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# Interorgan synthesis of arginine is down-regulated in tumor-bearing mice undergoing surgical trauma

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**Abstract**

Renal de novo arginine production has been suggested to be crucial for regulation of arginine production in disease. We investigated how the interorgan pathway for de novo arginine production is affected by the presence of malignant tumor and/or surgical trauma. Controls and methylcholanthrene-sarcoma–bearing mice were studied, both with and without undergoing laparotomy (n = 9–13 per group). One day after laparotomy, amino acid fluxes across the hindquarter, intestine, liver, and kidney were studied. In contrast to healthy mice, the liver of tumor-bearing mice took up citrulline (9 ± 3 vs 1 ± 2 nmol/[10 g min], $P < .05$), simultaneous with attenuated renal arginine output (4 ± 3 vs 12 ± 2 nmol/[10 g min], $P < .05$), despite increased intestinal conversion of glutamine to citrulline (15 ± 3 vs 8 ± 1 nmol/[10 g min], $P < .05$). In tumor-bearing mice undergoing surgery, intestinal citrulline output decreased (from 15 ± 3 to 8 ± 2 nmol/[10 g min], $P < .05$) and renal arginine output remained close to zero despite increased renal citrulline uptake (from 6 ± 2 to 12 ± 2 nmol/[10 g min], $P < .05$). In conclusion, the interorgan pathway for de novo arginine production was differently regulated depending on the pathophysiological situation. In methylcholanthrene-sarcoma–bearing mice, decreased de novo arginine production was accompanied by the presence of hepatic citrulline uptake, whereas tumor-bearing mice subjected to surgical trauma showed concomitant decreased intestinal citrulline output.


## 1. Introduction

In vivo, in the postabsorptive state, arginine production results from protein breakdown and endogenous synthesis via renal de novo arginine production [1]. An important step in de novo arginine production takes place in the gut, where glutamine (mainly derived from muscle) is converted to citrulline, which is subsequently converted to arginine in the kidney [2,3]. Although contributing only 10% to 20% of total arginine production in healthy circumstances [4,5], de novo renal arginine production has been shown to regulate arginine production in sepsis [6]. We therefore hypothesize that it may also regulate arginine production in other diseases, for example, in cancer, where we recently found indications for disturbances in arginine metabolism (low plasma arginine concentrations) [7].

A common treatment of cancer is surgical removal of the tumor. In the postoperative period, several immune processes rely on arginine as substrate. Arginine is necessary for a functional T-cell response after trauma [8] and is indispensable for wound healing [9]. Therefore, the de novo arginine production pathway may also be involved in amino acid metabolism after surgical trauma.

The aim of the current study was to investigate whether the pathway for de novo arginine production is disturbed in tumor-bearing mice undergoing surgical trauma. Because simultaneous measurements across several organs in humans are difficult in many situations for ethical reasons, we used a mouse model to study the in vivo relation between the amino acid fluxes across the organs that are

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part of the de novo arginine production pathway: muscle, intestine, liver, and kidney. In this way, we could visualize the complete interorgan axis involved in endogenous arginine synthesis. In addition, arginine production from protein breakdown was measured.

## 2. Methods

### 2.1. Animals

Male FVB mice were bred at the Centralized Animal Facilities of Maastricht University. The mice were fed standard laboratory chow (Hope Pharms, Woerden, the Netherlands) and subjected to standard 12-hour light-dark cycle periods. Room temperature was maintained at 22°C. Water was provided ad libitum throughout the experiment. Experiments were approved by the Ethical Committee of Animal Research of Maastricht University. Mice were randomly divided into 4 groups: controls (n = 12), controls undergoing laparotomy (n = 12), tumor-bearing mice (n = 9), and tumor-bearing mice undergoing laparotomy (n = 9).

### 2.2. Tumor model

Tumors were initially induced by subcutaneous injection of 1 mg methylcholanthrene (MCA; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in mice in our laboratory. These tumors were maintained in vivo by serially transplanting tumor tissue through a 15-gauge needle. The MCA tumor has been widely used as a model for cancer in metabolic studies in mice and rats and has the histologic characteristics of malignant sarcoma, with locally aggressive growth but without the tendency to metastasize [10-12]. In our hands, the tumor does not induce anorexia or weight loss. Control mice were sham implanted.

### 2.3. Surgical trauma

When the tumor reached 5% to 15% of carcass weight, a midline laparotomy was performed as a model for surgical trauma. In short, anesthesia was performed by a single intraperitoneal injection of a mixture of ketamine and medetomidine [13]. Mice were kept at 37°C using a rectal temperature controller and heat pads. A midline incision from the level of the superior iliac spines to the xyphoid process was made. Intestines were put aside and wrapped in wet gauze. Five minutes later, intestines were put back; and the abdomen was closed with wound clips (Autoclip; Clay Adams, Becton Dickinson, Parsippany, NJ). In total, the procedure of surgical trauma took about 15 minutes. Afterward, food was taken away from all groups of mice to prevent differences due to variations in food intake. Water was provided ad libitum.

### 2.4. Metabolic measurements

Twenty-four hours after laparotomy, measurements were performed essentially as described by Hallemeesch et al [13] with some adaptations. In short, anesthesia was induced by an intraperitoneal injection of a mixture of ketamine and medetomidine. Subsequently, anesthesia was maintained with a continuous subcutaneous infusion of a mixture of ketamine and medetomidine. Mice were kept at 37°C using a rectal temperature controller and heat pads. Catheterization of blood vessels for infusion of fluids or sampling of blood was performed using a 30-gauge needle fixed in a Silastic (Dow Corning, Midland, MI) tube. All catheters were filled with heparinized saline until blood sampling. To counteract the loss of body fluids by evaporation, saline was continuously infused via the jugular vein. For blood sampling, the carotid artery, portal vein, hepatic vein, right renal vein, and inferior caval vein (the latter just above the bifurcation) were catheterized. Plasma flows across organs were measured using an indicator-dilution technique with [glycyl-1-$^{14}$C]-*p*-aminohippuric acid ($^{14}$C-PAH; NEN Life Science Products, Boston, MA) as described in detail before [13]. In short, $^{14}$C-PAH was administered both via a colonic vein for blood flow measurements across portal-drained viscera and liver, and via the inferior abdominal aorta for blood flow across hindquarter. Plasma flow across the kidney was based on renal extraction of $^{14}$C-PAH.

In contrast with the original article by Hallemeesch et al [13], all organ measurements were performed simultaneously in one animal. In total, the procedure for catheterization of blood vessels took about 30 minutes. Afterward, 120 $\mu$L of blood was collected from the renal vein, carotid artery, hepatic vein, portal vein, and inferior caval vein, respectively, in that order. To reduce the dead volume, blood was pulled until the catheter was filled and all heparinized saline disappeared from the catheter, after which a clean needle and syringe were inserted into the catheter and blood was sampled. Blood sampling took about 2 minutes in total. After blood sampling, mice were killed by cervical dislocation when still under anesthesia.

### 2.5. Analysis

The $^{14}$C-PAH concentrations were determined in trichloroacetic acid deproteinized whole blood using a Winspectral 1414 counter (Wallac, EG&G, Breda, the Netherlands) [13]. Amino acid concentrations were determined in sulfosalicylic acid deproteinized plasma by high-performance liquid chromatography as described by Van Eijk et al [14].

### 2.6. Calculations

Blood flow across hindquarter, liver, and portal-drained viscera was calculated from the arterial-venous concentration difference of $^{14}$C-PAH across the organ of interest [13] based on the dilution principle as posed by Fick: the concentration of $^{14}$C-PAH downstream from the place where it is infused depends on the flow rate through the organ of interest. At steady state, the amount of indicator entering the blood stream equals the amount of indicator excreted. If the infusion rate and the concentration of the

indicator upstream and downstream are known, the flow rate can be calculated. Renal blood flow was calculated as renal extraction of $^{14}$C-PAH.

Hindquarter amino acid fluxes were calculated by multiplying the inferior caval venous-arterial concentrations differences with the mean hindquarter plasma flow. Assuming the hindquarter to be responsible for half of the total muscular compartment [15], values were multiplied by 2 to represent whole-body muscular fluxes. Portal-drained viscera amino acid fluxes were calculated by multiplying the portal venous-arterial concentration differences with the mean portal-drained viscera plasma flow. Portal-drained viscera amino acid fluxes were assumed to represent mainly intestinal metabolism. Splanchnic flux was calculated by multiplying the mean hepatic plasma flow with hepatic venous-arterial concentration differences. Hepatic amino acid fluxes were calculated by subtracting portal-drained visceral flux from the splanchnic flux. Renal amino acid fluxes were calculated by multiplying the renal venous-arterial concentrations differences with the mean renal plasma flow. A positive flux indicates net output, and a negative flux reflects net uptake.

Net protein breakdown in muscle and intestine was estimated from the respective phenylalanine fluxes. Net protein breakdown in liver and kidney was estimated from the respective valine fluxes. From a study measuring amino acid fluxes across the kidney in rats using valine and phenylalanine tracers [16] and a valine/phenylalanine amino acid composition ratio of 2.2 in mice kidneys (unpublished results), the calculated loss of valine into branched-chain amino acid transaminase activity after release from renal protein breakdown was not significant, suggesting that valine net balances can be validly used to estimate renal protein breakdown in rodents.

### 2.7. Statistical analysis

Results are presented as means ± SEM. Data are expressed per 10 g carcass weight, which was calculated by subtracting tumor weight from body weight including tumor. Analysis of variance (ANOVA) was used to test significant differences [17].

## 3. Results

### 3.1. Normal postoperative response

In healthy mice, muscular glutamine output and intestinal glutamine uptake and citrulline output were maintained after laparotomy (Table 1 and Fig. 1). The contribution of muscular glutamine output to intestinal glutamine uptake increased from 77% ± 9% to 99% ± 16%. In the liver, there was no net citrulline uptake both under control circumstances and after laparotomy (Fig. 1). In the kidneys of control mice, citrulline uptake and arginine output were maintained after laparotomy (Fig. 1). Both before and after surgery, renal citrulline uptake exceeded intestinal citrulline

Table 1
Amino acid fluxes across organs

| (nmol/[10 g min]) | CON/CON | CON/LAP | TUM/CON | TUM/LAP |
|---|---|---|---|---|
| *Muscle* | | | | |
| Blood flow (ml/[10 g min]) | 0.33 ± 0.04 | 0.38 ± 0.06 | 0.33 ± 0.07 | 0.45 ± 0.08 |
| GLU | −6 ± 3 | −2 ± 3 | −18 ± 4 * | −19 ± 7 |
| CIT | −6 ± 2 | −7 ± 1 | −5 ± 3 | −5 ± 1 |
| BCAA | −15 ± 16 | −26 ± 25 | −11 ± 15 | −8 ± 15 |
| PHE (PB) | 8 ± 1 | 10 ± 3 | 9 ± 4 | 13 ± 4 |
| *Intestine* | | | | |
| Blood flow (ml/[10 g min]) | 0.36 ± 0.08 | 0.37 ± 0.08 | 0.61 ± 0.20 | 0.51 ± 0.19 |
| GLU | 10 ± 2 | 9 ± 1 | 15 ± 4 | 6 ± 3 † |
| ALA | 81 ± 14 | 45 ± 14 † | 148 ± 22 * | 96 ± 22 † |
| PHE (PB) | 3 ± 1 | 3 ± 1 | 6 ± 2 | 5 ± 2 |
| *Liver* | | | | |
| Blood flow (ml/[10 g min]) | 0.73 ± 0.12 | 0.77 ± 0.14 | 0.83 ± 0.19 | 0.86 ± 0.21 |
| ARG | −25 ± 5 | −18 ± 4 | −29 ± 8 | −15 ± 4 † |
| VAL (PB) | 10 ± 3 | 6 ± 6 | −2 ± 2 * | −2 ± 5 |
| *Kidney* | | | | |
| Blood flow (ml/[10 g min]) | 0.79 ± 0.10 | 0.86 ± 0.13 | 0.96 ± 0.12 | 0.77 ± 0.09 |
| GLU | −32 ± 11 | −28 ± 14 | 0 ± 8 * | −26 ± 8 † |
| VAL (PB) | 3 ± 3 | −6 ± 5 | 3 ± 2 | −3 ± 3 |

Values are means ± SEM in nanomoles per 10 g carcass weight per minute. Organ amino acid fluxes were calculated by multiplying the venous-arterial concentrations differences with the mean organ plasma flow. Assuming the hindquarter to be responsible for half of the total muscular compartment, values were multiplied by 2 to represent whole-body muscular fluxes. A positive flux indicates net release, and a negative flux reflects net uptake. Net protein breakdown in muscle and intestine was estimated from the respective phenylalanine fluxes. Net protein breakdown in liver and kidney was estimated from the respective valine fluxes. CON indicates control; LAP, laparotomy; TUM, tumor; GLU, glutamine; CIT, citrulline; BCAA, branched-chain amino acids; PHE, phenylalanine; PB, protein breakdown; ALA, alanine; ARG, arginine; VAL, valine.

* Statistics with ANOVA: $P < .05$ tumor effect.

† Statistics with ANOVA: $P < .05$ laparotomy effect.

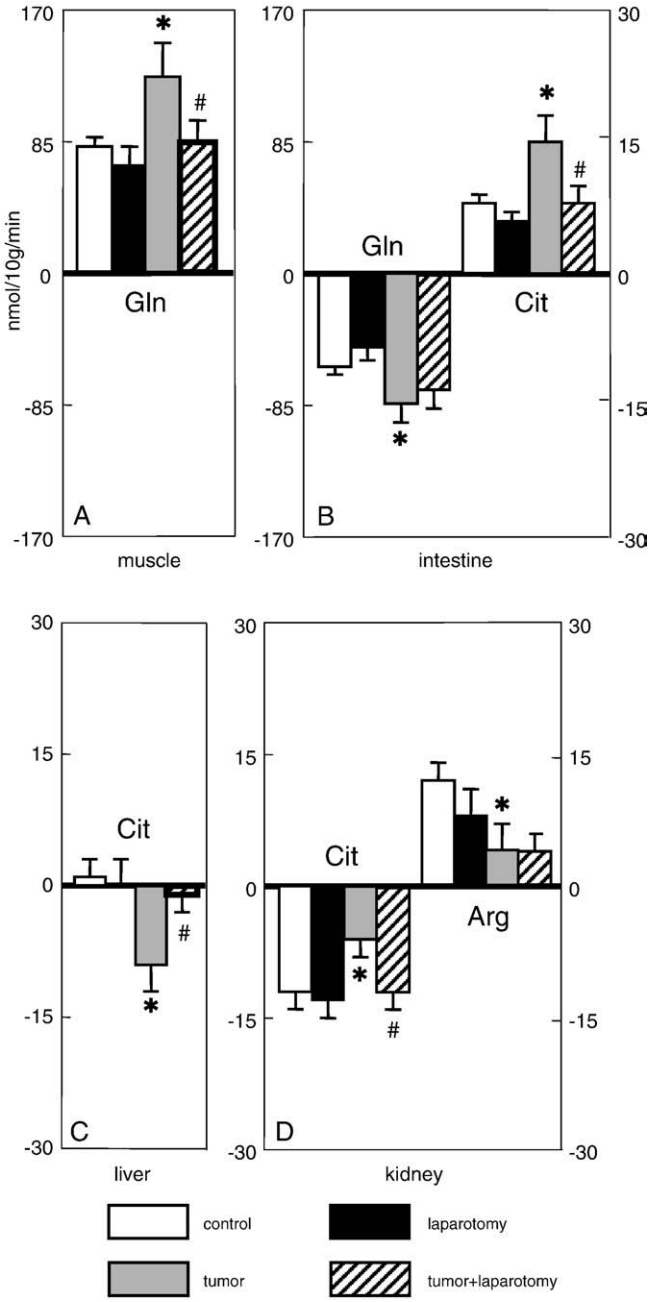


Fig. 1. De novo arginine pathway. Means ± SEM in nanomoles per 10 g carcass weight per minute. Positive values reflect net production; negative values reflect net uptake. Statistics with ANOVA: *$P$ < .05 vs tumor without laparotomy. B, The left axis displays glutamine flux; the right axis, citrulline flux.

output (Fig. 1). Net protein breakdown in muscle, intestine, liver, and kidney was not affected by laparotomy (Table 1).

### 3.2. Effects of tumor

The presence of tumor significantly increased muscular glutamine output and intestinal glutamine uptake and citrulline output (Fig. 1 and Table 1). The percentage of muscular glutamine output that was taken up by intestine was 76% ± 15%, similar to controls. In tumor-bearing mice, the liver took up citrulline from the circulation (Fig. 1). In relation, less citrulline was taken up by the kidneys; and renal arginine output was lower (Fig. 1). Net hepatic protein breakdown (Table 1) was reduced in tumor-bearing mice.

### 3.3. Postoperative response in the presence of tumor

In contrast with healthy mice, postoperative glutamine output from muscle was not maintained but decreased in the presence of tumor (Fig. 1). Glutamine uptake by the gut was maintained postoperatively (Fig. 1). The percentage of muscular glutamine output that contributed to intestinal glutamine uptake increased from 76% ± 16% to 93% ± 16% in the postoperative period. However, intestinal citrulline and glutamate output decreased; and hepatic citrulline uptake returned to zero (Fig. 1 and Table 1). In the kidneys of tumor-bearing mice, postoperative citrulline uptake increased; but arginine output remained unchanged (Fig. 1). Again, renal citrulline uptake exceeded intestinal citrulline output (Fig. 1). Postoperative net protein breakdown in muscle, intestine, liver, and kidney was not affected compared with healthy mice undergoing surgery (Table 1).

## 4. Discussion

The present study was performed to improve our understanding of the role of endogenous arginine synthesis in cancer and after surgical trauma. We observed that tumor-bearing mice up-regulated muscle glutamine output, intestinal glutamine uptake, and citrulline output but decreased renal citrulline uptake and arginine output. This coincided with the presence of hepatic citrulline uptake. When tumor-bearing mice underwent surgery, up-regulation of these pathways in muscle and intestine could not be maintained; and intestinal citrulline output decreased. Although citrulline was no longer taken up by the liver after surgery and renal citrulline uptake increased, these did not result in elevated renal arginine output. Thus, the de novo arginine production pathway was compromised in the presence of tumor, irrespective of the metabolic stress of surgery.

### 4.1. Effects of tumor

The presence of tumor increased muscular glutamine output with concomitant increased glutamate uptake, suggesting that more glutamate was converted to glutamine. Because this was not accompanied by increased phenylalanine output, increased protein breakdown was probably not a source for the enhanced glutamine output. Besides, glutamine output increased more than could be explained by the increased uptake of its precursors branched-chain amino acids and glutamate alone. Therefore, output from the intracellular glutamine pool was probably also increased in tumor-bearing mice. In a previous study in tumor-bearing rats, we observed a similar increase in glutamine production by muscle that was

accompanied by decreased intracellular glutamine concentrations [18], suggesting that membrane transport rates were increased. Thus, the presence of tumor probably up-regulated muscular amino acid metabolism.

At the same time, the gut took up and converted more glutamine, reflected by increased intestinal citrulline, glutamate (not significant), and alanine output. We calculated that intestinal glutamine uptake accounted for 76% of the glutamine output from muscle. Because glutamine is the most important fuel for intestinal cells, it indicates that intestinal metabolism was increased in the presence of a tumor.

Remarkably, we observed net hepatic citrulline uptake in tumor-bearing mice, extracting more than half of the citrulline released by the gut from the circulation. From previous flux studies, it was known that in healthy conditions there is only a small net uptake of citrulline by the liver in rats [19,20] and dogs [21]. Recently, our group described hepatic citrulline uptake in patients during gastrointestinal surgery [22,23]. The physiological significance of these observations remains to be elucidated.

In contrast to up-regulated intestinal conversion of glutamine to citrulline, renal uptake of citrulline and arginine output were decreased in tumor-bearing mice. Similarly to our results in endotoxemic mice [6], renal protein metabolism assessed by the flux of branched-chain amino acids across the kidney was unaffected, suggesting that decreased renal protein metabolism was not the source of decreased arginine output. Thus, the presence of tumor led to up-regulation of intestinal citrulline output, whereas renal citrulline uptake was down-regulated. Concomitantly, the liver took up citrulline in tumor-bearing mice.

### 4.2. Effects of surgical trauma

Control mice undergoing surgery without tumor maintained muscle glutamine output, intestinal glutamine uptake and citrulline output, and renal citrulline uptake and arginine output. In contrast, in tumor-bearing mice, laparotomy led to a reduction in glutamine output in muscle to levels similar to preoperative controls. This is in line with previous experiments in tumor-bearing rats undergoing surgery [24]. It was not related to reduced uptake of its precursors glutamate and branched-chain amino acids or net protein breakdown in muscle, suggesting that release from the intracellular glutamine pool was decreased in muscle.

Despite the postoperative decrease in muscular glutamine output, tumor-bearing mice maintained intestinal glutamine uptake after surgery. In fact, the contribution of muscular glutamine output to intestinal glutamine uptake increased from 76% to 93%. Therefore, it appears that the intestine of tumor-bearing mice was more dependent on muscular glutamine output after surgery. However, intestinal citrulline output was not maintained. Other products of intestinal glutamine metabolism such as glutamate and alanine were also released in smaller quantities. Apparently, enterocytes used more glutamine as fuel in the postoperative condition, leaving less to be converted to citrulline, alanine, and glutamate. Additional studies with specific tracers for these amino acids are necessary to confirm this assumption.

Strikingly, although intestinal citrulline output decreased, renal citrulline uptake increased in tumor-bearing mice undergoing laparotomy, illustrating that there was no direct relation between intestinal citrulline output and renal citrulline uptake in this situation. This is in keeping with another mice study showing no relation between intestinal citrulline production and renal citrulline uptake [25]. From the present data, it can be calculated that about half of renal citrulline uptake originated from extraintestinal sources. Besides intestine, both muscle and liver [19] have shown the capacity to synthesize citrulline. However, in our study, neither muscle nor liver released net quantities of citrulline, suggesting the presence of other sources of citrulline, presumably in cells of the immune system [26]. Despite the postoperative increase in renal citrulline uptake, renal arginine output into the circulation did not increase after surgery in tumor-bearing mice, which is in contrast with a study in rats with short bowel resections where a relation was present between renal citrulline uptake and renal arginine output [5]. Speculating on this discrepancy between renal citrulline uptake and arginine output, urinary excretion of arginine is unlikely because the fractional excretion of arginine in rats has been reported to be as small as 0.0003 [27]. Urinary excretion of arginine can thus be calculated to be about 0.007 nmol/(10 g min) and is too small to explain the 5-nmol/(10 g min) difference between renal citrulline uptake and arginine output. In addition, nitric oxide production is also probably too small to account for the difference. In a previous study from our group, renal nitric oxide production in control mice was only 1.9 nmol/(10 g min). Therefore, arginine probably has disappeared intracellularly into other pathways or was converted (eg, to urea, creatine, proline, or agmatine) and subsequently released either back to plasma or into urine. However, there was no compensatory efflux of amino acids other than arginine from the kidney.

A disadvantage of this study design is that it takes invasive measurements to study organ fluxes. Strictly taken, the experimental setup may be seen as a double-hit model: the first hit being laparotomy and the second hit being the experimental conditions of anesthesia and placement of catheters for arterial-venous fluxes. How this has affected the results remains speculative, but roughly 2 options are plausible: (1) the second hit has augmented the effects caused by the first hit because of greater metabolic stress, and (2) the second hit has attenuated the effects of the first hit because of exhaustion of endogenous amino acid sources.

In summary, in tumor-bearing mice, intestinal conversion of glutamine to citrulline increased, whereas renal citrulline uptake decreased in relation with increased hepatic citrulline uptake. Concomitantly, renal arginine output was reduced to

values approaching zero. When undergoing surgery, renal arginine output in tumor-bearing mice remained close to zero. At the same time, intestinal citrulline output decreased, despite increased renal citrulline uptake. Thus, our data demonstrate that the de novo arginine production pathway can be regulated at various organ sites depending on the pathophysiological situation. In all tumor-bearing groups, de novo arginine output by the kidney was reduced. In the preoperative situation, this occurred together with hepatic citrulline uptake; in the postoperative period, this coincided with decreased citrulline output by the gut.

It seems that renal arginine output was quite independent of gut citrulline production, independent of liver citrulline uptake, and independent of renal citrulline uptake. We recently published arterial plasma amino acid concentrations of these mice showing that citrulline concentrations were not different between the groups [28], thus suggesting that renal arginine output is also independent of circulating citrulline. This is in contrast with a study in rats where supplementation of glutamine increased arterial concentrations of citrulline and arginine together with increased renal citrulline uptake and renal arginine production [29]. On the other hand, in keeping with the present findings, a recent mice study showed no relation between intestinal citrulline production and renal citrulline uptake or renal arginine output [25]. Apparently, the interorgan relation between citrulline and arginine is more complicated than a mere substrate-driven one. In conclusion, in all tumor-bearing mice, de novo arginine output was decreased compared with control mice, suggesting a role for altered arginine metabolism in the tumor-bearing host.

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