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Nutrition and amino acid metabolism in critically ill patients

Mechteld
A.R. Vermeulen

Nutrition and amino acid metabolism in critically ill patients
Thesis, VU University Medical Center, Amsterdam, The Netherlands

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VRIJE UNIVERSITEIT

Nutrition and amino acid metabolism in critically ill patients

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promotor: prof.dr. P.A.M. van Leeuwen
copromotor: prof.dr. J.B. van Goudoever

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1. General Introduction and outline of the thesis

Mechteld A.R. Vermeulen, Marcel C.G. van de Poll,
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Petrousjka van den Tol, Petra G. Boelens, Paul A.M. van Leeuwen

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**Specific amino acids in the critically ill patient:
Exogenous glutamine/arginine: A common denominator?**

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Introduction

Glutamine, the most abundant free amino acid, regulates many biological functions in preserving cellular function [1]. In critically ill patients, rapid depletion of glutamine occurs in plasma and muscle. Plasma levels tend to drop for several weeks and are related to increased ICU mortality [2]. This state of glutamine depletion is mainly caused by the high consumption of glutamine by rapidly dividing cells such as enterocytes, bone marrow cells, and lymphocytes.
In the absence of adequate glutamine intake, compensatory release from muscle stores is unable to restore plasma levels. As a result, glutamine becomes essential in the critically ill because its availability relies on supplementary administration.

A number of trials using glutamine-enriched nutrition revealed a positive effect on infectious morbidity, length of ICU stay, and mortality in the critically ill [1, 3, 4].
However, some trials failed to show beneficial effects [5-7]. Traditionally, beneficial effects were explained as glutamine acting as a key respiratory fuel and nitrogen donor for rapidly dividing cells. However, recent translational research has revealed that this explanation is incomplete and oversimplified. Glutamine is now recognized as a modulator of the expression of many genes associated with metabolism, cell defence and repair, cytokine production and intracellular signalling pathways [8].

A positive effect of glutamine administration on morbidity was shown in a meta-analysis. This effect was more pronounced when glutamine was given parenterally. However, on the whole a larger number of studies were performed with parenterally supplied glutamine [1]. It still remains unclear whether the route of administration of glutamine can determine its beneficial effect.

Apart from the route of administration, another point of interest is whether the beneficial effects of glutamine may potentially be mediated by the metabolism of glutamine derivatives such as citrulline or arginine. A study performed in 1994 already indicated that citrulline metabolized from glutamine can serve as a substrate for arginine synthesis in the kidney [9].
Literature indicates that citrulline can serve as a precursor for arginine synthesis on the whole body level [10, 11]. As glutamine can generate arginine by the citrulline pathway in the kidney, supplementing glutamine may potentially be a more physiological way to administer arginine in the metabolically stressed patient. Also, it is not clear whether arginine metabolism is altered if the route of glutamine administration is different.
A study showed a higher plasma level of arginine when glutamine was parenterally supplied[12].

It is thus conceivable that the route of administration changes the course of the disease as important substrates such as arginine are possibly generated in different amounts by the parenteral route if compared to the enteral route.

This introduction will focus on the pathway from glutamine to arginine generation and elaborates whether the route of administration determines the metabolic fate of glutamine. Furthermore, we focus on the newly-discovered effects of glutamine relevant to the critically ill.

Arginine: functions of a semi-essential amino acid

Arginine is one of the most versatile amino acids in the human body. Since being targeted for research, considerable new insights on arginine functions and mechanisms have emerged over the past years.

Arginine serves as an essential component of protein, for synthesis of creatine, agmatine, proline, ornithine and polyamines. It serves as a modulator of lymphocyte proliferation and differentiation, and as an important intermediate in the urea cycle [13]. Arginine plays an important role in processes such as cell growth and proliferation, the healing of wounds, collagen synthesis, immune function and it stimulates the secretion of hormones such as insulin, prolactin, glucagon and growth hormone [14]. Importantly, arginine can serve as a substrate for nitric oxide generation.

Arginine is a semi-essential amino acid for humans and most mammals. As a semi-essential (conditionally indispensable) amino acid, arginine can be generated in sufficient amounts under normal conditions from de novo synthesis in the kidney whereas the body becomes dependent on additional exogenous arginine supply during times of metabolic stress. Dietary arginine uptake takes place in the small intestine. About 40% is already catabolized by the intestine before it enters the circulation [15]. During the fasting state, approximately 85% of the arginine entering the circulation is derived from protein turnover, and the remainder originates from de novo synthesis [15].

Arginine synthesis in the kidney takes place in the proximal tubule [16] and involves the conversion of citrulline into arginine. Arginine catabolism can take place through various metabolic pathways, which will be explained in more detail further in this introduction.

A crucial enzyme that facilitates the breakdown of arginine is arginase. There are two iso-enzymes: arginase I and II. The first serves the detoxification of ammonia in the urea cycle and the second catalyzes the metabolism of arginine mostly in the intestinal lumen [17]. Arginase activity yields urea and ornithine. Ornithine is further metabolized into citrulline and L-1-pyrroline-5-carboxylate, the latter leading to formation of L-proline, glutamate and polyamines [13]. Via ornithine and the polyamines, arginine is important for cell growth and differentiation [18]. Proline is important in collagen formation and plays a role in wound healing [13].

A large amount of the circulating arginine is used for protein synthesis and is involved in the formation of creatine, which serves as a precursor for creatinine, and agmatine [13, 18]. Agmatine appears to be an important cell signaling molecule, whereas more roles of agmatine in the human body remain under investigation [15].

Arginine is probably best known as the sole precursor for nitric oxide through its enzymatic conversion by nitric oxide synthase (NOS) with concomitant formation of NO and citrulline [18, 19]. NO is an important vasodilator that also prevents cellular adhesion to the vascular wall, inhibits platelet activation and retards the development of atherosclerosis in experimental models. Arginine derived NO plays an important role in the regulation of inflammation and immunity.

There are three isoforms of NOS: neuronal NOS in neurons (nNOS or NOS1), inducible NOS mainly in macrophages (iNOS or type 2) and endothelial NOS in endothelial cells (eNOS or type 3). Key differences between eNOS and nNOS versus iNOS are that the first two are always present in cells, are Ca^{2+} dependent and produce low levels of NO constitutively [19]. On the other hand, iNOS is rate limited by co-factors, and is highly inducible [13, 14]. Stimuli that can induce iNOS are T-helper 1 cytokines (IL-1, TNF,) and endotoxin [20]. In sepsis, iNOS is seen as an important pathogenic factor [21, 22].

By activating NOS and the forthcoming NO formation, immunity is enhanced by a variety of immune responders such as increased proliferation of lymphocytes and monocytes, enhanced T-helper cell formation, activation of macrophage cytotoxity, the reinforcement of natural killer cells, increased phagocytosis and enhanced cytokine production [14]. Thus, NOS derived NO has immunoregulatory functions [18].

NO produced from arginine by eNOS is important for preservation of organ blood flow by regulating vascular tone, influencing the interaction of white blood cells and platelets with the endothelium, and limiting the development of neointimal hyperplasia by reducing apoptosis of vascular smooth muscle cells [23].

Because of its arthrogenic role, depletion of NO is associated with cardiovascular pathology [23]. Nitric oxide is very important in regulating flow through major organs such as heart, liver, kidney and gut especially during stress. In relation to stressful events, specifically after major vascular surgery, arginine may become conditionally essential. Recent studies show decreased plasma levels and combined with the presence of endotoxemia, a low blood flow through the gut and liver was noticed [24]. It was postulated that low arginine levels per se did not influence flow, but the combination of both endotoxemia and low arginine levels may compromise the host. Thus a positive effect may be expected when arginine is supplemented during surgery. Several studies and a meta-analysis [25, 26] indicate that this indeed may be the case. However during critical illness extra arginine may detoriate the host and even a higher mortality may be the consequence [17, 27]. On the other hand, few studies are performed with solely arginine as supplementation. In a larger number of studies, arginine is supplemented in combination with omega fatty acids and nucleotides. It is not clear why extra arginine may give rise to a higher mortality.
Possible controversial effects and results may be explained by dose-dependent or case-related issues together with the contribution of NO to oxidative stress. This may be due to its capacity to highly react with superoxide, a free radical produced in the vascular wall by cellular oxidases that react with NO by forming peroxynitrate [28]. This may lead to damage to the host. Moreover it may diminish the cofactor BH_4 which supports the endothelial NOS. Diminished levels of BH_4 form a risk factor for cardiovascular disease [28].
Peroxynitrate may also generate nitrosylated

thiols that function as endogenous NO donors by inducing vasorelaxation and inhibiting platelet aggregation.

These factors have been suggested to play a role in the high mortality seen in critically ill patients when given extra arginine. However, no definite proof is presented yet. More studies are needed to prove whether arginine supplementation is really detrimental or that the those results are driven by a small amount of patients.

For instance, patients suffering from severe liver failure have shown plasma levels of arginine above 300 μmol, hence a few of these patients may have given rise to a wrong conclusion [29].

A competitive inhibitor of NOS is asymmetric dimethylarginine (ADMA). In critically ill patients, elevated ADMA levels were described and were considered a strong and independent risk factor for intensive care patients [18, 23, 30].

Glutamine: functions of a semi essential amino acid

Glutamine is one of the most abundant amino acids in the human body.
Its intracellular concentration is much higher than that of most other amino acids. Main functions of glutamine are mobilization of nitrogen stored in muscles, maintaining acid-base balance and serving as fuel for rapidly dividing cells such as enterocytes, lymphocytes, and macrophages [31-33].

The endogenous glutamine flux is determined by protein breakdown and by de novo synthesis. Most glutamine is synthesized in skeletal muscle and to lesser extent in brain, heart, lung and adipose tissue.

Glutamine is a nonessential amino acid under physiologic circumstances. However, during pathophysiological processes, such as sepsis, cancer, after trauma or surgery, glutamine demand by central organs such as the liver and the immune system is increased.

This leads to increased mobilization of the muscular glutamine pool. However the increased glutamine demand during metabolic stress may not be met by the increased endogenous flux. Thus glutamine can become an essential amino acid, warranting exogenous supplementation.

As a precursor for purine and pyrimidine, glutamine plays an important role in the preservation of the synthesis of DNA and mRNA necessary for proliferation of lymphocytes and repair of macrophages [34].
Neutrophils increase their phagocytic activity and rate of production of superoxide, when glutamine is offered in a dose-dependent manner [34, 35]. Furthermore glutamine improves neutrophil mitochondrial function.

Heat shock proteins

Heat shock proteins (HSPs) protect cells by preventing apoptosis and by acting as molecular chaperones to correct stress-related misfolding of proteins. A heat shock transcription factor (HSF-1) is activated in response to several stressors and increases the transcription of heat shock protein genes [36, 37]. The availability of glutamine in cell cultures is necessary for the expression of HSPs [38, 39]. Furthermore, glutamine supplementation enhances the expression of HSPs in in vitro and in vivo studies [40]. In an in vitro experiment, glutamine supplementation increased HSF-1 activation to protect cells against heat stress; this effect was lost in HSF-1 gene knockout cells [41]. In a mice model of abdominal sepsis, glutamine increased plasma levels of HSPs, decreased nuclear factor kappa B (NFκB) activation and reduced mortality; these effects were lost in HSF-1 gene knockout mice [42, 43].

In trauma patients, an early increase of HSP plasma levels has been associated with survival [44]. In surgical intensive care patients, glutamine-enriched parenteral nutrition increased HSP levels after 7 days, a finding that correlated with a decrease in ICU length of stay [45]. This effect of glutamine may be time dependent, since infusion of alanyl-glutamine in human experimental endotoxemia fails to increase early HSP expression within 8 hours [43, 46].

Apoptosis

Apoptosis or programmed cell death probably influences cellular survival in the critically ill, since anti-apoptotic treatments improve survival in septic mice [47, 48].
Glutamine deprivation in mammalian gut and immune cell lines elicits apoptosis through cell type specific signalling mechanisms, whereas glutamine supplementation prevents tumour necrosis factor alpha (TNF-α) induced apoptosis in a human colonic cell line [49, 50]. This glutamine effect may be regulated by the activation of extra-cellular signal-related kinase (ERK) pathways that have an anti-apoptotic effect.
In rat intestinal cells, glutamine supplementation does indeed activate ERK pathways [51].
Also, in other cell lines, like Jurkat T cells (human T-lymphocyte cell line) and neutrophils (in rats and humans) glutamine supplementation increases anti-apoptotic protein Bcl-2 expression [8]. Glutamine modulates intracellular signalling pathways involved in apoptotic changes.
This may become a target of treatment in human sepsis[43].

Cytokine production

One of the first indications that glutamine could be a modulator of inflammatory cytokine production was found by Houdijk et al. in trauma patients [52]. For patients in this trial, glutamine-enriched enteral nutrition reduced levels of soluble TNF receptors.
More recent studies have focussed on the effect of glutamine on NFκB signalling pathways, which are involved in the initiation of inflammatory cytokine response.
In a septic rat model, e.g., a single dose of glutamine attenuated nuclear binding of NFκB and prevented the degradation of its inhibitory protein IκBα [53]. This coincided with reduced levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-18) and improved survival.
These results were lost in HSP gene knock-out mice, which suggests a role for HSPs in

the inflammatory response [42]. In mice with abdominal sepsis, Yeh et al. showed that glutamine supplementation reduced IL-6 expression in the lung, kidney and gut [54]. Combined, these data suggest that glutamine is able to induce an anti-inflammatory response that may prove beneficial to the critically ill. However, until now this could not be shown in human studies. Luo et al. showed that 0.5g/kg/day of the dipeptide alanyl-glutamine, whether enterally or parenterally administered, failed to affect the T-lymphocyte subset (CD-3, CD-4, CD-8) number, compared to unsupplied ICU patients [6]. In agreement, van den Berg et al. did not detect differences in IFγ, TNFα, IL-2, IL-4, IL-5 and IL-10 in very low birth weight infants [55]. Furthermore, perioperative supplementation of glutamine to cardiopulmonary bypass patients did not appear to affect IL-1, IL-6, TNF-alpha, IL-8 [43, 56].

Cellular immunity

The expression of major histocompatibility complex class II antigens (HLA-DR) on monocytes is a prerequisite for effective antigen presentation to CD4+ T cells, which is an important component of immune response to infection [57]. Surgery, trauma and inflammation reduce HLA-DR expression on monocytes [58]. Spittler et al. showed that postoperative administration of glycyl-glutamine was accompanied by a smaller reduction in HLA-DR expression compared to a control group [58]. The preservation of HLA-DR expression on monocytes was also found in trauma patients receiving glutamine-enriched enteral nutrition [59].
Both parenteral and enteral glutamine administration seem to preserve HLA-DR expression on monocytes[43].

Glutamine and the gut

As glutamine is important fuel for the enterocyte, intestinal consumption is important for maintaining the integrity of the intestinal barrier with subsequent prevention of bacterial translocation and, through stimulation of the gut-associated immune system, prevention gut barrier atrophy [34, 43].

Intestinal integrity is secured by intact cellular linings and their tight junctions. It is jeopardized by glutamine depletion, oxidative stress, increased NO production, pro-inflammatory cytokines and prolonged parenteral nutrition (for a review see De Souza & Greene [60]). Gut barrier dysfunction may promote bacterial and endotoxin translocation, which induces systemic inflammatory response [61]. Gut-associated lymphoid tissue (GALT) adds to the protective mechanisms of gut barrier function. Harmful effects of bacterial translocation are prevented by GALT, by intra-epithelial lymphocytes, the lymphoid cells of the lamina propria, the Peyers patches and mesenteric lymph nodes.

In vitro and animal experimental studies indicate that the gut protective function of glutamine involves more than just providing oxidative fuel to the intestinal epithelium [61, 62]. It includes the release and preservation of glutathione (GSH), attenuation of inducible NO synthase (iNOS) expression maintaining tight junction functionality, rapid reduction of protein kinase C mediated hyperpermeability in intestinal cells, attenuation of cytokine release, increased HSP expression and reduced gut mucosal apoptosis [53, 63, 64]. TNFα associated bacterial translocation is reduced in vitro if glutamine is added [34].

Some clinical trials on glutamine supplementation show benefits in terms of reduced gut permeability and lower rates of gram negative bacteria in trauma and burn patients [65]. Unfortunately, studying bacterial translocation in humans is very difficult due to methodological limitations, which may explain the lack of hard evidence for an effect of glutamine [66].

Glutamine, oxidative stress and its relation to taurine and to acid base balance

The effect of glutamine on oxidative stress is achieved through conversion into glutamate, which can subsequently be incorporated into the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine). Glutathione is the most important regulator of the intracellular redox potential and it has a protective effect against oxidative injury [34]. Glutathione exists in a reduced and oxidized form. Oxygen-derived free radicals and peroxides are scavenged and reduced by reduced glutathione, leading to protection of the cell membrane[43].

In trauma, glutathione levels decrease in skeletal muscle, indicating an ongoing oxidative stressed situation. After supplementing glutamine, this decrease in glutathione levels was not observed, suggesting increased glutathione synthesis [67].

Glutamine may also regulate the plasma levels of taurine [68]. Taurine is especially abundant in the cytosol of inflammatory cells, such as neutrophils. A major function is to trap chlorinated oxidants by producing non-toxic, long-lived taurine chloroamine, hereby protecting the cell from self-destruction. As taurine migrates to destroyed tissues within the neutrophil cytosol, it serves as an excellent entrapment for free radicals (6). Taurine also serves as an osmoregulator [69]. By regulating cell membrane passage of solutes, and being extra potent when extracellular sodium increases, taurine prevents extracellular water retention. Taurine is generally considered a non-essential amino acid, but increased requirement is suggested by declined taurine levels in response to surgical injury, trauma, sepsis and other critical illnesses [70]. In response to enteral glutamine supplementation in trauma patients, an increase in taurine plasma concentration was observed [34, 68]. However the exact mechanism concerning this relation is not yet elucidated.

Glutamine plays an important role in acid base balance. Acidosis leads to increased interorgan transport of glutamine and its further breakdown by the kidney to serve nitrogen disposal [71].

Attenuation of inducible nitric oxide synthase expression

In rats, enteral glutamine supplementation reduced NO production by modulating iNOS gene expression [72]. Another study in rats undergoing cardiopulmonary bypass surgery, showed that parenteral glutamine decreased iNOS activity, reduced IL-6 and IL-8 levels, increased HSP expression and reduced myocardial and lung damage [73]. In rats with abdominal sepsis, parenteral glutamine reduced iNOS expression, inflammatory response and mortality [53]. The mechanisms by which glutamine reduces iNOS expression are not yet clear, but it seems that the glucosamine pathway and inhibition of

citrulline mediated NO production may be involved [74,43].

Preservation of insulin sensitivity

Glutamine has been found to preserve insulin sensitivity in animal studies and critically ill patients. Severe illness is accompanied by reduced insulin sensitivity, which leads to hyperglycaemia. Van den Berghe et al. showed that tight glucose control through the administration of insulin, reduced morbidity and mortality in ICU patients [75].

In dogs, Borel et al. showed that glutamine blunted the effect of insulin on glucose production and enhanced insulin-mediated glucose utilization [76]. In rats on a high fat diet, glutamine prevented weight gain by inducing insulin resistance in adipose tissue, thus preventing lipogenesis, while it improved liver and peripheral insulin sensitivity. Furthermore, decreased TNF-α production and NFκB activation were observed, suggesting that glutamine influences glucose and fat metabolism by reducing pro-inflammatory pathways [77].

Bakalar showed that in multiple trauma patients, parenteral nutrition enriched with glutamine preserved insulin sensitivity [78]. In a multicenter randomized controlled trial in 114 surgical and trauma ICU patients, glutamine supplementation resulted in reduced hyperglycaemic events [79]. The exact mechanism by which glutamine acts on insulin sensitivity is not known and this is currently under investigation.

Thus, glutamine not only serves as metabolic fuel, stored in the muscle and ready for action when it comes to starvation and cachexia, it also regulates directly and indirectly the immune response, by serving as fuel for immune cells or by promoting the capacity of the substrates.

Biochemical relations between glutamine and arginine

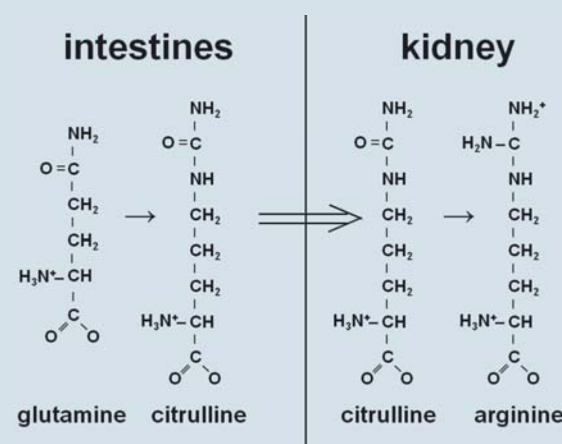
As described above, similar beneficial effects have been ascribed to glutamine-enriched and arginine-enriched nutrition. In this context it is interesting to pay some attention to the biochemical relations between glutamine and arginine.

Synthesis and catabolism of glutamine

Glutamine contains two nitrogenous groups per molecule, an amino-group and an amide group at the other end of the molecule It can be synthesized from glutamate and ammonia by the enzyme glutamine synthetase (GS, EC 6.3.2.1). This conversion occurs in the liver and skeletal muscle [80]. The endogenous whole body plasma turnover (uptake from and release into the plasma) of glutamine approximates 300 μmol/kg/h in adult humans [81].

Approximately 15% of this amount is metabolized by the intestinal mucosa and another 15% is taken up by the kidney [82]. The first step in glutamine degradation is deamidation, catalyzed by the enzyme glutaminase (GSE, EC 3.5.1.2), yielding equimolar amounts of glutamate and ammonia [83]. The intestines release all ammonia derived during this process to the portal vein, where after it is taken up by the liver to serve ureagenesis and glutamine synthesis. The remaining glutamate is only partly released into the portal vein, whereas an important part is deaminated in a transamination process with pyruvate, giving rise to α-ketoglutarate and alanine, which are subsequently released into the portal vein [83]. Approximately 12% of glutamate yielded by intestinal glutamine deamidation is eventually converted to citrulline in a multi-step intra-intestinal metabolic pathway [82]. In this pathway the amino-group and the carbon skeleton of the original glutamine molecule are preserved (Figure 1). Since the activity of citrulline metabolizing enzymes is low in the adult human intestine (see below) the major part of citrulline derived from intestinal glutamine metabolism is released into the portal vein.

Figure 1 - Conversion of glutamine to arginine via the intestinal-renal pathway.

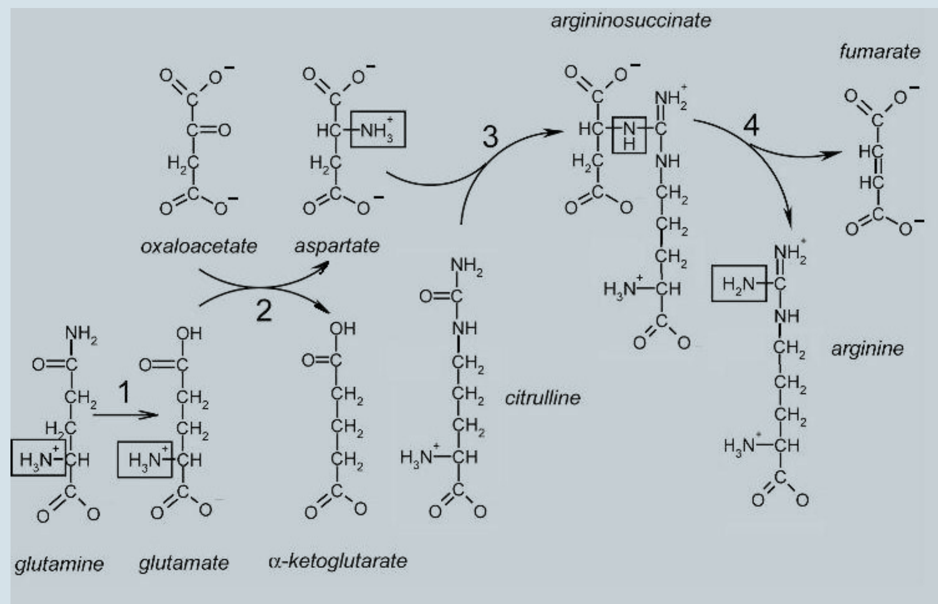


Intestinal breakdown of glutamine yields citrulline, which is released into the portal vein. Citrulline passes the liver roughly unchanged, reaching the systemic circulation. Thereafter it is taken up by the kidneys and converted to arginine. Throughout this pathway the general amino acid structure of the original molecule (amino and carboxyl-groups and α-carbon) is preserved, with reactions being limited to the side-chain of the molecule. Consequently glutamine becomes a true precursor for arginine donating its amino group, carboxyl group and part of its carbon backbone as a whole. Single arrow: intra-organ conversion, double arrow: inter-organ transport.

Synthesis and catabolism of arginine

The whole body flux of arginine into plasma approximates 65 µmol/kg/h and is predominantly derived from protein breakdown.
In post-absorptive humans about 15% of the circulating arginine pool is derived from endogenous de novo synthesis [10].
The immediate precursor for the endogenous synthesis of arginine is citrulline. The conversion of citrulline to arginine occurs by transamination of an aspartate amino group to the ureido group of citrulline, and is catalyzed by the enzymes argininosuccinate synthetase (ASS, EC 6.3.4.5) and argininosuccinate lyase (ASL, EC 4.3.2.1) [84] (Figure 2). It is a commonly held view that the kidney is the only organ that releases newly synthesized arginine into the circulation since it is the only organ known to take up citrulline and release arginine [71]. It should be noted that ASS and ASL are also widely expressed in for example hepatocytes, endothelial cells and macrophages [85].
In these cells arginine is being formed and broken down in intracellular cycles such as the urea cycle (liver) and the NO cycle (endothelial cells, macrophages) [86].
Although the synthesis and breakdown rate of arginine within such cycles may exceed the plasma turnover rate of arginine several-fold, compartmentalization of metabolites prohibits the net release of newly formed arginine to the circulation [87]. In neonates the gut is the major organ for systemic release of newly formed arginine and some of the sparse human studies have indicated that there is still rudimental ASS and ASL activity in the adult human gut, leading to intestinal arginine de novo synthesis [88].
However, we were unable to confirm these findings in a recent study concerning intestinal metabolism in human adults [82]. The same study showed stoichiometric renal citrulline uptake and arginine release at a rate of 2 – 3 µmol/kg/h, which thus approximates only 50% of the total flux of newly synthesized arginine into the plasma [82].

Figure 2 - Alternative route for the glutamine amino-group to serve as a precursor for arginine synthesis.



After uptake by most cells glutamine is largely deamidated to form glutamate by glutaminase (1). Glutamate is an important nitrogen donor in a large number of transamination processes, yielding amongst others substantial amounts of aspartate. The latter process is catalyzed by the enzyme aspartate aminotransferase (2). Arginine is synthesized from citrulline in a two-step reaction catalyzed by argininosuccinate synthetase (3) and argininosuccinate lyase (4). This process requires the input of an amino nitrogen atom derived from aspartate. Therefore glutamine becomes glutamate (1), glutamate donates is amino-nitrogen to aspartate via transamination (2). This amino nitrogen is subsequently incorporated in argininosuccinate (3) and arginine (4). The route of the amino nitrogen atom through this pathway is indicated by boxes.

The role of citrulline in the common pathway

Houdijk and colleagues observed an increase of plasma arginine levels in trauma patients receiving supplemental glutamine [52]. In line with these findings they showed that renal arginine synthesis increased in rats receiving a glutamine-enriched enteral diet [9].
As described earlier, it has been suggested that arginine-enriched diets yield similar clinical effects as glutamine-enriched diets in selected patient groups. Regarding the similar effects of these interventions and given the apparent relation between glutamine supplementation and arginine plasma concentration and renal synthesis, it can be

hypothesized that the beneficial effects of glutamine supplementation are partly mediated by the effects of glutamine on arginine metabolism. Several explanations can be given for this effect of glutamine on arginine metabolism. Given the connectivity between intestinal glutamine breakdown (yielding citrulline) and renal arginine synthesis (requiring citrulline), it is an attractive hypothesis that the relation between exogenous glutamine administration and increasing arginine plasma levels is mediated by an interorgan pathway. According to this hypothesis the glutamine carbon backbone is transported from the intestines to the kidney in the form of citrulline to become a precursor for the endogenous de novo synthesis of arginine [9, 13, 89, 90]. Integral part of this hypothesis is the assumption, based upon early ex vivo findings of Windmueller and Spaeth [90], that the liver is unable to extract citrulline from the portal circulation. Probably due to the attractiveness of this hypothesis it has frequently been presented by numerous authors as a given fact, although it actually has remained untested for several decades.

More importantly, the quantitative importance of the intestinal glutamine metabolism for the renal synthesis of arginine has remained unaddressed.

Using infusion of [amino-^{15}N] glutamine in mice, Boelens et al. were the first to show the actual existence of an interorgan pathway resulting in the conversion of glutamine to citrulline and arginine [91, 92]. Subsequent studies in humans confirmed these data and elucidated the quantitative importance of glutamine for newly synthesized plasma arginine [93]. Human data revealed that at least 65% of all newly synthesized plasma arginine is ultimately generated from the glutamine carbon skeleton. On the other hand it should be realized that in postabsorptive humans the flux through this pathway represents less than 5% of whole body glutamine plasma flux. Another important consideration that is important for critical appraisal of the interorgan hypothesis is the recent observation that in contrast to hitherto held views, the human liver extracts citrulline from the portal circulation in quantitatively important amounts [94].

Contribution of intestinal metabolism to the common pathway

In the intestinal-renal pathway of glutamine to arginine, citrulline is the pivotal interorgan transporter and it appears that the intestinal extraction and metabolism of glutamine is crucial to supply citrulline to the kidneys via the systemic circulation. Glutamine extraction by the intestines is relatively poorly correlated with systemic glutamine supply [88], whereas it has been shown that approximately 50% of glutamine is extracted and metabolized by the splanchnic organs after enteral administration [95]. These observations may indicate that the route of administration (enteral or intravenous) determines the extraction of exogenously supplied glutamine. In the light of the discussed

intestinal-renal transport of glutamine it may be expected that during enteral glutamine administration the intestinal conversion of glutamine to citrulline, which is the crucial step for this interorgan pathway, may be higher than during intravenous glutamine administration. In agreement with this hypothesis it has been shown in humans that enteral administration of glutamine (as the dipeptide alanyl-glutamine) leads to higher systemic citrulline levels than intravenous alanyl-glutamine administration [12].
Somewhat surprising, despite these higher systemic citrulline levels, enteral administration of alanyl-glutamine did not lead to higher systemic arginine levels. Although these studies clearly show that enteral glutamine administration stimulates intestinal glutamine metabolism, they do not provide evidence that enteral glutamine supplementation stimulates renal arginine synthesis morethan intravenous glutamine supplementation.
The reasons for these unexpected findings remain speculative, but it is clear that due to the high splanchnic extraction of enterally administered glutamine (first pass effect), enteral glutamine supplementation leads to lower systemic glutamine levels than intravenous glutamine supplementation [12].
The fact that arginine levels are apparently similar in patients and animals with high glutamine and low citrulline levels (after intravenous glutamine administration) on one hand, and low glutamine and high citrulline levels (after enteral glutamine administration) on the other hand, suggests that systemic citrulline levels are not the only determinant of systemic arginine concentration.
It may very well be that systemic arginine levels in patients receiving glutamine intravenously, can be explained by other mechanisms than the conversion of plasma citrulline to plasma arginine.

Other potential interactions between glutamine and arginine

Glutamine can serve arginine synthesis in a substrate-product relationship independent of its conversion to citrulline [96]. As pointed out above arginine is synthesized from citrulline by a transamination reaction involving aspartate, catalyzed by ASS and ASL. After being taken up, glutamine is rapidly converted to glutamate by most cells. Glutamate serves as an amino group donor in numerous transamination processes, and is an important precursor for aspartate, one of the most abundant intracellular amino acids. Taken these facts together it can be conceived that plasma glutamine serves as a nitrogen donor for arginine synthesis [96].
However, the quantitative importance of glutamine for this aspect of arginine synthesis is unclear, as is the question to what extent the availability of aspartate is rate limiting for the activity of ASS in arginine excreting cells.

On a further note, glutamine supplementation can change the activity of membrane transporters [97], which can enhance arginine efflux from arginine excreting cells [98]. The potential of glutamine to modulate plasma levels of amino acids by other mechanisms than simple substrate-product relationships has been shown before in vivo in patients receiving glutamine-enriched nutrition, who displayed increased plasma levels of taurine [68], a sulfur amino acid that cannot be generated from glutamine [99].

Parenteral versus enteral administration of glutamine

The route of administration of glutamine determines whether the gut is the first organ to receive glutamine. Glutamine is an important substrate for intestinal metabolism, as described in the glutamine section of this paper. It was already mentioned that approximately 50% of glutamine is extracted and metabolized by the splanchnic organs after enteral administration of glutamine in humans [95]. Apart from a local importance of glutamine in the intestines, glutamine is known to be the most important source of intestinally synthesized citrulline, which is the sole precursor for the biosynthesis of arginine.

Our group took special interest in further exploring the effect of the route of administration, enteral or parenteral, of glutamine as a free molecule or the dipeptide alanyl-glutamine, on citrulline and arginine metabolism. First, we compared the effects of parenteral and enteral administered dipeptide , the stable alternative for free glutamine and suitable substrate for application in clinical practice [100-102], on the plasma concentration of dipeptide, glutamine, glutamate, citrulline and arginine in patients prior to surgery [12]. Patients received 20 g of alanyl-glutamine intravenously or enterally on two consecutive days. After parenteral infusion a steady state plasma alanyl-glutamine response was measured, whereas no response was observed after enteral infusion, suggesting a 100% extraction of the dipeptide by the intestines and the liver. Both routes of administration of the dipeptide resulted in an increased plasma glutamine response, which was observed to be highest after parenteral infusion of the dipeptide (peak increment with IV: 468±57 vs. EN administration: 207±42). This finding illustrates the first pass splanchnic extraction of enterally provided glutamine. Splanchnic metabolism of enterally provided glutamine was further supported by the finding that the plasma concentration of glutamate was only enhanced with enteral administration of the dipeptide. Furthermore, the plasma concentration of citrulline was observed to be enhanced most with enteral administration of the dipeptide. As already mentioned in the biochemical elaboration of the pathway between glutamine and arginine, only parenteral infusion of alanyl-glutamine increased the plasma arginine concentration. Further exploration of the observed differences between intravenous and enteral administration of glutamine was done with help of stable isotopes.

Boelens et al. [91, 92] included 43 male Swiss mice in an experiment to study the pathway of glutamine into citrulline and arginine at whole body and organ level in mice, comparing two routes (parenteral or enteral) of administration and two forms of isotopically labeled glutamine (glutamine or alanyl-glutamine). Glutamine was converted into citrulline and arginine when [^{15}N]glutamine and alanyl-[^{15}N]glutamine were given by the enteral or parenteral route, demonstrating the metabolic pathway between glutamine and arginine. The contribution of glutamine to the de novo synthesis of citrulline and arginine, expressed as a percentage of the whole body turnover of citrulline, was observed to be higher in the enteral groups (Cit: Ala-Gln: 32%, Gln: 36% and Arg: Ala-Gln: 16%, Gln: 20%) compared to the intravenous groups (Cit: Ala-Gln: 15%, Gln: 24% and Arg: Ala-Gln: 6%, Gln: 16%) ($p<0.005$). Therefore, it was concluded that the route of administration affects the contribution of glutamine to the de novo synthesis of arginine in mice. However, absolute de novo synthesis of arginine was not affected by the route of administration. Also, renal synthesis of arginine was found to be similar for both routes of administration. These results agree with the observation that enhanced plasma levels of citrulline with enteral administration of glutamine in humans not necessarily result in enhancement of plasma levels of arginine [12]. The humans in the aforementioned study [12], and the mice in the study by Boelens et al. [91, 92] demonstrated high plasma levels of arginine. Therefore the kidney may not have been triggered to generate even more arginine. Prins et al. [103] showed in rats that low plasma levels of arginine trigger renal synthesis of arginine and Houdijk et al. [52] observed an increase in plasma arginine levels in trauma patients with glutamine enriched enteral nutrition, compared to low plasma levels of arginine at the onset of the study.

In human postabsorptive surgical patients the metabolic fate of glutamine was further investigated revealing that intravenously administered glutamine in tracer dosages was a precursor for citrulline and arginine [104]. 83% Of the citrulline (5.1 μmol/kg/h) was derived from glutamine, 11% (4.9 μmol/kg/h) of subsequent plasma arginine appearance was derived from citrulline, representing 76% of the total plasma turnover of citrulline. Hence, 64% of the arginine was de novo synthesis originated from citrulline that primarily originated form glutamine.

On organ level, 50% of the plasma citrulline was taken up by the kidneys (7.2 μmol/kg/h) and resulted roughly in equimolar arginine release (6.2 μmol/kg/h), representing 22% of the total renal production of plasma arginine (57% of the WB plasma turnover of citrulline into arginine)[104]. The renal arginine production was therefore observed to exceed the renal citrulline to arginine conversion. The reason remained unclear but authors guessed it might have been caused by direct glutamine to arginine conversion, since glutamine was also observed to be taken up by the kidneys. Furthermore, absolute glutamine uptake by the portally drained viscera accounted for 19% of plasma glutamine turnover (456 μmol kg/h).

Subsequently 13% of glutamine taken up by the intestines was converted to citrulline and net PDV citrulline flux equaled total citrulline production. The liver was observed to actively uptake and release glutamine, with a net balance of zero. Release was calculated to account for 30% of the plasma glutamine appearance (73 μmol/kg/h). Hepatic citrulline net flux that was not significantly different from zero, although little citrulline was seen to be taken up by the liver (release was not significant to zero). Arginine release by PDV or liver was not observed [105].

When comparing above mentioned intravenous postabsorptive tracer results with enterally administered tracers, the following conclusions could be made: Arterial glutamine enrichment was lower in the enteral glutamine tracer group, and within this group higher glutamine fractional extraction occurred by the PDV. Also higher enrichment of citrulline was observed in the enteral group, accompanied by greater intestinal release of citrulline. Furthermore, enrichments of arginine were observed, which tended to be higher in the enteral group [106].
Renal uptake of citrulline was observed along with net release of arginine. Renal results did not differ in both groups.

Glutamine versus Alanyl-glutamine

Enriching nutrition with glutamine is pharmacologically complicated because glutamine has a relative aqueous instability [107].
Alanyl-glutamine, a dipeptide which is stable in a watery solution, was shown to be a good alternative [100, 101]. In human volunteers 50% of an intravenous bolus of alanyl-glutamine was eliminated from plasma within 4 minutes and the disappearance of the dipeptide was accompanied by a prompt equimolar release of alanine and glutamine [100].

In the previously mentioned experiments with mice by Boelens [91, 92], the effect of the molecular form (free glutamine or alanyl-glutamine) of exogenously supplied glutamine on the metabolism of glutamine was also investigated with both routes (parenteral or enteral) of administration. The splanchnic extraction of enterally provided glutamine, was observed to be higher after dipeptide than after free glutamine administration (53% vs. 39%), suggesting a preference of the mouse gut or liver for enterally provided alanyl-glutamine. However, a lower intestinal production of [2-^{15}N]citrulline was observed with administration of the dipeptide. Furthermore, the de novo synthesis of arginine from citrulline at whole body level, expressed as a percentage of the whole turnover of citrulline, tended to be higher in the free glutamine groups (IV: IV: 16±1; EN: 20±2) when compared with the dipeptide groups (6±0.7; EN: 16±1). These observations suggest that intestinal metabolism of glutamine not necessarily explains the higher splanchnic extraction of the dipeptide. It can be speculated that the dipeptide was released partly intact by the intestines into the portal vein, to be further metabolized by the liver.
Anyhow, these findings support the hypothesis that the molecular form of glutamine affects the contribution of glutamine to the synthesis of arginine.

Quantitative studies in the ICU

Since Heyland's recent publication on glutamine and antioxidant supplementation in critically ill patients recently, the use of high dose glutamine in shock patients has been part of debate [108]. It has now been argued that safety is not guaranteed when high dosages (0.35 g/kg/d parenterally and 30 g/d enterally) of glutamine is administered to patients with multiple organ failure. Although (meta-)analyses in critically ill patients demonstrated that supplementation with glutamine as free molecule or dipeptide (up to 0.35g/kg/day) results in a favourable clinical outcome as reflected by a reduction in infectious morbidity (trauma and medical ICU patients), mortality and a reduction in length of hospital stay in severely ill patients [52, 79, 109-116], in critically ill patients the metabolic fate of glutamine is still unclear. Possible altered metabolism could be due to impaired enterocyte function because of injury, splanchnic ischemia, sepsis and starvation.
The quantitative stable isotope studies discussed in this chapter were performed in surgical postabsorptive mice and patients, but those results may differ in critically ill patients. Kao and Luijking investigated amino acid metabolism in septic ICU patients using stable isotope methodology. Kao showed an altered glutamine metabolism in fasted septic patients compared to healthy volunteers [117].
With enteral administration, they showed a more pronounced glutamine to citrulline conversion, as was observed earlier in non-critically ill patients [106]. Whole body citrulline production was lower in septic patients and both investigators observed diminished de novo arginine synthesis.
These findings strongly suggest that arginine availability is indeed at risk in septic patients [118, 119].

General conclusions

In summary, glutamine and arginine can both regulate similar functions in the human body. However specifically, they differ in utilizing their targets. Glutamine serves as fuel for the immune cells and can enhance the immune system through upgraded HLA-DR expression, enhanced neutrophil phagocytosis and an increase in HSP expression. Arginine enhances the immune system by modulation of lymphocyte proliferation and modification. Furthermore, arginine serves as a substrate for NO generation, through which it has an enhancing effect on processes such as lymphocyte proliferation and macrophage cytotoxity, as well as vasomodulatory effects. Glutamine may serve as a precursor for the important anti-oxidant glutathione.

As glutamine serves as a precursor of arginine through the citrulline-arginine pathway, glutamine may be of importance in regulating certain specific functions of arginine. Glutamine supplementation increases systemic arginine concentrations, suggesting a regulatory or biochemical relation between glutamine and arginine. Glutamine may become a precursor for arginine synthesis by donation of its carbon skeleton through an interorgan pathway involving intestinal conversion of glutamine to citrulline and renal conversion of citrulline to arginine.

It is known that excessive arginine supplementation may have adverse effects, probably due to NO-induced oxidative stress. As glutamine can be converted to arginine by the citrulline pathway in the kidney, supplementing glutamine may be a more physiological way to achieve the arginine benefits. Apart from a study by Heyland et al. using high dosages of enteral and intravenous glutamine next to anti-oxidants in shock patients, no studies showed any adverse effects, which might indicate a regulatory role of the body in optimizing plasma arginine concentrations [1, 108].

Alternatively glutamine may donate its amino nitrogen to arginine or enhance plasma levels of arginine by modulation of amino acid transporters. Enteral administration of glutamine enhances intestinal metabolism of glutamine and increases systemic citrulline levels. Interestingly this does not enhance systemic arginine levels compared with intravenous glutamine administration.

Rationale of this thesis

It is now clear that mice metabolism is unequal to human metabolism [104, 120, 121], that enteral glutamine administration has not the same effect as intravenous glutamine supply [106] and that the molecular from of glutamine also contributes to different quantities of its substrate citrulline and arginine.

However the role and metabolic fate of glutamine in the ICU is still not completely unravelled. In order to better understand function and fate of glutamine in this target group, this thesis discusses glutamine metabolism in greater detail with respect to the role of the dipeptide and the role of the gut, the effect of enteral feeding and the effect of a therapeutic dose of glutamine, and the consequences for protein metabolism and clinical parameters in ICU patients.

We know that differences exist between septic and relatively stable ICU patients and suggestions have been made that intestinal hyperpermeability, whether cause or effect, is related to the occurrence of sepsis, bacteraemia, and multiple organ failure (MOF) [66, 117-119, 122]. In vivo experiments, however, have not yet provided definitive evidence to support the claim that glutamine supplementation has a beneficial effect on gut permeability [60]. In contrast, in vitro experiments evidenced protective effect of glutamine on hyperpermeable cell lines [64, 123]. However it remains unclear whether the precise mechanism is mainly through (intra- or extracellular) conversion to glutamate. In Chapter 2 we present the results of an experimental model that allows differentiation between the effects of glutamine and glutamate on induced hyperpermeability in intestinal cell lines.

To further investigate the metabolic fate of alanyl-glutamine on organ level, we needed to verify previously used method on organ flow determination, since it is not clear whether Duplex Doppler Ultrasound (DDUS) during surgery or MRI before surgery is the tool to choose. In Chapter 3 we examined whether surgery and method (DDUS vs. MRI) affected flow values. In Chapter 4 and 5 we continued investigating the metabolic route of glutamine into citrulline and arginine. In Chapter 4, we present the results of a stable isotope study using labeled alanyl-glutamine in postprandial surgical patients. This study investigated both the enteral and the intravenous route of administration. Chapter 5 quantifies the arginine production from glutamine in surgical patients receiving a clinically relevant dose of intravenous glutamine dipeptide.

Further translating these and previous experiments into the clinical setting,

Chapter 6 discusses the results of an intervention study in which stable ICU patients were given an enteral dose of alanyl-glutamine, along with enteral and intravenous stable isotopes of glutamine, citrulline and arginine. To further unravel the ascribed beneficial effects of glutamine within the ICU,
Chapter 7 reveals the effects of an enteral dose of glutamine on protein metabolism in stable ICU patients, also studied by using stable isotope methodology. From stable to instable ICU patients, in Chapter 8 and 9 we studied plasma amino acid behavior in septic and cardiogenic shock patients.
In Chapter 8 we focused on arginine, its relation with markers of cardiac instability and asymmetric dimethyl-arginine (ADMA), the competitor for nitric oxide synthase-binding. Chapter 9 discusses the role of adequate enteral nutrition on amino acid plasma concentrations and amino acid deficiencies. We present a specific role for the sulfur amino acid taurine, that was in an earlier decade already observed to be related to glutamine metabolism [68].

By means of three introduced letters, Chapter 10 discusses the current opinion on glutamine metabolic studies and glutamine supply in the intensive care.

In Chapter 11 a general discussion and summary of the main results is given followed by a Dutch summary (Chapter 12).

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2. Glutamate reduces experimental intestinal hyperpermeability and facilitates glutamine support of gut integrity

Mechteld A.R. Vermeulen, Jeffrey de Jong, Mathijs J. Vaessen,
Paul A.M. van Leeuwen, Alexander P.J. Houdijk

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Abstract

Aim To assess whether glutamate plays a similar role to glutamine in preserving gut wall integrity.

Methods The effects of glutamine and glutamate on induced hyperpermeability in intestinal cell lines were studied. Paracellular hyperpermeability was induced in Caco2.BBE and HT-29CL.19A cell lines by adding phorbol -12,13-dibutyrate (PDB) apically, after which the effects of glutamine and glutamate on horseradish peroxidase (HRP) diffusion were studied. An inhibitor of glutamate transport (L-trans-pyrrolidine-2,4-dicarboxylic acid: trans-PDC) and an irreversible blocker (acivicin) of the extracellular glutamine to glutamate converting enzyme, γ-GT, were used.

Results Apical to basolateral HRP flux increased significantly compared to controls not exposed to PDB ($n = 30$, $P < 0.001$). Glutamine application reduced hyperpermeability by 19% and 39% in the respective cell lines. Glutamate application reduced hyperpermeability by 30% and 20%, respectively. Incubation of HT29CL.19A cells with acivicin and subsequent PDB and glutamine addition increased permeability levels. Incubation of Caco2.BBE cells with trans-PDC followed by PDB and glutamate addition also resulted in high permeability levels.

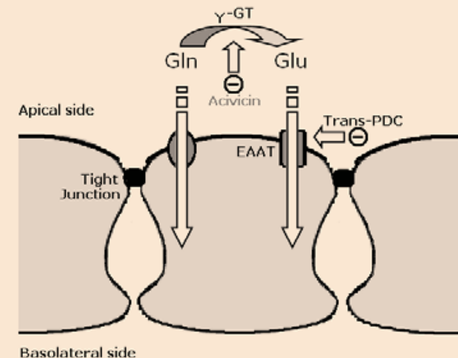
Conclusion Apical glutamate -similar to glutamine- can decrease induced paracellular hyperpermeability. Extracellular conversion of glutamine to glutamate and subsequent uptake of glutamate could be a pivotal step in the mechanism underlying the protective effect of glutamine.

Introduction

Intestinal hyperpermeability, whether cause or effect, seems to be related to the occurrence of sepsis, bacteraemia, and multiple organ failure (MOF) [1, 2]. Managing this change in gut physiology might contribute to substantial health improvement.
The semi-essential amino acid, glutamine, is thought to improve clinical outcome in these situations. It has been ascribed several properties that are supportive of intestinal cell function and relevant to cell survival [3].
Additionally, plasma and muscle glutamine concentrations drop dramatically in critically ill patients [4-6]. In vivo experiments, however, have not yet provided definitive evidence to support the claim that glutamine supplementation has a beneficial effect on gut permeability [7]. In contrast, in vitro experiments do show a positive influence of glutamine. Kouznetsova et al [8] induced hyperpermeability in the intestinal HT-29Cl.19A cell line and found that glutamine significantly reduced this increased permeability. Furthermore, Le Bacquer et al [9] demonstrated that glutamine helps to preserve adequate paracellular permeability levels in nutritionally deprived intestinal Caco-2 cells. The precise mechanisms underlying these findings remain to be clarified.Glutamate might play a pivotal role in the effects of glutamine therapy considering the metabolic fate of glutamine: it is mostly converted to glutamate, either intra- or extracellularly [10]. Welbourne et al [11] provide support for this theory by demonstrating that blocking the extracellular glutamine to glutamate converting enzyme γ-glutamyltransferase (γ-GT) and blocking glutamate uptake, both increase paracellular permeability in the proximal tubulelike LLC-PK_1-F^+ cells.

The aim of this study was to assess whether glutamate might play a similar role in the intestine (Figure 1). To do so, an experimental model that allows differentiation between the effects of glutamine and glutamate on induced hyperpermeability in intestinal cell lines was used.

Figure 1



Model illustrating intestinal cell lineage with tight junctions and EAAT transporter. The extracellular enzymatic conversion of glutamine to glutamate by γ-GT is shown on the apical side. The experimental design of the study using the γ-GT blocking enzyme, acivicin, and the blocker of the glutamate transporter, EAAT L-trans-pyrrolidine-2,4-dicarboxylic, is included in the figure.

Materials and methods

Study design

We created an experimental set-up using two intestinal cell lines: Caco2.BBE and HT-29CL.19A (both human colon adenocarcinoma derived cell lines). In culture, both cell lines exhibit polarity and apical brush-border membranes, similar to in vivo structure [12, 13]. Cells were therefore placed in a bicameral system to simulate a physiological situation in which they are exposed to distinct apical and basolateral compartments, and thereby allowing permeability experiments. Paracellular hyperpermeability was induced by adding phorbol-12, 13-dibutyrate (PDB) to the apical compartment after which the effects of glutamine and glutamate on horseradish peroxidase (HRP) diffusion were studied. To differentiate between the effect of glutamine andglutamate on permeability, an inhibitor of glutamate transport (L-trans-pyrrolidine-2,4-dicarboxylic acid: trans-PDC) and an irreversible blocker (acivicin) of the extracellular glutamine to glutamate converting enzyme, γ-GT, were used.

Cell culture

The HT29Cl.19A cell line, passage number 14-35 and the Caco2.BBE cell line, passage number 34-62, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The medium contained penicillin 40 mg/L, ampicillin 8 mg/L and streptomycin 9 mg/L. The cells were seeded in 12 cm^2 culture flasks which were placed in an incubator with a humidified atmosphere of 5% CO_2 and 95% O_2. The cells were subcultured on transparent filters (12 mm diameter; Falcon, Micronic, Lelystad, The Netherlands) for 14 (HT29Cl.19A) and 21 (Caco2.BBE) days to form confluent monolayers. The medium contained glutamine (2 mmol/L) and was replaced every other day.
During the last two days before the experiments, the cells were cultured in glutamine-free medium.

Flux experiments

The culture medium was discarded after cell cultivation and filters were rinsed with Ringer's solution (containing 117.5 mmol/L NaCl, 5.7 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$ and 27.8 mmol/L mannitol, kept at pH 7.4). Filters were placed in a bicameral system with 300 µL of Ringer's solution added to the apical chamber and 700 µL to the basolateral chamber.
The bicameral setup with filters was then placed in an incubator with humidified gas (5% CO_2, 95% O_2) where a temperature of 37°C was maintained.
After an equilibration period of 30 min, horseradish peroxidase (HRP, type IV; Sigma Chemical Co, St Louis, MO, USA) dissolved in Ringer's solution was added apically to reach a final concentration of 10^{-5} mol/L. For the next four hours, basolateral samples of 5 µL were taken, in triplicate, each hour and replaced by oxygenated Ringer's solution. The appearance of HRP in these samples was measured enzymatically. To this end, samples were mixed with 180 µL citrate buffer (0.1 mol/L citrate + 0.1 mol/L citric acid at pH 5.5) containing 3.6 µL bovine serum albumin (BSA 20 µL/mL).
Three samples of 25 µL were taken from the resulting mixture and were added to 200 µL substrate. Substrate was prepared by adding 340 µL TMB stock (3.3-5.5 tetramethylbenzidine) (6 mg/mL in H_20) and 200 µL 0.3% H_2O_2 to 20 mL citrate buffer.

Samples were then incubated for 30 min at normal room temperature, after which the positive samples were blue in colour. The reaction was stopped by adding 50 µL HCl (2 mol/L). The samples were read at 450 nm by a spectrophotometer. Data were recorded using Microplate Manager 5 Software, Bio-Rad Laboratories Ltd, United Kingdom. Experiments with PKC-mediated hyperpermeability were conducted by simultaneously adding 1 µmol/L of PDB and HRP to the apical chamber. The effects of L-glutamine (0.6 mmol/L) and L-glutamate (0.6 mmol/L) were (separately) studied by apical application with simultaneous PDB and HRP application. Acivicin experiments were conducted by incubating the cells with 10 µL of the following solution: 1.7 mg acivicin, dissolved in 50 µL HCl (2.0 mol/L) and 50 µL DES (buffering the medium). Inhibition of glutamate transporters EAAT 1-5 was achieved by pre-incubating the cells with 1 mmol/L trans-PDC.

Chemicals

Falcon filters were obtained from Micronic (Lelystad, The Netherlands), penicillin/streptomycin from Boehringer Mannheim (Almere, the Netherlands) and ampicillin from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). All other cell culture materials were obtained from Gibco (Breda, the Netherlands). Chemicals used for the Ringer's solution were obtained from Merck (Merck Nederland BV, Amsterdam).
PDB, L-glutamate, L-glutamine and trans-PDC were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands).

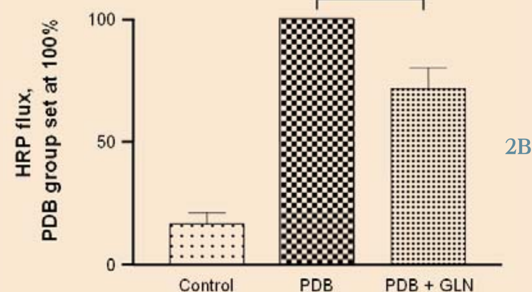
Data analysis

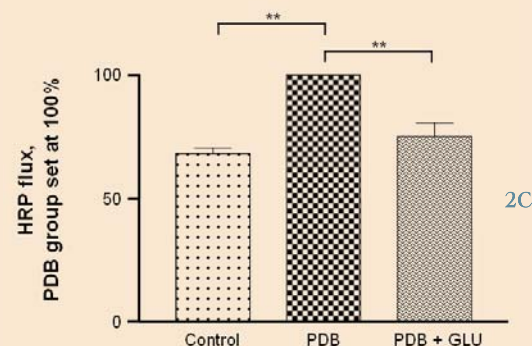
Statistical analyses of differences between groups were performed by one way ANOVA and the Tukey-Kramer test. A P-value < 0.05 was considered significant. HRP flux results are presented graphically as percentages of total flux. Total flux is defined by the HRP + PDB groups, which therefore represent the 100% mark. Graphpad Prism 3.03 for Windows ® (GraphPad Software Inc., California, USA) was used for analyses and graphical output.

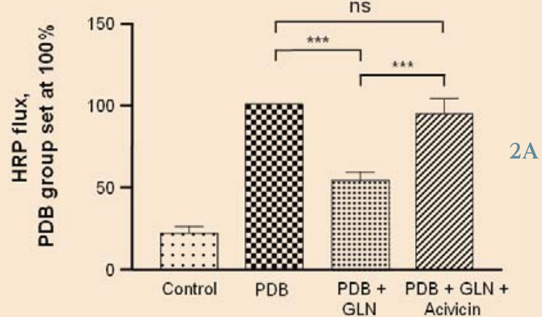
Results

Hyperpermeability was successfully induced by PDB stimulation: apical to basolateral HRP flux increased significantly in the HT29Cl.19A and the Caco2.BBE cell line (with a maximum after four hours) compared to controls not exposed to PDB (n = 30, P < 0.001). Cells in the PDB group defined the 100% mark, and all values were composed of triplicate measurements per group per experiment and were repeated 3-11 times. In HT29Cl.19A cells, glutamine application reduced hyperpermeability by 45% (n = 11, P < 0.001) (Figure 2A). In the Caco2.BBE cell line, glutamine application reduced hyperpermeability by 30% (n = 3, P < 0.05) (Figure 2B).

Glutamate application reduced hyperpermeability by 25% in the HT29Cl.19A cell line (n = 3, P < 0.01) (Figure 2C) and by 25% in the Caco2.BBE cell line (n = 4, P < 0.001) (Figure 2D).Incubation of HT29CL.19A cells with acivicin and subsequent PDB and glutamine addition resulted in high permeability levels which were not significantly different from the PDB group (n = 11; Figure 2A).Incubation of Caco2.BBE cells with trans-PDC and subsequent PDB and glutamate addition also resulted in high permeability levels, once again not significantly different from the PDB group (n = 4; Figure 2D).Control experiments revealed that acivicin and trans-PDC did not alter HRP permeability (results not shown).







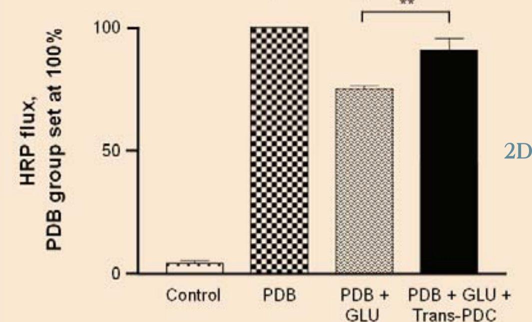


Figure 2

Effects of glutamine and glutamate on PDB-induced permeability in the two intestinal cell lines. A: in the HT29Cl.19A cell line, glutamine addition resulted in a 45% decrease in permeability. Acivicin nullified this effect; n=6. B: in the Caco2.BBE cell line, glutamine addition resulted in a 30% decrease in permeability; n=3. C: in the HT29Cl.19A cell line, glutamate addition resulted in a 25% decrease in permeability; n=3. D: in the Caco2.BBE cell line, glutamate addition resulted in a 25% decrease in permeability. Trans-PDC nullified this effect; n=4.

* P < 0.05, ** P< 0.01, *** P < 0.001.

Discussion

We found that both glutamine and glutamate can reduce an induced form of hyperpermeability in human colon derived cell lines. The effect of glutamine could be nullified by blocking the extracellular converting enzyme, γ-GT, whereas the effect of glutamate could be nullified by blocking the glutamate transporters EAAT1-5.

These results lead to two suggestions: firstly, the conversion of glutamine to glutamate is essential for its beneficial effect on permeability. Secondly, transport of glutamate into the cell is essential for the beneficial effect of glutamate on permeability.

Because the effect of trans-PDC on the protective action of glutamine was not studied, and similarly, the effect of acivicin on the protective action of glutamine was not studied, further research will be necessary to confirm these suggestions. Not all of the experiments were performed with both cell lines due to inherent differences between the two cell lineages. The HT29Cl.19A cell line proved unstable during later experiments compared to the Caco2.BBE cell line. Additionally, the Caco2.BBE cell line has been shown to possess EAATs [14], making it the favourable cell line for trans-PDC related experiments. Moreover, these inconsistencies might account for the observed differences in the reduction of induced hyperpermeability between the cell lines [15]. However, our study design was not focussed or powered on cell line comparison.

Glutamine and glutamate seem to reduce this hyperpermeability by acting on the paracellular permeability (tight junction) as opposed to transcellular permeability (endocytosis). PDB, induces a Protein Kinase C (PKC)-mediated hyperpermeability. This signal transduction pathway is also activated by clinically relevant mediators, including lipopolysaccharides [16]. PKC is thought to regulate tight junction (TJ) permeability via tightening and loosening of the cell's perijunctional actomyosin ring (PAMR) [17-19]. Furthermore, rinsing the PDB off the cells restored permeability levels to control values, indicating that the effect of PDB is not due to cell destruction (results not shown). In such, PDB addition creates a paracellular hyperpermeability which can be monitored by HRP diffusion from apical to basolateral compartments.

HRP needs to remain enzymatically active to be measured. Approximately 97% of the HRP that reaches the basolateral compartment via the transcellular pathway is degraded [20, 21] and loses its enzyme activity. The detection of enzymatically active HRP in this study therefore verified that we measured paracellular permeability.

Our results suggest that glutamine needs transamination to glutamate to exert its effect. In a broader scope, it would be interesting to quantify the transamination by glutaminase intracellularly. However, since blocking γ-GT extracellularly immediately showed a decrease in the effect of glutamine, the extracellular conversion seems important independent of intracellular mechanisms.

The protective effect of glutamine on gut mucosa is often thought to result from cell proliferation and attenuation of apoptosis [22]. Our study indicates that this is probably not the sole reason. HRP flux was inhibited within the four hour window of this study. Enterocyte proliferation, however, takes more than four hours, thus cell proliferation cannot (completely) explain the observed favourable effect. To exclude indirect effects of glutamine and glutamate metabolism, the measurement of metabolites by HPLC could pinpoint such effects.

Proliferation and maintaining the integrity of enterocytes requires an adequate supply of glutamine. Hence, plasma levels are normally maintained around 0.6 mmol/L [23, 24]. This physiological concentration was therefore used in the present study. For easier comparison the glutamate concentration was also set at 0.6 mmol/L, even though its physiological concentration approaches 24-80 µmol/L [23, 24].

The 0.6 mmol/L of glutamine and glutamate were applied to the apical chamber. In vivo, however, luminal concentrations of glutamine and glutamate commonly exceed 0.6 mmol/L after protein-rich meals [25]. It is, therefore, interesting to see that this concentration can already elicit advantageous effects. Future studies comparing different concentrations of glutamine and glutamate should be performed to optimally quantify dosage effects. To allow a comparison with catabolic patients, it would also be interesting to detect a minimum dose of glutamine and glutamate which still elicits a protective effect on hyperpermeability.

In summary, we have shown that apical glutamate -similar to glutamine- can decrease an induced paracellular hyperpermeability in two human colon derived cell lines. Because of the nature of the permeability inducing agent, PDB, glutamine and glutamate probably exert their effect through interaction with tight junctions. Furthermore, the extracellular conversion of glutamine to glutamate and the subsequent uptake of glutamate could be a pivotal step in the mechanism underlying the protective effect of glutamine. Yet, to certify this mechanism, the focus should be on different concentrations of apically applied glutamine and glutamate in different cell lines or in co-cultured cell lines, in parallel with research on intracellular conversion.

Author contributions:

Vermeulen M.A.R. and Vaessen M.J. performed data analyses, interpreted the data and wrote the manuscript; de Jong J. performed the experiments, designed the study, helped interpret data analyses and critically reviewed the manuscript; van Leeuwen P.A.M. critically reviewed the manuscript and helped interpret the data; Houdijk A.P.J. supervised all parts of the study and is responsible for all parts of the study; Vermeulen M.A.R. and de Jong J. contributed equally to this work

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3. Accurate perioperative flow measurement of the portal vein, hepatic artery and renal vein: a role for preoperative MRI?

Mechteld A.R. Vermeulen, Gerdien C. Ligthart-Melis,
René Buijsman, Michiel P.C. Siroen, Marcel C.G. van de Poll,
Petra G. Boelens, Cornelis H.C. Dejong, Cors van Schaik,
Mark B.M. Hofman, Paul A.M. van Leeuwen

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Abstract

Background

Quantification of abdominal blood flow is essential for a variety of gastrointestinal and hepatic topics such as liver transplantation or metabolic flux measurement, but those need to be performed during surgery. It is not clear whether Duplex Doppler Ultrasound during surgery or MRI before surgery is the tool to choose.

Objective

To examine whether preoperative evaluation of abdominal blood flow using MRI could prove to be a useful and reliable alternative for the perioperative sonographic approach.

Methods

In this study portal and renal venous flow and hepatic arterial flow were sequentially quantified by preoperative MRI, preoperative and perioperative Duplex Doppler Ultrasound (DDUS). 55 Patients scheduled for major abdominal surgery were studied and methods and settings were compared. Additionally, average patient population values were compared.

Results

Mean (±SD) plasma flow measured by perioperative DDUS, preoperative DDUS and MRI respectively was 433 ± 200/ 423 ± 162/ 507 ± 96 ml/min (portal vein); 96 ± 70/ 74 ± 41/ 108 ± 91 ml/min (hepatic artery); 248 ± 139/ 201 ± 118/ 219 ± 69 ml/min (renal vein).
No differences between the different settings of DDUS measurement were detected. Equality of mean was observed for all measurements. Bland Altman Plots showed widespread margins. Hepatic arterial flow measurements correlated with each other, but portal and renal venous flow correlations were absent.

Conclusion

Surgery and method (DDUS vs. MRI) do not affect mean flow values. Individual comparison is restricted due to wide range in measurements.

Since MRI proves to be more reliable with respect to inter-observer variability, we recommend using mean MRI results in experimental setups.

Introduction

Blood flow measurement using Duplex Doppler Ultrasound (DDUS) is a widely used technique for measuring blood flow in a variety of vessels. Blood flow quantification is an essential instrument for detecting or monitoring vascular pathology and evaluating therapeutic interventions [1-4]. Additionally, it is required for organ flux measurements, a measure of net exchange across organs (net flux = ([Vein] – [Artery] * plasma flow) [5, 6]. Techniques to visualize and assess blood flow both quantitatively and qualitatively have developed over the past decades. Besides the sonographic approach Magnetic Resonance Imaging (MRI) has proven to be a complementary way for quantifying blood flow non-invasively [2, 7-10].

A general disadvantage of the sonographic approach of blood flow measurement is inter- and intra-observer variability [11]. Since MRI proves to be more reliable with respect to inter-observer variability, this could represent a more accurate alternative for blood flow measurement [12]. Unfortunately immobility of the MRI machine disqualifies its use for e.g. perioperative purposes.

For a reliable measurement of organ flux, adequate sampling of the substance of choice and reliable blood flow measurements are of paramount importance for quantification of that particular substance across organs. However, flux calculation across organs that are drained by vessels that are relatively inaccessible (abdominal veins and arteries) is challenging. First, blood sampling cannot easily be performed in vessels covered by vital organs. To overcome this problem, sampling is often performed during abdominal surgery. Secondly as a consequence, flow measurement should also be performed during surgery.
For example, for splanchnic flow quantification, both the portal vein and the hepatic artery need to be examined. This is technically possible with DDUS, yet difficulties are involved: the perioperative approach of flow measurement can prolong the duration of surgery and measurements naturally have to be performed within a sterile setting.

Theoretically, MRI is superior to DDUS because of more pronounced accuracy and less inter-observer variability [12].
MRI, unfortunately, is currently not possible during surgery. Since the desired setting of flow measurement is during surgery, this technique is only suitable when it proves to be adequate in predicting perioperative blood flow.

The aim of this study is to examine whether a preoperative evaluation of blood flow using MRI could prove to be a useful and accurate alternative for the perioperative sonographic approach used at present in metabolic flux studies. To reinsure that possible differences reflect the method used instead of being a direct result of the surgical setting, DDUS measurements were performed both perioperatively and preoperatively.

Materials and methods

Subjects

All patients admitted to the VU University medical center (VUmc) scheduled for major abdominal surgery from January 2003 until July 2005 were included when they met inclusion criteria. These patients were studied prospectively as a separate part of the studies of Ligthart-Melis et al. and Siroen et al. [5, 6]. Subjects were thoroughly informed, whereupon written informed consent for all parts of the studies was obtained. Briefly, inclusion criteria were major abdominal surgery, age between 18 and 75. Patient characteristics are displayed in Table 1.

To resemble the preoperative situation, care was taken to measure the preoperative blood flow at a similar time of the day as the patient was scheduled for surgery. Furthermore, patients were assessed in the postabsorptive state.

The study was approved by the institutional board and the medical ethical review committee of our hospital and accorded to the ethical standards of the Helsinki Declaration as revised in 1983.

The analysis was performed on a vessel basis. In order to be included in the analysis, two of three measurements (perioperative DDUS, preoperative DDUS and MRI) had to be obtained in a specific patient, otherwise data points were excluded. Noteworthy, comparison between setting (DDUS before vs. during surgery) and method (preoperative MRI vs. preoperative DDUS) necessitated the presence of preoperative DDUS measurements. Data excluded in case of vascular anomalies were scored on the contrast-enhanced MRA sequence.

Flow measurement

<u>DDUS</u>

The principles of Doppler US are well described by Gill [13]. In this study, an Aloka Prosound SSD 5000 (Aloka Co., Ltd, Tokyo, Japan) was used for both the preoperative and perioperative DDUS measurements, using a 7.5 MHz probe. Measurements were performed by a senior radiologist or a Doppler US educated radiology assistant, with extensive amount of experience in Doppler examination of deep abdominal vessels. Doppler settings were optimized in each case according to common practice. The hepatic artery and portal and renal vein were assessed enabling calculation of splanchnic and renal metabolism. All vessels were first visualized in B-mode (black and white). Measurements were performed while the patient suspended his or her inspiration after which the Doppler spectrum was recorded. After correcting for the angle of insonation, the mean blood flow velocity was calculated online. Cross-sectional areas of the vessels were determined by drawing an area ellipse at the same location at which the velocity measurement was performed. For accurate velocity measurements, care was taken to keep the angle between the ultrasonic beam and blood flow direction below 60 degrees [14]. The Doppler sample volume was positioned in the centre of the vessel and varied in width to detect the clearest Doppler frequency shift signal. Measurements were taken during at least three cardiac cycles. Blood flow was measured in the hepatic artery, portal vein and renal vein in the postabsorptive state in supine position. Flow measurements of the portal vein were performed before hilar bifurcation. Hepatic arterial flow was measured after the gastroduodenal artery had branched off, but before bifurcating into the left and right hepatic arteries. In cases with aberrant left or right hepatic arteries, all vessels were measured separately.

Preoperative measurement was performed after a rest of 15 minutes, as recommended by Brown et al. [14].

Perioperative fw measurements took place after incision of the abdominal wall and exposure of the organs involved in the surgical procedure, but before start of organ resection. To prevent anesthetic effects on blood flow, the procedure was standardized

Table 1 - Characteristics of patients

Characteristics	N (%) / mean (± SD)	Diagnosis	N(%)
Sex: male/female (%)	41/14 (75/26)	Hepatocellular carcinoma	6 (11)
Age (y)	61 (±11)	Pancreatic cancer	1 (2)
Weight (kg)	82 (±14)	Sarcoma	1 (2)
BMI (kg/m2)	27 (±5)	Benign liver tumor	2 (4)
Underweight	1 (2)	Liver Metastasis	41 (75)
Normal	12 (22)	Colorectal origin	1 (2)
Overweight	32 (58)	Gastric origin	1 (2)
Obesity	9 (16)	Ovarian origin	1 (2)
Unknown	1 (2)	Melanoma	1 (2)
		GIST	1 (2)

Characteristics of patients, data are shown as percentages or mean ± SD.

as much as possible by preferably using isoflurane or sevoflurane [5, 6].

For both the preoperative and perioperative examination, the same probes were used and examinations were performed by the same operator.

MR flow quantification

The principles of phase-contrast MRI flow quantification have been described in detail by Debatin et al. [12]. The measurement protocol was standardized as follows: A 1.5 Tesla whole body MR system (Magnetom Sonata, Siemens, Erlangen Germany) was applied with a phased array surface RF receiving coil. For localization coronal, transversal, sagital, and sometimes oblique scouts were obtained, with an SSFP-pulse sequence with a spatial resolution of $1.6 \cdot 1.4 \cdot 5.0$ mm^3.

The position of flow measurement was determined on the localizer images. Hepatic arterial flow was measured after the gastroduodenal artery had branched off, if visible on the scouts. Both hepatic arterial flow and portal venous flow were measured before their hilar bifurcation.

The acquisition parameters for the MR phase contrast measurements were: an ECG triggered phase-contrast gradient echo pulse sequence, with a repetition time of 6 ms, an echo time of 3 ms, and a 25∞ excitation angle. For the portal vein and renal vein measurement the spatial resolution was $1.7 \cdot 1.2 \cdot 6.0$ mm^3 , and the velocity encoding value (V_{ENC}) was set at 30 cm/s. For hepatic artery measurements a slightly higher spatial resolution of $1.5 \cdot 1.2 \cdot 5.5$ mm^3 was applied with a V_{ENC} set at 80 cm/s. Patients were asked to hold their breath, after which flow measurements were taken during 19 heart cycles. After flow measurement completion, contrast was administered in order to detect any vascular anomalies, using contrast-enhanced MR [15].

Flow images were stored and analyzed using standard software (Argus, Siemens, Erlangen, Germany). Cross-sectional areas were drawn on the images to define the Region of Interest (ROI). Subsequently, a second ROI was drawn in a nearby area without any visible vessels; this ROI served as a control area. A cine-movie over the cardiac cycle was played to verify whether the ROI and reference ROI were drawn correctly. The volume flow was calculated by integration of the velocity over the cross-sectional area, and the cardiac cycle. Velocity values were compensated for stationary velocity offsets by use of the reference ROI.

Analyses were performed by two observers. In case of discrepancy, analysis was performed on mutual agreement.

Blood sampling and calculation for plasma flow

After blood flow measurements were performed using both techniques, plasma flow was calculated by correcting for corresponding (preoperative vs. perioperative) hematocrit:

plasma flow=blood flow · (1-hematocrit).

Statistics

Results of the plasma flow measurements are expressed as mean and Standard Deviation in case of normal distribution (SD).

Pearson Correlation test was used to detect correlations between the two settings (preoperative DDUS vs. perioperative DDUS) and to detect correlations between the different methods of measurement (preoperatively: DDUS vs. MRI).

Since a high degree of correlation does not entail good agreement between two methods, Bland-Altman plots (difference plots) were made. The limits of agreement during Bland-Altman analyses are specified as average difference ± 2SD (standard deviation of the difference).

Paired T-tests were performed to point out differences in the setting (preoperative DDUS vs. perioperative DDUS) and the methods (preoperatively: DDUS vs. MRI).

ANOVA (in case of equal variances) or Welch test (in case of unequal variances) was used to verify equality of means among setting and method.

Statistical analysis was performed with SPSS package software (SPSS 16.0 for Windows®). Statistical significance was defined as 2-tailed $P < 0.05$.

Table 2 – Mean plasma flow

	Perioperative DDUS	Preoperative DDUS	Preoperative MRI
Portal Vein	433 ± 200 n=43	423 ± 162* n=44	507 ± 96* n=24
Hepatic Artery	96 ± 70 n=41	74±41 n=38	108 ± 91 n=16
Renal Vein	248 ± 139 n=42	201 ± 118 n=34	219 ± 69 n=23

Mean plasma flow measured preoperatively by DDUS and by MRI and perioperatively by DDUS. Data are expressed in mean ± SD in ml/min.

* Data differ significantly at the p< 0.05 level

Results

Subjects and measurements

In total, 55 patients were subjected to flow assessment. Incomplete or unreliable measurements were excluded from analysis. Incomplete Duplex DDUS were either due to absent Doppler shift signal mainly due to overweight (8 patients), or to procedural difficulties during the surgical course (5 patients). The reliability of the DDUS measurements per vessel was judged by evaluating the angle of insonation (<60°) (details per vessel are expressed below). Incomplete MRI measurements were due to unavailability of the MRI-scanner (schedule difficulties or defects of the scanner) (22 patients), due to prosthetic devices interrupting magnetic signaling (1 patient), incorrect procedure of measurements (1 patient), claustrophobia (2 patients), or refusal of the patient (1 patient). MRI measurement reliability depended on breath hold adequacy. Retrospectively, reliability was again judged using the images. In case of strong image ghosting artifacts due to patient motion during the measurement, results were excluded from analyses.

Portal vein measurements

DDUS was performed during surgery in 50 patients, whereas in 5 patients measurements were not possible for above-mentioned reasons. Retrospectively, 7 of 50 measurements were performed in one of the portal vein's branches and were therefore excluded from analyses.

In 47 patients flow in the portal vein was measured prior to surgery, using DDUS; in 8 patients measurements were not possible for abovementioned reasons. Retrospectively, 3 of the 47 measurements turned out to be unreliable because flow was measured in one of the portal vein's branches.

MRI measurements of portal venous flow were performed in 28 patients but not in 27 patients; inadequate breath-hold further disqualified 4 patients of interpretation.

Consequently, flow comparison of the portal vein could be made in 33 cases with respect to preoperative DDUS and perioperative DDUS; in 21 cases a comparison between preoperative DDUS and preoperative MRI was possible.

Hepatic artery measurements

During surgery, DDUS flow measurement of the hepatic artery could be performed in 46 patients and failed in 9 patients. Unreliable measurements were observed due to coexistence of multiple hepatic arteries (n=3), mistakenly measuring maximum velocity instead of its mean (n=1), wrong measurement timing (after colonic resection: n=1).

The hepatic arterial flow was quantified by DDUS presurgically in 41 patients, whereas in 14 patients assessment was not possible for reasons discussed above. Existence of plural hepatic arteries was observed in 3 patients disallowing inclusion of those measurements.

Presurgical hepatic artery flow assessment using MRI could be performed in 23 patients. Again, 3 of the 23 patients' measurements were excluded due to hepatic artery anatomic anomaly. Consequently, hepatic artery flow could be compared in 32 cases for the preoperative DDUS and perioperative DDUS. In 11 cases a comparison between preoperative DDUS and preoperative MRI was possible.

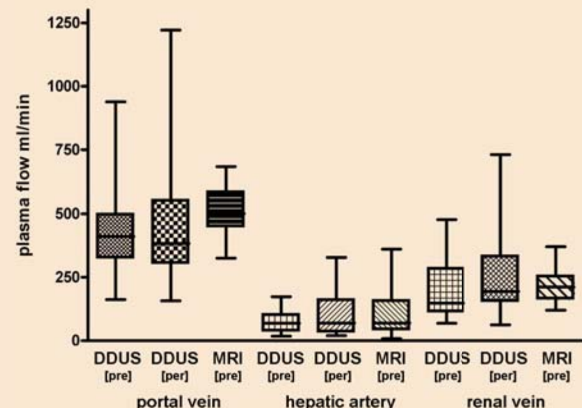


Figure 1

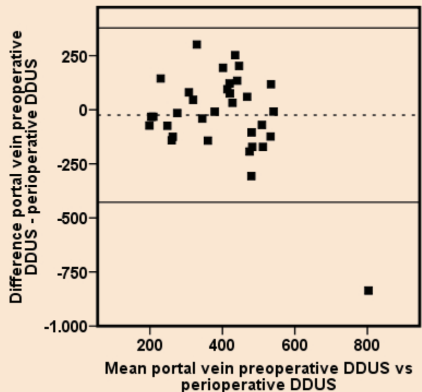
Plasma flow in ml/min in the portal vein, hepatic artery and renal vein. Boxes show range and median for perioperative and preoperative DDUS as well as for preoperative MRI measurements. DDUS: DD Ultrasound; [per]: perioperative, [pre]: preoperative

Renal vein measurements

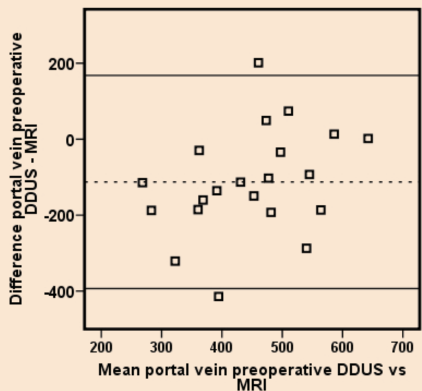
During surgery, flow in the renal vein was quantified by DDUS in 42 patients, whereas in 11 patients assessment was not possible. Eventually measurements of 2 patients had to be excluded due to invalidity. Renal venous flow measurement prior to surgery by DDUS could be performed in 36 patients.
In 19 patients measurements were not possible for reasons mentioned earlier. Altogether 2 measurements were observed being unreliable.

In 27 patients the flow in the renal vein was assessed before surgery using MRI; in 28 patients, MRI renal flow quantification was not successful. In 1 patient the measurement was retrospectively qualified unreliable.

Flow in the renal vein could be compared in 32 cases for the preoperative DDUS and perioperative DDUS. In 21 cases a comparison between preoperative DDUS and preoperative MRI was possible.



2A



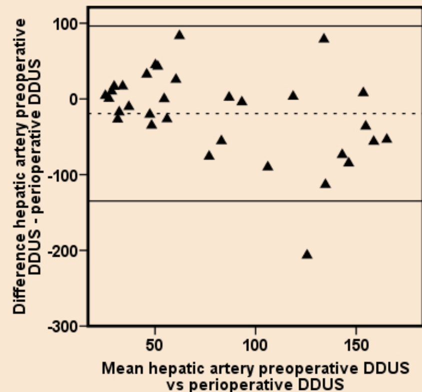
2B

Figure 2

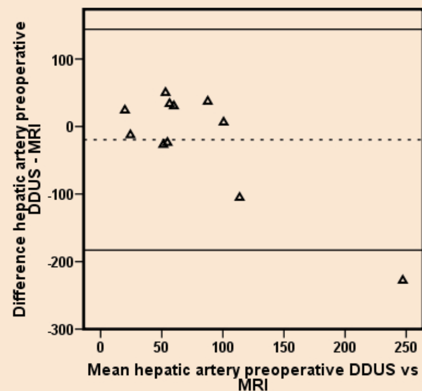
Bland-Altman plot for the portal venous flow showing mean difference and mean ± 2SD.

2A: Mean difference between perioperatively and preoperatively measured flow by DDUS is -25 ± 202 ml/min and limits of agreement are set at 378 and -428 ml/min.

2B: Mean difference measured by preoperative DDUS and MRI. Mean difference is -113 ± 140 ml/min and limits of agreement are set at 168 and -393 ml/min.



3A



3B

Figure 3

Bland-Altman plot for the hepatic artery showing mean and mean ± 2SD. Mean difference between preoperatively and perioperatively measured DDUS (3A) is -19 ± 58 ml/min and limits of agreement are set at 96 and -135 ml/min. Mean difference between preoperatively performed DDUS and MRI (3B) is -20 ± 81 ml/min and limits of agreement were calculated 143 and -183 ml/min.

Flow in the portal vein

Mean flow values are summarized in Table 2, flow value distribution is shown in Figure 1. Perioperative DDUS plasma flow measurements did not correlate with preoperative DDUS measurements (r=0.262, p=0.142). Equally, no significant correlation was observed between preoperative DDUS measurements and preoperative MRI measurements (r=0.360, p=0.109). Exchangeability was assessed using the Bland-Altman plot, displaying the difference between both methods (ml/min) at each mean plasma flow. Figure 2a shows the Bland-Altman plot for preoperative DDUS and perioperative DDUS. The pattern shown by this figure does not indicate any source of systematic error. The difference between both methods did not increase parallel with mean flow. A mean difference of -25 ± 202 ml/min was calculated, placing the limits of agreement at 378 and -428 ml/min. Figure 2b shows the Bland-Altman plot for the preoperative DDUS and the preoperative MRI. Again no systematic source of error was found and the difference between both techniques did not increase parallel with mean flow. A mean difference of -113 ± 140 ml/min was found. The limits of agreement were therefore set at 168 and -393 ml/min. The paired T-test did not express significant differences between setting (preoperative and perioperative DDUS) however significant differences between preoperative DDUSand preoperative MRI (p= 0.001) were detected.

When analyzing the variance, homogeneity was just not violated (p = 0.050). ANOVA indicated equality of means (p = 0.117).

Flow in the hepatic artery

Mean flow values are summarized in Table 2, flow value distribution is shown in Figure 1.

A significant correlation was found for plasma flow measured by preoperative DDUS and perioperative DDUS (r=0.50, p=0.004). Likewise, a correlation was found between preoperative DDUS and preoperative MRI (r=0.64, p=0.033). Subsequently, exchangeability was assessed using the Bland-Altman plots. Figure 3a shows the Bland-Altman plot for preoperative DDUS and perioperative DDUS. The patterns show a uniformly spread distribution, which does not indicate any source of systematic error. The difference between both techniques did not seem to depend on mean plasma flow. A mean difference of -19 ± 58 ml/min was

found, placing the limits of agreement at 96 and -135 ml/min. As shown by Figure 3b no systematic error could be detected concerning preoperative DDUS and preoperative MRI assessment. A mean difference of -20 ± 81 ml/min was calculated, therefore limits of agreement were set at 143 and -183 ml/min.

The paired T-test expressed no significant differences between preoperative and preoperative DDUS, and between preoperative DDUS and preoperative MRI. When analyzing the variance, homogeneity was violated (p = 0.001). Welch Test indicated equality of means (p = 0.130).

Flow in the renal vein

Mean flow values are summarized in Table 2, flow value distribution is shown in Figure 1.

When comparing the plasma flow values for the renal vein with respect to DDUS, no significant correlations were observed (r=-0.76, p=0.678). Likewise the correlation between preoperative DDUS and the perioperative MRI was not significant (r=0.316, p=0.152).

The Bland-Altman plot in Figure 4a shows the preoperative DDUS and perioperative DDUS. The pattern shows no signs of systematic error, although the difference seems to increase slightly when the mean plasma flow rises. A mean difference of -48 ± 177 ml/min was calculated; hence the limits of agreement were calculated between 305 and -401 ml/min.

Figure 4b shows the Bland-Altman plot which assesses the difference between preoperative DDUS and preoperative MRI.

No sources of systematic errors or increases in parallel with mean plasma flow are displayed. A mean difference of 2 ± 123 ml/min was calculated, which places the limits of agreement at 244 and -247 ml/min.
The paired T-test expressed no significant differences between preoperative and preoperative DDUS, and between preoperative DDUS and preoperative MRI. When analyzing the variance, homogeneity was violated (p = 0.013). Welch Test indicated equality of means (p = 0.282).

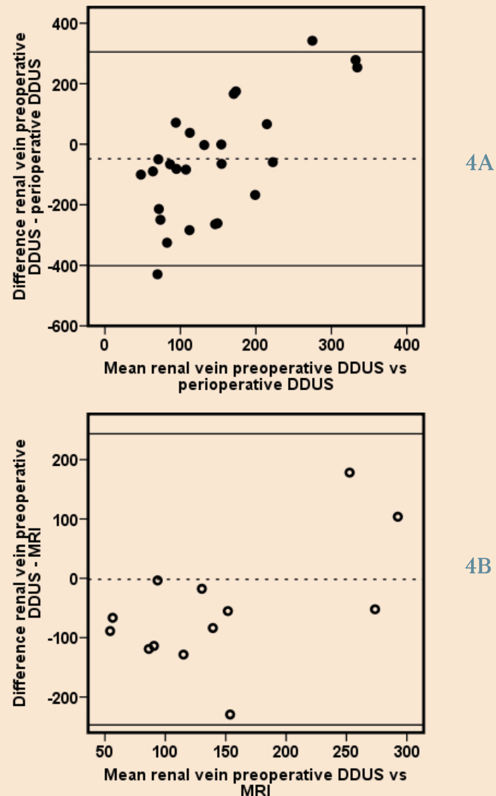


Figure 4

Bland-Altman plot illustrating mean difference and mean ± 2SD for renal venous flow as measured by perioperatively and preoperatively performed DDUS (4A). Mean difference is -48 ± 177 ml/min and limits of agreement are set at 305 and -401 ml/min. Mean difference between preoperatively performed DDUS and MRI (4B) is 2 ± 123 ml/min and limits of agreement were calculated 244 and -247 ml/min.

Discussion

In the present study, we compared DDUS with MRI in the context of measuring blood flow in intra-abdominal vessels. Equally, DDUS was performed pre- and perioperatively to evaluate the effect of setting of the measurement (i.e. intraoperative versus preoperative).

We observed no significant differences between the different settings of DDUS measurement for all vessels. As for MRI however, portal flow differed from preoperative DDUS. When comparing all measurements per vessel, no differences in means were observed.

However, absence of correlation in the setting (perioperative DDUS vs. preoperative DDUS) and method (preoperative DDUS vs. preoperative MRI) were observed. Individually, widespread margins in the Bland Altman agreement plots were calculated.

Perioperative vs. preoperative measurements

In our study the perioperative DDUS was considered as a reference standard, because this technique measured plasma flow in the exact setting in which we intended to analyze plasma concentrations for flux rate calculations. Furthermore measuring directly onto the vessel, which is allowed by perioperative DDUS, theoretically best estimates the angle of insonation, theoretically enabling more accurate flow measurement.

Remarkably, this seems to contrast the observed wide range and standard deviation of perioperative DDUS measurements (Table 2 and Figure 1). Perioperative DDUS portal and renal venous measurement showed the highest variability. Taken into account that the only correlations were found for hepatic artery measurements, this could imply that either perioperative portal and renal vein measurements are more difficult to perform accurately, or surgery induces an unpredictable effect on flow in the portal and the renal vein. Interestingly, the muscular anatomy of the arterial wall would naturally be more prone to be influenced by anesthetics and surgical stress, suggesting a wider range should theoretically have occurred for hepatic artery data, which was not the case.

In almost all subjects the overall effect of surgery (comparing DDUS pre- and preoperatively) was not identified systematically in the same direction.

Eventually, when assessing whether mean preoperative and perioperative measurements differ, we did not notice statistical significance, suggesting that mean DDUS - whether during or before surgery - is exchangeable.

DDUS and MRI measurements

Preoperative portal flow measurement by MRI and DDUS did produce different results when MRI instead of DDUS was used. This did not occur in comparing hepatic arterial flow and renal venous flow measurement with these two methods. Pearson correlation test shows the strongest relation for the hepatic artery. The correlations observed when evaluating the portal venous measurement were not significant although most results did show trends towards significance. Considering renal venous flow, correlations were not significant suggesting that individual flow measurements for this vein are not per se interchangeable.

Although DDUS is a widely used technique for non invasive measurement of blood flow in abdominal and non-abdominal vessels with the advantage that it can be used repeatedly, it has its limitations [11, 13, 14]. Disadvantages include the estimation of the angle of insonation, the difficulty in determining the cross-sectional area of the vessels and the high inter-observer variability [11, 13]. Since MRI already proved to be an alternative method for measuring flowin abdominal and non-abdominal vessels [2, 7-10, 16, 17], it might in fact be a more reliable technique of flow measurement as a result of the minor inter-observer variability, in comparison with DDUS measurement [11, 18]. Additionally, MRI flow measurement can be performed without being hindered by bowel gasses or patient habitus [9]. This is partly in agreement with our observations as reflected by the display of the range in MRI results in Figure 1 for portal and renal venous flow and by calculated unequal variances for renal venous flow.

The absence of a gold standard method makes a true comparison between both methods difficult. Whereas the use of microspheres for flow measurement in animals is used as the standard method, this technique is not suitable for human use. Nevertheless, previous research conducted by Nijeholt et al. demonstrates that MRI is preferred for absolute flow evaluation rather than DDUS. As a reference standard total liver blood flow was calculated and compared with reference values for hepatic blood flow, obtained by previous studies measuring indocyanine and D-sorbitol clearance [19, 20]. In this particular study a correlation between portal flow measured by MRI and DDUS was observed even though the study was limited to 8 healthy volunteers [9]. In contrast, Nanashima et al. evaluated MRI and DDUS flow measurements in the portal and hepatic vein in 75 consecutive patients and did not observe any correlation between both methods [2].

In order to create a reference standard for perioperative flow, metabolic studies using stable isotopes could prove useful. This could be executed by comparing flow dependent flux calculations with independent whole body rate of appearance values. Alternatively hepatic clearance could serve as a particular reference standard in the MRI / DDUS comparison when evaluating hepatic blood flow.

Bland-Altman Plots

The Bland-Altman plot is nowadays regarded as a very accurate method of assessing interchangeability of two methods.
The alternative often used for exchangeability assessment, the Pearson correlation test, can be misleading since the correlation coefficient r represents the strength of the correlation, but not the agreement.

The Bland-Altman plots that were made in order to assess exchangeability did not show any source of systematic error. Overall, no increase in difference was observed parallel with the increase in mean plasma flow, although this might be disputable for renal venous flow measurement by DDUS (Figure 4b). The limits of agreement reflect reliability by transforming the standard deviation (more precisely 2sd) into a concrete margin of agreement. Considering the magnitude of the error, the Bland Altman-plots reached moderately wide levels of agreement implying large variation. Conclusively, although interchangeability is legitimate (absence of errors), it adds a significant variance to the measured plasma flow.

Conclusion

In summary, considering both the absent or marginal correlations individually, as well as the widespread margins in the Bland Altman agreement plots, we do not recommend individual flow assessment. Since no differences were observed when assessing means it can be reasoned that mean values are in fact interchangeable. Since it can be theorized that MRI would be a more reliable method for measuring absolute flow, we therefore suggest that mean values for flow assessment may be performed in the preoper ative MRI setting, when perioperative flow indication is necessary for metabolic quantification.

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4. Enteral administration of alanyl-[2-15N]glutamine contributes more to the de novo synthesis of arginine than intravenous infusion of the dipeptide in humans

Gerdien C. Ligthart-Melis / Marcel C.G. van de Poll,
Mechteld A.R. Vermeulen, Petra G. Boelens, M. Petrousjka van den Tol,
Cors van Schaik, Jean-Pascal De Bandt, Nicolaas E.P. Deutz,
Cornelis H.C. Dejong and Paul A.M. van Leeuwen

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Abstract

Background We previously confirmed in humans the existence of a pathway of glutamine into citrulline and arginine, which is preferentially stimulated by luminally provided glutamine. However, because glutamine is unstable, we tested this pathway with a stable dipeptide of glutamine.

Objectives The objectives were to explore whether alanyl-glutamine contributes to the synthesis of arginine in humans and whether this depends on the route of administration.

Design The study was conducted under post-absorptive conditions during surgery. Sixteen patients received alanyl-[2-15N]glutamine enterally or intravenously together with intravenously administered stable-isotope tracers of citrulline and arginine. Blood was collected from an artery, the portal vein, a hepatic vein, and the right renal vein. Arterial and venous enrichments and (tracer) net balances of alanyl-glutamine and glutamine, citrulline, and arginine across the portal drained viscera, liver, and kidneys were determined. Parametric tests were used to test results (mean ± SEM). $P<0.05$ was considered significant.

Results Twice as much exogenous glutamine was used for the synthesis of citrulline when alanyl-glutamine was provided enterally (5.9 ± 0.6%) than when provided intravenously (2.8 ± 0.3%) ($P< 0.01$). Consequently, twice as much exogenous glutamine was used for the synthesis of arginine when alanyl-glutamine was provided enterally (5 ± 0.7%) than when provided intravenously (2.4 ± 0.2%) ($P < 0.01$). However, results at the organ level did not explain the differences due to route of administration.

Conclusions Alanyl-glutamine contributes to the de novo synthesis of arginine, especially when provided enterally. A stable-isotope study using a therapeutic dose of alanyl-glutamine is needed to investigate the clinical implications of this finding.

Introduction

Glutamine and arginine are 2 metabolically related amino acids that can enhance the recovery of seriously ill patients [1-3]. We showed in humans, under postabsorptive conditions, that plasma glutamine is the precursor for 64% of arginine generated by de novo synthesis from citrulline [4]. We also confirmed the concept that the intestinal conversion of glutamine leads to the release of citrulline from the gut, which, after its uptake from the bloodstream, is converted by the kidneys into arginine [5-8] in mice and in humans [4, 9-11]. The human intestine was observed to be responsible for the entire de novo synthesis of plasma citrulline from glutamine [11], and human kidneys were shown to take up more than 50% of circulating plasma citrulline and release equimolar amounts of arginine into plasma [4].

Importantly, the route of administration (enteral or intravenous) was observed to affect the metabolic fate of exogenous glutamine [9, 12]. Surgical patients who received [2-^{15}N]glutamine enterally had a greater intestinal fractional extraction of glutamine concomitant with greater intestinal release of [^{15}N]citrulline and greater plasma enrichments of [^{15}N]citrulline than did patients who received [^{15}N]glutamine intravenously [12]. These results suggest that enterally administered glutamine contributes more to the de novo synthesis of arginine than does intravenously administered glutamine. This is in line with previous observation in mice [9].

Free glutamine, however, is unstable in aqueous solutions [13]. Previous research has shown that glutamine-containing dipeptides (e.g., glycine-glutamine and alanyl-glutamine) are stable in aqueous solutions [14-17]. Therefore, these dipeptides are used instead of glutamine in intravenous nutrition [13]. Alanyl-glutamine is the most optimal dipeptide due to a superior clearance rate [17, 18]. However, alanyl-glutamine was observed to have a slightly different metabolic fate than free glutamine in mice [9, 10]. In addition, in preoperative patients, enterally provided alanyl-glutamine resulted in higher plasma concentrations of citrulline without increases in plasma concentrations of arginine, whereas the intravenous administration of the dipeptide enhanced plasma concentrations of arginine despite lower plasma concentrations of citrulline [19].

In the present human study, the metabolic fate of glutamine derived from alanyl-glutamine was explored with respect to the pathway of glutamine into citrulline and arginine after the enteral or intravenous administration of the dipeptide by using stable isotope tracers of glutamine, citrulline, and arginine. The application of the same

design as in previous human studies [4, 11, 12] enabled us to compare the effect of the molecule as well as the route of administration at the whole-body (WB) and organ (intestinal, hepatic, and renal) levels. Patients were studied during surgery in the postabsorptive state. The organ amino acid tracer net balances (TNBs) were examined to determine the effect of the route of administration of alanyl-[2-15N]glutamine at the organ level.

Results of this study provide a base for future studies with intervention doses of alanyl-glutamine, which will ultimately help us to understand the benefits and disadvantages of using alanyl-glutamine in clinical practice and to clarify the metabolic implications of the route of administration.

Subjects and methods

Patients

Sixteen patients undergoing gastrointestinal surgery at the VU University Medical Center (VUMC; Amsterdam, Netherlands) were included in the study. The reasons for surgery and the surgical procedures used are summarized in Table 1 and Table 2.

On the day of admission, routine blood tests were performed, and body composition was measured by using bioelectrical impedance analysis (Xitron 4200; Xitron Technologies, San Diego, CA). Patients with known parenchymal liver disease other than cancer or inborn errors of metabolism, type 1 diabetes mellitus, recent weight loss, clear cachexia, or other indications of metabolic disorders were excluded from the study. Patient characteristics are presented in Tables 1 and 2. Oral intake of foods and beverages other than water was stopped at 20.00h on the day before surgery.
All patients were transported to the surgical area at 07.30h the next day. All patients gave written informed consent. The Medical Ethical Committee of the VUMC approved the study. The study complied with the Declaration of Helsinki.

Study design

The metabolic study was conducted during surgery. Patients were studied in the postabsorptive state. Labeled glutamine was provided as the dipeptide L-alanyl-L-[2-^{15}N] glutamine. The dipeptide was provided at random, intravenously (IV group; n = 8) or enterally (EN group; n = 8) to explore possible differences in the metabolic fate of glutamine derived from the dipeptide due to the route of administration. Enteral alanyl-[2-^{15}N]glutamine was provided by a self-propelling nasojejunal tube (Bengmark tube; Nutricia, Zoetermeer, Netherlands), which was inserted 2 d before surgery by a qualified nurse. The position of the nasojejunal tube was checked by taking an X-ray. If necessary, the tube was manually maneuvered into the jejunum by a radiologist (n = 1). Stable isotopes of L-citrulline and L-arginine were provided intravenously.

Stable-isotopes tracers

The tracers (all > 98% mole percent enrichment) L-alanyl- [2-^{15}N]glutamine and L-[^{13}C] ureido[3,3,4-$^{2}H_3$]citrulline were purchased from Buchem BV/ARC Laboratories (Apeldoorn, Netherlands), and L-[guanidino-^{15}N2]arginine was purchased from Cambridge Isotope Laboratories (Woburn, MA). Sterile and pyrogen-free

Table 1

Baseline patient characteristics by route of administration (intravenous or enteral) of alanyl-[2-15N]glutamine[1].

	Intravenous	Enteral
Preoperative diagnosis	Colorectal liver metastasis (n=3) Melanoma liver metastasis (n=1) Hepatocellular carcinoma (n=2) Pancreatic head carcinoma (n=1) Intraabdominal liposarcoma (n=1)	Colorectal liver metastasis (n=6) Ovarian carcinoma liver metastasis (n=1) Gastrointestinal stroma tumor (n-1)
ASA score (n)		
1	2	1
2	5	7
3	1	
Male/Female	7/1	6/2
Age (y)	59±4	62±4
Height (cm)	181±4	178±4
Weight (kg)	87±4	86±6
FFM (kg)	65±3	63±5
BMI (kg/m^2)	27±1	27±1
Laboratory results, reference value values[3]		
Kidney function (mmol/l)		
Urea (3.0 – 7.5)	5.7±0.6	5.0±0.4
Creatinine (60 – 110)	98±6	95±5
Liver function:		
Bilirubine (< 20 μmol/L)	23±14	14±2
ASAT (10 – 40 U/L)	27±4	27±5
ALAT (5 – 45 U/L)	27±6	28±5
Other variables:		
Albumin (34 – 50 g/L)	38±1	41±1
CRP (< 8 mg/L)	14±7	4±1

No significant differences were observed between groups (t test for independent samples). ASA, American Society of Anesthesiologists; FFM, fat-free mass; ASAT, aspartate amino transferase; ALAT, alanine amino transferase, CRP, C-reactive protein. [1]n = 16. [2]Mean ± SEM (all such values). [3] From the laboratory at the VU University Medical Center. *Plasma concentrations of amino acids determined in samples taken before start of the tracer administration; no reference value available from the VUMC laboratory. Reference values are described by Cynober et al.[20]

† Significant difference between the intravenous and enteral group ($p < 0.05$)

stock solutions of the tracers were prepared by the Pharmacy of the VUMC and were kept at 220°C until the evening before surgery. The stock solutions were diluted with normal saline before the start of each tracer infusion.

Surgical procedure and anesthesia

To standardize metabolic and surgical conditions, all patients underwent surgery at the same time of the day and were operated on by the same surgical team. Anesthesia was induced by using sevoflurane, sufentanyl, and tracium. Patients received a thoracic epidural catheter for perioperative administration of analgesia; indwelling catheters were inserted into a jugular vein and a radial artery to monitor arterial and central venous blood pressure, pH, HCO_3^-, and glucose. No exogenous bicarbonate was supplied, and lactate-containing infuses were routinely avoided in patients undergoing liver surgery. Urine output was monitored by using transurethral catheterization. Body temperature was kept constant by using a Bair Hugger system (Arizant Health Care Inc, Eden Prairie, MN).

Tracer infusion, blood sampling, and organ blood flow measure-ments

After anesthesia was induced, a catheter was placed into an antecubital vein for isotope infusion. Blood was collected from the radial artery catheter. After baseline sampling and shortly after incision, a primed continuous infusion of the stable-isotope tracers was started and continued for 2.5 h (Table 3). One calibrated, volume-controlled pump (Graseby 3000; Graseby Medical Ltd, Watford, United Kingdom) was used for

	Intravenous	Enteral
Surgery	Liver segment resection or metastasectomy (n=5)	Liver segment resection or metastasectomy (n=5)
	pancreatico-duodenectomy (n=1)	resection gastro-intestinal stroma
	resection liposarcoma (n=1)	cancer (n=1)
	no resection (n=1)	no resection (n=2)
Laboratory results, reference valus[2]		
Glutamine (663 ± 31 μmol/L)	614±47	674±25
Citrulline (35 ± 3μmol/L)	36±3	40±4
Arginine (99 ± 6μmol/l)	95±6	106±6
PH (7.35-7.45)		
Baseline pH	7.43±0.01	7.40±0.02
pH at 90 min	7.41±0.01	7.39±0.01
pH at 150 min	7.39±0.01	7.37±0.01
HCO_3^- (22-28 mmol/L)		
Baseline HCO_3^-	24.2±0.4	24.9±0.4
HCO_3^- at 90 min	23.8±0.7	22.5±0.4
HCO_3^- at 150 min	22.3±0.5	22.6±0.5
Glucose (3.7-5.2 mmol/L)		
Baseline glucose	8.0±1.3	7.1±0.6
Glucose at 90 min	8.9±0.7	8.3±0.4
Glucose at 150 min	9.0±0.7	8.7±0.7
Blood loss during tracer protocol (ml)	451±81	673±213
Fluid provided during tracer protocol (ml)	3988±682	4850±582
Urine production during tracer protocol (ml)	125±37	229±50
Plasma flow (ml/kg/hr)[4]		
Kidneys, renal vein x 2	335±31	335±31
Intestines, portal vein	354±37	354±37
Hepatic artery	130±23	130±23
Liver , portal vein and hepatic artery	484±47	484±47

Table 2

Patient characteristics during surgery by route of administration (intravenous or enteral) of alanyl-[2-15N]glutamine[1].

[1] All values are mean +/- SEM; n = 16. No significant differences were observed between groups (t test for independent samples). [2] From the laboratory at the VU University Medical Center. [3] From the laboratory at the University Hospital of Maastricht; reference values of men between 50 and 59 y of age. [4] Mean blood flow values agree with data in the literature[21].

intravenous infusion of stable isotopes, and another Graseby 3000 pump (Graseby Medical Ltd) was used to enterally administer the labeled dipeptide.

During tracer administration, blood samples were drawn 30, 60, 90, 120, and 150 min after the beginning of tracer administration for the study of WB plasma amino acid turnover. After 1 h, when experience has shown that an isotopic steady state would be present, blood was drawn from the portal vein, a hepatic vein, and the right renal vein by direct puncture, and arterial blood sampling was done simultaneously to study intestinal, hepatic, and renal metabolism. These venous samples were obtained before organ transection in those patients undergoing liver resection. To quantify amino acid (tracer) fluxes across the portal drained viscera (PDV; representing the intestine), liver, and kidneys, blood flow was measured before bifurcations in the portal vein, the hepatic artery, and the right renal vein by means of color Doppler ultrasound (Prosound SSD 5000; Aloka Co, Ltd, Tokyo, Japan) before abdominal blood samples were taken. Time averaged mean velocities of the bloodstream and cross-sectional area of the vessels were measured as described previously [21]. Briefly, the vessel was traced by using a sterile transducer (UST- 579T-7.5, 5–10-MHz linear; Aloka Co, Ltd). For accurate measurements, care was taken to keep the angle <60°. The cross sectional area of the vessel was calculated by drawing an area ellipse at the same point at which the velocity was measured.

Blood flow was calculated by multiplying the time-averaged velocity of the bloodstream by the cross-sectional area of the vessel. Plasma flow was calculated from measured blood flow and hematocrit as follows: plasma flow = blood flow · (1 - hematocrit). Total renal flow was estimated by multiplying the flow through the right renal vein by 2 for each patient separately, assuming that both kidneys contribute equally.

Laboratory analysis and calculation of tracer enrichments

Blood was collected into chilled heparinized vacuum tubes and placed on ice. Within 1 h, blood was centrifuged (10 min, 2000 · g, 4°C) and 500 μL plasma was added to 20 mg dry

sulfosalicylic acid to precipitate plasma proteins. After vortex mixing, deproteinized plasma samples were snap frozen in liquid nitrogen and stored at 280/C until they were analyzed. The use of stable isotopes requires plasma sampling, because sampling should occur from a pool in which the tracers mix freely [22]. Before centrifugation, the hematocrit of each blood sample was determined by using a microcapillary centrifuge. The plasma concentration of alanyl-[2-^{15}N]glutamine was determined by the Department of Clinical Biochemistry of the Université Paris Descartes (France) as described previously [19]. Alanyl-[2-^{15}N]glutamine could be detected at a concentration of 1 μmol/L. Determination of plasma amino acid concentrations and tracer enrichments was performed by the laboratory of the University Hospital of Maastricht (Netherlands), as described previously [4, 11, 12, 23, 24], and were expressed as tracer-to-tracee ratio [(TTR) tracer = labeled substrate; tracee = unlabeled substrate] · 100 (TTR%). Isotopic enrichment was calculated by taking into account the contribution of overlapping isotopomer distributions of the tracee and tracers with lower masses to the measured TTR as described by Vogt et al. [25]. For the estimation of individual steady state values, arterial enrichment curves at each mass of each amino acid under study were fitted for each patient with the use of PRISM for WINDOWS software (version 4.03; GraphPad Software Inc, San Diego, CA). For calculations of organ metabolism, the enrichment of the arterial sample taken simultaneously with the sample from the corresponding vein was used in the calculations.

Table 3

	Infusion rate μmol/kg/h	Priming dosage μmol/kg
L-alanyl-L-[2-^{15}N]glutamine	10.02 ± 0.48	17.80 ± 0.83
L-[ureido-^{13}C-$^{2}H_3$]citrulline	0.17 ± 0.01	0.42 ± 0.02
L- [guanidino-$^{15}N_2$]arginine	2.75 ± 0.12	3.11 ± 0.15

Tracer dosages. All values are mean ± SEM.

Calculation of whole-body plasma turnover of glutamine, citrulline, and arginine and of the fraction of exogenously provided glutamine used for the de novo synthesis of arginine.

Whole-body plasma rate of appearance (= turnover) of glutamine, citrulline, and arginine, and the de novo synthesis of arginine.

The WB plasma rate of appearance (WB Ra; μmol · kg^{-1} · h^{-1}) of glutamine, citrulline, and arginine was calculated from the arterial TTR values (TTR-A) of [^{15}N] glutamine, [^{13}C-$^{2}H_3$]citrulline, and [$^{15}N_2$] arginine, respectively, and the known infusion rate of these tracers by using the following equation [26]:

[1] $\quad$ WB Ra = I/TTR-A

For [^{15}N]glutamine the infusion rate of alanyl-[2-^{15}N] glutamine was used, because a very high fraction of the dipeptide is immediately hydrolyzed into [2-^{15}N]glutamine and alanine [17, 18].

The WB Ra of glutamine was used to calculate the first-pass splanchnic extraction of glutamine derived from enterally provided alanyl-[2-^{15}N]glutamine. The splanchnic extraction of glutamine could be estimated from the difference between the Mean ($\bar{x}$) WB Ra Gln with intravenous (IV) or enteral (EN) administration of the dipeptide [9]:

[2] $\quad$ $SE = [1 - (\bar{x}\ IV\ WB\ Ra\ Gln / \bar{x}\ EN\ WB\ Ra\ Gln)] \cdot 100\%$

The WB plasma turnover of citrulline into arginine (de novo arginine synthesis) was calculated by using the following equation (4):

[3] $\quad$ $Q_{Cit \rightarrow Arg} = WB\ Ra_{Arg} * (TTR\text{-}A_{Arg\ M+4} / TTR\text{-}A_{Cit\ M+4})$

where WB Ra_{Arg} is the WB Ra of arginine, calculated from the TTR of the [^{15}N2]arginine tracer by using **Equation 1**, and Arg M+4 is [^{13}C-$^{2}H_3$]arginine coming from [^{13}C-$^{2}H_3$] citrulline (Cit M+4).

Contribution of intravenously or enterally provided alanyl-glutamine–derived glutamine to the de novo synthesis of arginine

First, the fraction of exogenously provided glutamine used for the synthesis of citrulline was calculated:

[4] $\quad$ $F_{Gln \rightarrow Cit} = TTR\text{-}A_{CitM+1} \cdot (WBRa_{Cit} / Gln_{M+1})$

where $TTR\text{-}A_{Cit\ M+1}$ is the arterial enrichment of [^{15}N]citrulline coming from [^{15}N]glutamine, WB Ra_{Cit} is the plasma WB Ra of citrulline calculated from the TTR of the infused [^{13}C-$^{2}H_3$ citrulline tracer by using **Equation 1**, and $I_{Gln\ M+1}$ is the infusion rate of [^{15}N] glutamine, which is assumed to be equal to the infusion rate of alanyl-glutamine as explained in relation to **Equation 1**.

Equation 3 can be mathematically derived from the equation that we used in previous publications to calculate the fraction of unlabeled glutamine that was used for the synthesis of citrulline [4, 9].

Subsequently, the fraction of citrulline used for the synthesis of arginine was calculated as follows:

$$[5]\quad F_{Cit \to Arg} = TTR\text{-}A_{ArgM+4} \cdot (WBRa_{Arg} / I_{CitM+4}) \cdot 100\%$$

where $TTR\text{-}A_{ArgM+4}$ is the arterial enrichment of [^{13}C-$^{2}H_3$]arginine coming from [^{13}C-$^{2}H_3$] citrulline (Cit M+4), WB Ra_{Arg} is the plasma WB Ra of arginine calculated from the TTR of the infused [$^{15}N_2$]arginine tracer by using Equation 1, and $I_{Cit M+4}$ is the infusion rate of [^{13}C-$^{2}H_3$]citrulline. **Equation 4** can be mathematically derived from the equation that we used in a previous publication to calculate the fraction of unlabeled citrulline that was used for the synthesis of arginine [9].

Finally, the fraction of exogenously provided glutamine used for the synthesis of arginine was calculated by combining both equations:

$$[6]\quad F_{Gln \to Arg} = F_{Gln \to Cit} \cdot F_{Cit \to Arg}$$

A similar approach was applied by us in previous publications to calculate the contribution of unlabeled glutamine to the de novo synthesis of unlabeled arginine [4, 9].

Calculation of intestinal, hepatic, and renal metabolism of the amino acids studied

Intestinal and renal (tracer) net balances of the amino acids studied

The amino acid NBs across the PDV, representing the intestines, and the kidneys, of alanyl-glutamine, glutamine, citrulline, arginine, and alanine were calculated by subtracting organ output {[portal or renal venous (V) amino acid concentration (in µmol/ L)] · plasma flow (F) in L· kg-1 · h-1)} from organ input {[arterial (A) amino acid concentration (in µmol/L)] · F}, according to the following equation (4):

$$[7]\quad NB = ([A] * F) - ([V] * F)$$

When the dipeptide was provided enterally, the intestinal NB of alanyl-glutamine and glutamine included the enteral infusion rate of alanyl-[2-^{15}N]glutamine:

$$[8]\quad \text{Intestinal NB Ala-Gln} = ([A_{Ala\text{-}Gln}] \cdot F_{PV} + I_{Ala\text{-}Gln}) - ([V_{Ala\text{-}Gln}] \cdot F_{PV})$$

$$[9]\quad \text{Intestinal NB Gln} = ([A_{Gln}] \cdot F_{PV} + I_{Ala\text{-}Gln} - ([A_{gln}] \cdot F_{PV})$$

where $[A_{Ala\text{-}Gln}]$ and $[A_{Gln}]$ are the arterial plasma alanylglutamine and glutamine concentrations, F_{PV} is the plasma flow in the portal vein, $I_{Ala\text{-}Gln}$ is the infusion rate of alanyl-glutamine, and $[V_{Ala\text{-}Gln}]$ and $[V_{Gln}]$ are the venous plasma alanyl-glutamine and glutamine concentrations. The TNBs of the glutamine, citrulline, and arginine tracers were calculated in the same way as the net balances (NBs), according to the following equation [4]:

$$[10]\quad TNB = ([A] \cdot TTR\text{-}A \cdot F) - ([V] \cdot TTR\text{-}V \cdot F)$$

where TTR-A is the arterial tracer enrichment, and TTR-V is the venous tracer enrichment. When the dipeptide was provided enterally, the intestinal TNB of [2-^{15}N]glutamine was adjusted in the same way as the NB of glutamine, by including the rate of enterally administered [2-^{15}N]glutamine derived from alanyl-[2-1^{5}N]glutamine:

$$[11]\quad \text{Intestinal TNB } [^{15}]Gln = ([A_{Gln}] \cdot TTR\text{-}A_{[15N]Gln} \cdot F_{PV} + I_{[Ala\text{-}Gln]}) \cdot I - ([V_{Gln}] \cdot TTR\text{-}PV_{[15N]Gln} \cdot F_{PV})$$

where $TTR\text{-}A_{[15N]Gln}$ is the arterial tracer enrichment of [^{15}N] glutamine, and $TTR\text{-}PV_{[15N]Gln}$ is the portal venous enrichment of [^{15}N]glutamine. To calculate the organ-specific fractional extraction (FE) of glutamine, citrulline, and arginine, the TNB of the concomitantly administered tracers was divided by the organ-specific input of the provided tracer, according to the following equation:

$$[12]\quad FE = TNB / ([A] \cdot TTR\text{-}A \cdot F)$$

To calculate the FE of alanyl-glutamine, the NB of alanylglutamine and the TNB of [^{15}N]glutamine were divided by the organ-specific input of the dipeptide or [^{15}N] glutamine, which included the infusion rate of the dipeptide tracer in the EN group.

Hepatic (tracer) net balances of the amino acids studied

Hepatic amino acid NBs and TNBs were calculated by subtractingPDV NBs from their corresponding splanchnic (PDV + liver) values, as described previously [11]. Splanchnic (SPL) NBs and TNBs were calculated by using the amino acid concentrations and TTRs of corresponding tracers in the hepatic vein (HV) and the combined flow of the portal vein (F_{PV}) and the hepatic artery (F_{HA}), according to the following equations:

$$[13]\quad SPL\ NB = ([A] \cdot (FHA + FPV)) - ([HV] \cdot FHA + FPV)$$

$$[14]\quad SPL\ TNB = ([A] \cdot TTR\text{-}A \cdot (FHA + FPV)) ([HV] \cdot TTR\text{-}HV \cdot (FHA + FPV))$$

The hepatic FE of the amino acids was calculated according to **Equation 12**.
The hepatic tracer input was calculated by adding up the hepatic input by the hepatic artery and the hepatic input by the portal vein, according to the following equation:

$$[15]\quad \text{Tracer input liver} = ([A] \cdot TTR\text{-}A \cdot FHA) + ([PV] \cdot TTR\text{-}PV \cdot FPV)$$

Statistical analysis

The results are presented as means ± SEMs. The one-factor analysis of variance for repeated measurements was used to test whether the arterial enrichments were in steady state. The one-sample t test was used to test whether arterial and venous enrichments, WB turnover, and NBs across organs of the amino acids, concomitant tracers, and the dipeptide differed from zero. Differences between the IV and EN groups were tested by using a t test for independent samples. We used EXCEL for WINDOWS software (version 2003; Microsoft Corp, Redmond, WA) to perform calculations and SPSS for WINDOWS software (version 14.0.1; SPSS Inc, Chicago, IL) to perform statistical tests. $P < 0.05$ was considered to indicate statistical significance.

Results

Sixteen patients were included in the study. Eight patients received alanyl-[2-15N] glutamine intravenously, and the other 8 patients received the labeled dipeptide enterally (Table 1). One patient in the IV group with pancreatic cancer had high concentrations of bilirubin (because of obstruction of the biliary tract) and of CRP before surgery. However, metabolic results of this patient were in line with the results of the other patients. Patient characteristics during surgery are summarized in Table 2.

For each vessel, the mean plasma flow results of all patients were used in calculations, because the results did not differ significantly from individual flow results, but variation was larger when individual flow results were applied.

Hydrolysis of alanyl-[2-15N]glutamine with intravenous or enteral administration of the dipeptide

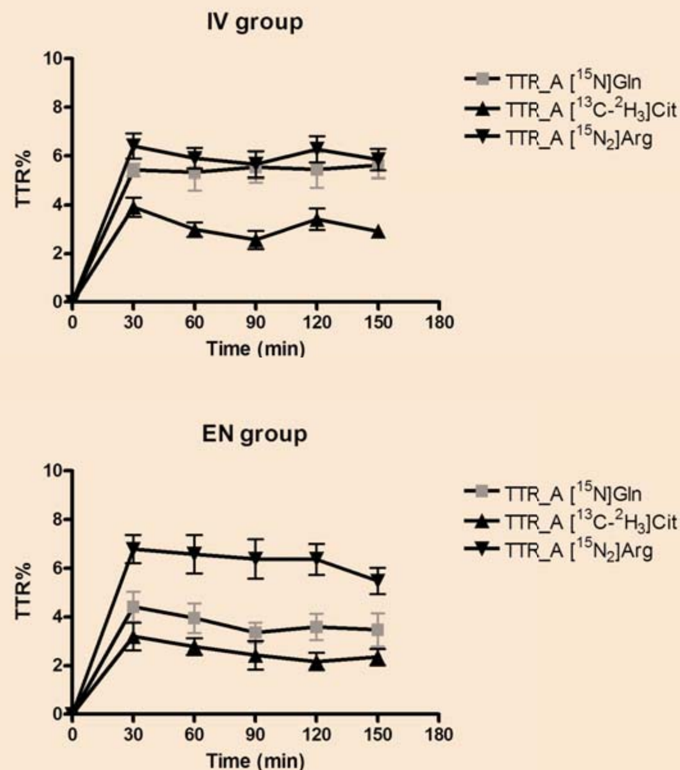
The intact dipeptide was barely detectable in any of the arterial (IV group: n = 2; EN group: n = 2) or portal venous blood (IV group: n = 2; EN group: n = 1), hepatic venous blood (IV group: n = 1; EN group: n = 1), or renal venous blood (IV group: n = 1; EN group: n = 2) plasma samples. No differences were observed between the IV and the EN groups.

When detected, plasma concentrations of alanyl-[2-15N]glutamine ranged between 1 and 5 μM. The absence of alanyl-glutamine in portal venous blood of all but one patient in the EN group indicated that the intestine is the site where the hydrolysis of enterally administered alanylglutamine takes place. On the other hand, the absence of alanyl-glutamine in the plasma samples of patients who received the dipeptide intravenously does not identify the site of hydrolysis of intravenously administered alanyl-glutamine. Overall, no difference was observed in the rate of hydrolysis between the EN and IV groups.

Route of administration affects the contribution of glutamine to the synthesis of citrulline and arginine at the whole-body level

Arterial plasma enrichment (TTR) was in steady state for all given tracers and for the conversion substrates [15N]citrulline, [15N] arginine, and [13C-2H3]arginine (Figures 1, 2, and 3). The TTRs of the metabolic products of [15N]glutamine metabolism— [15N] citrulline and [15N]arginine—were significantly different from zero in both groups ($P < 0.001$), which shows that the 15N label of glutamine derived from the dipeptide alanyl-[2-15N]glutamine found its way to citrulline and arginine with both routes of

Figure 1



Mean (±SEM) arterial plasma enrichments of the administered stable-isotope tracers at each time point: L-alanyl-L-[2-15N]glutamine–derived L-[2-15N]glutamine ([15N]Gln), L-[13C] ureido[3,3,4-2H3]citrulline ([13C-2H3]Cit), L-[guanidino-15N2]arginine, and ([15N2]Arg) after intravenous (IV group; n = 8) or enteral (EN group; n = 8) administration of alanyl-[2-15N] glutamine. TTR%, tracer-to tracee ratio (%). No significant change in the slope of arterial enrichment was observed between 30 and 120 min for any of the administered tracers. Therefore, the enrichment of all administered tracers was considered to be in steady state with both routes of administration of the dipeptide. To estimate individual steady state values, arterial enrichment curves were fitted for each patient, which resulted in one value for each enrichment per patient. The arterial enrichment of [15N]Gln was significantly different between the routes of administration of the dipeptide ($P = 0.02$).

administration (Figure 2). The [15N]glutamine enrichment was significantly lower in the EN group than in the IV group (Figure 1), which reflects the splanchnic extraction of alanyl-glutamine– derived glutamine when delivered enterally. On the other hand, the TTRs of [15N]citrulline and [15N]arginine were significantly higher in the EN group (Figure 2). This observation suggests that enterally provided alanyl-glutamine contributes more to the synthesis of citrulline and arginine than does intravenously provided alanyl-glutamine. No difference was observed in [13C-2H3]arginine enrichment, which is derived from [13C-2H3]citrulline between the 2 routes of administration of the dipeptide (Figure 3). This supports the notion that the higher plasma enrichment of [15N] arginine with enteral administration of alanyl-[2-15N]glutamine is due to the enteral route of administration.

With respect to the WB plasma turnover rates of unlabeled glutamine, citrulline, and arginine, the following observations were made. The WB Ra of glutamine was observed to be 201 ± 15 μmol·kg-1·h-1 in the IV group and 281 ± 31 μmol·kg-1·h-1 in the EN group. This difference again reflects the splanchnic extraction of [15N] glutamine with enteral administration of the dipeptide, which was calculated to be 29%. The WB Ra of citrulline (IV group: 6.0 ± 0.5 μmol·kg-1·h-1; EN group: 6.9 ± 0.8 μmol·kg-1·h-1), arginine (IV group: 47 ± 1;EN group: 42 ± 2) and the de novo synthesis of arginine (IV group: 4.9 ± 0.4 μmol·kg-1·h-1; EN group: 5.8 ± 0.8 μmol·kg-1·h-1) were comparable with both routes of administration.

The fraction of citrulline used for the synthesis of arginine (= de novo synthesis of arginine) was also comparable for both routes of administration (IV group: 85 ± 8%; EN group: 85 ± 7%).

However, twice as much exogenous glutamine was used for the synthesis of citrulline when alanyl-glutamine was provided enterally than when provided intravenously (Figure 4). Consequently, twice as much exogenous glutamine was used for the synthesis of arginine when alanyl-glutamine was provided enterally (Figure 4). These observations were highly significant (t test for independent samples: $P < 0.01$).

Intestinal net balances and tracer net balances of glutamine, citrulline, and arginine

Intestinal amino acid balances

The intestine took up a significant amount of glutamine and released a significant amount of citrulline (Table 5). A non-significant release of arginine was observed. The intestinal release of alanine tended to be higher with the enteral than with the intravenous administration of alanyl-glutamine (IV group: 26.3 ± 3.5 μmol·kg-1·h-1; EN group: -22.9 ± 8.4 μmol·kg-1·h-1; t test for independent samples: $P = 0.09$).

Intestinal tracer net balances

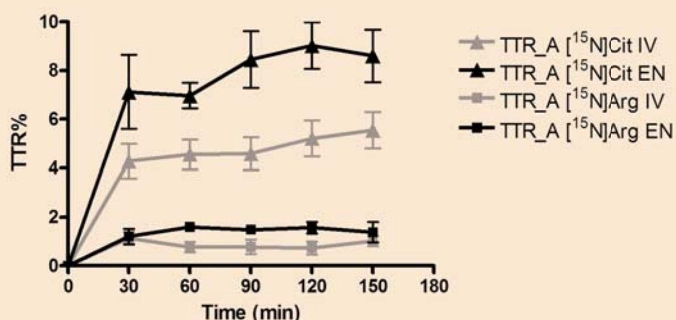
The intestinal TNB of [15N]glutamine and FE of glutamine was comparable for both routes of administration and was observed to be non-significant (Table 5). A significant intestinal uptake of [13C-2H3]citrulline and concomitant FE of citrulline was observed. At the same time, a significant intestinal release of [15N]citrulline (derived from

Table 4

	IV	EN
[15N]glutamine	5.28 ± 0.40	3.68 ± 0.45
[$^2H_3$-^{13}C]citrulline	3.08 ± 0.25	2.66 ± 0.33
[$^{15}N_2$]arginine	6.06 ± 0.31	6.49 ± 0.65
[$^2H_5$]phenylalanine	7.44 ± 0.78	7.49 ± 0.71
[$^2H_2$]tyrosine	2.22 ± 0.13	2.41 ± 0.25

Tracer enrichments. Fitted individual arterial steady state enrichments (TTR%) of given tracers with intravenous (IV) or enteral (EN) administration of alanyl-[15N]glutamine (mean ± SE)

Figure 2



Mean (+/-SEM) arterial plasma enrichments of [15N]citrulline (Cit) and arginine (Arg) with intravenous (IV group; n = 8) or enteral (EN group; n = 8) administration of L-alanyl-L-[2-15N] glutamine. TTR%, tracer to tracee ratio (%). No significant change in the slope of arterial enrichment was observed between 30 and 120 min (one-factor repeated-measures ANOVA) for any of the administered tracers for any of the substrates. Therefore, the TTRs of all [15N] substrates were considered to be in steady state with the 2 routes of administration of the dipeptide. To estimate individual steady state values, arterial enrichment curves were fitted for each patient (see Subjects and Methods), which resulted in one value for each enrichment per patient. The arterial enrichments of [15N]Cit and Arg were significantly different between the routes of administration of the dipeptide (P , 0.01, t test for independent samples).

[15N]glutamine) was shown. Furthermore, a significant release of [15N]arginine in the EN group.

Hepatic net balances and tracer net balances of glutamine, citrulline, and arginine

Hepatic amino acid balances

The liver was observed to release and take up nonsignificant amounts of glutamine with intravenous and enteral administration of the dipeptide, respectively (Table 5). A significant hepatic uptake of citrulline and arginine was shown. Furthermore, the liver was observed to take up significantly more alanine with enteral than with intravenous administration of the dipeptide (IV group: 18.3 ± 8.0 μmol·kg-1·h-1; EN group: 54.0 ± 12.0 μmol·kg-1·h-1; P = 0.03).

Hepatic tracer net balances

The hepatic TNB of [15N]glutamine indicated a significant uptake of [15N]glutamine with enteral administration of the dipeptide, in contrast with a nonsignificant release of [15N]glutamine with intravenous administration (Table 5). Therefore, the FE of glutamine was only present in the EN group. No hepatic FE of citrulline was observed, because the TNB of [13C-2H3]citrulline was zero.
A significant hepatic uptake of [15N2] arginine and concomitant FE of arginine was observed. The liver was not observed to release [15N] citrulline or arginine.

Renal net balances and tracer net balances of glutamine, citrulline, and arginine

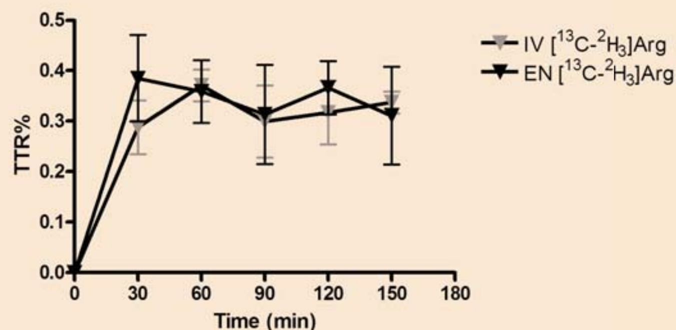
Renal amino acid balances

The kidneys were observed to take up citrulline and release arginine in significant amounts (Table 5).

Renal tracer net balances

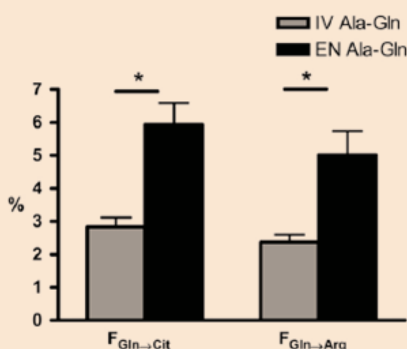
Renal citrulline uptake and arginine release were not statistically confirmed by the TNBs of [13C-2H3]citrulline and [15N2]arginine, although the TNB of [13C-2H3]citrulline indicated that the kidneys extracted significantly more citrulline with enteral than with intravenous administration of alanyl-[2-15N] glutamine (Table 5). Interestingly, despite the higher renal FE of citrulline with EN administration of the dipeptide, the renal release of [15N] and [13C-2H3] arginine was comparable with both routes of administration.
The renal fractional extraction of glutamine was negligible, which indicated that glutamine did not contribute to the synthesis of arginine at the kidney level.

Figure 3



Mean (+/-SEM) arterial plasma enrichments of [13C-2H3] arginine (Arg) with intravenous (IV group; n = 8) or enteral (EN group; n = 8) administration of alanyl-[2-15N]glutamine. TTR%, tracer-to tracee ratio (in %). No significant change in the slope of arterial enrichment was observed between 30 and 120 min (one-factor repeated-measures ANOVA). Therefore, the TTR of [13C-2H3]Arg was considered to be in steady state with both routes of administration of the dipeptide. To estimate individual steady state values, arterial enrichment curves were fitted for each patient (see Subjects and Methods), which resulted in one value for each enrichment per patient. No significant difference was observed between the mean TTRs with intravenous or enteral administration of the dipeptide (t test for independent samples).

Figure 4



The fraction (F) of exogenous glutamine (Gln) used for the de novo synthesis of citrulline (Cit) and arginine (Arg). Twice as much exogenous Gln was used for the synthesis of Cit when alanyl-glutamine (Ala-Gln) was provided enterally (EN group) than when provided intravenously (IV group). Consequently, twice as much exogenous Gln was used for the synthesis of Arg when Ala-Gln was provided enterally. *Significant difference between the IV and EN groups, P , 0.01 (t test for independent samples).

Table 5

	Portally drained viscera		Liver		Kidneys	
	IV	EN	IV	EN	IV	EN
Glutamine						
NB	24.1±6.9 *	28.1±7.9 *	-18.9±8.9	7.2±8.7	-3.3±10.7	7.1±8.9
[^{15}N]Gln tnb	1.6±1.0	6.2±2.4 *	-3.0±1.8	5.1±1.7 *†	-1.4±1.0	-0.3±0.7
FE Gln (%)	11.0±7.8	25.0±9.0 *	0.0±0.0	28±10 *†	0.0±0.0	0.0±0.0
Citrulline						
NB	-4.7±1.8 *	-3.8±1.3 *	3.1±1.8	2.7±1.3	1.7±0.7 *	2.3±0.6 *
[^{15}N]Cit tnb	-0.29±0.14	-0.22±0.11	-0.01±0.15	0.19±0.20	0.0±0.1	0.3±0.2
FE Cit (%)	19±10	28±8 *	0.0±0.0	0.1±11	0.0±0.0	24±14 †
Arginine						
NB	-0.56± 1.13	-1.09±1.03	2.7±2.2	7.8 ± 1.7 *	-4.6±1.6 *	-2.2±1.5
[^{15}N]Arg TNB	0.03±0.15	-0.17±0.05 *	0.08±0.15	0.25 ± 0.17	-0.12±0.13	-0.1±0.1
[$^{2}H_{3}$-^{13}C]Arg	0.03±0.01	0.03±0.02	0.05±0.04	0.12±0.02 *	-0.01± 0.02	0.01±0.01
FE Arg (%)	21±6 *	6±5	11±7	18±3 *	1.0±4.8	9.2±5.7
Alanine						
NB	-6.3±3.5	-22.9±8.4 *	18.3±8.0	54.0±12.0 *†	-9.7±5.2	-13.3±7.4

Organ net balance (NB) and tracer NB (TNB) of glutamine (Gln), citrulline (Cit), and arginine (Arg) and the fractional extraction (FE) of Gln, Cit, and Arg, which were calculated with the administered tracers[1] In μmol/kg/hr, or %. NB = net balance. Tnb = tracer net belance. FE = fractional extraction calculated using the tnb.

Ala-Gln = alanyl-glutamine.
Gln = glutamine. Cit = citrulline. Arg = arginine.
Ala = alanine. * = significantly different from 0.
† = significant difference between the intravenous and enteral group ($p < 0.05$).

Discussion

To our knowledge this is the first time that alanyl-glutamine– derived glutamine was shown to contribute to the synthesis of citrulline and arginine in humans and to contribute most when provided enterally. However, results at the organ level did not provide an explanation for the observation that enterally provided alanyl-glutamine contributed more to the de novo synthesis of arginine than did intravenous administration of the dipeptide.

Remarks with respect to the design of the study

The applied study design is identical to the design used in a prior human experiment [4, 11, 12], except for the use of the alanyl-[2-^{15}N] glutamine tracer in the present study. Patients were studied in the postabsorptive state and during abdominal surgery to gain access to the portal, hepatic, and renal veins. The reasons for and consequences of this design are discussed in a prior publication [4]. Briefly, surgery may cause a depression of WB protein metabolism and affect the acid-base status, which has a distinct effect on glutamine metabolism. Also, patients received large amounts of fluid during surgery, which may have diluted plasma. However, WB fluxes of glutamine and arginine were not observed to be affected. Also, the acid-base status (Table 2) and hematocrit values (data not shown) remained stable during the tracer protocol. Moreover, metabolic steady state was maintained during the study protocol, and venous blood was sampled before organ transection.

Plasma concentrations of glutamine were observed to be within a physiologic range (Table 2), which suggested that cancer cells present in the patients withdrew at most a limited amount of glutamine—a possibility discussed in a prior publication [19].

By which pathways does glutamine contribute to the synthesis of arginine?

When the [2-^{15}N] label is followed to investigate the contribution of glutamine to the synthesis of arginine, 2 and perhaps 3 possible metabolic pathways by which glutamine may contribute are included: 1) glutamine contributes to the synthesis of arginine by providing the carbon skeleton together with the a-amino group; and 2) glutamine contributes nitrogen to the guanidino group of arginine via aspartate after transamination of glutamate into α-ketoglutarate, which involves the transfer of the labeled amino group to aspartate;

and 3) glutamine is degraded and the labeled ammonia contributes nitrogen to the ureido group of citrulline and subsequently to the guanidino group of arginine within the urea cycle. However, the urea cycle is highly compartmentalized within the liver, yielding plasma release of urea but not of arginine [6].

In our previous study, the contribution of unlabeled glutamine to arginine synthesis was quantified by pathway A [4]. In the present study, we used the same approach to determine the fraction of alanyl-glutamine–derived glutamine used for the de novo synthesis of arginine and to explore whether this fraction is affected by the route of administration—intravenous or enteral.

Results at the whole-body level

The WB plasma turnover rates of glutamine (with intravenous administration of alanyl-[2-^{15}N]glutamine), citrulline, and arginine were in line with previous observations [4]. The WB plasma turnover of glutamine was higher with enteral administration of alanyl-[2-^{15}N]glutamine than with intravenous administration, which reflects the splanchnic extraction of enterally provided glutamine [12, 27].
The percentage of alanyl-glutamine–derived glutamine that was used to synthesize de novo arginine was observed to be twice as high with enteral than with intravenous administration of the dipeptide (EN group: 5 ± 0.7%; IV group: 2.4 ± 0.2%). Also, the plasma TTR of [^{15}N]arginine was significantly higher with enteral than with intravenous administration of the dipeptide, which supports the observation that enterally provided alanyl-glutamine contributes more to the synthesis of arginine than intravenously provided alanyl-glutamine.

This observation supports previous observations that luminally provided alanyl-glutamine contributes more to the synthesis of plasma arginine than does intravenously administered alanyl-glutamine– derived glutamine [9, 12].
When the observed fraction of exogenous glutamine used for the synthesis of arginine with intravenous or enteral administration of alanyl-glutamine is used to calculate how much arginine would be generated from an intervention dose of glutamine of 0.5 g·kg^{-1}·h^{-1} (provided as 0.8 g·kg^{-1}·h^{-1} alanyl-glutamine), the calculated de novo synthesis of arginine would be 2.4 μmol·kg^{-1}·h^{-1} with intravenous and 4.9 μmol·kg^{-1}·h^{-1} with enteral administration of 0.8 g alanyl-glutamine.
These are substantial amounts in relation to the absolute WB turnover of arginine (IV group: 47 ± 1 μmol·kg^{-1}·h^{-1}; EN group: 42 ± 2 μmol·kg^{-1}·h^{-1}) and the de novo rate of appearance of arginine (IV group: 4.9 ± 0.4 μmol·kg^{-1}·h^{-1}; EN group: 5.8 ± 0.8 μmol·kg^{-1}·h^{-1}) under current fasting conditions. A future study involving an intervention dose of exogenous alanyl-glutamine will be necessary to confirm the importance of exogenous glutamine, particularly when provided enterally, as a substrate for the de novo synthesis of arginine when the need for arginine is high (e.g. critical illness) [2, 28].

Results at the organ level

Results at the organ level should be interpreted with caution, because only one sample of each abdominal vessel was obtained for each patient and because organ amino acid and TNB depend on the accuracy of the flow measurement. Furthermore, organ TNBs approached zero, which complicated statistical testing of differences between groups.

The gut was shown to be the most important site for extraction and subsequent hydrolysis of enterally administered alanylglutamine.
The organ-specific extraction of the intravenously provided dipeptide could not be determined. Previous data show a higher intestinal release of [^{15}N]citrulline with enteral than with intravenous administration of [2-^{15}N]glutamine [12].

The results of the present study did not show a difference in the intestinal release of [^{15}N] citrulline with intravenous or enteral administration of the dipeptide. It can be hypothesized that alanine released from alanyl-glutamine within the intestine affected the intestinal use of [2-^{15}N]glutamine, for example, by diluting the [2-^{15}N] amino nitrogen pool.

Interestingly, enteral administration of the dipeptide resulted in a significant rate of appearance of [^{15}N]arginine in the portal vein. Arginine production by the gut has been reported by others in newborn mice [29], pigs [30], and, more importantly, in surgical patients [31, 32]. Enzymes responsible for the synthesis of arginine—argininosuccinate synthase and lyase—remain present in the intestines after birth and are inducible by the presence of glutamine and glucocorticoids [8, 33, 34]. Also, [^{15}N]citrulline was not a likely precursor for the intestinal release of [15N]arginine, because no intestinal uptake of [^{15}N]citrulline was observed with enteral administration of the dipeptide. The intestines were even observed to take up instead of release [^{13}C-^{2}H$_3$]arginine (coming from [^{13}C-^{2}H$_3$]citrulline), providing another argument against intestinal turnover of citrulline into arginine. We hypothesize that alanine released from alanyl-glutamine affected the direction of the conversion of glutamine toward arginine within the intestine.
This observation should be explored in a future stable-isotope study in surgical patients who receive a treatment dose of glutamine or alanyl-glutamine enterally or intravenously.

Neither the liver nor the kidney was observed to release significantly more [^{15}N] citrulline or arginine with enteral than with intravenous administration of the dipeptide. Therefore, the question remains as to where the higher arterial enrichment of [^{15}N]citrulline and [^{15}N] arginine originated from. We may have overlooked part of the synthesis of arginine in the intestine when this took place in the gut-associated immune system, because the gut-associated immune system is connected to the lymphatic system, which bypasses the portal vein. Others have shown that polymorphonuclear neutrophils convert glutamine into arginine [35]. Therefore, glutamine utilization by these and perhaps other cells of the immune system, such as lymphocytes, which are abundant in the gut-associated immune system, may also have contributed to the plasma WB Ra of [^{15}N]arginine and citrulline. Newsholme et al. [36] suggest that aspartate is an important end product of glutamine metabolism in the lymphocyte.
Therefore it can be speculated that glutamine after conversion into glutamate contributes to the synthesis of arginine and citrulline within the nitric oxide cycle by donating its amino nitrogen to aspartate. When this assumption is true, the nitric oxide cycle may in fact be less compartmentalized than its cyclic character suggests. More research is necessary to explore this hypothesis.

In conclusion, alanyl-glutamine-derived glutamine contributes to the synthesis of arginine when provided intravenously or enterally, but most when provided enterally. More studies are needed to investigate the clinical implication of this observation and to explore other possible sites in the human body where citrulline and arginine are synthesized from glutamine. From a clinical point of view, it is important to establish whether the administration of a treatment dose of alanyl-glutamine, particularly when provided enterally, enhances the synthesis of plasma citrulline and arginine in critically ill patients.

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The authors' responsibilities were as follows: G.C.L.M. and M.C.G.v.d.P.: conducted the study, interpreted the results, and wrote the manuscript; M.A.R.V.: helped write the manuscript; PGB: wrote the protocol for the study; M.P.v.d.T.: collected the abdominal blood samples; CvS: developed the protocol for the in vivo flow measurements; J.P.D.B.: measured plasma concentrations of alanylglutamine and helped write the manuscript; N.E.P.D.: helped design the study, interpret the results, and write the manuscript; C.H.C.D.: helped design the study, develop the model for the in vivo measurement in humans, interpret the results, and write the manuscript; and PAMvL: intellectual responsibility for the objective of the study and supervised all aspects of the project. None of the authors declared a potential conflict of interest.

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5. Intravenous glutamine supplementation enhances renal de novo arginine synthesis in humans; a stable isotope study

Nikki Buijs, Saskia J.H. Brinkmann, J. Efraim Oosterink, Joanna Luttikhold, Henk Schierbeek, Willem Wisselink, Albertus Beishuizen, Johannes B. van Goudoever, Alexander P.J. Houdijk, Paul A.M. van Leeuwen and Mechteld A.R. Vermeulen

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Abstract

Background Arginine plays a role in many different pathways in multiple cell types. Consequently, shortage of arginine, caused by pathological conditions like cancer or injury, has the potential to disturb many cellular and organ functions. Glutamine is the ultimate source for de novo synthesis of arginine in humans via the intestinal-renal axis. Therefore, we hypothesized that parenteral glutamine supplementation may stimulate the interorgan pathway of arginine production.

Objective To quantify arginine production from its precursor glutamine and to establish the contribution of the kidneys to de novo synthesis of arginine in patients during major abdominal surgery receiving intravenous supplementation of glutamine dipeptide.

Design Whole body and renal metabolism of glutamine, citrulline, and arginine was assessed by stable isotopes techniques in seven patients receiving a perioperative supplement of intravenous alanyl-glutamine (0.5 g/kg/day).

Results Plasma glutamine, citrulline and arginine concentrations increased significantly in patients receiving intravenous glutamine dipeptide. On whole body level, 91% of total citrulline turnover was derived from glutamine, while 49% of whole body citrulline turnover was used for de novo synthesis of arginine. The kidneys were responsible for 75% of whole body arginine production from citrulline.

Conclusions Glutamine and citrulline are important sources for de novo arginine synthesis. The kidneys are the main production site for endogenous arginine. When comparing these results with previous similar studies, our data suggest that an intravenous glutamine supplement doubles renal arginine production from citrulline.

Introduction

Arginine is a conditionally essential amino acid. This means the normal endogenous arginine production meets the need of the adult human body; however in pathophysiological conditions de novo arginine synthesis becomes inadequate. Without adequate supplementation of arginine or its precursors, arginine availability may decrease to levels jeopardizing normal biological responses [1, 2].
Arginine is a molecule of particular interest because it plays a role in many different pathways in multiple cell types. Arginine is an important regulator of protein synthesis and proteolysis and it serves as the precursor for nitric oxide, creatine, agmatine, polyamines, proline and glutamate [3].

Studies in animals and humans showed that arginine becomes an essential amino acid in several pathological conditions, such as cancer, critical illness, after traumatic of surgical injury and during infections [4,5].
This arginine deficiency state may lead to immunosuppression, impaired recovery, disturbed inflammatory response and diminished vascular function [2, 6]. Unfortunately, arginine supplementation showed controversial effects in critically ill patients [5]. Yet, it was also found that glutamine administration increases plasma concentrations of arginine [7-10]. Metabolic studies showed that glutamine is an important precursor for the synthesis of citrulline in the intestines and arginine by the kidneys (glutamine-citrulline-arginine intestinal-renal axis) [11-13], and also quantified using stable isotope methodology [12-16]. Moreover, glutamine supplementation has positive effects on clinical outcome in several diseases [17-20]. It has been suggested that a major part of glutamine's effects can be attributed to the formation of arginine from the administered glutamine [21]. However, data on clearly defined net contribution of glutamine to the synthesis of citrulline and arginine when extra glutamine is administered are lacking. When translating research, the development of optimal nutritional strategies relies on distinct insights in the effect of a supplemental component on metabolic pathways.
Especially now a recently published randomized trial showed controversial effects of high dose glutamine supplementation [22], in-depth metabolic data on the effects of glutamine supplementation are more desired than ever.
We hypothesized that a parenteral supplement of glutamine, provided as a dipeptide, stimulates citrulline formation and enhances de novo arginine synthesis in the kidneys in humans in the postabsorptive state.
We designed this stable isotope study in glutamine supplemented patients undergoing abdominal surgery to investigate the effects of extra glutamine on whole body and renal metabolism of glutamine, citrulline and arginine.

Subjects and methods

Patients

Whole body and renal amino acid kinetics were quantified in seven patients during abdominal surgery. Patients with parenchymal liver disease, renal failure, inborn metabolic disease, insulin dependent diabetes mellitus, recent weight loss, cachexia, or other indications of metabolic disorders were excluded from the study. After a baseline blood sample was drawn along with a blood sample for routine preoperative laboratory tests, a primed continuous intravenous infusion of 0.5 g/kg/day alanyl-glutamine (Dipeptiven®, Fresenius Kabi, Bad Homburg, Germany) was administered one day before surgery. The dosage of 0.5 g/kg/day alanyl-glutamine has been proven to be safe and is recommended by the manufacturer. Oral intake was allowed only until 12 h before surgery, except for water. Written informed consent was obtained from all patients. The study protocol was approved by the Medical Ethics Committees of the VU University Medical Center, Amsterdam, the Netherlands and was registered in the Netherlands trial register (NTR2914).

Study design

The study design consisted of 2.5 h tracer infusion and blood sampling during open abdominal surgery enabling steady state isotopomer calculations. The metabolic study was conducted during major abdominal surgery to quantify renal citrulline and arginine turnover. The tracer infusion and the blood sampling were performed during the first exploratory phase of the laparotomy, before invasive surgical intervention of abdominal or retroperitoneal anatomical structures. During the study period, an antecubital vein catheter was used for tracer infusion. This catheter was already in place for alanyl-glutamine infusion and clinical purposes. Blood was sampled from a radial artery catheter, installed according to standard perioperative protocol. In all patients, anesthesia and epidural analgesia was applied according to a standard protocol.

Stable isotope tracers

The tracers [2-15N]glutamine, [5-13C,2H4] citrulline, and [15N2]arginine were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). The Department of Clinical Pharmacy at the Erasmus Medical Center in Rotterdam, the Netherlands prepared sterile and pyrogen-free stock solutions of the tracers. The glutamine tracer was prepared the day before surgery, due to the limited stability of glutamine in solution (72 h). The stock solutions were diluted with a physiological saline solution just before the start of the tracer infusion.

Tracer infusion, blood sampling, and renal blood flow measurement

After baseline sampling, a primed, continuous intravenous infusion of the stable isotope tracers was administered and continued for 2.5 h. Tracer infusion was controlled by a calibrated, volume-controlled pump (Graseby 3000; Graseby Medical Ltd, Watford, United Kingdom).

Blood samples were drawn at 30, 60, 90, 120, and 150 min after the beginning of the tracer infusion. After approximately 120 min, at isotopic steady state, blood was drawn from both the radial artery catheter and the renal vein by direct puncture simultaneously, to study renal metabolism. The renal vein was sampled before organ clamping or transection.

Blood was collected in heparinized vacuum tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ, USA) and placed on ice. First, the hematocrit of the blood samples was measured. Blood was centrifuged (10 min, 1910 g, 4 °C), and 50 µL of the supernatant was deproteinized with 20 mg dry sulfosalicylic acid within 1 h after sampling. After mixing, samples were stored at -80 °C until analysis.

To quantify renal amino acid fluxes, renal blood flow was determined with a color Doppler ultrasound (Aloka Prosound SSD 5000; Aloka Co, Ltd, Tokyo, Japan) as described previously 12; time-averaged mean velocity of the bloodstream and cross-sectional area of the right renal vein were measured during the explorative phase of the operation. Blood flow was calculated by multiplying the cross-sectional area with the velocity of the bloodstream.
Plasma flow was calculated by this equation: plasma flow = blood flow(1-hematocrit). Total renal flow was estimated by multiplying plasma flowby 2. Mean renal plasma flow was used to calculate amino acid fluxes across the kidneys.

Mass spectrometric analysis

Plasma enrichments of the infused tracers and the tracer products were measured by liquid chromatography-mass spectrometry (LC/MS) 23. Briefly, 20 µL of hydrochloric acid, 20 µL of intern standard and 200 µL cation exchange solution (0.1 mg/mL Biorad AG 50W-X8 resin, 200-400 mesh) were added to 50 µL of deproteinized plasma.
After mixing and centrifugation, the supernatant was removed. The resin was washed with 1 mL water and the amino acids were extracted twice with 500 µL and 200 µL 6 mol/L ammonia respectively. The eluate was dried in a speedvac and re-dissolved in 80 µL acetonitrile, 20 µL 1 mol/L sodium carbonate, 200 µL water and 140 µL pyridine-ethanol (4:1). Derivatives of the amino acids were prepared by adding ethyl chloroformate and incubating for 5 min. After two extractions with ethyl acetate, the combined solutions of both the first (400 µL) and the second extraction (400 µL) were evaporated under a gentle stream of nitrogen at room temperature until it was almost dry and redissolved in 100 µL 20% methanol.
Analyses were performed on a LC/MS Velos Pro (Thermo Fisher, Bremen, Germany) by injecting 10 µL of sample extract on a 2.1-100 mm, 1.7µm Waters Acquity BEH C18 column. Elution was performed at a stable temperature of 40 ˚C using mobile phases consisting of 0.5 mmol/L tridecafluoroheptanoic acid

and 1 mL/L formic acid (A) and methanol with 0.5 mmol/L tridecafluoroheptanoic acid and 1 mL/L formic acid (B). Ion abundance was monitored in full scan using the zoom-scan modus for glutamine (m/z 245-260), citrulline (m/z 274-290), ornithine (m/z 303 - 320) and arginine (m/z 273 -290).

Calculations

All equations used for calculating the whole body and organ metabolism of glutamine, citrulline and arginine are described in Table 1. Isotope enrichments were expressed as mole percent excess (MPE), calculated as enrichment at steady state minus isotopic background measurements at baseline. Whole body turnover of glutamine, citrulline and arginine were calculated. Furthermore, whole body conversion rates for glutamine into citrulline and citrulline into arginine were determined.

Renal plasma flow and arterial-venous differences in amino acid concentrations and isotopic enrichments were used to obtain insight information in renal metabolism of citrulline and arginine. Arginine production from citrulline in the kidneys was calculated by using the arterial enrichment of citrulline [M+5] and the venous enrichment of arginine [M+5]. The renal output of arginine [M+5] was corrected for bypassing arginine.

All fluxes are presented in micromole amino acid per kilograms of total body weight of the human subject per hour.

Statistical analysis

Results are presented in mean ± SEM. Enrichment curves were fitted to determine a mean steady state value per individual using Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Q-Q plots of the data showed linearity, indicating that the distribution is consistent with the assumption of normality. The Student's t-test was used to determine significant differences in amino acid concentrations between the plasma sample before the start of parenteral alanyl-glutamine administration and during parenteral alanyl-glutamine supplementation just before the tracer infusion. The one-sample t-test was used to test whether the values were significantly different from zero. SPSS 20.0 for Windows software was used to perform statistical tests. P<0.05 was considered to indicate a significant difference.

Table 1 - Equations.

	Equation
Whole Body Turnover	$Q = i[(E_i/E_p) - 1]$
Whole Body endogenous glutamine flux	$Q_{Gln\text{-}endo} = \text{Total } Q_{Gln} - Q_{Gln\text{-}exo}$
Whole Body conversion rate glutamine to citrulline	$Q_{Gln\text{-}Cit} = E_{Cit\ M+1}/E_{Gln\ M+1} \times Q_{Cit\ M+5}$
Whole Body conversion rate citrulline to arginine	$Q_{Cit\text{-}Arg} = E_{Arg\ M+5}/E_{Cit\ M+5} \times Q_{Arg\ M+2}$
Whole Body conversion rate glutamine to arginine	$Q_{Gln\text{-}Arg} = E_{Arg\ M+1}/E_{Gln\ M+1} \times Q_{Arg\ M+2}$
Renal Net Balance (NB)	$NB = ([A]-[V]) \times F$
Renal Tracer Net Balance (TNB)	$TNB = ((E_A\ [A])-(E_V[V]) \times F$
Renal Fractional Extraction (FE)	$FE = \text{Tracer NB} / ([A] \times E_A \times F)$
Renal Disposal	TNB/E_V
Renal Production	Net renal influx - NB
Renal Arginine$_{M+5}$ Output	$([V] \times E_V \times F) - ([A] \times E_A \times F \times (1\text{-}FE))$
Renal arginine production from citrulline	$Q_{Citrulline \rightarrow Arginine} = \text{Arginine}_{M+5}\text{ Output} / ([A_{Cit}] \times E_{A\text{-}Cit\ M+5} \times F) \times ([A_{Cit}] \times F)$

Q, flux in µmol·kg^{-1}·h^{-1}. E, enrichment in mass percent excess (MPE). E_i, enrichment in infusate. E_p, enrichments in plasma at steady state. $Q_{Gln\text{-}endo}$, endogenous glutamine flux in µmol·kg^{-1}·h^{-1}. Total Q_{Gln}, whole body glutamine turnover during alanyl-glutamine infusion in µmol·kg^{-1}·h^{-}. $Q_{Gln\text{-}exo}$, exogenous flux of glutamine in µmol·kg^{-1}·h^{-}. $Q_{Gln\text{-}Cit}$, whole body glutamine to citrulline conversion rate in µmol·kg^{-1}·h^{-1}. $E_{Cit\ M+1}$, plasma enrichment of citrulline [M+1] in MPE. $_{EGln\ M+1}$, plasma enrichment of glutamine [M+1] in MPE. $Q_{Cit\ M+5}$, whole body flux of citrulline [M+5] µmol·kg^{-1}·h^{-1}. $Q_{Cit\text{-}Arg}$, whole body citrulline to arginine conversion rate in µmol·kg^{-1}·h^{-1}. $E_{Arg\ M+5}$, plasma enrichment of arginine [M+5] in MPE. $E_{Cit\ M+5}$, plasma enrichment of citrulline [M+5] in MPE. $Q_{Arg\ M+2}$, whole body flux of citrulline [M+5] in µmol·kg^{-1}·h^{-1}. NB, renal net balance in µmol·kg-1·h-1. TNB, renal tracer net balance in µmol·kg^{-1}·h^{-1}. FE, renal fractional extraction in percent. F, renal plasma flow. [A], arterial concentration. [V], venous concentration. E_A, arterial enrichment in MPE. E_V, venous enrichment.

Results

Baseline characteristics of the patients are shown in Table 2. The patients received 0.5 g/kg/day alanyl-glutamine for at least 15 h prior to the start of the tracer protocol. Tracer dosage for [5-13C,2H4]citrulline and [15N2]arginine were comparable with other studies (11;18;21); [2-15N]glutamine tracer dosage was corrected for the simultaneous alanyl-glutamine infusion to reach adequate enrichments (Table 3). We found significant enrichments of glutamine [M+1], citrulline [M+5] and citrulline [M+1] and arginine [M+2]. We also found significant enrichments of arginine in plasma at [M+1] and [M+5], which confirms the metabolic route from glutamine to citrulline and arginine.
No significant tracer enrichments for ornithine were detected. Arterial plasma enrichments of the infused tracers and the tracer products were observed to be in steady state (Figure 1).

Amino acid concentrations increase with a supplement of glutamine

The plasma concentrations of glutamine, citrulline and arginine significantly increased after the administration of intravenous 0.5 g/kg/day alanyl-glutamine compared to baseline (Figure 2).

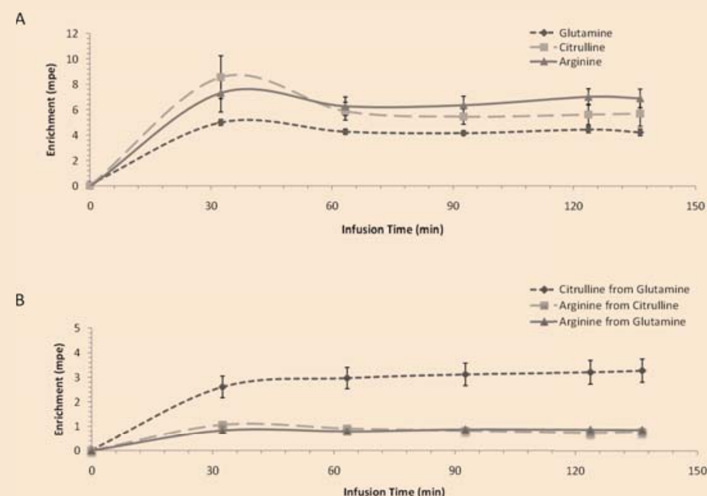
Whole body amino acid turnover

Whole body plasma turnover of glutamine, citrulline and arginine were 423±29, 9.5±1.0 and 30.4±2.2 µmol/kg/h, respectively. Whole body endogenous glutamine flux was 327±29 µmol/kg/h. Estimated whole body citrulline production from plasma glutamine was 8.1±0.9 µmol/kg/h, representing 91% of the total citrulline turnover. 49% of total citrulline turnover was used for de novo arginine synthesis on whole body level, which was 4.5±0.6 µmol/kg/h. Whole body arginine [M+1] production derived from glutamine [M+1] was 6.33 µmol/kg/h. Since this is more than 100% of the citrulline to arginine conversion rate, this is probably an overestimate caused by nitrogen recycling or channeling, as discussed later.

Renal metabolism

To determine organ specific amino acid handling, the enrichment of simultaneously taken arterial and venous samples was used. Arterial and venous plasma enrichments and concentrations are summarized in Table 4. Renal net balance of citrulline showed an uptake of 4.1±1.2 µmol/kg/h and this was accompanied by a net release of arginine of 4.0±0.7 µmol/kg/h, illustrating the role of the kidney in arginine metabolism (Figure 3). 85% of the net renal citrulline [M+5] uptake was utilized for net arginine [M+5] production. Total renal arginine production from citrulline was 3.0±0.7 µmol/kg/h, representing 72% of net renal citrulline uptake and 74% of net renal arginine release. The kidneys were responsible for 75% of de novo arginine synthesis on whole body level.

Figure 1



Steady state curve of primed, continuously infused tracers (A) and their products (B) in MPE (n=7). 60 minutes after the start of tracer infusion, all isotopomers are in equilibrium.

Table 2 – Patient baseline characteristics

	Values (n=7)
Sex	2 female; 5 male
Age (years)	64 ± 3
Height (cm)	178 ± 3
Weight (kg)	91 ± 5
Body Mass Index	29 ± 1
Amino Acid Concentration (μmol/L)[1]	
Glutamine	667 ± 30
Citrulline	30 ± 2
Arginine	69 ± 8
Preoperative Laboratory Results	
Creatinine (μmol/L)	84 ± 9
Urea (mmol/L)	6 ± 0.5
Glomerular Filtration Rate	>60
Billirubin (μmol/L)	7 ± 2
Albumin (g/L)	41 ± 3
Glucose (mmol/L)	6.9 ± 0.6
Surgical procedure	7 Infrarenal abdominal aorta aneurysm repair
Urine production during protocol (mL)	156 ± 40
Total fluid provided during protocol (mL)	1489 ± 199
Total renal Plasma flow ($mL \cdot kg^{-1} \cdot min^{-1}$)	5.58 ± 0.5

Values in mean ± SEM. 1Before alanyl-glutamine administration

Table 3 – Tracer dosage

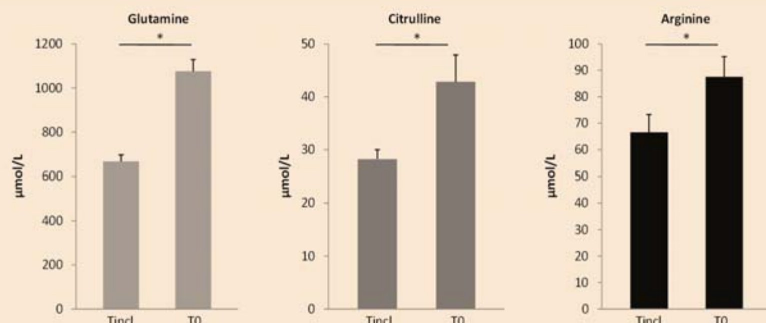
	Priming dose ($\mu mol \cdot kg^{-1}$)	Infusion dose ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)
L-[2-^{15}N]glutamine	23.82	20.98
L-[5-^{13}C-4,4,5,5$^{2}H_4$]citrulline	1.18	0.57
L-[$^{15}N_2$]arginine	2.73	2.16

Table 4 – Renal arterial and venous plasma amino acid concentrations and tracer enrichments

	Citrulline ($\mu mol \cdot L^{-1}$)	Arginine ($\mu mol \cdot L^{-1}$)	Citrulline[M+5] (MPE)	Arginine[M+2] (MPE)	Arginine[M+5] (MPE)
Arterial	33 ± 7	80 ± 7	6.11 ± 1.0	6.9 ± 0.8	0.7 ± 0.1
Venous	21 ± 4	91 ± 9	6.3 ± 1.0	5.9 ± 0.7	1.1 ± 0.1

Values in mean ± SEM (n=7). Amino acid concentrations and enrichments were measured by using mass spectrometry.

Figure 2



Plasma concentrations of glutamine, citrulline and arginine (mean ± SEM) at the time of inclusion (Tincl) and after the administration of intravenous 0.5 g/kg/day alanyl-glutamine just before the start of the tracer infusion (T0) (n=7). The Student's t-test was used to determine significant differences in amino acid concentrations between Tincl and T0. *$P < 0.05$.

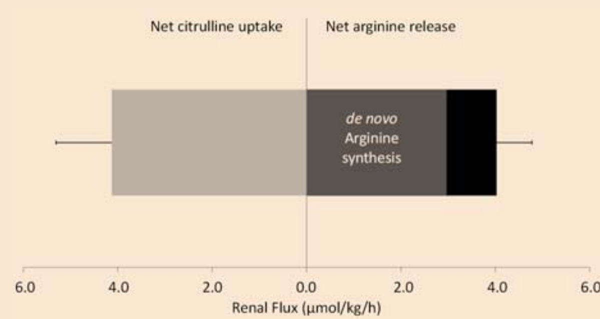


Figure 3

Renal net citrulline uptake and net arginine release in μmol/kg/h (n=7), from which 74% was derived from de novo arginine synthesis from citrulline (dark grey).

Discussion

This study shows the qualitative and quantitative effects of an intravenous supplement of glutamine on whole body and renal metabolism of glutamine, citrulline and arginine in humans in the postabsorptive state. The use of the [2-15N]glutamine tracer showed that almost all circulating citrulline has derived from glutamine. Approximately half of the circulating plasma citrulline was used for arginine production. The kidneys were responsible for 75% of whole body de novo arginine synthesis with a production rate of approximately 3.0 μmol/kg/h.

To illustrate the effect of the intravenous glutamine supplement on the metabolism of citrulline formation and arginine production, our results should be interpreted in the background of results obtained in previous stable isotope studies with a similar design. The study by Ligthart-Melis et al. is found to be comparable to the current study, because similar intravenous stable isotopes were used in human subjects in the postabsorptive state during abdominal surgery to determine renal metabolism [12]. In those patients, the whole body glutamine flux was 240±14 μmol/kg/h, while in our patients receiving a supplement of glutamine the whole body total glutamine flux was almost doubled and the endogenous glutamine flux approximately 40% higher. In both studies it was found that a similar half of circulating citrulline was used for de novo arginine synthesis. However, in our study the glutamine supplement caused an increase in citrulline levels and renal arginine production from citrulline was 3.0±0.7 μmol/kg/h compared to only 1.5±0.7 μmol/kg/h in the study without glutamine administration [12]. Thus, renal arginine production seems to be doubled in humans receiving 0.5 g/kg/day alanyl-glutamine intravenously, compared to previous results in humans without glutamine supplementation. This was also reflected by the significant increase of arginine plasma concentration during glutamine infusion compared to baseline in the current study.

Arginine is an important regulator of the immune system, cell homeostasis and protein synthesis and it plays important roles in multiple metabolic pathways [24]. In addition, arginine is the sole precursor for nitric oxide generation, a signaling agent with a crucial role in immunity, inflammation and organ perfusion [24, 25]. As demonstrated in previous studies and the present study, citrulline can be converted into arginine, mainly in the kidneys [12, 26]. This de novo arginine synthesis has been shown to be the main regulator of plasma arginine levels [11]. Plasma arginine levels can be affected in pathological conditions in which the disposal capacity of arginine is enhanced [4, 24, 27-30]. Thus in various pathological conditions de novo arginine synthesis appears to be insufficient. Systemic arginine deficiency contributes to immunosuppression, inflammation disorders and vascular dysfunction in sick patients, which may lead to concomitant morbidity and mortality [6]. Metabolic studies suggested that arginine deficiency is related to a decreased glutamine availability, affecting intestinal citrulline formation and subsequently arginine production [11]. Conversely, after glutamine supplementation an increase in citrulline and arginine plasma levels is found [7, 10, 31]. Other intermediates of the intestinal-renal axis have been suggested to enhance arginine production as well. For example, the supplementation of ornithine α-ketoglutarate resulted in increased citrulline and arginine concentrations, while ornithine supplementation did not [32]. Furthermore, citrulline regulates de novo arginine synthesis and citrulline supplementation could augment a similar effect on arginine production as shown in the current study. However, glutamine is still considered to be the premier precursor of arginine via the intestinal-renal axis. In fact, in this study we confirmed the existence of this precursor role and demonstrated that glutamine supplementation enhances renal de novo arginine synthesis from citrulline in comparison to previous published results.

Glutamine administration showed to be beneficial in several pathological conditions [7, 10]. Recently the administration of high doses of glutamine in critically ill patients with multi organ failure became controversial after possible adverse effects were found in these patients [22]. However, this clinical trial included patients with kidney failure and liver failure, which are both contraindications for glutamine supplementation. When combining available studies on glutamine supplementation, it is found that glutamine still may prevent and limit infections, improve recovery from injury and positively affect mortality [33, 34]. Since glutamine is the substrate for citrulline and arginine, (a part of) the effects of glutamine supplementation could be mediated by its derivatives. As described before, de novo arginine production from citrulline is the regulating factor in optimizing plasma arginine concentrations in the body. Excessive arginine supplementation could also have adverse effects, probably due to excessive formation of nitric oxide and oxidative metabolites, subsequently leading to oxidative stress [5]. Glutamine supplementation is suggested to be a more physiological way of correcting arginine levels and subsequently achieve both glutamine and arginine benefits. In the light of previous work, our results seem to support this by showing the qualitative and quantitative effects of the glutamine supplement on renal de novo arginine synthesis from citrulline.

Some specific patient populations may benefit most from a supplement of glutamine, since they show disease related low glutamine and arginine levels. In combination with data from the literature, our results indicate that in these specific patient populations intravenous glutamine supplementation could restore renal arginine production. However, the aim of this study was to investigate the effect of glutamine administration on arginine production on whole body level as well as in the kidneys under the most physiological condition possible.

The included patients did not have metabolic disorders, organ failures or aberrant diets; glutamine, citrulline and arginine levels were

in the normal range; and the tracer protocol was conducted in the exploratory phase of surgery. While our results suggest that an intravenous supplement of glutamine could correct depleted arginine levels in patients with a pathological induced arginine deficiency, quantitative and qualitative studies using a stable isotope technique in these specific patient populations should be performed in the future. Furthermore, the patients in this study had an average high BMI and were relatively of high age, representative of patients with abdominal aortic aneurysm, which may have influenced our metabolic measurements.

The use of stable isotope tracers to quantify glutamine to arginine metabolism was subject of discussion in the last years, since a study by Marini et al. showed that the use of a [2-15N]glutamine tracer may overestimate the quantitative contribution of glutamine to arginine in mice due to nitrogen recycling [15]. However, studies in humans showed that citrulline plasma levels increase after glutamine supplementation, which is confirmed by our study. This is only possible when a substantial part of the carbon skeleton of glutamine is used for citrulline formation. An excellent study by Tomlinson et al. evaluated this complexity of the glutamine to arginine pathway in humans in the fed state by using both an C-labeled and an N-labeled glutamine tracer [16]. Similar to our results, they found that the N-labeled tracer showed a contribution of glutamine [M+1] to arginine [M+1] synthesis that exceeded the citrulline to arginine conversion. Furthermore, they demonstrated that the labeled N-atom was found in various locations of the ornithine, citrulline and arginine molecules. Thus, although they demonstrated that the N-atom from glutamine is used for ornithine formation and the equivalent isotopomers of citrulline and arginine, the way of contribution to the formation of the isotopomers remains indefinite. Our results showed citrulline [M+1] and arginine [M+1] enrichments, but ornithine enrichments were not significant, indicating a complex contribution of the N-atom in this pathway.
Consequently, we agree with Tomlinson et al. that the N-labeled glutamine tracer can provide qualitative information on the glutamine to citrulline and arginine pathway, yet quantitative results should be interpreted with caution, since outcomes may overestimate the contribution of glutamine to citrulline and arginine formation.
More importantly, quantitatively they confirmed with a [1-13C]glutamine tracer that the carbon skeleton of glutamine is used for approximately 50% of de novo arginine synthesis in humans, supporting the existence of the glutamine-citrulline-arginine pathway [16]. With this in mind, future stable isotope studies to elucidate glutamine to arginine metabolism in humans are preferred to be performed using a carbon-labeled glutamine tracer.

Conclusion

This study was conducted to investigate the way in which a therapeutic dose of parenteral glutamine affects the synthesis of arginine on whole body level and in the kidneys specifically. Although the amino acid kinetics were examined in only seven patients, our data consistently showed that during glutamine supplementation the majority of the circulating citrulline has derived from glutamine and 49% is used for de novo arginine synthesis in the kidneys. The intravenous glutamine supplement resulted in significantly higher glutamine, citrulline and arginine plasma concentrations. This is the first quantitative study showing that renal arginine production from citrulline is enhanced in patients receiving an intravenous glutamine supplement when comparing these results with previously published data. In conclusion, an intravenous supplement of glutamine dipeptide enhances de novo arginine synthesis in the kidneys of humans in the postabsorptive state.

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The authors' responsibilities were as follows – N.B., S.J.H.B. and M.A.R.V. contributed to the study design. N.B., S.J.H.B. and A.B. contributed to the implementation of the study. N.B., S.J.H.B. and J.L. conducted the research and collected the data. W.W. supervised during surgical procedures. J.E.O. and H.S.: performed mass spectrometry and amino acid concentration analyses. N.B. and M.A.R.V.: performed calculations and statistical analysis. H.S., J.B.v.G. and A.P.J.H. helped with interpretation of the data. N.B., S.J.H.B. and J.E.O. drafted the manuscript. M.A.R.V., J.L., H.S., W.W., A.B., J.B.v.G. and A.P.J.H. critically revised the manuscript. P.A.M.v.L. had primary responsibility for all aspects of the study and for final content. P.A.M.v.L. reports receiving fees from Fresenius Medical Care for clinical consultation. None of the other authors had any conflict of interest to disclose. Fresenius Kabi provided alanyl-glutamine (Dipeptiven) for this study and the Vivax Foundation supported this work with a research grant; both entities did not participate in data collection, data analysis, data interpretation, or writing of the manuscript.

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Am Surg 2013;79:506-513.

6. Enteral glutamine administration in critically ill non-septic patients does not trigger arginine synthesis

Mechteld A.R. Vermeulen, Saskia J.H. Brinkmann, Nikki Buijs, Albertus Beishuizen, Pierre M. Bet, Alexander P.J. Houdijk, Johannes B. van Goudoever, Paul A.M. van Leeuwen

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Abstract

Glutamine supplementation in specific groups of critically ill patients results in favourable clinical outcome. Enhancement of citrulline and arginine synthesis by glutamine could serve as a potential mechanism. However while receiving optimal enteral nutrition, uptake and enteral metabolism of glutamine in critically ill patients remains unknown. Therefore we investigated the effect of a therapeutically relevant dose of L-glutamine, on synthesis of L-citrulline and subsequent L-arginine in this group. Ten versus ten critically ill patients receiving full enteral nutrition, or isocaloric isonitrogenous enteral nutrition including 0.5 g/kg L-alanyl-L-glutamine, were studied using stable isotopes. A cross-over design using intravenous and enteral tracers enabled splanchnic extraction (SE) calculations. Endogenous rate of appearance and SE of glutamine citrulline and arginine was not different (SE controls vs. alanyl-glutamine: glutamine 48 and 48%, citrulline 33 vs. 45%, arginine 45 vs. 42%). Turnover from glutamine to citrulline and arginine was not higher in glutamine-administered patients.

In critically ill non-septic patients receiving adequate nutrition and a relevant dose of glutamine there was no extra citrulline or arginine synthesis and glutamine SE was not increased. This suggests that for arginine synthesis enhancement there is no need for an additional dose of glutamine when this population is adequately fed.

Introduction

Previously, numerous clinical studies demonstrated that supplementation with glutamine as free molecule or dipeptide results in a favourable clinical outcome as reflected by a reduction in infectious morbidity (trauma [1, 2] and medical [2-5] patients), mortality [3, 6] and a reduction in length of hospital stay in severely ill patients [4, 7-9]. However, the use of high dose glutamine in shock patients has been part of debate [10]. The underpinning mechanism of the clinical effects of exogenous glutamine administration has not been completely elucidated yet. The effects of glutamine could be partially explained by the substrate that glutamine is for the synthesis of citrulline and arginine. Citrulline may act as a radical scavenger and is also a potent arginine precursor [11]. Arginine is of great importance for wound healing, the immune system and it is the precursor of nitric oxide (NO) [12-16]. During trauma and sepsis, plasma concentrations of arginine are decreased [17].
However, the action of arginine as a substrate for nitric oxide synthesis with potential subsequent hemodynamic instability and oxidative stress, may be responsible for the reported adverse events of arginine administration in severe critically ill patients [18-20]. Since endogenous glutamine can generate arginine by the citrulline pathway in the kidney, supplying glutamine may be a more physiologic and safe way to regulate arginine availability in the metabolically stressed ICU patient [21, 22].

However, in critically ill patients the metabolic fate of glutamine is still unclear. Possibly, generally altered metabolism could exist due to impaired enterocyte function because of injury, splanchnic ischemia, sepsis and starvation.

Considering the observation that that the gut preferentially takes up enterally provided glutamine, with subsequent higher intestinal release of citrulline, the precursor for arginine, we decided to provide L-alanyl-L-glutamine by the enteral route in this study, expecting to deliver glutamine most adequately to the interorgan pathway of glutamine into citrulline and arginine [23, 24]. Hence the objective of this clinical study was to investigate the effect of the enteral administration of a clinically relevant dose of L-glutamine, provided as L-alanyl-L- glutamine, on the synthesis of L-citrulline from L-glutamine and the subsequent synthesis of L-arginine from citrulline in critically ill nonseptic patients. Quantitatively, tracer methodology was used to determine exact turnover of these amino acids.
A cross-over study design using intravenous and enteral tracers was chosen enabling splanchnic extraction calculations. We hypothesized that after enteral glutamine supply splanchnic glutamine uptake would increase as well as subsequent increases in citrulline and arginine synthesis.

Patients and methods

Patients

Twenty critically ill patients considered stable were studied. All were expected to stay at the ICU for at least 5 days. Additional inclusion criteria were: Age: ≥ 18 years, BMI ≥ 18.5 and ≤ 35 ability to tolerate enteral nutrition, provided by postpyloric tube, meeting full protein/energy requirements based on indirect calorimetric measurements and a protein intake of 1.2-1.7 g/kg/day.

Exclusion criteria were: septic shock (defined according to the International Guidelines for Diagnosis of Sepsis [25]), need for high dose vasoactive medication such as norepinephrine higher than 0.2 μg/kg/min; PaO_2/FiO_2 ratio <200, PEEP >15 cm H_2O; liver failure (bilirubin levels > 100 μmol/L); hyperammonaemia (ammonia >50 μmol/L) ; kidney failure (renal replacement therapy or increase in serum creatinine levels to > 100 μmol/l), in the absence of primary underlying renal disease, associated with oliguria, (defined as urine output < 150 ml in the previous 8 hours), urea cycle defects; chronic corticosteroids use (> 7.5 mg/ day > 3 weeks); gastrointestinal malabsorption possibly interfering with intestinal absorptive function (celiac disease, Crohn's disease, presence of fistulas, major intestinal malabsorption disorder, or short bowel syndrome), pregnancy or lactation, admission after elective surgery, parenteral nutrition; use of medium chain triglycerides or glutamine/citrulline supplements.

Informed consent was obtained from all included patients or his/her legal representative. The Medical Ethical Committee of the VU University Medical Hospital approved the study protocol (MEtC VUmc 2009.083). The study complied with the Declaration of Helsinki (NTR 2285).

Study design

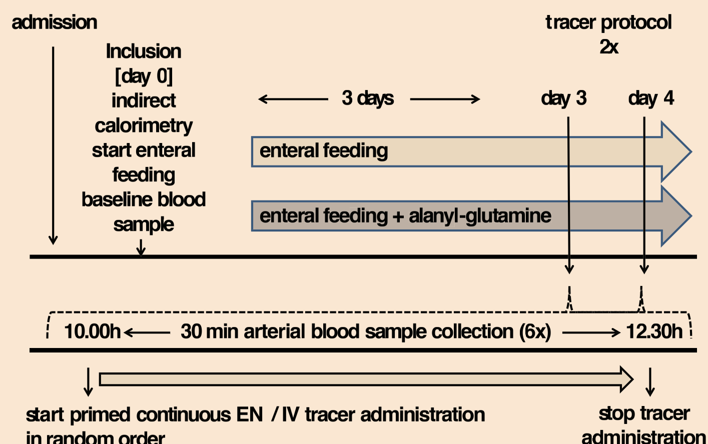
All twenty patients received enteral nutrition via postpyloric or nasogastric tube. Ten patients received an additional enteral dose of 0.5 g/kg/day L-alanyl-L-glutamine (ALA-GLN) (=0.325 g/kg glutamine/day) (Fresenius Kabi. Nederland B.V. Den Bosch, the Netherlands). Patients in the control group received isonitrogenous enteral nutrition without the additional glutamine (CON). Total nitrogen was compensated by using different enteral nutritional formulas containing different amounts of protein. Patients were investigated while being fed continuously. The study protocol is outlined in Figure 1.

Resting energy expenditure (REE) was measured with the Deltatrac Metabolic Monitor (Datex-Engstrom Division, Helsinki, Finland), calibrated every day. Energy requirement was measured within 24h hours before study or control feeding was started. During measurements, nutrition was not interrupted. Body height and weight were (self-)reported at admission. REE was measured for a minimum of one hour. Total Energy Expenditure (TEE) was calculated by adding 10 % (activity factor) above REE [26]. Nutrition was based on TEE and total protein was aimed for at 1.5-1.7 g/kg/day but at least not under 1.2 g/kg/day [27]. To achieve these goals we used the following enteral formulas: Nutrison Protein Plus® Nutrison Standaard®, (both Nutricia, Zoetermeer, the Netherlands) and Promote® (Abbott, Columbus, Ohio, US).

Table 1 - Enteral nutrition

	Energy (kcal/l)	Protein(g/l)	Glutamine(g/l)	Citrulline(g/l)	Arginine(g/l)
Nutrison Standard®	1000	40	4.6	0	1.6
Nutrison Protein Plus®	1250	63	7.19	0	2.5
Promote®	1000	63	2.3	0	2.3

Figure 1 - Study protocol



Data on nutrition and nutritional requirements are listed in Table 1 and Table S1.

Baseline characteristics and routine clinical blood variables were documented. APACHE II (Acute Physiology and Chronic Health Evaluation) scores were calculated as measures of severity of disease in ICU patients. A baseline blood sample was taken for amino acid concentration analysis.

All patients received stable isotopes both enterally and intravenously, on separate days (day 3 and 4, in random order). The enteral tracers were co-administered through a separate port on the tube, the intravenous tracers were administered in the antecubital vein. After 3 days of glutamine enriched or control feeding, if patients were considered stable, at approximately 10.00am an arterial baseline sample was collected to measure natural background enrichment followed by a primed continuous intravenous or enteral tracer infusion in random order.
Blood samples were collected at 30 minute intervals for 2.5 hours. The same protocol ran the following day with the alternative route of tracer administration. In case stability of the patient was not guaranteed or clinical situation did not allow research, the tracer protocol was postponed by a maximum of one day.

Blood was collected in prechilled heparinized vacuum tubes (BD Vacutainer, Franklin Lakes, NJ) and immediately placed on ice. Blood was centrifuged (10 minutes, 3000 rpm, 4°C), plasma was extracted and again centrifuged (10 minutes, 3000 rpm, 4°C) after which 500 µL of plasma was added to 20 mg dry sulfosalicylic acid (Across Inc, Geel, Belgium) to precipitate plasma proteins. After vortex mixing, deproteinized plasma samples were snap frozen in frozen carbon dioxide and stored at -80°C. until assayed.

Stable isotopes

Stable isotope tracers of L-[2-^{15}N]-glutamine, L-[5-^{13}C-4,4,5,5^{2}H$_4$]citrulline and L-[guanidino^{15}N$_2$]-arginine were used to investigate the effect of the enteral supplementation of glutamine on the metabolism of L-glutamine, L-citrulline and L-arginine, as well as the conversions of L-glutamine into L-citrulline and L-citrulline into L-arginine. Tracers will be noted as glutamine [M+1], citrulline [M+5] and arginine [M+2], respectively. Tracers were purchased from Cambridge Stable Isotope Laboratory (Woburn, MA, USA).
The Department of Clinical Pharmacy at the Erasmus Medical Center in Rotterdam, the Netherlands prepared sterile and pyrogen-free stock solutions of the tracers.
The glutamine tracer was prepared 1-2 days before tracer infusion, due to the limited stability of glutamine in solution (72 h).
The stock solutions were diluted with a physiological saline solution minutes before the start of the tracer administration.

The tracers were administered intravenously and enterally, to study splanchnic extraction of glutamine and to distinguish between the contribution of endogenous and exogenous L-glutamine to the metabolic interrelationship between L-glutamine, L-citrulline and L-arginine.

Tracers and amounts are listed in Table 2.
Tracer dosages were calculated by using previous results from Ligthart-Melis et al. and van de Poll et al. [24, 28]. Because these studies involved surgical patients in the postabsorptive state, a pilot was performed within the first two patients to confirm steady state within our continuously enterally fed critically ill patients [29]. This resulted in a small body weight related adjustment of the priming dose.

Laboratory analyses

Amino acid concentrations in plasma and infusates were measured using high-performance liquid chromatography, as described elsewhere [30]. Isotopic enrichment was expressed as tracer-to-tracee (labeled vs. unlabeled substrate) ratio (TTR, %), corrected for contribution of lower masses and for background TTR (determined in the baseline sample). Glutamine citrulline and arginine TTRs were measured by liquid chromatography-mass spectrometry [30].

Calculations

Isotopic enrichment was adjusted for natural enrichment and for the contribution of overlapping isotopomer distributions of the tracee and tracers with lower masses to the measured TTR as described by Vogt et al. [31]. Metabolic conversions were calculated using established calculations [32]. Since all tracers were administered during enteral nutrition (no matter which route of administration), adjustments were made for tracee infusion, as explained beneath in the calculations and also used by Buijs et al. [33].

For each amino acid studied, arterial enrichment curves were fitted for each patient with the use of PRISM software (version 4.03; GraphPad Software Inc, San Diego, CA). Steady state was calculated by curve fitting plateau calculations. Primarily a first order straight line was calculated (mean minus baseline). Hereafter an exponential decay function challenged the null hypothesis (first order straight line), when steady state was in fact more likely to have optimized following a plateau after correction for possible occurring under- or overpriming (this would be a line that decays to a plateau with a constant rate K).

The plasma rate of appearance (WBRA: µmol/kg/h) of glutamine, citrulline and arginine, and the known infusion rate of these tracers are based on the following equation:

1. WBRA = I(tracer) / TTR

In which I(tracer) is the known infusion rate of the tracers and TTR is the tracer/tracee ratio. Knowing that enteral feeding and alanyl-glutamine infusion affect the RA, the WBRA calculation includes the exogenous infusion rate of tracee:

2. WBRA = RA(endogenous) + I(tracee)

In which I(tracee) reflects the exogenous amino acid (AA) supply (amino acids given by enteral nutrition):

3. I(tracee) = I(AA) * [TTR(EN)/TTR(IV)]

With TTR(EN) being the TTR with enterally administered tracers, and TTR(IV) the TTR with intravenously administered tracers, corrected for splanchnic extraction of AA, reflected by splanchnic tracer extraction. True RA (RA(endogenous)) is therefore calculated as follows [33]:

4. RA(endogenous)= [I(tracer) / TTR(IV)] – [I(AA) * (TTR(EN)/TTR(IV))]

Calculation of the rate of WB plasma turnover (Q: μmol/kg/h) of glutamine into citrulline was performed by using the following equation from Castillo et al. adjusted for endogenous RA. [13]:

5. Q gln→cit = RA(endogenous)CIT * [TTR CITM+1 / TTR GLNM+1]

Where WBRACIT is the plasma WBRA of citrulline, calculated from the TTR of the infused CIT M+5 tracer by using equation 1., and CIT M+1 is the CIT M+1 coming from GLN M+1.

Likewise, calculation of the WB plasma turnover of citrulline into arginine (de novo synthesis) was performed by using the following equation:

6. Qcit→arg = RA(endogenous)ARG * [TTR ARG M+5 / TTR CIT M+5]

Where WBRA ARG is the WBRA of arginine, calculated from the TTR of ARG M+1 by using equation 1, and ARG M+5 is the ARG M+5 coming from CIT M+5.

Splanchnic extraction (%) of glutamine, citrulline and arginine was calculated as follows:

7. [1-(TTR(EN)/TTR(IV))] * 100

Statistical analyses

Data are expressed as mean ± standard error (SEM) in case of normally distributed data, and as median ± interquartile range (IQR) when data were not normally distributed (tested by Shapiro-Wilk normality test).

Independent sample T-Test or Mann Whitney Test was used to compare control group with alanyl-glutamine group, according to distribution.

One sample T-Test was used to test whether steady state of metabolic products differed from zero. Plasma values over time were compared using ANOVA and Bonferroni to determine specific time differences.

A p-value of <0.05 (2-tailed) was considered as statistical significant. Statistical analysis was performed with SPSS 17.0 for Windows® (SPSS Inc., Chicago, IL, USA).

Supplemental Table S1 - Amino Acid Concentration (g/l)

	Nutrison Standard®	Nutrison Protein Plus®	Promote®
Glutamine	4.6	7.2	2.3
Citrulline	0	0	0
Arginine	1.6	2.5	2.3
Alanine	1.4	2.1	1.8
Asparaginezuur	3.1	4.9	2.7
Cystine	0.1	0.2	0.3
Glycine	0.8	1.3	1.2
Histidine	1.3	2.0	1.6
Isoleucine	2.3	3.6	2.9
Leucine	4.2	6.6	5.5
Lysine	4.1	6.4	4.6
Methionine	1.3	2.1	1.6
Phenylalanine	2.2	3.5	3.0
Proline	4.0	6.3	6.3
Serine	2.6	4.1	3.4
Threonine	2.0	3.1	2.6
Tryptofaan	0.6	0.9	0.7
Tyrosine	2.4	3.8	3.2
Valine	2.9	4.5	3.7

Table 2 - Tracer dosages

	Prime (mg)		Infusate (μmol/kg/h)	
	CON	ALA-GLN	CON	ALA-GLN
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
GLN M+1	232.0 (13.8)	236.7 (8.0)	18.4 (1.6)	16.0 (1.5)
CIT M+5	627.2 (37.3)	639.8 (21.8)	1.2 (0.3)	1.0 (0.3)
ARG M+2	141.1 (8.4)	144.0 (4.9)	1.3 (0.1)	

Results

Twenty patients were successfully included: ten received enteral alanyl-glutamine isocalorically, isonitrogenously, compared to 10 control patients. Results of one patient (CON) were not completely obtained due to detubation and subsequent removal of enteral tube at the last day. One patient got discharged and had his parenteral tracer administration accidently interrupted (ALA-GLN), therefore only enteral results could be obtained. Patient characteristics are summarized in Table 3. Baseline characteristics were not significantly different when comparing the two groups.

Nutrition

Energy expenditure was similar in both groups. All patients received 100% of the caloric target during the tracer infusions. During the whole study period 16 out of 20 received an actual nutrition of >97% of the aimed 5 day nutrition (tube feeding stopped or was lowered during the 5 day course), one patient received 89%, one 80%, one 70% and one 66%. The last patient was eventually excluded due to unobtained steady state (described below). Mean energy intake during complete study period was 94.8 % (SE 2.3) of target nutrition.

Nutrison Standard® (lowest protein content) was used more often in patients in the alanyl-glutamine group, because the formula was chosen based on total amount of nitrogen (within the enteral formula) adjusted to the administered amount of alanyl-glutamine.

Although the alanyl-glutamine group received slightly more nitrogen, nitrogen per kg bodyweight was similar. Glutamine administration was as expected significantly higher in the alanyl-glutamine group. Citrulline administration was absent in both groups and total administered arginine was not different in both groups (Table 4).

Glutamine, citrulline and arginine metabolism

Alanyl-glutamine was not detectable in arterial plasma in both groups. Arterial plasma concentrations of glutamine, citrulline and arginine were not significantly different at D0 for both groups. Plasma concentrations of glutamine and arginine did not differ during the study period, or between groups. Plasma concentrations of citrulline increased significantly from baseline compared to 3-4 days in the control group (D0 28 ± 4 μmol/ml, D3: 34 ±3.3 μmol/ml, D4: 39 ±5.5 μmol/ml p=0.035), without differences between groups.

In one patient, an isotopic steady state for glutamine, citrulline and arginine tracers could not be reached with intravenous administration, therefore these results were excluded from analyses. This patient appeared to have higher bilirubin and creatinin levels (although not above exclusion level). Apart from this mentioned patient, steady state for the amino acid tracers could not be calculated for arginine M+2 (EN: 1 case) and arginine M+1 (EN 2 cases). These results were therefore excluded from analyses as well. Steady state curves are presented in Figure S1.

TTR% for almost all infused tracers were higher when intravenously administered compared to enteral infusion, in both groups (Table 5). TTR% of infused tracers was not significantly different between control and alanyl-glutamine group. TTR% of metabolic products differed in case of citrulline M+1 for both the intravenous and enteral experiments (Table 5).

The TTR% of the metabolic products of [15N]glutamine metabolism— [15N]citrulline and [15N]arginine—were significantly different from zero in both groups with either way of administration. However, TTR% of the metabolic product of L-[5-^{13}C-4,4,5,5$^{2}H_4$] citrulline metabolism - [5-^{13}C-4,4,5,5$^{2}H_4$] arginine - was below detection level in 6 and 7 control patients (iv and enteral tracer administration respectively) and 4 and 3 patients in the alanyl-glutamine group (iv and enteral tracer administration respectively).

Endogenous rates of appearance were not significantly different for all administered tracers in the alanyl-glutamine group as compared to the control group (Table 5).Splanchnic extraction of glutamine and citrulline was not significantly different for both groups: (Figure 2).

Whole body plasma turnover gln→ cit (Q: μmol*kg^{-1}*h^{-1}) was not significantly higher in glutamine-administered patients. In contrast, in control patients, 47.8% (±7.8) of the citrulline was derived from glutamine, versus 24.8% (±4.4; p=0.018) in the alanyl-glutamine group. The percentage of citrulline that served as substrate for arginine was 0% (range 0-10.8) versus 6.5% in the alanyl-glutamine group (range 1.3-12.5 ns).
The percentage of glutamine that was converted into arginine was 1.3% (range 1.0-1.4) versus 0.7% (range 0.3-1.3,ns). (Table 5, Figure 3).

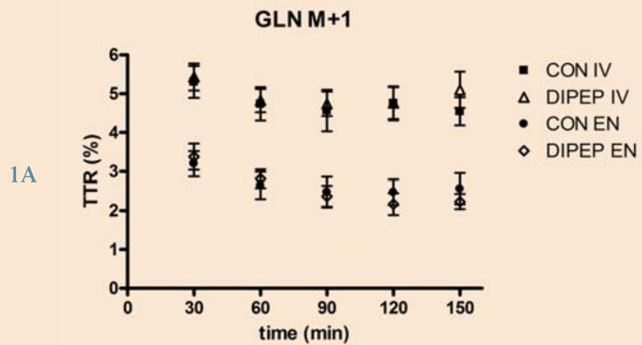
Table 3 - Baseline characteristics

	CON N (%) / mean (SEM) / median (IQR)	ALA-GLN N (%) / mean (SEM) / median (IQR)
Demographics		
Sex: male/female (%)	6/4 (60/40)	6/4 (60/40)
Age (y)	65 (6.4)	57 (5.4)
Length (cm)	172.6 (3.7)	176.8 (3.0)
Weight (kg)	73.2 (5.8)	77.7 (4.1)
BMI (kg/m2)	24.2 (1.0)	24.5 (3.3)
Clinical Assessment		
Type of ICU admission		
Respiratory insufficiency	6(60)	3(30)
Cardiogenic shock	1(10)	4(40)
Neurotrauma	1(10)	0
Multitrauma	1(10)	2(20)
Other	1(10)	1(10)
Apache II-score	27.0 (2.2)	25.3 (3.0)
Laboratory measurements at inclusion		
pH	7.42 (0.048)	7.44 (0.027)
pCO2	41.4 (10.3)	43.9 (9.7)
Bicarbonate (mmol/L)	32.9 (22.0-33.0)	30.5 (5.3)
Glucose (mmol/L)	6.6 (0.83)	7.5 (1.4)
Leukocytes (10*9 µmol/l)	11.0 (3.1)	11.8 (4.4)
Bilirubin (µmol/L)	8.5 (5.0-16.8)	9 (4.0-11.3)
Creatinine (µmol/L)	78 (9.7)	81.8 (13.7)
Urine production (ml/24h)	2301 (361)	2333 (295)

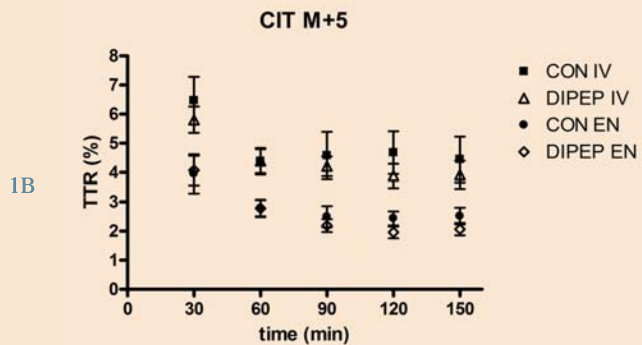
Table 4 - Nutritional Characteristics

	CON Mean (SEM)	ALA-GLN Mean (SEM)	Sig. between groups (P)
Carlorimetry			
REE (kcal/24h)	1847 (165)	1998 (85)	0.429
TEE (kcal/24h)	1998 (165)	2176 (80)	0.350
VCO2 (ml/min)	223 (17.0)	234 (10.7)	0.587
VO2 (ml/min)	269 (24.8)	292 (12.4)	0.577
RQ	0.84 (0.03)	0.81 (0.016)	0.237
Nutrition			
Nutrison Protein Plus®/ Nutrison	3/5/2	0/1/9	
Standard®/ Promote®	(30/50/20)	(0/10/90)	
Received % of nutritional target (study period)	98.5	100	0.136
median (IQR)	(84.3;100)	(98.8-100)	
Energy (kcal/d)	1999 (165)	2190 (88)	0.325
Received energy (kcal/d)	1844 (183)	2129 (67)	0.170
Nitrogen (g/d)	101.5 (7.2)	122.7 (5.8)	0.034
(g/kg/d)	1.41 (0.08)	1.59 (0.11)	0.066
Received Nitrogen (g/d)	93.1 (7.9)	119.4 (5.1)	0.014
(g/kg/d)	1.30 (0.11)	1.54 (0.02)	0.055
Received glutamine (g/d)	10.2 (1.0)	35.2 (0.17)	(<0.000)
(mmol/kg/d)	0.98 (0.09)	3.11 (0.03)	(<0.000)
Citrulline (g/d)	-	-	-
Received arginine (g/d)	3.6 (0.32)	3.2 (0.13)	0.278
(mmol/kg/d)	0.29 (0.08)	0.24 (0.01)	0.066
Baseline plasma glutamine (µmol/ml)	521 (66)	497 (37)	0.743
D3	539 (21)	518 (34)	0.592
D4	525 (32)	510 (38)	0.755
Baseline plasma citrulline (µmol/ml)	28 (4)	32 (3)	0.365
D3	33 (3)	37(2)	0.349
D4	39 (5)	38 (3)	0.865
Baseline plasma arginine (µmol/ml)	53 (6)	72 (8)	0.076
D3	64 (6)	62 (6)	0.833
D4	68 (7)	64 (5)	0.645

1A



1B



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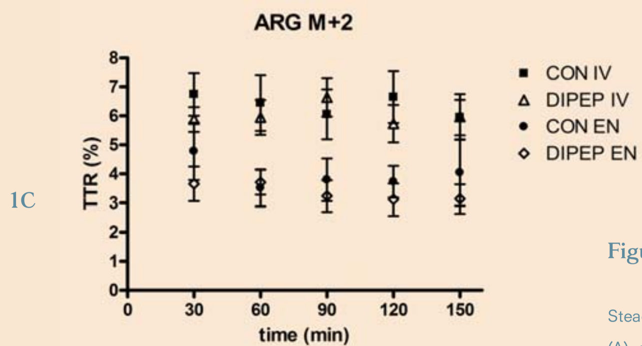
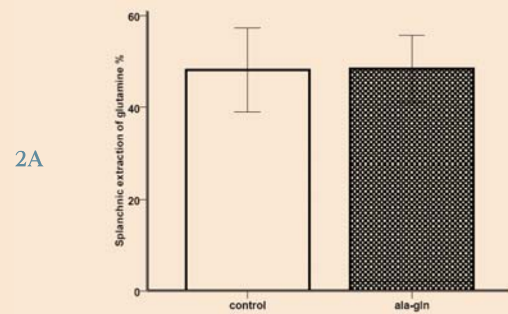


Figure S1 – Steady state curves

Steady state TTR% curves for glutamine M+1 (A), citrulline M+5 (B) and arginine M+2 (C)

2A



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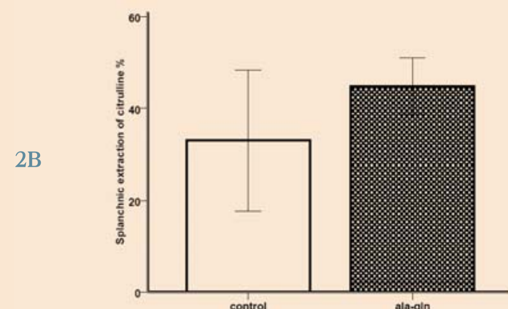

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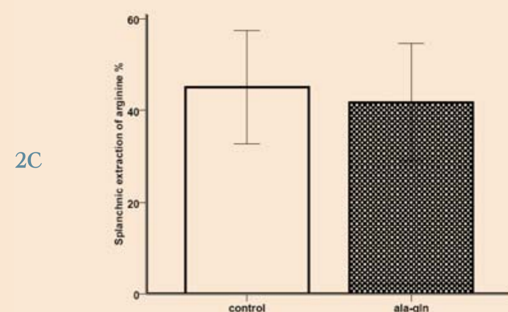

Figure 2 – Splanchnic extraction

Slanchnic extraction of glutamine (A), citrulline (B) and arginine (C) expressed in mean ± SE.

Table 5 – tracer dynamics and conversion rates

Tracer dynamics		CON Mean (SEM)/ Median (IQR)	ALA-GLN Mean (SEM)/ Median (IQR)	Difference CON vs. ALA-GLN (p)
Glutamine				
RA GLN M+1	IV	364.8 (57.5)	390.4 (40.0)	0.720
	EN	787.5 (91.6)	606.3 (88.3)	0.173
Endogenous RA GLN		335.3 (62.3)	322.04 (39.4)	0.856
TTR% GLN M+1	IV	4.88 (0.38)	4.88 (0.33)	0.997
	EN	2.54 (0.35)	2.44 (0.25)	0.820
Splanchnic extraction GLN		48.2 (4.6)	48.4 (3.6)	0.965
Citrulline				
RA CIT M+5	IV	26.5 (4.7)	28.5 (5.5)	0.780
	EN	51.7 (23.7;69.2)	29.5 (20.8;64.3)	0.462
Endogenous RA CIT		26.5 (4.7)	28.5 (5.5)	0.780
TTR% CIT M+1	IV	1.99 (0.33)	1.13 (0.16)	0.031
	EN	4.44 (0.47)	2.64 (0.46)	0.015
TTR% CIT M+2	IV	0.29 (0.14)	0.21 (0.08)	0.622
	EN	1.08 (0.21)	0.50 (0.19)	0.057
TTR% CIT M+5	IV	4.31 (0.85)	4.06 (0.34)	0.791
	EN	2.49 (2.25;2.94)	1.98 (1.82;2.29)	0.207
Splanchnic extraction CIT		33.0 (7.7)	44.8 (3.1)	0.185
Arginine				
RA ARG M+2	IV	76.6 (12.2)	75.0 (6.0)	0.907
	EN	135.4 (106.8; 240.3)	121.3 (17.0)	0.374
RA ARG M+2		26.9 (25.0;58.0)	31.8 (6.1)	0.336
TTR% ARG M+1	IV	0.44 (0.13)	0.35 (0.09)	0.583
	EN	0.97 (0.25)	0.83 (0.15)	0.624
TTR% ARG M+2	IV	6.33 (0.76)	6.00 (0.50)	0.717
	EN	3.73 (0.78)	3.26 (0.46)	0.593
TTR% ARG M+5	IV	0.33 (0.06)	0.35 (0.07)	0.898
	EN	0.25 (0.05)	0.30 (0.05)	0.500
Splanchniciextraction ARG		45.0 (6.2)	41.8 (6.4)	0.725

Conversion rates	Splanchnic extraction ARG	Splanchnic extraction ARG	
Q Gln→Cit	13.4 (4.2)	6.3 (1.3)	0.121
Q Cit→Arg	0 (0-2.2)	1.3 (0.5-3.1)	0.135
Q Gln→Arg	3.2 (1.0)	2.4 (0.9)	0.539
Q Gln→Cit % of Gln	4.45 (0.91)	2.06 (0.42)	0.026
Q Gln→Cit % of Cit	47.8 (7.8)	24.8 (4.4)	0.018
Q Cit→Arg % of Cit	0 (0-10.8)	6.5 (1.3-12.5)	0.370
Q Cit→Arg % of Arg	0 (0-7.4)	5.3 (2.2-10.4)	0.131
Q Gln→Arg % of Gln	1.3 (1.0-1.4)	0.7 (0.3-1.3)	0.370

RA in μmol/kg/h, endogenous RA in μmol/kg/h,
Splanchnic extraction in %, TTR in %, Q in μmol/kg/h.

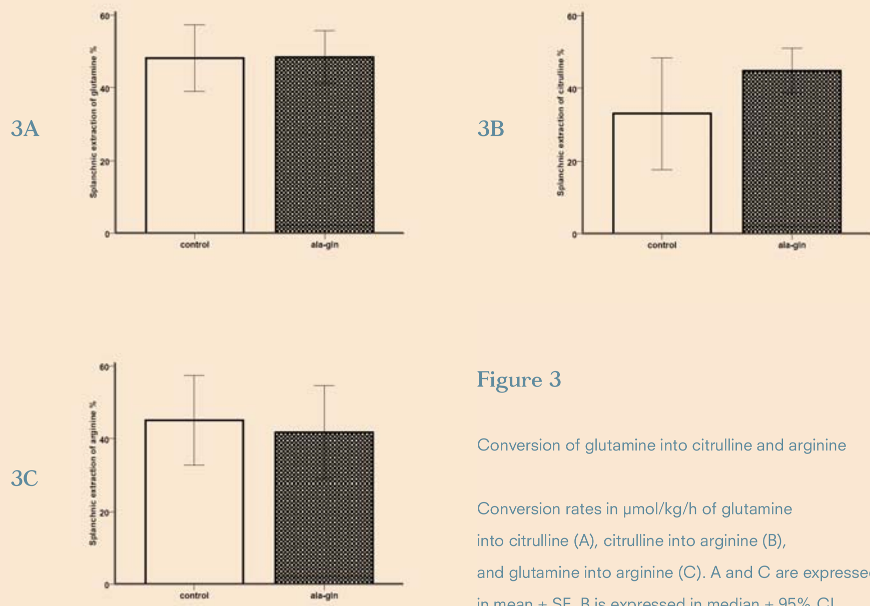

Figure 3

Conversion of glutamine into citrulline and arginine

Conversion rates in μmol/kg/h of glutamine into citrulline (A), citrulline into arginine (B), and glutamine into arginine (C). A and C are expressed in mean ± SE, B is expressed in median ± 95% CI.

Discussion

The primary aim of the present study was to quantify the effect of a therapeutically relevant dose of enteral L-glutamine on the synthesis of L-citrulline and subsequent L-arginine in critically ill patients receiving enteral nutrition. In contrast with our working hypothesis, we did not demonstrate a significantly higher turnover of glutamine into the substrates citrulline and arginine in this group.

Glutamine is one of the most abundant amino acid in the human body. In healthy adults, the small intestine is the major organ of glutamine utilization. Enterocytes extract both arterial glutamine and in a greater extent luminal glutamine.

Intestinal glutamine degradation starts with deamination into glutamate and ammonia. Ammonia is released into the portal vein, after which it can be taken up by the liver serving ureagenesis and glutamine synthesis. Glutamate is released into the portal vein, either as glutamate, as alanine and α-ketoglutarate after transamination with pyruvate, or it is converted to citrulline (approximately 12%, which is 60%–80% of the total citrulline) in which the amino-group and the carbon skeleton of the original glutamine molecule are preserved. The major part of this citrulline is released into the portal vein and subsequently taken up by the proximal tubular cells of the kidney for arginine de novo synthesis [22].

Citrulline itself is scarce in a regular human diet. It was first identified in the 1930s and its name is based on the juice of watermelon (citrullus vulgaris) [34]. In contrast to other amino acids, it is not used in protein synthesis, so it was long time considered to function solely as a metabolic intermediate, specifically in the urea cycle. However, with extensive research performed over the past decades, citrulline appears to play a considerable role in the regulation of nitrogen homeostasis and in the cardiovascular system as regulator of immunity.

After hepatic escape and renal extraction, an ammonia group from aspartate is incorporated to form arginine (catalysed by argininosuccinate synthase [ASS] and argininosuccinate lyase [ASL]) in the proximal convoluted tubules of the kidney. Citrulline is the only precursor for de novo arginine synthesis, of which the majority is executed in the kidneys. This pathway is called the "intestinal-renal axis". The synthesized arginine from citrulline accounts for 60% of the de novo whole-body arginine synthesis, however this only represents 5%–15% of the total circulating arginine [35]. This indicates that most of the plasma arginine is derived from proteolysis and food intake. This arginine pool is sufficient to provide the body's full arginine requirements in physiological conditions.

The relationship between glutamine and arginine has been subject of research in our group since the early nineties [1, 36]. Since then, extensive research has been evolved on using tracer methodology on this topic by us and others. Although the existence of the relationship between glutamine, arginine and citrulline is clear, we learned that: A) mice metabolism is unequal to human metabolism [35, 37, 38] B) enteral glutamine administration has not the same effect as intravenous glutamine supply [23], and C) critically ill patients behave differently as opposed to healthy volunteers [39-41]. At least three matters have remained unclear: does postabsorptive glutamine handling differ from the postprandial state; do critically ill patients metabolize an additional enteral dose of glutamine differently than (so far investigated) trace dosages and do differences exist between septic and relatively stable ICU patients? We attempted to provide the answers to the first two questions. Kao et al. and Luijking et al. investigated amino acid metabolism in septic ICU patients using stable isotope methodology. Kao shows an altered glutamine metabolism in fasted septic patients compared to healthy volunteers [39]. With enteral administration, they show a more pronounced glutamine to citrulline conversion, as was observed earlier in non critically ill patients [23]. Both investigators observed diminished de novo arginine synthesis. These findings strongly suggest that arginine availability is indeed at risk in septic patients [40, 42].

Since Heylands recent publication on glutamine and antioxidant supplementation in critically ill patients, concerns were raised about glutamine supplementation within their study population [10]. It has now been argued that safety is not guaranteed when high dosages (0.35 g/kg/d parenterally and 30 g/d enterally) of glutamine are administered to patients with multiple organ failure. Given the fact that liver and/or kidney failure impairs protein clearance, glutamine is probably best given to either surgical or medical critically ill patients, but should not be given in case of liver or kidney failure [43, 44].

Our results could be explained by a number of considerations: Primarily, the patients were well fed and not glutamine, citrulline or arginine deficient. Therefore the use of additional glutamine may not have been as effective as within truly depleted patients. Attributing to this, most severely ill (and possibly most depleted) patients could not be included, due to the five day study period in which dropout must be avoided.

Secondly, due to adapted nutritional formulas, control patients received an average of 10.4 grams of glutamine per day. Given the equal amounts of glutamine splanchnic extraction rates and the equal endogenous rates of appearances, the gut does not seem to metabolize glutamine differently when it comes to different amounts of enteral delivery.

The absence of glutamine promoting arginine synthesis was unexpected. In fact, control patients had a relatively larger glutamine into citrulline conversion rate (13.4 versus 6.3 µmol/kg/h) with significantly higher CIT M+1 TTR% in the control group. Since glutamine

and citrulline compete for the same transporter, (neutral amino acid system N transporter: SN1), similar or more citrulline splanchnic extraction can be explained with little glutamine supply [45, 46]. Some studies have previously demonstrated the capability of the liver to take up citrulline [28]. However, this uptake was associated with a release of the liver as well, so unidirectional uptake was never demonstrated.

Unfortunately, due to study design, we were unable to provide any insight neither on hepatic versus intestinal, nor on renal citrulline metabolism.

Remarkably, in our experiments, citrulline to arginine turnover and glutamine to arginine turnover were lower compared to Ligthart-Melis, with a conversion rate of 0-6.5% and 0.7-1.3% respectively, differing with a factor of 5-10% compared to earlier experiments. Again, splanchnic extraction and enteral administration partly accounts for this.

Remarkably, in the control group, the median conversion of citrulline to arginine was calculated zero while having higher glutamine to arginine conversion rates. This is because the TTR% of the metabolic product of L-[5-^{13}C-4,4,5,5$^{2}H_4$]citrulline metabolism - [5-^{13}C-4,4,5,5$^{2}H_4$]arginine - was below detection level in most of the control patients. The glutamine to arginine production probably finds its origin in the gut by the enzymes argininosuccinate synthase and argininosuccinate lyase. Circumstantial induction seems evident since this has been subject of discussion earlier [24, 47].

Furthermore, as we know renal citrulline metabolism is autoregulated [21]: in the presence of adequate arginine concentrations, arginine de novo synthesis is diminished, whereas at low concentrations renal arginine de novo synthesis is promoted. The faith of the "unused" citrulline is unclear, but it can be used for many systems.

Most importantly, our experiments prove that excessive arginine production after glutamine supplementation does not occur, hence the safety of 0.5 g/kg/day enteral alanyl-glutamine administration is proven in relatively stable critically ill patients without sepsis, kidney or liver failure.

Methodological perspective

Glutamine stable isotope studies have generated intense debate recently, since Marini et al. and Tomlinson et al. published multiple tracer results in mice and fed volunteers respectively [38, 48]. Marini found discrepancies between nitrogen and carbon labeled glutamine in mice implying that glutamine provides nonspecific carbon for the citrulline ureido group. Tomlinson found overestimation of the glutamine contribution to arginine synthesis with the labeled nitrogen, and found a contribution of 56% when using labeled carbon (compared to the 64% found earlier in fasted surgical patients).
In the light of the first study and earlier published results it can be concluded that there are interspecies differences. With Tomlinson's study, the overestimation of recycling tracers due to splanchnic extraction remains quantitatively unclear, because no correction was made with whole body rates of appearances calculated solely with intravenous tracers. Our results show 48% glutamine splanchnic extraction, with lower enterally administered glutamine M+1 TTR%, resembling results of Bourreille et al. [49] and lower glutamine to citrulline conversion rates (24.8-47.8%). Importantly the glutamine systemic delivery (endogenous infusion rate) after splanchnic extraction and corrected for steady state nutrient (tracee) delivery was not different in both groups. In contrast RA (not corrected) with enteral administration almost doubled intravenous administration (Table 5). Therefore, overestimation due to splanchnic extraction is proven by our experiments and future tracer studies should not use the dilution equations on solely enteral tracer experiments, as also addressed by Ligthart-Melis and Deutz [50]. This also implies that when correctly using the dilution equations it is still not definite which glutamine tracer should best be used for future studies. We suggest additional research on this topic using transition LC-MS/MS enabling differentiation between different fragments of the labeled amino acids, however this methods includes similar quantitative pitfalls. A multistep approach with multilabeled amino acids could probably be the golden standard, but then, inevitable setting associated bias (as discussed below) also disqualifies this approach.

Strength and limitations of the study

The cross over study design enabling correcting for splanchnic extraction and enteral feeding is a strength although it can also be seen as a weakness: Although patients were considered stable, within the ICU stability and clinical condition of patients can vary every minute. Therefore an approach with two separate study days does not cover small metabolic changes that may have occurred in the mean time. Additionally, different metabolic phases with different energy needs are observed within this patient population [27]. The initial metabolic phase after administration was covered by the three day administration of TEE-based nutrition with or without glutamine. Furthermore, by randomizing the administration order we attempted to outbalance potential metabolic differences. An alternative approach in which simultaneous administration through both routes is studies has the disadvantage of different tracer usage (often giving rise to different metabolic outcome), or (when given sequentially on the same day) different timing within circadian rhythm.

The study design disqualified the use of a control group, due to a five day continuous enteral feeding regime while being immobilized to mimic minimal basal energy expenditure.

The heterogeneity of the studied population means that interpretation should be with caution. It also means that this is a reflection of the exact population that is able to receive full enteral nutrition: not unstable, no bowel surgery, no sepsis, and no expected quick discharge. Therefore these results are useful as a pilot for larger investigation on enterally enriched nutrition.

Unfortunately two patients could not fulfil their second tracer study day. This is a risk that goes hand in hand with the clinical setting. Stable isotope studies are usually performed with 5-6 patients (per group), due to complexity and expenses of the method.

In conclusion, these results prove that in critically ill non-septic patients receiving optimal enteral nutrition including a clinically relevant dose of glutamine, the relationship between glutamine, citrulline and arginine is still present. However in the glutamine receiving group there was no extra citrulline or arginine synthesis and splanchnic glutamine extraction was not increased. Arginine synthesis was not promoted by glutamine administration indicating that in this population glutamine supplementation is safe. This also suggests that for arginine synthesis enhancement there is no need for an additional dose of glutamine when these patients are adequately fed. Furthermore, we proved that overestimation of calculated metabolic products can be reduced by correcting for splanchnic extraction and enteral nutrition.

Competing Interests:

Paul A. M. van Leeuwen has served as a speaker, a consultant, and an advisory board member for Fresenius Kabi. The study was partly financed by Fresenius Kabi. Other than this grant, Mechteld A. R. Vermeulen, Saskia J. H. Brinkmann, Albertus Beishuizen, Pierre M. Bet, Alexander P. J. Houdijk, and Johannes B. van Goudoever declare that there are no competing interests regarding this paper.

Authors' Contributions:

Mechteld A. R. Vermeulen designed and performed the study, performed calculations and statistical analyses, and wrote the paper. Saskia J. H. Brinkmann helped performing the study and critically reviewed the paper. Nikki Buijs critically reviewed the study. Albertus Beishuizen helped designing the study and performed the study; Pierre M. Bet was responsible for ad hoc tracer preparation and pharmaceutical handling of the tracer solutions. Alexander P. J. Houdijk helped designing the study; Johannes B. van Goudoever helped designing the study, performed calculations, and critically reviewed the paper. Paul A. M. van Leeuwen was responsible for all parts of the study. All authors read and approved the final paper.

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