DR ASGHAR GHASEMI (Orcid ID : 0000-0001-6867-2151)

REVIEW

Endogenous flux of nitric oxide: Citrulline is preferred to Arginine

Zahra Bahadoran[1], Ph.D., Parvin Mirmiran[2], Ph.D., Khosrow Kashfi[3], Ph.D., FRSC, FRSB, Asghar Ghasemi[4*], Ph.D.

[1]Nutrition and Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[2]Department of Clinical Nutrition and Human Dietetics, Faculty of Nutrition Sciences and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[3]Department of Molecular, Cellular and Biomedical Sciences, Sophie Davis School of Biomedical Education, City University of New York School of Medicine, New York, NY 10031, USA

[4]Endocrine Physiology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Short title: Citrulline and nitric oxide

*Correspondence to: Asghar Ghasemi

P.O. Box: 19395-4763

Phone: +98 (21) 224 32 500

Fax: +98 (21) 224 16 264, 224 02 463

Email address: Ghasemi@endocrine.ac.ir

No. 24, Sahid-Erabi St., Yemen St., Chamran Exp., Tehran, Iran.

Abstract

Both arginine (Arg) and its precursor citrulline (Cit) have received much interest during the past two decades because of their potential effects on whole-body nitric oxide (NO) production and augmentation of NO-dependent signaling pathways. However, the usefulness of Arg supplementation for NO production is questionable due to its high splanchnic first-pass metabolism (FPM), which limits its systemic availability. Both hepatic- and extra-hepatic arginases critically limit the availability of Arg for the NO synthase enzymes (NOSs) and therefore, a limited amount of oral Arg can reach the systemic circulation for NO synthesis. Arg has also some undesired effects including induction of arginase activity, an increase of urea levels, a decrease of cellular uptake of Cit, and decrease of recycling of Arg from Cit. In contrast, Cit has more availability as an NO precursor because of its high intestinal absorption, low FPM, and high renal reabsorption. At the cellular level, co-localization of Cit transport systems and the enzymes involved in the Cit-Arg-NO pathway facilitates channeling of Cit into NO. Furthermore, cells preferably use Cit rather than either intra- or extra-cellular Arg to improve NO output, especially in high-demand situations. In conclusion, available evidence strongly supports the concept that Cit leads to higher NO production and suggests that Cit may have a better therapeutic effect than Arg for NO-disrupted conditions.

Key words: Arginine, Citrulline, Nitric oxide.

1. Introduction

Arginine (Arg), a conditionally essential amino acid, has received significant research interest over the last two decades, because of its involvement in several metabolic pathways [1]. Although Arg has key roles in synthesis of proteins, creatine, polyamines, agmatine, and urea, as well as in metabolism of proline and glutamate in the body [1], the focus has been on its capability as a unique precursor of nitric oxide (NO) [2]. This property has led to its widely use as a complementary treatment in various NO-disrupted conditions such as, hypertension [3-5], preeclampsia [6], and endothelial dysfunction [7].

Despite lack of direct evidence, some effects of Arg supplementation on NO-related functions, for example, regulation of blood pressure and vascular function, has led to a dominant paradigm that oral administration increases its systemic availability and promotes NO production. This notion has now been challenged because there is strong evidence indicating limited bioavailability of orally ingested Arg [8,9], its plasma and cellular compartmentalization, and the competitive pathways utilizing Arg for synthesis of urea and ornithine [10,11].

Citrulline (Cit) is a neutral, non-essential, non-protein amino acid, which is an intermediary in the urea cycle, and also a precursor of Arg de novo synthesis and NO production [12]. Currently, Cit is receiving much attention as a natural NO precursor, especially for its potential cardiovascular and anti-hypertensive benefits and also for enhancing exercise performance and recovery [13-17]. This is because Arg supplementation is not as effective as Cit for NO production [18] and has some undesirable effects including induction of arginase activity [19,20], increase urea levels [21], a decrease in cellular uptake of Cit [22], decrease in recycling of Arg form Cit [20], suppression of eNOS expression and activity [23], and induction of cellular oxidative stress [23]. In addition, there is some evidence suggesting the existence of cellular "Arg tolerance" following long-term Arg exposure, a phenomenon that reduces the expected beneficial effects of long-term Arg supplementation [23]. Furthermore, there is some debate regarding the efficacy [24] and safety [25] of long-term Arg supplementation, for example, an increased risk of myocardial infarction and mortality rate have been reported following supplementation of 9 g/d of Arg for 6 months. [25] There are also numerous reports supporting the efficacy and safety of Cit [13,26,27] thus highlighting the importance of Cit as a precursor of Arg and NO. In this review, we summarize the evidence suggesting that supplementation with Cit is superior over Arg itself to improve Arg systemic bioavailability and NO production. If Cit is unequivocally shown to be superior over Arg as a safe and effective precursor for endogenous NO production, then its supplementation will receive further justification in medical practice.

2. Arg and Cit biosynthesis

Arg is provided by both exogenous dietary intake and endogenously through protein breakdown and de novo synthesis (Figure 1A). In healthy adult humans, Arg is considered to be a non-essential dietary amino acid because of its sufficient endogenous synthesis [28]. Dietary proteins are a major

source of Arg; seafood, nuts, seeds, alga, meats, rice protein concentrate, and soy protein are considered to be Arg-rich foods [12].

Plasma Arg level ranges 80-120 μmol L^{-1} in healthy adults [1,29]. Fluxes of plasma Arg in healthy humans on a normal diet have been estimated to be 63-70 and 70-95 μmol kg^{-1} h^{-1} in fasted and fed states, respectively [8,11]. Depending on Arg content, the contribution of diet to plasma Arg flux is as low as ~8–20% in the fasted state (6–11 μmol kg^{-1} h^{-1}) to as large as ~35–40% in postprandial state (~22–36 μmol kg^{-1} h^{-1}) [30-32].

In healthy adults ~10–15% [12,33] of the plasma Arg flux originates from de novo synthesis (~5-16 μmol kg^{-1} h^{-1}) [11,34, 30], which mainly occurs in the proximal tubules of the kidneys. About 50–80% of circulating Cit is taken up by the kidneys [35,36], of which 75% [37] to 100% [35] is converted to Arg, by the enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Other cells such as activated macrophages [38] and the endothelial cells [39] can take up Cit and use it as a substrate to synthesize Arg and NO.

Following a 7-day Arg-free diet, plasma flux of Arg (~52 μmol kg^{-1} h^{-1}) is enriched entirely by protein turn over (~36 μmol kg^{-1} h^{-1}) and de novo synthesis (16 μmol kg^{-1} h^{-1}) [30]. Following an Arg-rich diet, the absolute values for the contribution of protein turnover and de novo synthesis in the whole plasma Arg flux remain constant; however, because of increased whole plasma Arg value, the percent contribution for protein turnover is decreased from 70 to 40% and for de novo synthesis from 30 to 18% [30].

Plasma Cit is mainly supplied through endogenous synthesis with limited amounts obtained from usual dietary intake as its food source is relatively limited [14]. Although watermelon is a rich source of Cit (~1.6-3.5 g kg^{-1} fresh weight), it would take about 1-1.5 kg of fresh watermelon to provide the minimum effective amount of Cit, which is 3 g per day [14]. Cit content is higher in watermelon rind compared to its flesh, ~ 24.7 vs. 16.7 mg g^{-1} dry weight [40]. There is no recommended dietary allowance for Cit [12].

Mean plasma Cit concentration have been reported to be about 20–40 μmol L^{-1} [34,41-44] and whole body Cit flux is estimated to be roughly about 8.9 μmol kg^{-1} h^{-1} (6-10 μmol kg^{-1} h^{-1}) in healthy adults in the fasted state [41]. The intestine is the principle site of Cit biosynthesis [37] and 60–90% of plasma Cit flux comes from this source [45] (Figure 1B). Glutamine via glutaminase and pyrroline-5-carboxylate

synthase pathway is the main precursor of Cit biosynthesis (60–80%) in humans [33,35]. Proline via proline oxidase and ornithine aminotransferase (OAT), and Arg through arginase activity, are also involved in supplying the ornithine that is used for Cit biosynthesis in the enterocytes [46,47]. The ornithine is finally converted into Cit by the action of ornithine transcarbamylase (OTC) and carbamoyl phosphate synthetase I (CPS-I) [44]. High-protein diets and excess Arg intake downregulates CPS-I and OTC, reducing the conversion of ornithine to Cit [20,48].

Another potential source of systemic Cit is the Arg-NO pathway in NO-producing cells (Figure 2), which accounts for the release of Cit into the systemic circulation at a rate of 1 µmol kg^{-1} h^{-1} (~10% of plasma Cit flux, which is 9.5 µmol kg^{-1} h^{-1}) [11]. Cit can also be synthesized and released into circulation through degradation of asymmetric dimethylarginine (ADMA) via dimethylarginine dimethyloaminohydrolase (DDAH) [49,50] (Figure 2). The relative contribution of these potential sources to the supply of circulating Cit has not been quantified; however, a comparison of the net intestinal Cit release to that of the net renal uptake in the fasted state (i.e. 2.4±0.4 *vs.* 3.0±0.5 µmol kg^{-1} h^{-1}) [45], implies that the extra-intestinal tissues contributes about 20% (10–30%) of the plasma Cit. The extra-intestinal Cit biosynthetic pathways (i.e. citrulline-NO cycle and ADMA-DDAH pathway) seems to be crucial in supporting the local requirements of Cit in NO-producing cells rather than supplying to the systemic pool.

3. Systemic bioavailability of exogenous Arg and Cit

The main evidence justifying preference for Cit over Arg supplementation comes from preliminary isotope studies addressing gut-liver metabolism of amino acids [8,9,11,30-32,51,52], and more recent investigations evaluating pharmacokinetics of exogenous doses (from low to high-doses, i.e. 2-15 g per day) of Arg compared to that of Cit [18,53]. All evidence indicates a limited systemic bioavailability of oral Arg, alongside a more efficient and robust metabolism of Cit to elevate Arg flux.

3.1. Gut-liver metabolism of Arg

3.1.1. Arg absorption

Arg is absorbed in the small intestine, mainly from the jejunum, via a transport system that is shared with lysine, ornithine and cysteine [36], however, Arg has relatively poor absorption compared to the other amino acids [54]. Intestinal Arg absorption has saturable kinetics (*Km or* K_t= 3.8 mmol L^{-1}; V_{max}= 46.3 μmol min^{-1} 30 cm^{-1}); jejunal perfusion of high doses of Arg cease its intestinal absorption and

results in excessive secretion of water and electrolytes into the lumen, this is considered to be a nonspecific toxic reaction of Arg upon the mucosa [54]. When intestinal segments of rats were perfused with Arg at a dose of 20 mmol L^{-1}, the observed absorption rate was 9.8 µmol min^{-1} g^{-1} [55].

3.1.2. Splanchnic uptake of Arg

Arg is largely extracted by the splanchnic region, intestine, and liver [51,56] and mainly converted to urea and ornithine through first pass metabolism (FPM) [10,11] (Figure 3). Since arginase II activity in the intestine and arginase I activity in the liver are relatively high, 40–60% of exogenously absorbed Arg is removed by FPM within the intestinal–liver bed [10,11,51]. The rate of splanchnic uptake of dietary Arg is estimated to be 2.8–3.5 µmol kg^{-1} h^{-1}, where the rate of its dietary input is ~4.7 µmol kg^{-1} h^{-1} (obtained by intragastric doses of 5.3 µmol kg^{-1} [^{13}C]arginine or [M$_4$]arginine, over 3 min) [51]. Arg FPM is not a fixed fraction and the habitual dietary intake of proteins [57], body protein/Arg demand, and the amount of ingested Arg are all important modifying factors [20,58]. Increased ingested doses of Arg substantially promotes FPM by ~2-fold (from 32.5% in control diet providing 75 µmol kg^{-1} h^{-1} Arg to 60.4% in supplemented diet providing 375 µmol kg^{-1} h^{-1} Arg) [58].

Relative contribution of intestine and liver in Arg FPM has not been exactly quantified; however, existing of zone-specified hepatic utilization of Arg and well-structured compartmentalization of Arg metabolism within the hepatocytes [57,59-61], suggest that the contribution made by the liver is more important than that of the intestine. Animal experiments also indicate that the liver is the main site of Arg removal during FPM since lack of arginase II (the intestinal isoform of arginase) causes only a 10% reduction in Arg FPM from 75% into 65% [58].

3.1.3. Intestinal and hepatic metabolism of Arg

After entering the absorptive epithelial cells of the small intestine, Arg is mainly degraded by arginase II that is located in both the cytosol and the mitochondria of the enterocytes, and to a much lesser extent by the nitric oxide synthase (NOS) enzymes [62]. The intestinal arginase II is co-localized with ornithine-utilizing enzymes [i.e. OAT, OTC, and ornithine decarboxylase (ODC)] in the mitochondria, and the produced ornithine can therefore contribute to the synthesis of proline, polyamines, and Cit [9,51,63]. A minor fraction of Arg is converted to NO in the enterocytes [9].

The Arg presented to the liver is mainly converted to urea and ornithine within the periportal hepatocytes [61], where arginase I is co-localized with the urea cycle enzymes [59]. Because hepatic

arginase I activity is high and there is tight channeling of metabolites of the urea cycle to the next enzymes within the pathway [64,65], there is little or no net production and export of Arg by the liver [2]. The fraction of orally ingested Arg that is converted to urea during FPM is anything between 15% to 60% [10,11]. In healthy adults, the hepatic Arg flux within the urea cycle is estimated to be about 239 $\mu mol\ kg^{-1}\ h^{-1}$ in the fed state [11], which is mainly (~95%) obtained by dietary rather than plasma Arg [10,11]. The conversion of ingested Arg into urea occurs rapidly within early catabolic stage and reaches its maximum value within 30 min after ingestion; 90% of the whole-body urea production from dietary Arg occurs almost entirely during its first pass, that is within the first 2-h post-ingestion period [10]. In normal situations, the NO pathway as well as synthesis of glutamine and polyamine from ornithine do not substantially contribute to the hepatic catabolism of Arg [66,67]. Due to co-existing of arginase I with OAT and glutamine synthetase (GS) in the perivenous cells (hepatocytes in zone 3 of the hepatic acinus [60]), the complete Arg catabolic pathway resulting in CO_2 production, occurs in these cells [61].

3.1.4. Systemic availability of oral Arg

Due to substantial splanchnic FPM, limited amounts of intact ingested Arg (~10–30%) reaches the circulation [51,56,68]. However, in a recent study, the postprandial contribution of orally ingested Arg (acute doses of 1.8 and 4.9 g) to the total plasma pool of Arg was estimated to be 57% and 70%, respectively [10]; this estimation was obtained based on the [^{15}N-^{15}N-(guanido)]-arginine that appeared in the plasma and did not differentiate intact ingested Arg from that was recycled, and/or resynthesized from Cit by the kidney [10]. The Arg-Cit-Arg cycle via the intestinal-renal axis contributes to the fraction of Arg reaching the systemic circulation. The contribution of recycled-Arg to the total postprandial increased plasma Arg levels is estimated to be about 15% [10], which is close to the percentage of Cit produced by the intestinal trapped Arg within FPM (~17%) [9].

Taken together, oral intake of Arg is not an efficient strategy to potentiate NO synthesis. Multi-complex catabolic pathways that are activated upon exogenous Arg intake limit its accessibility to the systemic circulation and thus a large amount of oral Arg is required to provide a therapeutic plasma level; such high doses may increase blood nitrogen urea levels especially in cases where renal function is insufficient, as a major amount of ingested Arg is metabolized into urea and ornithine.

3.2. Gut-liver metabolism of Cit

3.2.1. Cit absorption

Orally ingested Cit can effectively be absorbed by the enterocytes, especially in the middle to the lower ileum, [69] and transported to the portal circulation using a Na$^+$–dependent transport system (B^{0+}) and Na$^+$–independent saturable transport (L and b$^{0,+}$) systems [22]. Such a broad set of transporters involved in intestinal Cit transport may be a compensation for the low Cit content in foods, and may explain why there is better bioavailability of Cit compared to Arg. In addition, it may also explain as to why high doses of Arg but not Cit cause osmotic diarrhea [22]. K_m of Cit uptake in a model of human intestinal epithelial cell was 0.46 and 0.67 mmol L^{-1} for the Na$^+$–dependent and Na$^+$–independent mechanisms; V_{max} was 3.58 and 2.16 nmol mg^{-1} min^{-1} in the Na$^+$–dependent and Na$^+$–independent uptake, respectively [22].

3.2.2. Splanchnic FPM and systemic availability of Cit

In contrast to Arg, Cit seems to effectively bypass the intestinal and liver metabolism [15,37] since absorbed Cit appears not to be metabolized by the enterocytes and hepatocytes [37]. Extraction of Cit is not significantly different from zero across the portal drained viscera (PDV) following intravenous infusion of L-[ureido-^{13}C-^{2}H2]Citrulline (infusion rate of 0.14 µmol kg^{-1}), indicating that net PDV Cit flux equals total Cit production [33]. Our initial understanding was that Cit passes through the liver without being taken up [37]; however, further studies showed that indeed the liver can take up about 55% of the Cit that is released by the intestine [33] or around 8-12% of the whole flux [45,70]. Nevertheless, the net hepatic exchange of Cit remains close to zero, because of the similar rates in its active hepatic uptake and active hepatic release, estimated to be about 5 µmol kg^{-1} h^{-1} [33]. How the Cit taken up by the liver is metabolized is not fully understood, however, its only fate appears to be conversion into Arg, thus serving as a substrate for NOS [45].

In summary, due to lack of significant splanchnic FPM, exogenous Cit appears almost entirely in the plasma [58]. In contrast to Arg, the effective net splanchnic exchange of Cit, that is low uptake and high release, bypasses intestinal-hepatic FPM and facilities down-stream Cit-Arg recycling. Thus, exogenous Cit is more efficient in increasing systemic Arg availability and NO production.

3.3. Pharmacokinetic of oral doses of Cit and Arg

An overview of the pharmacokinetics of Arg *vs.* Cit provides further evidence indicating how Cit results in a greater and sustained systemic level of Arg. About 1 h after an oral dose of 10 g of Arg, its

peak plasma concentration is about 287 μmol L^{-1}, which is 3-fold greater than that of the baseline [68]. Lower oral doses of Arg (up to 10 g) are eliminated from the plasma via non-renal clearance rate of 5 mL min^{-1} kg^{-1}, whereas higher doses are eliminated biphasically with a rapid renal clearance within 90 min (with a rate of 1.17 mL min^{-1} kg^{-1}) followed by a slower non-renal clearance (with a rate of 5 mL min^{-1} kg^{-1}) [68]. Comparison of the pharmacokinetic behavior of oral *vs.* intravenous doses of Arg provides further evidence to support the importance of FPM on its systemic bioavailability. Plasma concentration of Arg after an acute oral dose of 10 g was 3-fold higher than the baseline *vs.* a 90-fold increase in plasma concentration after an intravenous infusion of 30 g over 30 min indicating a limited systemic bioavailability after oral ingestion [68]. Peak plasma concentrations of Arg were about 3-fold higher following an infusion of 6 g of Arg compared to an equivalent orally ingested dose (822 μmol L^{-1} at T$_{max}$=22 min *vs.* 310 μmol L^{-1} at T$_{max}$=90 min) [71]. Similar findings in animals underscore the complex pharmacokinetic nature of oral compared to intravenous doses of Arg [72].

Following oral doses of Cit (2–15 g), its plasma concentration rapidly (T$_{max}$=38-56 min) reaches a maximum level (C$_{max}$=515-3849 μmol L^{-1}) [53]. In contrast to high doses of Arg that exceed the renal threshold for its reabsorption resulting in Arg overflow into the urine [68,71], renal Cit reabsorption is extremely powerful [53,73], and its urinary excretion remains as low as 5%, even at highest dose of 15 g (fractional Cit reabsorption rate ~99.0-91.9% for doses of 2-15 g) [53]. The prompt appearance and quantitatively massive increased plasma Cit following its oral consumption (~10-fold at the dose of 2 g to 100-fold at the dose of 15 g) alongside its gradual disappearance within 5-8 h post-loading, support a more efficient absorption and retention of orally ingested Cit, this is not the case for Arg.

Comparison of the pharmacokinetics of Cit and Arg imply that Cit is at least as efficient as Arg in increasing plasma Arg concentrations. Following oral Cit ingestion (an acute dose of 3 g) de novo Arg synthesis is substantially stimulated from 12.0±1.0 to 66.3±4.8 μmol kg^{-1} h^{-1} [74]. The peak plasma concentrations of Arg following oral doses of 2–15 g of Cit is 146–303 μmol L^{-1}, which was observed at times of 1.17–2.29 h [53]. Compared with the same dose of Arg [68], an acute dose of 10 g of Cit resulted in a similar maximum Arg concentration, i.e. about 280 μmol L^{-1} [53]. Furthermore, the actual body exposure to Arg, which was estimated using the area under the curve of baseline-adjusted plasma Arg *vs.* time, was about 2-fold higher following ingestion of Cit compared to Arg (AUC=1043 *vs.* 557.2 μmol L^{-1} h^{-1}) [53,68]. Such data indicates that the same dose of Cit supports systemic

availability of Arg more efficiently than Arg itself. Cit at one-half dose of Arg resulted in similar plasma concentrations of Arg after oral dosing [18]; 0.75 g of Cit and 1.6 g of Arg administered twice daily increased C_{max} (54 *vs.* 49 µmol L^{-1}) and AUC (271 vs. 289 µmol h L^{-1}) to the same extent [18]. Plasma concentration of Arg reached its maximum level more gradually following Arg administration compared to Cit (T_{max}=3.7 *vs.* 2.3 h) [18]; this delayed time to peak may indicate that plasma appearance of the ingested Arg is secondary to the Arg-Cit-Arg interorgan cycle. The plasma half-life of Arg (for an oral dose of 6 g) was estimated to be around 1 h [71]. For Cit this value is dose–dependent and ranges from 0.65–1.14 h (for oral doses of 2–15 g) [53]. Kinetic parameters of Arg and Cit in both steady-state and post-ingestion of the amino acids are summarized in Table 1.

In summary, compared to Arg, ingested doses of Cit have more efficient intestinal absorption, relatively no FPM and high renal re-absorption. The body seems, therefore, adopting a set of different economic approaches to trap and retain orally ingested Cit. These pathways enable Cit to be more efficient than Arg itself for increasing Arg flux.

4. Contribution of Arg *vs.* Cit to the whole-body NO flux

4.1.   Role of Arg in whole-body NO synthesis

The fraction of plasma Arg that contributes to circulatory NO is estimated to be as low as 0.5-2.8% in either fasted or postprandial conditions [10,11,52,74,75]. The conversion rate of plasma Arg to NO (estimated by the rate of conversion of the [15N]guanidino nitrogen of Arg to plasma [15N]ureido citrulline) has been estimated to be about 0.36 to 0.96 µmol kg^{-1} h^{-1} in healthy adults [11,74]. This contribution is independent of the amount of exogenous Arg reaching the systemic circulation. That is, regardless of increased whole body Arg flux and increased plasma concentrations by an Arg-rich diet, the fraction of plasma Arg pool used for NO synthesis remains unchanged under both Arg-rich and Arg-free diets (about 0.55% and 0.58%) [52]. Such data suggests that most of the plasma Arg is metabolized by other enzymatic pathways including arginase and arginine:glycine amidinotransferase, producing ornithine, urea, and creatine [76].

Contribution of dietary or exogenous Arg utilized as a substrate for NOS is negligible (ranged from zero to ~1%). Dietary Arg (40 g daily for 6 days) did not alter the urinary nitrate excretion, its plasma concentration, nor the conversion rate of [15N]arginine to NO [77]. Less than 0.1% of an ingested dose of [15N]-arginine (85 mg kg^{-1}) is recovered as urinary nitrate in 24-h [78] and only 1% of the ingested

doses of Arg, either as an acute dose or a 4-week supplementation, are converted into urinary ^{15}N-labled nitrate without any change in urinary cGMP level [79]. It has been reported that the fraction of an oral dose of Arg that contributes to the plasma NO flux is very small indeed, for example 0.087% of 1.8 g and 0.068% of 4.9 g [10]. Quantitatively, that translates to about 10 and 20 µmol of NO over a period of 12-h [10].

Whether intravenous or oral/intragastric Arg administration contributes more efficiently to the synthesis of NO within the whole body is not well understood. Using intravenous bolus injection of L-[^{15}N]arginine and subsequent analysis of L-[^{15}N]arginine in plasma and [^{15}N]nitrate in the urine, the contribution of Arg doses to NO production was estimated to be ~1.7–3.8% (average: 2.8%, 0.019 µmol h^{-1} kg^{-1}) [75], which is more than those estimated from oral doses [10,77-79]. Some evidence however indicates that intragastric but not intravenous doses, are likely to be associated with an increased NO generation, because a significant fraction of trapped Arg within the FPM is diverted towards the NO synthesis pathway [8]. Contribution of Arg to the whole-body NO production is ~4-fold higher by the intragastric rout compared to intravenous administration (15.1% *vs.* 3.7% of $^{15}NO_3$ excreted in the urine over 24-h; 1.95 *vs.* 0.6 µmol $^{15}NO_3$) [8]. About 0.34% of the dietary Arg trapped by the FPM within the splanchnic bed turns into NO, which accounts for ~16% of the whole daily endogenous NO synthesis [8]. This splanchnic NO production is mainly attributed to the intestine rather than the liver [80]. There is a substantial difference between the amount of NO produced from dietary Arg compared to that produced from plasma dietary Arg over 12-h, either at low (AUC= 10 *vs.* 0.15 µmol) or high (AUC= 20 *vs.* 0.35 µmol) doses of ingested Arg [10]. This may also provide further evidence on the importance of splanchnic Arg FPM for NO production. These observations appear to be in contrast with the common assumption regarding the effects of FPM on the capability of dietary Arg for NO production.

4.2. Role of Cit in whole-body NO synthesis

In contrast to Arg, Cit intake appears to provide more systemic available NO. An acute dose of 3 g of Cit effectively stimulates NO production rate by about 10-fold, from 0.36 to 3.57 µmol h^{-1} per kg of body weight or from 0.48 to 4.81 µmol h^{-1} per kg of fat free mass in young adults [74]. More quantitatively, 3 g of Cit resulted in 1340 µmol of NO produced over a period of 6-h (where the mean body weight of the subjects were 69.6 kg and the rate of NO synthesis attributed to Arg-derived

citrulline was 3.2 µmol h^{-1} per kg of body weight) [74]. When comparing the amount of NO produced from 5 g of ingested Arg (~20 µmol over 12-h) [10], it becomes apparent that Cit is about 200-fold more efficient in inducing NO production compared to Arg. Such estimations however need to be confirmed in well-designed isotopic studies investigating the contribution of similar doses of Arg *vs.* Cit in NO production simultaneously.

In healthy humans, following one week of oral ingestion of Cit (3.0 g twice daily) and Arg (either as sustained-release 1.6 g twice daily or immediate-release 1.0 g three times daily), only Cit significantly increased the markers of systemic NO production i.e. urinary nitrate excretion (from 92 to 125 µmol L^{-1}) and urinary cGMP concentrations (from 38 to 50 nmol mmol^{-1} creatinine) [18].

5. Cellular metabolism and preferential utilization of Arg *vs.* Cit for NO synthesis

Different patterns of cell-specific expression and sub-cellular localization of the enzymes involved in Arg and Cit metabolism, coupling among the enzymes, compartmentalization of Arg and Cit metabolism, the presence of various inter- and intracellular transport systems, as well as fluxes of substrates determine how cells metabolize these amino acids [1,12]. Some of these factors substantially limit utilization of extracellular Arg or make it conditionally-dependent available for NO production [81]. In contrast, NO-producing cells (e.g., endothelial cells and macrophages) have been well equipped by compartmentalized Cit-NO pathways [82,83], enabling them to prefer Cit over Arg for NO production regardless of Arg's source, intracellular or imported extracellular. This ability to be independent of extracellular Arg for NO production, is more critical in activated macrophages, which need to produce large amounts of NO to enhance immunity [81]. In such situations, exposing cells to Arg results in more production of ornithine (due to channeling of Arg toward arginase) rather than NO, whereas Cit effectively is directed to the NO pathway [81]. Figure 4 illustrates the proposed cellular compartments (Arg pools, Cit transporters and NOSs, with ASS-ASL) and mechanisms that enable the cells to preferentially utilize Cit rather Arg for NO production.

5.1. Cellular Arg pools and NO production

Two intracellular pools of Arg, named pool I and pool II, are detected in endothelial cells and macrophages (Figure 4). Pool I is exchangeable with extracellular cationic amino acids through the CAT system, whereas pool II does not rely on extracellular sources [84]. The iNOS activity in macrophages is suggested to be dependent on pool I and extracellular Arg when the pool I cannot

provide sufficient intracellular Arg [84]. Pool II is supported by Cit recycling pathways [39] and is accessible to eNOS in the endothelial cells (but not to iNOS in macrophages), this enables eNOS to be independent of extracellular Arg [84]. Pool II has two components; pool II-A participates in recycling of Cit to Arg and can be replenished by Cit, pool II-B is supplied by protein breakdown and is likely to accumulate the methyl-Arg and ADMA (as the main intracellular precursor of Cit) [39]. Taken together, Arg pool II (both II-A and II-B), which seems to be the main source of NO production under normal condition, is tightly dependent on Cit and it's recycling into Arg. Such precise intracellular compartments make freely distributed Arg, if any, not available for NO production especially by eNOS. It is speculated that lack of efficient Cit recycling and alterations in pool II or an impaired access of eNOS to pool II makes cellular NO production dependent to extracellular Arg, a situation that well explains the "Arg paradox" [39].

5.2. Cellular compartments of Cit-utilizing enzymes and NO production

Cell-specific compartmentalization of intracellular transport systems and enzymes involved in Cit-Arg-NO pathway facilitates channeling of Cit into NO synthesis instead of using Arg. For example, co-localization of ASS and ASL with all NOS isoforms, i.e. inducible NOS (iNOS) [82], endothelial NOS (eNOS), and neuronal NOS (nNOS) [85] as well as their co-induction in NO-producing cells may enable Cit to be more efficient than Arg to support NO production. ASL is the essential component of the compartment, maintaining the enzymes together to make a dynamic "NOS multi-protein complex", while lack of ASL results in cellular NO insufficiency, a situation that cannot be compensated with exogenous Arg [85].

Previously, it was speculated that the caveolar complex between the CAT-1 and eNOS provides a mechanism for the directed delivery of extracellular Arg to eNOS [86]. However, further studies have indicated that this compartment enhances NO production independent of Arg transport [87]. Caveolar co-localization of cationic amino acid transporters, CAT-1 and CAT-2B, with ASS-ASL and eNOS, which effectively couples endothelial NO production to the Cit-NO cycle [83], provides further evidence for preferential use of Cit but not Arg for NO production.

5.3. The economic approaches of the cell to preserve Cit for NO

Recycling of Cit from Cit-NO cycle, which is dependent on the compartment of NOS, ASL and ASS, effectively retains Cit upon synthesis of NO from Arg [11]. Intracellular Cit can also be recycled

through degradation of asymmetric dimethylarginine (ADMA), an Arg metabolite produced upon hydrolysis of intracellular proteins containing methylated-arginine via arginine methyltransferases (PRMTs) [49,50]. Since about 250 μmol intracellular ADMA is degraded daily by dimethylarginine dimethyloaminohydrolase (DDAH) into Cit and dimethylamine, cells can obtain a substantial amount of Cit through this way [88]. Existence of such a Cit-recycling pathway may imply that the cell prefers Cit rather than Arg for NO production.

5.4. Arg and the challenge of arginase

Cit is also suggested to be a more effective NO precursor than Arg, since Arg induces intracellular arginase, the enzyme that competes with NOS for the intracellular arginine pool [34]. In humans, orally ingested Arg (7 g three times daily for 3 days) significantly increases plasma urea levels (from 5.2 to 6.7 mmol L^{-1}) [21]. Besides intestinal and hepatic arginases that substantially decrease systemic Arg bioavailability and compartmentalize it into urea and ornithine production, Arg entered into the other cells may also be confined into the urea cycle (either completely or partially), which make it unavailable for NOS.

Arginase has emerged as an important regulator of NO bioavailability because of its competition with NOSs for Arg as their common substrate [89]. Although the affinity of the NOS enzymes for Arg is about three orders of magnitude greater than the arginases (Km = 2–20 μM $vs.$ Km = 1-5 mM), the V_{max} (the maximum activity) of the arginases is also about three orders of magnitude greater than that of the NOSs, resulting in a similar rate of substrate utilization within physiologic concentrations of Arg [2]. In endothelial cells, the amount of intracellular Arg catabolized by the arginase is ~200-fold more than those catabolized by NOSs [90]. In addition, in RBCs, which are considered to be as important as endothelial cells for supplying plasma NO levels, the predominant activity of arginase $vs.$ eNOS (RBC-NOS) is well documented [91,92].

Upregulated arginase expression and activity is involved in vascular dysfunctions because of decreased Arg supply for NO production [93]. Therefore, excessive arginase activity is associated with pathological cardiovascular conditions such as hypertension, atherosclerosis, myocardial ischemia, congestive heart failure, and vascular dysfunction in patients with diabetes mellitus [94,95]. Evidence indicates that arginase is co-expressed with NOS in NO-producing cells in the vasculature and a modest level of arginase is constitutively expressed in endothelial cells [93,96]; however, arginase

activity is increased by inflammatory factors (e.g., lipopolysaccharides, tumor necrosis factor alpha) or by aging [88].

Arg is an allosteric activator of arginase and accelerates the urea cycle; regulatory effects of Arg on ureagenesis is dependent on its nutritional status rather than on its local de novo synthesis through argininosuccinate synthase activation [19,28]. Induction of arginase limits Arg as a substrate for NOSs, and therefore affects cellular NO production [97]. In contrast, inhibition of arginases has been suggested to be an efficient way to maintain NO production and to prevent adverse effects related to impaired NO production in peripheral tissues [98]. Cit is a potent inhibitor of arginase in the liver, macrophages [20], and endothelial cells [99]; ingestion of Cit does not increase urea levels and instead improves nitrogen balance [73].

5.5.   *Arg and ADMA metabolism*

ADMA, an endogenous competitive inhibitor of NOSs, antagonizes NO production, therefore increased availability of Arg seems theoretically to be an effective strategy to restore NO formation. Targeting ADMA is of great interest because of its association with endothelial dysfunction and cardiovascular diseases [100,101]. Short-term use of Arg to reverse the competitive ADMA-induced inhibition on NOS has provided conflicting results. Following Arg supplementation, plasma ADMA concentration (~0.5 µmol L^{-1} [102]) remains unchanged [24] or even increases [103]. Theoretically, Arg regulates intra- and extracellular levels of ADMA at the level of synthesis or its cellular transport, therefore the higher the Arg concentration, the higher the ADMA concentration would be. Arg competitively inhibits DDAH and ADMA catabolism [104] and may saturate the y$^+$ transport system (CAT transporters) and limit cellular export of ADMA, resulting in increased intracellular ADMA levels and inhibition of NO production [105]. The antagonistic effect of ADMA on eNOS activity is suggested to be offset by increasing intracellular levels of Cit-derived Arg [106].

6. Metabolism of Arg *vs.* Cit in NO-disrupted conditions

Further evidence supporting the preference for Cit over Arg for NO production comes from focusing on Cit, Arg, and NO metabolism in pathologic conditions. For example, in patients with MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes), decreased NO synthesis is associated with decreased Cit flux, de novo Arg synthesis rate, and the percentage of Arg flux derived from de novo synthesis rather decreased Arg flux itself [34]. The argininosuccinic

aciduria (ASA), the second most common human urea cycle disorder, caused by congenital ASL deficiency, is related to decreased NO production and severe systemic hypertension despite high extracellular Arg flux [107,108]. Arg administration to ASA subjects (100 mg kg^{-1} per day, as a standard treatment) increases Arg and urea fluxes as well as Cit flux while ^{15}N transfer from infused ^{15}N$_2$-guanidino-labelled-Arg to ^{15}N-Cit, a marker of NO production, remains near zero (in healthy controls, the administered Arg resulted in ~5 μmol L^{-1} kg^{-1}) [85]. Such observations provide further evidence implying that recycling intracellular Cit into Arg is more critical than Arg availability for endogenous synthesis of NO [85,107].

In conditions with pathogenic elevated arginase activity (e.g., sickle cell disease, endotoxemia) [109-111], Arg supplementation is not suitable and enhances arginase activity [112,113]. In contrast, Cit supplementation is suggested as a promising alternative, because of its capability to bypass the arginase activity [111,114]. Intravenous infusion of similar doses (6.25 mg h^{-1}, during 6 hours) of Cit was superior over Arg in a mice model to increase plasma and tissue concentrations of Arg and Cit, and to restore intracellular NO production in the intestine [110]. Cit was also more effective than Arg in enhancing plasma (> 2-fold) and tissue (> 10-fold in liver and renal tissues) concentrations of Arg and NO production in arginase-injected mice [111]. Cit (100 μM) completely prevents glucose-induced arginase activity in bovine aortic endothelial cells and restore NOS activity [99]. Supplementation with a dose of 2 g/d Cit for 1 month also decreases arginase activity by 30% in patients with type 2 diabetes and elevates NO production [99]. Superiority of exogenous Cit over Arg may be extrapolated to other conditions characterized by elevated arginase activity including hemolysis, sepsis, asthma, and liver diseases.

Conclusion

As summarized in Table 2, current evidence indicates the preference for Cit over Arg administration for NO production. Because of more efficient intestinal absorption, low FPM, and high renal reabsorption, oral Cit compared with same dose of Arg provides more systemic available Arg in a sustainable manner. Cit is a more powerful NO precursor than Arg because: (1) exogenous Cit increases intracellular Arg pools, (2) Cit transporters and ASS-ASL enzymes are co-localized and co-induced with NOS enzymes, and (3) Cit recycling pathway is found in NO-producing cells. In contrast, imported Arg seems to be more available for arginase rather than the NOSs, which

effectively limits its accessibility for NO synthesis and shuttles Arg into other cellular pathways (e.g., production of ornithine and polyamines). The evidence strongly supports the hypothesis that extracellular sources of Arg can be utilized for other cellular requirements than NO production. On the other hand, side-effects (e.g., induction of arginase, disruption of ADMA metabolism, and inhibition of Cit recycling pathway), inhibit the usefulness of Arg supplementation for NO production. Since Cit is superior over Arg as a safe and effective precursor of NO production, its supplementation has further justification in medical practice.

References

1.   Morris SM, Jr. Arginine Metabolism: Boundaries of Our Knowledge. *The Journal of nutrition.* 2007;137(6):1602S-1609S.

2.   Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J.* 1998;336 (Pt 1)(Pt 1):1-17.

3.   Dong JY, Qin LQ, Zhang Z, et al. Effect of oral L-arginine supplementation on blood pressure: a meta-analysis of randomized, double-blind, placebo-controlled trials. *American heart journal.* 2011;162(6):959-965.

4.   McRae MP. Therapeutic Benefits of l-Arginine: An Umbrella Review of Meta-analyses. *Journal of chiropractic medicine.* 2016;15(3):184-189.

5.   Fisman EZ, Tenenbaum A, Shapira I, Pines A, Motro M. The nitric oxide pathway: is L-arginine a gate to the new millennium medicine? A meta-analysis of L-arginine effects. *Journal of medicine.* 1999;30(3-4):131-148.

6.   Zhu Q, Yue X, Tian QY, et al. Effect of L-arginine supplementation on blood pressure in pregnant women: a meta-analysis of placebo-controlled trials. *Hypertension in pregnancy.* 2013;32(1):32-41.

7.   Lucotti P, Monti L, Setola E, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. *Metabolism: clinical and experimental.* 2009;58(9):1270-1276.

8.   Castillo L, deRojas TC, Chapman TE, et al. Splanchnic metabolism of dietary arginine in relation to nitric oxide synthesis in normal adult man. *Proceedings of the National Academy of Sciences of the United States of America.* 1993;90(1):193-197.

9.   Wu G. Intestinal mucosal amino acid catabolism. *The Journal of nutrition.* 1998;128(8):1249-1252.

10.   Mariotti F, Petzke KJ, Bonnet D, et al. Kinetics of the utilization of dietary arginine for nitric oxide and urea synthesis: insight into the arginine-nitric oxide metabolic system in humans. *The American journal of clinical nutrition.* 2013;97(5):972-979.

11.   Castillo L, Beaumier L, Ajami AM, Young VR. Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. *Proceedings of the National Academy of Sciences of the United States of America.* 1996;93(21):11460-11465.

12.   Luiking YC, Castillo L, Deutz NE. Arginine, citrulline, and nitric oxide. In: *Modern Nutrition in Health and Disease: Eleventh Edition.* Wolters Kluwer Health Adis (ESP); 2012:477-486.

13. Papadia C, Osowska S, Cynober L, Forbes A. Citrulline in health and disease. Review on human studies. *Clinical nutrition (Edinburgh, Scotland).* 2017.

14. Allerton TD, Proctor DN, Stephens JM, Dugas TR, Spielmann G, Irving BA. l-Citrulline Supplementation: Impact on Cardiometabolic Health. *Nutrients.* 2018;10(7):921.

15. Romero MJ, Platt DH, Caldwell RB, Caldwell RW. Therapeutic use of citrulline in cardiovascular disease. *Cardiovascular drug reviews.* 2006;24(3-4):275-290.

16. Figueroa A, Jaime SJ, Morita M, Gonzales JU, Moinard C. L-Citrulline Supports Vascular and Muscular Benefits of Exercise Training in Older Adults. *Exercise and sport sciences reviews.* 2020;48(3):133-139.

17. Gonzalez AM, Trexler ET. Effects of Citrulline Supplementation on Exercise Performance in Humans: A Review of the Current Literature. *Journal of strength and conditioning research.* 2020;34(5):1480-1495.

18. Schwedhelm E, Maas R, Freese R, et al. Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: impact on nitric oxide metabolism. *British journal of clinical pharmacology.* 2008;65(1):51-59.

19. Morris SM, Jr. Regulation of enzymes of urea and arginine synthesis. *Annual review of nutrition.* 1992;12:81-101.

20. Cynober L, Le Boucher J, Vasson M-P. Arginine metabolism in mammals. *The Journal of Nutritional Biochemistry.* 1995;6(8):402-413.

21. Adams MR, Forsyth CJ, Jessup W, Robinson J, Celermajer DS. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. *J Am Coll Cardiol.* 1995;26(4):1054-1061.

22. Bahri S, Curis E, El Wafi F-Z, et al. Mechanisms and kinetics of citrulline uptake in a model of human intestinal epithelial cells. *Clinical Nutrition.* 2008;27(6):872-880.

23. Mohan S, Wu CC, Shin S, Fung HL. Continuous exposure to L-arginine induces oxidative stress and physiological tolerance in cultured human endothelial cells. *Amino acids.* 2012;43(3):1179-1188.

24. Walker HA, McGing E, Fisher I, et al. Endothelium-dependent vasodilation is independent of the plasma L-arginine/ADMA ratio in men with stable angina: Lack of effect of oral l-arginine on endothelial function, oxidative stress and exercise performance. *Journal of the American College of Cardiology.* 2001;38(2):499-505.

25. Schulman SP, Becker LC, Kass DA, et al. L-arginine therapy in acute myocardial infarction: the Vascular Interaction With Age in Myocardial Infarction (VINTAGE MI) randomized clinical trial. *Jama.* 2006;295(1):58-64.

26. Bahri S, Zerrouk N, Aussel C, et al. Citrulline: from metabolism to therapeutic use. *Nutrition (Burbank, Los Angeles County, Calif).* 2013;29(3):479-484.

27. Kaore SN, Amane HS, Kaore NM. Citrulline: pharmacological perspectives and its role as an emerging biomarker in future. *Fundamental & clinical pharmacology.* 2013;27(1):35-50.

28. Morris SM, Jr. Arginine Metabolism Revisited. *The Journal of nutrition.* 2016;146(12):2579S-2586S.

29. Lüneburg N, Xanthakis V, Schwedhelm E, et al. Reference intervals for plasma L-arginine and the L-arginine:asymmetric dimethylarginine ratio in the Framingham Offspring Cohort. *The Journal of nutrition.* 2011;141(12):2186-2190.

30. Castillo L, Ajami A, Branch S, et al. Plasma arginine kinetics in adult man: Response to an arginine-free diet. *Metabolism: clinical and experimental.* 1994;43(1):114-122.

31. Castillo L, Chapman TE, Sanchez M, et al. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. *Proceedings of the National Academy of Sciences of the United States of America.* 1993;90(16):7749-7753.

32. Castillo L, Sanchez M, Chapman TE, Ajami A, Burke JF, Young VR. The plasma flux and oxidation rate of ornithine adaptively decline with restricted arginine intake. *Proceedings of the National Academy of Sciences of the United States of America.* 1994;91(14):6393-6397.

33. van de Poll MCG, Ligthart-Melis GC, Boelens PG, Deutz NEP, van Leeuwen PAM, Dejong CHC. Intestinal and hepatic metabolism of glutamine and citrulline in humans. *J Physiol.* 2007;581(Pt 2):819-827.

34. El-Hattab AW, Hsu JW, Emrick LT, et al. Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Molecular genetics and metabolism.* 2012;105(4):607-614.

35. Ligthart-Melis GC, van de Poll MC, Boelens PG, Dejong CH, Deutz NE, van Leeuwen PA. Glutamine is an important precursor for de novo synthesis of arginine in humans. *The American journal of clinical nutrition.* 2008;87(5):1282-1289.

36. Barbul A. Arginine: biochemistry, physiology, and therapeutic implications. *JPEN Journal of parenteral and enteral nutrition.* 1986;10(2):227-238.

37.   Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. *The American journal of physiology.* 1981;241(6):E473-480.

38.   Bryk J, Ochoa JB, Correia MITD, Munera-Seeley V, Popovic PJ. Effect of Citrulline and Glutamine on Nitric Oxide Production in RAW 264.7 Cells in an Arginine-Depleted Environment. *Journal of Parenteral and Enteral Nutrition.* 2008;32(4):377-383.

39.   Simon A, Plies L, Habermeier A, Martine U, Reining M, Closs EI. Role of neutral amino acid transport and protein breakdown for substrate supply of nitric oxide synthase in human endothelial cells. *Circulation research.* 2003;93(9):813-820.

40.   Rimando AM, Perkins-Veazie PM. Determination of citrulline in watermelon rind. *Journal of chromatography A.* 2005;1078(1-2):196-200.

41.   Kao C, Hsu J, Bandi V, Jahoor F. Alterations in glutamine metabolism and its conversion to citrulline in sepsis. *Am J Physiol Endocrinol Metab.* 2013;304(12):E1359-1364.

42.   Crenn P, Coudray-Lucas C, Thuillier F, Cynober L, Messing B. Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. *Gastroenterology.* 2000;119(6):1496-1505.

43.   Nagasaka H, Yorifuji T, Egawa H, et al. Characteristics of NO cycle coupling with urea cycle in non-hyperammonemic carriers of ornithine transcarbamylase deficiency. *Molecular genetics and metabolism.* 2013;109(3):251-254.

44.   Curis E, Nicolis I, Moinard C, et al. Almost all about citrulline in mammals. *Amino acids.* 2005;29(3):177-205.

45.   van de Poll MC, Siroen MP, van Leeuwen PA, et al. Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. *The American journal of clinical nutrition.* 2007;85(1):167-172.

46.   Marini JC. Arginine and ornithine are the main precursors for citrulline synthesis in mice. *The Journal of nutrition.* 2012;142(3):572-580.

47.   Tomlinson C, Rafii M, Ball RO, Pencharz PB. Arginine can be synthesized from enteral proline in healthy adult humans. *The Journal of nutrition.* 2011;141(8):1432-1436.

48.   Cynober LA. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. *Nutrition (Burbank, Los Angeles County, Calif).* 2002;18(9):761-766.

49. Fulton MD, Brown T, Zheng YG. The Biological Axis of Protein Arginine Methylation and Asymmetric Dimethylarginine. *Int J Mol Sci.* 2019;20(13).

50. Leiper JM, Vallance P. The synthesis and metabolism of asymmetric dimethylarginine (ADMA). *European Journal of Clinical Pharmacology.* 2006;62(1):33-38.

51. Castillo L, Chapman TE, Yu YM, Ajami A, Burke JF, Young VR. Dietary arginine uptake by the splanchnic region in adult humans. *The American journal of physiology.* 1993;265(4 Pt 1):E532-539.

52. Castillo L, Sanchez M, Vogt J, et al. Plasma arginine, citrulline, and ornithine kinetics in adults, with observations on nitric oxide synthesis. *The American journal of physiology.* 1995;268(2 Pt 1):E360-367.

53. Moinard C, Nicolis I, Neveux N, Darquy S, Benazeth S, Cynober L. Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. *The British journal of nutrition.* 2008;99(4):855-862.

54. Hellier MD, Holdsworth CD, Perrett D. Dibasic amino acid absorption in man. *Gastroenterology.* 1973;65(4):613-618.

55. Mourad F, O'Donnell L, Andre E, et al. L-Arginine, nitric oxide, and intestinal secretion: studies in rat jejunum in vivo. *Gut.* 1996;39(4):539-544.

56. Deferrari G, Garibotto G, Robaudo C, Sala M, Tizianello A. Splanchnic exchange of amino acids after amino acid ingestion in patients with chronic renal insufficiency. *The American journal of clinical nutrition.* 1988;48(1):72-83.

57. O'Sullivan D, Brosnan JT, Brosnan ME. Catabolism of arginine and ornithine in the perfused rat liver: effect of dietary protein and of glucagon. *Am J Physiol Endocrinol Metab.* 2000;278(3):E516-521.

58. Agarwal U, Didelija IC, Yuan Y, Wang X, Marini JC. Supplemental Citrulline Is More Efficient Than Arginine in Increasing Systemic Arginine Availability in Mice. *The Journal of nutrition.* 2017;147(4):596-602.

59. Miyanaka K, Gotoh T, Nagasaki A, et al. Immunohistochemical Localization of Arginase II and Other Enzymes of Arginine Metabolism in Rat Kidney and Liver. *The Histochemical Journal.* 1998;30(10):741-751.

60. Brosnan ME, Brosnan JT. Hepatic glutamate metabolism: a tale of 2 hepatocytes. *The American journal of clinical nutrition.* 2009;90(3):857s-861s.

61. O'Sullivan D, Brosnan JT, Brosnan ME. Hepatic zonation of the catabolism of arginine and ornithine in the perfused rat liver. *Biochem J.* 1998;330 (Pt 2)(Pt 2):627-632.

62. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *The American journal of physiology.* 1995;269(3 Pt 2):R621-629.

63. Ozaki M, Gotoh T, Nagasaki A, et al. Expression of arginase II and related enzymes in the rat small intestine and kidney. *Journal of biochemistry.* 1999;125(3):586-593.

64. Watford M. The urea cycle: a two-compartment system. *Essays in biochemistry.* 1991;26:49-58.

65. Cheung CW, Cohen NS, Raijman L. Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. *The Journal of biological chemistry.* 1989;264(7):4038-4044.

66. Wettstein M, Gerok W, Haussinger D. Endotoxin-induced nitric oxide synthesis in the perfused rat liver: effects of L-arginine and ammonium chloride. *Hepatology (Baltimore, Md).* 1994;19(3):641-647.

67. Seiler N, Bolkenius FN, Knodgen B. The influence of catabolic reactions on polyamine excretion. *Biochem J.* 1985;225(1):219-226.

68. Tangphao O, Grossmann M, Chalon S, Hoffman BB, Blaschke TF. Pharmacokinetics of intravenous and oral L-arginine in normal volunteers. *British journal of clinical pharmacology.* 1999;47(3):261-266.

69. Vadgama JV, Evered DF. Characteristics of L-citrulline transport across rat small intestine in vitro. *Pediatric research.* 1992;32(4):472-478.

70. Neis EP, Sabrkhany S, Hundscheid I, et al. Human splanchnic amino-acid metabolism. 2017;49(1):161-172.

71. Bode-Boger SM, Boger RH, Galland A, Tsikas D, Frolich JC. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamic relationship. *British journal of clinical pharmacology.* 1998;46(5):489-497.

72. Wu G, Bazer FW, Cudd TA, et al. Pharmacokinetics and safety of arginine supplementation in animals. *The Journal of nutrition.* 2007;137(6 Suppl 2):1673s-1680s.

73. Rougé C, Des Robert C, Robins A, et al. Manipulation of citrulline availability in humans. *American Journal of Physiology-Gastrointestinal and Liver Physiology.* 2007;293(5):G1061-G1067.

74. Kim I-Y, Schutzler SE, Schrader A, et al. Acute ingestion of citrulline stimulates nitric oxide synthesis but does not increase blood flow in healthy young and older adults with heart failure. *Am J Physiol Endocrinol Metab.* 2015;309(11):E915-E924.

75.   Avogaro A, Toffolo G, Kiwanuka E, de Kreutzenberg SV, Tessari P, Cobelli C. L-arginine-nitric oxide kinetics in normal and type 2 diabetic subjects: a stable-labelled 15N arginine approach. *Diabetes.* 2003;52(3):795-802.

76.   Argaman Z, Young VR, Noviski N, et al. Arginine and nitric oxide metabolism in critically ill septic pediatric patients. *Critical care medicine.* 2003;31(2):591-597.

77.   Beaumier L, Castillo L, Ajami AM, Young VR. Urea cycle intermediate kinetics and nitrate excretion at normal and "therapeutic" intakes of arginine in humans. *The American journal of physiology.* 1995;269(5 Pt 1):E884-896.

78.   Leaf CD, Wishnok JS, Tannenbaum SR. L-arginine is a precursor for nitrate biosynthesis in humans. *Biochemical and biophysical research communications.* 1989;163(2):1032-1037.

79.   Böger RH, Tsikas D, Bode-Böger SM, Phivthong-ngam L, Schwedhelm E, Frölich JC. Hypercholesterolemia impairs basal nitric oxide synthase turnover rate: a study investigating the conversion of l-[guanidino-15N2]-arginine to 15N-labeled nitrate by gas chromatography–mass spectrometry. *Nitric oxide : biology and chemistry.* 2004;11(1):1-8.

80.   Poeze M, Bruins MJ, Kessels F, Luiking YC, Lamers WH, Deutz NEP. Effects of L-arginine pretreatment on nitric oxide metabolism and hepatosplanchnic perfusion during porcine endotoxemia. *The American journal of clinical nutrition.* 2011;93(6):1237-1247.

81.   Rapovy SM, Zhao J, Bricker RL, Schmidt SM, Setchell KDR, Qualls JE. Differential Requirements for L-Citrulline and L-Arginine during Antimycobacterial Macrophage Activity. *The Journal of Immunology.* 2015;195(7):3293-3300.

82.   Mori M, Gotoh T. Regulation of Nitric Oxide Production by Arginine Metabolic Enzymes. *Biochemical and biophysical research communications.* 2000;275(3):715-719.

83.   Flam BR, Eichler DC, Solomonson LP. Endothelial nitric oxide production is tightly coupled to the citrulline-NO cycle. *Nitric oxide : biology and chemistry.* 2007;17(3-4):115-121.

84.   Closs EI, Scheld JS, Sharafi M, Forstermann U. Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Molecular pharmacology.* 2000;57(1):68-74.

85.   Erez A, Nagamani SC, Shchelochkov OA, et al. Requirement of argininosuccinate lyase for systemic nitric oxide production. *Nature medicine.* 2011;17(12):1619-1626.

86. McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *The Journal of biological chemistry.* 1997;272(50):31213-31216.

87. Li C, Huang W, Harris MB, Goolsby JM, Venema RC. Interaction of the endothelial nitric oxide synthase with the CAT-1 arginine transporter enhances NO release by a mechanism not involving arginine transport. *Biochem J.* 2005;386(Pt 3):567-574.

88. Morris SM, Jr. Arginine metabolism in vascular biology and disease. *Vascular medicine (London, England).* 2005;10 Suppl 1:S83-87.

89. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clinical and experimental pharmacology & physiology.* 2007;34(9):906-911.

90. Wu G, Meininger CJ. Impaired arginine metabolism and NO synthesis in coronary endothelial cells of the spontaneously diabetic BB rat. *The American journal of physiology.* 1995;269(4 Pt 2):H1312-1318.

91. Eligini S, Porro B, Lualdi A, et al. Nitric oxide synthetic pathway in red blood cells is impaired in coronary artery disease. *PloS one.* 2013;8(8):e66945-e66945.

92. Bahadoran Z, Carlstrom M. Nitric oxide: To be or not to be an endocrine hormone? 2020;229(1):e13443.

93. Caldwell RW, Rodriguez PC, Toque HA, Narayanan SP, Caldwell RB. Arginase: A Multifaceted Enzyme Important in Health and Disease. *Physiol Rev.* 2018;98(2):641-665.

94. Pernow J, Jung C. Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? *Cardiovascular research.* 2013;98(3):334-343.

95. Beleznai T, Feher A, Spielvogel D, Lansman SL, Bagi Z. Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. *American Journal of Physiology-Heart and Circulatory Physiology.* 2011;300(3):H777-H783.

96. Huynh NN, Chin-Dusting J. Amino acids, arginase and nitric oxide in vascular health. *Clinical and experimental pharmacology & physiology.* 2006;33(1-2):1-8.

97. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *The Journal of clinical investigation.* 1997;100(9):2153-2157.

98. Shemyakin A, Kövamees O, Rafnsson A, et al. Arginase Inhibition Improves Endothelial Function in Patients With Coronary Artery Disease and Type 2 Diabetes Mellitus. *Circulation.* 2012;126(25):2943-2950.

99.   Shatanawi A, Momani MS, Aqtash R, Hamdan MH, Caldwell RB, Caldwell RW. Abstract 15199: L-Citrulline Supplementation Reduces Plasma Arginase Activity in Type 2 Diabetes Patients. *Circulation.* 2015;132(suppl_3):A15199-A15199.

100.   Liu X, Xu X, Shang R, Chen Y. Asymmetric dimethylarginine (ADMA) as an important risk factor for the increased cardiovascular diseases and heart failure in chronic kidney disease. *Nitric oxide : biology and chemistry.* 2018;78:113-120.

101.   Zhou S, Zhu Q, Li X, et al. Asymmetric dimethylarginine and all-cause mortality: a systematic review and meta-analysis. *Scientific reports.* 2017;7:44692.

102.   Teerlink T. HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences.* 2007;851(1-2):21-29.

103.   Jabecka A, Ast J, Bogdaski P, et al. Oral L-arginine supplementation in patients with mild arterial hypertension and its effect on plasma level of asymmetric dimethylarginine, L-citruline, L-arginine and antioxidant status. *European review for medical and pharmacological sciences.* 2012;16(12):1665-1674.

104.   Wang J, Sim AS, Wang XL, Wilcken DE. L-arginine regulates asymmetric dimethylarginine metabolism by inhibiting dimethylarginine dimethylaminohydrolase activity in hepatic (HepG2) cells. *Cellular and molecular life sciences : CMLS.* 2006;63(23):2838-2846.

105.   Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: Regulation and action. *Pharmacological Research.* 2009;60(6):448-460.

106.   McCarty MF. Asymmetric Dimethylarginine Is a Well Established Mediating Risk Factor for Cardiovascular Morbidity and Mortality-Should Patients with Elevated Levels Be Supplemented with Citrulline? *Healthcare (Basel, Switzerland).* 2016;4(3).

107.   Brunetti-Pierri N, Erez A, Shchelochkov O, Craigen W, Lee B. Systemic hypertension in two patients with ASL deficiency: a result of nitric oxide deficiency? *Molecular genetics and metabolism.* 2009;98(1-2):195-197.

108.   Nagamani SCS, Campeau PM, Shchelochkov OA, et al. Nitric-oxide supplementation for treatment of long-term complications in argininosuccinic aciduria. *Am J Hum Genet.* 2012;90(5):836-846.

109.	Little JA, Hauser KP, Martyr SE, et al. Hematologic, biochemical, and cardiopulmonary effects of L-arginine supplementation or phosphodiesterase 5 inhibition in patients with sickle cell disease who are on hydroxyurea therapy. *European journal of haematology.* 2009;82(4):315-321.

110.	Wijnands KA, Vink H, Briede JJ, et al. Citrulline a more suitable substrate than arginine to restore NO production and the microcirculation during endotoxemia. *PloS one.* 2012;7(5):e37439.

111.	Wijnands KA, Meesters DM, van Barneveld KW, et al. Citrulline Supplementation Improves Organ Perfusion and Arginine Availability under Conditions with Enhanced Arginase Activity. *Nutrients.* 2015;7(7):5217-5238.

112.	Morris CR, Morris SM, Jr., Hagar W, et al. Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? *American journal of respiratory and critical care medicine.* 2003;168(1):63-69.

113.	Morris CR, Kuypers FA, Larkin S, et al. Arginine therapy: a novel strategy to induce nitric oxide production in sickle cell disease. *British journal of haematology.* 2000;111(2):498-500.

114.	Waugh WH, Daeschner CW, 3rd, Files BA, McConnell ME, Strandjord SE. Oral citrulline as arginine precursor may be beneficial in sickle cell disease: early phase two results. *J Natl Med Assoc.* 2001;93(10):363-371.

115.	Clemmesen JO, Kondrup J, Ott P. Splanchnic and leg exchange of amino acids and ammonia in acute liver failure. *Gastroenterology.* 2000;118(6):1131-1139.

116.	Rashid J, Kumar SS, Job KM, Liu X, Fike CD, Sherwin CMT. Therapeutic Potential of Citrulline as an Arginine Supplement: A Clinical Pharmacology Review. 2020.

Table 1. Kinetic parameters of Arg and Cit metabolism

	Arg	Cit
Steady-state		
Plasma concentrations (μmol L^{-1})	80-120 [1,29]	20–40 [34,41-44]
Net splanchnic exchange (μmol min^{-1})	18-19 [56,115]	−12, −13 [56,115]
Renal clearance (ml min^{-1})	0.16 [73]	0.27 [73]
Urinary excretion (%)	0.11 [73]	0.20 [73]
Urinary concentrations (μmol/mmol of Cr)	1.2 [73]	0.9 [73]
Post-prandial		
Intestinal absorption		
V_{max}	46.3 μmol min^{-1} 30 cm^{-1} [54]	2.16-3.58 μmol min^{-1} g^{-1} [22]
K_m (mmol L^{-1})	3.8	0.46-0.67
Renal clearance (ml min^{-1} kg^{-1})	1.17 [68]	0.005 [73]
Systemic availability (%)	10-30 [51,56,68]	100
Half-life (h)	1 [71]	0.65–1.14 [53]
C_{max} (μmol L^{-1})	310 (a dose of 6 g) [71]	515-3849 (doses of 2-15 g) [53]
T_{max} (min)	90 (a dose of 6 g) [71]	38-56 (doses of 2-15 g) [53]

C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; V_{max}, the maximum rate of absorption; K_m, the Michaelis-Menten constant

Table 2. Preference for Cit over Arg as a natural NO precursor

	Cit	Arg	Reference
Intestinal absorption	More efficient	—	[22]
FPM (bypassing liver-intestine metabolism)	Limited or no	High	[15,37]
Renal reabsorption	More efficient	—	[53,73]
Providing systemic available Arg	Higher	—	[51,66,18,116]
Effect on arginase	Inhibition	Induction	[20, 99]
Utilization by NO-producing cells	More efficient	—	[47,48, 11]
Contribution to NO production	More efficient	—	[47,48, 11]
Supply intracellular Arg pools in the sub-cellular compartments associated with NO production	More efficient	—	[39, 39]
Utilization by the cells at a high-demand NO conditions	More efficient	—	[81]
Potentiating NO synthesis in pathologic conditions with elevated arginase activity	More efficient	—	[105,106, 110,111]
Safety and tolerability even at high doses	Higher	—	[13,24,25]

FPM, first pass metabolism; NO, nitric oxide; Arg, arginine; NOSs, nitric oxide synthases; MELAS syndrome, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
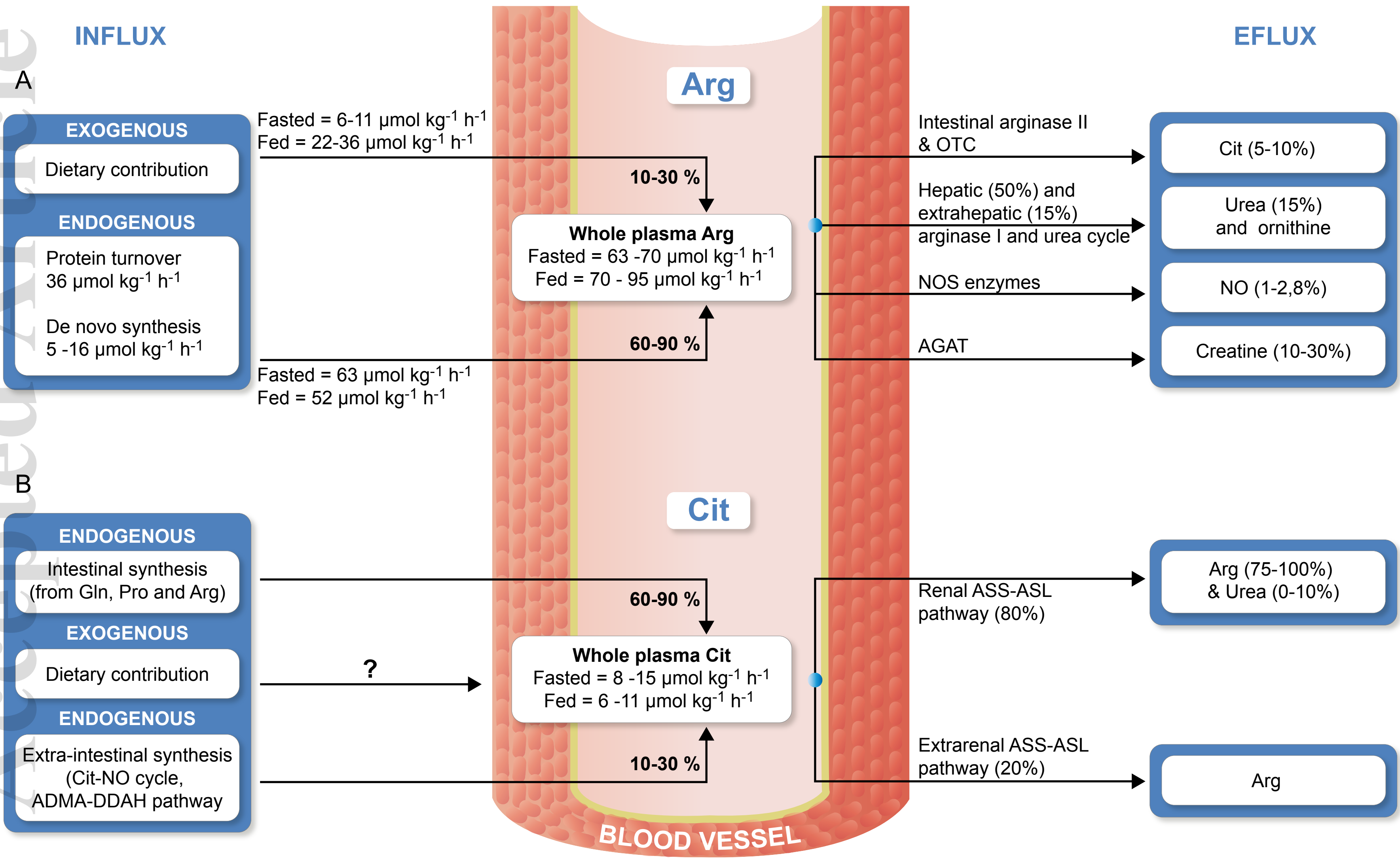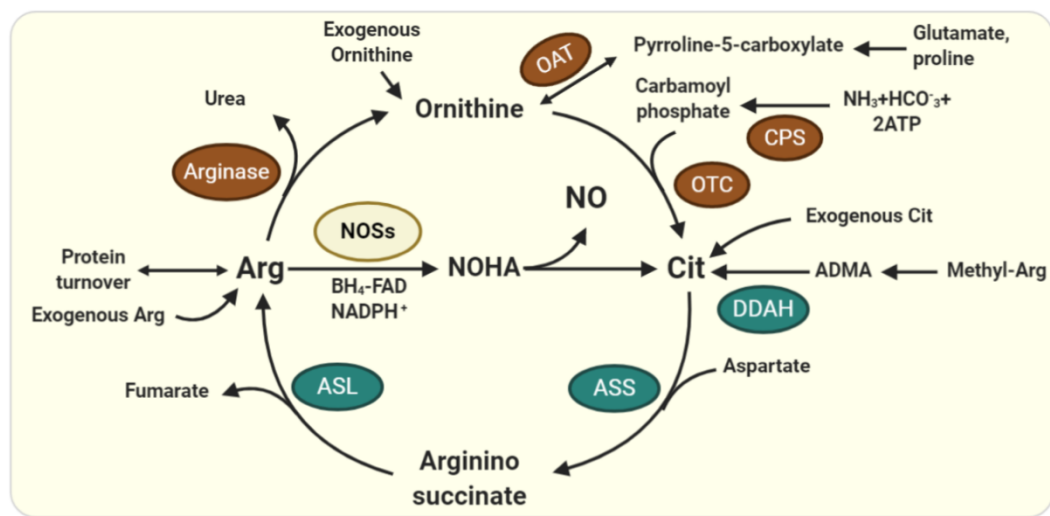
Legend to Figures:

FIGURE 1 A, Determinants of Arg plasma flux and Arg catabolic pathways. Both exogenous (10-30%) and endogenous sources (60-90%) contribute in Arg plasma flux. A large fraction of Arg flux is diverted into arginase I and urea cycle (50% by hepatic and 15% by extrahepatic arginase I); 10-30% of Arg fraction is utilized for synthesis of creatine (using l-arginine:glycine amidinotransferase; AGAT), and only a minor fraction is shuttled into NO production. B, Cit flux is supported mainly by the endogenous sources. The intestinal biosynthesis of Cit provides 60-90% of Cit flux and is mainly supported by the conversion of glutamine to Cit. Extraintestinal synthesis accounting for 10-30% of Cit flux, is mainly relayed on both Cit-NO cycle and ADMA-DDAH pathway. Cit flux is entirely used for Arg production via both renal and non-renal ASS-ASL pathways. ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase. Arg, Arginine; Cit, Citrulline, Gln, glutamine, Pro, proline.

FIGURE 2 Overview of Cit-NO cycle. ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; DDAH, dimethylarginine dimethylaminohydrolase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; ADMA, asymmetric dimethylarginine; CPS, carbamoyl phosphate synthetase; NO, nitric oxide; NOS, NO synthase; Arg, arginine; Cit, citrulline; NOHA, N(G)-hydroxy-L-arginine.

FIGURE 3 The fate of orally ingested doses of Arg. Ingested Arg is extracted and utilized during the first pass metabolism of the splanchnic region (intestinal and hepatic bed); Arg entered in the enterocytes is mainly confined into partial urea cycle by both cytosolic and mitochondrial arginases to produce urea, ornithine, Cit, proline, polyamines and CO_2. The Arg delivered into the liver by the portal vein, is subjected to further catabolism via arginase I and is mainly converted to urea; within the splanchnic metabolism, a minor fraction of the ingested dose of Arg is converted to NO. Finally, about 10-30% of ingested doses are available for circulation; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; GS, glutamine synthetase.

FIGURE 4 Arginine pools and cellular compartments of Cit transporters with NOSs and Cit utilizing enzymes (ASS-ASL). Extracellular Arg can only support intracellular Arg pool-I, which is accessible by iNOS rather than eNOS. Other Arg pools (pool-IIA and pool-IIB) are not freely exchangeable with extracellular space and are supported by intracellular synthesized Arg via ASS-ASL; these pools are therefore dependent to extracellular Cit or Cit recycled by Cit-NO cycle and ADMA-DDAH pathway. Effective compartment of CAT-1 and CAT-2B with eNOS and ASS-ASL makes extracellular Cit as an available source of NO production. ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; PRMTs, protein arginine methyltransferases; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase.

INFLUX

EFLUX

A

Arg

EXOGENOUS

Dietary contribution

Fasted = 6-11 µmol kg^{-1} h^{-1}
Fed = 22-36 µmol kg^{-1} h^{-1}

Intestinal arginase II
& OTC

Cit (5-10%)

10-30 %

ENDOGENOUS

Protein turnover
36 µmol kg^{-1} h^{-1}

De novo synthesis
5 -16 µmol kg^{-1} h^{-1}

Whole plasma Arg
Fasted = 63 -70 µmol kg^{-1} h^{-1}
Fed = 70 - 95 µmol kg^{-1} h^{-1}

Hepatic (50%) and
extrahepatic (15%)
arginase I and urea cycle

Urea (15%)
and ornithine

NOS enzymes

NO (1-2,8%)

60-90 %

AGAT

Creatine (10-30%)

Fasted = 63 µmol kg^{-1} h^{-1}
Fed = 52 µmol kg^{-1} h^{-1}

B

Cit

ENDOGENOUS

Intestinal synthesis
(from Gln, Pro and Arg)

Renal ASS-ASL
pathway (80%)

Arg (75-100%)
& Urea (0-10%)

60-90 %

EXOGENOUS

Dietary contribution

?

Whole plasma Cit
Fasted = 8 -15 µmol kg^{-1} h^{-1}
Fed = 6 -11 µmol kg^{-1} h^{-1}

ENDOGENOUS

Extra-intestinal synthesis
(Cit-NO cycle,
ADMA-DDAH pathway

10-30 %

Extrarenal ASS-ASL
pathway (20%)

Arg

BLOOD VESSEL

apha_13572_f2.tif

Oral Arg
(up to 9 g)

Intestine

Arg → Arginase II
urea cycle enzymes
Enterocyte cytosol → **Urea+ornithine**

Arginase II, OAT, OTC, ODC
Entrocyte mitochondria → **Pro, Cit, polyamines**

NOS enzymes → **NO**

Portal vein

Liver

Arg → Arginase I
urea cycle enzymes
Periportal hepatocytes → **Urea Ornithine**

Arginase I, OAT, GS
Perivenous hepatocytes → **Polyamines, Gln, CO2**

NOS enzymes → **NO**

Total splanchnic FPM of Arg
(40-60%) → **10-30% systemic availablity of Arg**

apha_13572_f3.tif

apha_13572_f4.tif