Summary, general conclusion and future perspectives

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In this chapter the results of the studies performed within the scope of this thesis are summarized and discussed, and a direction for future studies is given. Additional attention is paid to arginine, because all investigations within this thesis focus on the metabolic relationship between glutamine and arginine, which is assumed to be responsible for at least part of the beneficial effects of glutamine.

Introduction glutamine (Chapter 2)

The development of long-term total parenteral nutrition in the sixties of the past century may have contributed to the accumulation of publications about glutamine in the years after 1960 (number of Pubmed citations on glutamine before 1960: 261 (0.02% of all Pubmed citations) and after 1960: 27835 (0.17% of all Pubmed citations), because glutamine was not included in this intravenous nutrition due its instability in aqueous solutions. Since glutamine is a non-essential amino acid, it was assumed that no harm was done. However, time and research proved otherwise. As described in detail in chapter 2, glutamine has been established to be an important respiratory fuel and nucleotide substrate for the enterocyte and the (gut associated) immune system. Furthermore, recent discoveries on the mechanisms behind the beneficial effects of glutamine emphasize its role as a signalling molecule. For example, glutamine has been shown to support the immune system and blunt the inflammatory response at the same time, to preserve insulin sensitivity and to help organs withstand or recover from oxidative stress, by inducing the expression of protective heat shock proteins and by enhancing the availability of antioxidant substrates such as glutathione and taurine. Also, glutamine was shown to regulate the nitric oxide synthesis from arginine in endothelial cells, and to be an important precursor for the de novo synthesis of arginine in humans (this thesis). From a clinical perspective, based on an ongoing meta-analysis and up-to-date clinical trials, it is safe to state that supporting the parenteral fed, critically ill patient with ≥ 0.2 g glutamine · kg⁻¹ · day⁻¹ is beneficial because it improves survival. The benefits of enteral supplementation of glutamine seem established in trauma and burn patients. Currently, a large trial is ongoing involving 1200 critically ill patients receiving a combination of intravenous (0.35 g · kg⁻¹ · day⁻¹) and enteral glutamine (30 g/dag) (1).

Arginine

As described in the rationale, glutamine is believed to exert at least part of its beneficial effects by being an indirect precursor for the synthesis of arginine. Arginine is a versatile
amino acid which has received a great deal of attention over the past decades in critical care medicine, especially when arginine was shown to be the precursor of nitric oxide (NO). NO plays an important role in vasodilation, but also in the immune response, neurotransmission and adhesion of platelets and leucocytes (2;3). A meta-analysis of clinical trials showed that an enteral diet enriched with a combination of nutrients including arginine was associated with fewer infectious complications in elective surgical patients, but suggested that this kind of immunonutrition might be detrimental for critically ill patients (4). Therefore, authors recommended against routine administration of arginine-enriched nutrition in critically ill (4;5). However, others argue that the critical ill condition ‘sepsis’ may be an arginine-deficiency state. Arginine availibility is reduced in the septic state due to increased arginine catabolism and diminished endogenous arginine synthesis, which limits the arginine availability for NO production. Although increased NO production by inducible nitric oxide synthase (iNOS) in sepsis is linked to systemic hypotension, many characteristics of sepsis can be linked to locally diminished NO availibilty. For example, impaired NO synthesis by endothelial NOS (eNOS) may be related to loss of ability to autoregulate the microcirculation. Whereas iNOS is up-regulated, eNOS was shown to be down-regulated by inflammation. In-vitro studies show that NO production by eNOS can be stimulated by exogenous arginine when arginine stores were depleted, thereby improving the microcirculation (6;7).

**Glutamine and arginine & Focus of this thesis**

As described in the rationale of this thesis, it is speculated that glutamine exerts part of its beneficial effects by being a precursor for the synthesis of arginine. The studies described in this thesis investigate the metabolic pathway of glutamine into citrulline and arginine in mice and humans, in an attempt to lift a tip of the veil of this metabolic relationship between glutamine and arginine. The dipeptide alanyl-glutamine was included in our studies, because it is stable in aqueous solutions and already used as a therapeutic alternative for glutamine. Results of the studies presented in this thesis will now be discussed chronologically.

**Chapter 3**

Results show that administration of alanyl-glutamine by the intravenous or enteral route to patients enhanced the plasma concentration of glutamine with both routes of administration, but most when administered by the intravenous route. This observation shows that the gut as well as the rest of the human body will profit from the enteral supply of alanyl-glutamine. The more pronounced splanchnic metabolism of glutamine with enteral compared to intravenous administration of alanyl-glutamine was also illustrated by the absence of plasma alanyl-glutamine and the more pronounced increase in the
plasma concentrations of glutamate and citrulline with enteral administration of the dipeptide. Interestingly, intravenous administration of alanyl-glutamine enhanced the plasma concentrations of arginine, which was not the case with enteral administration of the dipeptide, despite the higher plasma availability of citrulline. The results of this study encouraged us to investigate the pathway of glutamine into citrulline and arginine with the help of stable isotopes, first in mice and subsequently in humans.

Chapter 4

One series of mice received free [2-15N]glutamine and the other series received the dipeptide alanyl-[2-15N]glutamine, by the intravenous or enteral feeding route. Results show that glutamine serves as substrate for \textit{de novo} citrulline and arginine synthesis in mice. However, when (alanyl-)

Chapter 5

The contribution of glutamine to the synthesis of arginine was established in 8 surgical patients under post absorptive conditions. The results from this study suggest that approximately 83% of the plasma citrulline turnover comes from plasma glutamine and that 76% of the plasma citrulline turnover is used for the \textit{de novo} synthesis of plasma arginine. Therefore, 64% of \textit{de novo} produced arginine was calculated to be derived from citrulline coming from glutamine. The 8 patients included in our study underwent major abdominal surgery, which facilitated access to the portal, hepatic and renal veins. Therefore, we were able to assess the turnover of glutamine, citrulline and arginine at intestinal, hepatic and renal level.

It was shown that the kidneys are indeed an important site for the uptake of citrulline and release of arginine. More than 50% of citrulline appearing in plasma was observed to be taken up by the kidneys, resulting in equimolar release of arginine in plasma. The calculated renal conversion of citrulline into arginine shows that this conversion is responsible for approximately 60% of the \textit{de novo} synthesis of plasma arginine from plasma citrulline at the whole body level. It can only be speculated where the other 40% of \textit{de novo} arginine is synthesized. The fact that argininosuccinate synthase and lyase are also widely expressed in other cell types, such as hepatocytes, endothelial cells and macrophages, may clarify this uncertainty. In these cells arginine is being formed and broken down in intracellular cycles like the urea cycle (liver) and the nitric oxide cycle (endothelial cells, macrophages). Although compartmentalization of metabolites within
Chapter 9

these cycles should prohibit the net release of newly formed arginine into the circulation (3), results from this study and another study by Wu et al. (2) suggest that the conversion of citrulline into arginine within one of these cycles might actually result in net release of arginine into the circulation.

Chapter 6

The results of the same study at intestinal and hepatic level are described in chapter 6. The absolute uptake of glutamine by the intestines accounted for 19% of total plasma glutamine turnover. Interestingly, tracer data revealed that glutamine disposal by the intestines is related to glutamine supply, a phenomenon that could not be detected before by measurement of net fluxes (8;9). This observation is of interest because it indicates that intestinal glutamine metabolism is regulated by glutamine supply in humans. Furthermore, the intestinal production of citrulline from glutamine equalled the total intestinal production of citrulline and the turnover of citrulline at whole body level, which makes glutamine the most important precursor of citrulline and the intestines the most important site for the synthesis of citrulline. The liver was observed to take up citrulline, although the net hepatic balance of citrulline was not significantly different from zero.

Chapter 7

The objective of the subsequent study was to investigate in 16 patients undergoing abdominal surgery the effect of the route of administration, intravenous or enteral, of glutamine on its intestinal conversion into citrulline, the renal conversion of glutamine-derived citrulline into arginine and the intestinal fractional extraction of glutamine. We observed that the plasma enrichment of [15N]glutamine was lower with enteral compared to parenteral administration of the glutamine tracer, reflecting the splanchnic extraction of enterally provided glutamine. Furthermore, when compared with intravenous administration, enteral administration of [15N]glutamine was observed to result in a higher intestinal fractional extraction of glutamine and a higher intestinal release of [15N]citrulline. To our knowledge this is the first time that the preference of the gut for enterally provided glutamine has been shown in humans by metabolic tracing. It can be suggested that the human gut preferably takes up glutamine from the enteral side, in order to secure important beneficial effects on the gut, as described in chapter 2 of this thesis. Because the plasma enrichment of [15N]glutamine was observed to be higher with parenteral administration of the glutamine tracer, it can be speculated that the maximum effect of glutamine administration is obtained with a combination of intravenous and enteral supply of glutamine, in order to secure the maximum systemic effect and the optimal local effect on the gut.
Chapter 8

The aim of this study was to explore how alanyl-glutamine contributes metabolically to the \textit{de novo} synthesis of arginine in humans, when provided by the enteral or intravenous route. By applying the same design as used in our previous human studies (10-12), the opportunity was created to compare the effect of the molecule, as well as the route of administration at the whole body and organ (intestinal, hepatic and renal) level. Results show that glutamine derived from alanyl-glutamine contributes to the \textit{de novo} synthesis of arginine, and that enterally provided alanyl-glutamine contributes most. Although alanyl-glutamine virtually disappeared in the intestines when provided by the enteral route, the fractional extraction of glutamine derived from alanyl-glutamine was not observed to be different with the route of administration. Most interestingly, the intestines were shown to release $[{^{15}\text{N}}]$arginine with enteral administration of alanyl-$[2-{^{15}\text{N}}]$glutamine. This release was not observed with enteral administration of $[{^{15}\text{N}}]$glutamine (10), although others have previously observed the intestinal release of unlabeled arginine in surgical patients in the post absorptive state (9). The surgery-induced stress response and the concomitant administration of alanine with enteral administration of the dipeptide may have induced the intestinal synthesis of arginine. Surprisingly, the higher plasma enrichment of $[{^{15}\text{N}}]$citrulline with enteral administration of the dipeptide could not be explained by the intestinal release of $[{^{15}\text{N}}]$citrulline. Also, the kidneys were not observed to contribute to the higher plasma enrichment of $[{^{15}\text{N}}]$arginine with enteral administration of the dipeptide, in spite of a higher supply with $[{^{15}\text{N}}]$citrulline. In fact, neither the liver nor the kidneys contributed to any of the observed differences at whole body level due to the route of administration of the dipeptide.

Most important findings & general conclusion

The connected investigations of this thesis yielded the following important results: glutamine is an important precursor for \textit{de novo} synthesis of arginine in humans, being responsible for 64% of \textit{de novo} synthesized arginine under post absorptive conditions. Glutamine is the most important precursor of citrulline and the intestines are the most important site for the synthesis of citrulline. When compared with intravenous administration, enteral administration of $[{^{15}\text{N}}]$glutamine was observed to result in a higher intestinal fractional extraction of glutamine and a higher intestinal release of $[{^{15}\text{N}}]$citrulline. Glutamine derived from alanyl-glutamine also contributed to the \textit{de novo} synthesis of arginine, but enterally provided alanyl-glutamine contributed most. Alanyl-glutamine virtually disappeared in the intestines when provided by the enteral route, but intestinal fractional extraction of glutamine derived from alanyl-glutamine was not significantly higher with enteral compared to intravenous administration of the dipeptide. Most interestingly, the intestines were shown to release $[{^{15}\text{N}}]$arginine with enteral
administration of alanyl-[2-\textsuperscript{15}N]glutamine. Finally, the results of the studies in mice and humans described in this thesis show that the route of administration, intravenous or enteral, and the molecular presentation of glutamine, as free molecule or the dipeptide alanyl-dipeptide, does affect the conversion of glutamine into citrulline and arginine, at the whole body and organ level. This observation should be taken into account in the design of future studies on the effects of glutamine, and in the application of glutamine in clinical practice. The different metabolic fate of enterally or intravenously provided (alanyl-)glutamine suggests the possibility of a differential beneficial effect related to the route of administration. Therefore, a combined treatment with enteral and intravenous glutamine may be most beneficial for the critically ill patient.

**Future perspectives**

The thesis confirms the importance of the relationship between glutamine and arginine. However, many more questions are raised by the results of these experiments and more research is necessary to make the knowledge applicable for clinical practice. Alanyl-glutamine seems to be the appropriate way to supplement the (critically) ill patient with glutamine because of its stability in aqueous solutions and superior clearing rate in plasma, when compared with other glutamine dipeptides. However, the outcome of this thesis suggests that free glutamine and glutamine derived from alanyl-glutamine have a different metabolic fate and are not simply exchangeable. Also, the human tracer studies described in this thesis were performed in the post absorptive state and during surgery, whereas the patients most likely to benefit from additional glutamine are critically ill, fed patients.

Therefore, studies recommended for the future should involve a treatment dose of alanyl-glutamine and preferably take place on the ICU. We are currently working on the design of 2 stable isotopes studies. First, we want to investigate the effect of a treatment dose of 0.5 g · kg\textsuperscript{-1} · day\textsuperscript{-1} of alanyl-glutamine by the intravenous or enteral feeding route on whole body and organ metabolism of glutamine, citrulline and arginine. This study will also take place during surgery to facilitate access to the portal, hepatic and renal vein. The other study aims to investigate the effect of additional alanyl-glutamine (0.5 g · kg\textsuperscript{-1} · day\textsuperscript{-1}) to enteral tube feeding in critically ill patients. To estimate the role of the intestines in the metabolic handling of glutamine in critically ill, patients participating in the protocol on the ICU will receive the glutamine tracer by the intravenous or enteral route, at random, on two consecutive days.

Other future studies should pay attention to the role of the intestines in the release of arginine and on the possible contribution of immune or endothelial cells to the plasma release of citrulline and arginine. These studies should probably include determination of
the expression of genes encoding for the enzymes involved in the synthesis of citrulline and arginine.

Also, studies are needed, which investigate the effects of a combination of intravenous and enteral administered (alanyl-)glutamine on clinical outcome in critically ill patients, because these patients are believed to profit most from the local protection of the gut integrity and modulation of the intestinal inflammatory response by glutamine in combination with systemic (protective) effects of glutamine. This hypothesis is supported by the recently commenced large-scale randomized, controlled trial in 1200 critically ill patients, with as primary goal to determine the effect of parenteral and enteral administration of a glutamine dipeptide and antioxidants on mortality (1).

Eventually, this research will contribute to a tailor-made nutrition for critically ill patients, taking into account individual needs including the state of the disease. For instance, a patient with kidney failure will benefit from additional arginine, whereas a patient with liver failure will not benefit from additional arginine. The composition of this tailor made nutrition should be flexible enough to include the right amount of calories and protein, additional substrates such as glutamine and/or arginine and additional micro-nutrients for each patient.

This tailor made nutrition should be developed step-wise, introducing one new substrate at the time, to avoid problems with the interpretation of the beneficial or adverse effects of additional substrates.

Reference List

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