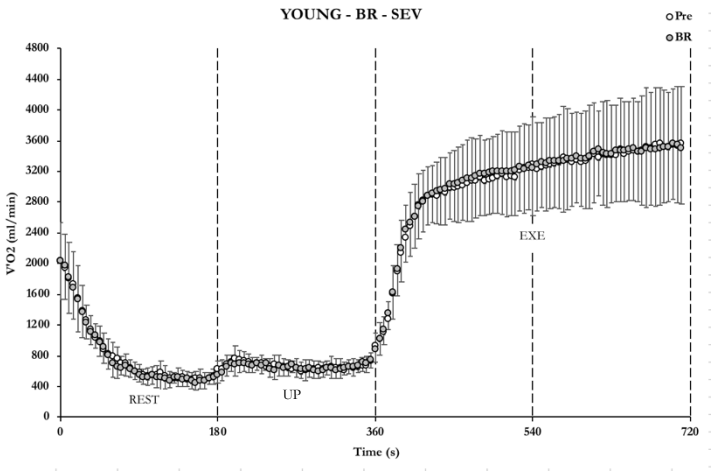
During SEV exercise (50%Δ; Workload @ Δ50%: 246.4 ± 48.22 W) there are no particular differences in metabolic parameters after BR related to the cardiodynamic phase (A_1, τ_1, TD_1), and to the main phase (A_2, τ_2, TD_2).

The only effects of BR can be observed on slow component phase (Phase III) where results a significant reduction of A_3 of about 18% between Pre BR and BR (381.2 ± 176.62 mL/min vs. 322.6 ± 172, 33 mL/min; p = 0.0341, ES = 0.335), with the same total O_2 consumption (SEV_{30s} – Average of the last 30 seconds).

Gain and net mechanical efficiency are not affected by any variation between the conditions before and after treatment with BR and PL.

YOUNG	MOD				SEV			
	BR		PL		BR		PL	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
EXESS (mL/min)	2112,5 ± 431,56	2096,2 ± 459,92	2080,6 ± 460,83	2067,4 ± 397,20	3508,7 ± 711,45	3502,9 ± 686,42	3469,4 ± 719,59	3427,0 ± 661,40
A2 (mL/min)	1051,49 ± 393,30	1068,54 ± 346,76	1081,30 ± 338,90	1109,51 ± 277,80	2052,67 ± 432,74	2088,84 ± 359,63	2070,63 ± 510,85	2051,62 ± 359,44
τ2 (s)	17,64 ± 5,36	20,63 ± 4,36	19,42 ± 3,87	19,04 ± 6,76	23,14 ± 4,09	24,25 ± 5,83	24,15 ± 3,82	23,21 ± 4,86
TD2 (s)	16,60 ± 4,19 *	12,93 ± 4,37	15,13 ± 4,26 *	14,88 ± 4,89 *	12,91 ± 3,52	11,99 ± 3,29	12,71 ± 2,94	12,83 ± 3,49
A3 (mL/min)					381,2 ± 176,62 *	321,6 ± 172,33	338,0 ± 145,55	315,2 ± 186,51
τ3 (s)					77,5 ± 15,73	75,2 ± 18,21	67,7 ± 6,98	65,2 ± 13,38
TD3 (s)					179,7 ± 11,13	183,4 ± 15,37	179,1 ± 9,27	185,3 ± 12,93
MRT (s)	25,6 ± 6,50	25,2 ± 5,82	26,7 ± 5,60	26,5 ± 5,54	59,4 ± 8,96	55,1 ± 8,88	56,2 ± 8,05	53,7 ± 8,82
Gain (mL/min/W)	13,7 ± 1,55	13,3 ± 1,60	13,6 ± 1,84	13,4 ± 1,10	12,1 ± 0,82	12,2 ± 0,70	11,7 ± 0,81	12,0 ± 0,90
[La] @ R (mM)	0,80 ± 0,21	1,02 ± 0,28	0,87 ± 0,26	1,12 ± 0,42	1,65 ± 0,54	1,78 ± 0,53	1,70 ± 0,65	2,00 ± 0,92

***Table 6**: $\dot{V}O_2$ kinetics in Young group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). EXE_{SS} corresponding to the $\dot{V}O_2$ (mL/min) in the last 30 s of exercise. A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of the main phase, (2) and slow component (3), estimated through fitting analysis. A_{TOT} (mL/min) is the value of $\dot{V}O_2$ total amplitude at net of baseline, while A_{UP} (mL/min) is the portion of O_2 consumed during warm up phase at net of the $\dot{V}O_2$ detected at the state of ($A_{UP} = UP_{SS} - R_{SS}$). Gain (mL/min/W) is the net gain calculated as the ratio between A_{TOT} ($EXE_{SS} - R_{SS}$) (mL/min). [La] is lactate concentration at rest.
*Significance legend (P <0.05): * difference to BR condition*



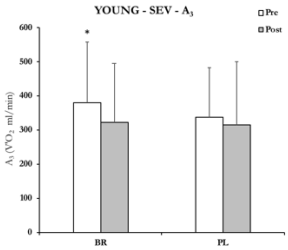


Figure 7 (left) *$\dot{V}O_2$ kinetics in Y group, during all SEV transition, with rest and UP phases.*
Figure 8 (up)*: A_3 (mL/min) in Y during SEV.*
*Significance legend (P <0.05): * difference to BR condition.*

8.3 *Young vs Old*

The results relative to the comparison between the two experimental groups (O and Y) were obtained with the statistical analysis Time x Age, within the same condition (Pre BR, BR, Pre PL and PL) transversely to age (O and Y). The values of the variables under examination are shown in brackets respecting the O-Y order.

8.3.1 Moderate intensity

In the MOD exercise domain, significant differences are found (P <0.0001) on MOD_{SS} $\dot{V}O_2$ values between O and Y in all experimental conditions (Pre BR: 1395.5 ± 302.28 mL/min vs. 2112.5 ± 431.56 mL/min; BR: 1324.8 ± 296.84 mL/min vs 2096.2 ± 459.92 mL/min; Pre PL : 1372.4 ± 249.4 mL/min vs 2080.6 ± 460.83 mL/min; PL: 1369.4 ± 280.16 mL/min vs 2067.4 ± 397.20 mL/min), reflecting the different work rate in terms of absolute mechanical power (Workload @ 80% GET: 69 ± 24.9 W and 122 ± 34.7 W).

This is also found in the values of $\dot{V}O_2$ defining A_2 (Pre BR: 622.8 ± 270.09 mL/min vs. 1051.5 ± 393.30 Ml/min, p = 0.0018; BR: 562.0 ± 243.42 mL/min vs. 1068.5 ± 346.76 mL/min, p = 0.0003; Pre PL: 602.1 ± 196.94 mL/min vs 1081.3 ± 338.90 mL/min, p = 0.0005; PL: 651.2 ± 241.01 vs 1109.5 ± 277.80 mL/min, p = 0.0009). There are no significant differences in τ_2 of despite average values of 4.4 s slower in O compared to Y (Pre BR: 21.9 ± 5.96 s vs 17.6 ± 5.36 s; BR: 23.8 ± 6.93 s vs 20.6 ± 4.36 s; Pre PL: 23.7 ± 4.37 s vs. 19.4 ± 3.87 s; PL: 25.1 ± 4.80 s vs. 19.0 ±

6.67 s). There is a difference of 5.8 s on the values of TD_2 in Post BR (18.8 ± 4.46 s vs. 12.9 ± 4.34 s; p = 0.0141), while in other conditions the delay times are similar between different ages (Pre BR: 21.9 ± 5.96 s vs 16.6 ± 4.19 s; Pre PL: 19.0 ± 4.46 s vs 15.1 ± 4.26 s; PL: 17.3 ± 5.78 s vs 15.1 ± 4.26 s).

The values of $\dot{V}O_2$ related to A_{UP}, even though averaging 25.1 mL/min more in Y, are not statistically significant in terms of differences between groups in all experimental conditions, with the exception of Pre PL (135.0 ± 56.63 mL/min vs. 198.8 ± 45.67 mL/min: p = 0.0050). Due to different mechanical power sustained by the two groups, the A_{TOT} of the $\dot{V}O_2$ are statistically different with a significance equal to p <0.0001 in all conditions (Pre BR: 1030, 5 ± 276.62 mL/min vs 1647, 8 ± 420.92 mL/min; BR: 984.5 ± 240.55 mL/min vs 1613.3 ± 428.19 mL/min; Pre PL: 1009.1 ± 228.49 mL/min vs 1657.0 ± 422.11 mL/min; PL: 1004.6 ± 254.43 mL/min vs 1618.6 ± 382.61 mL/min).

The results of Gain are affected by BR supplementation, showing BR values without significant differences between O and Y (14.2 ± 1.54 mL/min/W vs 13.3 ± 1.60 mL/min/W), unlike other experimental conditions (Pre BR: 15.5 ± 2.43 mL/min/W vs 13.7 ± 1.55 mL/min/W, p = 0.0338; Pre PL: 15.2 ± 2.04 mL/min/W vs 13.6 ± 4.26 mL/min/W, p = 0.0496; PL: 15.1 ± 2.83 mL/min /W vs 14.9 ± 4.89 mL/min/W, p = 0.0477) even if the values in BR are lower in both (O and Y).

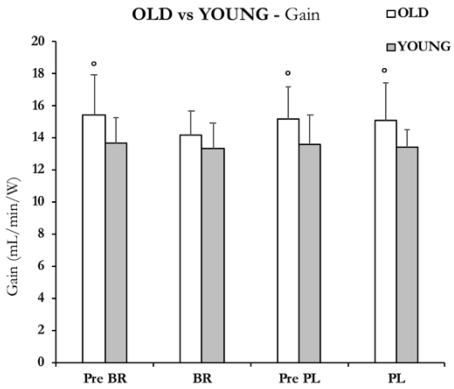


Figure 8; *Gain (mL/min/W) in O during MOD.*
Significance legend (P <0.05): ° difference to BR condition

8.3.2 Severe Intensity

Even in the SEV exercise domain the different mechanical power (Workload @ Δ50%: 152, 7 ± 25.08 W vs 246.4 ± 48.22 W) sustained by the two groups, determines $\dot{V}O_2$ values in operation significantly higher in Y compared to O. This is particularly

evident in SEV_{30s} in all experimental conditions with $p < 0.0001$ (Pre BR: 2338.0 ± 283.39 mL/min vs. 3508.7 ± 711.45 mL/min; BR: 2299.4 ± 308.04 mL/min vs. 3502.9 ± 686.42 mL/min; Pre PL: 2340.4 ± 301.48 mL/min vs 3469.4 ± 719.59 mL/min; PL: 2297.9 ± 284.84 mL/min vs 3427.0 ± 661.40 mL/min), in A_2 on all values, before and after treatments (Pre BR: 1344.9 ± 316.13 mL/min vs 2052.7 ± 432.74 mL/min; BR: 1326.4 ± 343.22 mL/min vs. 2088.8 ± 359.63 mL/min; Pre PL: 1332.6 ± 275, 15 mL/min vs. 2070.6 ± 510.85 mL/min; PL: 1326.7 ± 267.87 mL/min vs. 2051.6 ± 359.44 mL/min), and A_{TOT} (Pre BR: 1927.3 ± 283.28 mL/min vs 2997.0 ± 695.42 mL/min; BR: 1919.1 ± 283.28 mL/min vs 3008.0 ± 657, 95 mL/min; Pre PL: 1935.8 ± 282.99 mL/min and 2947.3 ± 702.55 mL/min; PL: 1903.7 ± 264.45 mL/min vs 2949.4 ± 631, 05 mL/min).

Time constant (τ_2) has statistically significant differences between O and Y (Pre BR: 31.6 ± 6.80 s vs 23.1 ± 4.09 s, $p = 0.0042$; Pre PL: 33.3 ± 6.88 s vs 24.2 ± 3.82 s, $p = 0.018$; PL: 32.4 ± 6.60 s vs. 23.2 ± 4.86 s, $p = 0.0016$) with the exception of BR (29.8 ± 4.68 s vs 24.2 ± 5.83 s) although there are 5.5 s more O. Furthermore, a difference of 4.3 s emerges from the values of TD_2 in BR (16.3 ± 4.05 s vs 12.0 ± 3.29 s; $p = 0.0405$).

9. Results – Peripheral oxygenation (NIRS)

Here are reported results of O group of the NIRS data ([HHb], [HbO], [THb] and SAT – [HHb] fitting parameters – AUC of $\Delta[HHb]/\Delta\dot{V}O_2$ ratio).

Results are divided by age, for each group of age the two different exercise intensities (MOD and SEV) are examined. Finally, a comparison between the different ages is made.

9.1 *Old*

Here are reported the result related to O group. Results are divided by intensities.

9.1.1 Moderate intensity

In group O during MOD exercise there are no significant differences due to BR considering the steady states of the four measurements made [HHb], [HbO], [THb] and SAT, in none of the phases of exercise, rest (R_{MSS}), unloaded pedalling (UP_{MSS}) and exercise (MOD_{SS}).

No significant differences are found even considering the parameters calculated through the fitting analysis of [HHb]. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

Finally, no significant differences are found even in the area under the curve (AUC) calculated after finding the $\Delta[HHb]/\Delta\dot{V}O_2$ ratio.

9.1.2 Severe Intensity

In group O during the SEV exercise, as occurs during the MOD exercise, there are no significant differences due to BR considering the stationary states of the four measurements made [HHb], [HbO], [THb] and SAT, in none of the phases of exercise, rest (R_{SSS}), free load (UP_{SSS}) and exercise (SEV_{SS}).

No significant differences are found even considering the parameters calculated through the fitting process of [HHb]. Amplitude (A_2) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

Finally, no significant differences are found even in the area under the curve (AUC) calculated after finding the $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio.

OLD		MOD				SEV			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SAT (%)	Rss	63,12 ± 6,76 °	64,75 ± 4,08 °	64,29 ± 5,53	63,98 ± 6,01	66,55 ± 6,31 °	68,35 ± 4,68 °	68,10 ± 5,36 °	67,91 ± 5,89 °
	UPss	66,27 ± 5,78 °	67,97 ± 4,40	67,69 ± 4,92	66,81 ± 4,82	70,53 ± 5,09 °	71,39 ± 3,15 °	72,02 ± 4,61 °	70,97 ± 4,45 °
	EXE	61,69 ± 5,84	61,83 ± 5,35	63,11 ± 5,03	62,97 ± 5,87	57,74 ± 7,47	57,54 ± 7,34	59,35 ± 8,41	58,57 ± 8,86

***Table 7**: NIRS data in Old group*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SAT is saturation
Significance legend (P <0.05): °difference to Y group.

HHb OLD	MOD				SEV			
	BR		PL		BR		PL	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A_1	8.67 ± 6.09	10.17 ± 8.24	9.76 ± 7.42	9.59 ± 8.06	14.74 ± 8.87	17.07 ± 12.34	16.76 ± 12.27	17.79 ± 14.04
τ_1 (s)	4.59 ± 1.19	5.04 ± 2.30	5.30 ± 2.67	3.71 ± 2.25	6.25 ± 2.80	6.02 ± 1.80	6.15 ± 2.57	7.52 ± 2.51
TD_1 (s)	9.53 ± 3.28	7.10 ± 4.48	8.22 ± 3.62	9.75 ± 2.91	5.50 ± 2.24	5.02 ± 2.98	4.95 ± 2.62	6.45 ± 3.91
MRT	14.12 ± 3.37	12.14 ± 5.14	13.52 ± 3.89	13.46 ± 3.16				
A_2 (mL/min)					1.51 ± 0.49	1.39 ± 0.69	2.51 ± 1.73	2.25 ± 2.03
τ_2 (s)					50.02 ± 33.77	29.00 ± 24.15	45.70 ± 21.32	37.51 ± 21.50
TD_2 (s)					182.19 ± 52.71	137.04 ± 63.52	137.95 ± 61.21	181.59 ± 78.67

***Table 8**: [HHb] kinetics parameters in Old group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of first (1), and second component (2), estimated through fitting analysis. MRT is mean response time, calculated as sum of τ_1 and TD_1

AUC OLD	BR		PL	
	Pre	Post	Pre	Post
MOD	23.72 ± 18.54	20.09 ± 6.30	20.47 ± 18.63	20.39 ± 10.46
SEV	20.34 ± 8.09	22.93 ± 9.43	21.61 ± 5.87	20.51 ± 11.36

***Table 9**: Area under curve in O group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). AUC is area under curve calculated after ⊿[HHb]/ΔV̇O 2 Ratio

9.2 *Young*

Here are reported the result related to Y group. Results are divided in MOD and SEV.

9.2.1 Moderate intensity

Similarly, to what happens in O also in Y there are no significant differences during MOD exercise in the stationary states of [HHb], [HbO], [THb] and SAT, in any of the phases of the exercise, R_{MSS}, UP_{MSS} and MOD_{SS}, and in the parameters of [HHb] calculated by the fitting. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

With regard to AUC of Δ[HHb]/ $\Delta\dot{V}O_2$ ratio, on the other hand, there is a tendency to decrease in the BR phase compared to the other 3 phases (BR 8.62 ± 10.73 vs Pre BR 11.38 ± 8.66, Pre PL 10.59 ± 5.81, PL 13.04 ± 7.55), but this difference is not significant, probably due to the high value of SD in the BR phase.

9.2.2 Severe Intensity

In Y, there are no significant differences during the SEV exercise in the stationary states of [HHb], [HbO] and [THb], in any of the phases of the exercise, R_{SSS}, UP_{SSS} and SEV_{SS}, and in the parameters of [HHb] calculated from the fitting. Amplitude (A_2) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

On the other hand, a statistically significant increase in SAT in the BR phase appears compared to the Pre PL and PL phases (BR 62.27 ± 9.32, vs Pre PL 57.85 ± 7.53, $p = 0.0184$, PL 56.67 ± 7.02, $p = 0.0013$), and an insignificant increase compared to Pre BR (BR vs Pre BR 62.27 ± 9.32 vs 60.60 ± 5.26) (Figure 9)

Finally, no significant differences are found in AUC calculated after finding the Δ[HHb]/$\Delta\dot{V}O_2$ ratio.

YOUNG		MOD				SEV			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SAT (%)	Rss	68,50 ± 2,85	69,07 ± 4,52	68,04 ± 4,39	67,09 ± 3,58	74,92 ± 3,73	74,52 ± 5,31	75,53 ± 3,46	73,17 ± 3,20
	UPss	70,39 ± 3,04	70,97 ± 3,98	70,21 ± 3,48	69,32 ± 3,26	76,23 ± 3,33	77,14 ± 4,23	76,15 ± 4,22	74,91 ± 2,64
	EXE	63,56 ± 4,03	61,74 ± 5,87	61,81 ± 5,38	61,16 ± 6,66	60,60 ± 5,26	62,27 ± 9,32	57,85 ± 7,53 *	56,67 ± 7,02 *

***Table 10**: NIRS data in Young group*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SAT is saturation.
*Significance legend (P <0.05): *difference to BR condition*

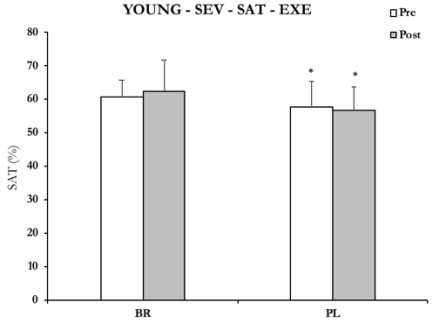


***Figure 9**: SAT during SEV in Young group*
*Significance legend (P <0.05): *difference to BR condition*

HHb YOUNG	MOD				SEV			
	BR		PL		BR		PL	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A_1	10.92 ± 6.61	12.50 ± 6.33	13.70 ± 7.39	14.01 ± 9.16	18.34 ± 10.49	18.72 ± 10.08	21.12 ± 10.89	21.73 ± 11.69
τ_1 (s)	5.45 ± 2.60	5.99 ± 1.87	6.39 ± 1.92	6.16 ± 4.68	7.60 ± 1.43	7.03 ± 2.47	7.73 ± 1.62	7.56 ± 1.88
TD_1 (s)	8.84 ± 2.23	7.42 ± 2.58	8.18 ± 3.00	7.36 ± 3.70	3.72 ± 2.02	3.66 ± 2.11	4.18 ± 2.32	4.01 ± 2.25
MRT	14.29 ± 3.02	13.41 ± 3.04	14.57 ± 2.37	13.53 ± 4.16				
A_2					2.80 ± 1.60	4.60 ± 2.43	3.18 ± 2.66	4.30 ± 5.26
τ_2 (s)					139.86 ± 84.70	139.16 ± 92.58	112.58 ± 73.57	82.61 ± 54.59
TD_2 (s)					111.44 ± 37.86	116.28 ± 44.54	114.07 ± 42.07	131.37 ± 52.99

***Table 11**: [HHb] kinetics parameters in Young group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of first (1), and second component (2), estimated through fitting analysis. MRT is mean response time, calculated as sum of τ_1 and TD_1

AUC YOUNG	BR		PL	
	Pre	Post	Pre	Post
MOD	11.38 ± 8.66	8.62 ± 10.73	10.59 ± 5.81	13.04 ± 7.55
SEV	14.17 ± 7.18	17.80 ± 6.15	13.79 ± 4.89	15.81 ± 6.51

***Table 12**: Area under curve in Y group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). AUC is area under curve calculated after ⊿[HHb]/ΔVO 2 Ratio

9.3 *Young vs Old*

The results relative to the comparison between the two experimental groups (O and Y) were obtained with the statistical analysis Time x Age, within the same condition (Pre BR, BR, Pre PL and PL) transversely to age (O and Y). The values of the variables under examination are shown in brackets respecting the O-Y order.

9.3.1 Moderate intensity

In the comparison of O and Y in the NIRS data during the MOD exercise, no significant differences emerge in the fitting parameters of [HHb] and in AUC calculated from the $\Delta[HHb]/\Delta\dot{V}O_2$ ratio. In AUC seems to be higher in O, but the difference between O and Y is not significant.

Instead, some significant differences emerge in the steady states of SAT during R in Pre BR and BR (Pre BR 63.12 ± 6.76 vs 68.50 ± 2.85 %, p = 0.0109; BR 64.75 ± 4, 08 vs 69.07 ± 4.52 %, p = 0.0404) and during UP in the Pre BR phase (66.27 ± 5.78 vs 70.39 ± 3.04%, p = 0.0242), with SAT in all conditions greater in Y compared with O.

There are also differences in [HHb] in R and UP in the Pre BR phase (R: 36.71 ± 9.54 vs. 27.23 ± 9.16, p = 0.0137; UP: 31.67 ± 6.99 vs 24.50 ± 7.57, p = 0.0140), with [HHb] greater in O in all conditions.

There are no significant differences between O and Y during exercise phase.

9.3.2 Severe Intensity

As in the MOD exercise, even in the SEV exercise there are no significant differences in the fitting parameters of [HHb] and in the AUC calculated from the $\Delta[HHb]/\Delta\dot{V}O_2$ ratio. In AUC seems to be higher in O, but the difference between O and Y is not significant.

There are significant differences, however, in SAT, which is greater in Y, in the steady states in R and UP in all experimental phases (R: Pre BR 66.55 ± 6.31 vs 74.92 ± 3.73 % p < 0.0001; BR 68.35 ± 4.68 vs 74.52 ± 5.31 % p = 0.0036; Pre PL 68.10 ± 5.36 vs 75.53 ± 3.46 %, p = 0.0005; PL 67.91 ± 5.89 vs 73.17 ± 3.20 %, p = 0.0127; UP: Pre BR 70.53 ± 5.09 vs 76.23 ± 3.33 %, p = 0.0019; BR 71.39 ± 3.15 vs 77.14 ±

4.23 %, p = 0.0018; Pre PL 72.02 ± 4.61 vs 76.15 ± 4.22 %, p = 0.0241; PL 70.97 ± 4.45 vs 74.91 ± 2.64, p = 0.0311)

In addition, there are significant differences in [HHb] during R and UP in the phases of Pre BR and BR (R: Pre BR 35.97 ± 10.44 vs 23.46 ± 8.60 p = 0.0012; BR 34.13 ± 9.04 vs 24.96 ± 7.59, p = 0.0171; UP: Pre BR 29.43 ± 6.90 vs 20.56 ± 5.91 p = 0.0024; BR 28.71 ± 6.14 vs 20.73 ± 4.65 p = 0.0064), with [HHb] greater in O in all conditions.

There are no significant differences between O and Y during exercise phase.

10. Results – Blood pressure

Here are reported results of O group of the observed parameters (SYS, DIA, MAP and TPR).

Results are divided by age, for each group of age the two different exercise intensities (MOD and SEV) are examined. Finally, a comparison between the different ages is made.

10.1 *Old*

Here are reported the result related to O group. Results are divided in MOD and SEV.

10.1.1 Moderate intensity

During MOD exercise in O, the main differences due to BR supplementation are observed in MAP (Pre BR vs BR 103.3 ± 7.1vs 98.35 ± 11 mmHg, p = 0.0249, ES = 0.535) with a reduction of ~5% and TPR (Pre BR vs BR 1.24 ± 0.22 vs 1.16 ± 0.21, p = 0.0458) with a reduction of ~6,5% during R phase. Always in R phase, in TPR there is also difference between BR and PL conditions (BR vs PL 1.16 ± 0.21 vs 1.25 ± 0.30, p = 0.0226) with TPR in BR slower ~6,5%.

In DIA differences after BR supplementation was observed only in UP (Pre BR vs BR 77.86 ± 11.80 vs 83.71 ± 9.10, p=0.0190).

In SYS there are no significant differences between conditions during MOD exercise.

10.1.2 Severe Intensity

During SEV exercise in O, differences due to BR supplementation are observed in SYS (Pre BR vs BR 220.96 ± 28.58 vs 210.59 ± 34.50 mmHg, p = 0.0336) with a reduction of ~4.7% during exercise.

In DIA there are no significant differences between conditions during SEV exercise

During SEV exercise in MAP during R phase there is a reduction of ~5,8% due to BR (Pre BR vs BR 105.84 ± 12.37 vs 99.70 ± 11 mmHg, p = 0.0056). In MAP there is also differences in UP after BR and versus PL condition (Pre BR vs BR 110.23 ±

14.53 vs 104.73 ± 11.22 mmHg, p = 0.0331, BR vs PL 104.73 ± 11.22 vs 112.62 ± 11 mmHg, p = 0.0025), and during exercise SEV (Pre BR vs BR 155.74 ± 23.38 vs 141.85 ± 20.12 mmHg, p <0.0001, BR vs PL 141.85 ± 20.12 vs 150.10 ± 22.58 mmHg, p = 0.026). During UP reductions are ~5% after BR and ~7% compared with PL. During SEV exercise reductions are ~9% after BR and ~5.5% compared with PL.

TPR have significant differences during R and UP, after BR and compared with PL (R: Pre BR vs BR 1.29 ± 0.39 vs 1.18 ± 0.26, ~8.5%, p = 0.0046; BR vs PL 1.18 ± 0.26 vs 1.26 ± 0.30, ~6.3 %, p = 0.0415 – UP: Pre BR vs BR 1.19 ± 0.26 vs 1.11 ± 0.25, ~6.7%, p = 0.0216; BR vs PL 1.11 ± 0.25 vs 1.18 ± 0.22, ~6 %, p = 0.0461).

OLD		MOD				SEV			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SYS (mmHg)	R_{SS}	138,77 ± 12,91	131,67 ± 12,29	138,77 ± 12,91	134,62 ± 14,25	141,45 ± 15,88	142,68 ± 18,17 °	141,45 ± 15,88	139,51 ± 15,98
	FW_{SS}	144,43 ± 17,87	152,34 ± 15,81 °	144,43 ± 17,87	146,53 ± 15,95 °	148,16 ± 20,73	151,77 ± 20,94	148,16 ± 20,73	151,19 ± 15,03
	EXE	141,45 ± 15,88 °	142,68 ± 18,17	141,45 ± 15,88 °	139,51 ± 15,98 °	220,96 ± 28,58 *°	210,59 ± 34,50 °	220,96 ± 28,58 *°	212,53 ± 30,24 °
DIA (mmHg)	R_{SS}	82,99 ± 4,31 °	85,32 ± 7,15 °	82,99 ± 4,31 °	82,47 ± 8,29 °	79,16 ± 12,96	80,66 ± 9,27	79,16 ± 12,96	80,77 ± 13,09
	FW_{SS}	77,86 ± 11,80 *°	83,71 ± 9,10	77,86 ± 11,80 *	80,61 ± 9,65	80,99 ± 13,08	83,46 ± 8,71	80,99 ± 13,08	83,14 ± 12,61
	EXE	84,40 ± 16,12	86,89 ± 11,86	84,40 ± 16,12	84,66 ± 18,91	103,43 ± 14,49	107,32 ± 10,00	103,43 ± 14,49	103,97 ± 13,93
MAP (mmHg)	R_{SS}	103,31 ± 7,11 *°	98,35 ± 11,00	103,31 ± 7,11 *°	101,56 ± 8,33 °	105,84 ± 12,37 *	99,70 ± 11,01	105,84 ± 12,37 *	103,72 ± 13,21
	FW_{SS}	106,09 ± 9,76	105,46 ± 10,94 °	106,09 ± 9,76	106,23 ± 9,72 °	110,23 ± 14,53 *	104,73 ± 11,22	110,23 ± 14,53 *	112,62 ± 11,00 *°
	EXE	118,95 ± 12,80	114,08 ± 14,66	118,95 ± 12,80	115,71 ± 18,53	155,74 ± 23,38 *°	141,85 ± 20,12	155,74 ± 23,38 *°	150,10 ± 22,58 *°
TPR	R_{SS}	1,24 ± 0,22 *°	1,16 ± 0,21 °	1,24 ± 0,22 *°	1,25 ± 0,30 *°	1,29 ± 0,39 *°	1,18 ± 0,26 °	1,29 ± 0,39 *°	1,26 ± 0,30 *°
	FW_{SS}	1,11 ± 0,23 °	1,11 ± 0,19 °	1,11 ± 0,23 °	1,14 ± 0,22 °	1,19 ± 0,26 *°	1,11 ± 0,25 °	1,19 ± 0,26 *°	1,18 ± 0,22 *°
	EXE	0,77 ± 0,16 °	0,72 ± 0,16 °	0,77 ± 0,16 °	0,73 ± 0,18 °	0,79 ± 0,15 °	0,77 ± 0,28 °	0,79 ± 0,15 °	0,73 ± 0,12 °

***Table 13**: Blood pressure in Old group*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre PI, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SYS is systolic pressure, DIA is diastolic pressure, MAP is mean pressure and TPR are total peripheral resistances.
*Significance legend (P <0.05): *difference to BR condition, °difference to young group*

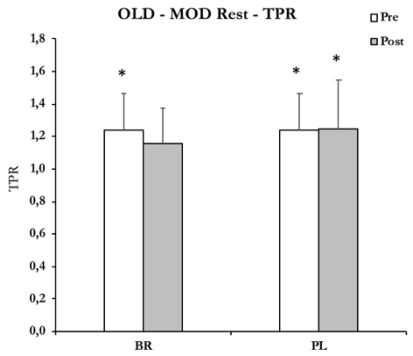


***Figure 10**: TPR during rest MOD in Old group*
*Significance legend (P <0.05): *difference to BR condition*

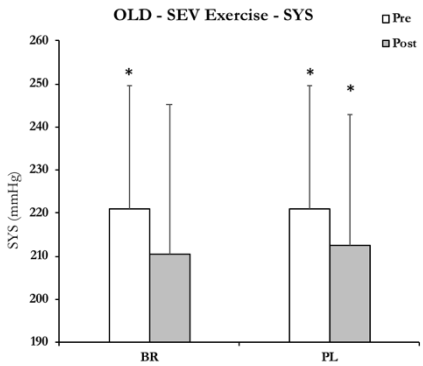


***Figure 11** SYS during SEV exercise in Old group. Significance legend (P <0.05): *difference to BR condition*

10.2 *Young*

Here are reported the result related to Y group. Results are divided in MOD and SEV.

10.2.1 Moderate intensity

During MOD exercise in young, there are significant differences only on TPR, during R and UP. During R the difference is between BR and PL (BR vs PL 0.84 ± 0.18 vs 0.93 ± 0.18, ~9.7 %, p = 0.0215). During UP there are differences in both, after BR and compared with PL (Pre BR vs BR 0.80 ± 0.18 vs 0.73 ± 0.18, ~8.7%, p = 0.0363; BR vs PL 0.73 ± 0.18 vs 0.83 ± 0.20, ~12 %, p = 0.0035).

There are no differences in SYS, DIA and MAP in various phases during MOD exercise.

10.2.2 Severe Intensity

During SEV exercise in young, there are significant differences after BR during UP in MAP (Pre BR vs BR 103.29 ± 8.94 vs 100.39 ± 9.02, ~3%, p = 0.0328) and compared with PL in TPR during R and UP (R: BR vs PL 0.78 ± 0.16 vs 0.86 ± 0.17, ~9.3 %, p = 0.0398 – UP: BR vs PL 0.67 ± 0.16 vs 0.75 ± 0.17, ~10.6 %, p = 0.016).

There are no differences in SYS and DIA in various phases during SEV exercise.

YOUNG		MOD				SEV			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SYS (mmHg)	R_{SS}	125,43 ± 12,10	124,03 ± 9,64	125,43 ± 12,10	122,17 ± 11,70	131,06 ± 12,42	127,87 ± 13,85	131,06 ± 12,42	126,97 ± 15,52
	FW_{SS}	133,03 ± 10,94	127,64 ± 13,34	133,03 ± 10,94	129,06 ± 13,24	141,17 ± 11,88	139,56 ± 12,46	141,17 ± 11,88	138,30 ± 13,79
	EXE	165,64 ± 13,89	160,25 ± 15,64	165,64 ± 13,89	163,51 ± 16,11	190,96 ± 11,96	191,80 ± 19,14	190,96 ± 11,96	187,11 ± 15,89
DIA (mmHg)	R_{SS}	74,03 ± 7,78	73,21 ± 7,63	74,03 ± 7,78	73,11 ± 6,66	77,37 ± 7,47	75,38 ± 9,38	77,37 ± 7,47	76,79 ± 7,44
	FW_{SS}	76,68 ± 7,97	72,39 ± 10,83	76,68 ± 7,97	74,78 ± 7,53	80,31 ± 7,75	77,91 ± 8,70	80,31 ± 7,75	79,19 ± 8,09
	EXE	87,24 ± 9,00	83,68 ± 9,21	87,24 ± 9,00	85,27 ± 7,39	102,32 ± 10,06	99,59 ± 9,38	102,32 ± 10,06	98,40 ± 9,89
MAP (mmHg)	R_{SS}	93,40 ± 9,38	91,86 ± 7,78	93,40 ± 9,38	91,81 ± 8,19	97,19 ± 8,68	93,97 ± 9,79	97,19 ± 8,68	95,17 ± 9,63
	FW_{SS}	98,16 ± 9,38	92,64 ± 11,34	98,16 ± 9,38	95,42 ± 9,14	103,29 ± 8,94 *	100,39 ± 9,02	103,29 ± 8,94 *	101,43 ± 10,27
	EXE	115,45 ± 10,57	110,72 ± 10,17	115,45 ± 10,57	113,27 ± 9,36	138,32 ± 10,32	133,98 ± 10,16	138,32 ± 10,32	133,53 ± 12,13
TPR	R_{SS}	0,90 ± 0,19	0,84 ± 0,18	0,90 ± 0,19	0,93 ± 0,18 *	0,79 ± 0,14	0,78 ± 0,16	0,79 ± 0,14	0,86 ± 0,17 *
	FW_{SS}	0,80 ± 0,18 *	0,73 ± 0,18	0,80 ± 0,18 *	0,83 ± 0,20 *	0,70 ± 0,15	0,67 ± 0,16	0,70 ± 0,15	0,75 ± 0,17 *
	EXE	0,49 ± 0,10	0,45 ± 0,07	0,49 ± 0,10	0,47 ± 0,08	0,44 ± 0,11	0,42 ± 0,08	0,44 ± 0,11	0,42 ± 0,09

***Table 13**: Blood pressure in Young group*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SYS is systolic pressure, DIA is diastolic pressure, MAP is mean pressure and TPR are total peripheral resistances.
*Significance legend (P <0.05): *difference to BR condition*

10.3 *Young vs Old*

The results relative to the comparison between the two experimental groups (O and Y) were obtained with the statistical analysis Time x Age, within the same condition (Pre BR, BR, Pre PL and PL) transversely to age (O and Y). The values of the variables under examination are shown in brackets respecting the O-Y order.

10.3.1 Moderate intensity

In the comparison between O and Y in the MOD exercise the main results to be highlighted are on SYS in the operating phase, on DIA and MAP during R and on TPR in all conditions.

SYS during MOD exercise phase in O reaches lower levels than Y and this difference is significant in all conditions (PreBR 141.45 ± 15.88 vs 165.64 ± 13.89, p = 0.0073; PrePL 141.45 ± 15.88 vs 165.64 ± 13.89 p = 0.0073, PL 139.51 ± 15.98 vs 163.51 ± 16.11 p = 0.0078) except that of BR, where the difference between O and Y is not significant (BR 142.68 ± 18.17 vs 160.25 ± 15.64).

DIA during R is at higher values in O than Y in all conditions (PreBR 82.99 ± 4.31 vs 74.03 ± 7.78, p = 0.0224; BR 85.32 ± 7.15 vs 73.21 ± 7.63, p = 0.0221; PrePL82.99 ± 4.31 vs 74.03 ± 7.78, p = 0.0224, PL 82.47 ± 8.29 vs 73.11 ± 6.66 p = 0.0171)

MAP during R in O reaches higher levels than Y and this difference is significant in all conditions (Pre BR 103.31 ± 7.11 vs 93.40 ± 9.38, p = 0.0365; Pre PL 103.31 ± 7.11 vs 93.40 ± 9.38, p = 0.0365; PL 101.56 ± 8.33 vs 91.81 ± 8.19, p =0.0395) except that of BR, where the difference between O and Y is not significant (98.35 ± 11.00 vs 91,86 ± 7,78)

TPR are always higher in O than Y, in all phases and conditions.

10.3.2 Severe Intensity

In the comparison between O and Y in the MOD exercise the main results to be highlighted are on SYS and MAP in the exercise phase, and on TPR in all conditions.

SYS during SEV exercise phase in O reaches higher levels than Y and this difference is significant in all conditions (PreBR 220.96 ± 28.58 vs 190.96 ± 11.96, p =

0.0009; BR 210.59 ± 34.50 vs 191.80 ± 19.14, p = 0.0366; PrePL 220.96 ± 28.58 vs 190.96 ± 11.96, p = 0.0009, PL 212.53 ± 30.24 vs 187.11 ± 15.89 p = 0.0049).

DIA has no significant differences in any phase and condition.

MAP during SEV exercise in O reaches higher levels than Y and this difference is significant in all conditions (Pre BR 155.74 ± 23.38 vs 138.32 ± 10.32, p = 0.0076; Pre PL 155.74 ± 23.38 vs 138.32 ± 10.32, p = 0.0076; PL 150.10 ± 22.58 vs 133.53 ± 12.13, p =0.0110) except that of BR, where the difference between O and Y is not significant (141.85 ± 20.12 vs 133.98 ± 10.16)

TPR are always higher in O than Y, in all phases and conditions.

11. General discussion

The present study aims to investigate the effects of NO_3^- supplementation, carried out by administration of beetroot juice (8.0 mmol of NO_3^- in 250 mL for 8 days), on metabolic responses due to the instantaneous onset of exercise in two intensity domains (Moderate and Severe). Effects on $\dot{V}O_2$ consumption and in cardiovascular system were explored, in elderly (O: 68 ± 4.6 years; n = 10) and young (Y: 25 ± 3.9 years; n = 10) subjects.

On $\dot{V}O_2$ kinetics the main results obtained can be summarized as follows: 1) improvement of efficiency in moderate exercise (80%GET) in the elderly (O); 2) reduction of the amplitude of the slow component in severe exercise (Δ50%) in young subjects (Y).

In the microvascular system, no clear results are observed, except a tendency to improve saturation in SEV in Y. There are no improvements in the matching between Δ[HHb] and $\Delta\dot{V}O_2$ and there are no clear changes in the parameters of [HHb] kinetics.

In blood pressure, main changes were observed in elderly subjects, where lower MAP and TPR were observed in R and UP phases during MOD exercise, and during exercise in SEV transition.

11.1 *Old*

11.1.1 O_2 consumption and NIRS

Following nitrate supplementation, significant increases in plasma NO_3^- and NO_2^- concentrations occur, with values similar to those reported in previous studies (Larsen et al., 2007; Webb et al., 2008; Vanhatalo et al., 2010).

The fact that the plasma levels of nitrite, closely related to the increase in the bioavailability of NO, are only slightly lower than those found in young people suggests that contrary to what is expected, age-related changes in oral bacterial colonization are poorly accentuated (Kelly et al., 2013)

One of the most relevant aspects emerging from the present study is the significant reduction of O_2 consumption at submaximal intensity following BR supplementation in the O group. The response of this parameter is in fact influenced by the treatment with nitrates both in the low-intensity UP phase (0 W - 30 rpm), and at the steady-state of the 80% GET square wave metabolic transition, with a reduction in

this last case of 5.3% compared to baseline. This is consistent with the size of the variation with as reported in the literature (Bailey et al., 2009; Vanhatalo et al., 2010; Larsen et al., 2011), although with different reference to the age of the sample as representative of the young population (26 ± 7 years).

From the energetic point of view, it should be considered that the reduction of $\dot{V}O_2$, and therefore of the resynthesis of ATP through oxidative phosphorylation (energetically more consistent process), is not compensated by an increase in the glycolytic contribution. In fact, the values of accumulation of blood lactate (ΔLa) show the absence of significant differences between the Pre and Post BR conditions.

As regards the parameters of the kinetics of the $\dot{V}O_2$, however, no changes in the time constant (τ_2) were found of Phase II in response to the metabolic transition in the moderate intensity domain. . Therefore, there were no reduction in the dependence of non-oxidative metabolic processes, unlike those reported by Kelly and colleagues, (Kelly et al., 2013) on elderly subjects (men: 64 ± 4 years, and women: 63 ± 2 years). Between Pre and post BR there seems to be a slight speeding of adaptation time (~ 2 s) of the $\dot{V}O_2$ to the metabolic perturbation given by the immediate onset of the exercise, despite the absence of statistical significance of the difference.

The mechanisms underlying what is reported, in particular the reduction of $\dot{V}O_2$ to steady state in moderate exercise, following the intake of NO_3^-, are currently unclear. However, the involvement of NO as a cellular signaling molecule in the modulation of a multiplicity of processes implicated in exercise physiology is well established, in particular on the regulation of endothelium-dependent vasodilatation, mitochondrial respiration and aspects of muscular contractility (Stamler et al. al., 2001). Given the results obtained in the condition under examination, a potential NO intervention is hypothesized, deriving from the sequence of exogenous nitrate reductions, at the cellular rather than the vascular level, in terms of traceability to a greater efficiency of the energy metabolism in the oxidation processes of substrates related to the synthesis of ATP. This is plausible if we consider the affinity of nitric oxide gas with mitochondrial cytochrome C oxidase, then its interaction with the terminal electron acceptor of the electron transport chain which, in physiological concentrations, causes transient inhibition of cellular respiration in competition with O_2 (Larsen et al. 2011). Therefore, a kinetic constraint is established. That in turn affects the reduction of ˙VO2 perceived by the cell as a mild hypoxia. This determines

a cascade of functional signals to the down regulation of the nucleotide adenine translocase (ANT) responsible for mitochondrial proton conductance. So a reduction of H+ loss, is established (Clerk et al., 2007; Hinkle, 2005).. In this regard, the best metabolic efficiency observed could be related to the reduction of energy dissipation of the proton gradient in heat. In this sense, slower τ_2 following NO_3^- supplementation, which may contribute to the contraction of the mild cell hypoxia necessary for the initiation of the P/O optimization processes, suggests a potential difference in sensitivity or latency between the mechanisms that regulate the adaptation of the system to the metabolic perturbations induced by the onset of the exercise starting from the rest condition. Furthermore, the vascular effect of NO_3^-, as the role of NO as secondary messenger in the synthesis of GMPc starting from GTP, able to modulate the relaxation of smooth arteriolar muscle (Ferguson et al., 2015; Jones 2014), could not to be highlighted because of the absence of important functional and structural impairment at the level of the peripheral district and of muscle perfusion. In fact, the values of the time constant of the elderly sample examined are lower than those reported in the literature in elderly adults (DeLorey 2004; Scheuermann et al., 2002). It should in fact be considered that the characteristics of the subjects recruited in the O group are only partially representative of the belonging population, due to the general state of good (ACSM, 2009) physical conditioning ($\dot{V}O_{2max}$ = 36 ± 7 mL/min/kg, for an average age of 67 ± 4.3 years old). Probably due to this condition the effect supplementation may be reduced and not consistent with previous work (Kelly et al., 2013).

The reduction of the consumed O_2 induced by BR supplementation consequently leads to a significant improvement in Gain in terms of reduction (6.5%) according to an effect size similar to that reported by Larsen et al., (Larsen et al., 2007), attesting an action of inorganic anions on the global economy of the system.

The absence of statistically significant differences on the parameters of the kinetics of $\dot{V}O_2$ in SEV exercise could be traced, in elderly subjects, to the physiological selective loss of rapid motor units (Granacher, Zahner, and Gollhofer 2008) and therefore to a lower consistency of the potential effect on of them of the NO_3^-. The literature supports, following studies carried out on young subjects, that the action of supplementation is appreciable in terms of improving the efficiency of type II fibers as regards the greater perfusion therefore the ability to use O_2 and with it to produce ATP

with greater efficiency given by the bioactivation of NO_3^- in conditions of tissue acidosis and relative hypoxia (Behnke et al., 2005; Breese et al., 2013; Ferguson et al., 2013). The results obtained by the present study, in relation to the high intensity exercise, do not show the effects of alteration of the efficiency given by the treatment as the absence, in fact, of improvements of this parameter. This could indicate that, unlike what occurs for slow fibers, rapid ones are not affected by NO_3^- mediation.

In NIRS data on vastus lateralis muscle no evident results were observed in O during MOD exercise. No significant differences were found at steady states for

SAT, [THb], [HbO] and [HHb], and also no improvements in the matching between Δ[HHb]/$\Delta\dot{V}O_2$ has been found. Parameters of the [HHb] kinetic did not show a net change. These results are in contrast with results of Bailey and colleagues (Bailey et al., 2009), that showed a reduction in [HHb] after 6 days of BR supplementation during moderate intensity exercise. Concerning muscle O_2 saturation, our results suggest that there were no changes in O_2 extraction required to perform moderate intensity exercise, despite the improvement in energy cost..

Considering this data in O during MOD there are no clear evidences on BR effect. Reduction of $\dot{V}O_2$ induced by the intake of NO_3^- could ultimately be attributed in part to effects on the intrinsic contractile properties of skeletal muscle in terms of reducing ATP expenditure necessary to support the production of force. One of the most expansive, in term of energy, process of muscle contraction is the pumping of Ca^{2+} from the sarcoplasmic reticulum channels, which can represent up to 50% of the total expenditure (Hernandez et al., 2012). The action of NO on sarcoplasmic reticulum ion channel receptors improve the management of intracellular Ca^{2+} transients and increase release of cytosolic Ca^{2+}, but also its re-uptake. A higher NO level protects the channels from the release of ions following oxidation, preventing the excess, and this is configured as an aspect that positively affects the energy cost of catch of Ca^{2+} (Haider et al., 2014).

11.1.2 Blood pressure

Aging causes inevitable adaptation of the organs and systems, with relative impairment of the metabolic and circulatory function with respect to the young person. The initial hypothesis of observing major changes in the most "compromised" organism was therefore confirmed.

During MOD, elderly showed a significant change in MAP, which on average decreased of 5mmHg, and a decrease in TPR (6.3%) after BR supplementation. There were no significant changes due to treatment in SYS. Similar reductions have also been reported in the literature, in particular Lee (Lee et al. 2015) described how 15-day BR juice supplement (6.4 mmol/day) favors the decrease of TPR and MAP both at rest and during all exercise intensities. Unlike our study, however, Lee also found a significant reduction in SYS values.

In the recovery phase after MOD (~55% $\dot{V}O_{2max}$), the TPR and MAP values were significantly different after BR, all the other variables have remained unchanged. Aerobic activity has caused A variation of the general homeostasis of the organism, with a consequent increase in oxygen transport and blood flow to the muscle in response to the metabolic needs imposed by the applied mechanical load. In this phase (post-exercise), the mechanism linked to the reduction of MAP and TPR is mainly attributable to the effects of NO_{3-} on the circle. We know that at the end of an exercise follows a recovery phase during which the flow of blood to the tissues remains high even if the mechanical request decreases. In this condition, the shear stress of the arteriolar wall is maintained, a mechanism for which NO release is stimulated. Nitric oxide is an intracellular signaling molecule that acts by generating a cascade of effects by activating guanil-cyclase in many cell types, increasing levels of cyclic GMP and making ion channels open (Pontieri, Venezuela, and Scavone 1998) This is the mechanism by which vasodilation is performed. After supplementation, being present at circulatory level a greater availability of nitrates (Larsen et al., 2007), the release of the vasodilator molecule should be facilitated causing a decrease in vascular resistance, increasing the flow to the tissue, which should be accompanied to an improvement of the metabolite wash-out. (Toth et al. 2007; Bentley et al. 2017).

In support of this effect we can see once again a significant reduction after BR of MAP and TPR in UP warm up before SEV exercise.

During SEV exercise, due to the treatment, we have a reduction of SYS and this reduction in SYS is reflected by reduction in MAP. The decrease of SYS is not due to the reduction of TPR, that result unchanged, so there may have been a reduction in cardiac output (CO) which is could explained by the reduction of heart rate (HR) (Note: HR data is not reported in thesis, but in this condition in O during SEV exercise results slower after BR – Pre BR 139.6 ± 14.1 vs BR 134 ± 21.5 bpm, p=0.0098).

Regarding this depressive effect after BR heart rate during severe exercise (EXE2) and we tried to advance a hypothesis explaining the significant reduction. In literature, it was seen that the inhibition of NO production is connected with a modulatory effect on the solitary tract nucleus and on the modulation of HR given by the baroreflex and therefore that this has a direct positive chronotropic effect (Pontieri, Venezuela, and Scavone 1998). Schneider and colleagues (Schneider et al. 2017) observing a reduction in pressure after muscular exercise with ischemia (useful for isolating the blood pressure response and evaluating without external conditioning if metaboreflex is present) suggest that the BR supplementation is able to attenuate muscle metaboreflex during exercise in elderly adults. Then, we can assume that our elderly subjects during BR while performing SEV exercise, may have an improvement in blood flow and, at the end of the exercise, they may have a greater wash-out of the metabolites and consequently attenuated metaboreflex. If the metaboreflex is attenuated, the stimulus induced by this on the nucleus of the solitary tract is reduced, consequently also the stimulus to the sympathetic SN and therefore the heart rate may be depressed.

11.2 *Young*

11.2.1 O_2 consumption and NIRS

Following nitrate supplementation, significant increases in plasma NO_3^- and NO_2^- concentrations occur, with values similar to those reported in previous studies (Larsen et al., 2007; Webb et al., 2008; Vanhatalo et al., 2010).

Differently from previous studies (Larsen et al., 2007; Bailey et al., 2009; Bailey et al., 2010; Vanhatalo et al., 2010), in the Y group there are no differences in the values of $\dot{V}O_2$ steady-state in moderate exercise dependent on BR supplementation and with them, Gain reductions or efficiency improvements. It is not clear which factors are due to the absence of effects of nitrate treatment on the kinetics of $\dot{V}O_2$, except for the reduction of Phase II delay time (TD_2) in BR compared to Pre BR, because the functional characteristics ($\dot{V}O_{2max}$) of the subjects recruited are similar on average to those of the experimental groups of previous works. Although in the sample of subjects a couple of them had a VO_{2max} greater than 60 ml/kg/min, which may be a factor that negatively affects the effect of supplementation (Porcelli et al., 2015). However,

even if these subjects are excluded from the analysis, different significance was not observed.

The physiological responses observed in high-intensity exercise suggest a possible influence of nitrates on the alteration of the amplitude of the $\dot{V}O_2$ kinetics. In fact, there is a slight increase of A_2 equal to about 1.6%, despite the difference is not significant, and the significant reduction of A_3 (18%), with average values of $\dot{V}O_2$ in the last 30 s of SEV exercise unchanged. In this condition, the energetic contribution by non-oxidative mechanisms is negligible, since the differences in the accumulation of blood lactate (ΔLa) are statistically devoid of significance.

In NIRS data on vastus lateralis during SEV exercise, no significant results are observed, in particular in τ_1 and on [HHb] at steady state. These results are in contrast with observation of Breese and colleagues (Breese et al. 2013) of faster τ_1, that suggest BR supplementation might have facilitated muscle O_2 extraction in transition from MOD to SEV exercise. In our results, this improvement does not appear. Our findings, instead, agree with another recent study of Breese (Breese et al. 2017) and colleagues in which appears that at high intensity cycling BR treatment does not alter the spatial heterogeneity of the dynamic balance between muscle O_2 delivery relative to O_2 utilization during heavy submaximal exercise (Breese et al. 2017). In the same study, however, authors observed that average [HHb] in three different muscle is significant higher after BR, but there are no differences if muscle are considered individually. So, probably, the observation of a single muscle may not be enough to measure an improvement in muscle O_2 utilization.

Another hypothesis, as a speculation based on literature, of a possible effect of nitrates on the increase of oxidative efficiency following the partial inhibition of cytochrome C oxygenase, with non-selective action on the slow motor units. In fact, the increase of A_2 could be due to the optimization of the P/O ratio of the type I fibers, in association with the progressive improvement of the same mechanism on the fast fibers, probably responsible for the reduction of A_3, although the reduction effect of NO_3^- on calcium channels of the sarcoplasmic reticulum in terms of reduction of energy expenditure related to the management of intracellular ions transients cannot be excluded.

11.2.2 Blood pressure

On Y subjects, it was founded only a slight reduction in TPR during UP prior to moderate exercise, then, vascular system in the early stages of exercise activates a tissue and muscle circulatory response that seems to benefit from BR supplementation. All the other subsequent phases of the protocol did not show significant differences. Literature data shows some effects on MAP and TPR on Y subjects (Lee et al. 2015), that are no evident in our conditions.

11.3 *Old vs Young*

11.3.1 O_2 consumption and NIRS

In comparison between subgroups, there are implications that assume functional relevance in O for a potential use of BR supplementation for the attenuation of the limitations to the exercise proper to aging.

According to the literature (DeLorey 2004; De Roia et al., 2012; Kelly et al., 2013), the data obtained in the present study confirm a slowing of the time constant of Phase II of the kinetics of $\dot{V}O_2$ according to age at the onset of exercise at the cycle ergometer. Compared to what reported in previous studies, the average values of τ_2 of O are inferior. Moreover, differences in τ_2, in MOD, are meaningless in all experimental conditions. These results probably are due high $\dot{V}O_{2max}$ of our sample of subjects.
There is, therefore, no greater dependence on no oxidative processes in the transition from a reduced metabolic rate to a higher one in elderly subjects than in uncompromising models such as young people. It should be emphasized that the action of nitrate in terms of "rejuvenation" of the Old metabolic system is detectable mainly, always with absence of significant differences between subgroups, on the Gain in MOD.

In NIRS data main difference in comparison between O and Y is observed in the SAT in the phase of R and of UP after the MOD exercise, where it is higher in the Y, indicating a faster recovery rate at the microvascular level in the Y compared to the O

11.3.2 Blood pressure

In comparison between O and Y subjects is necessary to underline how the MAP values in the elderly at rest, after supplementation, become comparable, approaching

the values of the young. In fact, in the Pre BR condition, we can notice a significant difference of ~10 mmHg (103.31 O vs 93.40 Y mmHg; p = 0.0365), while after supplementation the difference in basal MAP is reduced, although there remains a difference of ~5 mmHg (103.31 O vs 98.35 Y), losing its significance. Similar effects on MAP are observed during SEV exercise, with reduction after BR, that make difference with Y of ~8 mmHg (141.85 O vs 133.98 Y) without significance.

Basal SYS has no significant difference between O and Y in R and UP phases. During MOD exercise SYS is higher in Y, probably due to heavier load (69 O vs 122 Y mmHg) in Y than O. When intensity of exercise is SEV trend of SYS is opposite, higher in O then Y, although the load is always higher in Y(152,6 in O vs 246.4 in Y W), but probably near maximal intensity the effect of higher TPR in O is more influential.

TPR are always higher in O in comparison with Y, in all phases and intensities of exercise, confirming that the system is more "compromise" due to modifications related to age, like loss of stiffening and thickening of the arterial wall with the consequent reduction of the lumen of the duct, which determines the increase of the peripheral resistances.

The overall extent of improvement of vascular response following nitrate supplementation, seems indicative for the adoption of an appropriate diet that provides for the proper integration of foods rich in nitrates especially in the elderly person. The vascular advantage is also due to the subsequent metabolic advantage with consequent improvement in exercise capacity and fatigue tolerance.

Nitrate supplementation proves to be a useful means to promote vascular response in baseline conditions and in recovery phases after moderate stress.

11.4 *Study limits*

From the presented study emerge a series of non-negligible aspects that can be considered as limits in particular with respect to the size of the effect under examination and to the statistical power of the results.

Firstly, the reduced number of the subjects (Old: n = 10, Young: n = 10), in association with the inter-individual variability that characterizes each of the two subgroups, could prevent the achievement of statistical significance in the different comparisons between variables. In particular, elderly subjects, whose recruitment has not

always proved to be easy, tend to be unrepresentative of the population to which they belong in terms of their fitness level on average higher than that normally expected ($\dot{V}O_{2max}$, τ_2, MRT). They are physically active and they pay attention to the health benefits of exercise and diet, and these aspects may have reduced the potential impact of NO_3^- supplementation by masking its effects in particular on the vascular level. There may therefore have been limited opportunities for nitrates to positively influence exercise physiology due to insufficient impairment of the systems involved.

Any training effects that may have been induced by the overall duration of the experimental design were excluded from the randomization of the treatment assignment.

12. Conclusion

Nitrates supplementation to which the subjects recruited in the present study undergo has indeed determined an elevation of the plasma concentrations of this ion and consequently the bioavailability of NO of which it is a precursor.

This intervention in elderly, hypothesized able to influence the physiological responses to exercise, inducted a reduction in energy demand (5.3%) at the steady-state of the moderate exercise. In the same group, at high intensity exercise does not seem to benefit from the treatment resulting in an absence of changes in efficiency and kinetic parameters. The results obtained suggest that the mechanisms involved in the modulation of the responses to the exercise in relation to the higher bioavailability of nitrates are mainly cellular rather than vascular.

Young subjects seem to show a relative effect of nitrates on the development of the slow component in high-intensity exercise, which is reduced (18%) without modifying the overall energy demand.

On blood pressure, as we expected, main effects of nitrate supplementation are observed mainly in elderly subjects rather than in young subjects.

The modulation of the vascular response observed following the administration of beetroot juice has mainly affected rest and low intensity exercise (unloaded pedalling warm up), rather than during exercise. The changes were seen above regarding mean arterial pressure and total peripheral vascular resistance.

Young people do not seem to benefit from significant vascular effects but there is still a modulatory effect of mean resting arterial pressure.

Considering that the nitrates are part of our diet, for which they are constituted as NO reserves that can be implemented from the outside, it is possible to "facilitate" the production of NO based on the diet. An appropriate nutritional intervention through NO_3^- could be a useful strategy adopted in the elderly, natural and economic, with positive effects on fatigue tolerance, and able to encourage the practice of daily physical activities.

SECTION THREE

STUDY TWO

Nitrates supplementation and

Priming exercise

Summary of the section

In this section are analyzed effects of NO_3^-, through beetroot juice supplementation, and the combination with priming exercise, a high intensity warm-up, before a moderate intensity exercise. Experimental protocol includes two different transitions from unload to moderate intensities of exercise, with the second one anticipated by a severe intensity warm up.

After an introduction on priming effect, data of oxygen consumption, oxygen extraction of at the muscular level and blood pressure parameters in combination with beetroot supplementation are reported and analyzed.

List of abbreviations

NO_3^- Nitrates

NO_2^- Nitrites

NO Nitric oxide

BR Beetroot

PL Placebo

MOD Moderate intensity of exercise transition

SEV Severe intensity of exercise transition

MOD2 Moderate intensity of exercise transition after priming exercise

PE Priming exercise **NIRS** Non-invasive near-infrared spectroscopy

R Rest phase

UP Unloaded pedalling warm up phase

EXE Exercise phase

ES Effect size

$\dot{V}O_2$ Oxygen consumption

$\dot{V}CO_2$ Carbon dioxide production

A Amplitude of kinetics

TD Time delay of kinetics

τ Time constant of kinetics

[La] Lactate concentration

SAT Saturation of hemoglobin

[HHb] Concentration of deoxygenated hemoglobin

[HbO] Concentration of oxygenated hemoglobin

[THb] Total concentration of hemoglobin

SYS Systolic pressure

DIA Diastolic pressure

MAP Mean arterial pressure

TPR Total peripheral resistance

HR Heart rate

13. Introduction

13.1 *Priming effect*

Over the years the study of the metabolic response at exercise onset from resting state, $\dot{V}O_2$ kinetics, has been used as a paradigm to try to understand the mechanisms that control and/or limit the oxidative metabolism. The results obtained and the reflections on them have raised the question of where the main limitation was, whether at the central level (O_2 delivery) or at the peripheral level (O_2 utilization). The PE priming exercise (PE) is a tool to try and find an answer to this question.

PE is a high-intensity warm-up phase (> GET) that has been shown to modify the $\dot{V}O_2$ response in the subsequent transition from the resting state to moderate intensity exercise (Murias, Kowalchuk, and Paterson 2011; De Roia et al., 2012). This occurs in terms of an increase in the rate of regulation of oxidative metabolism and therefore in reduction of the time constant of phase II (Murias, Kowalchuk, and Paterson 2011; De Roia et al, 2012), in old population, but, also, in young adults that presenting initial values of $\tau > 20$ s (Scheuermann et al., 2002), these evidences, since, PE is believed to produce an acute improvement of oxygen delivery (Gerbino, Ward, and Whipp 1996) and muscle perfusion (DeLorey, Kowalchuk, and Paterson 2005; Brendon J Gurd 2005; B. J. Gurd et al. 2006), has been interpreted as indirect demonstration of a larger role of muscle O_2 delivery in the limitation of oxidative metabolism in older than in younger due to age characteristic decline of the oxidative capacity of the muscles (Granacher, Zahner, and Gollhofer 2008).

Gurd and colleagues, (Brendon J Gurd 2005) reports a similar effect in association with increased muscle oxygenation and mitochondrial complex activity of pyruvate dehydrogenase (PHD), highlighting that the enzymes involved in oxidative metabolism are subject to an acute, short-term adaptation, and with it that the metabolic inertia plays a role in limiting the energy system in question to the onset of physical exercise.

More recently (Murias et al., 2011; De Roia et al., 2012) it has been shown that the acceleration of the kinetics of the $\dot{V}O_2$ depends by an improvement in the matching between the delivery of O_2 to the muscle and its use (reduction Δ[HHb]/ $\dot{V}O_2$ Ratio), following PE, suggesting the importance of increasing muscle perfusion in improving adaptation to metabolic transition. Moreover, in study by De Roia and colleagues (De Roia et al., 2012) in old subjects were observed and opposite changes in the [HHb]

kinetics parameters, increase of time constant (τ_1) and reduction of time delay (TD_1) after PE.

According to Gerbino and colleagues, ((Gerbino, Ward, and Whipp 1996)), PE determines an increase in muscle flow following the vasodilatory effect given by local acidification, which is higher in the elderly than in the young. Other authors report, acutely to the PE, a rapid increase in the vascularization of the microcirculation dependent on endothelial vasodilation and flow mediated ((Gerbino, Ward, and Whipp 1996)). Based on this, factors related to both the transport and the use of O_2 are probably involved in the regulation of the kinetics of the $\dot{V}O_2$, although it seems that under conditions of time constants> 20 s the speed of adaptation is limited above all by the contribution of O_2 to the level of the microcirculation of active tissues (Murias, Kowalchuk, and Paterson 2011)

14. Materials and Methods

14.1 *Aim of the study*

The aim of this study is to investigate the effects of nitrate supplementation on muscle oxidative metabolism after PE cicloergometer.

The slowing of the $\dot{V}O_2$ kinetics in older adults is commonly attributed to limitations in muscle delivery of O_2 resulting in increased metabolic disturbance and reduced exercise tolerance. For this reason, the interest of the investigation is aimed at comparing the effects induced by nitrates compared to those obtained with the PE, since it is known that the latter acts by increasing the rate of regulation of oxidative metabolism in moderate work carried out after high-intensity exercise. If, unlike the PE, supplementation does not modify the parameters of the kinetics of the $\dot{V}O_2$, we hypothesize that the action of the nitrates is not predominantly located on the vascular mechanisms.

14.2 *Subjects*

The study participants were 20 volunteered, healthy, subjects divided in two groups: 10 old (67 ± 4.3 years) and 10 young (25 ± 3.9 years). During subjects' selection phase were recruited 28 men, but 4 refused to participate, 3 were excluded after preliminary medical examination and 1 drop out during first supplementation phase. The remaining 20 subjects participate in the study after given their informed and written consensus.

Inclusion criteria to participate at the study were: a normal clinical exam, absence of orthopedic, muscle-skeletal, metabolic, cardiovascular or respiratory pathology. ,

Exclusion criteria were: abnormal clinical exam, presence of orthopedic, muscle-skeletal, metabolic, cardiovascular or respiratory pathology, obesity (BMI ≥ 30 kg/m^2), the age limits.

All procedures were approved by the Department of Neurological and Movement Sciences' ethical committee for research on human subjects.

OLD	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
O1	75	182	88	2718	31	230	148	55	163
O2	63	180	72	2796	39	210	167	45	138
O3	65	182	91	2211	24	194	155	53	140
O4	65	173	77	2153	28	188	183	69	130
O5	67	161	63	2697	43	206	141	60	141
O6	72	172	71	2549	36	221	147	90	172
O7	71	166	66	2620	40	198	155	64	147
O8	67	164	58	1959	34	166	157	62	125
O9	61	163	58	2718	47	230	145	59	161
O10	65	176	78	3161	41	258	150	131	216
Mean	67	172	72	2558	36	210	155	69	153
St. Dev.	4.3	8.0	11.5	355.6	7.0	26.0	12.3	24.9	26.6

YOUNG	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
Y1	26	175	65	4082	62.8	404	183	181	285
Y2	27	183	85.5	4247	49.7	411	188	154	301
Y3	29	182	80	4501	56.3	414	170	161	324
Y4	29	173	70	4291	61.3	357	189	130	279
Y5	30	169	76.5	3256	42.6	270	186	100	207
Y6	25	169	66	3267	49.5	304	189	102	216
Y7	21	166	60	2673	44.6	242	190	80	172
Y8	20	178	66	3598	54.5	296	187	92	222
Y9	21	184	74	3551	48.0	327	190	132	238
Y10	22	173	69	3308	47.9	304	174	89	220
Mean	25	175	71	3677	52	333	185	122	246
St. Dev.	3.9	6.4	7.8	582.8	6.8	61.1	7.0	34.7	48.2

Table 1,2: *The table shows the individual data of subjects examined, Old (up) and Young (down). The values of age (Age, years) of the anthropometric parameters have been reported: height (Height, cm) and body mass (Weight, Kg), of the maximum metabolic power, absolute ($\dot{V}O_{2max}$, mL/min) and relative ($\dot{V}O_{2max}$, mL/min/Kg, , of the maximum mechanical power (Power max, W) and of the maximum heart rate (Hrmax, bpm) detected in the preliminary test, and of the Workloads (W) of the two intensity domains (Moderate: 80 % GET, and Severe: ⊿ 50%, W)*

14.3 *Study design and protocol*

The study has a double-blind crossover design with Nitrate (BR) or Placebo (PL) supplementation. The protocol included a preliminary day of test (D0) in which subjects performed a ramp incremental test (EXP1) and after that 3 testing days (D1. D2. D3) with $\dot{V}O_2$ kinetics tests (EXP3) repeated in 4 experimental phases (BDC1. PS1. BDC2. PS2).

In the first phase (BDC1) basal conditions were measured. In the second phase (PS1) the conditions after first period of supplementation (randomly selected between NO_3^- or PL) were recorded.

After at least 10 days of washout. the third phase was performed (BDC2) where basal conditions were measured again. In the fourth and last phase (PS2) the conditions after second period of supplementation (opposite of the first period) were determined.

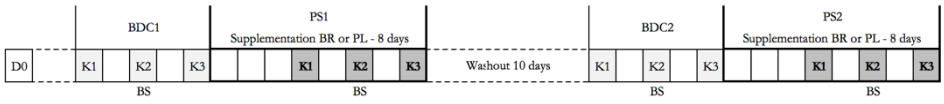


Figure 1: *The representation schematically summarizes order of test. After the preliminary evaluations (D0) follow the four experimental phases (BDC1, PS1, BDC2 and PS2) in each of which the kinetic evaluation protocol is repeated in non-consecutive days (K1, K2 and K3). All subjects underwent 8 days of supplementation with NO_3^- and PL, according to a balanced randomization. In PS1 and PS2 the kinetic evaluation protocol is repeated again. PS1 and BDC2 are separated by 10 days of washout. BS indicated blood sample, that is taken for the determination of the blood concentrations of nitrates and nitrites.*

14.3.1 Supplementation

The BR supplementation was made by beetroot juice (BR) (250 ml/day – Azienda agricola "Aureli" – Ortucchio (AQ) - Italy). The juice was provided in two different formulations: one with high concentration (~8.0 mmol) of NO_3^- and one with low concentration (~0.8 mmol) of NO_3^- (used as a placebo (PL)). The PL was identical in color, taste, smell and texture to the NO_3^- rich BR juice. Supplementation was distributed by an experimenter not involved in laboratory tests and/or in data analysis and the subjects and all the experimenters involved didn't know what supplementation was provided (if BR or PL). The matching of assumptions was known only at the end of data analysis.

This is considered a medium-term supplementation design that lasts for 8 days (Porcelli et al. 2015, Wylie et al. 2013).

with ingestion of a daily dose of 250 ml of juice before breakfast. The measurements of the kinetics started on the third day of treatment. The kinetics protocol took place on average 2.5/3 h after the supplementation. In each phase the same cadence of supplementation/test was repeated..

The subjects independently provided the supplementation following a sheet of instructions delivered to them. They were also warnings on foods to avoid rich in nitrates (spinach, beetroot, salad, rocket and Chinese cabbage) and to avoid the use of antibacterial mouthwash.

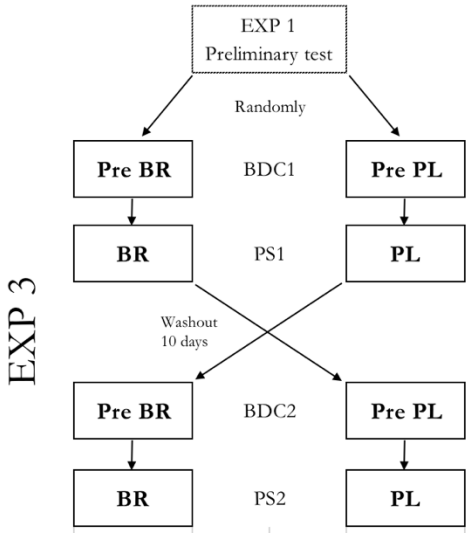


Figure 2: *The representation schematically summarizes the experimental design that structures the presented study, of a longitudinal type in a double-blind crossover. After the preliminary evaluations (EXP1) subjects randomly divided in two groups (BR or PL) and perform first two experimental phases (BDC1 and PS1).*
After 10 days of washout they crossed their condition and change supplementation and perform last two phases (BDC2 and PS2)

14.4 *EXP1 – Preliminary ramp incremental test*

To determine peak of oxygen consumption $\dot{V}O_{2max}$. gas exchange threshold (GET), power output (PO), power output peak (PO_{peak}) and maximal heart rate (HR_{max}) a ramp incremental (RI) test was performed.

RI protocol included 3 min of measurement of baseline condition, where subject remained sit on bike without moving. After that, the subject start cycling at 30 W(warm-up), for 3 min, with self-selected cadence. This cadence was recorded and was maintained during all subsequent tests using visual feedback and verbal encouragement from the experimenters. Warm-up was followed by RI protocol with different workload increments every minute (15, 20, 25, 30 W/min – 2W/8s, 2W/6s. 5W/12s. 3W/6s) in order to maintain entire test duration between 16 and 18 min. Test ended with exhaustion of the subject, and howeevr when the criteria for maximal test were reached ($\dot{V}O_2$ plateau, HR ~ HRmax, [la] >10mM). Failure to maintain the indicated cadence to within 5 rpm (for longer than 5s) during testing despite strong verbal encouragement was considered as the criterion for exhaustion.

In order to obtain a more reliable measure of $\dot{V}O_{2max}$ a verification trial test (VER) was also executed: after 2 min of recovery subjects start pedaling again at 20 W, after 5 min the workload was augmented to constant-work rate equal to 105% of the mechanical power achieved at the end of the ramp test until exhaustion. (Poole, Wilkerson and Jones AM. 2008)

14.5 *EXP2 – $\dot{V}O_2$ Kinetics test*

To measure physiological adaptations at the onset of exercise a $\dot{V}O_2$ kinetics (EXP2) test was assessed.

EXP2 was performed on cycle ergometer (Excalibur Sport – Lode B.V. – Groningen. The Netherlands) and the protocol provided two square wave transitions of 6 minutes duration, at the same moderate intensity (MOD and MOD2 – 80%GET). The second transition (MOD2), was performed after one step at severe intensity (SEV – 50%Δ). Tests were executed in three days (D1, D3, D5).

After 3 minutes of basal condition measurement, subjects start pedaling at 30 rpm (round per minute) for another 3 minutes to warm-up. At the 6th minute the moderate

intensity transition started: the workload became equivalent to 80% GET (80% GET represents the imposed mechanical load in order to reach a metabolic intensity of 80% of GET - gas exchange threshold-) .The subject kept his a fix pedaling cadence corresponding to that determined during the RI. The transition lasted 6 minutes. At the end the transition at 50%Δ was performed and at the thirtieth minute of exercise the moderate intensity transition MOD2 started again: the workload became equivalent to 80% GET. The transition lasted 6 minutes.

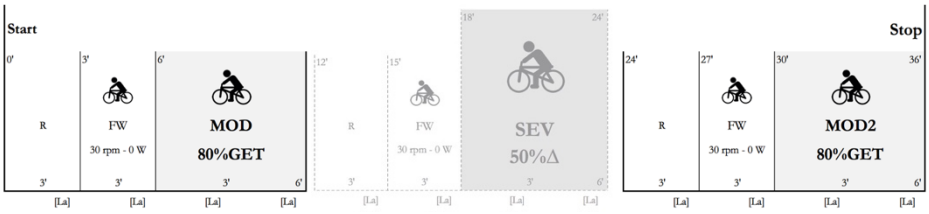


Figure 3: *The representation schematically summarizes the experimental protocol PE. [La] indicates the measurement of lactate concentrations in the last minute of each phase*

14.6 *Measures and instruments*

In all the tests the followingmeasurs were done:

- Pulmonary gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) (Quark CPET – Cosmed srl – Rome. Italy).
- Oxygenated [HbO] and deoxygenated [HHb] hemoglobin concentration on vastus lateralis muscle (VL) by Near InfraRed Spectroscopy (NIRS – OxiplexTS™ – ISS Inc. – Champaign. IL. USA)
- Blood pressure (Portapres® – Finapres medical system B.V. – Enschede. The Netherlands).
- Lactate [La] and Glucose [Glu] concentration (Biosen C-line – EKF Diagnostic – Barleben. Germany), by capillary blood collection (10 μL) from the earlobe performed every 3 minutes, 30s before changing phase.
- Blood samples were collected by venous sampling to (5 + 5 mL) glass EDTA tubes.

To perform the tests was used

- Cycle ergometer (Excalibur Sport cycle, Lode – Groningen, The Netherlands)

14.6.1 Quark CPET- Cosmed, Rome, Italy

Gas exchanges ($\dot{V}O_2$, $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) were measured breath-by-breath using the metabolimeter with a facial mask.

The concentrations of inhaled and exhaled gases were sampled at a frequency of 100 Hz via a capillary line connected to the mask and quantified by respectively paramagnetic analyzers for O_2 with response time of 120 ms and infrared rays (NDIR technology) for CO_2 with a response time of 100 ms. The measurement of the volume of the respiratory flows was carried out by a flowmeter consisting of a bidirectional digital turbine inside which a movable vanity unit, free to rotate around its axis, rotates at speed and in a direction proportional to the flow of air from which it is invested. The number of rotations was transduced into the parameters of interest by an optoelectronic system with infrared LED diodes based on the frequency of detection of the passage of the blades, integrated and processed by a microcomputer.

Prior to each test, the gas concentration and volume transducer analyzers of the turbine were calibrated using a mixture of a gas with known concentrations, according to the manufacturer's instructions, (FO_2: 0.16; FCO_2: 0.05) and a 3.0 L syringe. Concentration data e volume were aligned temporally, breath-by-breath, taking into account the delay in the passage of the gas to the capillary then the discrepancy between the time of acquisition of the signal by the analyzer and the flow meter, through the calibration of delays.

14.6.2 Portapres® – FMS, Amsterdam, The Netherlands

Non-invasive monitoring of the pressure profile was performed by continuous recording of the pressure pulse with cuff placed at the level of the phalanx distal of the middle or ring finger of the right hand using the photoplethysmographic method.

The mean arterial pressure values (MAP) were calculated as the mean of the integral of any data detected by the Beatscope software (FMS), making the correction for the height difference between the heart and the fingertips and the individual factors of the subject (anthropometric data, age, sex), as indicated by the manufacturer.

14.6.3 NIRS – OxiplexTS™ – ISS Inc. – Champaign. IL. USA

The changes in the oxygenation state at the level of the microcirculation of the muscular tissue of the lateral vastus were measured using a non-invasive method using NIRS (Near Infrared Spectroscopy) spectroscopy. This instrument detects in real time, at a sampling rate of 100Hz, the absolute (micromolar) concentrations of oxyhemoglobin [HbO], deoxyhemoglobin [HHb], total hemoglobin [THb] and tissue oxygenation index (SAT) whose values are expressed and analyzed, second by second, as average data. NIRS light is emitted in the muscle at wavelengths between 690 and 830 nm using light sources and receivers placed at distances of 1.50 - 3.04 cm, with the intake of cellular water at a constant concentration of 70%.

The NIRS probe was positioned after the treatment of the skin surface (degreased, slightly abraded and depilated), at the lower third of the vastus lateralis, calculated as the midpoint of the distance between large trochanter and lateral epicondyle of the femur of the right leg, secured with adhesive tape. Velcro and elastic straps were used to ensure no microspacing of the device and its isolation from external light, minimizing interference during acquisition.

The NIRS probe was calibrated before each test session using a calibration block with known absorption and dispersion coefficients of the known NIRS electromagnetic wave, a procedure performed according to the manufacturer's recommendations.

14.6.4 Lactacidometer

Blood lactate concentrations ([La], mM) and glucose ([Gly], mM) were detected on arterialized capillary blood samples (10 μL) taken from the earlobe. Values were obtained using an electrochemical system (Biosen C_line, EKF Diagnostic, Barleben, Germany).

14.6.5 Kit for blood samples

The evaluation of the plasma concentration of nitrates (NO_3^-) and nitrite (NO_2^-) was carried out on blood samples obtained by venous sampling (5 + 5 mL), for each of the experimental phases (BDC1, PS1, BDC2 and PS2). The intervention was conducted, before the experimental session, by medical staff.

The analysis of the samples, collected in glass tubes containing EDTA anticoagulant, was performed by Borgo Roma hospital chemical laboratory.

14.6.6 Excalibur Sport cycle, Lode – Groningen, The Netherlands

All the tests were performed on an electromagnetic brake cycle ergometer, connected and managed by the metabolimeter (Quark CPET - Cosmed, Rome, Italy).

The electromechanical characteristics of the ergometer allow the application of the workload in 50 ms. The signals of the pedaling frequency (rpm) and of the load (W) were digitized into parallel to a 16-channel analog-to-digital converter (MP100, Biopac Systems, Goleta, CA) and stored on a computer at a frequency of 100Hz.

15. Data analysis

15.1 *Nitrate and Nitrite concentrations*

The blood concentration of nitrates and nitrites was evaluated on plasma with a colorimeter kit (Nitrate/Nitrite Colorimetric Assay Kit - Cayman). The plasma fraction was prepared by ultra-filtration using filters with a 10 KDa cutoff (Amicon). For the test, 10 ul of filtrate were used and the supplier's indications were followed. The reading was done with a reader for 96-well plates at a wavelength of 540 nm (Gralis - Buoty Diagnostics)

15.2 *Maximal oxygen consumption ($\dot{V}O_{2max}$)*

During RI in D0 $\dot{V}O_{2max}$ was determined and it was calculated as the average of the $\dot{V}O_2$ recorded in the last 30 seconds before exhaustion. As maximal power output (PO_{peak}) was considered the last completed load before the end of test.

The results $\dot{V}O_{2max}$ was compared with one recorded during VER. $\dot{V}O_{2max}$ of VER was calculated as the average the $\dot{V}O_2$ recorded in the final 10 seconds before exhaustion. If the difference between two $\dot{V}O_{2max}$ was more of 100 ml$\dot{V}O_2$/min it was calculated average between them, otherwise $\dot{V}O_{2max}$ determined after RI was used.

15.3 *Thresholds*

In order to determine the aerobic threshold (GET), data was individually edited to remove outlier data (more than 4 SD from the local mean) and aligned to the onset of RI. After that Wasserman method was applied. GET has been identified by visual inspection, by three independent expert reviewers and averaging their results as the $\dot{V}O_2$ at which CO_2 output ($\dot{V}CO_2$) began to increase out of proportion in relation to $\dot{V}O_2$ with a systematic rise in the minute ventilation ($\dot{V}E$)-to-$\dot{V}O_2$ relation and end-tidal PO_2 whereas the ventilatory equivalent of $\dot{V}CO_2$ ($\dot{V}E$ /$\dot{V}CO_2$) and end-tidal PCO_2 is stable (Beaver, Wasserman, and Whipp 1986)

On the basis of $\dot{V}O_{2max}$ and GET the PO used in the EXP2 (80%GET - 50%Δ) were defined. To define the PO, it was used the relation between PO and $\dot{V}O_2$ during

RI. The linear regression between the two parameters was applied and with the equation of the regression line the PO corresponding to 80%GET and to 50%Δ has been calculated.

15.4 *Kinetics parameters*

$\dot{V}O_2$ during EXP2 was measured breath by breath. Single data was individually edited to obtain every second data from breath by breath data. Then linear interpolation second by second was made, through the Spline function (Hughson, Sherrill, and Swanson 1988) which allows to calculate the value of the parameters in the instants of time in which no breaths have been registered. Data were then examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1, PS1, BDC2 and PS2) and intensity of exercise.

On the single data set were calculated $\dot{V}O_2$ values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) averaging the last 30 seconds of each corresponding phase. It was also calculated the amplitude of unloaded pedalling (A_{UP}) as difference between UP_{SS} and R_{SS}. Moreover, the single data set was used for the analysis of $\dot{V}O_2$ kinetics at the onset of exercise. It was calculated net $\dot{V}O_2$ relating to the 360 seconds of exercise subtracting to each value of $\dot{V}O_2$ during exercise the value of UP_{SS}.

Next step was visual data fitting using the algorithm of Levenberg Marquardt (LM) specially implemented in Labview 8.2 (National Instrument. Austin. TX). LM is an interactive regression technique considered standard for solving multivariable non-linear problems, based on an exponential mathematical model with two (phase I and phase II) components (phase I, phase II) [1] (Lador et al.. 2006; Whipp & Wasserman. 1972). In this way have been obtained values of the amplitude (A). time constant (τ) and time delay (TD) that corresponding to the best fit of the values of the data collected.

Equation used by LM are the subsequent:

$$Y(t) = H(t - TD_1)[A_1 (1 - e^{(t-TD_1)/\tau_1)}] + H(t - TD_2)[A_2 (1 - e^{(t-TD_2)/\tau_2)}] \quad [1]$$

Where: Y(t) is $\dot{V}O_2$ during exercise. $A_1 - A_2$ are amplitudes of first – second component. $\tau_1 - \tau_2$ are time constants of first – second component, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). $TD_1 - TD_2$ are time delays of first – second – component.

Referring to equation [1]. $H(t - TD_{1.2.})$ is related to Heaviside function. defined as:

$$H(t - TD) = \begin{cases} 0 \; if \; t < TD \\ 1 \; if \; t \geq TD \end{cases} \quad [2]$$

It was calculated also mean response time (MRT), a parameter that returns an index of the speed of adjustment of the $\dot{V}O_2$. This index is useful in order to obtain indications regarding the time necessary to the oxidative metabolism to adapt at the variation of energy demands.

$$MRT = [(\tau_1 + TD_1 * A_1) + (\tau_2 + TD_2 * A_2)]/(A_1 + A_2) \quad [3]$$

Where: Y(t) is MRT. $A_1 - A_2$ are amplitudes of first – second component. $\tau_1 - \tau_2$ are time constants of first – second component, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). $TD_1 - TD_2$ are time delays of first – second – component.

Finally, the Gain, defined as the ratio between $\dot{V}O_2$ necessary to sustain a given mechanical output and the respective power (W) was calculated. As $\dot{V}O_2$ it was considered the difference between total amplitude (A_{TOT}) and of O_2 consumed at rest (BAS_{SS}). Gain was calculated as follows:

$$Gain_{MOD} \; (mL/min/\; W) = A_{TOT}/Workload \; @ \; 80\%GET \quad [4]$$

15.5 *NIRS Parameters*

After collecting data second by second with NIRS (OxiplexTS™ – ISS Inc. – Champaign. IL, USA), data were exported with OxiTS™ software (OxiplexTS™ – ISS Inc. – Champaign. IL, USA). Data were then examined in order to exclude artifacts

represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1, PS1, BDC2 and PS2) and intensity of exercise.

On the single data set were calculated concentration of deoxygenated hemoglobin [HHb], oxygenated hemoglobin [HbO], total hemoglobin ([THb]) and saturation (SAT) values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) and during exercise moderate (MOD_{SS}) or severe (SEV_{SS}), averaging the last 30 seconds of each corresponding phase.

Next step was visual data fitting of [HHb] data, using the algorithm of Levenberg Marquardt (LM) specially implemented in Labview 8.2 (National Instrument. Austin. TX), LM is an interactive regression technique considered standard for solving multi-variable nonlinear problems, based on an exponential mathematical model with two (phase I and phase II). In this way have been obtained values of the amplitude (A). time constant (τ) and time delay (TD) that corresponding to the best fit of the values of the data collected.

Equation used by LM are the subsequent:

$$Y(t) = H(t - TD_1)[A_1 (1 - e^{(t-TD_1)/\tau_1)}] \quad [5]$$

Where: Y(t) is HHb during exercise. A_1 is amplitudes. τ_1 is time constants, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). TD_1 are time delays.

Referring to equation [4]. $H(t - TD_1)$ is related to Heaviside function. defined as:

$$H(t - TD) = \begin{cases} 0 \; if \; t < TD \\ 1 \; if \; t \geq TD \end{cases} \quad [2]$$

It was calculated also mean response time (MRT_{HHb}), a parameter that returns an index of the speed of adjustment of the HHb based on TD and τ.

$$MRT_{HHb} = \tau_1 + TD_1 \quad [6]$$

Where: τ_1 is time constants, that represent time necessary to complete 63% of the total amplitude observed (Hughson et al. 1988). TD_1 is time delays.

After that the randomization of the subjects was unveil to perform the appropriate matching (BR or PL) and the average and standard deviation were obtained.

15.6 *Δ[HHb]/ΔV̇O₂ Ratio*

In order to get an index of matching of microvascular blood flow and O_2 distribution and muscle O2 utilization, $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio was calculated. This ratio is characterized by an overshoot in the firsts seconds of exercise, during the on-transient phase. A reduction of overshoot A suggests a better matching of microvascular blood flow and O_2 distribution and muscle O_2 utilization (Murias et al. 2011).

To calculate the ratio the second-by-second amplitude of [HHb] (A_{HHb}) and amplitude of phase II (A_2) of $\dot{V}O_2$ kinetic data were normalized for each subject (0–100% of the response). Normalized A_2 was left shifted by TD_2 for each subject, to remove cardiodynamic phase so the onset of exercise coincided with the beginning of phase II of $\dot{V}O_2$ kinetic and is aligned with the beginning of [HHb] data signal. Data were further averaged into 5-s bins for statistical comparison of the rate of adjustment for [HHb] and $\dot{V}O_2$ kinetic. After that area under curve (AUC)was calculated, from the beginning of the signal to 150s to ensure that both signal, [HHb] and $\dot{V}O_2$, had already reached 100% of their amplitude (Murias et al. 2011).

15.7 *Blood pressure*

After collecting data beat by beat with Portapres® (FMS, Amsterdam, The Netherlands) the data was exported using BeatScope® (FMS, Amsterdam, The Netherlands). Then linear interpolation second by second was made, through the Spline function (Hughson et al. 1988) which allows to calculate the value of the parameters in the instants of time in which no breaths have been registered. Data were then examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean.

After these analysis processes the data of the 3 repetitions of the 3 different days of the same experimental phase were aligned with the beginning of the rest (R) phase preceding each effort at constant load MOD and SEV and mediated in order to obtain, for each subject, a single data set for each experimental condition (BCD1. PS1. BDC2 and PS2) and intensity of exercise.

On the single data set were calculated systolic pressure (SYS), diastolic pressure (DIA), mean arterial pressure (MAP) and total peripheral resistance (TPR) values at steady state at rest (R_{SS}) and steady state during unloaded pedalling (UP_{SS}) and during exercise moderate (MOD_{SS}) or severe (SEV_{SS}), averaging the last 30 seconds of each corresponding phase. After that the randomization of the subjects was unveil to perform the appropriate matching (BR or PL) and the average and standard deviation were obtained. Due to signal troubles (artifacts, low quality - signal/noise ratio), basal data are averaged in order to obtain a unique more reliable value.

15.7.1 Limits

In the acquisition of data with the Portapres® (PP) for the elderly, some signal problems have been found. The data were corrected by means of data collected by a parallel measurement carried out with both PhysioFlow® (PF) and Tango®. this in order to be sure that the estimate of the cardiac output with the PP was reliable, and for parallel measurement of arterial pressure at the brachial level. The correction coefficient was obtained and applied to the data obtained by PP. The pressure signal obtained from the PP beat by beat was calibrated through a factor obtained during R by a measure of independent brachial pressure (Tango monitor).

At R, a correction factor was calculated for cardiac output:

$$F_{COR} = Q_{PF}/Q_{PP} \quad [5]$$

Then the cardiac output signal was multiplied by the factor of correction (5)

$$CO_{Pp} = Q_{PP}*F_{COR} \quad [6]$$

Consequently, TPR were recalculated starting from the correct CO_{PP} signal (Tam et al., 2004).

15.8 *Statistics*

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, USA). After verifying the type of data distribution, using the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test, a two-way ANOVA test was applied, considering PE (MOD and MOD2) and treatment (Pre and Post BR, Pre and PL), for repeated measurements.

Multiple comparison in the post-hoc analysis was performed using the Fisher Test LSD and, when appropriate, the recommended corrections for parametric data (Tukey, Bonferroni and Sidak).

Statistical significance was accepted for $P < 0.05$. The results are expressed as mean ± standard deviation (Mean ± SD). On main relevant data significantly different Cohen's d effect size was calculated. (Sawilowsky 2009)

16. Results – Nitrite and Nitrite concentrations

The first results that are reported are those related to the plasma concentration of nitrates [NO_3^-] and nitrites [NO_2^-].

16.1 *Old*

In elderly subjects supplementation with BR resulted in a significant increase ($P < 0.0001$) in [NO_3^-] compared to the concentrations found in the other conditions. Values of increasing are approximately 93.5% between Pre BR and Post BR (39.87 ± 22.55 µM vs 615.06 ± 317.38 µM), 92.8% between Post BR and Pre PL (615.06 ± 317.38 µM vs 44.03 ± 43.33 µM) and 86.5% between Post BR and PL (615.06 ± 317.38 µM vs 82.94 ± 35.52µM). As for [NO_3^-] also [NO_2^-] significantly increasing after BR compared to the other conditions. The increasing corresponds to 46.1% between Pre BR and Post BR (0.244 ± 0.01 µM vs 0.453 ± 0, 22 µM; $p = 0.0003$), 47% between Post BR and Pre PL (0.453 ± 0.22 µM vs 0.240 ± 0.21 µM, $p = 0.0131$) and 62.3% between Post BR and PL (0.453 ± 0.22 µM vs 0.171 ± 0.12 µM; $p = 0.0017$).

16.2 *Young*

As for elderly subjects, supplementation with BR in young has also resulted in a significant increase in plasma levels of both [NO_3^-] and [NO_2^-] in comparison to concentrations without supplementation.

In [NO_3^-] the improvements given by supplementation were: 92.4% between Pre BR and Post BR (24.32 ± 15.34 µM vs 321.56 ± 246.73 µM), 91.3% between Post BR and Pre PL (321.56 ± 246.73 µM vs 27.85 ± 27.35 µM) and 85.1% between Post BR and PL (321.56 ± 246.73 µM vs 47.73 ± 18.69 µM).

In [NO_2^-], the increases were 44.4% between Pre BR and Post BR (0.301 ± 0.09 µM vs 0.542 ± 0.24 µM, $p = 0.0099$), of 42, 9% between Post BR and Pre PL (0.542 ± 0.24 µM vs 0.309 ± 0.17 µM, $p = 0.0131$) and 52.7% between Post BR and PL (0.542 ± 0.24 µM vs 0.256 ± 0.19 µM; $p = 0.0017$).

OLD	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	39.87 ± 22.55	651.06 ± 317.38*	44.03 ± 43.33	82.94 ± 35.52
[NO_2^-] (µM)	0.244 ± 0.10	0.453 ± 0.22*	0.240 ± 0.21	0.171 ± 0.12

YOUNG	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	24.32 ± 15.34	321.56 ± 246.74*	27.85 ± 27.35	47.73 ± 18.69
[NO_2^-] (µM)	0.301 ± 0.09	0.542 ± 0.23*	0.309 ± 0.17	0.256 ± 0.19

***Table 3,4**: The table shows [NO_3^-] and [NO_2^-] in Old (up) and Young (down). * indicated differences from other condition, $p<0.05$.*

etabolic transitions in the moderate and subsequently with it (MOD2),

was obtained, for each studied group (O and Y) following the statistical analysis Time x Moderate, within the same condition (Pre BR and BR, Pre PL and PL).

The values of the variables under examination are shown in brackets respecting the order MOD and MOD2.

17.1 *Old*

17.1.1 Moderate

The effect of BR supplementation, in relation MOD exercise (80% GET; Workload @ 80% GET: 69 ± 24.9 W), is significant on the $\dot{V}O_2$ at steady-state (MOD_{SS} – Average of the last 30 seconds) with a statistically significant difference between the Pre BR and BR conditions (1395.5 ± 41.02 mL/min vs. 1324.8 ± 73.81 mL/min, p = 0.0420) equal to 70.7 mL/min (5.3%).

The same occurs on $\dot{V}O_2$ of A_{UP} between Pre BR and BR (142.8 ± 52.65 mL/min vs. 91.9 ± 48.68 mL/min, p = 0.0081) with a variation of 50.9 mL/min (35.6%). Also $\dot{V}O_2$ of A_{TOT} show significant differences between Pre BR and BR (1030.5 ± 276.62 mL/min vs. 948.5 ± 240.55 mL/min, p = 0.0139) with a decrease of 82 mL/min (~ 8%) after treatment.

BR supplementation has positive effects on Gain (mL/min/W) showing reductions of 1.29 mL/min/W (9.1%) between Pre BR and BR values (15.5 ± 2.43 mL/min/W vs 14.2 ± 1.54 mL/min/W, p = 0.0022), and 1.01 mL/min/W (6.5%) between BR and Pre PL (14.2 ± 1.54 mL/min/W vs 15.2 ± 2.04 mL/min/W; p = 0.0265).

There are no significant effects depending on the treatment of the kinetic parameters related to the cardiodynamic phase (A_1, τ_1, TD_1), and to the main phase (A_2, τ_2, TD_2).

17.1.2 Moderate 2

In MOD2 (80% GET, Workload @ 80% GET: 69 ± 24.9 W) carried out after the high metabolic effort (PE), BR effects are identified on the UP values (Pre BR 572.5 ± 80.10 mL/min vs. BR 536.7 ± 55.30 mL/min; p = 0.0345) according to a statistically significant difference of 35.8 mL/min (~6.6%) and on the $\dot{V}O_2$ at the

steady state $MOD2_{SS}$ which is reduced by 78.2 mL/min (~5.7%) after BR (Pre BR 1450.3 ± 329.01 mL/min vs BR 1372.1 ± 309.31 mL/min, p = 0.0257, ES = 0.245).

In MOD2 BR influence is also significantly visible A_2 that decreased by 83.8 mL/min (~15%) after BR (Pre BR 636.4 ± 288.38 mL/min vs. BR 552.6 ± 236.02 mL/min, p = 0.049), while no variation on Gain is found.

17.1.3 Priming effect (PE) - MOD vs MOD2

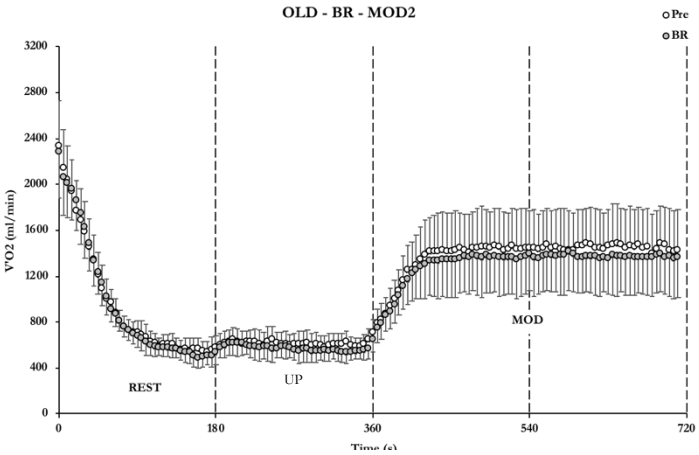
From the comparison between the values found in O in MOD and MOD2, it appears that the $\dot{V}O_2$ relative to R and UP is higher after PE in all experimental conditions (R: Pre BR: 365,0 ± 41,01 mL/min vs 572,5 ± 80,10 mL/min, p<0.0001; BR 376,4 ± 73,81 mL/min vs 536,7 ± 55,30 mL/min, p<0.0001; Pre PL 363,3 ± 53,58 mL/min vs 565,9 ± 76,30 mL/min, p<0.0001; PL 364,8 ± 45,82 mL/min vs 547,8 ± 78,03 mL/min, p<0.0001; UP: Pre BR: 507.9 ± 74.84 mL/min vs 609.2 ± 72.88 mL/min, p = 0.0042; BR: 468.3 ± 77.67 mL/min vs. 551.6 ± 60.95 mL/min, p = 0.0177; Pre PL: 498.2 ± 76.28 mL/min vs 569.3 ± 75.76 mL/min, p = 0.0420; PL: 498.3 ± 82.36 mL/min vs 589.0 ± 90, 15 mL/min, p = 0.0100).

There are no statistically significant differences between the values of steady states both before and after the treatments (Pre BR: 1395.5 ± 302.28 mL/min vs. 1450.3 ± 329.01 mL/min; BR: 1324.8 ± 296.84 mL/min vs. 1372.1 ± 309.31 mL/min; Pre PL: 1372.4 ± 249.4 mL/min vs 1432.5 ± 282.4 mL/min; PL: 1369.4 ± 280.16 mL/min vs. 1424.8 ± 295.09 mL/min) despite an average difference of 54.4 mL/min.

Absolute value of [La] is higher in all conditions after PE (Pre BR: 1,12 ± 0,37 mL/min vs 6,44 ± 2,11 mL/min, p<0.0001; BR 1,14 ± 0,40 mL/min vs 5,80 ± 1,91 mL/min, p<0.0001; Pre PL 1,14 ± 0,36 mL/min vs 6,07 ± 2,08 mL/min, p<0.0001; PL 1,07 ± 0,24 mL/min vs 5,83 ± 1,86 mL/min, p<0.0001).

OLD	MOD						MOD2					
	BR			PL			BR			PL		
	Pre	Post		Pre	Post		Pre	Post		Pre	Post	
EXEss (mL/min)	1395,5 ± 302,28	*°	1324,8 ± 296,84	°	1372,4 ± 249,42	*°	1369,4 ± 280,16	*°	1450,3 ± 329,01	*°	1372,1 ± 309,31	°	1432,5 ± 282,42	*°	1424,8 ± 295,09	*°
A2 (mL/min)	622,78 ± 270,09		562,00 ± 243,42		602,10 ± 196,94		651,19 ± 241,01		636,36 ± 288,38		552,65 ± 236,02		615,94 ± 253,45		607,38 ± 256,93	
τ2 (s)	21,89 ± 5,96		23,82 ± 6,93		23,70 ± 4,37		25,12 ± 4,79		22,21 ± 5,87		20,41 ± 4,91		19,69 ± 5,60		18,79 ± 6,45	#
TD2 (s)	19,97 ± 6,29		18,79 ± 4,46		19,01 ± 6,71		17,31 ± 5,78		13,77 ± 4,13		17,11 ± 4,96		15,93 ± 5,04		15,65 ± 5,35	
MRT (s)	29,7 ± 6,33		29,8 ± 9,74		29,6 ± 7,39		31,6 ± 5,81		27,2 ± 6,16		25,9 ± 5,68		25,1 ± 7,22		25,6 ± 6,31	
Gain (mL/min/W)	15,5 ± 2,43	*°	14,2 ± 1,54		15,2 ± 2,04	*°	15,1 ± 2,37	*°	12,9 ± 1,24	#	12,3 ± 1,55		12,9 ± 1,44	#	13,2 ± 2,40	#
[La] @ R (mM)	1,12 ± 0,37		1,14 ± 0,40		1,14 ± 0,36		1,07 ± 0,24		6,44 ± 2,11	#	5,80 ± 1,91	#	6,07 ± 2,08	#	5,83 ± 1,86	#

***Table 5**: $\dot{V}O_2$ kinetics in Old group.*
Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). EXE_{SS} corresponding to the $\dot{V}O_2$ (mL/min) in the last 30 s of exercise. A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of the main phase, (2) and slow component (3), estimated through fitting analysis. A_{TOT} (mL/min) is the value of $\dot{V}O_2$ total amplitude at net of baseline, while A_{UP} (mL/min) is the portion of O_2 consumed during warm up phase at net of the $\dot{V}O_2$ detected at the state of (A_{UP} = UP_{SS} - R_{SS}). Gain (mL/min/W) is the net gain calculated as the ratio between A_{TOT} (EXE_{SS} - R_{SS}) (mL/min). [La] is lactate concentration at rest.
*Significance legend (P <0.05): * difference to BR condition, ° to Young , # to MOD*



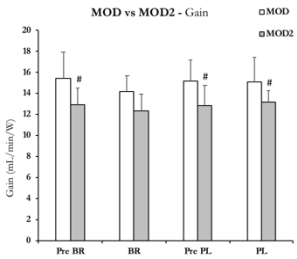


***Figure 5 (left)** $\dot{V}O_2$ kinetics in Old group, during all MOD2 transition, with R and UP phase*
***Figure 6 (up)**: Gain (mL/min/W) in O pre PE e PE (MOD vs MOD2)*
Significance legend (P <0.05): # difference to MOD

17.2 *Young*

17.2.1 Moderate

The effect of BR supplementation, in MOD (80% GET; Workload @ 80% GET: 122.1 ± 34.68 W), reduce significantly only TD_2 (Pre BR 16.6 ± 4.19 s vs BR 12.9 ± 4.37 s; p = 0.0498), that results anticipated by 3.7 s. There are no significant differences on the values of $\dot{V}O_2$ at MOD_{SS}, on kinetic parameters, and on Gain.

17.2.2 Moderate 2

In Y in MOD2 (80% GET, Workload @ 80% GET: 122.1 ± 34.6 W) carried out after the high metabolic effort (PE), there are no evident effects after BR, neither on steady states at end of exercise neither ok kinetics parameters neither on Gain.

17.2.3 Priming effect - MOD vs MOD2

In the Y, results that emerge from the comparison between the values found in MOD and MOD2 show statistically significant differences on $\dot{V}O_2$ in R and UP in all experimental conditions (R: Pre BR: 464,8 ± 64,69 mL/min vs 806,9 ± 180,49 mL/min, p<0.0001; BR 482,8 ± 83,69 mL/min vs 811,4 ± 201,73 mL/min, p<0.0001; Pre PL 423,5 ± 76,47 mL/min vs 810,3 ± 215,88 mL/min, p<0.0001; PL 448,8 ± 48,80 mL/min vs 784,1 ± 187,16 mL/min, p<0.0001; UP: Pre BR: 644.0 ± 48.18 mL/min vs 831.2 ± 163.40 mL/min, p = 0.0054; BR: 616.1 ± 66.44 mL/min vs. 810.6 ± 143.60 mL/min, p = 0.0036 Pre PL: 622.3 ± 79.64 mL/min vs. 805.6 ± 178.59 mL/min, p = 0.0066; PL: 592.7 ± 53.84 mL/min vs 796.2 ± 175.21 mL/min, p = 0.0021).

The values steady states between MOD and MOD2 in all experimental conditions are similar (Pre BR: 2112.5 ± 431.56 mL/min vs. 2230.9 ± 455.66 mL/min; BR : 2096.2 ± 459.92 mL/min vs 2205.4 ± 475.55 mL/min, Pre PL: 2080.6 ± 460.83 mL/min vs 2176.5 ± 474.08 mL/min; PL : 2067.4 ± 397.20 mL/min vs 2205.5 ± 454.16 mL/min), as well as those of A_2 (Pre BR: 1051.5 ± 393.30 mL/min vs 1011.4 ± 314.71 mL/min; BR: 1068.5 ± 346.76 mL/min vs. 1100.7 ± 299.10 mL/min; Pre PL: 1081.3 ± 338.90 mL/min vs 986.4 ± 307.40 mL/min; PL: 1109.5 ± 277.80 mL/min vs. 1040.9 ± 285.37 mL/min) and A_{TOT} (Pre BR: 1647.8 ± 420.92 mL/min vs. 1423.9 ± 335.26 mL/min; BR: 1613.3 ± 428.19 mL/min and 1394.1 ± 317.55

mL/min; Pre PL: 1657.0 ± 422.11 mL/min and 1366.2 ± 340.13 mL/min; PL: 1618.6 ± 382.61 mL/min vs. 1421.3 ± 318.96 mL/min).

There are statistically significant differences, instead, between τ_2 with an average reduction of 4.9 s following the PE (BR: 20.6 ± 4.36 s vs 16.2 ± 2.78 s, p = 0, 0388; Pre PL: 19.4 ± 3.87 s vs. 14.7 ± 3.53 s, p = 0.0274; PL: 19.0 ± 6.76 s vs. 13.7 ± 4.59 s, p = 0.0120), except for the condition of Pre BR in which the values are similar (17.6 ± 5.36 s vs 16.7 ± 4.85 s). TD_2 significantly decreases in MOD2 in PreBR (16.6 ± 4.19 s vs 11.9 ± 3.32 s; p = 0.0095) and in BR (12.9 ± 4.37 s vs 9.4 ± 2.52 s; p = 0.0489).

Gain values are significantly decreased after PE in all experimental conditions (Pre BR: 13.7 ± 1.55 mL/min/W vs 11.9 ± 1.48 mL/min/W, p = 0.0073; BR: 13.3 ± 1.60 mL/min/W vs 11.6 ± 1.24 mL/min/W, p = 0.0098; Pre PL: 13.8 ± 1.84 mL/min/W vs 11.3 ± 1.48 mL/min/W, p = 0.0004; PL: 13.4 ± 1.10 mL/min/W vs 11.8 ± 1.28 mL/min/W, p = 0.0172).

Absolute value of [La] is higher in all conditions after PE (Pre BR: 0,80 ± 0,21 mL/min vs 9,11 ± 2,67 mL/min, p<0.0001; BR 1,02 ± 0,28 mL/min vs 9,26 ± 2,67 mL/min, p<0.0001; Pre PL 0,87 ± 0,26 mL/min vs 8,59 ± 2,93 mL/min, p<0.0001; PL 1,12 ± 0,42 mL/min vs 8,68 ± 3,21 mL/min, p<0.0001)

YOUNG	MOD				MOD2			
	BR		PL		BR		PL	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
EXE_{SS} (mL/min)	2112,5 ± 431,56	2096,2 ± 459,92	2080,6 ± 460,83	2067,4 ± 397,20	2230,9 ± 455,66	2205,4 ± 475,55	2176,5 ± 474,08	2205,5 ± 454,16
A_2 (mL/min)	1051,49 ± 393,30	1068,54 ± 346,76	1081,30 ± 338,90	1109,51 ± 277,80	1011,37 ± 314,71	1100,68 ± 299,10	986,44 ± 307,40	1040,85 ± 285,37
τ_2 (s)	17,64 ± 5,36	20,63 ± 4,36	19,42 ± 3,87	19,04 ± 6,76	16,65 ± 4,85	16,25 ± 2,78 #	14,73 ± 3,53 #	13,68 ± 4,59 #
TD_2 (s)	16,60 ± 4,19 *	12,93 ± 4,37	15,13 ± 4,26 *	14,88 ± 4,89 *	11,90 ± 3,32 #	9,40 ± 2,52 #	14,20 ± 3,94	12,47 ± 3,54
MRT (s)	25,6 ± 6,50	25,2 ± 5,82	26,7 ± 5,60	26,5 ± 5,54	21,7 ± 5,93	20,5 ± 3,88	21,1 ± 5,89	20,4 ± 6,01
Gain (mL/min/W)	13,7 ± 1,55	13,3 ± 1,60	13,6 ± 1,84	13,4 ± 1,10	11,9 ± 1,48 #	11,6 ± 1,24 #	11,3 ± 1,48 #	11,8 ± 1,28 #
[La] @ R (mM)	0,80 ± 0,21	1,02 ± 0,28	0,87 ± 0,26	1,12 ± 0,42	9,11 ± 2,67 #	9,26 ± 2,67 #	8,59 ± 2,93 #	8,68 ± 3,21 #

***Table 5**: $\dot{V}O_2$ kinetics in Young group.*
*Values are expressed as mean ± SD divided in moderate intensity MOD and severe intensity (SEV) and for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). EXE_{SS} corresponding to the $\dot{V}O_2$ (mL/min) in the last 30 s of exercise. A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of the main phase, (2) and slow component (3), estimated through fitting analysis. A_{TOT} (mL/min) is the value of $\dot{V}O_2$ total amplitude at net of baseline, while A_{UP} (mL/min) is the portion of O_2 consumed during warm up phase at net of the $\dot{V}O_2$ detected at the state of ($A_{UP} = UP_{SS} - R_{SS}$). Gain (mL/min/W) is the net gain calculated as the ratio between A_{TOT} ($EXE_{SS} - R_{SS}$) (mL/min). [La] is lactate concentration at rest.Significance legend (P <0.05): * difference to BR condition, # to MOD*

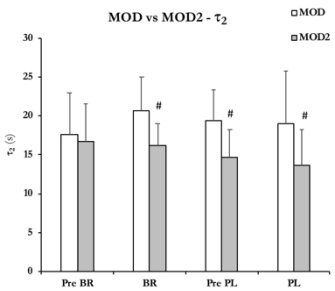


Figure 8
Time constant τ_2 in Y pre PE e PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

18. Results – Microvascular effects (NIRS)

The comparison of the data related to the metabolic transitions in the moderate intensity domain carried out without PE (MOD) and subsequently with it (MOD2), was obtained, for each studied group (O and Y) following the statistical analysis Time x Moderate, within the same condition (Pre BR and BR, Pre PL and PL).

The values of the variables under examination are shown in brackets respecting the order MOD and MOD2.

18.1 *Old*

Here are reported the result related to O group. Results are divided in MOD, MOD2 and comparison between MOD and MOD2.

18.1.1 Moderate

In group O during MOD exercise there are no significant differences due to BR considering the steady states of the four measurements made [HHb], [HbO], [THb] and SAT, in none of the phases of exercise, rest (R_{MSS}), unloaded pedalling (UP_{MSS}) and exercise (MOD_{SS}).

No significant differences are found even considering the parameters calculated through the fitting analysis of [HHb]. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

Finally, no significant differences are found even in the area under the curve (AUC) calculated after finding the $\Delta[HHb]/\Delta\dot{V}O_2$ ratio.

18.1.2 Moderate 2

In group O during MOD2 exercise there are no significant differences due to BR considering the steady states of the four measurements made [HHb], [HbO], [THb] and SAT, in none of the phases of exercise, rest (R_{M2SS}), unloaded pedalling (UP_{M2SS}) and exercise ($MOD2_{SS}$).

No significant differences are found even considering the parameters calculated through the fitting analysis of [HHb]. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

Finally, no significant differences are found even in the area under the curve (AUC) calculated after finding the $\Delta[HHb]/\Delta\dot{V}O_2$ ratio.

18.1.3 Priming effect (PE) - MOD vs MOD2

From the comparison between the values found in O in MOD and MOD2, it appears that PE influences the steady states of SAT during R and UP, [HbO] during R and UP and [HHb] during R. No significant effects due to PE were found during exercise phase. No differences were found from the comparison between MOD and MOD2 in [THb].

It appears that SAT relative to R and UP is greater after PE in all experimental conditions with p<0,0001 (R: Pre BR 63.12 ± 6.76 vs 78.44 ± 6.17; BR 64.75 ± 4.08 vs 79.93 ± 2.55; Pre PL 64.29 ± 5.53 vs 79.05 ± 3.56; PL 63.98 ± 6.01 vs 79.13 ± 4.36; UP: Pre BR 66.27 ± 5.78 vs 75.89 ± 4.84; BR 67.97 ± 4.40 vs 77.06 ± 3.56; Pre PL 67.69 ± 4.92 vs 76.58 ± 3.59; PL 66.81 ± 4.82 vs 77.28 ± 3.68).

Also in [HbO] after PE values are greater during R and UP (R: Pre BR 62.34 ± 10.93 vs 91.23 ± 20.13, p = 0.0025; BR 64.03 ± 13.59 vs 96.51 ± 24.37, p = 0.007; Pre PL 58.49 ± 15.18 vs 85.83 ± 23.35, p = 0.0042; PL 57.42 ± 14.41 vs 85.71 ± 23.33, p = 0.0031; UP: Pre BR 62.55 ± 12.43 vs 82.08 ± 13.12, p = 0.0227; BR 64.55 ± 15.08 vs 86.71 ± 21.00, p = 0.0099; Pre PL 58.34 ± 16.04 vs 76.78 ± 17.61, p = 0.0313; PL 58.00 ± 14.89 vs 78.78 ± 22.37, p = 0.0154).

In [HHb] only R was influenced by PE with a decrease (Pre BR 36.71 ± 9.54 vs 25.30 ± 9.06, p = 0.0031; BR 34.89 ± 8.14 vs 24.07 ± 6.12, p = 0.0050; Pre PL 32.24 ± 8.65 vs 22.54 ± 6.27, p = 0.0116; PL 33.05 ± 11.95 vs 22.08 ± 6.95, p = 0.0044).

In [HHb] parameters calculated with fitting, PE slows down τ_1 (Pre BR 4.59 ± 1.19 vs 8.66 ± 3.62, p = 0.0139; BR 5.04 ± 2.30 vs 8.11 ± 2.56, p = 0.0304; Pre PL 5.30 ± 2.67 vs 8.84 ± 4.70, p = 0.0259; PL 3.71 ± 2.25 vs 9.99 ± 4.70, p <0.0001) and reduces TD_1 (Pre BR 9.53 ± 3.28 vs 3.62 ± 2.63, p = 0.0005; BR 7.10 ± 4.48 vs 3.00 ± 2.79, p = 0.0236; Pre PL 8.22 ± 3.62 vs 2.78 ± 2.23, p = 0.0014; PL 9.75 ± 2.91 vs 3.01 ± 2.23, p = 0.0004).

OLD		MOD				MOD2			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SAT (%)	Rss	63,12 ± 6,76 °	64,75 ± 4,08 °	64,29 ± 5,53	63,98 ± 6,01	78,44 ± 6,17 #	79,93 ± 2,55 #	79,05 ± 3,56 #	79,13 ± 4,36 #
	UPss	66,27 ± 5,78 °	67,97 ± 4,40	67,69 ± 4,92	66,81 ± 4,82	75,89 ± 4,84 #°	77,06 ± 3,56 #	76,58 ± 3,59 #°	77,28 ± 3,68 #
	EXE	61,69 ± 5,84	61,83 ± 5,35	63,11 ± 5,03	62,97 ± 5,87	65,85 ± 6,15	65,88 ± 5,98	67,18 ± 5,77	67,03 ± 5,73

***Table 7**: NIRS data in Old group*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SAT is saturation
Significance legend (P <0.05): °difference to Y group, # difference to MOD

HHb		MOD				MOD2			
OLD		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
A_1		8.67 ± 6.09	10.17 ± 8.24	9.76 ± 7.42	9.59 ± 8.06	11.25 ± 6.22	13.19 ± 11.45	10.80 ± 8.97	11.90 ± 8.97
τ_1 (s)		4.59 ± 1.19	5.04 ± 2.30	5.30 ± 2.67	3.71 ± 2.25	8.66 ± 3.62 #	8.11 ± 2.56 #	8.84 ± 4.70 #	9.99 ± 4.70 #
TD_1 (s)		9.53 ± 3.28	7.10 ± 4.48	8.22 ± 3.62	9.75 ± 2.91	3.62 ± 2.63 #	3.00 ± 2.79 #	2.78 ± 2.23 #	3.01 ± 2.23 #
MRT		14.12 ± 3.37	12.14 ± 5.14	13.52 ± 3.89	13.46 ± 3.16	12.29 ± 3.86	11.11 ± 2.22	11.63 ± 3.45	13.00 ± 3.45

***Table 8**: [HHb] kinetics parameters in Old group.*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL). A, τ, TD, respectively amplitude (mL/ min), time constant (s) and time delay (s) of first (1), and second component (2), estimated through fitting analysis. MRT is mean response time, calculated as sum of τ_1 and TD_1
Significance legend (P <0.05): # difference to MOD

AUC OLD	BR		PL	
	Pre	Post	Pre	Post
MOD	23.72 ± 18.54	20.09 ± 6.30	20.47 ± 18.63	20.39 ± 10.46
MOD2	10.73 ± 6.39	8.07 ± 9.84	10.11 ± 8.42	9.93 ± 9.35

***Table 9**: Area under curve in O group.*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL).
AUC is area under curve calculated after $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio

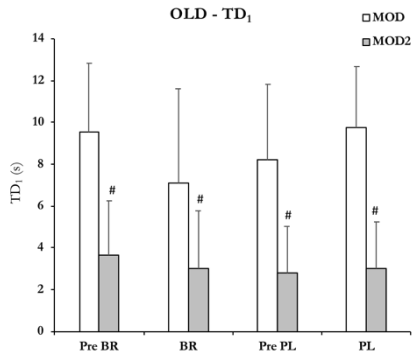


Figure 9
Time delay (TD_1) of [HHb] in O pre PE e PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

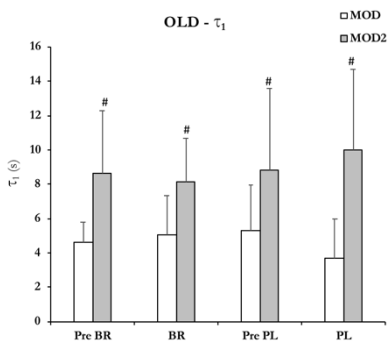


Figure 10
Time constant (τ_1) of [HHb] τ_1 in O pre PE e PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

18.2 *Young*

Here are reported the result related to O group. Results are divided in MOD, MOD2 and comparison between MOD and MOD2.

18.2.1 Moderate

Similarly, to what happens in O also in Y there are no significant differences during MOD exercise in the stationary states of [HHb], [HbO], [THb] and SAT, in any of the phases of the exercise, R_{MSS}, UP_{MSS} and MOD_{SS}, and in the parameters of [HHb] calculated by the fitting. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

With regard to AUC of Δ[HHb]/ $\Delta\dot{V}O_2$ ratio, on the other hand, there is a tendency to decrease in the BR phase compared to the other 3 phases (BR 8.62 ± 10.73 vs Pre BR 11.38 ± 8.66, Pre PL 10.59 ± 5.81, PL 13.04 ± 7.55), but this difference is not significant, probably due to the high value of SD in the BR phase.

18.2.2 Moderate 2

In group Y during MOD2 exercise there are no significant differences due to BR considering the steady states of the four measurements made [HHb], [HbO], [THb] and SAT, in none of the phases of exercise, rest (R_{M2SS}), unloaded pedalling (UP_{M2SS}) and exercise ($MOD2_{SS}$).

No significant differences are found even in the area under the curve (AUC) calculated after finding the Δ[HHb]/$\Delta\dot{V}O_2$ ratio.

Considering the parameters calculated through the fitting analysis of [HHb], differences were found in τ_1 between BR and Pre BR and Pre PL conditions (BR 9.33 ± 3.64 vs Pre BR 11.638 ± 4.79, p =0.0453, ES = 0.543; vs Pre PL 11.6402 ± 6.02, p <0.0451), with τ_1 diminished by ~2 s. Amplitude (A_1) is reported, but has not been statistically analyzed, because it was not possible to normalize it.

18.2.3 Priming effect (PE) - MOD vs MOD2

By means of a comparison between the values found in O in MOD and MOD2, it appears that PE influences the steady states of SAT, [HbO], [HHb] during R and

UP. No significant effects due to PE were found during exercise phase. No differences were found from the comparison between MOD and MOD2 in [THb].

It appears that SAT relative to R and UP is greater after PE in all experimental conditions with p<0,0001(R: Pre BR 68.50 ± 2.85 vs 81.82 ± 4.03; BR 69.07 ± 4.52 vs 79.68 ± 5.82; Pre PL 68.04 ± 4.39 vs 82.13 ± 3.14; PL 67.09 ± 3.58 vs 81.64 ± 3.26 – UP: Pre BR 70.39 ± 3.04 vs 80.99 ± 3.32; BR 70.97 ± 3.98 vs 80.42 ± 4.80; Pre PL 70.21 ± 3.48 vs 81.00 ± 2.89; PL 69.32 ± 3.26 vs 80.22 ± 3.46).

Also in [HbO] after PE values are greater during R and UP (R:Pre BR 59.01 ± 18.21 vs 83.72 ± 28.82, p = 0.0095; BR 63.17 ± 15.84 vs 88.66 ± 28.05, p = 0.0075; Pre PL 63.10 ± 21.28 vs 90.71 ± 30.67, p = 0.0038; PL 60.15 ± 16.20 vs 91.81 ± 29.03, p = 0.0009 – UP: Pre BR 59.23 ± 19.83 vs 77.31 ± 24.73, p = 0.0348; BR 62.71 ± 16.56 vs 84.69 ± 23.58, p = 0.0105; Pre PL 62.12 ± 19.20 vs 83.66 ± 26.06, p = 0.0121; PL 60.50 ± 15.86 vs 83.00 ± 22.76, p = 0.0088).

In [HHb] BR and PE seems to have combined effects because in both, R and UP, there are decrease value of [HHb] in comparison between MOD and MOD2 in all phases except BR (R: Pre BR 27.23 ± 9.16 vs 18.14 ± 6.69, p = 0.0181; Pre PL 29.76 ± 11.95 vs 19.28 ± 6.50, p = 0.0066; PL 29.54 ± 8.75 vs 20.21 ± 6.48, p = 0.0153 – BR 27.88 ± 6.84 vs 21.57 ± 6.45 – UP: Pre BR 24.50 ± 7.57 vs 17.91 ± 6.13, p = 0.0235; Pre PL 25.73 ± 7.04 vs 19.34 ± 5.60, p = 0.0282; PL 26.71 ± 7.88 vs 20.17 ± 5.72, p = 0.0247 – BR 25.10 ± 5.58 vs 19.80 ± 5.15).

Also in [HHb] parameters calculated with fitting, BR and PE seems to have combined effects on τ_1 because τ_1 slows down in all conditions, but in BR τ_1 in MOD2 has no significant differences from MOD (Pre BR 5.45 ± 2.60 vs 11.64 ± 4.79, p = 0.0009; Pre PL 6.39 ± 1.92 vs 11.64 ± 6.02, p = 0.0045; PL 6.16 ± 4.68 vs 10.14 ± 4.60, p = 0.0233 – BR 5.99 ± 1.87 vs 9.33 ± 3.64). Finally, PE reduces TD_1 (Pre BR 8.84 ± 2.23 vs 2.12 ± 1.32, p <0.0001; BR 7.42 ± 2.58 vs 3.05 ± 2.15, p = 0.0017; Pre PL 8.18 ± 3.00 vs 3.10 ± 1.68, p = 0.0002; PL 7.36 ± 3.70 vs 2.20 ± 2.43, p = 0.0021)

YOUNG		MOD				MOD2			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SAT (%)	Rss	68,50 ± 2,85	69,07 ± 4,52	68,04 ± 4,39	67,09 ± 3,58	81,82 ± 4,03 #	79,68 ± 5,82 #	82,13 ± 3,14 #	81,64 ± 3,26 #
	UPss	70,39 ± 3,04	70,97 ± 3,98	70,21 ± 3,48	69,32 ± 3,26	80,99 ± 3,32 #	80,42 ± 4,80 #	81,00 ± 2,89 #	80,22 ± 3,46 #
	EXE	63,56 ± 4,03	61,74 ± 5,87	61,81 ± 5,38	61,16 ± 6,66	66,44 ± 5,74	65,67 ± 6,04	65,09 ± 7,16	64,53 ± 6,26

***Table 10**: NIRS data in Young group*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SAT is saturation.
Significance legend (P <0.05): # difference to MOD

HHb YOUNG	MOD				MOD2			
	BR		PL		BR		PL	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A_1	10.92 ± 6.61	12.50 ± 6.33	13.70 ± 7.39	14.01 ± 9.16	15.88 ± 9.02	15.96 ± 8.00	18.01 ± 9.05	18.74 ± 11.81
τ_1 (s)	5.45 ± 2.60	5.99 ± 1.87	6.39 ± 1.92	6.16 ± 4.68	11.64 ± 4.79 *#	9.33 ± 3.64	11.64 ± 6.02 #	10.14 ± 4.60 *#
TD_1 (s)	8.84 ± 2.23	7.42 ± 2.58	8.18 ± 3.00	7.36 ± 3.70	2.12 ± 1.32 #	3.05 ± 2.15 #	3.10 ± 1.68 #	2.20 ± 2.43 #
MRT	14.29 ± 3.02	13.41 ± 3.04	14.57 ± 2.37	13.53 ± 4.16	13.76 ± 4.95	12.37 ± 3.45	14.74 ± 5.13	12.34 ± 5.28

***Table 11** [HHb] kinetics parameters in Young group.*
*Values are expressed as mean ± SD divided in pre PE (MOD) and PE (MOD2) for each intensity the four experimental phases (Pre BR, BR, Pre Pl, PL). A, τ, TD, respectively amplitude (mL/min), time constant (s) and time delay (s) of first (1), and second component (2), estimated through fitting analysis. MRT is mean response time, calculated as sum of τ_1 and TD_1. Significance legend (P <0.05): # difference to MOD, * to BR condition*

AUC YOUNG	BR		PL	
	Pre	Post	Pre	Post
MOD	11.38 ± 8.66	8.62 ± 10.73	10.59 ± 5.81	13.04 ± 7.55
MOD2	7.17 ± 6.04	7.18 ± 1.69	8.35 ± 7.12	7.59 ± 4.35

***Table 12**: Area under curve in Young group.*
Values are expressed as mean ± SD divided in pre PE (MOD) and PE (MOD2) in the four experimental phases (Pre BR, BR, Pre Pl, PL).
AUC is area under curve calculated after ⊿[HHb]/Δ$\dot{V}O_2$ Ratio.

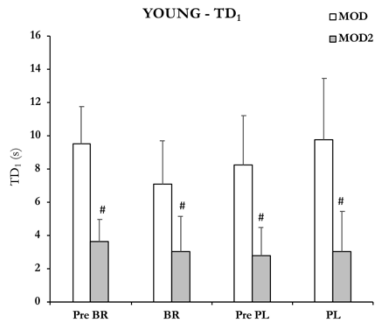


Figure 11
Time delay (TD_1) of [HHb] in Y pre PE e PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

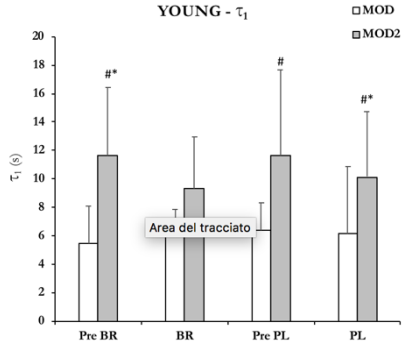


Figure 12
Time constant (τ_1) of [HHb] τ_1 in Y pre PE e PE (MOD vs MOD2)
*Significance legend (P <0.05): # difference to MOD, * difference to BR*

19. Results – Vascular effects

The comparison of the data related to the metabolic transitions in the moderate intensity domain carried out without PE (MOD) and subsequently with it (MOD2), was obtained, for each studied group (O and Y) following the statistical analysis Time x Moderate, within the same condition (Pre BR and BR, Pre PL and PL).

The values of the variables under examination are shown in brackets respecting the order MOD and MOD2.

19.1 *Old*

Here are reported the result related to O group. Results are divided in MOD, MOD2 and comparison between MOD and MOD2.

19.1.1 Moderate

During MOD exercise in O, the main differences due to BR supplementation are observed in MAP (Pre BR vs BR 103.3 ± 7.1vs 98.35 ± 11 mmHg, p = 0.0249) with a reduction of ~5% and TPR (Pre BR vs BR 1.24 ± 0.22 vs 1.16 ± 0.21, p = 0.0458) with a reduction of ~6,5% during R phase. Always in R phase, in TPR there is also difference between BR and PL conditions (BR vs PL 1.16 ± 0.21 vs 1.25 ± 0.30, p = 0.0226) with TPR in BR slower ~6,5%.

In DIA differences after BR supplementation was observed only in UP (Pre BR vs BR 77.86 ± 11.80 vs 83.71 ± 9.10, p=0.0190).

In SYS there are no significant differences between conditions during MOD exercise.

19.1.2 Moderate 2

During MOD2 exercise in O, the main differences due to BR supplementation are observed in SYS during R with a reduction in BR respect to other condition (BR 148.56 ± 25.78 vs Pre BR 164.50 ± 18.05, p =<0.0001; vs Pre PL 164.50 ± 18.05, p <0.0001; vs PL 159.29 ± 21.54, p = 0.0039) and in MAP during R (BR 103.18 ± 15.39 vs Pre BR 114.80 ± 10.23, p <0.0001; vs Pre PL 114.80 ± 10.23, p <0.0001; vs PL 111.42 ± 11.43, p = 0.0002).

In MAP was observed same results also in UP (BR 101.74 ± 16.50 vs Pre BR 108.84 ± 16.28, p = 0.0062; vs Pre PL 108.84 ± 16.28, p = 0.0062; vs PL 107.29 ± 13.09, p = 0.0318).

In TPR there are significant differences due to BR during UP (BR 0.94 ± 0.24 vs Pre BR 1.02 ± 0.24, p = 0.0313) with TPR in BR slower ~7,9%.

In DIA no significant differences after BR supplementation was observed.

19.1.3 Priming effect (PE) - MOD vs MOD2

From the comparison between the values found in O in MOD and MOD2 were observed differences due to PE that raise SYS during R and exercise ($MOD2_{SS}$) (R: Pre BR 138.77 ± 12.91 vs 164.50 ± 18.05, p = 0.0003; BR 131.67 ± 12.29 vs 148.56 ± 25.78, p = 0.0159; Pre PL 138.77 ± 12.91 vs 164.50 ± 18.05, p = 0.0003; PL 134.62 ± 14.25 vs 159.29 ± 21.54, p = 0.0005 – $MOD2_{SS}$: Pre BR 141.45 ± 15.88 vs 163.15 ± 28.36, p = 0.0160; BR 142.68 ± 18.17 vs 163.21 ± 19.08, p = 0.0225; Pre PL 141.45 ± 15.88 vs 163.15 ± 28.36, p = 0.0160; PL 139.51 ± 15.98 vs 164.61 ± 22.07, p = 0.0054).

In MAP at R, BR and PE seems to have combined effects because MAP is significant higher in MOD2 in all conditions (Pre BR 103.31 ± 7.11 vs 114.80 ± 10.23, p = 0.0154; Pre PL 103.31 ± 7.11 vs 114.80 ± 10.23, p = 0.0154; PL 101.56 ± 8.33 vs 111.42 ± 11.43, p = 0.0371) except after BR (BR 98.35 ± 11.00 vs 103.18 ± 15.39).

TPR due to PE are slower at R (Pre BR 1.24 ± 0.22 vs 0.98 ± 0.28, p = 0.0174; BR 1.16 ± 0.21 vs 0.95 ± 0.34, p = 0.0481; Pre PL 1.24 ± 0.22 vs 0.98 ± 0.28, p = 0.0174; PL 1.25 ± 0.30 vs 0.97 ± 0.23, p = 0.0098).

In DIA no significant differences due to PE was observed.

OLD		MOD				MOD2			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SYS (mmHg)	Rss	138,77 ± 12,91	131,67 ± 12,29	138,77 ± 12,91	134,62 ± 14,25	164,50 ± 18,05 *°#	148,56 ± 25,78 °#	164,50 ± 18,05 *°#	159,29 ± 21,54 *°#
	FWss	144,43 ± 17,87	152,34 ± 15,81 °	144,43 ± 17,87	146,53 ± 15,95 °	146,96 ± 27,07 °	143,38 ± 25,02	146,96 ± 27,07 °	147,30 ± 22,17 °
	EXE	141,45 ± 15,88 °	142,68 ± 18,17	141,45 ± 15,88 °	139,51 ± 15,98 °	163,15 ± 28,36 #	163,21 ± 19,08 °#	163,15 ± 28,36 #	164,61 ± 22,07 °#
DIA (mmHg)	Rss	82,99 ± 4,31 °	85,32 ± 7,15 °	82,99 ± 4,31 °	82,47 ± 8,29 °	81,18 ± 6,74	82,43 ± 9,58 °	81,18 ± 6,74	82,64 ± 7,64 °
	FWss	77,86 ± 11,80 *°	83,71 ± 9,10	77,86 ± 11,80 *	80,61 ± 9,65	79,18 ± 10,90	81,83 ± 11,12	79,18 ± 10,90	80,80 ± 8,86
	EXE	84,40 ± 16,12	86,89 ± 11,86	84,40 ± 16,12	84,66 ± 18,91	81,59 ± 12,13	85,41 ± 8,96	81,59 ± 12,13	83,35 ± 10,48
MAP (mmHg)	Rss	103,31 ± 7,11 *°	98,35 ± 11,00	103,31 ± 7,11 *°	101,56 ± 8,33 °	114,80 ± 10,23 *°#	103,18 ± 15,39 °	114,80 ± 10,23 *°#	111,42 ± 11,43 *°#
	FWss	106,09 ± 9,76	105,46 ± 10,94 °	106,09 ± 9,76	106,23 ± 9,72 °	108,84 ± 16,28 *°	101,74 ± 16,50	108,84 ± 16,28 *°	107,29 ± 13,09 *°
	EXE	118,95 ± 12,80	114,08 ± 14,66	118,95 ± 12,80	115,71 ± 18,53	115,49 ± 17,12	109,53 ± 13,45	115,49 ± 17,12	114,37 ± 14,65 °
TPR	Rss	1,24 ± 0,22 *°	1,16 ± 0,21 °	1,24 ± 0,22 *°	1,25 ± 0,30 *°	0,98 ± 0,28 °#	0,95 ± 0,34 °#	0,98 ± 0,28 °#	0,97 ± 0,23 °#
	FWss	1,11 ± 0,23 °	1,11 ± 0,19 °	1,11 ± 0,23 °	1,14 ± 0,22 °	1,02 ± 0,24 *°	0,94 ± 0,24 °	1,02 ± 0,24 *°	0,98 ± 0,23 °
	EXE	0,77 ± 0,16 °	0,72 ± 0,16 °	0,77 ± 0,16 °	0,73 ± 0,18 °	0,74 ± 0,16 °	0,71 ± 0,21 °	0,74 ± 0,16 °	0,68 ± 0,13 °

***Table 13**: Blood pressure in Old group*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL). R_{SS}, UP_{SS} and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SYS is systolic pressure, DIA is diastolic pressure, MAP is mean pressure and TPR are total peripheral resistances.
*Significance legend (P <0.05): *difference to BR condition, °difference to young group, # difference to MOD*

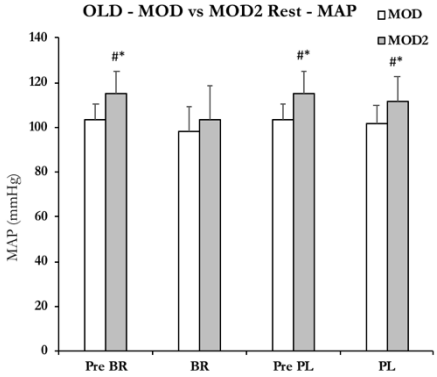


Figure 13
MAP in Old group in rest pre PE and PE (MOD vs MOD2)
*Significance legend (P <0.05): # difference to MOD, * difference to BR*

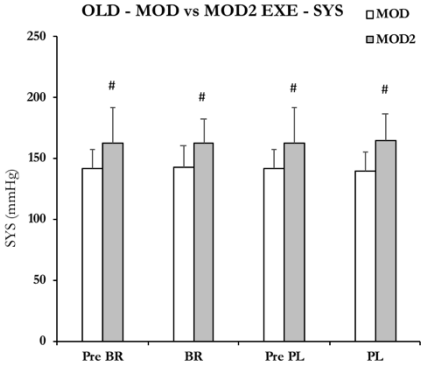


Figure 14
SYS in Old group in exercise pre PE and PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

19.2 *Young*

Here are reported the result related to O group. Results are divided in MOD, MOD2 and comparison between MOD and MOD2.

19.2.1 Moderate

During MOD exercise in young, there are significant differences only on TPR, during R and UP. During R the difference is between BR and PL (BR vs PL 0.84 ± 0.18 vs 0.93 ± 0.18, ~9.7 %, p = 0.0215). During UP there are differences in both, after BR and compared with PL (Pre BR vs BR 0.80 ± 0.18 vs 0.73 ± 0.18, ~8.7%, p = 0.0363; BR vs PL 0.73 ± 0.18 vs 0.83 ± 0.20, ~12 %, p = 0.0035).

There are no differences in SYS, DIA and MAP in various phases during MOD exercise.

19.2.2 Moderate 2

During MOD2 exercise in Y, no significant differences were observed in all parameters of blood pressure measured, SYS, DIA, MAP and TPR in all phases, rest (R_{M2SS}), unloaded pedalling (UP_{M2SS}) and exercise ($MOD2_{SS}$).

19.2.3 Priming effect - MOD vs MOD2

From the comparison between the values found in Y in MOD and MOD2, differences were observed due to PE only during exercise ($MOD2_{SS}$) in SYS, and in TPR at R and during UP.

During $MOD2_{SS}$ SYS are slower due to PE, on contrary to what happens in the O (Pre BR 165.64 ± 13.89 vs 145.99 ± 10.08, p = 0.0289; BR 160.25 ± 15.64 vs 139.12 ± 12.24, p = 0.0189; Pre PL 165.64 ± 13.89 vs 145.99 ± 10.08, p = 0.0289; PL 163.51 ± 16.11 vs 140.46 ± 11.95, p = 0.0105).

TPR due to PE are slower during R (Pre BR 0.90 ± 0.19 vs 0.52 ± 0.11, p = 0.0004; BR 0.84 ± 0.18 vs 0.51 ± 0.10, p =0.0020; Pre PL 0.90 ± 0.19 vs 0.52 ± 0.11, p = 0.0004; PL 0.93 ± 0.18 vs 0.55 ± 0.14, p = 0.0003). Moreover, during UP, TPR are slower due to PE (Pre BR 0.80 ± 0.18 vs 0.56 ± 0.09, p = 0.0180; Pre PL 0.80 ± 0.18 vs 0.56 ± 0.09, p = 0.0180; PL 0.83 ± 0.20 vs 0.54 ± 0.14, p = 0.0050) but after

BR there are no significant difference due to reduction on MOD (BR 0.73 ± 0.18 vs 0.55 ± 0.12).

In DIA and MAP no significant differences due to PE was observed.

YOUNG		MOD				MOD2			
		BR		PL		BR		PL	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
SYS (mmHg)	Rss	125,43 ± 12,10	124,03 ± 9,64	125,43 ± 12,10	122,17 ± 11,70	130,17 ± 13,23	131,09 ± 16,71	130,17 ± 13,23	130,07 ± 18,63
	FWss	133,03 ± 10,94	127,64 ± 13,34	133,03 ± 10,94	129,06 ± 13,24	128,06 ± 12,29	133,07 ± 12,98	128,06 ± 12,29	126,71 ± 12,84
	EXE	165,64 ± 13,89	160,25 ± 15,64	165,64 ± 13,89	163,51 ± 16,11	145,99 ± 10,08 #	139,12 ± 12,24 #	145,99 ± 10,08 #	140,46 ± 11,95 #
DIA (mmHg)	Rss	74,03 ± 7,78	73,21 ± 7,63	74,03 ± 7,78	73,11 ± 6,66	73,56 ± 8,22	73,46 ± 10,52	73,56 ± 8,22	73,82 ± 10,28
	FWss	76,68 ± 7,97	72,39 ± 10,83	76,68 ± 7,97	74,78 ± 7,53	74,80 ± 6,45	76,02 ± 8,74	74,80 ± 6,45	72,97 ± 8,14
	EXE	87,24 ± 9,00	83,68 ± 9,21	87,24 ± 9,00	85,27 ± 7,39	81,78 ± 7,69	78,35 ± 7,65	81,78 ± 7,69	77,75 ± 7,49
MAP (mmHg)	Rss	93,40 ± 9,38	91,86 ± 7,78	93,40 ± 9,38	91,81 ± 8,19	93,63 ± 10,08	93,49 ± 12,27	93,63 ± 10,08	93,98 ± 13,79
	FWss	98,16 ± 9,38	92,64 ± 11,34	98,16 ± 9,38	95,42 ± 9,14	94,35 ± 8,48	96,21 ± 9,75	94,35 ± 8,48	93,03 ± 10,39
	EXE	115,45 ± 10,57	110,72 ± 10,17	115,45 ± 10,57	113,27 ± 9,36	105,91 ± 7,58	100,98 ± 8,78	105,91 ± 7,58	101,30 ± 8,31
TPR	Rss	0,90 ± 0,19	0,84 ± 0,18	0,90 ± 0,19	0,93 ± 0,18 *	0,52 ± 0,11 #	0,51 ± 0,10 #	0,52 ± 0,11 #	0,55 ± 0,14 #
	FWss	0,80 ± 0,18 *	0,73 ± 0,18	0,80 ± 0,18 *	0,83 ± 0,20 *	0,56 ± 0,09 #	0,55 ± 0,12	0,56 ± 0,09 #	0,54 ± 0,14 #
	EXE	0,49 ± 0,10	0,45 ± 0,07	0,49 ± 0,10	0,47 ± 0,08	0,43 ± 0,10	0,40 ± 0,07	0,43 ± 0,10	0,41 ± 0,11

***Table 13**: Blood pressure in Young group*
Values are expressed as mean ± SD divided in MOD (pre Priming Effect, PE) and MOD2 (PE) in the four experimental phases (Pre BR, BR, Pre Pl, PL). Rss, UPss and EXE corresponding to average of last 30" of each phase at rest, at the end of the freewheeling warm up and in the last 30 s of exercise. SYS is systolic pressure, DIA is diastolic pressure, MAP is mean pressure and TPR are total peripheral resistances.
*Significance legend (P <0.05): *difference to BR condition, # difference to MOD*

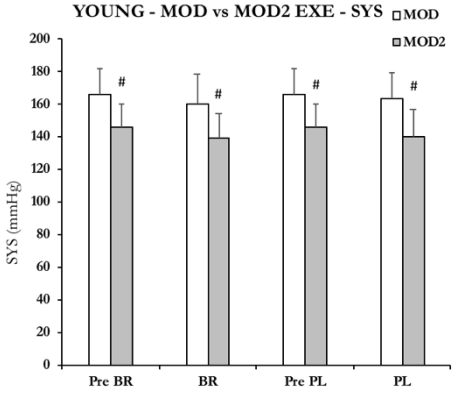


Figure 15
SYS in Young group in exercise pre PE and PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD

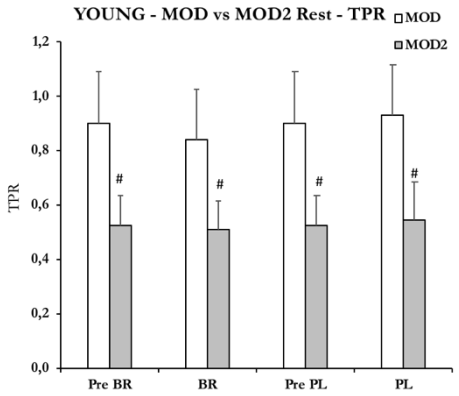


Figure 16
TPR in Young group in rest pre PE and PE (MOD vs MOD2)
Significance legend (P <0.05): # difference to MOD.

20. General discussion

20.1 *PE effects and Nitrate in old*

20.1.1 O_2 consumption and NIRS

The PE is constituted as an intervention aimed at improving the contribution of O_2 to the muscle (Murias, Kowalchuk, and Paterson 2011) considered able to influence the $\dot{V}O_2$ response in the transition to moderate exercise.

PE leads to augmented O_2 consumption at R and during UP and this is common outcome in all conditions. This in not followed of any significant increase in speed of kinetics (τ_2) (except in PL condition). An average decrease of 3.4 s was observed, despite of what is observed in the literature (Murias, Kowalchuk, and Paterson 2011; De Roia et al, 2012).

About the variables measured by NIRS, instead, we observed a common trend that is reported also in the literature (De Roia et al, 2012). For instance, HHb kinetic parameters, are decreased for TD_1 (average -4.24 s) and for τ_1 (average 5.55 s) in all conditions. This is in agreement with previous studies on elderly subject (DeLorey et al 2004, De Roia et al, 2012).) The decrease in TD_1 is retained related to the early mismatch between O_2 consumption, by the muscle, and O_2 delivery. And this is attributable to a quicker activation of cell enzymes (mitochondrial enzymes and/or PDH) following heavy-intensity warm-up (Gund et al. 2009). PE leads to a better O_2 extraction. The higher values of τ_1 are explained by the slower rate of adjustment of muscle O_2 consumption compared with the adjustment of O_2 delivery. So, during MOD2, O_2 delivery increase relatively swiftly compared to O_2 extraction during exercise (relatively to MOD).

De Roia and colleagues (De Roia et al, 2012), observed an improvement of matching between O_2 delivery and O_2 consumption, with the decrease of the overshoot in the $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio. We observe the same decrease in area under curve of $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio, although this result was not significant, probably due to small size and to great variability of the sample (SD is very high). NIRS data depict also that the steady state of SAT and [HbO] is higher after PE both in the R and UP phases of MOD2, confirming a better muscle oxygenation, induced by the PE, before exercise.

In the elderly, BR effects that are observed in MOD2 are similar to those found in MOD: significant reduction in O_2 consumption at steady state ($MOD2_{SS}$). Reduction registered is 78.2 mL/min (~5.7%) after BR (Pre BR 1450.3 ± 329.01 mL/min vs BR 1372.1 ± 309.31 mL/min, p = 0.0257). Furthermore, there are no effects due to BR supplementation concerning NIRS data, neither in the area under the curve, nor in the kinetic parameters of [HHb], nor in the stationary states of SAT, [THb], [HHb] and [HbO]. Therefore, despite a more acidic environment due to greater [La] and the need to buffer it, which results in a lowering of PH, the effect of BR supplementation leads to very similar results, even if the NO_3^- - NO_2^- - NO pathway it should be more active (Lundberg et al., 2008). Then, it is not understandable where the NO_3^- supplement acts after PE.. This consideration is relative to the similar results obtained at the steady state comparing MOD and MOD2 exercise. And this last consideration leads to support the hypothesis that the "energetic action" of NO_3^- involves mainly cellular mechanisms rather than vascular modulation.

BR is able to Also the Gain on both, MOD and MOD2 results similar, bringing values closer to those found by young people, confirming even after PE this "rejuvenation" due to BR supplementation.

20.1.2 Blood pressure

In comparison between MOD and MOD2 on blood pressure parameters in the O, changes emerge during the R phase in SYS, MAP and TPR. In particular, SYS and MAP are affected by the increase they undergo during the PE, and do not return to baseline values, remaining elevated. However, this does not occur in the condition of BR supplementation, where the NO_3^- seem to facilitate, probably due to the vasodilatory effect of the NO, the return speed at baseline of the SYS and MAP, which in fact differ in the BR condition compared to the other conditions. In the MAP, the BR condition in MOD2, in fact, is not significantly different from MOD (98.35 vs 103.18 mmHG), while in SYS, although lower than the other conditions, the significant difference remains with MOD (131.67 vs 148.56). To underline then how the SYS falls in the UP phase, reaching values comparable to MOD, and then rises again to levels significantly higher than MOD (average = 22.26) during exercise phase. This phenomenon is not observed in young people, where, instead, the SYS in MOD2 decreases compared to MOD (average -20.87) and no changes are observed in the exercise phase

on TPR, as already reported in the literature (De Roia et al 2012). In this phase no effects due to BR supplementation are observed. TPR, instead, are significant lower after PE during R phase before MOD2.

20.2 *PE effects and Nitrate in Young*

20.2.1 O_2 consumption and NIRS

As for the elderly, even in Y PE leads to augmented O_2 consumption at R and during UP common to all conditions. Differently from O, however, in Y PE speeds the $\dot{V}O_2$ kinetics, with average increase of 4.8 s in τ_2, in all experimental conditions except for Pre BR. An increasing in the speed of adaptation of the oxidative metabolism in the transition to moderate intensity domain was found, despite the $\dot{V}O_2$ response present values of τ_2 around 20s (mean time constant cleavage of phosphocreatine), indicated by the literature as a quantity that hardly allows the influence on regulation of kinetics by improvements of the local distribution of O_2 induced by PE (Poole et al, 2012).

Gain values in MOD2, as for MOD, are not influenced by BR supplementation, but is significantly lowered after PE, indicating that muscle activation does not bring improvements on it.

Also, in Y, NIRS data were in agreement with that are reported in the literature (De Roia et al, 2012) in the [HHb] kinetic parameters, with a decrease in TD_1 (average -4.69 s) and a slowdown of τ_1 (average 5.66 s). But here, in the time constant there is a difference induced by BR supplementation, highlighting an effect of NO_3^- on the speed of adaptation of [HHb], which is improved. In fact, in the condition of BR supplementation $\tau_1$became not significant different after PE, making it comparable to MOD (5.99 vs 9.33).

Therefore, it is hypothesized that in the Y, a more acidic environment in MOD2 compared to MOD has been establish, due to the higher concentration of [LA], and this influence the activity of NO_3^- - NO_2^- - NO pathway compared to the elderly, thus causing a better adaptations on vascular system (better O2 delivery) and/or a strongest interaction with hemoglobin (deoxyhemoglobin) (Lundberg et al., 2008), leading to a

faster O_2 extraction after BR supplementation (faster activation of mitochondrial enzymes and/or PDH).

Similarly to what is found in the elderly and as reported in the literature (De Roia et al, 2012), it was observed a tendency to an improvement of matching between O_2 delivery and O_2 consumption, with the decrease of area under curve, although limited, in the $\Delta[HHb]/\Delta\dot{V}O_2$ Ratio, except in BR condition, where area under curve value in MOD2 is very close to MOD. Decreasing in area under curve, however, is not significant, probably due to small size and to great variability of the sample (SD is very high).

In NIRS data it is also noted that the steady state of SAT and [HbO] is higher after PE both in the R and UP phases of MOD2, confirming a better muscle oxygenation, induced by the PE, before exercise.

20.2.2 Blood pressure

In comparison between MOD and MOD2 on blood pressure parameters in the Y, changes emerge during in TPR during R and UP and in SYS during exercise.

In TPR there is a significant decrease in all the conditions in the R phase after PE, thus modifying the peripheral resistance at the muscle level in the recovery phase after PE (average = 0.37). During the UP phase the responses are similar to those observed in R, with the exception of the BR phase, where there is no difference between MOD and MOD2, due to the decrease of the TPR in MOD, not in MOD2, where there are no differences after BR supplementation. During the exercise, there is a decrease in TPR, but not significant after PE.

In SYS an opposite effect due to PE is observed to one observed in O, in fact, while in O SYS increase during the exercise phase after PE (average = 22.26), in Y the SYS decreases in MOD2 with respect to MOD (average = -20.87). This decrease is probably due to lower TPR.

20.3 *Study limits*

The present study springs out a series of non-negligible aspects that can be considered as limits in particular with respect to the size of the effect under examination and to the statistical power of the results.

Firstly, the reduced number of the sample (Old: n = 10, Young: n = 10), in association with the inter-individual variability that characterizes each of the two subgroups, could prevent the achievement of statistical significance in the different comparisons between variables. In particular, elderly subjects, whose recruitment has not always proved to be easy, tend to be unrepresentative of the population to which they belong in terms of their fitness level on average higher than that normally expected ($\dot{V}O_{2max}$, τ_2, MRT). They are physically more active than usual people of the age and they pay attention to the health benefits of exercise and diet. All these aspects may have reduced the potential impact of NO_3^- supplementation by masking its effects in particular at vascular level. Therefore, there may have been little chance for nitrates to positively influence exercise responses due to insufficient impairment of the systems involved.

Any training effects that may have been induced by the overall duration of the experimental design were excluded from the randomization of the treatment assignment.

21. Conclusion

Supplementation of nitrates to which the subjects recruited in the present study underwent, has indeed determined an elevation of the plasma concentrations of this ion and consequently the bioavailability of NO of which it is a precursor.

Priming Exercise could be a useful paradigm for discriminating the location of nitrate intervention.

In elderly, priming exercise show similar results observed in no priming condition on energy demand (-5.3% in MOD and -5.7% in MOD2) at the steady-state after supplementation. The results obtained suggest that the mechanisms involved in the modulation of the responses to priming exercise could not be influenced by nitrates.

In young, instead, priming exercise, that leading to speeding of $\dot{V}O_2$ kinetics could have also a better microvascular effect, moreover time constant of the kinetics of deoxy hemoglobin became faster in nitrates supplementation. All this consideration underlines a better mismatching between O_2 delivery and O_2 utilization by the muscle. These effect are probably stronger in acidic environment.

Main results on blood pressure due to priming exercise are on systolic pressure, which shows an opposite trend between the elderly and the young. In the elderly, during exercise phase, systolic pressure is higher after priming exercise in comparison with non-priming one, while in the young it is lowered. In the elderly this tendency is counteracted by nitrate supplementation.

To clarify the effects of nitrates linked to the priming exercise, further in-depth studies would be needed.

SECTION FOUR

STUDY THREE

Nitrates supplementation and

Energy Cost of walking

Summary of the section

In this section nitrate supplementation effects , on energy cost of walking are analyzed. Experimental protocol includes a series of 4 minutes walking at different speeds and slopes.

After an introduction on energy cost of walking, experimental data of energy cost of walking in young and old are reported and analyzed.

List of abbreviations

NO_3^-	Nitrates
NO_2^-	Nitrites
NO	Nitric oxide
BR	Beetroot
PL	Placebo
$\dot{V}O_2$	Oxygen consumption
$\dot{V}CO_2$	Carbon dioxide production
EC	Energy cost

22. Introduction

The energy cost (EC) is the energy spent per unit distance to transport a Kg of body mass ((P. Di Prampero 1986)). It is expressed in milliliters of oxygen (or kilocalories) spent to transport one kilogram of the body for the space of one meter or one kilometer.

EC varies, depending on the form of locomotion used, in fact if we consider a more or less similar muscle power, as that of high-level athletes, the speeds are very different and vary from 7 km/h (100m freestyle) more than 70 km/h (track cycling). These differences are due to all the intrinsic characteristics of each form of locomotion. Therefore, every form of locomotion has its own characteristic EC.

Not all the energy produced by our body is actually transformed into external mechanical work, since most of it is dispersed in heat. This heat dispersion makes the efficiency lower, in fact the efficiency of human locomotion varies between 20 and 30% (P. E. Di Prampero 1985).

In general, the energy that is produced is used to: overcome the air or water resistance, overcome gravitational forces (raise/lower body center of gravity), overcome the inertial forces (acceleration/deceleration of body center of mass), win the friction of the point of contact with the ground (wheel, pads), muscle contraction necessary to maintain posture, support cardiac and respiratory muscle activity, overcoming the internal load (energy spent to overcome the resistance to the movement of the limbs)

The energy spent related to the speed of locomotion allows to calculate EC, using following formula (Di Prampero et al., 1986):

$$\boldsymbol{EC = \frac{\dot{E} * 20.92}{v}} \qquad [1]$$

Where:
$\boldsymbol{\dot{E}}$ *'is the metabolic power, ie the energy expenditure per unit of time, ie the consumption of oxygen ($\dot{V}O_2$) per minute (L /min or mL/min). It can also be normalized for body weight (L/min/kg or mL/min/kg);* ***20.92*** *is the energetic equivalent of O_2 (1 kcal = 4.185 kJ; 1 l of O_2 = 5 kcal = 20.92 kJ), which has units of measure kJ/L;* $\boldsymbol{v}$ *is the speed (km/h, m/s). In the case of walking, it is the walking speed on a treadmill.*

Experimentally, therefore, to obtain the energy cost of a certain form of locomotion, the energy spent at a steady state is measured at different rates.

Analyzing the characteristic EC of walking, where the resistance effect of the air is negligible, a graph with a characteristic U-shape is obtained; this graph shows a minimum EC at intermediate speeds (which are usually the self-selected walking speed) and higher EC for low and high speeds (before the spontaneous transition to running).

All the works mentioned here have been carried out on flat, but similar behaviors can be observed when walking is carried out on inclined terrain, with different slopes (Minetti et al., 2002). In uphill walking EC for the same speed increases as the inclination increases, but at the same slope still follows the characteristic U-shape, with intermediate walking speed, lower than those on flat, which have the minimum energy cost. This increase in EC at a given velocity depending on the slope indicates an increase in intensity in locomotion and this implies a different muscle activation with a probable greater involvement of type II motor units.

As seen before, various studies have been performed on the effects of nitrate supplementation (BR) on exercise and performance. No one, until now, has measured the EC of treadmill walking at various walking speeds and slopes after BR supplementation. Studies performed so far have shown a significantly reduced oxygen consumption in walking at a constant speed. After BR supplementation compared with placebo (PL) there is a significant decrease in the consumption of O_2 ($\dot{V}O_2$) in the 4 km/h walking in young subjects (22 ± 4 years) (Lansley et al., 2011). On the other hand, a work conducted by Kelly showed that in elderly subjects (64 ± 4 years), no variation in the O_2 consumption was found during walking at the same speed after BR supplementation (Kelly et al., 2013). The latter work is the only one until now, that has studied the relationship between healthy elderly subjects, BR and walking.

23. Materials and Methods

23.1 *Aim of the study*

The aim of the study is to investigate the effects at metabolic level induced by nitrate supplementation (NO_3^-), and the comparison within a population of elderly subjects (60-75 years), and young people (20-35 years old) during exercise locomotion. The study was developed in order to describe the trend of energy cost of locomotion on a treadmill at different intensities administered by varying the speed and slope of the instrument. The increase in the metabolic cost of walking in old age means that older adults walk at a slower rate to achieve the same energy expenditure. Based on this evidence, the elderly manifests a higher metabolic cost of the path compared to the young population at the same intensity. We hypothesized that, there is a decrease in the energy cost of locomotion following dietary supplementation of nitrates. And that the decrease in the energy cost of locomotion should be significant al moderate intensities for elderly subjects. In the young, on the contrary, it is hypothesized a possible decrease of the CE of the path particularly at high intensity (up to 20% at higher speeds).

23.2 *Subjects*

The study participants were 20 volunteered, healthy, subjects divided in two groups: 10 old (67 ± 4.3 years) and 10 young (25 ± 3.9 years). During subjects' selection phase were recruited 28 men, but 4 refused to participate, 3 were excluded after preliminary medical examination and 1 drop out during first supplementation phase. The remaining 20 subjects participate in the study after given their informed and written consensus.

Inclusion criteria to participate at the study were: a normal clinical exam, absence of orthopedic, muscle-skeletal, metabolic, cardiovascular or respiratory pathology. ,

Exclusion criteria were: abnormal clinical exam, presence of orthopedic, muscle-skeletal, metabolic, cardiovascular or respiratory pathology, obesity (BMI ≥ 30 kg/m^2), the age limits.

All procedures were approved by the Department of Neurological and Movement Sciences' ethical committee for research on human subjects.

.

OLD	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
O1	75	182	88	2718	31	230	148	55	163
O2	63	180	72	2796	39	210	167	45	138
O3	65	182	91	2211	24	194	155	53	140
O4	65	173	77	2153	28	188	183	69	130
O5	67	161	63	2697	43	206	141	60	141
O6	72	172	71	2549	36	221	147	90	172
O7	71	166	66	2620	40	198	155	64	147
O8	67	164	58	1959	34	166	157	62	125
O9	61	163	58	2718	47	230	145	59	161
O10	65	176	78	3161	41	258	150	131	216
Mean	67	172	72	2558	36	210	155	69	153
St. Dev.	4.3	8.0	11.5	355.6	7.0	26.0	12.3	24.9	26.6

YOUNG	Age (years)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml/min)	$\dot{V}O_{2max}$/kg (ml/min/kg)	Power max (W)	HR_{max} (bpm)	80% GET (W)	50%Δ (W)
Y1	26	175	65	4082	62.8	404	183	181	285
Y2	27	183	85.5	4247	49.7	411	188	154	301
Y3	29	182	80	4501	56.3	414	170	161	324
Y4	29	173	70	4291	61.3	357	189	130	279
Y5	30	169	76.5	3256	42.6	270	186	100	207
Y6	25	169	66	3267	49.5	304	189	102	216
Y7	21	166	60	2673	44.6	242	190	80	172
Y8	20	178	66	3598	54.5	296	187	92	222
Y9	21	184	74	3551	48.0	327	190	132	238
Y10	22	173	69	3308	47.9	304	174	89	220
Mean	25	175	71	3677	52	333	185	122	246
St. Dev.	3.9	6.4	7.8	582.8	6.8	61.1	7.0	34.7	48.2

***Table 1,2**: The table shows the individual data of subjects examined, Old (up) and Young (down). The values of age (Age, years) of the anthropometric parameters have been reported: height (Height, cm) and body mass (Weight, Kg), of the maximum metabolic power, absolute ($\dot{V}O_{2max}$, mL/min) and relative ($\dot{V}O_{2max}$, mL/min/Kg, , of the maximum mechanical power (Power max, W) and of the maximum heart rate (Hrmax, bpm) detected in the preliminary test, and of the Workloads (W) of the two intensity domains (Moderate: 80 % GET, and Severe: ⊿ 50%, W)*

23.3 *Study design and protocol*

The study is a double-blind crossover design with Nitrate (BR) or Placebo (PL) supplementation. The protocol consested of a preliminary day of test (D0) in which subjects performed a ramp incremental test (EXP1); followed by 3 alternate testing days (D1, D2, D3) in which $\dot{V}O_2$ kinetics tests were performed (EXP2). This plan was repeated in 4 experimental phases (BDC1, PS1, BDC2, PS2).

In the first phase (BDC1) (basal data collection) basal conditions were measured. In the second phase (PS1) (post supplementation) the conditions after first period of supplementation (randomly selected between NO_3^- or PL) were recorded.

After at least 10 days of washout, the third phase was performed (BDC2) where basal conditions were measured again. In the fourth and last phase (PS2) the conditions after second period of supplementation (opposite of the first period) were determined.

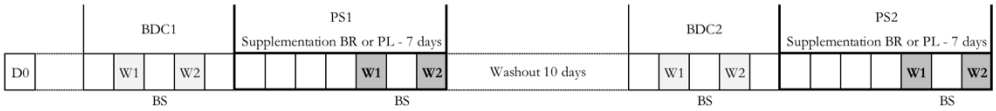


***Figure 1:** The representation schematically summarizes order of test. After the preliminary evaluations (D0) the four experimental phases are followed (BDC1, PS1, BDC2 and PS2) in each of which the execution of the walking evaluation protocol is repeated in non-consecutive days (W1 and W2). All subjects underwent 8 days of supplementation with NO3- and PL, in PS1 and PS2, according to a balanced randomization. PS1 and BDC2 are separated by 10 days of washout. BS indicated blood sample, that is taken for the determination of the blood concentrations of nitrates and nitrites.*

23.3.1 Supplementation

The BR supplementation was made by beetroot juice (BR) (250 ml/day – Azienda agricola "Aureli" – Ortucchio (AQ) - Italy). The juice was provided in two different formulations: one with high concentration (~8.0 mmol) of NO_3^- and one with low concentration (~0.8 mmol) of NO_3^- (used as a placebo (PL)). The PL was identical in color, taste, smell and texture to the NO_3^- rich BR juice. Supplementation was distributed by an experimenter not involved in laboratory tests and/or in data analysis and the subjects and all the experimenters involved didn't know what supplementation was provided (if BR or PL). The matching of assumptions was known only at the end of data analysis.

This is considered a medium-term supplementation design that lasts for 8 days (Porcelli et al. 2015, Wylie et al. 2013).

with ingestion of a daily dose of 250 ml of juice before breakfast. The measurements of the kinetics started on the third day of treatment. The kinetics protocol took place on average 2.5/3 h after the supplementation. In each phase the same cadence of supplementation/test was repeated.

The subjects independently provided the supplementation following a sheet of instructions delivered to them. They were also warnings on foods to avoid rich in nitrates (spinach, beetroot, salad, rocket and Chinese cabbage) and to avoid the use of antibacterial mouthwash.

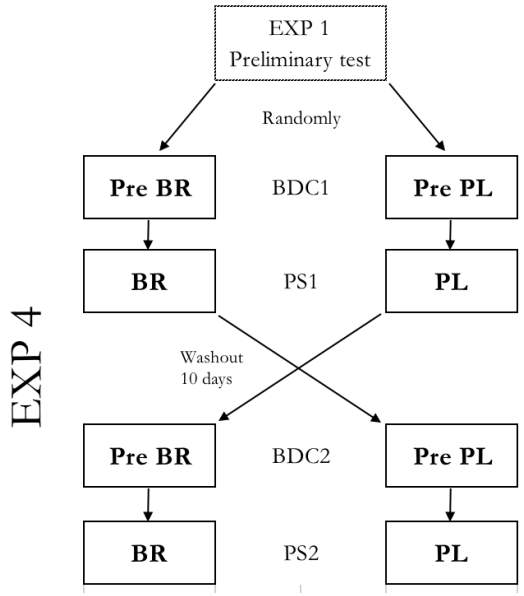


Figure 2: *The representation schematically summarizes the experimental design that structures the presented study, of a longitudinal type in a double-blind crossover. After the preliminary evaluations (EXP1) subjects randomly divided in two groups (BR or PL) and perform first two experimental phases (BDC1 and PS1).*
After 10 days of washout they crossed their condition and change supplementation and perform last two phases (BDC2 and PS2)

23.4 *EXP1 – Preliminary ramp incremental test*

To determine peak of oxygen consumption $\dot{V}O_{2max}$. gas exchange threshold (GET), power output (PO), power output peak (PO_{peak}) and maximal heart rate (HR_{max}) a ramp incremental (RI) test was performed.

RI protocol included 3 min of measurement of baseline condition, where subject remained sit on bike without moving. After that, the subject start cycling at 30 W(warm-up), for 3 min, with self-selected cadence. This cadence was recorded and was maintained during all subsequent tests using visual feedback and verbal encouragement from the experimenters. Warm-up was followed by RI protocol with different workload increments every minute (15, 20, 25, 30 W/min – 2W/8s, 2W/6s. 5W/12s. 3W/6s) in order to maintain entire test duration between 16 and 18 min. Test ended with exhaustion of the subject, and however when the criteria for maximal test were reached ($\dot{V}O_2$ plateau, HR ~ HRmax, [la] >10mM). Failure to maintain the indicated cadence to within 5 rpm (for longer than 5s) during testing despite strong verbal encouragement was considered as the criterion for exhaustion.

In order to obtain a more reliable measure of $\dot{V}O_{2max}$ a verification trial test (VER) was also executed: after 2 min of recovery subjects start pedaling again at 20 W, after 5 min the workload was augmented to constant-work rate equal to 105% of the mechanical power achieved at the end of the ramp test until exhaustion. (Poole, Wilkerson and Jones AM 2008)

23.5 *EXP4 – Energy Cost (EC) of walking*

The test on EC of walking were carried out in two sessions on a treadmill (Hp/Cosmos Saturn®) for each of the four experimental phases (BDC1, PS1, BDC2, PS2). This was done in non-consecutive days and approximately at the same time. In each experimental session a different working protocol was repeated according to the type of path that the subjects faced with progressive and constant increases in speed at different inclinations of treadmill.

To highlight the response of the subjects following BR supplementation, the cardiopulmonary and metabolic responses to walk at different slopes that correspond to different work intensities were analyzed (-10 and -20%, 0%, +10 and +20 %).

In the first session, lasting approximately 70 minutes, after an initial period of data collection at rest, the subjects walked to the first slope (0%) to 2 km/h for 4 min before moving on to the next speed of 3 km/h and then a subsequent increase of 1 km/h every 4 min up to a speed of 6 km/h. After 10 minutes of rest the subjects walked with a negative slope (-10%) to 3 km/h with increases of 1 km/h every 4 min up to 5 km/h to perform after 5 minutes of recovery subjects performed same test at the next slope (-20%).

In the second session, usually done at least after 48 hours, duration approximately 80 minutes, after an initial period of data collection at rest, the subjects walked to the first positive slope (10%) at 2 km/h for 4 min before moving on to the next speed of 3 km/h and then a subsequent increase of 0.5 km/h every 4 min up to a speed of 5 km/h. After 10 minutes of rest the subjects faced the next slope (20%) at 2 km/h with increases of 0.5 km/h every 4 min up to 4.5 km/h.

Each speed subjected to the subjects was maintained for 4 min, in order to reach after about 3 min. a steady state of metabolic parameters; this allowed the analysis to obtain medium and stable parameters related to the specific walking speed.

All the variations foreseen by the protocol have been reported in advance to the subjects through verbal information provided by the experimenters and commands at the moment of beginning or end of the various phases.

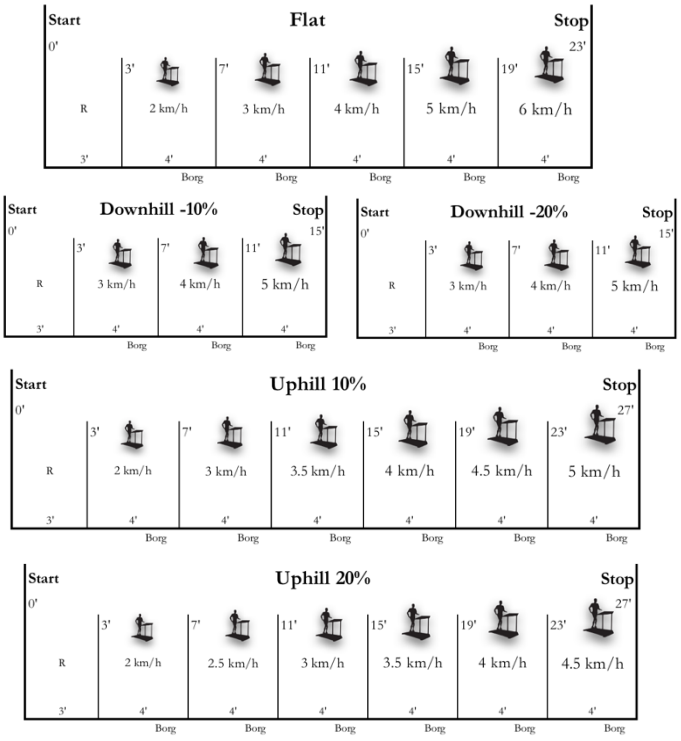


Figure 3: *The representation schematically summarizes the experimental protocols of EC of walking.*

23.6 *Measures and instruments*

During the test the following parameters were detected:

- Pulmonary gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) (K5b2 – Cosmed srl – Rome. Italy).
- Rate of perceived exertion (RPE) with Borg scale 6-20, detected in the last 10 s of each phase of walk at constant load

23.6.1 K5B2 - Cosmed, Rome, Italy

Gaseous exchanges ($\dot{V}O_2$, $\dot{V}CO_2$) and pulmonary ventilation ($\dot{V}E$) were measured breath-by-breath using the metabolimeter with a facial mask. The concentrations of inhaled and exhaled gases were sampled at a frequency of 100 Hz via a capillary line connected to the mask and quantified by respectively paramagnetic analyzers for O_2 with response time of 120 ms and infrared rays (NDIR technology) for CO_2 with a response time of 100 ms. The measurement of the volume of the respiratory flows was carried out by a flowmeter consisting of a bidirectional digital turbine inside which a movable vanity unit, free to rotate around its axis, rotates at speed and in a direction proportional to the flow of air from which it is invested. The number of rotations was transduced into the parameters of interest by an opto-electronic system with infrared LED diodes based on the frequency of detection of the passage of the blades, integrated and processed by a microcomputer.

Prior to each test, the gas concentration and volume transducer analyzers of the turbine were calibrated using a mixture of gases with known concentrations, according to the manufacturer's instructions, (FO_2: 0.16; FCO_2: 0.05) and a 3.0 L syringe. Concentration data e volume were aligned temporally, breath-by-breath, taking into account the delay in the passage of the gas to the capillary then the discrepancy between the time of acquisition of the signal by the analyzer and the flow meter, through the calibration of delays.

23.6.2 Hp/Cosmos Treadmill – Saturn® 300/100r

All tests were carried out on Hp/cosmos - Saturn® treadmill with speeds up to 40 km/h and slope range between -27% and + 27%.

The speed given to patients was set in the User Terminal control panel. This type of software allows to set the working program and the duration of the experimental protocol speed domains; the progressive speeds have been entered automatically.

The safety of the subjects has been guaranteed by the arc/cosmos device which prevents the fall in case of error, loss of coordination and concentration. This instrument, if activated, immediately brakes the belt motor (11 Kw) stopping the treadmill.

24. Data Analysis

24.1 *EC Of walking*

The $\dot{V}O_2$ data measured breath-by-breath were recorded by the metabolimeter during each energy cost assessment tests. An average of the last two minutes of breath--by-breath samples at each walking speed was done. At each speed, the steady state condition have to be reached, so, it was needed to reach at least the third minute of walking, on data of $\dot{V}O_2$, heart rate and respiratory quotient (R –$\dot{V}CO_2$ / $\dot{V}O_2$).

Data were exported and examined in order to exclude artifacts represented by the values not included in the interval defined by the four 4 SD on the local mean. Following these operations, the values of $\dot{V}O_2$ at rest (baseline) were calculated as the average of the data for the third minute, while those of the effort as an average of the last 30 s at each speed administered. The results are therefore average values related to the metabolic and ventilator parameters for each speed domain.

As previously mentioned, the energy spent related to the speed of locomotion makes it possible to calculate the EC. Therefore, the EC was calculated using the formula [1] developed by Di Prampero (Di Prampero, 1986):

$$\boldsymbol{EC = \frac{\acute{\dot{E}} * 20.92}{v}} \qquad [1]$$

*Where: $\dot{\boldsymbol{E}}$ 'is the metabolic power, ie the energy expenditure per unit of time, ie the consumption of oxygen ($\dot{V}O_2$) per minute (L /min or mL/min). It can also be normalized for body weight (L/min/kg or mL/min/kg); **20.92** is the ventilatory equivalent of O_2 (1 kcal = 4.185 kJ; 1 l of O_2 = 5 kcal = 20.92 kJ), which has units of measure kJ/L; **v** is the speed (km/h, m/s). In the case of walking, it is the walking speed on a treadmill.*

The metabolic expense rate (Ė in W/kg) was calculated from the net values of $\dot{V}O_2$ (the value of total energy expenditure minus basal $\dot{V}O_2$ (rest conditions)) assuming an energy equivalent of 20.9 kJ/LO_2 (corresponding to a non-protein respiratory quotient of 0.96).

To obtain a comparison between the subjects, the EC was normalized for body weight so it was expressed in J/Kg*m. Then EC was calculated as:

$$\boldsymbol{EC} = \frac{\left(\dot{V}O_{2SS} - \dot{V}O_{2BAS}\right)}{\boldsymbol{v}} * \boldsymbol{k} * \frac{\mathbf{1}}{\boldsymbol{BM}} \quad [2]$$

Where: $\dot{V}O_{2SS}$ is O_2 consumption calculated at steady state of each step. $\dot{V}O_{2BAS}$ is O_2 consumption calculated at rest, before starting of exercise. ***k*** *is a conversion factor to express EC in J/Kg*m, and it correspond at (20.92*60)/1000 = 1.2552.* ***BM*** *is body mass*

24.2 *Statistical analysis*

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, USA). After verifying the type of data distribution, using the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test, a two-way ANOVA test was applied, considering as factor speed at same slope and treatment (Pre and Post BR, Pre and PL), for repeated measurements.

Subsequently, in order to directly compare the effects of age, treatment and speed in a single test a 3-way-Anova 2x2x2 analysis was performed where these 3 parameters were considered, age (O, Y) treatment (Pre and Post BR, Pre and PL) and speed (3 and 4 km/h – only speeds common to all slopes). Also in this situation Multiple comparison in the post-hoc analysis was performed using the Fisher Test LSD and, Statistical significance was accepted for $p<0.05$.

Finally, the minimum cost at each slope for each subject was calculated and a 2-way-Anova analysis was performed for repeated measurements considering the treatment factor (Pre and Post BR, Pre and PL) and the slope factor (-20%, -10%, 0, + 10%, + 20%).

After each ANOVA, post-hoc analysis with multiple comparison was performed using uncorrected Fisher's LSD test and statistical significance was accepted for $p<0.05$. The results are expressed as mean ± standard deviation (Mean ± SD). On main relevant data significantly different Cohen's d effect size was calculated. (Sawilowsky 2009)

25. Results – Nitrite and Nitrite concentrations

The first results that are reported are those related to the plasma concentration of nitrates [NO_3^-] and nitrites [NO_2^-].

25.1 *Old*

In elderly subjects supplementation with BR resulted in a significant increase ($P < 0.0001$) in [NO_3^-] compared to the concentrations found in the other conditions. Values of increasing are approximately 93.5% between Pre BR and Post BR (39.87 ± 22.55 µM vs 615.06 ± 317.38 µM), 92.8% between Post BR and Pre PL (615.06 ± 317.38 µM vs 44.03 ± 43.33 µM) and 86.5% between Post BR and PL (615.06 ± 317.38 µM vs 82.94 ± 35.52µM). As for [NO_3^-] also [NO_2^-] significantly increasing after BR compared to the other conditions. The increasing corresponds to 46.1% between Pre BR and Post BR (0.244 ± 0.01 µM vs 0.453 ± 0, 22 µM; $p = 0.0003$), 47% between Post BR and Pre PL (0.453 ± 0.22 µM vs 0.240 ± 0.21 µM, $p = 0.0131$) and 62.3% between Post BR and PL (0.453 ± 0.22 µM vs 0.171 ± 0.12 µM; $p = 0.0017$).

25.2 *Young*

As for elderly subjects, supplementation with BR in young has also resulted in a significant increase in plasma levels of both [NO_3^-] and [NO_2^-] in comparison to concentrations without supplementation.

In [NO_3^-] the improvements given by supplementation were appoximately: 92.4% between Pre BR and Post BR (24.32 ± 15.34 µM vs 321.56 ± 246.73 µM), 91.3% between Post BR and Pre PL (321.56 ± 246.73 µM vs 27.85 ± 27.35 µM) and 85.1% between Post BR and PL (321.56 ± 246.73 µM vs 47.73 ± 18.69 µM).

In [NO_2^-], the increases were 44.4% between Pre BR and Post BR (0.301 ± 0.09 µM vs 0.542 ± 0.24 µM, $p = 0.0099$), of 42, 9% between Post BR and Pre PL (0.542 ± 0.24 µM vs 0.309 ± 0.17 µM, $p = 0.0131$) and 52.7% between Post BR and PL (0.542 ± 0.24 µM vs 0.256 ± 0.19 µM; $p = 0.0017$).

OLD	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	39.87 ± 22.55	651.06 ± 317.38*	44.03 ± 43.33	82.94 ± 35.52
[NO_2^-] (µM)	0.244 ± 0.10	0.453 ± 0.22*	0.240 ± 0.21	0.171 ± 0.12

YOUNG	BR		PL	
	Pre	Post	Pre	Post
[NO_3^-] (µM)	24.32 ± 15.34	321.56 ± 246.74*	27.85 ± 27.35	47.73 ± 18.69
[NO_2^-] (µM)	0.301 ± 0.09	0.542 ± 0.23*	0.309 ± 0.17	0.256 ± 0.19

***Table 3,4**: The table shows [NO_3^-] and [NO_2^-] in Old (up) and Young (down). * indicated differences from other condition, $p<0.05$.*

26. Results – EC of walking

Contrary to what has been hypothesized, only limited differences of the EC of walking emerged due to BR supplementation. Furthermore, there were no significant differences in speed induced by BR, so these results have not been reported here. Below, however, the effect of the BR for each slope is analyzed.

26.1 *Walking on flat*

The flat walking protocol provided for 5 speeds (2, 3, 4, 5, 6 km/h). Each speed was maintained for 4 minutes. These are the results for old and young.

26.1.1 Old

The effect of BR supplementation is evident on the EC (Avg last 30"), at speed of 2 km/h, with a statistically significant difference (p = 0.0195) between the conditions of Pre BR and BR (3.54 ± 1.24 vs 3.01 ± 0.81 J/kg*m) equivalent to 0.53 J/kg*m (~ 15%). There are no significant effects dependent on the treatment on EC in the elderly at speeds of 3 km/h between the conditions of Pre BR and BR (2.77 ± 0.62 vs 2.41 ± 0.70 J/kg*m; p = 0.1166), 4 km/h between Pre BR and BR conditions (2.65 ± 0.60 vs 2.42 ± 0.64 J/kg*m; p = 0.2915), 5 km/h between Pre BR and BR conditions (2.85 ± 0.49 vs 2.65 ± 0.63 J/kg*m; p = 0.3856), 6 km/h between Pre BR and BR conditions (3.32 ± 0.53 vs 3.19 ± 0.61 J/kg*m; p = 0.5667) There are no significant differences in Placebo conditions.

26.1.2 Young

The effect of BR supplementation is evident on the EC (Avg last 30 "), at speed of 2 km/h, with a statistically significant difference (p = 0.0045 *) between the conditions of Pre BR and BR (2.63 ± 0.95 vs 2.11 ± 0.51 J/kg*m, ES = 0.682) equivalent to 0.52 J/kg*m (~ 20%). same occurs on the values of the EC related to the speed of 3 km/h between Pre BR and BR (2.36 ± 0.54 vs. 1.83 ± 0.25 J/kg*m; p = 0.0043, ES = 1.260) with a variation of 0.53 J/kg*m (~22.4%), at speed of 4 km/h between the conditions of Pre BR and BR (2.35 ± 0.47 vs. 1.96 ± 0.25 J/kg*m; p = 0.0351, ES = 1.036) with a variation of 0.39 J/kg*m (~16.4%), 5 km/h between Pre BR and BR

conditions (2.52 ± 0.47 vs. 2.12 ± 0.19 J/kg*m; p = 0.0291, ES = 1.116) with a variation of 0.40 J/kg*m (~15.8%), 6 km/h between the conditions of Pre BR and BR (2.93 ± 0.51 vs 2.53 ± 0.18 J/kg*m; p = 0.0268, ES = 1.046) with a variation of 0.41 J/kg*m (~13. 9%).

26.1.3 Old vs Young

In the comparison between O and Y the EC trend was analyzed at speeds of 3 and 4 km/h. From this analysis it emerges that in during flat walking a significant difference is seen between O and Y subjects following BR supplementation at speed of 3 km/h (1.83 ± 0.25 vs 2.41 ± 0.66 J/kg*m; p = 0.0126) with a variation of 0.59 J/kg*m (~ 24.4%). There are no significant effects from the comparison between the elderly and the young at different conditions at the other speeds of the level walk given to the subjects.

EC (J/kg*m)	YOUNG				OLD			
Flat	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
2 km/h	2,63 ± 0,95	2,11 ± 0,51 *	2,48 ± 0,59	2,39 ± 0,75	3,54 ± 1,17	3,01 ± 0,76 *	3,00 ± 0,70	2,96 ± 0,59
3 km/h	2,36 ± 0,54	1,83 ± 0,25 *	2,15 ± 0,42	2,20 ± 0,44	2,77 ± 0,58	2,41 ± 0,66 #	2,40 ± 0,66	2,57 ± 0,63
4 km/h	2,35 ± 0,47	1,96 ± 0,25 *	2,17 ± 0,53	2,11 ± 0,42	2,65 ± 0,56	2,42 ± 0,60	2,34 ± 0,62	2,48 ± 0,54
5 km/h	2,52 ± 0,47	2,12 ± 0,19 *	2,36 ± 0,51	2,28 ± 0,47	2,85 ± 0,46	2,65 ± 0,59	2,56 ± 0,58	2,75 ± 0,52
6 km/h	2,93 ± 0,51	2,53 ± 0,18 *	2,76 ± 0,48	2,66 ± 0,54	3,32 ± 0,50	3,19 ± 0,58	3,02 ± 0,57	3,17 ± 0,48

***Table 1**: EC of walking on flat in Young and Old. Significance legend (P <0.05): *difference to pre BR condition. # difference to Young*

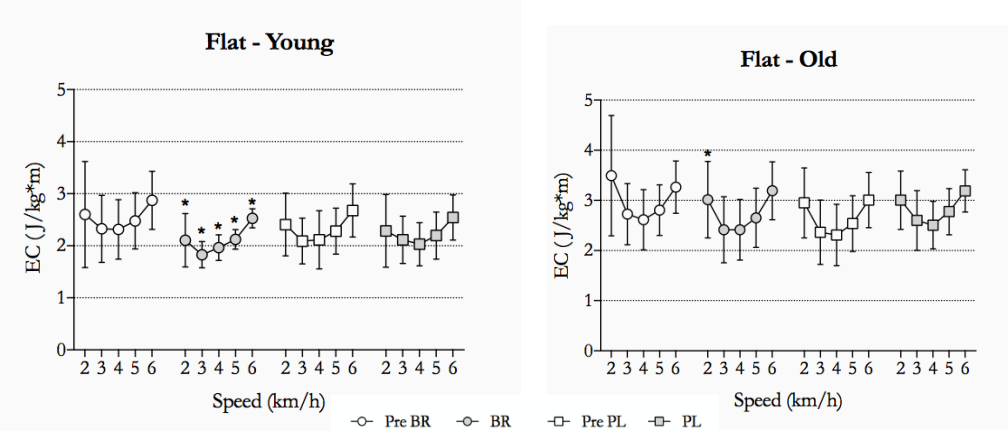


***Figure 1**: EC of walking on flat in Young (left) and Old (right). Significance legend (P <0.05): *difference to pre BR condition.*

26.2 *Downhill*

The protocol for downhill walking provided 3 speeds with a negative slope of 10% (3, 4, 5 km/h) and 3 speeds with a negative gradient of 20% (3, 4, 5 km/h). Each speed was maintained for 4 minutes. These are the results for old and young.

26.2.1 Old

The effect of BR supplementation does not demonstrate significant treatment effects on EC in elderly at a 10% negative slope at a rate of 3 km/h between Pre BR and BR conditions (1.36 ± 0.42 vs 1.13 ± 0.53 J/kg*m; p = 0.1123), 4 km/h between Pre BR and BR conditions (1.35 ± 0.35 vs 1.12 ± 0.52 J/kg*m, p = 0.1154), 5 km/h between Pre BR and BR conditions (1.38 ± 0.37 vs 1.68 ± 0.72 J/kg*m, p = 0.3509).

The same result was observed at a 20% negative slope at speed of 3 km/h between the Pre BR and BR conditions (1.90 ± 0.47 vs. 1.68 ± 0.72 J/kg*m; p = 0.2770), 4 km/h between Pre BR and BR conditions (1.99 ± 0.47 vs. 1.88 ± 0.76 J/kg*m; p = 0.5909), 5 km/h between Pre BR and BR conditions (2.15 ± 0.51 vs 2.21 ± 0.89 J/kg*m; p = 0.7553). There are no significant differences in Placebo conditions.

26.2.2 Young

The effect of BR supplementation does not demonstrate significant treatment effects on EC in Y at a 10% negative slope at a rate of 3 km/h between Pre BR and BR conditions (1.22 ± 0.32 vs 1.00 ± 0.46 J/kg*m; p = 0.0894), 4 km/h between Pre BR and BR conditions (1.20 ± 0.32 vs 1.11 ± 0.35 J/kg*m; p = 0.4759), 5 km/h between the Pre BR and BR conditions (1.25 ± 0.36 vs 1.18 ± 0.37 J/kg*m, p = 0.5899).

The same result was observed at a negative slope of 20% at speed of 3 km/h between the conditions of Pre BR and BR (2.18 ± 0.39 vs 1.97 ± 0.62 J/kg*m; p = 0.2349), 4 km/h between Pre BR and BR conditions (1.93 ± 0.57 vs 1.91 ± 0.44 J/kg*m; p = 0.8975), 5 km/h between Pre BR and BR conditions (2.21 ± 0.62 vs 2.02 ± 0.48 J/kg*m; p = 0.2814). There are no significant differences in Placebo conditions.

26.2.3 Young vs Old

In the comparison between old and young there is no significant difference in descent at any speed or slope. There are no significant differences in Placebo conditions.

EC (J/kg*m)	YOUNG				OLD			
-10%	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
3 km/h	1,22 ± 0,32	1,00 ± 0,46	1,06 ± 0,42	0,92 ± 0,49	1,36 ± 0,39	1,13 ± 0,50	1,20 ± 0,55	1,31 ± 0,58
4 km/h	1,20 ± 0,32	1,11 ± 0,35	1,08 ± 0,30	1,08 ± 0,43	1,35 ± 0,33	1,12 ± 0,49	1,23 ± 0,50	1,38 ± 0,60
5 km/h	1,25 ± 0,36	1,18 ± 0,37	1,19 ± 0,29	1,24 ± 0,38	1,38 ± 0,35	1,24 ± 0,47	1,33 ± 0,55	1,44 ± 0,56

***Table 2**: EC of walking downhill 10% in Young and Old.*

EC (J/kg*m)	YOUNG				OLD			
-20%	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
3 km/h	2,18 ± 0,89	1,97 ± 0,62	1,92 ± 0,52	2,01 ± 0,70	1,90 ± 0,44	1,68 ± 0,68	1,51 ± 0,47	1,87 ± 0,66
4 km/h	1,93 ± 0,57	1,91 ± 0,44	1,93 ± 0,61	1,99 ± 0,76	1,99 ± 0,44	1,88 ± 0,72	1,68 ± 0,59	1,93 ± 0,63
5 km/h	2,21 ± 0,62	2,02 ± 0,48	2,02 ± 0,55	2,00 ± 0,66	2,15 ± 0,48	2,21 ± 0,84	1,93 ± 0,60	2,17 ± 0,68

***Table 3**: EC of walking downhill 20% in Young and Old.*

26.3 *Uphill*

The protocol for the uphill walking provided 6 speeds with a positive gradient of 10% (2, 3, 3.5, 4, 4.5, 5 km/h) and 6 speeds with a 20% positive slope (2, 2.5, 3, 3.5, 4.5 km/h). Each speed was maintained for 4 minutes. These are the results for old and young.

26.3.1 Old

The effect of BR supplementation does not demonstrate significant treatment effects on EC in elderly at positive slope of 10%, at speed of 2 km/h between the conditions of Pre BR and BR (6.38 ± 0.57 vs 5.91 ± 0.86 J/kg*m; p = 0.0683), 3 km/h between the Pre BR and BR conditions (5.90 ± 0.53 vs 5.65 ± 0.65 J/kg*m; p = 0.3233), 3.5 km/h between Pre BR and BR conditions (5.96 ± 0.54 vs 5.66 ± 0.48 J/kg*m, p = 0.2396), 4 km/h between Pre BR and BR conditions (5.98 ± 0.40 vs 5.66 ± 0.58 J/kg*m; p = 0.2098), 4.5 km/h between Pre BR and BR conditions (6.21 ± 0.37 vs 5.81 ± 0.63 J/kg*m; p = 0.1181), 5 km/h between the Pre BR and BR conditions (6.15 ± 0.50 vs 5.97 ± 0.54 J/kg*m, p = 0.4748).

The effect of BR supplementation is evident at the 20% positive slope on the EC (Avg last 30 "), at speed of 2 km/h, with a statistically significant difference (p = 0.0045) between conditions of Pre BR- and BR- (10.02 ± 1.36 vs 9.23 ± 1.17 J/kg*m; p = 0.0477). No statistically significant differences at speed 2.5 km/h between Pre BR and BR conditions (9.87 ± 1.41 vs 9.24 ± 1.19 J/kg*m; p = 0.1118), 3 km/h between Pre BR and BR conditions (9.73 ± 1.26 vs 9.35 ± 1.43 J/kg*m; p = 0.3412), 3.5 km/h between Pre BR and BR conditions (9.63 ± 1.04 vs 9.28 ± 1.22 J/kg*m; p = 0.3849) There are no significant differences in Placebo conditions.

26.3.2 Young

The effect of BR supplementation does not demonstrate significant treatment effects on EC in Y at 10% positive slope, at speed of 2 km/h between Pre BR and BR conditions (5.82 ± 1.48 vs 6.00 ± 1.04 J/kg*m; p = 0.3701), 3 km/h between Pre BR and BR conditions (5.55 ± 1.09 vs 5.61 ± 0.79 J/kg*m; p = 0.7785), 3.5 km/h between Pre BR and BR conditions (5.66 ± 0.96 vs 5.46 ± 0.42 J/kg*m, p = 0.3208), 4 km/h between Pre BR and BR conditions (5.48 ± 0.86 vs 5.47 ± 0.40 J/kg*m, p = 0.9613),

4.5 km/h between Pre BR and BR conditions (5.73 ± 0.90 vs 5.61 ± 0.50 J/kg*m; p = 0.5705), 5 km/h between the Pre BR and BR conditions (5.86 ± 0.84 vs 5.71 ± 0.49 J/kg*m, p = 0.4554).

The same result was observed at 20% positive slope at speed of 2 km/h between the Pre BR and BR conditions (9.52 ± 1.39 vs 9.17 ± 0.97 J/kg*m; p = 0.2176), 2.5 km/h between Pre BR and BR conditions (9.41 ± 1.23 vs 8.99 ± 0.80 J/kg*m; p = 0.1396), 3 km/h between Pre BR and BR conditions (9.33 ± 1.04 vs 9.26 ± 0.75 J/kg*m; p = 0.8081), 3.5 km/h between Pre BR and BR conditions (9.28 ± 1.07 vs 9.32 ± 0.76 J/kg*m; p = 0.8892), 4 km/h between Pre BR and BR conditions (9.38 ± 1.07 vs 9.29 ± 0.73 J/kg*m; p = 0.7642), 4.5 km/h between Pre BR and BR conditions (9.45 ± 1.19 vs 9.43 ± 0.78 J/kg*m; p = 0.9601).

26.3.3 Young vs Old

In the comparison between Old and young there is no significant difference in descent at any speed or slope.

EC (J/kg*m)	YOUNG				OLD			
10%	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
2 km/h	5,82 ± 1,48	6,00 ± 1,04	6,19 ± 1,25	5,57 ± 0,93	6,38 ± 0,54	5,91 ± 0,81	5,95 ± 0,86	6,18 ± 1,09
3 km/h	5,55 ± 1,09	5,61 ± 0,79	5,74 ± 0,87	5,53 ± 0,96	5,90 ± 0,50	5,65 ± 0,61	5,58 ± 0,81	5,84 ± 1,08
3,5 km/h	5,66 ± 0,96	5,46 ± 0,42	5,66 ± 0,86	5,44 ± 0,75	5,96 ± 0,51	5,66 ± 0,46	5,53 ± 0,65	5,86 ± 1,00
4 km/h	5,48 ± 0,86	5,47 ± 0,40	5,63 ± 0,87	5,44 ± 0,76	5,98 ± 0,38	5,66 ± 0,55	5,50 ± 0,63	5,88 ± 0,99
3,5 km/h	5,73 ± 0,90	5,61 ± 0,50	5,74 ± 0,82	5,75 ± 0,76	6,21 ± 0,35	5,81 ± 0,59	5,73 ± 0,65	6,06 ± 1,06
5 km/h	5,86 ± 0,84	5,71 ± 0,49	5,75 ± 0,74	5,75 ± 0,71	6,15 ± 0,47	5,97 ± 0,51	5,72 ± 0,71	6,20 ± 1,01

***Table 4**: EC of walking uphill 10% in Young and Old.*

EC (J/kg*m)	YOUNG				OLD			
20%	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
2 km/h	9,52 ± 1,39	9,17 ± 0,97	9,57 ± 1,24	9,26 ± 1,17	10,02 ± 1,28	9,23 ± 1,11	9,49 ± 0,85	9,93 ± 1,41
2,5 km/h	9,41 ± 1,23	8,99 ± 0,80	9,43 ± 1,29	9,04 ± 1,41	9,87 ± 1,33	9,24 ± 1,13	9,19 ± 1,13	10,03 ± 1,20
3 km/h	9,33 ± 1,04	9,26 ± 0,75	9,45 ± 1,22	9,07 ± 1,17	9,73 ± 1,19	9,35 ± 1,35	9,15 ± 1,00	10,15 ± 1,16
2,5 km/h	9,28 ± 1,07	9,32 ± 0,76	9,55 ± 1,22	9,12 ± 1,21	9,63 ± 0,98	9,28 ± 1,15	9,14 ± 1,03	9,84 ± 1,20
4 km/h	9,38 ± 1,07	9,29 ± 0,73	9,58 ± 1,01	9,25 ± 1,20				
2,5 km/h	9,45 ± 1,19	9,43 ± 0,78	9,78 ± 1,07	9,45 ± 1,07				

***Table 5**: EC of walking uphill 20% in Young and Old.*

26.4 *EC and slopes*

The results of the energy cost according to the slope do not show particular treatment effects on minimum EC for each slope. There is only a significant reduction in young people during flat (2.17 ± 0.54 vs 1.68 ± 0.33 J/kg*m; p = 0.0172) and in elderly at +20% (9.29 ± 0.94 vs. 8.85 ± 0.94 J/kg*m; p = 0.0499). Furthermore, there is no significant change in speed at minimum EC.

EC (J/kg*m)	YOUNG				OLD			
	Pre BR	BR	Pre PL	PL	Pre BR	BR	Pre PL	PL
-20%	1,81 ± 0,57	1,76 ± 0,50	1,83 ± 0,52	1,83 ± 0,72	1,80 ± 0,32	1,67 ± 0,66	1,49 ± 0,48	1,81 ± 0,69
-10%	1,10 ± 0,33	0,96 ± 0,41	0,92 ± 0,37	0,92 ± 0,48	1,25 ± 0,34	1,06 ± 0,43	1,18 ± 0,53	1,22 ± 0,46
0%	2,17 ± 0,54	1,68 ± 0,33 *	1,99 ± 0,49	1,97 ± 0,47	2,55 ± 0,47	2,36 ± 0,63	2,32 ± 0,64	2,46 ± 0,56
10%	5,24 ± 1,15	5,25 ± 0,42	5,38 ± 0,73	5,26 ± 0,74	5,76 ± 0,53	5,40 ± 0,53	5,38 ± 0,66	5,68 ± 0,99
20%	8,92 ± 1,13	8,83 ± 0,75	9,16 ± 1,17	8,77 ± 1,17	9,29 ± 0,94	8,85 ± 0,94 *	8,87 ± 0,83	9,60 ± 1,19

***Table 6**: Minimal EC of walking at different slopes in Young and Old. Significance p<0.05 * different to Pre BR*

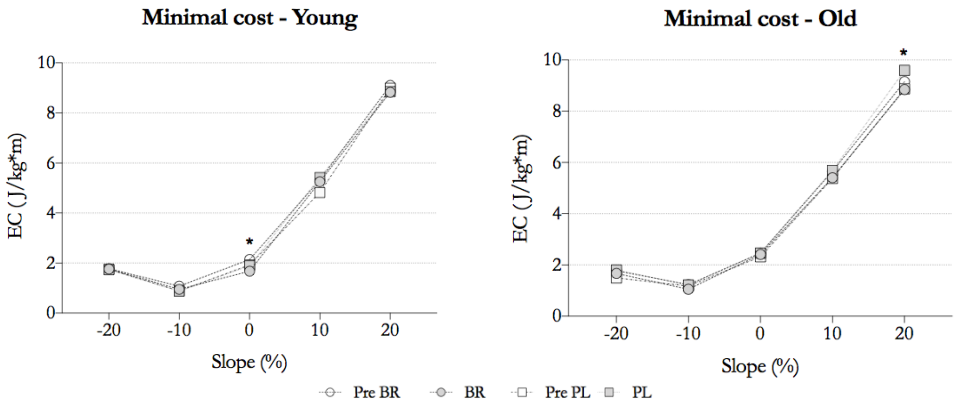


***Figure 2**: Minimal EC of walking at different slopes in Young and Old. Significance p<0.05 * different to Pre BR*

27. Discussion

The present study aims to investigate the effects of NO_3^- supplementation on EC of walking by administering beetroot juice (~8.0 mmol of NO_3^- in 0.25 L for 8 days) in elderly subjects (Old: 68 ± 4.6 years; n = 10) and young (Young: 25 ± 3.9 years; n = 10). EC variations were explored in 5 different slopes and, for each of them, the EC at increasing speed was observed and analyzed. The main results obtained can be summarized as follows: 1) reduction of the rising EC in elderly at speed of 2 km/h to a positive gradient of 10%; 2) reduction of the EC on flat in young people for all the speeds considered.

No one, until now, has measured the EC of walking on treadmill at various speeds.

27.1 *Old*

Following nitrate supplementation, significant increases in plasma NO_3^- and NO_3^- concentrations occur, with values similar to those reported in previous studies (Larsen et al., 2007; Webb et al., 2008; Vanhatalo et al., 2010). The fact that the plasma levels of nitrite, closely related to the increase in the bioavailability of NO, are only slightly lower than those found in young people suggests that contrary to what is expected, age-related changes in oral bacterial colonization are not very strong (Kelly et al., 2013)

27.1.1 Effects of BR supplementation

One of the most relevant aspects emerging from the present study is the significant reduction of O_2 consumption in the uphill slope at a 20% positive slope following NO_3^- supplementation in Old group. The response of this parameter is, in fact, influenced by the treatment with nitrates, with a reduction at the speed of 2 km/h with a statistically significant difference of 7.9%.

Kelly and colleagues, are the only ones who has studied the relationship between healthy elderly subjects, NO_3^- supplementation and locomotion. In elderly subjects (64 ± 4 years), no variation in O_2 consumption was found during walk at the same speed after supplementation of NO_3^- (Kelly et al., 2013).

No other studies were found to compare our results with, because most of them were performed on cycle ergometer, where it can measure efficiency rather than EC. One of these studies, on young subjects, measured efficiency at 5 different intensities

measured as percentage of to the $\dot{V}O_{2max}$ (45, 60, 70, 80 and 85%), finding an improvement (+10%, +3%, respectively) of the efficiency in the 4 lower intensities of exercise after supplementation of NO_3^- and not PL (Larsen et al., 2007). The data of the present study therefore seem to have a direction contrary to those just underlined, highlighting a reduction in the EC in elderly in high-intensity exercise.

The vascular effect of nitrates, as the role of NO as secondary messenger in the synthesis of GMPc starting from GTP, able to modulate the relaxation of arteriolar smooth muscle (Ferguson et al. 2015,Jones 2014), may not be highlighted due to the absence of important functional and structural impairment in the peripheral district and muscle perfusion. In fact, it should be considered that the characteristics of the subjects recruited in the O group are only partially representative of the belonging population as to the general state of good physical conditioning ($\dot{V}O_{2max}$ = 36 ± 7 ml/min/kg), for this reason the effect of supplementation may be reduced and not consistent with previous work (Kelly et al., 2013)

27.2 *Young*

Supplementation of nitrates in young subjects leads to an effective increase in the plasma concentrations of nitrates and nitrites and with them the bioavailability of NO.

27.2.1 Effects of BR supplementation

One of the most relevant aspects that emerges from the present study is the significant reduction of O_2 consumption in flat march following NO_3^- supplementation in the Young group. The response of this parameter is in fact influenced by the treatment with nitrates, with a reduction at speed of 2 km/h of 19.9%. The same occurs on values at speed of 3 km/h with a variation of 22.4%, at speed of 4 km/h with a variation of 16.4%, at speed of 5 km/h with a variation of 15.8%, and at speed of 6 km/h with a variation of (13.9%).

Studies performed so far have shown a significantly reduced oxygen consumption in walking at a constant rate. After supplementation of NO_3^- compared with placebo (PL) there is a significant decrease in the consumption of O_2 ($\dot{V}O_2$) in the 4 km/h walking in young subjects (22 ± 4 years) (Lansley et al., 2011). This speed in young people could be considered 'self selected speed' and the present study shows that the

reduction of EC values, on flat, following treatment of NO_3^- in young people, 22.4%, is obtained at the spontaneous speed of walk.

The mechanisms underlying what is reported, in particular the reduction of $\dot{V}O_2$ to steady state in moderate exercise, following the intake of nitrates, are currently unclear. However, the involvement of NO as a cellular signaling device in the modulation of a multiplicity of processes implicated in exercise physiology is amply established, in particular on the regulation of endothelium-dependent vasodilatation, mitochondrial respiration and aspects of muscular contractility (Stamler et al. al., 2001).

The speed of the $\dot{V}O_2$ kinetics in high-intensity exercise, but not in moderate exercise (Breese et al., 2013), the scarcity of effects observed in the elderly (Kelly et al., 2013) or in subjects with a $V'O_2max$ very high (Porcelli et al., 2015), which are characterized by a greater presence of slow type I fibers to the detriment of fast type II (Pette and Staron 2000), have led to hypothesize that the NO_3^- supplementation may have more effect on fast fibers than slow ones.

Hernandez and colleagues, have shown an increase in strength following NO_3^- supplementation in an isolated muscle (Fast Extensor of Fingers - ELD) with fast fibers, for stimulation frequencies up to 50 Hz (Hernandez et al., 2012). Similarly, a slow muscle (Soleus) muscle that has not undergone any change for each stimulation frequency after BR. Data of the present study would indicate a direction contrary to the one described above because the exercise intensity at which the main changes in EC in young population are highlighted involves a percentage of the $\dot{V}O_{2max}$ which is positioned below the first aerobic threshold (30-40% vs 60-70% $\dot{V}O_{2max}$) or low intensity exercise. A reduction in EC of walking could be related to the optimization of the P/O ratio of the type I fibers even if the effect of nitrates on the calcium channels of the sarcoplasmic reticulum cannot be excluded in terms of reduction of energy expenditure linked to the management of intracellular ion transients. This consideration is supported by Larsen studies which showed a better efficiency in oxidative phosphorylation (P/O ratio), with a 19% improvement after NO_3^- supplementation and an increase in ATP production of 23% (Larsen et al. 2011).

27.3 *Comparison with Minetti study*

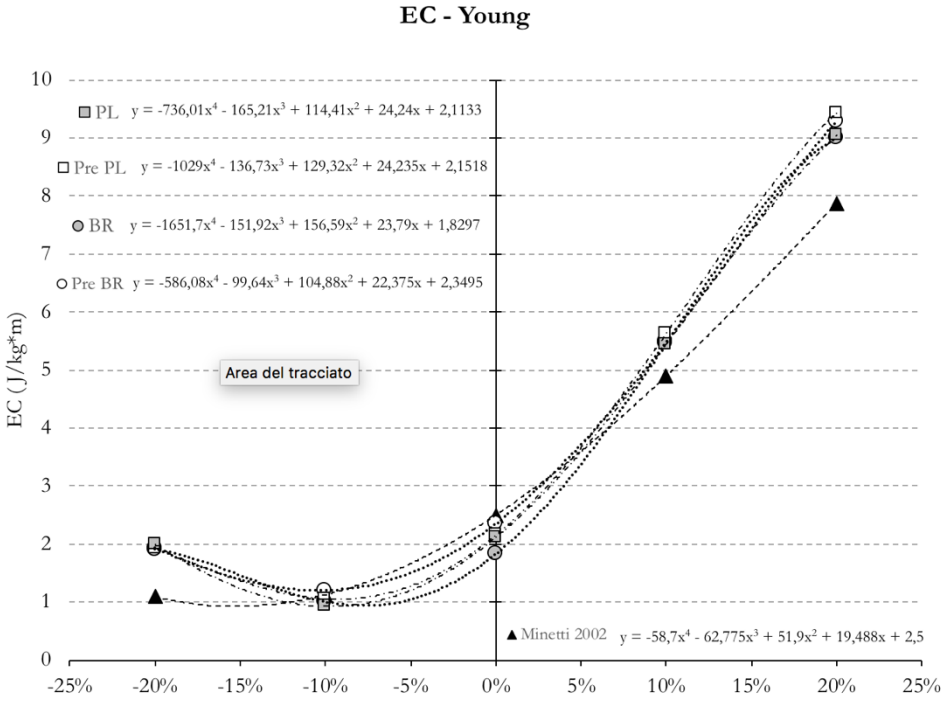
Considering the variability of the EC of walking obtained at different speeds and, under different slope conditions, a comparison was made with the study by Minetti and colleagues (Minetti et al., 2002) which reports the equations to estimate the energy cost at the various slopes.

Minetti in this work has found that there is an equation that relates EC of walking according to the slope. The equation is as follows:

$$EC = 280.5i^5 - 58.7i^4 - 76.8i^3 + 51.9i^2 + 19.6i + 2.5 \qquad (R^2 = 0.999)$$

*Where **EC** is the EC of walking and **i** is the inclination.*

Our results show that in relation to the prediction equation reported above, the elderly subjects of the present study show data overlap in the conditions of walking on flat and at the negative slope of 10%. In the walking on flat the young subjects taken into consideration by the present study show an EC that moves away from the value of the subjects studied by Minetti et al., as consequence of the reduction of the EC values of walking on flat after BR supplementation of the. However, on extreme slopes, (-20%, +10% and 20%) the trend of the EC of walking moves away from the specific EC curve of the subjects studied by Minetti (Minetti et al., 2002) This suggests that the athletes investigated by Minetti et al., specialized in mountain endurance races, developed a more economical condition during downhill greater than 10%; the same result is observed during uphill climb equal to and greater than 10%. Since the movement of center of mass at extreme gradients, which could reduce the overall mechanical work, can be changed little, a possible explanation reported by Minetti of the greater economy could be the decrease of the contractions necessary for the stabilization during downhill.



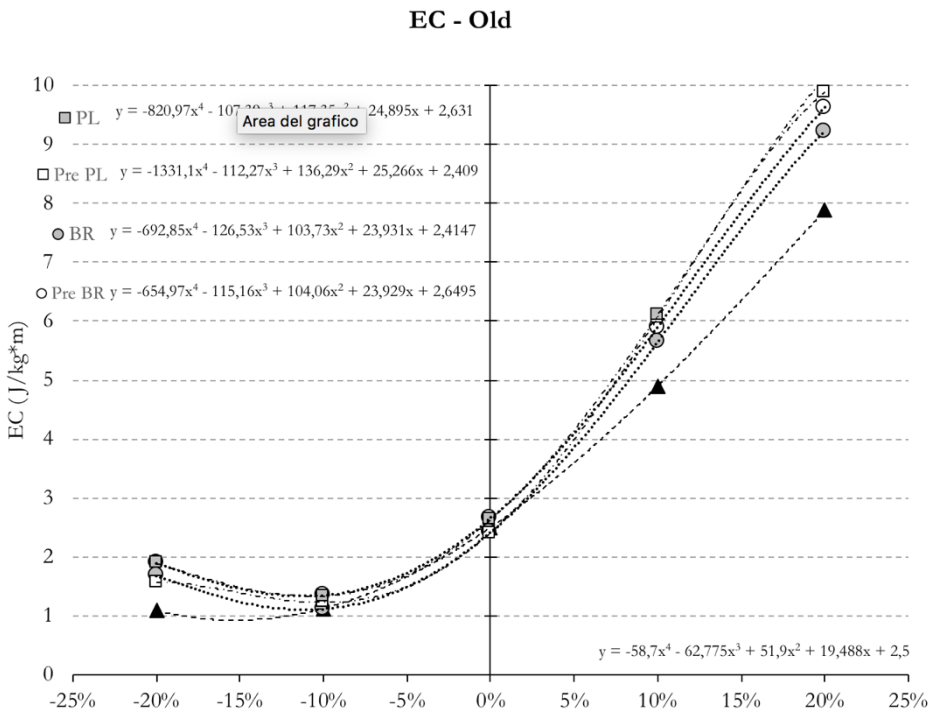


***Figure 3 - 4**: Minimal EC of walking at different slopes in Young (up) Old (down) in comparison with study of Minetti and colleagues. It can be observed from the graphical point of view as to the most extreme gradients taken into consideration, ie -10%, + 10% and + 20% the EC course of the path moves away from the specific CE of the subjects analyzed in the study of Minetti et to the. (Minetti et al., 2002).*

27.4 *Study limits*

From the presented study emerge a series of non-negligible aspects that can be considered as limits in particular to the size of the effect under examination and to the statistical power of the results.

Firstly, the reduced number of the sample (Old: n = 10, Young: n = 10), in association with the inter-individual variability that characterizes each of the two subgroups, could prevent the achievement of statistical significance in the different comparisons between variables. In particular, elderly subjects, whose recruitment has not always proved to be easy, tend to be not very representative of the population they belong to because of their fitness level that is average higher than that normally expected ($\dot{V}O_{2max}$).

They are physically active and pay attention to the health benefits of exercise and diet, and these aspects may have reduced the potential impact of NO_3^- supplementation by masking the effects in particular in vascular level. Any training effects that may have been induced by the overall duration of the experimental design were excluded from the randomization of the treatment assignment.

28. Conclusion

Supplementation of nitrates to which the subjects recruited in the present study have been subjected has indeed determined an elevation of the plasma concentrations of this ion and consequently the bioavailability of NO of which it is a precursor.

This intervention, hypothesized able to influence the physiological responses to exercise, proved to be effective in reducing EC values of the flat walking at all speeds given (at speed of 2 km/h of 19.9% to 3 km/h of 22.4%, to 4 km/h of 16.4%, to 5 km/h of 15.8%, and to 6 km/h of 13, 9%) in young. This reduction highlights the reduced values following NO_3^- supplementation compared to the EC registered values of flat walking on the study of Minetti (Minetti et al. 2002). In the same group, the exercise at negative (-10%, -20%) and positive (+ 10%, + 20%) slopes does not seem to benefit from the treatment resulting in an absence of changes in the EC of walking. The results obtained suggest that the mechanisms involved in the modulation of the responses to the exercise in relation to the higher bioavailability of nitrates could be related to the optimization of the P/O ratio in type I fibers even if the effect of the nitrates on calcium channels of the sarcoplasmic reticulum, in terms of reduction of energy expenditure related to the management of intracellular ion transients, cannot be excluded.

The elderly seems to show a relative effect of the nitrates in the uphill walk at a 20% positive slope following the NO_3^- supplementation of 7.9%.

Data of present study seem to have a direction contrary to those reported on previous studies, performed, however, on cycle ergometer, highlighting a reduction of EC of walking in the elderly in high-intensity exercise.

However, a vascular effect of the nitrates may not be evident due to the characteristics of the subjects recruited in old group, which are only partially representative of the belonging population because of good physical conditioning ($\dot{V}O_{2max}$ = 36 ± 7 ml/min/kg); this is why the effect of supplementation can be reduced and not consistent with previous work (Kelly et al. 2013).

Bibliography

ACSM's guidelines for exercise testing and prescription. n.d. "ACSM, 2009. Exercise and Physical Activity for Older Adults."

Araújo, Adriana L de, Léia CR Silva, Juliana Ruiz Fernandes, and Gil Benard. 2013. "Preventing or Reversing Immunosenescence: Can Exercise Be an Immunotherapy?" *Immunotherapy* 5 (8):879–93.

Bailey, Stephen J., Jonathan Fulford, Anni Vanhatalo, Paul G. Winyard, Jamie R. Blackwell, Fred J. DiMenna, Daryl P. Wilkerson, Nigel Benjamin, and Andrew M. Jones. 2010. "Dietary Nitrate Supplementation Enhances Muscle Contractile Efficiency during Knee-Extensor Exercise in Humans." *Journal of Applied Physiology* 109 (1):135–48.

Bailey, Stephen J., Paul Winyard, Anni Vanhatalo, Jamie R. Blackwell, Fred J. DiMenna, Daryl P. Wilkerson, Joanna Tarr, Nigel Benjamin, and Andrew M. Jones. 2009. "Dietary Nitrate Supplementation Reduces the O2 Cost of Low-Intensity Exercise and Enhances Tolerance to High-Intensity Exercise in Humans." *Journal of Applied Physiology* 107 (4):1144–55.

Baker, John E., Jidong Su, Xiangping Fu, Anna Hsu, Garrett J. Gross, James S. Tweddell, and Neil Hogg. 2007. "Nitrite Confers Protection against Myocardial Infarction: Role of Xanthine Oxidoreductase, NADPH Oxidase and KATP Channels." *Journal of Molecular and Cellular Cardiology* 43 (4):437–44.

Bassett, D R, and E T Howley. 2000. "Limiting Factors for Maximum Oxygen Uptake and Determinants of Endurance Performance." *Medicine and Science in Sports and Exercise* 32 (1):70–84.

Beaver, W. L., K. Wasserman, and B. J. Whipp. 1986. "A New Method for Detecting Anaerobic Threshold by Gas Exchange." *Journal of Applied Physiology* 60 (6):2020–27.

Behnke, Brad J., Michael D. Delp, Patrick J. Dougherty, Timothy I. Musch, and David C. Poole. 2005. "Effects of Aging on Microvascular Oxygen Pressures in Rat Skeletal Muscle." *Respiratory Physiology & Neurobiology* 146 (2–3):259–68.

Behnke, Bradley J., and Michael D. Delp. 2010. "Aging Blunts the Dynamics of Vasodilation in Isolated Skeletal Muscle Resistance Vessels." *Journal of Applied Physiology* 108 (1):14–20.

Bell, Christopher, Donald H Paterson, John M Kowalchuk, Andrew P Moy, David B Thorp, Earl G Noble, Albert W Taylor, and David A Cunningham. 2001. "Determinants of Oxygen Uptake Kinetics in Older Humans Following Single-Limb Endurance Exercise Training." *Experimental Physiology* 86 (5):659–65.

Benjamin, Nigel, Fionnuala O'Driscoll, Hamish Dougall, Callum Duncan, Lorna Smith, Michael Golden, and Hamish McKenzie. 1994. "Stomach NO Synthesis." *Nature* 368 (6471):502–502.

Bentley, Robert F., Jeremy J. Walsh, Patrick J. Drouin, Aleksandra Velickovic, Sarah J. Kitner, Alyssa M. Fenuta, and Michael E. Tschakovsky. 2017. "Dietary Nitrate Restores Compensatory Vasodilation and Exercise Capacity in Response to a Compromise in Oxygen Delivery in the Noncompensator Phenotype." *Journal of Applied Physiology* 123 (3):594–605.

Bescos, Raól, Ferran A. Rodríguez, Xavier Iglesias, Miguel D. Ferrer, Elena Iborra, and Antoni Pons. 2011. "Acute Administration of Inorganic Nitrate Reduces VO2peak in Endurance Athletes." *Medicine and Science in Sports and Exercise* 43 (10):1979–86.

Biagi, P. 2009. "Il Cuore Senile E Lo Scompenso Cardiaco Diastolico Nell'anziano." *Italian Journal of Medicine*.

Boit, Mariasole Da, Stephen J. Bailey, Steven Callow, Fred J. DiMenna, and Andrew M. Jones. 2014. "Effects of Interval and Continuous Training on O2 Uptake Kinetics during Severe-Intensity Exercise Initiated from an Elevated Metabolic Baseline." *Journal of Applied Physiology* 116 (8):1068–77.

Bond, Vernon, Bryan H Curry, Richard G Adams, Richard M Millis, and Georges E Haddad. 2014. "Cardiorespiratory Function Associated with Dietary Nitrate Supplementation." *Appl Physiol Nutr*

Metab 39 (2):168–72.

Bondonno, Catherine P., Alex H. Liu, Kevin D. Croft, Natalie C. Ward, Ian B. Puddey, Richard J. Woodman, and Jonathan M. Hodgson. 2015. "Short-Term Effects of a High Nitrate Diet on Nitrate Metabolism in Healthy Individuals." *Nutrients* 7 (3):1906–15.

Breese, Brynmor C., Melitta A. McNarry, Simon Marwood, Jamie R. Blackwell, Stephen J. Bailey, and Andrew M. Jones. 2013. "Beetroot Juice Supplementation Speeds O2 Uptake Kinetics and Improves Exercise Tolerance during Severe-Intensity Exercise Initiated from an Elevated Metabolic Rate." *AJP: Regulatory, Integrative and Comparative Physiology* 305 (12):R1441–50.

Breese, Brynmor C., David C. Poole, Dai Okushima, Stephen J. Bailey, Andrew M. Jones, Narihiko Kondo, Tatsuro Amano, and Shunsaku Koga. 2017. "The Effect of Dietary Nitrate Supplementation on the Spatial Heterogeneity of Quadriceps Deoxygenation during Heavy-Intensity Cycling." *Physiological Reports* 5 (14):e13340.

Bryan, N.S., and J Loscalzo. 2011. Nitrite and Nitrate in Human Health and Disease.

Buffa, Roberto, Giovanni U Floris, Paolo F Putzu, and Elisabetta Marini. 2011. "Body Composition Variations in Ageing." *Collegium Antropologicum* 35 (1):259–65.

Burnley, Mark, and Andrew M. Jones. 2007. "Oxygen Uptake Kinetics as a Determinant of Sports Performance." *European Journal of Sport Science* 7 (2). Taylor & Francis Group:63–79.

Carlsson, S, N P Wiklund, L Engstrand, E Weitzberg, and J O Lundberg. 2001. "Effects of pH, Nitrite, and Ascorbic Acid on Nonenzymatic Nitric Oxide Generation and Bacterial Growth in Urine." *Nitric Oxide : Biology and Chemistry* 5 (6):580–86.

Casaburi, R., J. Daly, J. E. Hansen, and R. M. Effros. 1989. "Abrupt Changes in Mixed Venous Blood Gas Composition after the Onset of Exercise." *Journal of Applied Physiology* 67 (3):1106–12.

Cermak, Naomi M, Martin J Gibala, and Luc J C Van Loon. 2012. "Nitrate Supplementation ' S Improvement of 10-Km Time-Trial Performance in Trained Cyclists." *International Journal of Sport Nutrition and Exercise Metabolism*, no. 3:64–71.

Clerc, Pascaline, Michel Rigoulet, Xavier Leverve, and Eric Fontaine. 2007. "Nitric Oxide Increases Oxidative Phosphorylation Efficiency." *Journal of Bioenergetics and Biomembranes* 39 (2):158–66.

Cosby, Kenyatta, Kristine S. Partovi, Jack H. Crawford, Rakesh P. Patel, Christopher D. Reiter, Sabrina Martyr, Benjamin K. Yang, et al. 2003. "Nitrite Reduction to Nitric Oxide by Deoxyhemoglobin Vasodilates the Human Circulation." *Nature Medicine* 9 (12):1498–1505.

Coyle, E F. 1995. "Integration of the Physiological Factors Determining Endurance Performance Ability." *Exercise and Sport Sciences Reviews* 23:25–64.

Cruz-Jentoft, A. J., J. P. Baeyens, J. M. Bauer, Y. Boirie, T. Cederholm, F. Landi, F. C. Martin, et al. 2010. "Sarcopenia: European Consensus on Definition and Diagnosis: Report of the European Working Group on Sarcopenia in Older People." *Age and Ageing* 39 (4):412–23.

Cruz-Jentoft, Alfonso J, Francesco Landi, Eva Topinková, and Jean-Pierre Michel. 2010. "Understanding Sarcopenia as a Geriatric Syndrome." *Current Opinion in Clinical Nutrition and Metabolic Care* 13 (1):1–7.

Dahm, Christina C., Kevin Moore, and Michael P. Murphy. 2006. "Persistent S-Nitrosation of Complex I and Other Mitochondrial Membrane Proteins by S-Nitrosothiols but Not Nitric Oxide or Peroxynitrite: Implications for the Interaction of Nitric Oxide with Mitochondria." *Journal of Biological Chemistry* 281 (15):10056–65.

Dejam, André, Christian J Hunter, and Mark T Gladwin. 2007. "Effects of Dietary Nitrate on Blood Pressure." *New England Journal of Medicine* 356 (15):1590–1590.

DeLorey, Darren S. 2004. "Effect of Age on O2 Uptake Kinetics and the Adaptation of Muscle Deoxygenation at the Onset of Moderate-Intensity Cycling Exercise." *Journal of Applied Physiology* 97 (1):165–72.

DeLorey, Darren S., John M. Kowalchuk, and Donald H. Paterson. 2005. "Adaptation of Pulmonary O2 Uptake Kinetics and Muscle Deoxygenation at the Onset of Heavy-Intensity Exercise in Young and Older Adults." *Journal of Applied Physiology* 98 (5):1697–1704.

Doherty, Daniel H., Michael P. Doyle, Shawn R. Curry, Rita J. Vali, Timothy J. Fattor, John S. Olson, and Douglas D. Lemon. 1998. "Rate of Reaction with Nitric Oxide Determines the Hypertensive Effect of Cell-Free Hemoglobin." *Nature Biotechnology* 16 (7):672–76.

duManoir, Gregory R., Darren S. DeLorey, John M. Kowalchuk, and Donald H. Paterson. 2010. "Kinetics of VO2 Limb Blood Flow and Regional Muscle Deoxygenation in Young Adults during Moderate Intensity, Knee-Extension Exercise." *European Journal of Applied Physiology* 108 (3):607–17.

Duncan, Callum, Hamish Dougall, Peter Johnston, and Susan Green. 1995. "Chemical Synthesis of Nitric Oxide in the Stomach from Dietary Nitrate in Humans." *Nature Medicine* 1 (6):546–51.

Ferguson, Scott K., Daniel M. Hirai, Steven W. Copp, Clark T. Holdsworth, Jason D. Allen, Andrew M. Jones, Timothy I. Musch, and David C. Poole. 2013. "Effects of Nitrate Supplementation via Beetroot Juice on Contracting Rat Skeletal Muscle Microvascular Oxygen Pressure Dynamics." *Respiratory Physiology and Neurobiology* 187 (3):250–55.

Ferguson, Scott K, Clark T Holdsworth, Jennifer L Wright, Alex J Fees, Jason D Allen, Andrew M Jones, Timothy I Musch, and David C Poole. 2015. "Microvascular Oxygen Pressures in Muscles Comprised of Different Fiber Types: Impact of Dietary Nitrate Supplementation HHS Public Access." *Nitric Oxide* 48:38–4309.

Frontera, Walter R., Dongwon Suh, Lisa S. Krivickas, Virginia A. Hughes, Richard Goldstein, and Ronenn Roubenoff. 2000. "Skeletal Muscle Fiber Quality in Older Men and Women." *American Journal of Physiology-Cell Physiology* 279 (3):C611–18.

Furchgott, R F, and S Bhadrakom. 1953. "Reactions of Strips of Rabbit Aorta to Epinephrine, Isopropylarterenol, Sodium Nitrite and Other Drugs." *The Journal of Pharmacology and Experimental Therapeutics* 108 (2):129–43.

Gaesser, G A, and D C Poole. 1996. "The Slow Component of Oxygen Uptake Kinetics in Humans." *Exerc Sport Sci Rev.*

Gago, Bruno, Jon O. Lundberg, Rui M. Barbosa, and João Laranjinha. 2007. "Red Wine-Dependent Reduction of Nitrite to Nitric Oxide in the Stomach." *Free Radical Biology and Medicine* 43 (9):1233–42.

Gerbino, A., S. A. Ward, and B. J. Whipp. 1996. "Effects of Prior Exercise on Pulmonary Gas-Exchange Kinetics during High-Intensity Exercise in Humans." *J Appl Physiol* 80 (1):99–107.

Giansante, C, and N Fiotti. 2006. "Insights into Human Hypertension: The Role of Endothelial Dysfunction." *Journal of Human Hypertension* 20:725–26.

Giraldez, R R, A Panda, Y Xia, S P Sanders, and J L Zweier. 1997. "Decreased Nitric-Oxide Synthase Activity Causes Impaired Endothelium-Dependent Relaxation in the Postischemic Heart." *The Journal of Biological Chemistry* 272 (34):21420–26.

Gladwin, M. T., F. P. Ognibene, L. K. Pannell, J. S. Nichols, M. E. Pease-Fye, J. H. Shelhamer, and A. N. Schechter. 2000. "Relative Role of Heme Nitrosylation and Beta -Cysteine 93 Nitrosation in the Transport and Metabolism of Nitric Oxide by Hemoglobin in the Human Circulation." *Proceedings of the National Academy of Sciences* 97 (18):9943–48.

Godber, B L, J J Doel, G P Sapkota, D R Blake, C R Stevens, R Eisenthal, and R Harrison. 2000. "Reduction of Nitrite to Nitric Oxide Catalyzed by Xanthine Oxidoreductase." *The Journal of Biological Chemistry* 275 (11):7757–63.

Gouspillou, Gilles, Isabelle Bourdel-Marchasson, Richard Rouland, Guillaume Calmettes, Jean-Michel Franconi, Véronique Deschodt-Arsac, and Philippe Diolez. 2010. "Alteration of Mitochondrial Oxidative Phosphorylation in Aged Skeletal Muscle Involves Modification of Adenine Nucleotide Translocator." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1797 (2):143–51.

Granacher, Urs, Lukas Zahner, and Albert Gollhofer. 2008. "Strength, Power, and Postural Control in Seniors: Considerations for Functional Adaptations and for Fall Prevention." *European Journal of Sport Science* 8 (6). Taylor & Francis Group :325–40.

Grassi, Bruno, Harry B. Rossiter, and Jerzy A. Zoladz. 2015. "Skeletal Muscle Fatigue and Decreased Efficiency: Two Sides of the Same Coin?" *Exercise and Sport Sciences Reviews* 43 (2):75–83.

Gurd, B. J., S. J. Peters, G. J. F. Heigenhauser, P. J. Leblanc, T. J. Doherty, D. H. Paterson, and J. M. Kowalchuk. 2006. "Prior Heavy Exercise Elevates Pyruvate Dehydrogenase Activity and Speeds O2 Uptake Kinetics during Subsequent Moderate-Intensity Exercise in Healthy Young Adults." *Journal of Physiology* 577 (3):985–96.

Gurd, Brendon J. 2005. "Prior Heavy-Intensity Exercise Speeds V{middle dot}O2 Kinetics during Moderate-Intensity Exercise in Young Adults." *Journal of Applied Physiology* 98 (4):1371–78.

Haider, Georg, and Jonathan P. Folland. 2014. "Nitrate Supplementation Enhances the Contractile Properties of Human Skeletal Muscle." *Medicine and Science in Sports and Exercise* 46 (12):2234–43.

Hardman, and Stensel. 2013. Physical Activity and Health: The Evidence Explained.

Henneman, Elwood, George Somjen, and David O. Carpenter. 1965. "Excitability and Inhibitability of Motoneurons of Different Sizes." *Journal of Neurophysiology* 28 (3):599–620.

Hernández, Andrés, Tomas A Schiffer, Niklas Ivarsson, Arthur J Cheng, Joseph D Bruton, Jon O Lundberg, Eddie Weitzberg, and Håkan Westerblad. 2012. "Dietary Nitrate Increases Tetanic [Ca 2+] I and Contractile Force in Mouse Fast-Twitch Muscle." *Journal of Physiology* 590 (15):3575–83.

Hinkle, Peter C. 2005. "P/O Ratios of Mitochondrial Oxidative Phosphorylation." *Biochimica et Biophysica Acta - Bioenergetics*.

Hughson, R. L., D. L. Sherrill, and G. D. Swanson. 1988. "Kinetics of VO2 with Impulse and Step Exercise in Humans." *J Appl Physiol* 64 (1):451–59.

Ignarro, L J, G Cirino, A Casini, and C Napoli. 1999. "Nitric Oxide as a Signaling Molecule in the Vascular System: An Overview." *Journal of Cardiovascular Pharmacology* 34 (6):879–86.

Ignarro, L J, H Lippton, J C Edwards, W H Baricos, A L Hyman, P J Kadowitz, and C A Gruetter. 1981. "Mechanism of Vascular Smooth Muscle Relaxation by Organic Nitrates, Nitrites, Nitroprusside and Nitric Oxide: Evidence for the Involvement of S-Nitrosothiols as Active Intermediates." *The Journal of Pharmacology and Experimental Therapeutics* 218 (3):739–49.

Jones, Andrew M. 2014. "Dietary Nitrate Supplementation and Exercise Performance." *Sports Medicine (Auckland, N.Z.)* 44 Suppl 1 (Suppl 1):S35-45.

Jones, Andrew M., Daryl P. Wilkerson, Katrien Koppo, Sally Wilmshurst, and Iain T. Campbell. 2003. "Inhibition of Nitric Oxide Synthase by L-NAME Speeds Phase II Pulmonary ??VO2 Kinetics in the Transition to Moderate-Intensity Exercise in Man." *Journal of Physiology* 552 (1):265–72.

Kelly, James, Jonathan Fulford, Anni Vanhatalo, Jamie R Blackwell, Olivia French, Stephen J Bailey, Mark Gilchrist, Paul G Winyard, and Andrew M Jones. 2013. "Effects of Short-Term Dietary Nitrate Supplementation on Blood Pressure, O2 Uptake Kinetics, and Muscle and Cognitive Function in Older Adults." *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 304 (2):R73-83.

Kindig, Casey A. 2004. "Effects of Acute Creatine Kinase Inhibition on Metabolism and Tension Development in Isolated Single Myocytes." *Journal of Applied Physiology* 98 (2):541–49.

Lador, Frédéric. 2005. "Simultaneous Determination of the Kinetics of Cardiac Output, Systemic O2 Delivery, and Lung O2 Uptake at Exercise Onset in Men." *AJP: Regulatory, Integrative and Comparative Physiology* 290 (4):R1071–79.

Lansley, Katherine E., Paul G. Winyard, Jonathan Fulford, Anni Vanhatalo, Stephen J. Bailey, Jamie R. Blackwell, Fred J. DiMenna, Mark Gilchrist, Nigel Benjamin, and Andrew M. Jones. 2011. "Dietary Nitrate Supplementation Reduces the O2 Cost of Walking and Running: A Placebo-Controlled Study." *J Appl Physiol (1985)* 110 (3):591–600.

Lansley, Katherine E, Paul G Winyard, Stephen J Bailey, Anni Vanhatalo, Daryl P Wilkerson, Jamie R Blackwell, Mark Gilchrist, Nigel Benjamin, and Andrew M Jones. 2011. "Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance." *Medicine and Science in Sports and Exercise* 43 (6):1125–31.

Larsen, F. J., E. Weitzberg, J. O. Lundberg, and B. Ekblom. 2007. "Effects of Dietary Nitrate on Oxygen Cost during Exercise." *Acta Physiologica* 191 (1):59–66.

Larsen, Filip J., Björn Ekblom, Kent Sahlin, Jon O. Lundberg, and Eddie Weitzberg. 2006. "Effects of

Dietary Nitrate on Blood Pressure in Healthy Volunteers." *New England Journal of Medicine* 355 (26):2792–93.

Larsen, Filip J., Eddie Weitzberg, Jon O. Lundberg, and Björn Ekblom. 2010. "Dietary Nitrate Reduces Maximal Oxygen Consumption While Maintaining Work Performance in Maximal Exercise." *Free Radical Biology and Medicine* 48 (2). Elsevier Inc.:342–47.

Larsen, Filip J, Tomas A Schiffer, Sara Borniquel, Kent Sahlin, Björn Ekblom, Jon O Lundberg, and Eddie Weitzberg. 2011. "Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans." *Cell Metabolism* 13 (2):149–59.

Lee, Jae-Seok, Charles L. Stebbins, Eunji Jung, Hosung Nho, Jong-Kyung Kim, Myoung-Jei Chang, and Hyun-Min Choi. 2015. "Effects of Chronic Dietary Nitrate Supplementation on the Hemodynamic Response to Dynamic Exercise." *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 309 (5):R459–66.

Li, Haitao, Hongmei Cui, Xiaoping Liu, and Jay L. Zweier. 2005. "Xanthine Oxidase Catalyzes Anaerobic Transformation of Organic Nitrates to Nitric Oxide and Nitrosothiols: Characterization of This Mechanism and the Link between Organic Nitrate and Guanylyl Cyclase Activation." *Journal of Biological Chemistry* 280 (17):16594–600.

Lidder, Satnam, and Andrew J Webb. 2013. "Vascular Effects of Dietary Nitrate (as Found in Green Leafy Vegetables and Beetroot) via the Nitrate-Nitrite-Nitric Oxide Pathway." *British Journal of Clinical Pharmacology* 75 (3):677–96.

Liu, Alex H., Catherine P. Bondonno, Kevin D. Croft, Ian B. Puddey, Richard J. Woodman, Lisa Rich, Natalie C. Ward, Joseph A. Vita, and Jonathan M. Hodgson. 2013. "Effects of a Nitrate-Rich Meal on Arterial Stiffness and Blood Pressure in Healthy Volunteers." *Nitric Oxide - Biology and Chemistry* 35 (November):123–30.

Lowery, Erin M., Aleah L. Brubaker, Erica Kuhlmann, and Elizabeth J. Kovacs. 2013. "The Aging Lung." *Clinical Interventions in Aging*.

Lundberg, J M O, E Weitzberg, J M O Lundberg, and K Alving. 1994. "Intragastric Nitric Oxide Production in Humans: Measurements in Expelled Air." *Gut* 35 (11):1543–46.

Lundberg, J O, E Weitzberg, J A Cole, and N Benjamin. 2004. "Nitrate, Bacteria and Human Health." *Nat Rev Microbiol* 2 (7):593–602.

Lundberg, Jon O., and Mirco Govoni. 2004. "Inorganic Nitrate Is a Possible Source for Systemic Generation of Nitric Oxide." *Free Radical Biology and Medicine* 37 (3):395–400.

Lundberg, Jon O., Eddie Weitzberg, and Mark T. Gladwin. 2008. "The Nitrate-Nitrite-Nitric Oxide Pathway in Physiology and Therapeutics." *Nature Reviews Drug Discovery* 7 (2):156–67.

Lundberg, Jon O, Mattias Carlström, Filip J Larsen, and Eddie Weitzberg. 2011. "Roles of Dietary Inorganic Nitrate in Cardiovascular Health and Disease." *Cardiovascular Research*.

Maione, D., A. F.G. Cicero, S Bacchelli, E R Cosentino, D Degli Esposti, D N Manners, E R Rinaldi, et al. 2015. "The vo2-on Kinetics in Constant Load Exercise Sub-Anaerobic Threshold Reflects Endothelial Function and Dysfunction in Muscle Microcirculation." *Physiological Research* 64 (6):807–19.

McKay, Bryon R., Donald H. Paterson, and John M. Kowalchuk. 2009. "Effect of Short-Term High-Intensity Interval Training vs. Continuous Training on O2 Uptake Kinetics, Muscle Deoxygenation, and Exercise Performance." *Journal of Applied Physiology* 107 (1):128–38.

Mensinga, Tjeert T, Gerrit J Speijers, and Jan Meulenbelt. 2003. "Health Implications of Exposure to Environmental Nitrogenous Compounds." *Toxicological Reviews* 22 (1):41–51.

Minetti, Alberto E., Christian Moia, Giulio S. Roi, Davide Susta, and Guido Ferretti. 2002. "Energy Cost of Walking and Running at Extreme Uphill and Downhill Slopes." *Journal of Applied Physiology* 93 (3):1039–46.

Miura, T, T Takeuchi, H Sato, N Nishioka, S Terakado, Y Fujieda, and C Ibukiyama. 1998. "Skeletal Muscle Deoxygenation during Exercise Assessed by near-Infrared Spectroscopy and Its Relation to Expired Gas Analysis Parameters." *Japanese Circulation Journal* 62 (9):649–57.

Moncada, Salvador, and Annie Higgs. 1993. "The L-Arginine-Nitric Oxide Pathway." *New England Journal of Medicine* 329 (27):2002–12.

Murias, Juan M., John M. Kowalchuk, and Donald H. Paterson. 2011. "Speeding of VO2 Kinetics in Response to Endurance-Training in Older and Young Women." *European Journal of Applied Physiology* 111 (2):235–43.

Murias, Juan M., Matthew D. Spencer, Darren S. DeLorey, Brendon J. Gurd, John M. Kowalchuk, and Donald H. Paterson. 2011. "Speeding of VO2 Kinetics during Moderate-Intensity Exercise Subsequent to Heavy-Intensity Exercise Is Associated with Improved Local O2 Distribution." *Journal of Applied Physiology* 111 (5):1410–15.

Murphy, Margaret, Katie Eliot, Rita M Heuertz, and Edward Weiss. 2012. "Whole Beetroot Consumption Acutely Improves Running Performance." *Journal of the Academy of Nutrition and Dietetics* 112 (4). Elsevier:548–52.

Nadtochiy, Sergiy M., Lindsay S. Burwell, and Paul S. Brookes. 2007. "Cardioprotection and Mitochondrial S-Nitrosation: Effects of S-Nitroso-2-Mercaptopropionyl Glycine (SNO-MPG) in Cardiac Ischemia–reperfusion Injury." *Journal of Molecular and Cellular Cardiology* 42 (4):812–25.

Nagababu, Enika, Somasundaram Ramasamy, Darrell R. Abernethy, and Joseph M. Rifkind. 2003. "Active Nitric Oxide Produced in the Red Cell under Hypoxic Conditions by Deoxyhemoglobin-Mediated Nitrite Reduction." *Journal of Biological Chemistry* 278 (47):46349–56.

Oelze, M., S. Kroller-Schon, S. Steven, E. Lubos, C. Doppler, M. Hausding, S. Tobias, et al. 2014. "Glutathione Peroxidase-1 Deficiency Potentiates Dysregulatory Modifications of Endothelial Nitric Oxide Synthase and Vascular Dysfunction in Aging." *Hypertension* 63 (2):390–96.

Østergaard, Louise, Edgaras Stankevicius, Malene R. Andersen, Yvonne Eskildsen-Helmond, Thomas Ledet, Michael J. Mulvany, and Ulf Simonsen. 2007. "Diminished NO Release in Chronic Hypoxic Human Endothelial Cells." *American Journal of Physiology-Heart and Circulatory Physiology* 293 (5):H2894–2903.

Peri, Laura, Donatella Pietraforte, Giuseppe Scorza, Aurora Napolitano, Vincenzo Fogliano, and Maurizio Minetti. 2005. "Apples Increase Nitric Oxide Production by Human Saliva at the Acidic pH of the Stomach: A New Biological Function for Polyphenols with a Catechol Group?" *Free Radical Biology & Medicine* 39 (5):668–81.

Pette, Dirk, and Robert S. Staron. 2000. "Myosin Isoforms, Muscle Fiber Types, and Transitions." *Microscopy Research and Technique* 50 (6):500–509.

Plavnik, Fl, Sa Ajzen, Dmj Christofalo, Csp Barbosa, and O Kohlmann. 2007. "Endothelial Function in Normotensive and High-Normal Hypertensive Subjects." *Journal of Human Hypertension* 21:467–72.

Pontieri, V, MK Venezuela, and C Scavone. 1998. "Role of Endogenous Nitric Oxide in the Nucleus Tratus Solitarii on Baroreflex Control of Heart Rate in Spontaneously Hypertensive Rats." *Journal of Hypertension* 16 (12 Pt 2):1993–99.

Poole, D. C., W. Schaffartzik, D. R. Knight, T. Derion, B. Kennedy, H. J. Guy, R. Prediletto, and P. D. Wagner. 1991. "Contribution of Exercising Legs to the Slow Component of Oxygen Uptake Kinetics in Humans." *Journal of Applied Physiology* 71 (4):1245–60.

Poole, David C., Thomas J. Barstow, Paul Mcdonough, and Andrew M. Jones. 2008. "Control of Oxygen Uptake during Exercise." *Medicine and Science in Sports and Exercise*.

Poole, David C., and Andrew M. Jones. 2012. "Oxygen Uptake Kinetics." *Comprehensive Physiology* 2 (2). Hoboken, NJ, USA: John Wiley & Sons, Inc.:933–96.

Poole, David C., Daryl P. Wilkerson, and Andrew M. Jones. 2008. "Validity of Criteria for Establishing Maximal O2 Uptake during Ramp Exercise Tests." *European Journal of Applied Physiology* 102 (4):403–10.

Poole, David C, David C Poole, Casey A Kindig, Brad J Behnke, and Andrew M Jones. 2005. "Oxygen Uptake (O2) Kinetics in Different Species: A Brief Review." *Equine and Comparative Exercise Physiology* 2 (1):1–15.

Porcelli, Simone, Matthew Ramaglia, Giuseppe Bellistri, Gaspare Pavei, Lorenzo Pugliese, Michela Montorsi, Letizia Rasica, and Mauro Marzorati. 2015. "Aerobic Fitness Affects the Exercise Performance Responses to Nitrate Supplementation." *Medicine and Science in Sports and Exercise* 47 (8):1643–51.

Prampero, PE Di. 1986. "The Energy Cost of Human Locomotion on Land and in Water." *International Journal of Sport Nutrition and Exercise Metabolism* 7 (2):55–72.

Prampero, Pietro Enrico Di. 1985. La Locomozione Umana Su Terra, in Acqua, in Aria.

Pringle, Jamie S. M., Jamie S. M. Pringle, Æ Jonathan H Doust, Æ Jonathan H Doust, Helen Carter, Helen Carter, Æ Keith Tolfrey, et al. 2003. "Oxygen Uptake Kinetics during Moderate, Heavy and Severe Intensity 'submaximal' Exercise in Humans: The in Uence of Muscle Bre Type and Capillarisation." *European Journal of Applied Physiology* 89 (3):289–300.

Rassaf, Tienush, Ulrich Flögel, Christine Drexhage, Ulrike Hendgen-Cotta, Malte Kelm, and Jürgen Schrader. 2007. "Nitrite Reductase Function of Deoxymyoglobin: Oxygen Sensor and Regulator of Cardiac Energetics and Function." *Circulation Research* 100 (12):1749–54.

Roia, Gabriela De, Silvia Pogliaghi, Alessandra Adami, Christina Papadopoulou, and Carlo Capelli. 2012. "Effects of Priming Exercise on the Speed of Adjustment of Muscle Oxidative Metabolism at the Onset of Moderate-Intensity Step Transitions in Older Adults." *AJP: Regulatory, Integrative and Comparative Physiology* 302 (10):R1158–66.

Sawilowsky, Shlomo S. 2009. "New Effect Size Rules of Thumb." *Journal of Modern Applied Statistical Methods* 8 (2):597–99.

Scheuermann, Barry W., Chris Bell, Donald H. Paterson, Thomas J. Barstow, John M. Kowalchuk, Alessandro Mezzani, Bruno Grassi, et al. 2011. "Oxygen Uptake Kinetics for Moderate Exercise Are Speeded in Older Humans by Prior Heavy Exercise." *Journal of Applied Physiology* 92 (2):609–16.

Schneider, Aaron C, William E Hughes, Nicholas T Kruse, Kenichi Ueda, and Darren P Casey. 2017. "Blood Pressure Responsiveness to Muscle Metaboreflex Activation in Older Adults Following Dietary Nitrate Supplementation." *The FASEB Journal* 31 (1_supplement). Federation of American Societies for Experimental Biology:1012.11-1012.11.

Shephard, Roy J. 1993. "Aging, Respiratory Function, and Exercise." *Journal of Aging and Physical Activity* 1 (1):59–83.

Shiva, Sruti, Zhi Huang, Rozalina Grubina, Junhui Sun, Lorna A Ringwood, Peter H. MacArthur, Xiuli Xu, Elizabeth Murphy, Victor M Darley-Usmar, and Mark T Gladwin. 2007. "Deoxymyoglobin Is a Nitrite Reductase That Generates Nitric Oxide and Regulates Mitochondrial Respiration." *Circulation Research* 100 (5):654–61.

Shiva, Sruti, Xunde Wang, Lorna A Ringwood, Xueying Xu, Susan Yuditskaya, Vidhya Annavajjhala, Hiroaki Miyajima, Neil Hogg, Zena Leah Harris, and Mark T Gladwin. 2006. "Ceruloplasmin Is a NO Oxidase and Nitrite Synthase That Determines Endocrine NO Homeostasis." *Nature Chemical Biology* 2 (9):486–93.

Sieck, Gary C. 2017. "Physiology in Perspective: Aging and Underlying Pathophysiology." *Physiology* 32 (1):7–8.

Sobko, Tanja, Claude Marcus, Mirco Govoni, and Shigeru Kamiya. 2010. "Dietary Nitrate in Japanese Traditional Foods Lowers Diastolic Blood Pressure in Healthy Volunteers." *Nitric Oxide* 22 (2):136–40.

Stamler, J S, and G Meissner. 2001. "Physiology of Nitric Oxide in Skeletal Muscle." *Physiological Reviews* 81 (1):209–37.

Taaffe, Dennis R. 2006. "Sarcopenia: Exercise as a Treatment Strategy." *Australian Family Physician* 35 (3):130–33.

Tam, Enrico, Marcel AZABJI Kenfack, Michela Cautero, Federic Lador, Guglielmo Antonutto, Pietro Enrico Di Prampero, Guido Ferretti, and Carlo Capelli. 2004. "Correction of Cardiac Output Obtained by Modelflow® from Finger Pulse Pressure Profiles with a Respiratory Method in Humans." *Clinical Science* 106 (4):371–76.

Tannenbaum, S R, and P Correa. 1985. "Nitrate and Gastric Cancer Risks." *Nature* 317 (6039):675–76.

Thomas, D. D. 2001. "The Biological Lifetime of Nitric Oxide: Implications for the Perivascular Dynamics of NO and O2." *Proceedings of the National Academy of Sciences* 98 (1):355–60.

Toth, A, Miklos Pal, Marcos Intaglietta, and Paul C. Johnson. 2007. "Contribution of Anaerobic Metabolism to Reactive Hyperemia in Skeletal Muscle." *Am.J Physiol Heart Circ.Physiol* 292 (6):H2643–53.

Tripatara, P., N. S.A. Patel, A. Webb, K. Rathod, F. M.J. Lecomte, E. Mazzon, S. Cuzzocrea, M. M. Yaqoob, A. Ahluwalia, and C. Thiemermann. 2007. "Nitrite-Derived Nitric Oxide Protects the Rat Kidney against Ischemia/Reperfusion Injury In Vivo: Role for Xanthine Oxidoreductase." *Journal of the American Society of Nephrology* 18 (2):570–80.

Tschakovsky, M. E., and R. L. Hughson. 1999. "Interaction of Factors Determining Oxygen Uptake at the Onset of Exercise." *Journal of Applied Physiology* 86 (4):1101–13.

Vanhatalo, Anni, Stephen J. Bailey, Jamie R. Blackwell, Fred J. DiMenna, Toby G. Pavey, Daryl P. Wilkerson, Nigel Benjamin, Paul G. Winyard, and Andrew M. Jones. 2010. "Acute and Chronic Effects of Dietary Nitrate Supplementation on Blood Pressure and the Physiological Responses to Moderate-Intensity and Incremental Exercise." *AJP: Regulatory, Integrative and Comparative Physiology* 299 (4):R1121–31.

Vanhatalo, Anni, Jonathan Fulford, Stephen J Bailey, James R Blackwell, Paul G Winyard, and Andrew M Jones. 2011. "Dietary Nitrate Reduces Muscle Metabolic Perturbation and Improves Exercise Tolerance in Hypoxia." *Journal of Physiology* 589 (22):5517–28.

Vijg, Jan, and Yousin Suh. 2013. "Genome Instability and Aging." *Annual Review of Physiology* 75 (1):645–68.

Webb, Andrew J., Nakul Patel, Stavros Loukogeorgakis, Mike Okorie, Zainab Aboud, Shivani Misra, Rahim Rashid, et al. 2008. "Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite." *Hypertension* 51 (3):784–90.

Weiss, By Soma, Robert W Wilkins, and Florence W Haynes. 1937. "The Nature of Circulatory Collapse Induced by Sodium Nitrite." *The Journal of Clinical Investigation* 16 (3). American Society for Clinical Investigation:73–84.

Whipp, B J, and K Wasserman. 1972. "Oxygen Uptake Kinetics for Various Intensities of Constant-Load Work." *Journal of Applied Physiology* 33 (3):351–56.

Wilkerson, Daryl P, Giles M Hayward, Stephen J Bailey, Anni Vanhatalo, Jamie R Blackwell, and Andrew M Jones. 2012. "Influence of Acute Dietary Nitrate Supplementation on 50 Mile Time Trial Performance in Well-Trained Cyclists." *European Journal of Applied Physiology* 112 (12):4127–34.

Williams, Alexandra M., Donald H. Paterson, and John M. Kowalchuk. 2013. "High-Intensity Interval Training Speeds the Adjustment of Pulmonary O2 Uptake, but Not Muscle Deoxygenation, during Moderate-Intensity Exercise Transitions Initiated from Low and Elevated Baseline Metabolic Rates." *Journal of Applied Physiology* 114 (11):1550–62.

Wylie, Lee J., Magni Mohr, Peter Krustrup, Sarah R. Jackman, Georgios Ermdis, James Kelly, Matthew I. Black, Stephen J. Bailey, Anni Vanhatalo, and Andrew M. Jones. 2013. "Dietary Nitrate Supplementation Improves Team Sport-Specific Intense Intermittent Exercise Performance." *European Journal of Applied Physiology* 113 (7):1673–84.

Xu, Zhelong, Xiang Ji, and Philip G. Boysen. 2004. "Exogenous Nitric Oxide Generates ROS and Induces Cardioprotection: Involvement of PKG, Mitochondrial K_{ATP} Channels, and ERK." *American Journal of Physiology-Heart and Circulatory Physiology* 286 (4):H1433–40.

Zhang, Z, D P Naughton, D R Blake, N Benjamin, C R Stevens, P G Winyard, M C Symons, and R Harrison. 1997. "Human Xanthine Oxidase Converts Nitrite Ions into Nitric Oxide (NO)." *Biochemical Society Transactions* 25 (3):524S.

Zweier, J L, P Wang, A Samouilov, and P Kuppusamy. 1995. "Enzyme-Independent Formation of Nitric Oxide in Biological Tissues." *Nature Medicine* 1 (8):804–9.