Perioperative glutamine supplementation restores disturbed renal arginine synthesis after open aortic surgery: a randomized controlled clinical trial

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**ABSTRACT**

**Background:** Postoperative renal failure is a common complication after open repair of an abdominal aortic aneurysm. The amino acid arginine is formed in the kidneys from its precursor citrulline, and citrulline is formed from glutamine in the intestines. Arginine enhances the function of the immune and cardiovascular system, which is important for recovery after surgery. We hypothesized that renal arginine production is diminished after ischemia-reperfusion injury caused by clamping of the aorta during open abdominal aortic surgery and that parenteral glutamine supplementation might compensate for this impaired arginine synthesis.

**Methods:** This open-label clinical trial randomized patients who underwent clamping of the aorta during open abdominal aortic surgery to receive a perioperative supplement of intravenous alanyl-glutamine (0.5 · kg⁻¹ · day⁻¹; group A, n = 5) or no supplement (group B, n = 5). One day after surgery, stable isotopes and tracer methods were used to analyze the metabolism and conversion of glutamine, citrulline, and arginine.

**Results:** Whole-body plasma flux of glutamine, citrulline, and arginine was significantly higher in group A than in group B (glutamine, 391 ± 34 vs 258 ± 19; citrulline, 5.7 ± 0.4 vs 2.8 ± 0.4; and arginine, 50 ± 4 vs 26 ± 2 μmol · kg⁻¹ · h⁻¹, respectively; p < 0.01), as was synthesis of citrulline from glutamine (4.8 ± 0.7 vs 1.6 ± 0.3 μmol · kg⁻¹ · h⁻¹), citrulline from arginine (2.3 ± 0.3 vs 0.96 ± 0.1 μmol · kg⁻¹ · h⁻¹), and arginine from glutamine (7.7 ± 0.4 vs 2.8 ± 0.2 μmol · kg⁻¹ · h⁻¹), respectively (p < 0.001 for all).
Conclusion: Production of citrulline and arginine is severely reduced after clamping during aortic surgery. This study shows that an intravenous supplement of glutamine increases the production of citrulline and arginine and compensates for the inhibitory effect of ischemia-reperfusion injury.

Keywords: acute kidney injury, ischemia reperfusion injury, glutamine, citrulline, arginine
INTRODUCTION

Patients with an abdominal aortic aneurysm are treated by placement of a prosthetic graft in the aorta to prevent rupture of the aneurysm. One of the procedural options in patients with a juxtarenal aneurysm (aneurysms that involve the infrarenal abdominal aorta adjacent to or including the lower margin of the renal artery origins) is an open aortic aneurysm repair. During this procedure, the aorta will be clamped above, below, or between the renal arteries, impairing adequate renal perfusion and leading to ischemia-reperfusion injury.

Acute postoperative renal failure is a common complication after open repair of an abdominal aneurysm (occurrence of 6%-7%), which may lead to multiorgan failure and higher 30-day mortality. Up to 3.3% of the patients require dialysis, which results in lower quality of life and higher health care costs (18, 21). Renal failure may result in a diminished renal production of the amino acid arginine from citrulline and could have consequences for the whole-body availability of arginine. Nijveldt et al. demonstrated in rats and in humans that arginine levels dropped during abdominal aneurysm repair, possibly as a consequence of ischemia-reperfusion injury of the kidney (40, 48).

Arginine is the precursor of the vasodilator nitric oxide (NO) (38, 62), which acts as a mediator in host immune defense and mediates the protective effects of endothelium in the cardiovascular system by acting as a vasodilator (9, 39). Glutamine is a substrate for the de novo production of arginine by using the “glutamine-citrulline-arginine pathway.” The synthesis of arginine from glutamine occurs via this intestinal-renal axis (12, 27, 54). Glutamine is converted into citrulline...
In the intestines, after which citrulline is transported to the kidneys, where arginine production takes place (34, 47).

Recently, arginine and glutamine supplementation have received significant attention because of the potential positive effects on morbidity, wound healing, hospital length of stay, and mortality when given perioperatively (5, 31, 38, 58, 62). When patients are critically ill, however, arginine supplementation may result in a negative outcome, probably due to excessive formation of NO and oxidative metabolites that subsequently leads to oxidative stress (3, 50).

Glutamine supplementation is suggested to be a safe, more physiologic way of correcting arginine levels and subsequently achieving glutamine and arginine benefits (20, 60, 66). This was confirmed by results showing that a supplement of glutamine enhanced de novo synthesis of arginine during surgery (3) and that glutamine supplementation was protective for ischemia-reperfusion injury in the intestine and liver and in renal function in rats (24, 36, 65). Esposito et al. observed that glutamine reduces renal dysfunction and tissue injury associated with ischemia-reperfusion injury in the kidney (14). Glutamine also improves immunologic status, shortens hospital stay after elective abdominal aortic reconstruction, and has positive effects on clinical outcome in several other diseases (2, 23, 26, 31, 41). Vermeulen et al. (56) suggested that a major part of glutamine’s effects can be attributed to the formation of arginine from the administered glutamine. The safety of glutamine in critically ill patients is a topic of an ongoing debate; however, most of the patients in whom glutamine is potentially not safe are septic, mechanically ventilated, and have multiorgan failure (7, 19), which does not apply to the patients in our study.
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Only a few studies have used stable isotope techniques to determine the qualitative and quantitative metabolic pathway of glutamine conversion to citrulline and arginine in adults (22, 27-29, 53, 54). Previous studies have shown that almost all citrulline in rodents and humans is derived from intestinal conversion of glutamine and that the kidneys take up citrulline for de novo synthesis of arginine (12, 27, 54).

This study was conducted to quantitatively demonstrate that the production of arginine is diminished after clamping during aortic surgery and that a parenteral supplement of glutamine, provided as a dipeptide, stimulates citrulline formation and enhances de novo synthesis of arginine in humans after a period of renal ischemia. We therefore designed this stable isotope study with glutamine-supplemented patients and a control group to quantify arginine production from its precursor glutamine after a period of renal ischemia and to establish the contribution of intravenous supplementation of glutamine dipeptide to the synthesis of arginine.

METHODS

Patients

This open-label randomized controlled trial included 10 patients. An independent clinician used numbered envelopes to randomly assign patients to receive a 0.5 g · kg⁻¹ · day⁻¹ alanyl-glutamine supplement or no supplement. Patients underwent open surgical repair of an abdominal aortic aneurysm at the VU University Medical Center, Amsterdam, or at St. Antonius Hospital, Nieuwegein, The Netherlands.
Patients aged 35 to 75 years were considered eligible for the study. Exclusion criteria were pre-existing kidney and liver failure, pregnancy, use of corticosteroids within 4 weeks before surgery, insulin-dependent diabetes mellitus, celiac or Crohn disease, or another major cause of intestinal malabsorption and malnourishment. Patients with a body mass index (BMI) of <20 kg/m² were considered to be at risk for malnourishment (13).

Liver enzymes and renal function were assessed before the start of the study because both organs are involved in the metabolism of glutamine, citrulline, and arginine. Liver failure was defined as bilirubin levels >100 µmol/L and kidney failure as an increase in serum creatinine levels to >100 µmol/L. Patients were admitted to the hospital 1 day before surgery. Patient characteristics are listed in Table 1.

Informed consent was obtained from all patients before inclusion. The study protocol was approved by the ethical committees of the VU University Medical Center and St. Antonius Hospital, Nieuwegein, and registered in the trial register (Study ID number: NTR2914).

Study design

A preoperative blood sample was drawn to measure the baseline concentration of amino acids (glutamine, citrulline, and arginine) and for routine preoperative laboratory tests. Patients were not allowed to eat or drink after 8:00 P.M. on the evening before surgery until postoperative day 1 of the study. Patients were allocated to one of the following groups: group A received a continuous intravenous infusion of 0.5 g·kg⁻¹·day⁻¹ alanyl-glutamine (Dipeptiven®, Fresenius
Kabi, Bad Homburg, Germany) that was initiated the evening before surgery and continued until
the end of the tracer infusion the day after surgery. Figure 1 provides a schematic overview of
the study. The infusion was primed and started before the operation to obtain a balance between
the glutamine infusion and whole-body flux and to not suddenly increase and change the
glutamine pool during the tracer protocol. The infusion was given until after the operation to
reach a therapeutic effect. Group B received no supplement.

An intravenous catheter was placed in an antecubital vein and an arterial catheter in the radial
artery as part of standard perioperative care. The arterial catheter was used to draw blood
samples. Anesthesia and epidural analgesia were applied preoperatively in all patients according
to standard protocol.

Surgery was performed by three experienced surgeons. Operation started the day before the
tracer protocol, through a midline incision. The intestines were moved aside to free the aorta.
The aorta was exposed and clamped proximal from the aneurysm, suprarenally (n = 5) or
infrarenally (n = 5) for a period of time (Results and Table 1). The aneurysm was incised, and a
prosthetic graft was placed in the aorta. The clamp was removed from the aorta to restore the
distal blood flow, resulting in reperfusion of both kidneys. The operation was completed
according to guidelines and protocol, and patients were transferred to the intensive care unit
(ICU).

Stable isotope tracers
The stable isotope methodology is thoroughly explained in the book by Wolfe et al. and in previous studies of our group (6, 27-29, 61). In brief, stable isotope tracers behave the same as the amino acids of interest, but one atom in the tracer is replaced with an isotope that has an extra neutron. The tracer quantifies the endogenous presence of an amino acid and the conversion rate of one amino acid into another at the whole-body level. The tracers, L-[2-15N]glutamine, L-[5-13C-4,4,5,5$^2$H$_4$]citrulline, and L-[15N$_2$]arginine, were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA) and are from this point noted as glutamine [M+1], citrulline [M+5], and arginine [M+2], respectively.

The Department of Clinical Pharmacy at the Erasmus Medical Center in Rotterdam prepared sterile and pyrogen-free stock solutions of the tracers. The glutamine tracer was prepared the day before surgery, owing to the limited stability of soluble glutamine of only 72 hours. The stock solutions were diluted with normal saline just before the start of the tracer infusion. The tracer dosages for citrulline [M+5] and arginine [M+2] were comparable with other studies, and the glutamine [M+1] tracer dosage was corrected for the simultaneous alanyl-glutamine infusion to reach adequate enrichments (6, 27, 28, 53) (Table 2).

Tracer infusion, blood sampling, and measurements

The tracer protocol started after 24 hours after the first operative incision. A baseline blood sample was first drawn from the radial artery catheter, and a calibrated, volume-controlled pump (Graseby 3000, Graseby Medical Ltd, Watford, United Kingdom) was used to administer a primed-continuous infusion of the stable isotopes through the intravenous catheter. The infusion was continued for 2.5 hours. During the stable isotope tracer-administration, blood samples were
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taken at 30, 60, 90, 120, and 150 minutes after the infusion was started. A plasma isotopic
enrichment is in steady state after 90 to 120 minutes (6, 27-29, 54).

**Blood sampling and analysis**

High-performance liquid chromatography was used to measure amino acid concentrations in
deproteinized samples and infusates, as previously described (55). Blood was collected in
heparinized vacuum tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) and placed on
ice. First, the hematocrit of the blood samples was measured. Blood was centrifuged (10 minutes,
3000 rpm, 4°C), and 50 μL of the supernatant was deproteinized with 20 mg dry sulfosalicylic
acid within 1 hour after sampling. After mixing, samples were stored at –80°C until analysis.

**Mass spectrometric analysis**

Plasma enrichments of the infused tracers and the tracer products were measured by liquid
chromatography-mass spectrometry (LC/MS) according to a recently developed novel method
(44). Briefly, 20 μL hydrochloric acid, 20 μL internal standard, and 200 μL cation exchange
solution (0.1 mg/mL AG 50W-X8 resin [Bio-Rad], 200-400 mesh) were added to 50 μL
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
of 6 mol/L ammonia, respectively. The eluate was dried in a SpeedVac (Genevac Ltd) and
redissolved in 80 μL acetonitrile, 20 μL of 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 minutes. After two extractions with ethyl acetate, the combined solutions of the first (400 µL) and second (400 µL) extractions were evaporated under a gentle stream of nitrogen at room temperature until almost dry and redissolved in 100 µL of 20% methanol. Analyses were performed with LC/MS (Velos Pro; Thermo Fisher) by injecting 10 µL of the sample extract on a 2.1- to 100-mm, 1.7-mm Waters Acquity BEH C18 column. Elution was performed at a stable temperature of 40ºC by using mobile phases consisting of 0.5 mmol/L tridecafluoroheptanoic acid and 1 mL/L formic acid (phase A) and methanol with 0.5 mmol/L tridecafluoroheptanoic acid and 1 mL/L formic acid (phase B). Ion abundance was monitored in full scan by 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) (6).

Calculations

The equations used for calculating the whole-body metabolism of glutamine, citrulline, and arginine are described in Table 3. Enrichment curves were fitted to determine a mean steady-state value on the different time points per individual by using Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). For all patients, we found a steady state after 60 minutes of infusion for glutamine, citrulline, and arginine. Isotope enrichments were calculated as enrichment at steady state minus isotopic background measurements at baseline and are expressed as mole percent excess. Whole-body flux of glutamine, citrulline, and arginine were calculated, and whole-body conversion rates for glutamine into citrulline and citrulline into arginine were determined.
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Statistical analysis

Previous studies have shown that a small number of patients is sufficient to observe significant conversions of glutamine into citrulline and arginine on the whole-body level (6, 27-29). A sample size of 5 patients in each group was thus calculated to provide a power of 1.0 ($\alpha \leq 0.05$) to determine a difference between the intervention and the control group. (Program PS: Power and Sample Size Calculation by Dupont and Plummer, Department of Biostatistics, Vanderbilt University, version 2.1.31, 2004). The Shapiro-Wilk normality test and Q-Q plots were used to test results at distribution. Normally distributed data are expressed as mean ± standard error. Non-normally distributed data are expressed as median and interquartile range (IQR). The independent sample $t$ test or Mann-Whitney $U$ test was used to compare the control group with the alanyl-glutamine group, according to distribution. The one-sample $t$ test was used to test whether the steady state of metabolic products differed from 0. A two-tailed $p$ value of $<0.05$ was considered statistically significant. Statistical analysis was performed with SPSS 17.0 software (IBM Corp., Armonk, NY USA).

RESULTS

Patients

Ten patients were included between June 2011 and September 2012: 5 patients (group A) received 0.5 g · kg$^{-1}$ · day$^{-1}$ alanyl-glutamine for at least 42 hours before the start of the tracer protocol, and 5 control patients (group B) did not receive a supplement. Baseline characteristics of the patients are reported in Table 1. The BMI of patients did not reveal malnourishment. Compared with preoperative values, creatinine plasma levels in both groups were significantly
higher and the estimated glomerular filtration rates were significantly lower after the operation ($p < 0.05$). Clamping time was almost doubled in the patients in group B, which was caused by a prolonged clamping time in 1 patient. The Mann-Whitney $U$ test found no significant difference (median, 36.5 minutes; IQR, 26-64 minutes; $p = 0.10$). When the patients were divided according to suprarenal and infrarenal clamping, regardless of glutamine supplementation, no significant differences other than described were found in baseline characteristics. Q-Q plots of the amino acid concentrations and fluxes showed linearity, indicating that the distribution was consistent with the assumption of normality.

**Alanyl-glutamine infusion**

Patients in group A received an average of $37 \pm 5$ grams of alanyl-glutamine per day. This resulted in approximate amounts of $19 \pm 0.4$ grams in the 18 hours before surgery at day 0, $43 \pm 0$ grams in the 42 hours before the tracer protocol at day + 1, and $3.9 \pm 0.5$ grams during the 2.5-hour tracer infusion. Alanyl-glutamine was also infused during the tracer protocol to keep glutamine metabolism with the supplement in steady state.

**Amino acid concentrations**

Before surgery, baseline plasma concentrations of glutamine, citrulline and arginine concentrations did not differ between the groups. After surgery, plasma concentrations decreased significantly in both groups compared with baseline ($p < 0.05$, see Table 1). Plasma concentrations of glutamine, citrulline, and arginine were significantly higher postoperatively in group A than in group B ($p < 0.05$).

**Enrichments of isotopes**
We found significant enrichments of the infused isotopes of glutamine [M+1], citrulline [M+5], and arginine [M+2] in both groups \( (p < 0.001) \), with a slight overshoot at the beginning of the steady-state plateau due to a small overestimated amount of the priming doses. Their metabolic products, citrulline [M+1], arginine [M+1], and arginine [M+5], were significantly different from zero \( (p < 0.001) \), which states that the labels of glutamine [M+1] and citrulline [M+5] are metabolized into citrulline and arginine molecules. Arterial plasma enrichments of the infused tracers and the tracer products were observed to be in steady state for both groups (Figure 2).

**Whole-body amino acid flux**

Whole-body plasma flux of glutamine in group A was 487.1 ± 34.3 µmol · kg\(^{-1}\) · h\(^{-1}\). When corrected for the alanyl-glutamine infusion of 0.5 g · kg\(^{-1}\) · day\(^{-1}\), the endogenous glutamine flux was 391.1 ± 34 µmol · kg\(^{-1}\) · h\(^{-1}\). The endogenous flux was calculated by subtracting the glutamine given with this infusion (exogenous flux) from the flux to obtain endogenous flux only. Whole-body plasma flux was 5.7 ± 0.4 µmol · kg\(^{-1}\) · h\(^{-1}\) for citrulline and 49.6 ± 3.9 µmol · kg\(^{-1}\) · h\(^{-1}\) for arginine in group A. These fluxes were significantly higher for all amino acids in group A compared with group B (total glutamine, 258.8 ± 19.2; citrulline, 2.8 ± 0.4; and arginine, 25.9 ± 1.7 µmol · kg\(^{-1}\) · h\(^{-1}\), respectively; \( p < 0.001 \); Figure 3).

**Citrulline production from glutamine**

Estimated whole-body citrulline [M+1] synthesis from plasma glutamine [M+1] in group A was 4.8 ± 0.7 µmol · kg\(^{-1}\) · h\(^{-1}\), representing 85% of the total citrulline flux. In group B, citrulline [M+1] synthesis was 1.6 ± 0.3 µmol · kg\(^{-1}\) · h\(^{-1}\) (56% of whole-body citrulline flux), which was significantly less than in group A \( (p < 0.01) \); Figure 4).
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Arginine production from citrulline

Estimated whole-body arginine [M+5] synthesis from plasma citrulline [M+5] in group A was 2.3 ± 0.3 µmol · kg\(^{-1}\) · h\(^{-1}\), representing 40% of the total citrulline flux. In group B, 35% of the total citrulline flux was used for arginine production of 0.96 ± 0.1 µmol · kg\(^{-1}\) · h\(^{-1}\), which was significantly lower than in group A (\(p < 0.001\); Figure 4).

Arginine production from glutamine

Whole-body production of arginine [M+1] from glutamine [M+1] appeared to be 7.7 ± 0.4 µmol · kg\(^{-1}\) · h\(^{-1}\), which represents more than 15% of the total arginine flux. In group B, 11% of all arginine was derived from glutamine (2.8 ± 0.2 µmol · kg\(^{-1}\) · h\(^{-1}\)). This difference was significant in favor of group A (\(p < 0.001\)). Arginine production from glutamine appeared to be more than 100% of the citrulline-to-arginine conversion rate. This was probably an overestimation caused by nitrogen recycling or channeling, which will be further outlined in the Discussion.

DISCUSSION

The primary aim of the present study was to demonstrate the detrimental effects of renal ischemia-reperfusion injury on whole-body metabolism of glutamine, citrulline, and arginine in patients in the early postoperative, postabsorptive state and to investigate whether a perioperative supplement of glutamine would prevent this decrease. In line with our hypothesis, we demonstrated for the first time that plasma concentrations of glutamine, citrulline, and arginine were significantly decreased after ischemia-reperfusion injury by aortic clamping \textit{in vivo}. The whole-body flux of glutamine, citrulline, and arginine was significantly higher when patients
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received a supplement of alanyl-glutamine. The whole-body flux of citrulline and arginine from
 glutamine was significantly higher in the group supplemented with glutamine than in the control
group, and the de novo synthesis of arginine was doubled in the patients receiving glutamine.

Besides being the precursor of NO and therefore important for the immune and cardiovascular
system, arginine is of great importance for wound healing (11). Arginine levels drop when
patients are exposed to surgical injury, cancer, critical illness, or infection, and then arginine
becomes an essential amino acid (37, 57). Arginine deficiency may lead to immunosuppression,
impaired recovery, disturbed inflammatory response, and diminished vascular function (37, 62).

Fasting before surgery results in depletion of glycogen stores, dehydration, muscle wasting, a
weakened immune response, unnecessary production of inflammatory mediators, and a drop in
glutamine, citrulline, and arginine levels (36). Patients in the present study fasted during the
entire study protocol. The metabolic response to surgical stress and critical illness is
characterized by muscle protein breakdown and mobilization of amino acids to be used by the
immune system, gut, and liver, as reflected in our results that showed a decrease in the plasma
concentrations of all amino acids. This is also reflected by the difference in the estimated whole-
body flux of arginine (M+2) (25.9 ± 1.7 µmol · kg⁻¹ · h⁻¹) compared with the much lower de
novo production of arginine from citrulline (0.96 ± 0.1 µmol · kg⁻¹ · h⁻¹). The whole-body flux is
representative for the rate of the appearance and disappearance at the time of measurement in
steady state.
When patients undergo an open aortic aneurysm repair, additional ischemia-reperfusion injury occurs in the kidneys. In brief, tissue ischemia occurs during cross-aortic clamping. This causes an anaerobic cellular metabolism, leading to acidosis, decreased cellular glycogen and adenosine triphosphate levels that cause reduced cell membrane function, vascular endothelial adhesions, and upregulation of clotting pathways (1). The most detrimental part of this cascade is the production of oxygen free radicals when cellular metabolism shifts from anaerobic back to aerobic after clamping. After restoration of blood flow, these free radicals cause further damage to the ischemic tissue by neutrophil influx and depletion of NO, aggravating interstitial edema, vasoconstriction, accumulation of toxins, and production of proinflammatory mediators (25, 33).

This ischemia-reperfusion process causes serious damage to tissues and diminishes cell function.

To demonstrate that the pathway of glutamine to arginine is disturbed primarily by ischemia-reperfusion injury and not by the effect of surgery itself, we compared our results with those of a study of similar design by Ligthart-Melis et al. (27) in which stable isotopes were used to quantify the metabolism of glutamine, citrulline, and arginine. They used intravenous stable isotopes in patients in the postabsorptive state during abdominal surgery to determine renal metabolism (27). The whole-body glutamine flux in their surgical patients was similar to our control group. These levels were in the normal reference range, as previously described (8, 9, 27). Whole-body citrulline flux was $6.2 \pm 0.6 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, whereas the flux in our patients was less than half of this ($2.8 \pm 0.2 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Whole-body arginine flux appeared to be decreased as well to almost half compared with the results of Ligthart-Melis et al. ($42 \pm 2.9$ vs $26 \pm 1.7 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, respectively). Conversion rates of glutamine to citrulline ($5.1 \pm 0.7$ vs $1.55 \pm 0.3 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and citrulline to arginine ($4.9 \pm 0.9$ vs $0.96 \pm 0.11 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) were also significantly lower in our patients.
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were seriously reduced after ischemia-reperfusion. Thus, citrulline and arginine fluxes seem to be substantially diminished after ischemia and reperfusion of the kidneys and the gut.

The present study shows that the first step in the intestinal-renal axis, the conversion of glutamine to citrulline in the enterocyte, is disturbed after open abdominal aneurysm repair. The intestine is very susceptible to ischemia due to the unique anatomic arrangement of the mucosal microcirculation and labile cells (17, 30). Subsequent reperfusion of the intestine results in further development of hemorrhagic mucosal damage by oxygen free radicals and activation of phospholipase A2 (51).

Although the superior mesenteric artery, which supplies the small intestine with oxygen-rich blood, is not directly clamped during elective open aneurysm repair, the intestine is exposed to ischemia secondary to altered blood flow (42). Mesenteric traction and cooling caused by intestinal manipulation exacerbates intestinal ischemia secondary to vasoactive amine release and anatomic distortion of small blood vessels (30). In addition, ischemia, trauma, and intestinal cooling result in an impaired mucosal barrier leading to endotoxin translocation (49). Dysmotility of the small intestine was noted in a large group of surgical patients undergoing abdominal aortic aneurysm repair (29). This leads to impaired absorption of enteral nutrition, suggesting that the small bowel does not function properly after abdominal aneurysm repair (15, 35). Studies have shown that a functional competent enterocyte is required to convert glutamine into citrulline (10, 45, 46). Our results suggest that enterocyte function is indeed disturbed during open aneurysm repair because the synthesis of citrulline is approximately 70% lower than in other surgical patients (50).
The second step in the intestinal-renal axis takes place in the kidneys. It was demonstrated that the kidneys show (temporary) damage postoperatively, even when the aorta is clamped infrarenally (16, 63), which has direct consequences for the availability of arginine. Prins et al. (48) found decreased renal arginine production in rats after ischemia-reperfusion injury, which could contribute to or slow the recovery from the low plasma levels of arginine as seen during surgery and after clamping. Nijveldt et al. (40) showed low arginine plasma levels after aortic surgery in patients exposed to ischemia-reperfusion injury as a result of clamping.

We used stable isotopes to quantitatively demonstrate that ischemia-reperfusion injury results in a decrease in arginine fluxes of 44% compared with patients who were under normal surgical conditions without ischemia-reperfusion injury and of up to 90% compared with healthy individuals (8, 27). The likelihood of kidney damage after clamping in our study group was confirmed by the significantly worse rate of creatinine clearance and estimated glomerular filtration compared with preoperative values (43). However, the number of patients was too small to draw definite conclusions regarding this hypothesis.

To assess whether a perioperative supplement of alanyl-glutamine restores low levels of amino acids after ischemia-reperfusion injury, we compared our results with a study by Buijs et al. (6) in which glutamine, citrulline, and arginine fluxes were determined with stable isotopes peroperatively. We found that the whole-body flux of glutamine postoperatively did not differ from their peroperative results (6). Thus, levels of glutamine appeared to be preserved after ischemia-reperfusion injury when a alanyl-glutamine supplement was given perioperatively. The
conversion of glutamine to citrulline was 8.1 ± 0.9 µmol · kg⁻¹ · h⁻¹ in the peroperative study, which was almost twice as high as in our intervention group postoperatively (4.8 ± 0.7 µmol · kg⁻¹ · h⁻¹, p < 0.01). Citrulline-to-arginine conversion seemed to be twice as high compared with our group exposed to renal ischemia-reperfusion injury (4.5 ± 0.60 vs 2.29 ± 0.31 µmol · kg⁻¹ · h⁻¹). This proves that de novo synthesis of citrulline and arginine is diminished even when a supplement of glutamine is given, probably due to damaged enterocytes and kidney damage. However, plasma concentrations of glutamine, citrulline, and arginine were significantly higher in the intervention group. Compared with our control group, this supplement of glutamine is capable of preserving glutamine-to-citrulline fluxes in the intestine and citrulline-to-arginine fluxes in the kidney as well as keeping glutamine levels at perioperative values (p < 0.01).

The results of this study are relevant for clinical practice because they provide more insight into the detrimental effects of ischemia-reperfusion injury and how to overcome these. Studies have shown intravenous glutamine administration is safe (60, 66). In connection with surgery, glutamine administration improves recovery from injury, positively affects survival, and reduces infectious complications and hospital and ICU lengths of stay (3, 64, 66). In addition, the ESPEN Guidelines on Enteral Nutrition in Surgery still recommend giving arginine preoperatively in abdominal surgery because of the reduced postoperative morbidity and length of stay (4, 52, 59).

This study has some limitations. Firstly, we did not measure organ fluxes to calculate the specific contribution of the intestine and kidney because this was not possible in postoperative setting. However, Buijs et al. and Lighthart-Melis et al. extensively demonstrated the contribution of these two organs to this conversion (6, 27-29, 54). Secondly, different clamping methods were used (suprarenal and infrarenal), which could have influenced the rate and amount of ischemia-
reperfusion injury to the kidney. However, ischemia-reperfusion injury to the kidney still occurs with infrarenal clamping (16, 63). When the groups were divided by infrarenal and suprarenal clamping, no significant differences were seen. The difference between groups in clamping time was almost doubled but was not significant. Furthermore, the patients in this study were relatively older and representative of patients with abdominal aortic aneurysm. BMI was not significantly different between the groups, but more overweight people were in group B. This might have influenced our metabolic measurements.

Similar to the results of Buijs et al., we also found an overestimate in the quantitative contribution of glutamine to arginine. Debate about the interpretation of the use of this glutamine \([\text{M+1}]/[2^{-15}\text{N}]\) glutamine tracer is ongoing. We are aware that this tracer is also converted into \(L-[5^{-15}\text{N}]\) glutamine, as demonstrated by Marini et al., (32) who showed that the use of a \([2^{-15}\text{N}]\) glutamine tracer may overestimate the quantitative contribution of glutamine to arginine in mice due to nitrogen recycling. Tomlinson et al. (53) found, similar to our results and the results of Buijs et al., that the N-labeled tracer showed a contribution of glutamine \([\text{M+1}]\) to arginine \([\text{M+1}]\) synthesis that exceeded the citrulline-to-arginine conversion. However, they also demonstrated that overestimation of arginine production is possible because the labeled \(N\)-atom was found in various locations of the ornithine, citrulline, and arginine molecules. Our study, however, was designed to determine the first-pathway effects of aortic clamping on citrulline and arginine metabolism and whether parenteral glutamine, as a supplement of 0.5 g · kg\(^{-1}\) · day\(^{-1}\), could enhance this synthesis, hence our chosen methods (6, 44). Our aim was not to analyze the metabolism of the different fragments of the molecules of glutamine, citrulline, and arginine. We also did not intend to study the biochemical transitions of nitrogen on the amino acid molecules,
because various studies have already showed this precursor pathway in humans by using the stable isotope method (6, 27, 53). However, the quantitative results of the N-labeled glutamine tracer should be interpreted with caution because outcomes may overestimate the contribution of glutamine to citrulline and arginine formation.

Conclusions

This is the first study to quantify the whole-body fluxes of glutamine, citrulline, and arginine and the conversion of glutamine to citrulline and of citrulline to arginine after ischemia-reperfusion injury of the kidneys. We demonstrate that a perioperative supplement of alanyl-glutamine is capable of preserving whole-body and organ fluxes of glutamine, citrulline, and arginine to perioperative values in patients in the postoperative and postabsorptive state exposed to ischemia-reperfusion injury of the kidneys caused by clamping during abdominal aortic surgery.

Acknowledgements:
The authors thank Sigrid de Jong for her help with the laboratory work, Joanna Luttikhold for helping to collect the data, and Ernst Cancrinus for operating on some of the patients.

Contributions of the authors:
SJHB, NB, and MARV: contributed to the study design; SJHB, NB, and AB: contributed to the implementation of the study; SJHB, NB: conducted the research and collected the data; WW and JPdV: supervised during surgical procedures; JEO and HS: performed mass spectrometry and amino acid concentration analyses; SJHB, NB, and MARV: performed calculations and statistical analysis; HS: helped with interpretation of the data; SJHB, NB, MARV, and JEO:
Glutamine restores disturbed renal arginine synthesis

drafted the manuscript; HS, WW, JPDV, and AB: critically revised the manuscript; and PAMvL: had primary responsibility for all aspects of the study and for final content.

Disclosure and grants:
PAMvL reports receiving fees from Fresenius Medical Care for clinical consultation. None of the other authors report a conflict of interest related to the study. Fresenius Kabi provided alanyl-glutamine (Dipeptiven) for this study, and the Vivax Foundation supported this work with a research grant; however, neither entity participated in data collection, data analysis, data interpretation, or in writing the manuscript.


Glutamine restores disturbed renal arginine synthesis


Glutamine restores disturbed renal arginine synthesis


Glutamine restores disturbed renal arginine synthesis


Figures captions

Figure 1. A schematic overview of the study design. ICU: intensive care unit.

Figure 2. (1) Steady state curve of primed-continuous infused tracers, displayed as mole percent excess (MPE) for group A and B (both groups: n = 5). (D) Steady-state curve of the products of the infused tracers, displayed as MPE for group A and B (n = 5 for both groups). Enrichments of the infused tracers and the tracer products were observed to be in steady state for both groups.

Figure 3. Estimated whole-body flux of total endogenous + exogenous glutamine, citrulline, and arginine (mean ± standard error of the mean; n = 5 for both groups). *p < 0.01.

Figure 4. Estimated whole-body synthesis de novo of citrulline [M+1] from glutamine [M+1] and arginine [M+5] from citrulline [M+5] (mean ± standard error of the mean; n = 5 for both groups). *p < 0.01.
### Table 1. Baseline patient characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A Ala-Gln supplementation (n = 5)</th>
<th>Group B Controls (n = 5)</th>
<th>p#</th>
</tr>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>67 ± 3</td>
<td>72 ± 3</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>74 ± 9</td>
<td>82 ± 7</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>175 ± 5</td>
<td>175 ± 5</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>Body mass index (kg/m²)</strong></td>
<td>23 ± 2</td>
<td>27 ± 2</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Amino acid concentration (µmol/L) T incl1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>880 ± 32</td>
<td>773 ± 34</td>
<td>0.06</td>
</tr>
<tr>
<td>Citrulline</td>
<td>35 ± 4</td>
<td>32 ± 2</td>
<td>0.57</td>
</tr>
<tr>
<td>Arginine</td>
<td>86 ± 5</td>
<td>82 ± 11</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>Hours from surgery to start tracer protocol</strong></td>
<td>23 ± 1</td>
<td>24 ± 1</td>
<td></td>
</tr>
<tr>
<td><strong>Amino acid concentration (µmol/L) T0</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>746 ± 48*</td>
<td>465 ± 16*</td>
<td>&lt;0.001</td>
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<tr>
<td>Citrulline</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Arginine</td>
<td>40 ± 4*</td>
<td>30 (23-33)*</td>
<td>0.05</td>
</tr>
<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>93 ± 20</td>
<td>93 (72-139)</td>
<td>0.99</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>6 ± 1</td>
<td>7 ± 1</td>
<td>0.82</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate (mL/min/1.73 m²)</td>
<td>54 (43-60)</td>
<td>62 ± 6</td>
<td>0.33</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>8 ± 2</td>
<td>6 ± 1</td>
<td>0.37</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>27 ± 2</td>
<td>22 ± 3</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Glutamine restores disturbed renal arginine synthesis

Glucose (mmol/L) | 6 ± 0.2 | 6 ± 0.4 | 0.43

Postoperative laboratory results

<table>
<thead>
<tr>
<th>Substance</th>
<th>Group A</th>
<th>Group B</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (µmol/L)</td>
<td>114 ± 23*</td>
<td>156 ± 30*</td>
<td>0.30</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>11 ± 2*</td>
<td>11 ± 1*</td>
<td>0.80</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate (mL/min/1.73 m²)</td>
<td>50 (33-60)*</td>
<td>33 ± 4*</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Clamping method

<table>
<thead>
<tr>
<th>Method</th>
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<th>Group B</th>
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</thead>
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<tr>
<td>Suprarenal</td>
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<td>3</td>
</tr>
<tr>
<td>Infrarenal</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clamping time (min)</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31 ± 3</td>
<td>60 (24-110)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total blood loss during surgery (mL)</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>760 ± 136</td>
<td>1110 ± 165</td>
</tr>
</tbody>
</table>

Tincl: time of inclusion, before operation; T0: the day after operation after administration of intravenous

0.5 g · kg⁻¹ · day⁻¹ alanyl-glutamine (only in group A) just before the start of the tracer infusion;

Independent sample t-test or Mann Whitney test was used to determine significant differences (p<0.05),

data are expressed as mean ± standard error of the mean and as median ± interquartile range (IQR), respectively;

*Significant difference between preoperative and postoperative values.

Table 2. Tracer dosage

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Notation</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[2-¹⁵N]glutamine</td>
<td>Glutamine [M+1]</td>
<td>23.8</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.3</td>
<td>16.7</td>
</tr>
<tr>
<td>L-[5-¹³C-4,4,5,5²H₄]citrulline</td>
<td>Citrulline [M+5]</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>L-[¹⁵N₂]arginine</td>
<td>Arginine [M+2]</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Variable</td>
<td>Equation</td>
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</tr>
<tr>
<td>----------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body flux</td>
<td>( Q = i[(E_i/E_p) - 1] )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body endogenous glutamine flux</td>
<td>( Q_{\text{Gln-endo}} = \text{Total } Q_{\text{Gln}} - Q_{\text{Gln-exo}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body conversion rate glutamine to citrulline</td>
<td>( Q_{\text{Gln-Cit}} = E_{\text{Cit } M+1}/E_{\text{Gln } M+1} \times Q_{\text{Cit } M+5} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body conversion rate citrulline to arginine</td>
<td>( Q_{\text{Cit-Arg}} = E_{\text{Arg } M+5}/E_{\text{Cit } M+5} \times Q_{\text{Arg } M+2} )</td>
<td></td>
<td></td>
</tr>
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<td>Whole-body conversion rate glutamine to arginine</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

\( Q \), flux in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( E \), enrichment in mass percent excess (MPE). \( E_i \), enrichment in infusate. \( E_p \), enrichments in plasma at steady state. \( Q_{\text{Gln-endo}} \), endogenous glutamine flux in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). Total \( Q_{\text{Gln}} \), whole body glutamine flux during alanyl-glutamine infusion in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( Q_{\text{Gln-exo}} \), exogenous flux of glutamine in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( Q_{\text{Gln-Cit}} \), whole body glutamine to citrulline conversion rate in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( E_{\text{Cit } M+1} \), plasma enrichment of citrulline \([M+1]\) in MPE. \( E_{\text{Gln } M+1} \), plasma enrichment of glutamine \([M+1]\) in MPE. \( Q_{\text{Cit } M+5} \), whole body flux of citrulline \([M+5]\) \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( Q_{\text{Cit-Arg}} \), whole body citrulline to arginine conversion rate in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \). \( E_{\text{Arg } M+5} \), plasma enrichment of arginine \([M+5]\) in MPE. \( E_{\text{Cit } M+5} \), plasma enrichment of citrulline \([M+5]\) in MPE. \( Q_{\text{Arg } M+2} \), whole body flux of arginine \([M+2]\) in \( \mu mol \cdot kg^{-1} \cdot h^{-1} \).
Day -1: 
- Check incl/excl criteria + informed consent

Day 0: 
- Group A: start alanyl-glutamine
- Surgery

Day +1: 
- Start tracer protocol ICU
- 2.5 hours
- Stop tracer protocol and alanyl-glutamine infusion
- Blood sampling
Citrulline production from glutamine

Arginine production from citrulline

- Group A
- Group B
<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A</th>
<th>Group B</th>
<th>p#</th>
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<td>Ala-Gln supplementation</td>
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</tr>
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<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
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<td>Sex</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.30</td>
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### Results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Urea (mmol/L)</td>
<td>11 ± 2*</td>
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</tr>
<tr>
<td>Clamping method</td>
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<tr>
<td>Suprarenal</td>
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</tr>
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<td>2</td>
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<td>0.14</td>
</tr>
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Tincl: time of inclusion, before operation; T0: the day after operation after administration of intravenous 0.5 gr · kg⁻¹ · h⁻¹ alanyl-glutamine (only in group A) just before the start of the tracer infusion; Independent sample T-Test or Mann Whitney Test was used to determine significant differences (p<0.05), data are expressed as mean ± standard error (SEM) and as median ± interquartile range (IQR), respectively; * significant difference between pre- and postoperative values.
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<tr>
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<th>Notation</th>
<th>Group A</th>
<th></th>
<th></th>
<th>Group B</th>
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<td>Priming dose</td>
<td>Infusion dose</td>
<td>Priming dose</td>
<td>Infusion dose</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(µmol · kg⁻¹)</td>
<td>(µmol · kg⁻¹ · h⁻¹)</td>
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<td>Citrulline [M+5]</td>
<td>1.5</td>
<td>0.9</td>
<td>1.3</td>
<td>0.6</td>
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<td>L-[¹⁵N₂]arginine</td>
<td>Arginine [M+2]</td>
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<td>3.1</td>
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Table 3. Equations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Equation</th>
</tr>
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<tr>
<td>Whole-body turnover</td>
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</tr>
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</tr>
<tr>
<td>Whole-body conversion rate citrulline to arginine</td>
<td>( Q_{\text{Cit-Arg}} = \frac{E_{\text{Arg}}}{E_{\text{Cit}}} \times Q_{\text{Arg}} )</td>
</tr>
<tr>
<td>Whole-body conversion rate glutamine to arginine</td>
<td>( Q_{\text{Gln-Arg}} = \frac{E_{\text{Arg}}}{E_{\text{Gln}}} \times Q_{\text{Arg}} )</td>
</tr>
</tbody>
</table>

*Q*, flux in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). *E*, enrichment in mass percent excess (MPE). *E_p*, enrichment in infusate. *E_p*, enrichments in plasma at steady state. \( Q_{\text{Gln-endo}} \), endogenous glutamine flux in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). Total \( Q_{\text{Gln}} \), whole body glutamine turnover during alanyl-glutamine infusion in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). \( Q_{\text{Gln-exo}} \), exogenous flux of glutamine in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). \( Q_{\text{Gln-Cit}} \), whole body glutamine to citrulline conversion rate in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). \( E_{\text{Cit}} \), plasma enrichment of citrulline \([M+1]\) in MPE. \( E_{\text{Gln}} \), plasma enrichment of glutamine \([M+1]\) in MPE. \( Q_{\text{Gln-Arg}} \), whole body glutamine to arginine conversion rate in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). \( E_{\text{Arg}} \), plasma enrichment of arginine \([M+5]\) in MPE. \( E_{\text{Cit}} \), plasma enrichment of citrulline \([M+5]\) in MPE. \( Q_{\text{Arg}} \), whole body flux of arginine \([M+2]\) in \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \).