

# Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism<sup>1-3</sup>

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## ABSTRACT

**Background:** The liver plays a central role in amino acid metabolism. However, because of limited accessibility of the portal vein, human data on this subject are scarce.

**Objective:** We studied hepatic amino acid metabolism in noncirrhotic fasting patients undergoing liver surgery.

**Design:** Twenty patients undergoing hepatectomy for colorectal metastases in a normal liver were studied. Before resection, blood was sampled from a radial artery, portal vein, hepatic vein, and renal vein. Organ blood flow was measured by duplex ultrasound scan.

**Results:** The intestine consumed glutamine and released citrulline. Citrulline was taken up by the kidney. This was accompanied by renal arginine release, which supports the view that glutamine is a precursor for arginine synthesis through an intestinal-renal pathway. The liver was found to extract citrulline from this pathway at a rate that was dependent on intestinal citrulline release ( $P < 0.0001$ ) and hepatic citrulline influx ( $P = 0.03$ ). Fractional hepatic extractions of citrulline (8.4%) and arginine (11.5%) were not significantly different. Eighty-eight percent of arginine reaching the liver passed it unchanged. Splanchnic citrulline release could account for one-third of renal citrulline uptake.

**Conclusions:** This is the first study of hepatic and interorgan amino acid metabolism in humans with a normal liver. The data indicate that glutamine is a precursor of ornithine, which can be converted to citrulline by the intestine; citrulline is transformed in the kidneys to arginine. Hepatic citrulline uptake limits the amount of gut-derived citrulline reaching the kidney. These findings may have implications for interventions aimed at increasing systemic arginine concentrations. *Am J Clin Nutr* 2007;85:167-72.

**KEY WORDS** Glutamine, citrulline, arginine, gut, liver, kidney, interorgan amino acid exchange, humans

## INTRODUCTION

The liver plays a central role in amino acid metabolism. Because of its anatomical relation to the gut, the liver receives compounds absorbed or released by the gut through the portal vein before they gain access to the systemic circulation. Most data about hepatic amino acid metabolism originate from animal research, and, although many similarities exist between these studies, inconsistencies between studies suggest that important qualitative and quantitative differences also exist among species (1-4). The *in vivo* study of hepatic amino acid uptake and release requires blood sampling from among others the portal vein which

is difficult to access in humans. As a consequence human studies on the role of the liver in this context are scarce. In fact, the available human data come from studies in patients with liver disease and a transjugular intrahepatic portosystemic shunt (4, 5), through which the portal vein can be accessed by a percutaneous transluminal approach. To our knowledge no *in vivo* data about hepatic amino acid metabolism in humans with a normal liver exist. In patients undergoing surgery for colorectal liver metastases, the function and structure of the nontumorous parenchyma is generally preserved. In addition, the hepatic veins, portal vein, and renal veins are relatively easily accessible for blood sampling. We studied interorgan amino acid exchange among the gut, liver, and kidneys in patients without portosystemic shunting, cirrhosis, or both undergoing liver surgery. On the basis of our current research interest, special attention was paid to the metabolism of glutamine, citrulline, and arginine (6-8). Arginine is a pluripotent amino acid (9), and supplementation may prove beneficial in certain conditions (8). It is generally assumed that arginine synthesis is the endpoint of an interorgan pathway that involves the conversion of glutamine to glutamate, ornithine, and citrulline by the intestine and the conversion of citrulline to arginine by the kidneys (6, 7, 10-14). The relation among glutamine, citrulline, and arginine provides a basis for the theory that the established clinical effects of glutamine (15) may partly rely on its role as a precursor of arginine.

The effectiveness of enteral arginine supplementation has been questioned, because the high hepatic activity of the enzyme arginase (EC 3.5.3.1), which breaks down arginine to urea, is thought to prohibit the release of enteral-administered arginine to the systemic circulation (16, 17). In contrast, citrulline is believed to pass the liver without significant uptake (7, 10, 12, 16).

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**TABLE 1**  
Demographic characteristics of the subjects<sup>1</sup>

Variable	Value (n = 15 M, 5 F)
Age (y)	65 (37–79)
Body mass (kg)	78 (61–96)
Height (cm)	173 (153–192)
Bilirubin (μmol/L)	8 (5–13)
Aspartate aminotransferase (IU/L)	21 (16–53)
Prothrombin time (s)	1 (1–1.2)
Creatinine (μmol/L)	88 (72–99)
Urea (mmol/L)	5.3 (3.1–6.1)
Albumin (g/L)	39 (31–45)
C-reactive protein (mg/L)	4 (1–35)

<sup>1</sup> All values are median; range in parentheses.

In this light it has been suggested that enteral citrulline as a precursor for renal arginine synthesis may be more effective than enteral arginine to raise systemic arginine concentrations, without inducing hepatic nitrogen loss (12, 18). Insight into the interorgan conversion of glutamine to citrulline and of citrulline to arginine in humans is needed to clarify normal human physiology. This knowledge may be applied to optimize dietary supplementation of these amino acids.

## SUBJECTS AND METHODS

### Patients

The study population consisted of 20 patients undergoing partial liver resection because of colorectal metastases. No patients were jaundiced, and none of them had been on chemotherapy, radiotherapy, or medical treatment for their tumors in the 4 wk preceding the operation. Patients were excluded if the tumor volume exceeded 10% of total liver volume. Preoperatively, serum aspartate aminotransferase, creatinine, glucose, and albumin were measured routinely by the local clinical chemistry laboratory. Patients consumed their standard oral nutrition in the prestudy period. Oral intake was ceased at 2000 on the day before surgery, and all patients were transported to the operating theater at ≈0800. Patient characteristics are summarized in **Table 1**. Permission for the study was granted by the Medical Ethical Committee of the VU University Medical Center. Written informed consent was obtained from each participant.

### Anesthesia

Anesthesia was applied according to institutional routines with the use of isoflurane and propofol. All patients had a thoracic epidural catheter inserted for preoperative and postoperative analgesia and indwelling catheters in a jugular vein and a radial artery for monitoring arterial and central venous blood pressure. Urine output was monitored by transurethral catheterization. Body temperature was kept constant with the use of a Bair Hugger system (Arizant Healthcare Inc, Eden Prairie, MN).

### Surgical procedure and blood sampling

Surgical procedures were conducted as described previously (19) and were commenced with a bilateral subcostal incision. After dissection of the teres hepatis ligament, the procedure was continued with dissection of the falciform ligament and further

mobilization of the liver as appropriate to deal with the surgical disorder followed by intraoperative ultrasonography (IOUS). Immediately after IOUS, blood was sampled from the right renal vein, the portal vein, and one of the hepatic veins by direct puncture. Simultaneously, arterial blood was drawn from the radial artery catheter.

### Duplex flow measurement

During IOUS organ blood flow was measured by means of color Doppler ultrasound scanning (Aloka Prosound SSD 5000; Aloka Co, Ltd, Tokyo, Japan). Time-averaged mean velocities of the bloodstream and cross-sectional area of the portal vein, hepatic artery, and right renal vein were measured as described before (20). The renal vein, portal vein, and hepatic artery were traced with the use of a sterile transducer (5–10 MHz linear UST-579T-7,5; Aloka). For accurate measurements, care was taken to keep the angle between the ultrasonic beam direction and blood flow direction below 60°. The cross-sectional area of the vessel was calculated by drawing an area ellipse at the same point as where the velocity was measured. Portal venous and hepatic arterial blood flows were measured proximal to their hilar bifurcations. In case of an accessory hepatic artery, both arteries were assessed.

### Blood processing and laboratory analysis

Blood samples were transferred to prechilled heparin-containing blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). A glass capillary was filled with arterial whole blood and centrifuged at high speed (10 000 × g) for 5 min at room temperature to determine hematocrit with the use of a hematocrit reader. The remainder of the heparinized blood was centrifuged at 4 °C at 4000 × g for 10 min to separate plasma. Plasma was processed and amino acids were measured with the use of HPLC as described previously (21).

### Calculations for plasma flow

Blood flows in the portal vein, renal vein, and hepatic artery were calculated by multiplying the time-averaged velocity of the bloodstream with the cross-sectional area of the vessel. Plasma flows were calculated from measured blood flow and hematocrit [plasma flow = blood flow × (1 – hematocrit)]. Splanchnic (hepatic) plasma flow was calculated as portal plasma flow plus hepatic arterial plasma flow. Total renal plasma flow was estimated by multiplying right renal plasma flow with a factor of 2.

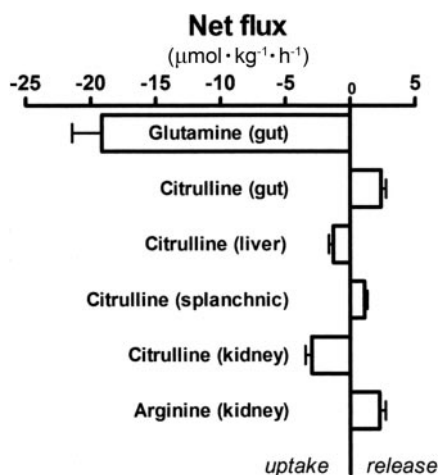
### Calculations for amino acid flux

Organ amino acid fluxes were calculated as organ plasma flow multiplied by the product of subtracting arterial plasma amino acid concentration from venous plasma amino acid concentration. Portal, renal, and hepatic venous plasma concentrations of amino acid were used to calculate fluxes across the intestine, kidneys, and splanchnic area (comprising portal-drained viscera and the liver), respectively. Hepatic amino acid fluxes were calculated by subtracting intestinal amino acid flux from corresponding splanchnic amino acid fluxes. Positive fluxes indicate net amino acid release; negative fluxes indicate net uptake.

### Calculations for amino acid influx and fractional extractions

Amino acid influx, the rate at which an amino acid is supplied to an organ by the circulation, was calculated by multiplying





**FIGURE 1.** Mean ( $\pm$ SEM) fluxes through the interorgan pathway of arginine synthesis postabsorptively in humans undergoing surgery ( $n = 20$ ). All fluxes are significantly different from zero ( $P < 0.05$ , one sample  $t$  test). Glutamine is taken up by the intestine. Intestinal citrulline release accounts for 12% of intestinal glutamine uptake. The liver consumes citrulline in quantities equaling 55% of intestinal citrulline release. Renal citrulline uptake exceeds splanchnic citrulline release 3-fold and approximates renal arginine release.

arterial plasma amino acid concentrations with portal or renal plasma flow (intestinal and renal influx, respectively). Hepatic amino acid influx was calculated as (portal plasma flow  $\times$  portal amino acid concentration) + (hepatic arterial plasma flow  $\times$  arterial amino acid concentration). In cases of negative amino acid flux (net amino acid uptake), fractional amino acid extractions were calculated from net amino acid uptake (net uptake = negative net flux  $\times -1$ ) and amino acid influx as described by Remesy et al (1): (fractional extraction = amino acid uptake/net influx  $\times 100\%$ ). This calculation enables the expression of organ-specific uptake of an amino acid in relation to its influx and represents the percentage of amino acid influx that is actually taken up from the bloodstream.

## Statistics

Data are presented as means  $\pm$  SEMs unless stated otherwise. Differences in arteriovenous concentrations were tested against zero with the use of a one-sample  $t$  test with a theoretical mean of zero. Correlations were calculated with the use of Pearson's test. A  $P$  value  $< 0.05$  was used to indicate statistical significance.

Statistical calculations were performed with the use of PRISM 4.0 for WINDOWS (GraphPad Software Inc, San Diego, CA).

## RESULTS

### Plasma flow

Mean plasma flows in the portal vein and hepatic artery were found to be  $320 \pm 42$  and  $110 \pm 23$  mL/min, respectively. Hepatosplanchnic plasma flow amounted to  $430 \pm 47$  mL/min. Renal plasma flow (2 kidneys) equaled  $606 \pm 112$  mL/min. These values are in accordance with our previous data (20).

### Amino acid flux

#### Intestinal glutamine metabolism

Glutamine was taken up by the gut (**Figure 1**) with a fractional extraction of  $12.0 \pm 1.5\%$ . No correlation was observed between arterial glutamine concentrations and intestinal glutamine uptake ( $r^2 = 0.005$ ). Citrulline was produced by the intestine, and intestinal citrulline release accounted for  $\approx 13\%$  of intestinal glutamine metabolism (Figure 1). Citrulline release did not correlate with glutamine uptake ( $r^2 = 0.013$ ).

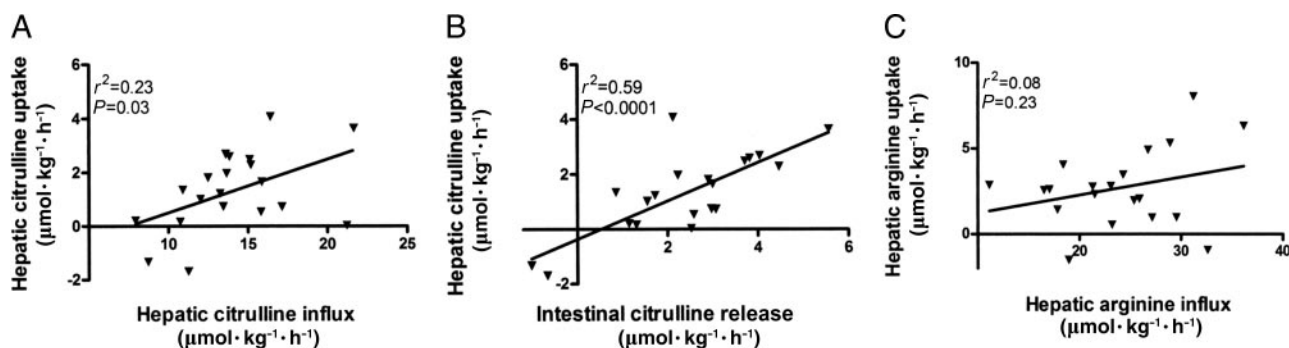
#### Citrulline exchange between the intestine and the liver

Surprisingly, the liver was found to take up citrulline (Figure 1). Hepatic citrulline uptake appeared to depend on hepatic citrulline influx ( $r^2 = 0.23$ ) (**Figure 2A**) and correlated strongly with intestinal citrulline release ( $r^2 = 0.59$ ) (Figure 2B). As a consequence of hepatic citrulline uptake, net splanchnic citrulline release was considerably less than intestinal citrulline release and equaled only  $1.1 \pm 0.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  (45% of intestinal citrulline release) (Figure 1).

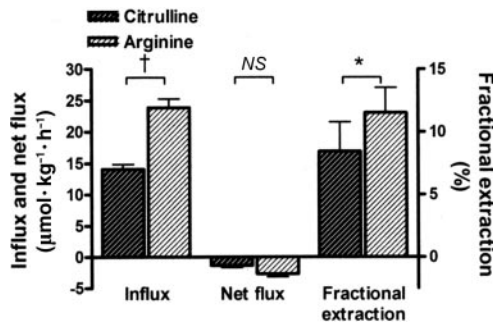
#### Arginine exchange between the intestine and the liver

Intestinal arginine flux was not significantly different from zero, whereas the splanchnic area as a whole (and hence the liver) significantly removed arginine from the circulation. Hepatic arginine uptake did not correlate with arginine influx ( $r^2 = 0.08$ ) (Figure 2C).

Data on absolute and fractional uptake of citrulline and arginine by the liver are presented in **Figure 3**. The influx of arginine to the liver ( $23.9 \pm 1.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) exceeded the influx of citrulline ( $14.0 \pm 0.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). In addition, the absolute



**FIGURE 2.** Correlation between hepatic influx and hepatic uptake of citrulline and arginine postabsorptively in humans undergoing surgery ( $n = 20$ ). There was a significant correlation between hepatic citrulline uptake and hepatic citrulline influx (A) and between hepatic citrulline uptake and intestinal citrulline release (B), which suggests that hepatic citrulline uptake depends on its influx. A similar correlation between hepatic arginine uptake and arginine influx is lacking (C; Pearson's test).



**FIGURE 3.** Mean ( $\pm$ SEM) hepatic influx (portal + arterial), net flux, and fractional extraction of citrulline and arginine postabsorptively in humans ( $n = 20$ ) undergoing surgery. †\*Significant difference between citrulline and arginine (paired  $t$  test):  $^{\dagger}P < 0.0001$ ,  $^{*}P = 0.026$ . NS ( $P = 0.349$ ).

uptake of arginine by the liver ( $2.7 \pm 0.5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) was significantly higher than the absolute uptake of citrulline ( $1.3 \pm 0.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). From influx and absolute uptake, the fractional hepatic extractions of arginine and citrulline were calculated ( $11.5 \pm 2.0\%$  and  $8.4 \pm 2.3\%$ , respectively). These values were not significantly different.

#### Renal citrulline and arginine metabolism

Renal citrulline uptake correlated significantly with arterial citrulline concentrations ( $r^2 = 0.63$ ,  $P < 0.0001$ ; fractional extraction:  $23.9 \pm 3.3\%$ ). Renal citrulline uptake was not significantly different from intestinal citrulline release ( $P = 0.36$ ), but because of hepatic citrulline extraction, which reduced net splanchnic citrulline release, renal citrulline uptake ( $3.0 \pm 0.5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) exceeded splanchnic citrulline release ( $1.1 \pm 0.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) almost 3-fold ( $P = 0.008$ , renal citrulline uptake compared with splanchnic citrulline release). A significant correlation ( $r^2 = 0.26$ ,  $P = 0.026$ ) was observed between renal citrulline uptake and arginine release. The rates of renal citrulline uptake and arginine release were not significantly different. Fluxes of amino acids through the intestinal-hepatic-renal pathway of glutamine-citrulline-arginine conversion are graphically summarized in Figure 1. Intestinal, hepatic, and renal amino acid fluxes are summarized in **Table 2**.

**TABLE 2**

Net amino acid flux across the intestine, liver, splanchnic area, and kidneys postabsorptively in humans undergoing surgery ( $n = 20$ )<sup>1</sup>

Amino acid	Intestine	Liver	Splanchnic area		Kidneys
			$\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$		
Glutamine	$-19.1 \pm 2.3^2$	$7.9 \pm 3.3^2$	$-11.2 \pm 2.9^2$	$-13.5 \pm 3.5^2$	
Citrulline	$2.4 \pm 0.4^2$	$-1.3 \pm 0.3^2$	$1.1 \pm 0.2^2$	$-3.0 \pm 0.5^2$	
Arginine	$0.1 \pm 0.2$	$-2.7 \pm 0.5^2$	$-2.6 \pm 0.5^2$	$2.2 \pm 0.5^2$	
Ornithine	$0.0 \pm 0.3$	$0.8 \pm 0.3^2$	$0.8 \pm 0.3^2$	$0.9 \pm 0.4^2$	
Alanine	$5.8 \pm 1.1^2$	$-28.2 \pm 3.1^2$	$-22.4 \pm 2.8^2$	$7.2 \pm 1.6^2$	
EAA	$-3.0 \pm 2.5$	$-7.9 \pm 3.1^2$	$-10.8 \pm 2.7^2$	$2.8 \pm 2.3$	
BCAA	$-1.3 \pm 1.0$	$-1.6 \pm 1.3$	$-2.7 \pm 1.0^2$	$1.1 \pm 0.7$	
AAA	$-0.8 \pm 0.5$	$-2.0 \pm 0.7^2$	$-2.8 \pm 0.6^2$	$0.8 \pm 0.8$	
$\alpha\text{AN}$	$-13.7 \pm 7.3$	$-19.5 \pm 9.1^2$	$-33.1 \pm 8.4^2$	$7.1 \pm 8.1$	

<sup>1</sup> EAA, essential amino acids; NEAA, nonessential amino acids;  $\alpha\text{AN}$ ,  $\alpha$  amino nitrogen, sum of amino acids; BCAA, branched-chain amino acids; AAA, aromatic amino acids.

<sup>2</sup> Significantly different from zero,  $P < 0.05$  (one-sample  $t$  test).

## DISCUSSION

This is the first study about amino acid exchange among the intestine, the liver, and the kidney in humans without parenchymal liver disease. We found intestinal uptake of glutamine which is a confirmation of previous data obtained in humans without cirrhosis (4, 22). Intestinal uptake of glutamine was accompanied by citrulline release. The splanchnic area released citrulline, and citrulline was taken up by the kidneys. Renal citrulline uptake in turn was followed by arginine release. Thus, the present study confirms in humans the existence of the glutamine-citrulline-arginine pathway in vivo. In addition it shows some new findings that are potentially important for everyday clinical practice.

Intestinal glutamine uptake accounted for  $\approx 6\%$  of whole-body plasma appearance of glutamine (23). In line with the aforementioned human studies we found no correlation between arterial glutamine concentrations and glutamine uptake by the portal-drained viscera (4, 22), although there probably is a relation between glutamine influx and glutamine uptake in the ileum (22). The intestine released citrulline, an important product of glutamine metabolism. Citrulline is not incorporated into protein but is converted to arginine (12). This process occurs in several cell types (24) but generally serves intracellular nitric oxide production rather than systemic arginine release (24). The kidneys are seen as the only organs that significantly release newly synthesized arginine into the plasma (7), reflected by the renal citrulline uptake and arginine release found in the present study.

Interestingly, the liver consumed citrulline. Moreover, the rate at which citrulline was released from the splanchnic area to the systemic circulation was considerably smaller than renal citrulline uptake. This suggests that in fasted humans, other processes than intestinal glutamine metabolism provide citrulline for renal arginine synthesis. Apart from its intestinal formation from glutamine, citrulline can be formed from arginine by the activity of nitric oxide synthase (NOS; EC 1.14.13.39). Stable isotope studies show that NOS activity accounts for the release of citrulline into the systemic circulation at a rate of  $1 \mu\text{mol}/(\text{h} \cdot \text{kg})$  (25), which apparently equals splanchnic citrulline release. Thus, systemic NOS activity may be equally important for systemic citrulline release as the intestine.


Renal synthesis and systemic concentrations of arginine can be enhanced by administration of citrulline or ornithine as a precursor of arginine (18) but probably also by (enteral) administration of glutamine as a precursor of citrulline. Increasing plasma arginine concentrations may improve important physiologic processes, including organ perfusion, immune function, protein synthesis, and wound healing (9). It has been suggested that enteral supplementation of arginine is ineffective because the liver may act as an arginine trap as a result of high arginase activity (16). Against the background of the assumption that citrulline passes the liver without significant uptake (10, 16, 18), it has been proposed that enteral administration of citrulline, contrary to the administration of arginine, enhances systemic arginine concentrations without inducing hepatic nitrogen loss (16, 18). The present study confirms that the liver takes up arginine, but more importantly it shows that citrulline is also extracted by the liver. Consequently, hepatic citrulline extraction may reduce the appearance of enteral-delivered citrulline in the systemic circulation. Fractional hepatic extractions of citrulline (8.4%) and arginine (11.5%) were not significantly different,

although fractional citrulline extraction tended to be somewhat lower.

Hepatic arginine uptake has been ascribed to the activity of arginase, the final enzyme of the urea cycle. However, there is little exchange between extracellular (and even cytosolic) amino acids and amino acids that are formed and broken down within the urea cycle (26, 27), suggesting that plasma arginine does not gain access to hepatic arginase. The absence of a relation between hepatic arginine influx and uptake also suggests that hepatic arginine uptake is an orchestrated process that is not limited by substrate supply but rather by substrate demand. This supports the view that hepatic arginine uptake does not simply result from arginine capture by arginase, but rather serves specific purposes (eg, protein synthesis).

The available literature on hepatic citrulline metabolism is scarce. The only study that specifically concerned hepatic citrulline metabolism was performed in isolated, perfused rat livers and showed that <10% of radioactive-labeled citrulline was removed from the perfusate after  $\approx$ 40 passes (10). In vivo hepatic citrulline uptake has been observed before in rats (1) but not in mice (2) and pigs (3). In a recent study from our group (4) hepatic citrulline uptake was observed in metabolically stable patients with liver cirrhosis. Because hepatic citrulline metabolism is a virtually unexplored area, its physiologic importance is unclear. The only known fate of citrulline is its conversion to arginine by the subsequent actions of the enzymes argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) (12, 24). It, therefore, seems reasonable to assume that hepatic citrulline uptake serves de novo synthesis of arginine which may enhance arginine availability for intracellular processes such as nitric oxide synthesis. In fact, this hypothesis was formulated previously by Pastor et al (26), but they qualified it as unlikely "because citrulline is poorly taken up by hepatocytes." Judging from the present results this is not the case. All patients were studied in the fasted state, which hampers direct translation of the present findings to situations of enteral supplementation of glutamine, citrulline, or arginine or ad libitum eating. In the first place enterally administered amino acids are subject to first-pass intestinal metabolism that modifies the amino acid composition of a feed before it is released into the portal vein (28). Intestinal arginine extraction depends on the amount of protein in the habitual diet (7, 16, 17). In subjects ingesting sufficient amounts of protein, 60–100% of enteral-administered arginine is released into the portal vein unchanged. It has been suggested that, only after prolonged protein deprivation, enzymes are expressed in the gut that convert arginine to citrulline on absorption (29, 30). Effectively this means that during enteral arginine administration to nondepleted subjects or during ad libitum eating, the gut becomes an arginine-releasing organ. Human data on intestinal citrulline extraction are lacking, but a recent mouse experiment applying stable isotopes showed that, despite the intestine being net producers of citrulline, they also take up a considerable amount of citrulline from the circulation (46% of intestinal influx) (31). This finding suggests that the gut can take up and metabolize citrulline, but it does not necessarily mean that intestinal substrate utilization is similar if supplied by the gut lumen or by the circulation. In addition, feeding stimulates hepatic protein synthesis and hence hepatic amino acid uptake, but, because this is accompanied by an increased portal amino acid flux, it remains to be seen whether the fractional hepatic amino acid

extraction is different in the fed state compared with the fasted state.

In conclusion, this is the first study of the role of the liver in interorgan amino acid metabolism in humans with a structurally normal liver that did not involve portosystemic shunting. The data support the existence of an interorgan pathway of arginine synthesis involving intestinal glutamine to citrulline conversion and renal citrulline to arginine conversion. A substantial proportion of renal citrulline uptake is derived from other than splanchnic processes, presumably systemic NOS activity. The liver takes up citrulline, which limits the release of gut-derived citrulline to the systemic circulation. This may impair the efficacy of enteral citrulline supplementation. 

MCGvdP participated in the data collection, analyzed the data, and wrote the manuscript. MPCS, GCM, and PGB participated in the data collection and critically reviewed the manuscript. PAMvL participated in the study design and critically reviewed the manuscript. PBS advised on data interpretation and critically reviewed the manuscript. NEPD participated in the study design, advised on data interpretation, and critically reviewed the manuscript. CHCD participated in the study design, advised on data interpretation, and critically reviewed and revised the manuscript. None of the authors had a potential financial or other personal interest from the presented results.

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