

The Effect of Nitric-Oxide-Related Supplements on Human Performance

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Contents

Abstract	1
1. Introduction	2
2. Synthesis of Nitric Oxide (NO) from the NO Synthase-Dependent Pathway	3
2.1 L-Arginine: Sources and Metabolism	3
2.1.1 Ergogenic Effect of L-Arginine Supplements Alone	4
2.1.2 Ergogenic Effect of L-Arginine Supplements in Combination with Other Components	5
2.2 L-Citrulline: Sources and Metabolism	7
2.2.1 Ergogenic Effect of L-Citrulline Supplements Alone	9
2.2.2 Ergogenic Effect of L-Citrulline Supplements with Malate	9
3. Synthesis of NO from the NOS-Independent Pathway	10
3.1 Nitrate and Nitrite: Sources and Metabolism	10
3.1.1 Ergogenic Effect of Sodium Nitrate Supplementation	11
3.1.2 Ergogenic Effect of Nitrate Supplementation in the Form of Beetroot Juice	11
4. Other Components Related to NO Synthesis	13
4.1 Glycine Propionyl-L-Carnitine (GPLC)	13
4.1.1 Ergogenic Effect of GPLC	13
4.2 2-(Nitrooxy) Ethyl 2-Amino-3-Methylbutanoate	14
5. Side Effects of NO Supplements	14
6. Conclusion and Future Perspectives	14

Abstract

Nitric oxide (NO) has led a revolution in physiology and pharmacology research during the last two decades. This labile molecule plays an important role in many functions in the body regulating vasodilatation, blood flow, mitochondrial respiration and platelet function. Currently, it is known that NO synthesis occurs via at least two physiological pathways: NO synthase (NOS) dependent and NOS independent. In the former, L-arginine is the main precursor. It is widely recognized that this amino acid is oxidized to NO by the action of the NOS enzymes. Additionally, L-citrulline has been indicated to be a secondary NO donor in the NOS-dependent pathway, since it can be converted to L-arginine. Nitrate and nitrite are the main substrates to produce NO via the NOS-independent pathway. These anions can be reduced *in vivo* to NO and other bioactive nitrogen oxides. Other molecules, such as

the dietary supplement glycine propionyl-L-carnitine (GPLC), have also been suggested to increase levels of NO, although the physiological mechanisms remain to be elucidated.

The interest in all these molecules has increased in many fields of research. In relation with exercise physiology, it has been suggested that an increase in NO production may enhance oxygen and nutrient delivery to active muscles, thus improving tolerance to physical exercise and recovery mechanisms. Several studies using NO donors have assessed this hypothesis in a healthy, trained population. However, the conclusions from these studies showed several discrepancies. While some reported that dietary supplementation with NO donors induced benefits in exercise performance, others did not find any positive effect. In this regard, training status of the subjects seems to be an important factor linked to the ergogenic effect of NO supplementation. Studies involving untrained or moderately trained healthy subjects showed that NO donors could improve tolerance to aerobic and anaerobic exercise. However, when highly trained subjects were supplemented, no positive effect on performance was indicated. In addition, all this evidence is mainly based on a young male population. Further research in elderly and female subjects is needed to determine whether NO supplements can induce benefit in exercise capacity when the NO metabolism is impaired by age and/or estrogen status.

1. Introduction

Nitric oxide (NO) is a labile lipid soluble gas synthesized at several locations in the body. The endogenous formation and biological significance of NO were revealed in a series of studies in the 1980s and for these seminal discoveries, three American researchers were subsequently awarded the Nobel Prize in Physiology or Medicine in 1998. Soon after the identification of NO as a signalling molecule in mammals, it was reported that specific nitric oxide synthase (NOS) enzymes catalyze a complex enzymatic reaction leading to NO formation from the substrates L-arginine and molecular oxygen.^[1] Later, an alternative NOS-independent pathway of NO synthesis was discovered, based on the simple reduction of nitrate and nitrite,^[2,3] the main oxidation products of NO. During this period, interest in the biological role of NO has led a revolution in pharmacological and physiological research. Currently, NO is known to regulate important functions as a mediator in noradrenergic and non-cholinergic neurotransmission; in learning and memory, synaptic plasticity; and neuroprotection.^[4]

In exercise physiology, NO has also received much interest, and supplements of NO are thought to be an ergogenic aid.^[5] This fact is based on the evidence that NO is an important modulator of blood flow and mitochondrial respiration during physical exercise.^[6] In addition, it is suggested that the increase of blood flow derived from NO synthesis may improve recovery processes of the activated tissues.^[7] These supposed benefits are claimed in most sport supplements, which are currently sold in the market and linked with stimulation of NO production. However, a careful examination of the composition of NO-stimulating supplements shows that, in many cases, they are 'cocktails' of a great variety of ingredients such as creatine, carbohydrates, amino acids, vitamins, minerals, etc. It is known that some of these components (creatine, carbohydrates and amino acids) may have an ergogenic effect in themselves.^[8-10] In addition, the scientific evidence behind these 'cocktails' of supplements related with NO stimulation is very scarce. Only one study has evaluated the effect of some of these products, indicating that their effectiveness at increasing NO and/or improving

performance is very limited.^[11] In comparison with data reported in scientific studies, it has been suggested that the amounts of NO ingredients (mainly L-arginine and L-citrulline) that contain commercial NO-stimulating supplements are extremely low and ineffective to induce changes in NO.^[11]

For this reason, most studies involving NO donors have used pharmaceutical products to assess the effect on human performance.^[12-18] Furthermore, there are some recent studies that have also assessed the effect of natural foods rich in NO donors, such as beetroot juice.^[19-23] Results from these studies show great controversy. Some of them showed that dietary NO supplements may enhance human performance in healthy subjects,^[19,24,25] but others did not find any positive effect.^[16,18,26] One reason to explain this fact could be the large methodological differences between studies: duration of treatment, exercise protocol and training status differ significantly between studies, making a comparison between them difficult. Additionally, many studies have used NO donors in combination with other components such as malate, glutamate, aspartate, etc., in an attempt to increase the bioavailability of NO donors. This fact adds more difficulty because some of these additional products may participate in the independent NO-synthesis pathways in the body.

Accordingly, this review focuses on pathways and donors of NO synthesis and elucidates the effect of NO supplements on human performance. Scientific articles were retrieved based on an extensive search in MEDLINE (1980–2011) and Google Scholar (1990–2011) databases. Computer search engines used the following combined keywords: 'L-arginine', 'L-citrulline', 'nitrate', 'glycine-propionyl-L-carnitine', 'supplementation', 'nitric oxide', 'exercise', 'performance'. After using these initial keywords, the search engines were limited to human studies excluding research with animals, as well as in humans in pathological states. As a result, 42 articles related to the effects of dietary ingredients linked with NO and performance in response to exercise, were considered. References cited in the retrieved articles were also considered in this review.

2. Synthesis of Nitric Oxide (NO) from the NO Synthase-Dependent Pathway

L-arginine amino acid participates in the NOS-dependent pathway in a reaction catalyzed by specific NOS enzymes^[4] (figure 1). Additionally, it has been suggested that L-citrulline could be an alternative donor of NO, due to the fact that it can increase the levels of L-arginine.

2.1 L-Arginine: Sources and Metabolism

L-arginine is considered a conditional essential proteinogenic amino acid that is a natural constituent of dietary proteins. L-arginine is relatively high in seafood, watermelon juice, nuts, seeds, algae, meat, rice protein concentrate and soy protein isolate.^[27] The typical dietary intake of L-arginine is approximately 4–5 g per day. Furthermore, L-arginine could be endogenously synthesized, mainly in the kidney, where L-arginine is formed from L-citrulline.^[28] The liver is also able to synthesize considerable amounts of L-arginine, although this is completely reutilized in the urea cycle.^[28] Normal plasma L-arginine concentrations depend upon the age of the individual and homeostasis is primarily achieved via its catabolism.^[29] The usual mean \pm standard deviation range of plasma L-arginine in humans has been determined between 70 and 115 $\mu\text{mol} \cdot \text{L}^{-1}$.^[30] Extracellular L-arginine can be quickly taken up by endothelial cells; in the presence of molecular oxygen and nicotinamide adenosine dinucleotide phosphate, L-arginine is subsequently oxidized to NO.^[1,4] This is a complex reaction, which is catalyzed by NOS enzymes that contain a binding site for L-arginine. There are three isoforms of NOS that have been recognized: type I (neuronal NOS; nNOS), type II (inducible NOS; iNOS) and type III (endothelial NOS; eNOS). eNOS and iNOS are constitutive enzymes that are controlled by intracellular Ca^{2+} /calmodulin. nNOS is inducible at the level of gene transcription, Ca^{2+} independent and expressed by muscle activity^[31] the aging process,^[32] as well as by macrophages and other tissues in response to inflammatory mediators.^[33]

L-arginine participates in other metabolic pathways independent of NO synthesis. For in-

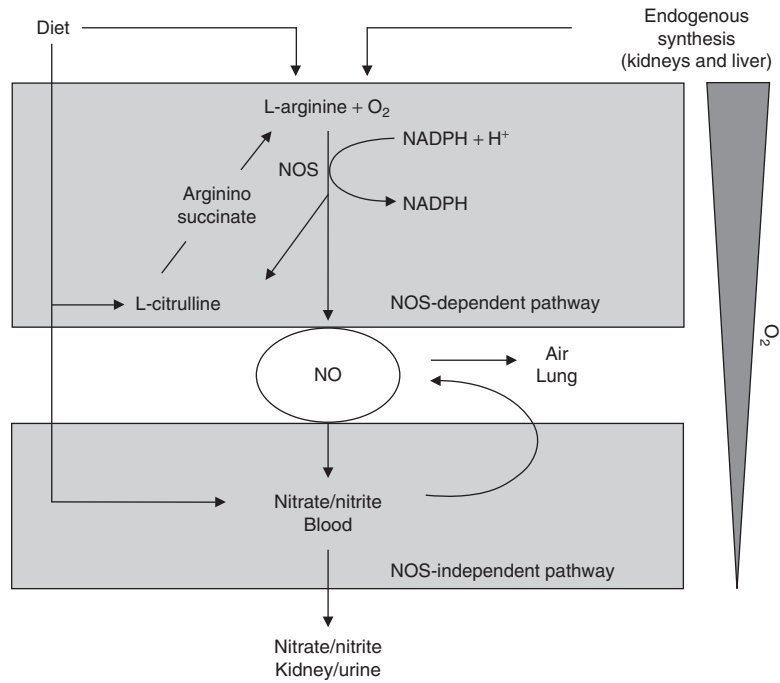


Fig. 1. Metabolic pathways of NO synthesis in humans. **NADPH** = nicotinamide adenine dinucleotide phosphate-oxidase; **NO** = nitric oxide; **NOS** = NO synthases.

stance, L-arginine is essential for the normal function of the urea cycle, in which ammonia is detoxified through its metabolism into urea.^[34] L-arginine is also a potent hormone secretagogue. L-arginine infusion at rest increases plasma insulin, glucagon, growth hormone (GH), prolactin and catecholamines concentrations.^[35] Such hormonal changes affect the metabolism. For instance, insulin and GH are important anabolic hormones with a remarkable degree of synergy in regulating glucose and fat metabolism. While insulin facilitates glucose entry into cells and an increase in glycogen stores, GH stimulates lipolysis and reduces glucose oxidation to maintain blood glucose levels.^[36] Thus, it has been suggested that GH and insulin release may enhance exercise performance by increasing fatty acid oxidation and sparing glycogen stores.^[36] In addition, GH also causes the release of insulin-like growth factor (IGF)-1 that increases amino acid uptake and protein synthesis.^[37] These ef-

fects could also improve performance through increased muscle mass and strength.^[37]

2.1.1 Ergogenic Effect of L-Arginine Supplements Alone

Seven studies have analyzed the effect of L-arginine supplementation alone.^[12-18] Two of these studies were carried out in healthy, but not well trained, males^[12,14] and one in healthy, postmenopausal women.^[13] In this study, females were supplemented with high doses of L-arginine ($14.2 \text{ g} \cdot \text{day}^{-1}$) for 6 months. After this period, a significant increase in the maximal power in relation with body mass ($\text{power} \cdot \text{kg}^{-1}$) measured as peak jump power (counter-movement jump) was found.^[13] In male studies, it has been indicated that L-arginine supplementation could enhance the respiratory response. Koppo et al.^[12] showed a significant increase in speed in phase II of pulmonary oxygen consumption ($\dot{V}\text{O}_2$) at the onset of moderate intensity endurance cycle exercise after 14 days'

L-arginine supplementation ($6\text{ g}\cdot\text{day}^{-1}$). Faster $\dot{V}\text{O}_2$ kinetics of phase II reduces the O_2 deficit that follows the onset of exercise and can reduce intracellular perturbation (e.g. increased lactic acid, decreased phosphocreatine).^[38] This fact could be interesting in order to enhance tolerance to endurance exercise, mainly in subjects with slow $\dot{V}\text{O}_2$ kinetics (time it takes to reach 63% of steady state $[\tau] > 30$ seconds). However, all these findings were not linked with NO synthesis since the above studies did not report data related to NO markers, such as plasma ratio of L-arginine: L-citrulline and/or plasma levels of nitrate and nitrite. On the other hand, Olek et al.^[14] assessed the effect of an acute dose of L-arginine in a low dose (2 g) 60 minutes before exercise. They showed that this amount of L-arginine did not induce any increase in the total work performed or mean power output during Wingate cycle tests (30 seconds), or $\dot{V}\text{O}_2$ either.^[14] Additionally, plasma levels of nitrate/nitrite were unchanged after L-arginine supplementation compared with placebo.

The remaining studies were performed in well trained athletes using different types of athletic populations such as judo athletes,^[17,18] tennis players^[16] and cyclists.^[15] Despite analyzing supplements during different durations (between 1 and 28 days) and doses (between 6 and 12 g), no benefit was indicated in parameters linked with performance, such as power in a cycle ergometer test^[18] or $\dot{V}\text{O}_2$ during a treadmill test.^[15,16] Additionally, the levels of some exercise metabolites (lactate and ammonia) were unchanged after L-arginine supplementation compared with placebo.^[17,18] Moreover, three of these studies analyzed the level of plasma nitrate/nitrite as NO markers showing that they did not increase after dietary L-arginine ingestion.^[16-18] The other study did not include data regarding NO metabolites.^[15]

Apart from dietary supplementation, other studies have analyzed the effect of intravenous infusion of L-arginine in an attempt to increase its bioavailability,^[39,40] since dietary L-arginine bioavailability is only about 60%. This fact is due to the high activity of arginases in the liver.^[41] Arginases are enzymes that participate in the fifth and final step of the urea cycle, competing with NOS for L-arginine.^[42] In athletes, there is evidence that

exhaustive exercise increases arginase activity in lymphocytes nearly 6-fold, limiting L-arginine availability for lymphocyte iNOS activity.^[43] However, despite the fact that the bioavailability of intravenous infusion of L-arginine could be high compared with dietary consumption, no positive effect in parameters of performance, such as maximal workload during an incremental cycle ergometer test or the amount of work completed in a 15-minute test after intravenous L-arginine infusion, has been reported.^[39,40]

2.1.2 Ergogenic Effect of L-Arginine Supplements in Combination with Other Components

There are several studies that have shown an increase in exercise performance after L-arginine supplementation in combination with other components in untrained or moderately trained subjects. For instance, a recent study of Bailey et al.^[24] showed that L-arginine ($6\text{ g}\times 3$ days) in combination with other amino acids and vitamins induced a decrease in $\dot{V}\text{O}_2$ (L-arginine: $1.48\pm 0.12\text{ L}\cdot\text{min}^{-1}$; placebo: $1.59\pm 0.14\text{ L}\cdot\text{min}^{-1}$; $p < 0.05$) in a low to moderate bout of exercise (6 minutes at $82\pm 14\text{ W}$); and an increase in time to exhaustion (L-arginine: 707 ± 232 seconds; placebo: 562 ± 145 seconds; $p < 0.05$) during an incremental cycling test.^[21] In another recent study, Camic et al.^[25] found an increase in power output (5.4%) during an incremental test to exhaustion (cycle ergometer) when L-arginine (3 g) in combination with grape seed extract was administered for 28 days. Similarly, in elderly males, Chen et al.^[44] found that supplementation of L-arginine ($5.2\text{ g}\cdot\text{day}^{-1}\times 21$ days) with L-citrulline and antioxidants increased power output (~21%) during an incremental cycle ergometer test until exhaustion. These surprising findings have been related to an increase in gas exchange threshold after L-arginine supplementation.^[44,45] It has been suggested that the attenuation of metabolic products such as potassium, ammonia and lactate, may be the result of increased clearance from the circulation related to NO synthesis and increased blood flow.^[45] However, it is only speculation, since there is evidence indicating that higher doses of dietary L-arginine ($> 10\text{ g}$) are ineffective to increase blood flow in healthy humans.^[46,47]

Other studies have also reported benefits of a mixture of L-arginine supplements in strength and power performance in moderately trained subjects. Campbell et al.^[48] indicated a significant increase of one-maximum repetition (1-RM) of bench press, as well as peak power during a 30-second Wingate test after L-arginine supplementation ($6 \text{ g} \cdot \text{day}^{-1} \times 56 \text{ days}$) in combination with α -ketoglutarate. Furthermore, Buford et al.^[4] and Koch^[49] and Stevens et al.^[50] showed that an acute dose of L-arginine (6 g of L-arginine) in the form of α -ketoisocaproic increased the mean power performed during Wingate tests (10 seconds) and work sustained during continuous isokinetic concentric/eccentric knee extension repetitions, respectively.

In well trained athletes, two studies have assessed dietary L-arginine supplementation in combination with aspartate. In the first, Colombani et al.^[51] supplemented ($15 \text{ g} \cdot \text{day}^{-1} \times 14 \text{ days}$) endurance-trained runners. They showed that the plasma level of somatotrophic hormone (STH), glucagon, urea and arginine were significantly increased, and the level of plasma amino acids was significantly reduced after a marathon run following L-arginine supplementation. The conclusion of this study was that there was no metabolic or performance benefit derived from L-arginine. More recently, similar findings were reported by Abel et al.^[52] They supplemented endurance-trained cyclists with L-arginine and aspartate at high (5.7 g of L-arginine; 8.7 g of aspartate) and low (2.8 g of L-arginine; 2.2 g of aspartate) doses for 28 days. After an incremental endurance exercise test (cycle ergometer) in laboratory conditions, no modification was found in endurance performance ($\dot{V}O_2$ peak [$\dot{V}O_{2\text{peak}}$], time to exhaustion), or in endocrine (concentration of growth hormone, glucagon, cortisol and testosterone) or in metabolic (concentration of lactate, ferritin and urea) parameters.^[52]

Therefore, including all studies with L-arginine supplementation alone and with other components (tables I and II), no study in well trained athletes reported benefits in human performance.^[15-18,51,52] One important factor that may explain the reduced effect of L-arginine in well trained athletes, could be explained by the physiological and metabolic adaptation derived from chronic physical training. The effect of exercise

training on the enhancement of endothelial function has been well established.^[69] Repetitive exercise over weeks results in an upregulation of endothelial NO activity. This is not a localized but rather a systemic response in endothelial function when large muscle mass is regularly activated, as in aerobic exercise.^[70] Perhaps benefits in pulmonary, cardiovascular and neuromuscular systems induced by long-term training may overcome any potential effects of dietary L-arginine supplementation in well trained athletes. However, there are other factors that may also reduce the effect of dietary L-arginine, such as the L-arginine:lysine ratio. The amino acid lysine competes with L-arginine for entry into cells and also inhibits arginase activity.^[71] Under normal feeding conditions, the total amount of L-arginine in the diet should not be more than 150% greater than that of lysine (namely, L-arginine:lysine <2.5).^[72]

In addition, in most of the above mentioned studies, there is a lack of data concerning NO metabolites. Only Bailey et al.^[24] analyzed the plasma levels of nitrite, reporting a significant increase after L-arginine supplementation. However, only one study^[24] states that there is too little scientific evidence to corroborate that dietary L-arginine supplementation increases NO synthesis in healthy humans. Some of the benefits shown in the previous studies could be related to other metabolic pathways independent of NO synthesis, as well as to the other ingredients included in L-arginine supplements. For example, there is evidence that L-arginine supplementation in combination with glutamate and aspartate is effective at reducing blood levels of ammonia,^[55,56] as well as blood lactate.^[40,57] during exercise. This response could explain the results reported by the aforementioned studies of Camic et al.^[45] and Stevens et al.^[50] Moreover, L-arginine is known to actively participate in the synthesis of creatine.^[73] Diets supplemented with L-arginine increase intramuscular creatine phosphate concentrations between 1% and 2% in laboratory animals; thus, this may enhance the response to anaerobic exercise.^[74] This finding may be a suitable response to the study of Buford et al. and Koch^[49] who indicated that a supplement of

Table 1. Studies with nitric oxide supplements that reported an increase in performance

Substance	Dose per day	Duration (days)	Other components	Design	Sample size	Training status	Effects	References
L-arg	14.2 g	180		DB, R	23	U	↑ Maximal power	13
L-arg	6.0 g	3	Vitamins and amino acids	DB, CO	9	M	↑ Efficiency and time to exhaustion	24
L-arg	1.5 g	28	Grape seed extract	DB, R	50	U	↑ Work capacity	25
L-arg	1.5 g	28	Grape seed extract	DB, R	41	U	↑ Increase of gas exchange threshold and power output	45
L-arg	5.2 g	21	L-citrulline and antioxidants	DB, R	16	M	↑ Power output	44
L-arg	6.0 g	56	α-ketoglutarate	DB, R	35	M	↑ Increase of 1-RM	48
L-arg	6.0 g	1	Glycine α-ketoisocaproic	DB, R	19	M	↑ Power performance	49
L-arg	6.0 g	1	Glycine α-ketoisocaproic	DB, R, CO	13	U	↑ Work sustained during anaerobic exercise	50
L-citr	8.0 g	1	Malate	DB, R, CO	41	M	↑ Work capacity	53
Nitrate	5.5 mmol	6	Beetroot juice	DB, R, CO	8	M	↑ Efficiency and time to exhaustion	19
Nitrate	5.1 mmol	6	Beetroot juice	DB, R, CO	7	M	↑ Increase time-to-task failure	20
Nitrate	6.2 mmol	6	Beetroot juice	DB, R, CO	9	M	↑ Efficiency and time to exhaustion	22
Nitrate	6.2 mmol	1	Beetroot juice	DB, R, CO	9	M	↑ Power output	23
Nitrate	5.2 mmol	15	Beetroot juice	DB, R, CO	8	U	↑ Increase efficiency and peak power	21
GPLC	4.5 g	1		DB, R, CO	24	M	↑ Peak power and reduced power decrement	54

1-RM=one-repetition maximum; **CO**=crossover; **DB**=double blind; **GPLC**=glycine propionyl-L-carnitine; **L-arg**=L-arginine; **L-citr**=L-citrulline; **M**=moderately-trained subjects; **R**=randomized; **U**=untrained subjects; ↑ indicates improvement in performance.

glycine-arginine-α-ketoisocaproic acid (GAKIC) enhances performance during repeated bouts of anaerobic cycling performance.

In summary, current evidence of L-arginine supplementation in sports performance suggests that (i) L-arginine, mainly in combination with other components, could induce some benefit in untrained or moderately trained subjects, improving tolerance to aerobic and anaerobic physical exercise. However, as the studies do not show a well defined relationship between dietary L-arginine supplementation and NO synthesis, the benefit in exercise performance shown in some studies could be derived from other ingredients of supplements, as well as other meta-

bolic pathways independent of NO synthesis; and (ii) in well trained athletes, there is a lack of data indicating that L-arginine supplementation induces benefits in performance. A recent review analyzing the potential ergogenic effects of acute and chronic L-arginine supplementation did not reach a clear conclusion as to the benefits in exercise performance either.^[75]

2.2 L-Citrulline: Sources and Metabolism

The organic compound L-citrulline, is a non-essential α-amino acid. Its name is derived from *Citrullus*, the Latin word for watermelon from which it was first isolated in 1930, and which

Table II. Studies with NO supplements that reported no or negative effects on performance

Substance	Dose per day	Duration (days)	Other components	Design	Sample size	Training status	Performance	Other findings	References
L-arg	6.0g	14		DB, CO	7	M	NM	Increase of phase II pulmonary VO ₂	12
L-arg	2.0g	1		DB, R, CO	6	M	None		14
L-arg	12.0g	28		DB, R	18	H	None		15
L-arg	20.5g	3		DB, R	9	H	NM		16
L-arg	≈7.6g	1		R	15	H	NM	Increase of glucose and insulin	17
L-arg	6.0g	3		DB, R	10	H	None		18
L-arg	30.0g ^a	1		DB, R	9	H	None	Increase of glucose	39
L-arg	3.0g ^a	1		DB, R	8	M	None	Lower blood lactate and ammonia	40
L-arg	5.7g	28	L-asp	R	30	H	None		52
L-arg	15.0g	14	L-asp	DB, CO	20	H	NM	Increase of somatotrophic hormone, glucagon and urea	51
L-arg	20.0g	1	L-glut	DB, CO	3	U	NM	Lower ammonia	55
L-arg	5.0g	10	L-asp	DB, R	15	U	NM	Lower ammonia	56
L-arg	3.0g	21	L-asp	DB	16	M	NM	Lower blood lactate and VO ₂	57
L-citr	6.0g	1	Malate	DB, R	17	H	NM	Increased levels of NO metabolites	58
L-citr	9.0g	1		DB	17	M	↓	Decrease of time to exhaustion	59
L-citr	6.0g	15	Malate	?	18	U	NM	Increase ATP production	60
L-citr	6.0g	1	Malate	DB, R	17	H	NM	Increase of plasma nitrite	61
Nitrate	10.0mg•kg ⁻¹	1	SN	DB, R, CO	11	H	None	Reduce VO _{2peak}	26
Nitrate	0.1mmol•kg ⁻¹	3	SN	DB, R, CO	9	M	None	Increase efficiency	62
Nitrate	0.1mmol•kg ⁻¹	2	SN	DB, R, CO	9	M	None	Reduce VO _{2peak}	63
Nitrate	0.1mmol•kg ⁻¹	3	SN	DB, R, CO	14	M	NM	Increase mitochondrial efficiency	64
GPLC	4.5g	1		DB, R, CO	19	M	None	Decrease of malondialdehyde	11
GPLC	3.0g	28		DB, R, CO	15	M	NM	Increased levels of NO metabolites	65
GPLC	1.5–4.5g ^b	56		DB, R	30	U	NM	Increased levels of NO metabolites	66
GPLC	1.5–4.5g ^b	56		DB, R, CO	32	U	None		67
2-ethyl	–	1		R, CO	10	M	NM	No changes in plasma nitrate/nitrite	68

^a Intravenous supplementation.
^b Data is presented in ranges where stated.
2-ethyl = 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate; **ATP** = adenosine triphosphate; **CO** = crossover; **DB** = double blind; **GPLC** = glycine propionyl-L-carnitine; **H** = highly trained subjects; **L-arg** = L-arginine; **L-asp** = L-aspartate; **L-citr** = L-citrulline; **L-glut** = L-glutamate; **M** = moderately trained subjects; **NM** = none measured; **R** = randomized; **SN** = sodium nitrate; **U** = untrained subjects; **VO₂** = oxygen consumption; **VO_{2peak}** = peak VO₂; ↓ indicates decrease in performance; – indicates no supplement composition shown therefore amount not shown.

is the main dietary source of this amino acid.^[76] L-citrulline is also produced endogenously via the following two main pathways: (i) it is synthesized from glutamine in enterocytes, by condensation of ornithine and carbamyl phosphate in a reaction catalyzed by ornithine carbamyl-transferase,^[77,78] and (ii) L-citrulline is produced via the conversion of L-arginine to NO in a reaction catalyzed by NOS enzymes (figure 1). The normal value of L-citrulline reported in healthy populations is approximately $25 \mu\text{mol} \cdot \text{L}^{-1}$,^[79] although lower values have recently been found ($10\text{--}15 \mu\text{mol} \cdot \text{L}^{-1}$) in professional cyclists.^[58]

The dietary interest for this amino acid has substantially increased in the last decade as a result of the importance of L-citrulline as a precursor of L-arginine.^[80,81] It is interesting, because, unlike L-arginine, it bypasses the hepatic metabolism and is not a substrate of arginase enzymes. For this reason, it has been indicated that systemic administration of L-citrulline could be a more efficient way to elevate extracellular levels of L-arginine by itself.^[82] Dietary L-citrulline is taken up and released by enterocytes in the portal circulation, bypasses metabolism by periportal hepatocytes and is transported to the kidneys where around 80% is catabolized to L-arginine by cells of the proximal tubules.^[83] Apart from the function as a precursor of L-arginine, it is known that L-citrulline is an essential component participating in the urea cycle in the liver.^[77]

2.2.1 Ergogenic Effect of L-Citrulline Supplements Alone

Only one study has been carried out involving L-citrulline supplementation without the addition of other products. In this study, Hickner et al.^[59] assessed the effect of one dose of L-citrulline administered 3 hours (3 g) or 24 hours (9 g) before an incremental treadmill test until exhaustion in young healthy subjects. Contrary to the hypothesis of the authors, the results showed that L-citrulline supplementation impaired exercise performance measured as time to exhaustion compared with placebo. To explain this surprising response, it was indicated that L-citrulline ingestion might reduce nitric-oxide-mediated pancreatic insulin secretion or increase insulin clearance. This hypothesis was

based on the lower plasma insulin levels found after L-citrulline ingestion.^[59] Additionally, lower levels of plasma NO markers (nitrates/nitrites) were also indicated following L-citrulline supplementation compared with placebo.

2.2.2 Ergogenic Effect of L-Citrulline Supplements with Malate

The other studies that have analyzed the effect of L-citrulline combined this amino acid with malate, which is an intermediate component of the tricarboxylic acid cycle (TCA). The first of these studies examined the rate of adenosine triphosphate (ATP) production during an exercise of finger flexions using ^{31}P -magnetic resonance spectroscopy (^{31}P -MRS).^[60] This study concluded that $6 \text{g} \cdot \text{day}^{-1}$ of L-citrulline with malate for 16 days resulted in a significant increase (34%) in the rate of oxidative ATP production during exercise, and a 20% increase in the rate of phosphocreatine recovery after exercise.^[60] However, there is some criticism around this research, because it used a very simple design without a placebo group or a blind condition. More recently, two studies conducted by the same research group showed an increase in plasma NO metabolites in well trained endurance athletes after a cycling competition; these athletes were supplemented with only one dose of L-citrulline with malate (6 g) 2 hours before exercise.^[58,61] In addition, an increase in plasma arginine availability was linked with substrate for NO synthesis, as well as polymorphonuclear neutrophils (PMNs).^[58] PMNs play an important role in the defense against infections, the inflammatory response, and muscle repair and regeneration.^[84,85] Unfortunately, these findings were unable to be associated with variables of exercise performance because of the characteristics of the study design. Many factors, such as strategy, environmental conditions, nutrition, drafting and breakdown of material, can affect the results during field sport events, limiting the use of these data to assess the association between dietary supplement and performance. Another recent study by Pérez-Guisado and Jakeman^[53] showed that a single dose of L-citrulline with malate (8 g) increased work capacity by an average of 19%, measured as the number of repetitions

performed until exhaustion during a flat barbell bench-press test at 80% of 1-RM. However, this finding cannot be related to NO delivery because plasma NO markers were not determined in this study.^[53]

Taking all this overview together, it is evident that there is a lack of data linking an increase in exercise performance to an increase in NO production derived from L-citrulline supplementation (tables I and II). Performance enhancement reported by L-citrulline in combination with malate could be explained by the interaction of these molecules in other metabolic pathways independent of NO production. For example, L-citrulline increases levels of plasma L-arginine indirectly; it could also enhance the synthesis of creatine, since it has been reported that L-arginine supplementation stimulates an increase in intramuscular creatine concentration.^[74] Therefore, this mechanism may improve the response to anaerobic exercise. In addition, malate may be involved in the beneficial effects on energy production because it is an intermediate of TCA.^[59,86] It has been suggested that hyperactivation of aerobic ATP production coupled to a reduction in anaerobic energy supply, may contribute to the reduction in fatigue sensation reported by the subjects.^[87]

In short, the conclusions that we can extract from the studies using L-citrulline as a dietary supplementation in sport are as follows:

- Dietary supplementation with L-citrulline alone does not improve exercise performance.
- Addition of malate to dietary L-citrulline supplements may increase levels of NO metabolites.
- However, this response has not been related to an improvement in athletic performance.

3. Synthesis of NO from the NOS-Independent Pathway

The NOS-independent pathway is a novel pathway that was discovered by two independent research groups during the 1990s.^[2,3] Nitrate and nitrite are the main precursors for NO synthesis in this alternative system. Interestingly, the NOS-dependent pathway is O₂ dependent; whereas the nitrate/nitrite-NO pathway is gradually activated as O₂ tension falls^[88] (figure 1).

3.1 Nitrate and Nitrite: Sources and Metabolism

The main providers of nitrate in the diet of humans are vegetables such as lettuce, spinach or beetroot.^[89] Drinking water can also contain considerable amounts of nitrate. It has been estimated that nitrate consumption derived from food and beverages is on average 100–150 mg • day⁻¹ in adults.^[90] However, the amount of nitrate in food has been regulated for a long time and there is currently an acceptable daily intake (ADI) for humans of 5 mg sodium nitrate or 3.7 mg nitrate • kg⁻¹ of body weight, which equals 222 mg for a 60 kg adult. This is due to the fact that nitrate has been considered a carcinogenic substance and a toxic residue in our food and water. The supposed carcinogenic mechanism is the nitrite-dependent formation of nitrosating agents, which can react with dietary amines, forming nitrosamines, substances with known carcinogenic properties.^[91] However, despite extensive research, no causal link between dietary nitrate intake and gastric cancer in humans has been found.^[92]

Apart from the diet, nitrate and nitrite is generated endogenously in our bodies. The NO generated by L-arginine and NOS enzymes is oxidized in the blood and tissues to form nitrate and nitrite.^[4] Thus, the NOS-dependent pathway significantly contributes to the overall nitrate and nitrite production, which indicates an active recycling pathway for generating NO in the human body. The normal plasma level of nitrate is within the 20–40 μM range, while the nitrite level is substantially lower (50–1000 nM), although many factors such as training and diet can modify these levels.^[93]

Nitrate circulating in plasma distributes to the tissues and has a half life of approximately 5 hours. By not yet fully defined mechanisms, circulating nitrate is actively taken up by the salivary glands and concentrated in the saliva (10- to 20-fold higher than in the blood).^[94] In the oral cavity, facultative anaerobic bacteria on the surface of the tongue reduces nitrate to nitrite by the action of nitrate reductase enzymes.^[95] In the absence of O₂, these bacteria use nitrate as an alternative electron acceptor to gain ATP. When

swallowed, one part of nitrite in the saliva is metabolized to NO locally in the acidic environment of the stomach, but the other part of swallowed nitrite is absorbed intact to increase circulating plasma nitrite.^[93] Such nitrite can be converted to NO and other bioactive nitrogen oxides in the blood and tissues under appropriate physiological conditions.^[3] These findings demonstrate that a complete reverse pathway (nitrate–nitrite–NO) exists in mammals.

3.1.1 Ergogenic Effect of Sodium Nitrate Supplementation

This alternative pathway of NO generation has not gone unnoticed in exercise physiology. Currently, four studies have assessed the effect of dietary nitrate supplementation in the form of sodium nitrate.^[26,62-64] The first was carried out by Larsen et al.,^[62] which showed that the ingestion of sodium nitrate ($0.1 \text{ mmol} \cdot \text{kg}^{-1} \times 3 \text{ days}$) reduced $\dot{V}O_2$ ($\sim 160 \text{ mL} \cdot \text{min}^{-1}$) during work rates at mean intensities of 40–80% of $\dot{V}O_{2\text{peak}}$ performed on a cycle ergometer. Gross efficiency, defined as the ratio of mechanical work output to the metabolic energy input, was also significantly improved ($\sim 0.4\%$). This highly surprising effect occurred without changes in other cardiorespiratory parameters (ventilation, carbon dioxide production, heart rate and respiratory exchange ratio) or lactate concentration, which suggests that energy production became more efficient after dietary nitrate consumption. Interestingly, in the second study by Larsen et al.,^[63] it was reported that $\dot{V}O_2$ at maximal intensity of exercise ($\dot{V}O_{2\text{peak}}$) was also significantly reduced ($\sim 100 \text{ mL} \cdot \text{min}^{-1}$) after nitrate supplementation ($0.1 \text{ mmol} \cdot \text{kg}^{-1} \times 2 \text{ days}$). Despite this decrease in $\dot{V}O_{2\text{peak}}$, exercise performance measured until time to exhaustion during an incremental exercise test did not decrease compared with placebo (nitrate: 564 ± 30 seconds; placebo: 524 ± 31 seconds; $p > 0.05$). To explain this physiological response during endurance exercise, it was suggested that nitrate and nitrite modulated mitochondrial respiration via NO synthesis, since both studies showed a significant increase in plasma NO metabolites (nitrate and nitrite) after nitrate treatment.^[62,63] This hypothesis was investigated by the same research group in an interesting recent

study.^[64] They reported that human mitochondrial efficiency, measured *in vitro* as the amount of O_2 consumed per ATP produced, termed P/O ratio, was significantly improved after sodium nitrate ingestion, compared with placebo using a similar amount of supplementation as in previous studies ($0.1 \text{ mmol} \cdot \text{kg}^{-1} \times 3 \text{ days}$).^[64] Nevertheless, despite these interesting findings, these studies did not report an enhancement in specific parameters of sports performance such as power output, time to exhaustion or total work performed.

While all the above studies assessed moderately trained subjects, one recent study by another independent research group assessed the effect of nitrate supplementation in well trained endurance athletes.^[26] Following acute supplementation of sodium nitrate ($10 \text{ mg} \cdot \text{kg}^{-1}$ of body mass) 3 hours before exercise, 11 trained cyclists and triathletes completed a cycle ergometer test performing four intermittent workloads at submaximal intensities (between 2 and $3.5 \text{ W} \cdot \text{kg}^{-1}$ of body mass) and one continuous incremental test until volitional exhaustion.^[26] Interestingly, this study showed that plasma nitrate levels increased at the same level after only one dose of nitrate ingestion ($10 \text{ mg} \cdot \text{kg}^{-1}$ of body mass) compared with 2-days' supplementation ($8.5 \text{ mg} \cdot \text{kg}^{-1}$ of body mass $\cdot \text{day}^{-1}$).^[63] Furthermore, results of exercise tests showed that, contrary to previous studies,^[62,63] $\dot{V}O_2$ at low-to-moderate intensities and increase in gross efficiency were not improved. However, in agreement with Larsen et al.,^[62,63] at maximal intensity of exercise, the $\dot{V}O_{2\text{peak}}$ was significantly reduced ($\sim 180 \text{ mL} \cdot \text{min}^{-1}$) without a decrease in time to exhaustion after nitrate supplementation.

3.1.2 Ergogenic Effect of Nitrate Supplementation in the Form of Beetroot Juice

Five studies by the same research group have used dietary nitrate supplementation in the form of beetroot juice to assess the effect on human performance.^[19-23] Interestingly, to isolate the effects of dietary nitrate from the other potentially active ingredients found in beetroot juice (betaine, quercetin and resveratrol), a process was developed to selectively remove the nitrate from beetroot juice using a commercially available resin.^[22] In the first of these studies, Bailey et al.^[19] showed a significant en-

hancement of $\dot{V}O_2$ kinetics after supplementation with $500 \text{ mL} \cdot \text{day}^{-1}$ of nitrate-rich beetroot juice (468 mg of sodium nitrate $\cdot \text{day}^{-1}$) for 6 days. During low- to moderate-intensity exercise (cycle ergometer exercise), there was a 19% reduction in the amplitude of the pulmonary response. In addition, it was shown that the $\dot{V}O_2$ -slow component was reduced ($\sim 23\%$) and the ~~time to exhaustion~~ during an incremental cycle ergometer test was extended ($\sim 14\%$) after beetroot juice supplementation compared with placebo.^[19] In an attempt to extend these findings to other forms of exercise (walking and running on a treadmill), the same research group performed another study using the same protocol of beetroot juice ingestion ($500 \text{ mL} \cdot \text{day}^{-1}$ equivalent to 527 mg of sodium nitrate $\times 6$ days).^[22] This study concluded that beetroot supplementation induced similar changes in respiratory response in a treadmill exercise compared with the previous data in a cycle ergometer test.^[19]

However, the effects of beetroot juice derived from nitrate seem to be fast, and acute ingestion of food rich in nitrate can affect the cardiovascular response in a few hours.^[96] This fact was analysed in the study of Vanhatalo et al.^[21] In this research, subjects ingested only one dose of beetroot juice (500 mL equivalent to 434 mg of sodium nitrate) 2.5 hours before a cycle ergometer test that included two moderate workloads at 90% of the gas exchange threshold followed by a ramp test. Moreover, subjects performed the same test after 5 and 15 days of beetroot juice ingestion and placebo. The steady-state $\dot{V}O_2$ during moderate-intensity exercise was significantly reduced 2.5 hours after supplementation and remained low on day 5 and 15 compared with placebo. However, contrary to the previous studies, the gas exchange threshold, maximal $\dot{V}O_2$ ($\dot{V}O_{2\text{max}}$) and peak power output were not affected 2.5 hours post-ingestion or after 5 days of supplementation. Surprisingly, these parameters showed a significant increase (peak power: $\sim 3\%$; $\dot{V}O_{2\text{max}}$: $\sim 4\%$) after 15 days of beetroot juice ingestion. However, several factors such as training or resting conditions, as well as diet (subjects did not follow a nitrate-restricted diet at any time during the study period) could be the reason for these changes after 15 days of beetroot

juice ingestion. Furthermore, in another very recent study by Lansley et al.^[23] following the same protocol of supplementation (500 mL of beetroot juice equivalent to 527 mg of sodium nitrate 2.5 hours before exercise), a significant improvement of average power output (5%) and mean completion time (2.8%) was indicated during 4 and 16.1 km cycle ergometer time trials compared with placebo.

To explain all these findings derived from beetroot juice ingestion, Bailey et al.^[20] suggested that the nitrate content of beetroot juice could play an important role in the reduction of ATP turnover in contracting myocytes. With the utilization of ^{31}P -MRS, these authors reported that the decrease of O_2 cost at moderate and high intensities after beetroot ingestion ($500 \text{ mL} \cdot \text{day}^{-1}$ equivalent to 468 mg of sodium nitrate $\times 6$ days) was accompanied by a reduction in muscle phosphocreatine of a similar magnitude.^[20] From this viewpoint, one of the most costly energy processes during skeletal muscle contraction is sarcoplasmic reticulum calcium pumping, which may account for up to 50% of the total ATP turnover.^[97] There is evidence that small elevations of NO improves muscle metabolism, preventing excess calcium release and subsequently modulates the ATP cost of force production.^[98] Interestingly, in beetroot juice studies, plasma nitrite levels measured as NO markers showed a significant increase after beetroot juice ingestion. Therefore, this is another alternative metabolic pathway to mitochondrial respiration indicated by Larsen et al.,^[64] which may explain the reduction of O_2 demands during exercise derived from ingestion of food rich in nitrate.

Nevertheless, in all studies involving beetroot juice supplementation, moderately trained but not well trained subjects participated. In only one study that evaluated nitrate supplementation (sodium nitrate) in well trained endurance athletes, no reduction of O_2 consumption was found, or gross efficiency at ~~low-to-moderate~~ intensities of exercise either.^[26] This study concluded that at ~~low-to-moderate~~ intensities of exercise, dietary nitrate supplementation could have a low effect in well trained endurance athletes compared with moderately trained subjects. Further research is needed in

highly trained athletes to assess the effect of sodium nitrate or beetroot juice supplementation on performance.

In conclusion, in the field of exercise physiology, studies indicate that nitrate supplementation (i) ~~could~~ be effective at enhancing exercise efficiency and tolerance to exercise in untrained or moderately-trained subjects; and (ii) have shown that there is a lack of data assessing the effect of nitrate supplementation in the form of sodium nitrate as well as beetroot juice in well trained athletes (tables I and II). Results derived from the only study that assessed well trained endurance athletes, concluded that sodium nitrate does not enhance efficiency at low to moderate intensities of exercise.

4. Other Components Related to NO Synthesis

4.1 Glycine Propionyl-L-Carnitine (GPLC)

Glycine propionyl-L-carnitine (GPLC) is a new United States Pharmacopeial Convention-grade dietary supplement that consists of a molecular bonded form of propionyl-L-carnitine and one of the carnitine precursor amino acids, glycine. This molecule has also been proposed to improve NO metabolism^[65] via two mechanisms: first, it has been reported in animal studies that the protective action of GPLC is derived from its antioxidant action, which may prevent vessels from peroxidative damage.^[99] In accordance with this fact, other authors have suggested that the lower release of reactive oxygen species could be linked with a decrease in NO breakdown.^[100] Second, eNOS gene expression has been demonstrated to increase within cultured human endothelial cells following carnitine incubation.^[101] Thus, it has also been hypothesized that GPLC could stimulate NO synthesis via eNOS expression.^[102]

Separating the components of GPLC, glycine is considered a glucogenic amino acid, in that it helps to regulate blood glucose levels, and is also important in the formation of creatine. Interestingly, glycine has been shown to have its own independent vasodilatory effects in rats.^[103] On the other, hand L-carnitine in combination with

propionyl (propionyl-L-carnitine) is a pharmaceutical agent that has been examined primarily as a treatment in clinical populations with apparent muscle carnitine deficiencies.^[104,105]

4.1.1 Ergogenic Effect of GPLC

Recent studies assessed the effect of GPLC as a NO donor in sport exercise with different conclusions. First, Bloomer et al.^[65] showed an increase in plasma NO metabolites (nitrate/nitrite) in active males after GPLC supplementation ($4.5 \text{ g} \cdot \text{day}^{-1} \times 4$ weeks). These findings were confirmed in the second study by the same research group.^[66] However, contrary results were found in the third study published by Bloomer et al.^[11] They showed that an acute dose (4.5 g) of GPLC did not increase NO markers. This controversy was attributed to the fact that in the latter study,^[11] a single dose of GPLC was provided prior to exercise, whereas in the first two studies, GPLC was administered for 4 and 8 weeks, respectively.^[65,66] Nevertheless, an important limitation of the studies performed by Bloomer et al.^[11,65,66] was the lack of evidence indicating some benefit of GPLC in exercise performance. Two recent studies assessed this issue showing different results. Smith et al.^[67] showed that ingestion of $3 \text{ g} \cdot \text{day}^{-1}$ of GPLC for 8 weeks did not enhance peak power, mean power or total work during a 30-second Wingate test. In contrast, Jacobs et al.^[54] indicated that only one dose of GPLC (4.5 g) 90 minutes before performing a test consisting of five 10-second Wingate cycle sprints separated by 1-minute of active recovery periods, significantly improved peak power ($\sim 5.2\%$) and reduced power decrement ($\sim 5.2\%$) through sprints, compared with placebo. In addition, lactate measures taken 14 minutes post-exercise were 16.2% ($p < 0.05$) lower with GPLC.^[54] However, these findings were not linked to NO delivery.

In summary, current scientific evidence of GPLC supplementation indicates that (i) in healthy and moderately trained subjects, GPLC could induce a mild increase in plasma NO metabolites, although the mechanism behind this response has not been defined; and (ii) evidence seems to indicate that the ergogenic effect of GPLC could be very limited; only one study indicated

benefits in exercise performance after GPLC supplementation.^[54] However, this finding could not be related to NO production as a result of the absence of analysis of NO markers

4.2 2-(Nitrooxy) Ethyl 2-Amino-3-Methylbutanoate

Recently, a new molecule 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate has been claimed to increase NO delivery in the body, with this being more efficient and effective than the traditional NO donors.^[68] However, to the best of our knowledge, there is only one study that has assessed the acute effect of 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate in plasma NO markers, measured as nitrate/nitrite in moderately trained resistance males.^[68] This study concluded there was no effect on circulating nitrate and nitrite within 1-hour post-ingestion of two tablets (no data was given on dose).

5. Side Effects of NO Supplements

Dietary supplementation with L-arginine and L-citrulline is not lacking in side effects, with gastrointestinal disturbances such as nausea, vomiting or diarrhoea as the most common adverse effects.^[106] However, there is great inter-individual variation in the tolerance of these amino acids; high doses ($>9 \text{ g} \cdot \text{day}^{-1}$) can increase the risk of gastrointestinal distress. It has been suggested that smaller, divided doses might lead to fewer side effects.^[34] In addition, the pre-existing proabsorptive or prosecretory state of the intestine may be important. The combination of secretory state and the extra stimulus provided by an acute dose ($>9 \text{ g} \cdot \text{day}^{-1}$) of L-arginine and/or L-citrulline may overwhelm the reserve-absorption capacity of the colon.^[106]

As has been indicated previously, the amount of inorganic nitrate in food and water has been strictly regulated because of their proposed role in the development of malignancies such as methaemoglobinemia and cancer;^[107] however, this view is currently changing. It is now thought that the nitrate concentrations commonly encountered in food and water are unlikely to cause methaemoglobinemia.^[108,109]

Moreover, an effect of exogenous nitrite on cancer seems less likely, because large amounts of nitrite are formed endogenously. Fasting saliva contains $\approx 2 \text{ mg} \cdot \text{L}^{-1}$, and after consumption of an amount of nitrate equivalent to 200 g of spinach, nitrite concentration in saliva may rise to as much as $72 \text{ mg} \cdot \text{L}^{-1}$. This is much higher than the ADI of $4.2 \text{ mg} \cdot \text{day}^{-1}$. Interestingly, all the above studies assessing nitrate supplementation in sports performance used amounts that were possible to achieve with natural foods, such as beetroot juice or green leafy vegetables (lettuce, spinach).

Studies have not reported adverse effects related with GPLC and 2-(nitrooxy) ethyl 2-amino-3-methylbutanoate supplementation. Nevertheless, this lack of data does not mean that this supplement is completely safe. For instance, it is known that amounts larger than $2 \text{ g} \cdot \text{day}^{-1}$ of L-carnitine may induce slight gastrointestinal distress.^[110,111]

6. Conclusion and Future Perspectives

The current available data indicate that L-arginine supplementation, mainly in combination with other components, may be effective in moderately trained or untrained subjects for enhancing cardiorespiratory adaptation and tolerance to endurance exercise,^[24,25,45] although a relationship between these findings and NO synthesis has not been established. On the other hand, L-citrulline in combination with malate could be a more efficient way to elevate extracellular levels of L-arginine by itself and with plasma NO markers. However, despite these effects, there is a lack of data indicating an improvement in exercise performance after L-citrulline supplementation. Therefore, it seems that some of the benefits shown after L-arginine and L-citrulline supplementation could be derived from the other ingredients included in supplements, as well as other metabolic pathways, independently of NO synthesis, which these amino acids participate in. Alternatively, a NOS-independent pathway has been reported by nitrate and nitrite oxidation. There is evidence that nitrate supplementation reduces the O_2 cost of endurance exercise, increasing the efficiency of energy production. An explanation for this intriguing physiological response has been linked

with an increase in mitochondrial efficiency,^[64] as well as ATP turnover functions.^[20]

While some of the benefits linked with NO donors have been shown in moderately trained subjects, in well trained athletes, scientific data show that the effect of these supplements is low. Thus, it seems that the training status is an important factor linked with the effectiveness of dietary NO donors. One reason to explain this fact may be attributable, in part, to the positive effect of exercise in the regulation of NO metabolism. While short-term training rapidly increases NO bioactivity, if training is maintained, the short-term functional adaptation is succeeded by NO-dependent structural changes, leading to arterial remodelling and structural normalization of shear.^[112] This structural remodelling and consequent normalization of shear obviates the need for ongoing functional dilatation, including enhanced NO dilator system function. The conclusion that we can extract is that training performed by competitive athletes has a greater effect on improving the NO system compared with NO supplementation. This fact raises the intriguing possibility of a threshold effect – volume and intensity – for training and mechanisms associated with NO production. Further investigation is needed to elucidate where this limit of physical exercise lies.

Moreover, almost all studies analysing NO donors and exercise performance were carried out, mainly, in young male subjects. It is known that vascular function and NO availability is impaired with age; thus, other studies should be planned in order to assess the effect of NO supplements in healthy adults (>40 years). Additionally, almost all research has been focused on endurance performance and few data exist concerning the effect of NO supplements in the regulation of hypertrophy and stimulation of satellite cells. This point needs more attention, not only for sports performance, but also for muscle mass losses associated with age and convalescence periods after injuries. Finally, gender differences have not been analysed, although it is recognized that there are structural/morphological differences between adult males and females for many, if not all, organ systems, which may have a significant impact on physiolog-

ical function.^[113] The female reproductive system is highly sensitive to physiological stress, and reproductive abnormalities including delayed menarche, primary and secondary amenorrhoea and oligomenorrhoea occur in 6–79% of women engaged in athletic activity.^[114] The prevalence of observed irregularities varies with athletic discipline and level of competition.^[115] It has also been indicated that amenorrhoea is associated with altered endothelial function.^[116] We consider this point needs further research to analyse the potential effects of NO supplementation and physical exercise, specifically in females.

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