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Nitrate supplementation as an ergogenic aid within trained and elite endurance athletes with a VO_2 above 51.9 mL/kg.min

Systematic Review

In Ökotrophologie

Submitted by

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Contents

1. Introduction

This systematic review aims to investigate the role of dietary nitrates as an ergogenic aid within a trained endurance athlete population as defined by possessing a VO2 max above or equal to 51.9 mL/kg.min. This meta-analysis has been developed in compliance with the Cochranes Handbook for Systematic Review of Interventions and is accordingly broken down into four separate subsections, namely background, methods, results and discussion (JPT *et al.*, 2019). Within the background, the current body of evidence concerning the use of dietary nitrates as ergogenic aids within athlete populations will be elucidated to provide a theoretical framework upon which to base further analysis of results stemming from the current review. Following this, within the methods section, the search strategy employed during bibliographic searching as well as the pre-determined exclusion criteria, which were implemented during following the screening phases will be presented. Within the results section, the systematic exclusion of literature will be illustrated as a PRISMA Flowchart, whilst extracted data contained within randomized control that passed the inclusion criteria will be shown for comparison within a PICO table and quantified with the use of forest plots. Finally, within the discussion section, the study design and findings of each RCT will be assessed and the overall finding of this systematic review discussed and compared to the results of other systematic reviews.

1.1 Background

The use of nitrate-based intervention strategies within patient groups suffering from such pathologies such as hypertension-induced cardiovascular disease has been well documented, which are based on the potent hypotensive properties of nitric oxide (Yu, Gao and Ma, 2011; Koch *et al.*, 2016). Concurrent to these studies sports research investigating the use of exogenous nitrates in competitive athlete populations have indicated a significant treatment effect on relevant performance related outcome such as increased TT performance (Lansley *et al.*, 2011; Cermak, Gibala and Loon, 2012; Muggeridge *et al.*, 2013; McQuillan *et al.*, 2017a), increase time to exhaustion via attenuation of the VO2 slow component (Bescos *et al.*, 2012; Balsalobre-Fernández *et al.*, 2018) and increased exercise efficiency as measured by the reduction of VO2 uptake (Cermak, Gibala and Loon, 2012; Flueck *et al.*, 2015). However at this time evidence supporting the ergogenic properties of nitrate-based interventions has not been consistently reported in all studies, especially within elite and trained athlete populations (MacLeod *et al.*, 2015; Shannon *et al.*, 2017). Hence the current meta-analysis aims to

investigate the role of chronic and acute nitrate-based intervention strategies on performance-related outcomes within athlete population with a VO2 max above or equal to 51,9 mL/kg.min.

1.1.1 Inorganic Nitrates and Sources

Nitrates (NO_3^-) and nitrites (NO_2^-) fulfill a crucial role as nitric oxide (NO) precursors within the human organism specifically within hypoxic, acidic environments (Nnate and Ngozi, 2016; Pawlak-Chaouch *et al.*, 2016). NO is involved in numerous physiological roles as a ubiquitous signaling molecule within the human organism (Pawlak-Chaouch *et al.*, 2016). Naturally occurring nitrate anions are widely employed within the field of agriculture and play a significant role in the biological activity and growth processes of plants as a part of the nitrogen cycle (Nnate and Ngozi, 2016). Hence, naturally occurring dietary sources of exogenous nitrates include green leafy and root vegetables such as spinach, rocket, cress, lettuce, celery, radish and beetroot, which have been documented to contain the highest relative concentration of nitrates of any root vegetable (Mcmahon, Leveritt and Pavey, 2017). Aside from plant-based sources, the consumption of cured meats preserved with nitrate salts as well as drinking water contaminated with runoff from nitrate enriched fertilizer may also play a role in increasing nitrate levels within the body, at times resulting in the initiation of pathogenic processes, such as infantile cyanosis (Nnate and Ngozi, 2016).

1.1.2 Nitric Oxide Metabolism

Upon ingestion of inorganic nitrates, these physiologically inert anions are reduced to their reactive counterparts - nitrite by the facultative anaerobic bacteria which inhabit the posterior tongue (Cuenca *et al.*, 2018). The presence of nitrite within the mouth has been reported to play an important role as an antibacterial agent with an estimated 25% of nitrites within the body being recirculated into the mouth via acinar cells located within the salivary glands as a part of the enterosalivary cycle (Cuenca *et al.*, 2018). Upon ingestion nitrite anions may enter the stomach and react with the hydrochloric acid present to form nitrous acid (HNO_2), which is then be absorbed into the duodenal wall under hypoxic and acidic conditions (Nnate and Ngozi, 2016). Upon entry into the bloodstream, the nitrite anions quickly disassociate and where they may be reduced by circulating NO_2 reductase containing proteins such as hemo- and myoglobin as well as molybdenum-containing enzymes such as xanthine oxidoreductase or by cytochrome C located within the third complex of the electron transport chain as shown in the formulas below to form bioactive nitric oxide (NO) (Nnate and Ngozi, 2016).

$$HbFe^{2+} + NO_2^- + H^+ \rightarrow HbFe^{3+} + NO^{\cdot} + OH^-$$
$$MbFe^{2+} + NO_2^- + H^+ \rightarrow MbFe^{3+} + NO^{\cdot} + OH^-$$
$$NO_2^- + Mo^{4+} + H^+ \rightarrow NO^{\cdot} + Mo^{5+} + OH^-$$

Being a ubiquitous, amphiphilic, paramagnetic signaling molecule, NO has been shown to be able to pass directly through the cell membrane and deliver its "message" to surrounding tissues by directly activating intracellular cyclic Guanylyl Monophosphatase (cGMP) (Nnate and Ngozi, 2016). However, given that biologically active NO as a part of the reactive nitrate species, it is highly unstable and only possesses a short half-life, dependent on surrounding partial pressure of NO and tissue oxygenation status(Koch *et al.*, 2016). Therefore due to its relatively short half-life, typically lasting from a few seconds to several minutes, it must be preserved for later use through binding to deoxyhemoglobin, which possesses a high NO binding affinity (Nnate and Ngozi, 2016) or within muscle tissue (Mcmahon, Leveritt and Pavey, 2017). The binding of NO to deoxyhemoglobin inhibits future binding of oxygen to the ferric group, thereby causing a left shift in the oxygen disassociation curve (Nnate and Ngozi, 2016). In order to release the NO from the hemoglobin pocket another member of the reactive nitrogen species, peroxynitrite ($ONOO^-$), must react with this nitrosyl-heme intermediate, thereby oxidizing the ferrous binding site from Fe_2^+ to Fe_3^+ and decreasing its binding affinity and releasing the NO anion into the surrounding tissue. Subsequently, the methemoglobin will be reduced by the NADH reductase system to refresh it for later use (Nnate and Ngozi, 2016). Should this not be the case oxygen-binding capacity would be greatly impeded due to the significantly reduced binding affinity of ferric (Fe3+) vs. ferrous (Fe2+) for oxygen (Nnate and Ngozi, 2016).

Within this branch of nitrogen metabolism, the current body of research currently suggests the existence of two distinct metabolic pathways that act to serve nitric oxide synthesis, thereby meeting nitric oxide demand within both aerobic and one anaerobic conditions (Nnate and Ngozi, 2016). The aerobic pathway is distinguished by a state of normoxia and associated neutral serum pH resulting from the recruitment of the electron transport chain for ATP-synthesis via oxidative phosphorylation along with corresponding inhibition of hypoxia-induced glycolytic lactic acid synthesis (Power and Edward T. Howley, 2014; Nnate and Ngozi, 2016). Within a state of normoxia, studies have illustrated that NO is synthesized by Nitric Oxide Synthase (NOS) located within specific cell species including macrophage and endothelial cells,

which cleave one NO molecule from the L-Arginine side-chain thereby forming NO and L-citrulline as illustrated in diagram 1 (Wimalawansa, 2008; Nnate and Ngozi, 2016).

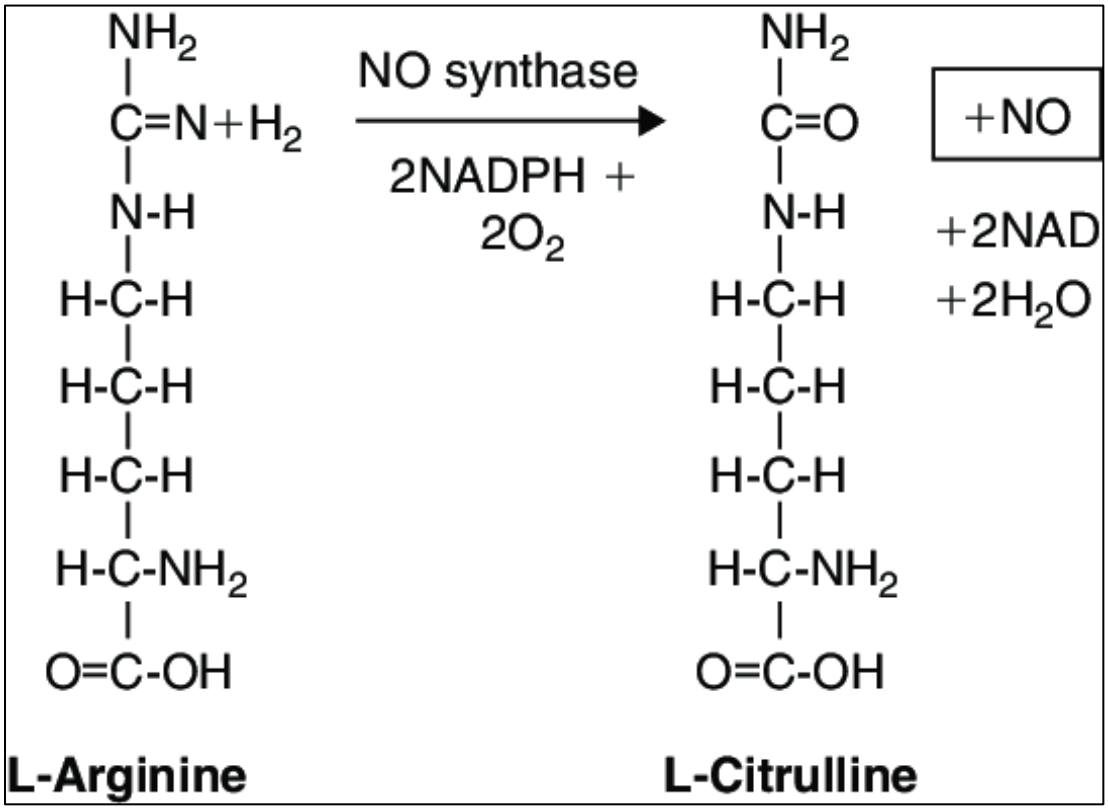

Diagram 1: NO Synthase cleaving NO from L-Arginine (Wimalawansa, 2008)

Due to training-induced increases in intramuscular capillary density and associated improvements in microvascular perfusion (Laughlin and Roseguini, 2008) studies suggest that trained endurance athletes may primarily rely on this form of NO synthesis during bouts of exercise within the realms of aerobic metabolism (Pawlak-Chaouch *et al.*, 2016). However anaerobic metabolism, by contrast, is generally categorized by a marked increase in blood CO_2 concentrations together with a corresponding decrease in blood pH due to the formation of lactic acid via in the absence of oxygen within the glycolytic pathway and further spurred on by the reaction of excess CO2 to carbonic acid within the bloodstream (Power and Edward T. Howley, 2014). Studies have illustrated that hypoxic, acidic environments favor the NO_3^--NO_2^--NO pathway (NNN-pathway) over the aforementioned eNOS pathway and may even go as far so as to reduce eNOS activity (Bescos *et al.*, 2011). Based on this model, exogenous dietary sources of NO_3^- may have an important role to play as NO-precursors in maintaining NO supply during bouts of heavy and intense exercise or provide the winning edge to endurance athletes

who are approaching their maximum VO2 during the end phases of a sporting event or competition.

1.1.3 Nitric Oxide Functions

Upon reduction by facultative anaerobic bacterial cultures within the oral cavity and hypoxic duodenal absorption, research indicates that nitric oxide fulfills a staggering number of physiological functions within the human organism, several of which have recently been identified as relevant performance-related outcomes within competitive athlete populations (Mcmahon, Leveritt and Pavey, 2017). These outcomes include increased nitrate induced vasodilation, reductions in phosphocreatine degradation, increases in mitochondrial biogenesis and respiration, improvements in glucose uptake and increases in calcium pump efficiency within skeletal muscle (Bescos *et al.*, 2012; Hoon *et al.*, 2013; Pawlak-Chaouch *et al.*, 2016; Mcmahon, Leveritt and Pavey, 2017; Balsalobre-Fernández *et al.*, 2018). In the following sections, the proposed mechanisms responsible for each of the aforementioned physiological responses will be elucidated as reported by the current body of evidence.

1.1.3.1 Increased vasodilation

Proposed mechanisms involved in NO-induced vasodilation may be seen in diagram 1. Upon crossing the ventilatory threshold the body increasingly begins to rely on anaerobic metabolism for ATP fixation and synthesis, thereby increasing levels of lactic acid produced via glycolytic pathways (Power and Edward T. Howley, 2014). Lactic acid synthesis, as well as work-induced hypoxia, result in decreased tissue and serum pH thereby increasing O_2/hemoglobin binding affinity and causing a left shift in the oxygen disassociation curve (Power and Edward T. Howley, 2014). Within hypoxic, acidic environments endothelial Nitric Oxide Synthase (eNOS) activity has been shown to decrease, rendering the NOS pathways inactive and in exchange favoring the Nitrate-Nitrite-Nitric Oxide pathway (NNN pathway) described above (Nnate and Ngozi, 2016) in order to synthesize the required amounts of NO. Within hypoxic, acidic environments induced by anaerobic metabolism NO begins to proliferate into vascular smooth muscle cells and directly acting on and activating cyclic guanylyl cyclase, which begins dephosphorylating cyclic guanylyl triphosphate (cGTP) to form cyclic monophosphate (cGMP) and thereby initiates a signal cascade, which elicits arteriolar smooth muscle relaxation resulting in improving microvascular perfusion (Bahadoran *et al.*, 2017). As eNOS activation is oxygen-dependent (Mcmahon, Leveritt and Pavey, 2017), it seems plausible that during bouts of heavy and severe exercise, which overcome the lactate threshold, the NNN-pathway may

be able to circumvent this rate-limiting factor and help to attenuate the ergolytic effects associated with work-induced hypoxic acidosis.

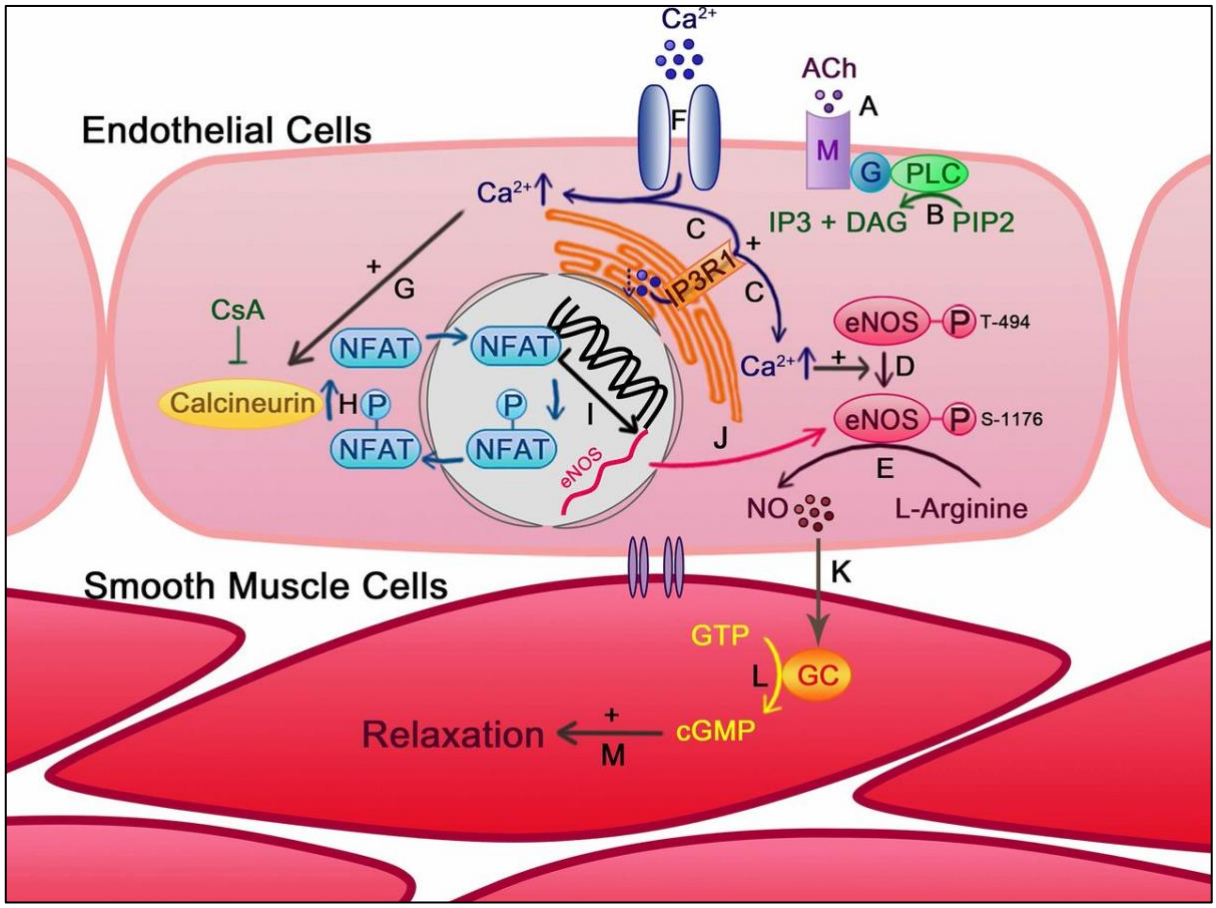

Diagram 2: NO-induced smooth muscle relaxation (Yuan *et al.*, 2016)

1.1.3.2 Increased mitochondrial biogenesis

The presence of NO within the mitochondria and serum has been shown by several studies to be an important proponent in mitochondrial biogenesis (Jornayvaz and Shulman, 2010). Whereas the exact molecular mechanisms, by which this process occurs remains to be fully elucidated, studies have indicated that the increase in mitochondrial concentrations of NO result in activation of inter-mitochondrial cyclic guanylyl cyclase and signal transduction to the cell nucleus via the activation of PGC-1α gene transcription (Jornayvaz and Shulman, 2010). Additionally, NO competes with O_2 for reduction by cytochrome C between the third and fourth complex of the electron transport chain (ETC) as well as playing an important role as a signaling molecule in regulating heme O_2 binding affinity (Nisoli and Carruba, 2006). Therefore it has been speculated that NO plays an important role in regulating rates of mitochondrial oxidative phosphorylation and biogenesis, especially in hypoxic states as well as improving electron transport chain efficiency by preventing proton pump "slippage" (Nisoli and Carruba, 2006).

1.1.3.3 Increased mitochondrial respiration

A model of the ETC may be seen in diagram 3. As already stated above the presence of NO_2^- has been known to inhibit cytochrome C activity, which operates by transporting electrons between redox center of the third and fourth complexes of the ETC thereby promoting the expulsion of H^+ protons into the intermembrane space. By maintaining a constant electrochemical gradient H^+ ions pass down their electrochemical potential differences via the access and egress channels within the c-subunit of the V-complex, driving it in an anti-clockwise motion and providing the necessary kinetic energy to achieve ATP fixation within the β-subunits (Pawlak-Chaouch *et al.*, 2016). During heavy and severe bouts of exercise skeletal muscle increasingly begin to rely on glycolytic metabolism for ATP-generation due to a state of hypoxia and its associated decreases in serum-pH, both of which elicit NO_2^- proliferation into the mitochondrial inter-membrane space where it competes with oxygen for reduction by cytochrome C. Studies have indicated that where nitrite has been considered a largely inert molecule within the human organism, cytochrome-C activity is thought to be able to reduce nitrite to NO thus preventing electron transport to the fourth complex (Nnate and Ngozi, 2016). In the absence of electrons flowing via its redox centers proton pumping within the fourth complex comes to a halt, thereby reducing oxygen consumption, whilst increasing inter-mitochondrial NO reduction (Basu *et al.*, 2008). This may partly explain improvements in athletic performance due to an optimization of mitochondrial oxygen perfusion and distribution during states of hypoxia. It has also been suggested that NO may enhance the efficiency of oxidative phosphorylation by reducing "slippage" of the mitochondrial proton pumps or attenuating the expression of uncoupling proteins (Clerc et al., 2007), reducing the total ATP cost of muscle force production (Bailey et al., 2010), or some combination of the three, thereby improving metabolic efficiency. Moreover, animal studies have illustrated a significant difference in inter-mitochondrial NO_2^- reduction between Type I and Type II muscle fibers, with fast-twitch fibers generating a significantly higher level of response to NO_2^- perfusion than slow-twitch fibers, which did not exhibit any significant changes in muscular contractility (Hernández *et al.*, 2012). Hence some authors have speculated that individual participant muscle fiber composition may be an important rate-limiting within nitrogen metabolism that remains to be fully elucidated in future studies (Laughlin and Roseguini, 2008).

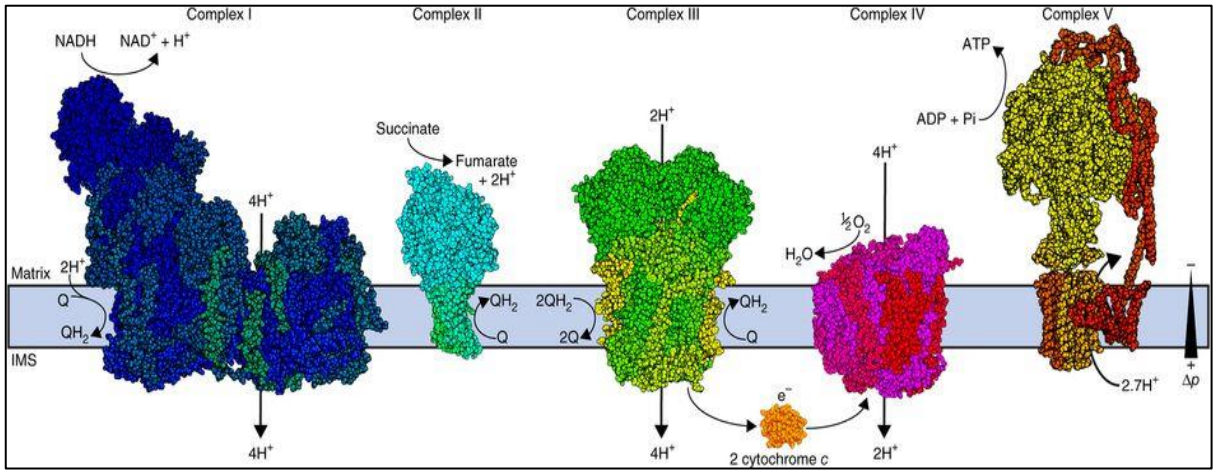

Diagram 3: Electron Transport Chain (Letts and Sazanov, 2017)

1.1.3.4 Improved glucose uptake

NO, apart from being a potent vasodilator has also been illustrated to play a central role in glucose uptake, as was shown in an in vivo diabetes study involving diabetic patients who performed an ergometer fitness test at 60% of their VO_2 max (Kingwell *et al.*, 2002). Patients who received a NOS-inhibitor exhibited a substantially lower glucose uptake than those who did not receive the intervention, indicating that NO may have an important role to play within glucose uptake (Kingwell *et al.*, 2002). Researchers have speculated that NO influence on glucose uptake may take place via the upregulation of GLUT4 translocation and generation (Kingwell *et al.*, 2002). It remains to be seen, however, whether or not the same effects are transferrable to trained and elite athlete populations, due the powerful sensitizing effects physical training has on tissue and whether the uprating of GLUT4 translocation and glucose uptake are relevant outcomes for athletes at this level (Power and Edward T. Howley, 2014).

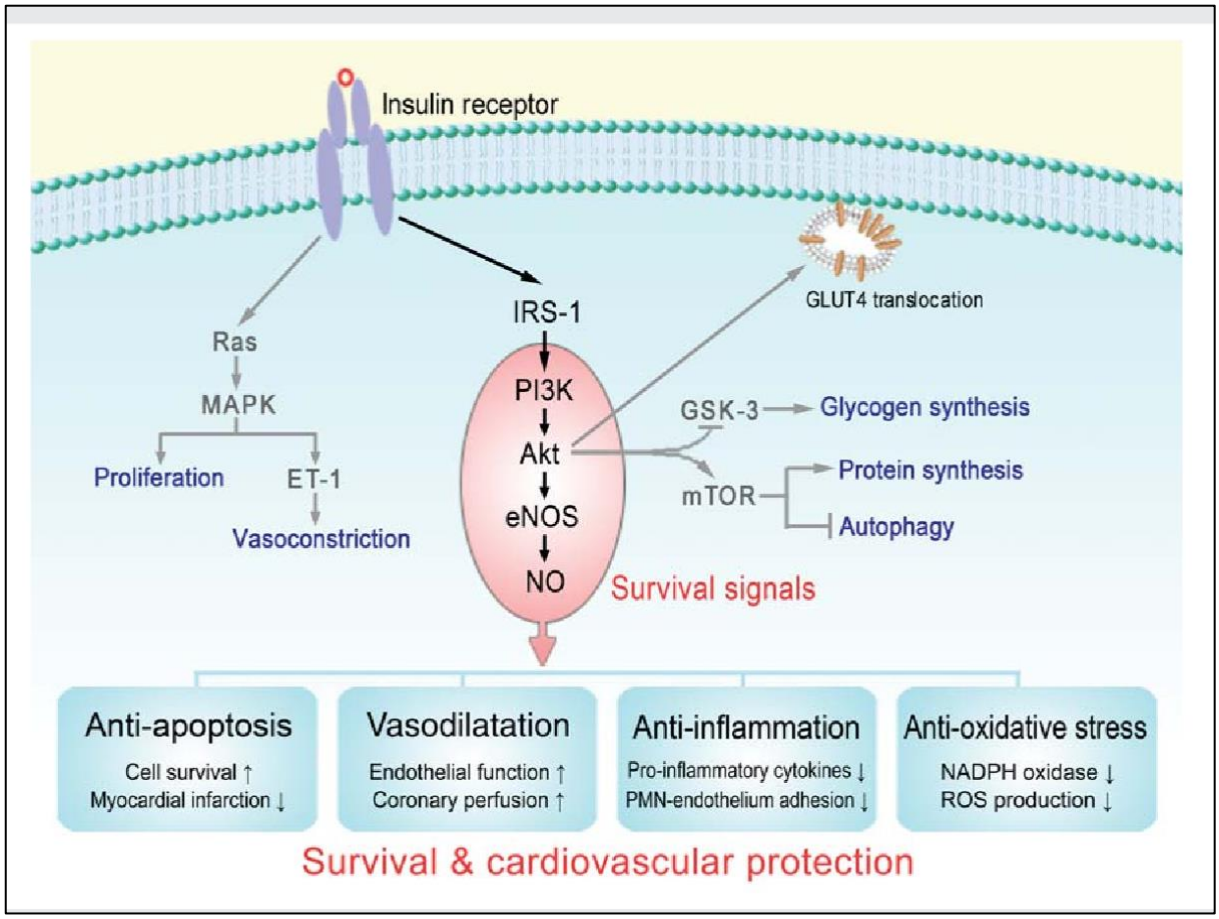

Diagram 4: The role of NO in glucose uptake (Yu, Gao and Ma, 2011)

1.1.3.5 Augmented calcium handling

In diagram 5 an illustration of the inner workings of one single sarcolemma may be seen. The presence of NO within the serum is hypothesized to be a potent proponent in optimizing muscle pump efficiency within the sarcoplasmic reticulum (SR) by reducing ATP cost within ATPase calcium pumps during the muscle relaxation phase (Hernández *et al.*, 2012). This is an attractive prospect due to the ATP attributed to calcium pumping, with some authors hypothesizing that calcium pumping may contribute to up to 50% of all ATP consumption within the muscle cell (Bescos *et al.*, 2012). In animal studies, these effects have been noted to be greater within fast-twitch muscle fibers, with slow-twitch fibers experiencing no significant difference in force production (Hernández *et al.*, 2012). Studies observing the differentiated reactions of human muscle fibers as well as differing types of muscle fiber compositions within the framework of an in vivo study to the presence of nitric oxide have not been published to date but would provide a novel insight into possible ergogenic, muscle-fiber-specific effects of nitrate-based interventions (Mcmahon, Leveritt and Pavey, 2017).

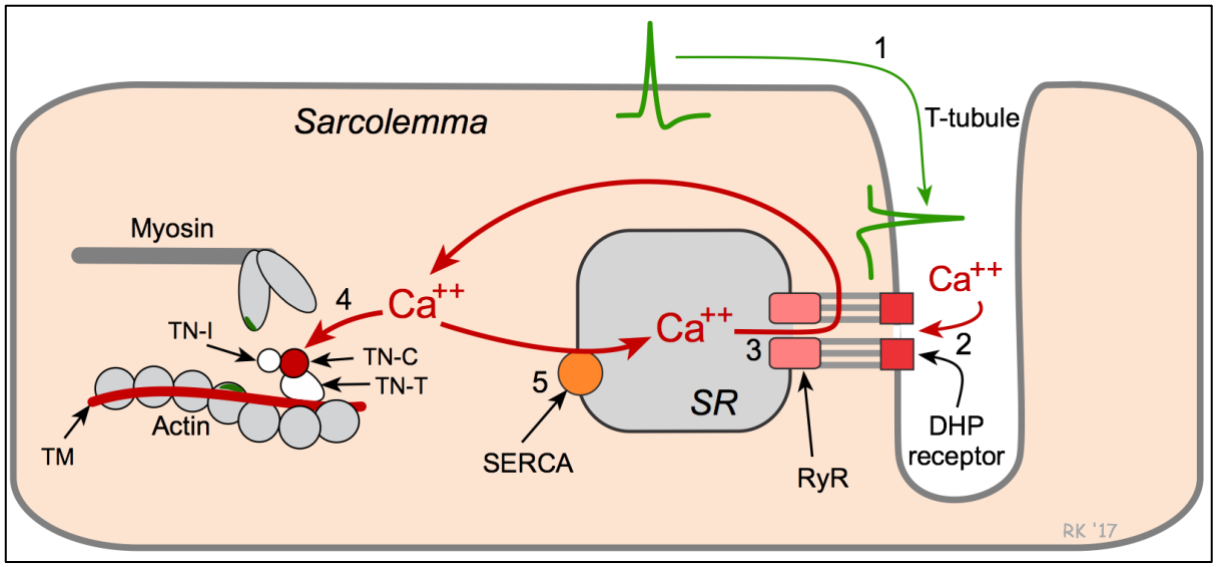

Diagram 5: Calcium-induced muscle contraction within skeletal muscle (Klabunde, 2017)

1.1.4 Nitric Oxide Half-Life

Being a member of the reactive nitrogen species (RNS), nitric oxide possesses a relatively short half-life typically, lasting for only a couple of seconds before it reacts with circulating superoxide (O_2^-) to form peroxynitrite (Nnate and Ngozi, 2016), which has been in previous studies to be a selective oxidant and a proponent involved in increased cellular toxicity (Koppenol *et al.*, 1992). Consequently, the toxic effects of peroxynitrite must be neutralized via a series of reactions with either oxyhemo- or oxymyoglobin to form $2H_2O$ and O_2 or be neutralized via reaction with CO_2 forming NO_3^-, CO_3^- and $2H^+$ (Nnate and Ngozi, 2016). Studies have indicated that nitric oxide half-life has been shown to inversely correlate to its own serum concentration (Kelm, 1999).

1.1.5 Potential risks of excessive nitrate supplementation

Potentially an excessive NO-presence within the body may compromise oxidative phosphorylation by impeding oxygen delivery to peripheral tissue producing a state of hypoxia as well as by irreversibly inhibiting cytochrome C activity and impeding hydrogen ion transport required to maintain the electrical potential difference powering complex V, which at best would diminish athletic performance and at worst result in cellular apoptosis (Nnate and Ngozi, 2016).

Noteworthy is that excessive nitrate consumption through dietary sources or nitrate contaminated water has been known to cause cyanosis via acute methemoglobinemia in infants, resulting due to the reduction of hemo- to methemoglobin and the associated left shift in oxygen disassociation curve. The reduced oxygenic binding affinity of ferric (Fe_3^+) compared

to ferrous iron (Fe_2^+) has been shown to cause peripheral hypoxia (Nnate and Ngozi, 2016), suggesting that the use of exogenous nitrates as an ergogenic aid should be used with care so as not to overdose and harm rather than impair athletic performance.

1.2 Relevance and Study Aim of Systematic Review

Within the competitive sports world, a difference ranging from +0,5% to +1,6% is deemed as constituting a significant improvement in performance (Paton and Hopkins, 1999). Due to marginal differences in performance dictating the difference between victory or defeat, various forms of nutritional-based supplementation seem to have become an attractive option to athletes, who wish to achieve a competitive edge (Domínguez *et al.*, 2017). For example, the difference between the first and twelfth place at the 2012 Olympics 10000 m mens' run was only 0,66% (Mcmahon, Leveritt and Pavey, 2017). With such results as were found by (Lansley *et al.*, 2011) that documented a 2,7% increase in time trial performance while supplementing with nitrate enriched beetroot juice, nitrate supplementation may provide an attractive option for athletes to enhance performance through dietary intervention.

There currently exists a growing body of scientific evidence supporting a broad spectrum of supplementation forms, ranging from nitrate salts to beetroot juice. Within this context, nitrates have received a growing degree of interest as ergogenic aids due to a significant body of evidence supporting their efficacy (Domínguez *et al.*, 2017). Whereas the exact mechanisms; by which nitrate supplementation enhances performance-related outcomes; remain to be fully elucidated, factors dictating treatment efficacy are relatively well documented and include: NO3 dose, training level, athlete status, supplementation duration, normal dietary NO3-intake and type of exercise protocol (Domínguez *et al.*, 2017; Garnacho-Castaño *et al.*, 2018). Consequently, not all studies have come to the same findings, leading to some disagreement on the efficacy of nitrates as an ergogenic aid, especially within trained and elite athlete populations (Hoon *et al.*, 2013; Pawlak-Chaouch *et al.*, 2016; Domínguez *et al.*, 2017; Mcmahon, Leveritt and Pavey, 2017). Conversely, studies indicating the ergogenic effects of nitrate supplementation within population of recreational and healthy athletes indicate a high degree of efficacy, suggesting that fitness status as well population-specific differences act as confounding variables within studies by increasing interparticipant variance and thereby reducing study power of significance (Domínguez *et al.*, 2017). To date several meta-analyses have been conducted, investigating nitrate supplementation in a broadly defined population. Such studies recruited participants from both trained and recreational sporting backgrounds

(Hoon *et al.*, 2013; Pawlak-Chaouch *et al.*, 2016; Domínguez *et al.*, 2017; Mcmahon, Leveritt and Pavey, 2017). This may, however, be problematic and a potentially confounding variable as numerous studies have documented differences in the ways that specific populations react to nitrate treatment, noting that trained and elite athletes often require higher doses to achieve same effects as their recreational compatriots (Garnacho-Castaño *et al.*, 2018). Hence this systematic review seeks to investigate and compare the efficacy of acute and chronic nitrate supplementation within professional/competitive endurance athlete population during submaximal and maximal bouts of endurance athletes exercise.

2. Main Text

2.1 Methods

2.1.1 Search protocol

PICO is an acronym that allows researchers to clearly define the four major aspects of a given research question, namely which participants, interventions, comparisons and outcomes that would be implemented within the ideal target study. These are essential elements that are involved in bibliographical searching as a part of evidence-based practice, which forms one of the cornerstones for the writing of systematic reviews (Khan *et al.*, 2003). Therefore, the PICO method was used in the writing of this systematic review to define the research question as well as form the foundation of the bibliographical searching of evidence. This allows for studies to be compared on common terms to thereby qualitatively assess their key findings on the level of a meta-analysis. Furthermore, this systematic review was based on the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and Cochranes Handbook for Systematic Reviews of Interventions, which have been described in great detail elsewhere (Higgins, O'Connor and Green, 2008; Liberati *et al.*, 2009; JPT *et al.*, 2019). Bibliographical searching took place with the use of the "PubMED with Full Text" Database between the 16.01.2020 and 20.01.2020, incorporating the following search term:

(((((((Nitrates) OR Nitrites) OR Nitric Oxide) OR NO) OR Beetroot))) AND ((((((((((O2 Uptake) OR Ergogenic) OR Metabolic Rate) OR Blood Flow) OR Circulation) OR Performance) OR Enhancement)) AND ((((Exogenous) OR Dietary) OR supplement*) OR nutritional)) AND ((((((athlete) OR professional) OR sports person) OR sportsman) OR sportswomen) OR player)))*

Bibliographic searching consisted of four phases – Identification, Screening, Eligibility and Inclusion. During the identification phase the database PubMed was used during bibliographic searching. No other databases were implemented during the writing of the current review. Subsequent to the identification phase, intervention abstracts and titles were used to screen each of the studies and assess in terms of their relevance. Studies that passed this stage passed into the eligibility phase, where the full-text article was read and assessed for its applicability on the basis of pre-determined exclusion criteria. Finally, studies that passed the exclusion criteria entered into the inclusion phase and were qualitatively and quantitatively assessed with the use of the PICO table shown in table 3 and forest plots.

2.1.2 Types of interventions

This systematic review considered randomized controlled interventions conducted within the last 10 years, which assessed various modes of inorganic dietary nitrate delivery such as nitrate enriched beetroot juice, nitrate salts or other exogenous nitrate forms within professional and competitive athlete populations possessing a VO2 max above or equal to 51,9 mL/kg.min. The target study population of this systematic review was defined as professional and trained endurance athletes with a broad range of endurance sport backgrounds ranging from triathletes to swimmers to mid- and long-distance track athletes. Data from interventions investigating the effects of dietary nitrate supplementation on the exercise intensity domains – moderate (<60% of VO_2 max), heavy (from 60 – 80% VO_2 max) and severe (>80% of VO_2 max) – was extracted and pooled into selective sub-groups upon successful passing of the pre-determined exclusion criteria. If the trials employed different modes of nitrate delivery, eg: nitrate salts vs. nitrate enriched beetroot juice, then these results were individually extracted and separated into subgroups within the PICO table. Furthermore, data originating from interventions that implemeted chronic and acute forms of supplementations was extracted into two subsets, while noting total NO3 dosage contained per bolus (in mmol) as well as total intervention duration and the time before trial commencement in chronic and acute strategies respectively. Interventions investigating the several different modes of delivery of exogenous nitrate, in the form of nitrate enriched beetroot juices, sodium nitrate salts and nitrate enriched capsules, on performance related outcomes, were considered for entry into the current systematic review and the type of nitrate intervention noted within the PICO table. As was the case in the overwhelming majority of previous systematic reviews in this field of study,

no limiting factor was placed on the mode of supplement delivery, given that the nitrate intervention was not given in conjunction with known nitrate antagonists such as caffeine or other forms of supplementation such as L-citrulline or L-arginine, which may have acted as confounding variables (Hoon *et al.*, 2013; Pawlak-Chaouch *et al.*, 2016). Furthermore, studies that did not include at least one unadulterated nitrate intervention within their trial rounds were excluded from entry into the current review. To ascertain if exogenous nitrate modulate factors controlling exercise efficiency and so as not to exclude any potentially significant effects of nitrate supplementation, interventions were allowed to include a broad range of exercise protocols ranging from incremental exercise to exhaustion (TTE), repeated sprints, time trials (TT), distance trials (DT) or constant work rate trials. Furthermore, trials investigating the effects of nitrate intervention using several different modalities such as cycle, running and rowing ergometers while incorporating elite athletes from several different disciplines such as triathletes, long-distance track athletes, rowers, etc. were considered for entry into the current systematic review. The following data was gathered from each of the studies that successfully passed entry criteria: sample size, participant VO_2 max (mL/kg.min), participant gender, study design, nitrate form, dosage size (mmol), intervention duration (days), time before trial commencement (hours), percentile changes in circulating nitrate/nitrite (%), exercise protocol, in-test VO_2 kinetics (mL/min), mean power output (Watts), TT performance (minutes/seconds) and percentile changes between experimental and control groups as well as any nitrate-induced adverse effects in the trial participants.

2.1.3 Type of outcome measures

Potential outcomes were selected based on preliminary research during bibliographic searching, which predicted them as being relevant markers for exercise economy (Mcmahon, Leveritt and Pavey, 2017). The primary outcome measures of the current review related to VO2 uptake (mL/min), TT performance (s) and TTE (s). Performance data originating from separate trial protocols and modes of nitrate delivery was stratified and entered into the PICO table. This data formed the basis for the generation of percentile treatment effect values (Δ%) as well as for quantitative analysis with the use forest plots generated with commercially available software (Review Manager 5, 2014). Circulating concentrations of nitrate and nitrite were extracted from each intervention and expressed as an overall percentile difference in comparison to their baseline values. Where studies did not include data pertaining to changes

in circulating nitrite and nitrate levels, the author attempted to establish contact with the research team responsible for the intervention. Upon completion of data entry, the total effect

2.1.4 Exclusion criteria

Studies were selected based on systematic exclusion criteria, which may be viewed below in *Table 1*. Studies were considered for excluded from the current meta-analysis based on the following pre-determined exclusion criteria: a) did not originate from peer-reviewed publication b) non-RCT study design, b) did not measured outcomes relevant for exercise efficiency such as fluctuations in VO_2 uptake, maximum power output (MPO), average power output (APO), time to exhaustion (TTE), distance traveled (DT) or reductions in time trial (TT) times, c) recruited participants with a mean VO2 max below 51,9 mL/kg.min, d) conducted intervention in populations with a history of chronic diseases such as Type-I or Type-II diabetes mellitus, ischemic heart disease or cancer e) Conducted nitrate intervention in conjunction with known nitrate antagonists and/or no solely nitrate-based intervention. As a final inclusion criterion, the Physiotherapy Evidence Database (PEDro) scale was employed as a means of assessing the robustness of each study design. The PEDro has been determined to be a fairly to substantially accurate way of assessing the quality of RCTs (Maher *et al.*, 2003) and was therefore included to qualitatively assess each study design. Implementation of the PEDro scale has been described in-depth elsewhere (Verhagen, 1999). Studies with a PEDro score of 5 and higher were deemed eligible for entry. Studies passing the eligibility phase were included for entry into this systematic review and were subsequently fully assessed. Following this study variables were then extracted and entered into a PICO table for later comparison and discussion.

2.1.5 Administrative Software

Upon successful entry into the eligibility section, each article was downloaded as full text and administered with a commercially available bibliography manager by the name of Mendeley. This program was also used for the generation of citations and bibliography within the scope of the current review. Furthermore, the Cochranes Review Manager 5 (Review Manager 5, 2014) was employed for systematic inclusion and exclusion of studies during the eligibility phase.

2.1.6 Statistical analysis

A commercially available bibliography manager by the name of Review Manager V5.3 was used to conduct statistical data analysis. Upon inclusion into this systematic review, study IDs were

entered into the RevMan 5 software (Review Manager 5, 2014), including the last name of the author and the year of publication. In the case of two or more studies sharing the same study ID, the study was specified by listing a defining study characteristic in brackets. The data source was set to "Published data only (unpublished not sought)" and the year of publication confirmed. If necessary, identifiers were added to the study at this point, after which the study was entered into the study pool. For the generation of the forest charts the "New Comparison Wizard" was opened and the name of the comparison defined, after which outcomes were added under the new comparison with data type being set to continuous. At this point, the type of outcome, as well as each of the study groups, were defined by the author. The statistical method and analysis model were set to inverse variance and fixed effects respectively whereas Standard Mean Difference (SMD) was implemented as a means of effect measurement. Subsequently, the analysis details were conducted in totals and subtotals utilizing a study confidence interval and total confidence interval both equal to 95%. Finally, graph labels were defined with studies being sorted by year of publication.

1. **Study Design**	- Non-RCT studies (Systematic Reviews, Reviews, etc.) and from - Did not originate from a peer-reviewed journal
2. Participants	- Populations with a history of chronic disease - Population average VO2 max <51.9 mL/min.kg - Participant age <18 or >65 yrs. - Animal studies
3. Intervention	- Direct treatment combination with known nitrate antagonists (eg: caffeine) - L-Arginine/L-Citrulline supplementation - No separate nitrate intervention
4. Control	- No placebo or placebo was not taste and odor disguised
5. Outcome	- Non-performance related variables
6. PEDro Score	- PEDro score < 5

Table 1: Exclusion criteria applied during bibliographic searching

2.2 Results

2.2.1 Trial selection

Initial bibliographic searching yielded a total of 188 results, all of which then passed into the screening phase. No other literature sources were identified during the identification phase and correspondingly there were no duplicates to be removed during the screening. During this process presets one through three were applied, thereby yielding a total of 66 results, all of which entered into the eligibility phase.

1. Article type: Randomized Control Trial
2. Date of publication: last 10 years, species: human
3. Age: 19 – 65 years

Upon application of the pre-determined exclusion criteria shown in table 2, a total of sixteen studies passed into the inclusion phase and were retrieved as full text via the PubMed database. Bibliographic searching was conducted as stipulated by the PRISMA evidence based minimum set for reporting of systematic reviews and has been illustrated in the form of a flow chart below in diagram 1 (Liberati *et al.*, 2009). Following successful entry into the inclusion phase, the data of each of the respective studies were extracted and illustrated into the PICO table shown below in table 3. A total of 50 studies were excluded based on the following exclusion criteria: a) participants with a VO2 max below 51,9 mL/kg.min (n=29), b) use of nitrate agonists within intervention (n=2), c) use of L-arginine supplementations in conjunction with nitrate intervention (n=3), d) studies did not measure outcome related to exercise economy (n=16). Reasons for study exclusion have been illustrated as an overview in table 2.

Criteria	Reason for exclusion	Number of studies excluded
1	Participant VO_2 max below 51.9 mL/kg.min	29
2	Use of nitrate agonists (caffeine)	2
3	Use of L-arginine supplementation	3
4	Study did not measure VO_2 outcomes	16
Total		50

Table 2: Reasons for exclusion of 50 studies from the current review

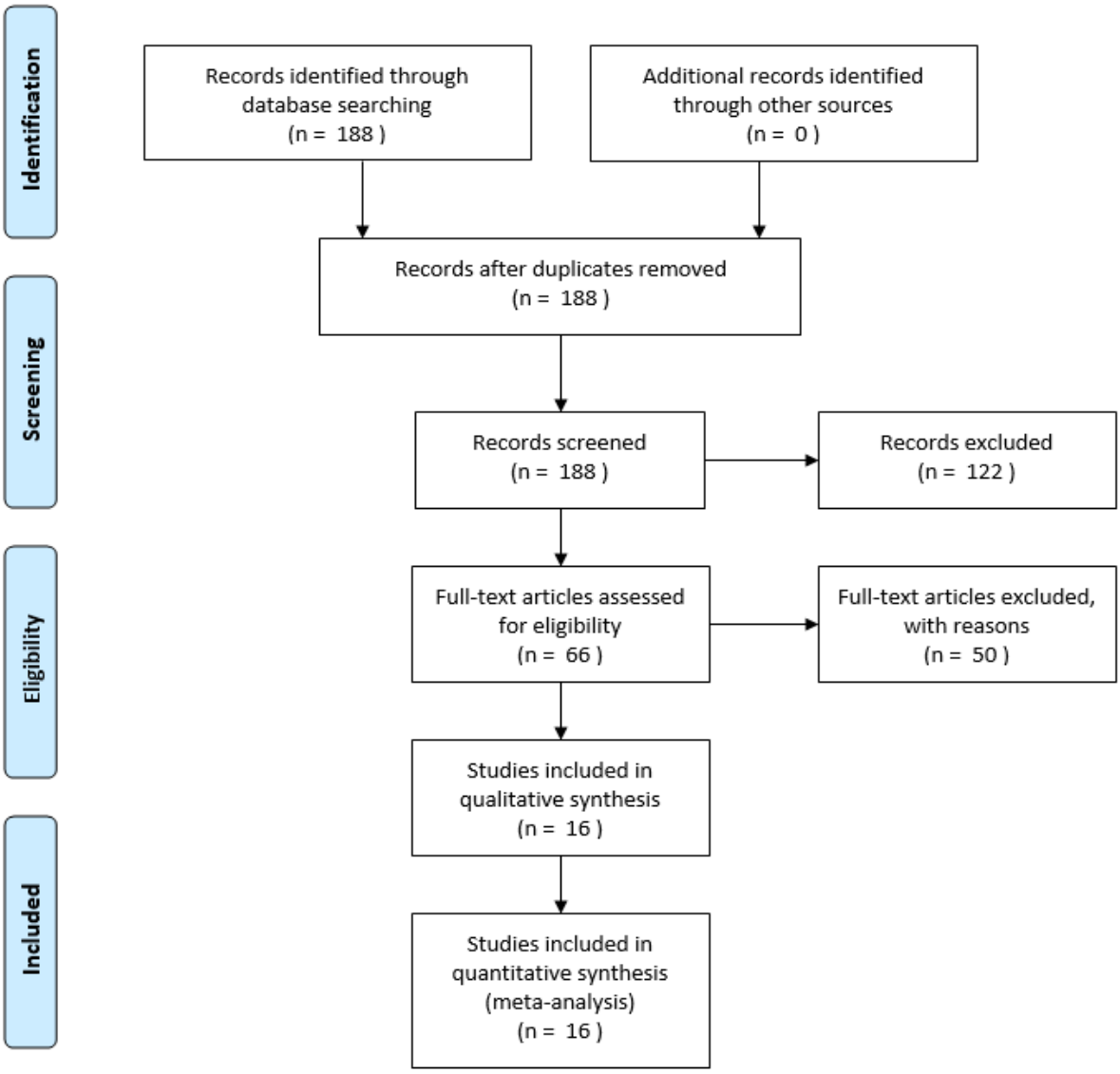

Diagram 6: PRISMA Flowchart of results acquired through bibliographical search

2.2.2 Study characteristics

Characteristics of each the included studies have been illustrated below in table 3. The included interventions show a preference for the inclusion of double-blinded, cross-over study designs, with only two studies opting not to utilize them (Flueck *et al.*, 2015; Balsalobre-Fernández *et al.*, 2018). Three further studies made implemented a repeat measure-based system (Cermak *et al.*, 2012; Cermak, Gibala and Loon, 2012) with one final study choosing to employ a Latin square study design (Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017). All trials with the exception of one conducted their interventions in normobaric, normoxic conditions (MacLeod *et al.*, 2015; Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017) and showed a general tendency to prefer the cycle ergometer within their testing protocols (Bescos *et al.*, 2011, 2012; Lansley *et al.*, 2011; Cermak *et al.*, 2012; Cermak, Gibala and Loon, 2012;

Muggeridge *et al.*, 2013; Christensen, Nyberg and Bangsbo, 2013; Flueck *et al.*, 2015; McQuillan *et al.*, 2017a, 2018; Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017; Lowings *et al.*, 2017; Balsalobre-Fernández *et al.*, 2018; Garnacho-Castaño *et al.*, 2018). A general trend was observed amongst studies into the current meta-analysis to favor the use of time trials (Bescos *et al.*, 2011; Lansley *et al.*, 2011; Cermak *et al.*, 2012; Christensen, Nyberg and Bangsbo, 2013; Muggeridge *et al.*, 2013; Boorsma, Whitfield and Spriet, 2014; MacLeod *et al.*, 2015; Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017; McQuillan *et al.*, 2017a; Shannon *et al.*, 2017), whereas two trials implemented the use of distance trials (Bescos *et al.*, 2011; Cermak *et al.*, 2012), one the use of a power output protocol (McQuillan *et al.*, 2018) and final three trials, the implementation time to exhaustion protocols (Bescos *et al.*, 2012; Balsalobre-Fernández *et al.*, 2018; Garnacho-Castaño *et al.*, 2018). All studies included the use of double-blinding with the exception of one study that only instituted the use of single-blinding (Flueck *et al.*, 2015).

2.2.3 Participants

Within the 16 trials, a total of 161 trained and/or elite athletes with a mean VO_2 max above or equal to 51.9 mL/kg.min were treated with an oral nitrate intervention and tested for improvements in performance-related outcomes as defined by the aforementioned variables of exercise economy. The largest population of athletes was comprised of 91 cyclists who competed a professional, club or recreational level (Lansley *et al.*, 2011; Bescos *et al.*, 2012; Cermak *et al.*, 2012; Cermak, Gibala and Loon, 2012; Hoon *et al.*, 2015; McQuillan *et al.*, 2017a)(Christensen, Nyberg and Bangsbo, 2013); followed by a combined total of 30 national and elite long-distance runners and track athletes (Boorsma, Whitfield and Spriet, 2014; Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017; Balsalobre-Fernández *et al.*, 2018), 20 professional triathletes (Shannon *et al.*, 2017; Garnacho-Castaño *et al.*, 2018) and finally by a group of 8 elite kayakers (Muggeridge *et al.*, 2013). On average the number of participants recruited ranged from 8 to 20 with the mean sample size consisting of 10 participants per study. Interestingly all included studies exclusively recruited male athletes who possessed an overall mean VO_2 max of 64.25 ± 9 mL/kg.min ranging from values of 51,9 mL/kg.min to 80 mL/kg.min. In the broad majority of cases, the beetroot juice intervention was reported to be well tolerated amongst most athletes, however rare cases of gastrointestinal discomfort were noted in some participants (Muggeridge *et al.*, 2013), with cases by the name of beeturia or a red discoloration of the urine, as well as discoloration of fecal matter were commonly

documented in several studies (Cermak *et al.*, 2012; Cermak, Gibala and Loon, 2012; Christensen, Nyberg and Bangsbo, 2013; Boorsma, Whitfield and Spriet, 2014; McQuillan *et al.*, 2017a, 2018; Balsalobre-Fernández *et al.*, 2018). Nitrate supplementation did not lead to any lasting detrimental effects on health or performance prior to trial commencement in any of the interventions.

2.2.4 Supplementation (dosages, chronic/acute, form)

Six studies employed the use of chronic nitrate supplementation (Bescos *et al.*, 2012; Cermak, Gibala and Loon, 2012; Christensen, Nyberg and Bangsbo, 2013; McQuillan *et al.*, 2017a; Balsalobre-Fernández *et al.*, 2018) by contrast to the remaining nine studies that exposed their participants to a single bolus of nitrate followed by the measurement of performance-related outcomes. The last study employed both the employed a hybrid form incorporating both chronic and acute supplementation models within its study design (Boorsma, Whitfield and Spriet, 2014). The mean daily consumption of the chronic subgroup was equal to 8.3 ± 4.5 mmol NO_3^- with supplementation taking place over a mean timeframe of 6.28 ± 4.3 days. By comparison within the eleven acute studies, participants supplemented using a single bolus containing a mean 9.24 ± 4.6 mmol of NO_3^- an average of 2.77 ± 0.3 hrs before trial commencement. Most studies preferred the use of commercially available nitrate enriched beetroot supplements as a nitrate carrier (Lansley *et al.*, 2011; Cermak *et al.*, 2012; Cermak, Gibala and Loon, 2012; Muggeridge *et al.*, 2013; Christensen, Nyberg and Bangsbo, 2013; Boorsma, Whitfield and Spriet, 2014; MacLeod *et al.*, 2015; Shannon *et al.*, 2017; Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017; McQuillan *et al.*, 2017a, 2018; Balsalobre-Fernández *et al.*, 2018; Garnacho-Castaño *et al.*, 2018), whereas two studies employed the use of nitrate salt derivatives such as $NaNO_3$ and KNO_3 (Bescos *et al.*, 2011, 2012). One final study employed a comparative cross-over study design incorporating both an enriched beetroot juice preparation and $NaNO_3$ supplementation (Flueck *et al.*, 2015). All studies documented a significant treatment interaction as measured by the increase in circulating plasma nitrite and nitrate levels, documenting a mean percentile increase of 52.8 ± 35.2 % and 1431.1 ± 634.8 % respectively. Chronic studies generally reported the highest increase in circulating NO2 and NO3 concentrations as may be seen in table 3 below.

2.2.5 PICO Table

Reference	PEDro Score	Sample Size and Sex	VO_2 max/peak	Study Design	NO_3^- Dose	Percentage NO_2^- /NO_3^- change	Exercise Protocol	Trial Result	Difference (%)
(Balsalobre-Fernández *et al.*, 2018)	9	12 M	71,8 ml/kg.min	DB	BRJ (~6.5 mmol NO3) Chronic (15 d.)	Not published	TTE (Incremental) Treadmill	TTE PG: 1230 ± 73.5 s NG: 1269 ± 53.6 s	+3.17*
(Bescos et al., 2012)	10	11 M	65,1 mL/kg.min	DB, CO	NaNO3 (10 mg/kg) Acute (3 hrs.)	↑ 733% (NO3-) ↑ 15.77% (NO2-)	TTE (Incremental) Cycle ergometer	TTE PG: 409 ± 106.1 s NG:416±106.1 s	+1.71*
(Bescos et al., 2011)	10	13 M	60 mL/kg.min	DB, CO	NaNO3 (10 mg/kg) Chronic (3 d)	↑ 79% (NO2-)	40-min DT Cycle ergometer	DT PG: 26.3 ± 1.2 km NG: 26.4 ± 1.1 km	0
(Boorsma, Whitfield and Spriet, 2014)	8	8 M .	80 ml/kg.min	DB, CO	140 mL BRJ (~13 mmol NO3) Chronic (8 d.)	↑ 1562% (NO3-)	1500 m TT Indoor track	TT PG: 251.4 ± 7.6 s NG: 250.5 ± 4.3 s	-0.2
(Boorsma, Whitfield and Spriet, 2014)	8	8 M	80 ml/kg.min	DB, CO	140 mL BRJ (~13 mmol NO3) Acute (2.5 hrs.)	↑ 2251% (NO3-)	1500 m TT Indoor track	TT PG: 250.4 ± 7.0 s NG: 250.7 ± 7.6 s	-0.12
(Boorsma, Whitfield and Spriet, 2014)	8	8 M	80 ml/kg.min	DB, CO	210 mL BRJ (~19,5 mmol NO3) Chronic (3 d)	↑ 1562% (NO3-)	Sub-maximal Treadmill Run	SMTR (at 65% VO2) PG: 3315 ± 240 mL/min NG: 3367 ± 225 mL/min	+1.57
(Boorsma, Whitfield and Spriet, 2014)	8	8 M	80 ml/kg.min	DB, CO	210 mL BRJ (~19,5 mmol NO3) Acute (2,5 hrs.)	↑ 1562% (NO3-)	Sub-maximal Treadmill Run	SMTR (at 65% VO2) PG: 3265 ± 242 mL/min NG: 3239 ± 214 mL/min	-0.8
(Cermak, Gibala and Loon, 2012)	9	12 M	58 ± 2 ml/kg.min	DB, RM, CO	140 ml BRJ (~8 mmol NO3) Chronic (6d)	↑ 1907% (NO3-)	60 mins (45% Wmax) Cycle ergometer	VO2 Uptake PG: 2.0 ± 0.07 L/min NG: 1.93 ± 0.05 L/min	-3.5*

(Cermak, Gibala and Loon, 2012)	9	12 M	58 ± 2 ml/kg.min	DB, RM, CO	140 ml BRJ (~8 mmol NO3) Chronic (6d)	↑ 1907% (NO3-)	60 mins (65 Wmax) Cycle ergometer	VO2 Uptake PG: 3.1 ± 0.09 L/min NG: 2.94 ± 0.10 L/min	-5.16*
(Cermak, Gibala and Loon, 2012)	9	12 M	58 ± 2 ml/kg.min	DB, RM, CO	140 ml BRJ (~8 mmol NO3) Chronic (6d)	↑ 1907% (NO3-)	10 km TT Cycle ergometer	TT PG:965 ± 75.7 s NG: 953 ± 75.7 s	+1.24*
(Cermak et al., 2012)	9	20 M	60 ml/kg.min	DB, CO, RM	140 ml BRJ (~8,7 mmol NO3) Acute (2,5 hrs.)	↑ 96% (NO2-)	1 hr. DT Cycle ergometer	TT PG: 3900 ± 395.2 s NG: 3930 ± 295.2 s	+0.77
(Christensen, Nyberg and Bangsbo, 2013)	8	10 M	72 mL/kg.min	DB, CO	0,5 L BRJ Chronic (6 d.)	↑ 297% (NO3-)	400 kcal TT Cycle ergometer	TT PG: 1117 ± 167 s NG: 1100 ± 163 s	-1.52
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~3 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 2.03 ± 0.21 L/min	-3.33
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~6 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 1.94 L/min	-7.62
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~12 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 1.99 L/min	-5.23
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~3 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.39 ± 0.33 L/min	-3.14
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~6 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.29 ± 0.62 L/min	-6.00*
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	BRJ (~12 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.37 ± 0.84 L/min	-8.00

(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~3 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 2.03 ± 0.15 L/min	-3.33
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~6 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 2.12 ± 1.15 L/min	+1.00
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~12 mmol NO3) Acute (3 hrs.)	Not published	5 mins (MI) Cycle ergometer	VO2 Uptake PG: 2.10 ± 0.23 L/min NG: 2.06 ± 0.15 L/min	-2.00
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~3 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.44 ± 0.28 L/min	-1.71
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~6 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.56 ± 0.39 L/min	+1.71
(Flueck et al., 2015)	7	12 M	59,4 mL/min.kg	SB, CO	NaNO3 (~12 mmol NO3) Acute (3 hrs.)	Not published	5 mins (SI) Cycle ergometer	VO2 Uptake PG: 3.50 ± 0.32 L/min NG: 3.54 ± 0.17 L/min	+1.14
(Garnacho-Castaño et al., 2018)	9	12 M	54.8 ± 3.1 mL/kg.min	DB, CO	70 mL BRJ (~6,5 mmol NO3) Acute (3 hrs.)	Not published	Endurance test (30 mins at VT1) Cycle ergometer	VO2 Uptake PG: 2.4 ± 0.4 L/min NG: 2.4 ± 0.5 L/min	0.00
(Garnacho-Castaño et al., 2018)	9	12 M	54.8 ± 3.1 mL/kg.min	DB, CO	70 mL BRJ (~6,5 mmol NO3) Acute (3 hrs.)	Not published	15 min TT (VT2) Cycle ergometer	VO2 Uptake PG: 3.4 ± 0.3 L/min NG: 3.3 ± 0.4 L/min	-2.94
(Lansley et al., 2011) T A	9	9 M	56 mL/kg.min	DB, CO	0,5 L BRJ (~6,2 mmol NO3) Acute (2,5 hrs.)	↑ 105% (NO2-)	4 km TT Cycle ergometer	4 km TT PG: 387 ± 25.2 s NG: 376 ± 21 s	-2.84*
(Lansley et al., 2011)	9	9 M	56 mL/(kg.min	DB, CO	0,5 L BRJ (~6,2 mmol NO3) Acute (2,5 hrs.)	↑ 105% (NO2-)	16.1 km TT Cycle ergometer	16.1 km TT PG: 1662 ± 126 s NG: 1614 ± 108 s	-2.88*

(MacLeod et al., 2015)	7	11 M	60 ml/kg.min	SB	70 mL BRJ (~6 mmol NO3) Acute (2,5 hrs.)	↑ 441% (NO3-)	10 km TT on ergometer (normoxia)	10 km TT PG: 954 ± 47 s NG: 961 ± 54 s	+0.73
MacLeod et al., 2015)	7	11 M	60 ml/kg.min	SB	70 mL BRJ (~6 mmol NO3) Acute (2,5 hrs.)	↑ 441% (NO3-)	10 km TT on ergometer (hypoxia)	10 km TT PG: 1023 ± 49 s NG: 1018 ± 52 s	-0.49
(McQuillan et al., 2018)	10	8 M	64 mL/kg.min	DB CO	140 mL BRJ (~8 mmol NO3) Chronic (3 d.)	Not published	PO Cycle ergometer	Power Output PG: 336 ± 45 W NG: 337 ± 50 W	+0.3
(McQuillan *et al.*, 2017b)	10	8 M	63 mL/kg.min	DB, CO	70 mL BRJ (~4 mmol NO3) Chronic (8 d.)	Not published	4 km TT Cycle ergometer	TT Time PG: 344.8 ± 14 s NG: 343.6 ± 14.3 s	-0.7*
(Muggeridge et al., 2013)	10	8 M	51.9 mL/kg.min	DB, CO	70 mL BRJ (~5 mmol NO3) Acute (3 hrs.)	↑ 32% (NO2-)	16.1 km TT Cycle ergometer	TT PG: 1702 ± 15 s NG: 1664 ± 14 s	-2.23*
(Shannon et al., 2017)	10	8 M	62.3 mL/kg.min	DB	140 mL BRJ (~12,5 mmol) Acute (3 hrs.)	Not published	1500 m TT Treadmill	TT PG: 325.7 ± 38.8 s NG: 319.6 ± 36.2 s	-2.79*
(Shannon et al., 2017)	10	8 M	62.3 mL/kg.min	DB	140 mL BRJ (~12,5 mmol) Acute (3 hrs.)	Not published	10 km TT Treadmill	TT PG: 2649.9 ±319.8 s NG: 2643 ± 324.1 s	-0.26
(Timothy Arnold, James Oliver and Maria Lewis-Jones, 2017)	9	10 M	66 mL/kg.min	DB, CO, RM	BRJ (~7 mmol NO3) Acute (2,5 hrs.)	Not published	10 km TT At 2500 m simulated alt. Treadmill	TT PG: 2876 ± 265 s NG: 2862 ± 233 s	-0.49

Table 3: PICO table of results gathered from data extraction of included studies (M – male, DB – double-blind, CO – cross-over, RM – repeat-measure, LS- Latin-square, SB – single-blind, BRJ – beetroot juice, TTE – time to exhaustion, TT – time trial, DT – distance-trial, PO – Power Output, Wmax – maximum power output, MI – moderate intensity, SI – severe intensity, significant result)*

2.2.6 Performance outcomes

As shown below in diagram 7, chronic and acute nitrate supplementation significantly lowered overall effect estimate for VO_2 uptake below the line of no effect, thereby favoring the intervention (P=0.46, I^2=0%). The standard mean difference from 18 trials was 0,3 indicating a statistically significant small to moderate reduction of VO2 uptake (95 % CI -0.5–-0.1 p<0,005). A randomized effects analysis indicated no heterogeneity within intervention results (I^2, df=17, P=0.46).

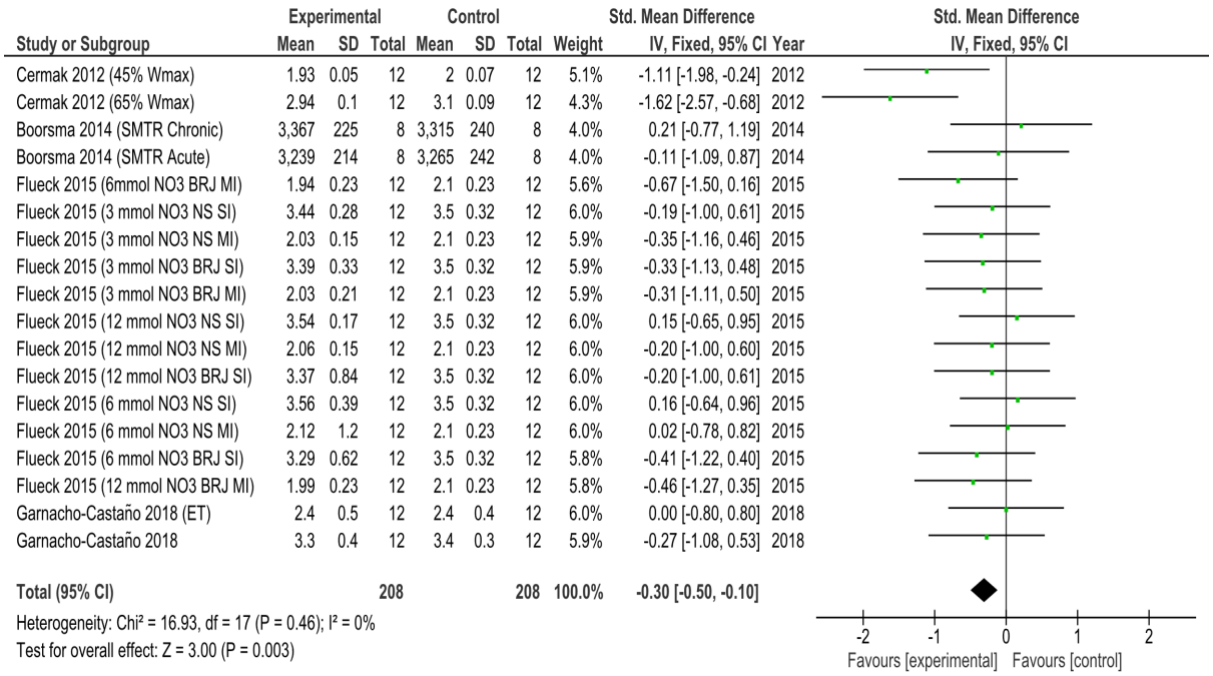

Diagram 7: The effects of chronic and acute nitrate supplementation on VO2 Uptake (Wmax – maximum power output, SMTR – submaximal treadmill running, BRJ – beetroot juice, MI – moderate intensity, SI – severe intensity, NS – nitrate salts, ET – endurance test, SD – standard

The standardized mean difference of the 15 interventions pertaining to TT performance are shown in diagram 8 and equated to – 0,18 (95% CI -0.42–0.06) The total estimated effect therefore equated to a trivial to small increase in TT performance, which crossed the line of no effect, indicating a non-significant treatment interaction (95%, p>0,05). A randomized effects analysis indicated no heterogeneity within the intervention results (I^2=0% df=14 P=0.57).

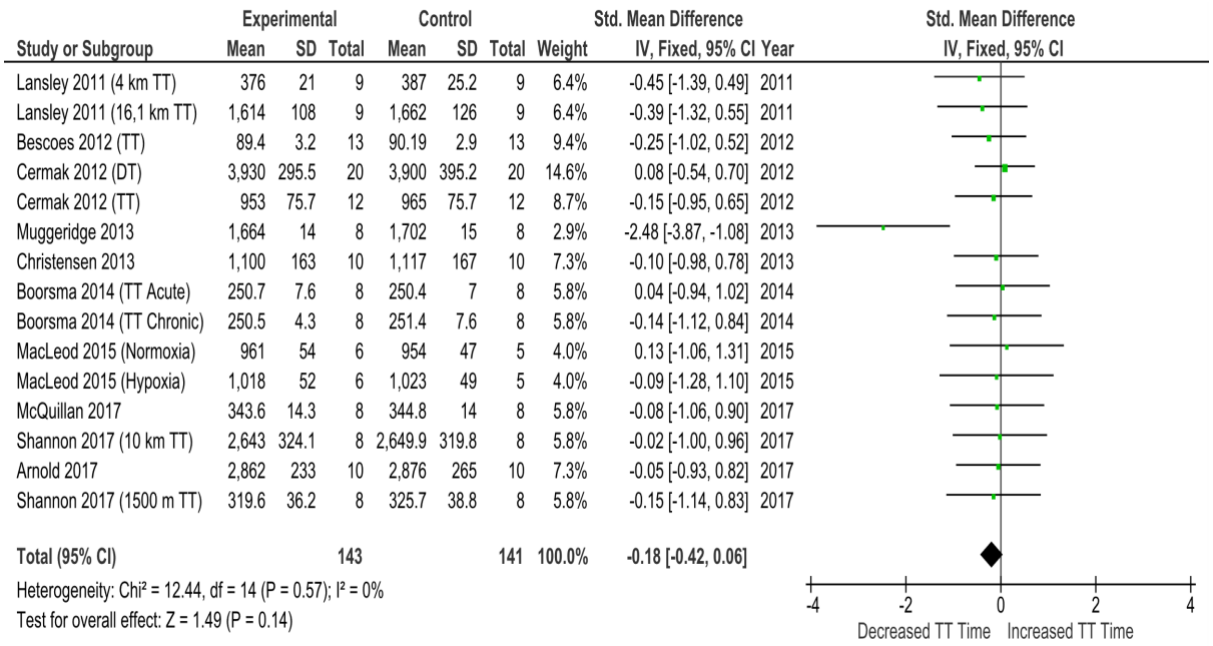

Diagram 8: The effects of chronic and acute nitrate supplementation on TT completion time (TT – time trial, DT – distance trial, SD – standard deviation, CI – confidence interval)

2.2.7 Risk of study bias

According to the Cochrane Risk of Bias tools, trials included, the current meta-analysis contained a relatively low risk of study bias. The majority of studies included into this review implemented double blinding (n=14) with the exception of two trials, which only applied single blinding in their study designs, thereby increasing the risk of study bias due to staff having direct access to group allocation (Flueck *et al.*, 2015; MacLeod *et al.*, 2015). All study designs employed the use of randomization and control groups as well as using a nitrate-depleted placebo, which did not appear or taste different from the intervention treatment. Furthermore, several studies implemented a feedback system, in which participants were asked to identify if they had taken the placebo or the intervention to verify placebo quality (Boorsma, Whitfield and Spriet, 2014).

2.2.8 PEDro Score

Interventions included into the current meta-analysis achieved an average PEDro score of 9.0 ± 1.0. As stated above, some study designs only implemented single blinding between assessors and participants, thereby increasing the risk of study bias, for which their PEDro score was reduced to 7 (Flueck *et al.*, 2015; MacLeod *et al.*, 2015). Nonetheless the average PEDro score suggests a good to excellent level of study design and robustness.

2.3 Discussion

2.3.1 Summary of key findings

The primary aim of this systematic review was to assess the efficacy of chronic and acute nitrate supplementation within a trained endurance athlete population as defined as possessing a VO_2 max above or equal to 51.9 mL/kg.min The key findings indicated a statically significant reduction of exercise-induced oxygen expenditure within trained endurance athletes ($p<0.05$). These findings are in line with other similar systematic reviews and provide further evidence that nitrate supplementation elicits a small to moderate treatment effect by improving overall exercise efficiency within elite and trained athlete populations. Contrary to results published by several trials (Lansley *et al.*, 2011; Cermak *et al.*, 2012; McQuillan *et al.*, 2017b) the key findings from this review indicated that neither chronic nor acute nitrate supplementation had any significant treatment effects on time trial performance ($p>0.05$). These findings are in line with other systematic reviews, which documented a trivial however non-significant difference in TT performance (Domínguez *et al.*, 2017; Mcmahon, Leveritt and Pavey, 2017). Finally, studies that investigated TTE indicated a statistically significant treatment effect ($p<0.05$) on overall participant work capacity (Bescos *et al.*, 2012; Balsalobre-Fernández *et al.*, 2018).

2.3.2 Nitrate Absorption

Several studies that were taken into consideration for entry into the current meta-analysis noted differing serum concentrations of bioactive nitric oxide despite using similar dosages and delivery forms (Lansley *et al.*, 2011; Muggeridge *et al.*, 2013; Hoon *et al.*, 2014). Some authors have hypothesized the existence of a threshold value in circulating nitrate and nitrite that may be required for the intervention to fully expound its ergogenic properties (Cermak *et al.*, 2012). However other aspects apart from the dosage size, such as participant training status as well as several other factors relating to age, gender, anthropometrics, muscle fiber composition, oral facultative anaerobic bacteria profile, muscle fiber oxygenation status as well as mitochondrial function may play a central role in modulating duodenal uptake of the nitrate substrates as well as predetermining the efficacy of nitric-oxide-based intervention strategies. For example one study examining the effects of an acute dose of beetroot juice containing a total of 4.1 mmol of NO3 reported a mere 297% increase in circulating nitrate (Christensen, Nyberg and Bangsbo, 2013). By contrast other trials have achieved far greater gains in nitrate

and nitrite serum concentrations, sometimes as high as a 2000% percent increase in circulating nitrate levels, while using dosages of a similar size and type before commencement (Lansley *et al.*, 2011; Bescos *et al.*, 2012; Boorsma, Whitfield and Spriet, 2014; MacLeod *et al.*, 2015). Training status may be a plausible modulating factor as some authors have described differences in nitrate metabolism between recreational and elite athlete populations, reporting a substantially higher increase in plasma nitrates and nitrites concentrations post-intervention within highly trained athletes. (Jonvik *et al.*, 2018). This may partly explain the relatively large degree of standard deviation that was observed in serum concentration of circulating nitrate and nitrite during testing between studies (Jonvik *et al.*, 2018), however, it does not explain why untrained participants experienced the highest degree of ergogenic effects in contrast to their trained compatriots. Moreover, gender may also play a significant role in nitrate uptake as studies have shown that women are significantly more sensitive to nitrate supplementation than their male counterparts (Jonvik *et al.*, 2018). More trials investigating the use of nitrates in elite female athlete populations would offer an attractive comparative field of study in understanding gender-specific differences in nitrogen metabolism and its ergogenic effects on exercise economy. Besides training status and gender, other modulating factors for circulating nitrate and nitrite concentrations have been suggested to be genetic or relate to the participant's muscle oxygenation status, muscle fiber composition and mitochondrial function (Wylie *et al.*, 2013). Some studies have also indicated that skeletal muscle possesses the unique ability to act as a reservoir for NO precursors, thereby inferring that the effects surrounding nitrate supplementation may last for more than 24 hours after the last dosage (Kramer *et al.*, 2016). This characteristic of skeletal muscle to sequester circulating NO may be relevant in adapting future modes of nitrate delivery whilst elucidating the advantages and disadvantages of chronic and acute nitrate supplementation strategies.

2.3.3 Phytonutrients and vitamins

In terms of dietary controls, the study data collected suggested that nitrate uptake and the resulting increase in circulating levels of bioavailable NO may be augmented by the presence of specific naturally occurring phytonutrients and vitamins such as polyphenols, betacyanin and vitamin C (Lansley *et al.*, 2011; Flueck *et al.*, 2015; Cuenca *et al.*, 2018). It is postulated that the presence of these micro- and phytonutrients may help to facilitate the proliferation of nitrous acid through the duodenal epithelial cell wall, after which it quickly disassociates and thereby promotes an increase circulating NO2 (Lansley *et al.*, 2011; Flueck *et al.*, 2015; Nnate

and Ngozi, 2016; Mcmahon, Leveritt and Pavey, 2017). More studies investigating the role of various other dietary constituents as modulating factors in duodenal absorption of nitrate-based substrates may help to further elucidate the underlying mechanisms as well as allowing for evidence-based augmentations and recommendations to be devised for athletes, to maximize the ergogenic potential of nitrate supplementation. In terms of study design, many of the trials did not include any restrictions of dietary nitrates, with the result that the dietary habits of participants with a high consumption of naturally occurring nitrates may have acted as confounding variable in studies and further served to have reduced trial significance (Bescos *et al.*, 2011; Lansley *et al.*, 2011). This compounded with a relatively small sample sizes endemic to these population groups alongside the relatively high heterogeneity observed between participants may have served to obscure possibly meaningful outcomes by decreasing their statistical significance. Providing participants with a dietary list of nitrate-containing foods prior to the trial as well as preparation of standardized meals as was the case in studies conducted by some studies, constitutes both an elegant and practical way to reduce diet-related confounding variables (Cermak *et al.*, 2012; Callahan *et al.*, 2017).

2.3.4 Gut microbiome

Recently a great deal of research and interest has been devoted to the field of the human microbiome, specifically in relation to the pathologies of non-contagious chronic disease such as atherosclerosis, pulmonary hypertension and cardiovascular heart disease in conjunction with their role as facilitators of the nitrate-nitrite-NO pathway (NNN-pathway) and the corresponding vasodilatory effects (Koch *et al.*, 2016). As has been shown in previous research, the enterosalivary circulation plays a central role as a nitrate reservoir, in sequestering approximately 25% of the body's nitrate store within the salivary glands (Cuenca *et al.*, 2018). Intensive study to date has revealed the presence of over 50 – 100 billion bacteria consisting of over 700 bacterial species within the oral cavity, which build a broad array of micro-ecologies within various regions in the mouth (Koch *et al.*, 2016). It is these facultative anaerobic bacterial cultures that rely on naturally occurring dietary nitrates as a fuel source within the nitrogen cycle and convert the physiologically inert nitrate molecules via several steps involving nitrate denitrification via the NNN-pathway into the highly reactive NO (Koch *et al.*, 2016). The current body of evidence suggests that the reduction of physiologically inert nitrate to nitrite via molybdenum-dependent nitrate reductases exists as the initial and primary rate regulating step within the realms of anaerobic nitrogen metabolism (Koch *et al.*, 2016; Nnate and Ngozi,

2016). Hence, a compromised gut microbiome has been linked to a multitude of cardio-pulmonary diseases, such as those listed above, due to the inability of the human organism to naturally metabolize nitrate, thus setting a broad number of pathologies into motion due to these absence of the vasodilatory effects exerted by nitric oxide (Koch *et al.*, 2016). Equally, these micro-organisms may also play a role within performance-related outcomes by acting as modulating factors governing the scale of duodenal nitrite uptake. Therefore the reported heterogeneity in NO2 uptake may partially be explained by the presence of vastly different bacterial micro-ecosystems within the participant's oral-cavity, which act as the initial and primary rate-limiting step in duodenal nitric acid uptake via the NNN-pathway (Koch *et al.*, 2016). This phenomenon was reported in several RCT studies (Boorsma, Whitfield and Spriet, 2014; Hoon *et al.*, 2014; Flueck *et al.*, 2015; Shannon *et al.*, 2017). Generally, most study designs to date include the recommendation for athletes to refrain from the use of chewing gum and antibacterial mouth, which are shown to impede the growth of nitrate-reducing bacterial colonies (Lansley *et al.*, 2011; Muggeridge *et al.*, 2014; MacLeod *et al.*, 2015; Mcmahon, Leveritt and Pavey, 2017). However, none of the studies included within the current meta-analysis introduced pre-trial testing for baseline levels of nitrate-reducing bacterial colonies and may have therefore missed an important rate modulating factor in understanding the underlying nitrogen metabolism that formed the basis of their intervention. As studies documenting the taxonomy of bacterial cultures within the human microbiome, specifically in the more accessible oral cavity, are becoming more readily available, future research into the ergogenic nature of nitrate supplementation should devote more attention to both homogenizing bacterial cultural baselines between participants as well as comparing heterogeneous groups to one another to further future understanding of the fine interplay between commensal bacterial colonies and maximal efficacy of nitrate-based intervention strategies (Koch *et al.*, 2016).

2.3.5 Discussing decreases in VO_2 Uptake

A statistical meta-analysis of the interventions revealed a significant small to moderate treatment effect on VO2 Uptake. Researchers commonly use VO2 Uptake as a measure for metabolic rate as determined by indirect calorimetry to reflect whole-body oxidative metabolism (Pawlak-Chaouch *et al.*, 2016). Nitrate supplementation has been shown through numerous studies to significantly reduce VO_2 Uptake by increasing oxygen efficiency through methods described within the background sections of this review (Hoon *et al.*, 2013;

Domínguez *et al.*, 2017; Mcmahon, Leveritt and Pavey, 2017). The findings of the current meta-analysis are therefore consistent with the findings of other reviews, indicating that nitrate supplementation is very likely to significantly lowered VO_2 uptake during submaximal and maximal bouts of endurance trials (Pawlak-Chaouch *et al.*, 2016; Mcmahon, Leveritt and Pavey, 2017). VO_2 Uptake and by proxy metabolic rate can be seen as an important parameter for determining the overall exercise economy, either by lowering the amount of energy required to conduct the same amount of work or by increasing the workload for the same amount of energy (Pawlak-Chaouch *et al.*, 2016). The results of the current study further provide evidence for the ergogenic effects of nitrate-based interventions on exercise economy.

2.3.6 Time Trials

Pooled data collected from studies within the current meta-analysis; as well as from other systematic reviews gathered; suggest that nitrate supplementation does not significantly improve time trial performance in endurance athletes, however, there is a trend to trivially reduce TT completion time (Hoon *et al.*, 2013; Mcmahon, Leveritt and Pavey, 2017). Effectiveness in TT performance is of importance due to the high ecological validity that time trials possess in their recreation of real-world competitive scenarios that athletes are faced with (Lansley *et al.*, 2011). Hence, it would seem that time trial would give a realistic impression of nitrate supplementation's efficacy as an ergogenic aid. Experimental studies indicate that recreational athletes enjoy an increased benefit from nitrate supplementation compared to their trained compatriots (Bescos *et al.*, 2011; Boorsma, Whitfield and Spriet, 2014), which may come about due to training-induced changes in capillary density within skeletal muscle that inhibits the proliferation of hypoxic, acidic environments, that have been hypothesized to be required for activation of the NNN-pathway and inhibition of the normoxic L-Arginine-eNOS pathways (Bescos *et al.*, 2011; Nnate and Ngozi, 2016; Garnacho-Castaño *et al.*, 2018). Should the above hypothesis ring true then recreational athletes would be far more susceptible to the ergogenic effects of nitrate interventions due to increased levels of training-induced acidity and hypoxia associated with bouts of sport within untrained populations, thus favoring the NNN-pathway, which is dependent on exogenous nitrate substrates. However, intermuscular angiogenesis alone cannot explain why trained athletes who possess the relatively similar VO_2 max values react differently to nitrate treatment, as was seen in 1500 m and 10 km time trials conducted with elite track athletes with a mean VO_2 max of 80 mL/kg.min (Boorsma, Whitfield and Spriet, 2014). Upon evaluation of study results, two subgroups were identified as

responders and non-responders (Boorsma, Whitfield and Spriet, 2014). Responders and non-responders have been reported by several other studies suggesting that other factors such as muscle fiber type and composition may have a crucial role in dictating levels of efficacy of nitrate supplementation (Bescos *et al.*, 2011; Boorsma, Whitfield and Spriet, 2014; Flueck *et al.*, 2015; Callahan *et al.*, 2017). In animal studies, type II muscle fibers, which have a higher capacity for anaerobic metabolism, have been illustrated to possess a higher sensitivity to the presence of nitric oxide, by comparison, to type I muscle fibers (Hernández *et al.*, 2012). In the light of current studies, it seems possible that type II muscle fibers may react most acutely to the presence of NO and its precursors, given their high concentration of mitochondria and concurrent predisposition to favor anaerobic metabolism in ATP-synthesis (Hernández *et al.*, 2012). By increasing microvascular perfusion into skeletal muscle, reducing excessive oxygen usage within the ETC through cytochrome C inhibition and improving intermuscular calcium pump efficiency it seems plausible that type II muscle fibers may benefit the most from the presence of NO and its precursors, by comparison, to type I fibers, which show a preference for aerobic metabolism and the corresponding L-Arginine- NOS pathway (Bescos *et al.*, 2011; Hernández *et al.*, 2012; Nnate and Ngozi, 2016; Callahan *et al.*, 2017). During bouts of submaximal endurance exercise, oxygen efficiency decreases as part of the VO_2 slow component, which may be attributed to the gradual recruitment of less efficient type II muscle fibers (Ghiarone *et al.*, 2017). This could also help in elucidating the differences between responders and non-responders, which may be partly explained due to a difference in participant muscle fiber composition. Translated into practice this would mean participants with higher levels of type II muscle fibers may enjoy greater levels of benefits from nitrate consumption. Additionally, this may also partly explain why trained endurance athletes enjoy less benefits as a result of nitrate supplementation, which may be attributed to higher levels of type I muscle fiber recruitment to type II muscle fibers for long-distance events combined with associated training-induced increases in levels of eNOS activity, both of which diminish the importance of the NNN-pathway and by proxy nitrate treatment efficacy (Garnacho-Castaño *et al.*, 2018). The results of a study conducted with participants who received acute nitrate treatment and demonstrated a significant increase in power output during the first 15 seconds of a 30s all-out Windgate sprint, however not in the second 15-second division (Cuenca *et al.*, 2018). This may indicate that nitrate supplementation affects anaerobic metabolism function by primarily acting on type II muscle fibers via the same mechanisms

described above and resulting in increased power output during the first 15 seconds (Cuenca *et al.*, 2018). Hence studies investigating the relationship between athlete muscle fiber composition and nitrate metabolism would be of particular interest in future research to fully elucidate if the efficacy of nitrate supplementation on "responders" vs "non-responders". To these ends, non-invasive methods of determining muscle fiber composition that avoid the use of invasive muscle biopsies such as those which test muscle carnosine content may play an invaluable part within future research in this area (Baguet *et al.*, 2011).

2.3.7 Time to exhaustion

During bibliographic searching, only two trials were found that included time to exhaustion as their primary outcome and met inclusion criteria, both of which reported a significant improvement in time to exhaustion within trained athlete populations (Bescos *et al.*, 2012; Balsalobre-Fernández *et al.*, 2018). These results are in keeping with the findings of other systematic reviews, which documented a trivial to small improvements to TTE in recreational and trained athletes (Hoon et al., 2013; Mcmahon, Leveritt and Pavey, 2017). The improvement in TTE performance may be attributed to an overall reduction of oxygen cost via mechanisms described above whilst maintaining an equal amount of work, hence resulting in an improvement in exercise economy that would tend to favor protocols testing for exercise capacity rather than performance (Mcmahon, Leveritt and Pavey, 2017). Such factors like the reduced oxygen cost of ATP synthesis via the prevention of hydrogen slippage at complex V and the NO-induced inactivation of cytochrome C at the third complex of the electron transport chain (Koch *et al.*, 2016; Nnate and Ngozi, 2016; Mcmahon, Leveritt and Pavey, 2017), combined with the increased intramuscular blood perfusion exercised due to the vasodilatory effects of bioactive nitric oxide on endothelial smooth muscle cells may all help to increase exercise capacity by reducing oxygen cost during sub-maximal and maximal bouts of exercise.

This may be due to inherent qualities that are associated with sports research within trained and athlete populations, i.e.: population sizes of elite athletes are significantly smaller than those of recreational athlete populations as well as the fact that improvements enhancements within these groups tend to be smaller and more difficult to detect alongside inter-participant differences such as those noted within responders and non-responders studies. These factors may act together and contribute to reducing the power of significance. Hence more studies that minimize inter-participant baseline differences in terms of physical characteristics such as

VO_2 max, muscle fiber composition, bacterial colony profile and training background may help to improve trial sensitivity in future studies. Moreover, systematic standardization of testing protocols may help to reduce the differences between study protocols and help to increase the power of significance in future meta-analysis.

2.3.8 Study limitations

Due to the nature and limited scope of this meta-analysis, only one reviewer was employed during bibliographic searching and selection of studies, which were chosen based on the aforementioned exclusion criteria. Hence the current meta-analysis may be subject to author bias and would require a second reviewer to improve the reliability of its results. To diminish the risk of author bias this meta-analysis was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (JPT *et al.*, 2019) and bibliographic results were administered with the help of commercially available software – Cochrane's literature manager. A second limitation was that only athletes with a VO2 max of 51,9 mL/kg.min were taken into account within the current review, thereby removing the possibility of comparing the ergogenic effects of nitrate intervention strategies between trained and untrained populations. This may offer an interesting field for future study.

3. Summary

In summary, the findings introduced in this systematic review, the chronic and acute use of nitrate supplementation significantly raised percentile circulating nitrate and nitrite serum levels and increasing TTE while reducing VO_2 uptake within a trained population with a recorded VO_2 max above 51.9 mL/min. Despite decreases VO_2 uptake, nitrate supplementation did not significantly increase TT performance, however, a general trend to increase TT performance in each of the three trial protocols was noted. Thus the findings of the current meta-analysis are in accord with other systematic reviews to date, further underscoring the likely to very likely ergogenic effects that exogenous nitrate supplementation exercises on trained athlete population (Hoon *et al.*, 2013; Domínguez *et al.*, 2017; Mcmahon, Leveritt and Pavey, 2017). Further study is required to fully elucidate inter-study differences in outcomes relating to nitrate substrate uptake, TTE performance and TT as well to adapt effective guidelines for nitrate supplementation.

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5. Affidavit

Statement of Authorship I hereby declare that I am the sole author of this bachelor thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution in order to obtain a degree.

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(Place, Date) (Signature)