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# GLUTAMINE KINETICS IN CRITICALLY ILL PATIENTS

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# Glutamine kinetics in critically ill patients

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*Becoming is not about arriving somewhere or achieving a certain aim. I see it instead as forward motion, a means of evolving, a way to reach continuously toward a better self. The journey does not end.*

Michelle Obama



## ABSTRACT

Critical illness, defined as a life-threatening organ failure, entails an extreme stress that results in a pathophysiology of its own. The abnormal metabolism of these patients causes a severe muscle wasting that is poorly understood. One hypothesis is that the muscle is sacrificed to provide the body with key metabolic substrates, such as the amino acid glutamine.

Both low and high plasma glutamine concentrations at Intensive Care Unit (ICU) admission are associated with a higher mortality. However, the mechanisms behind these associations are unknown. It has been suggested that there is an increased demand for glutamine in critical illness and that its availability is crucial for proper function of the immune system and the intestine. Hence, several intervention studies on glutamine supplementation in the ICU have been conducted. However, design and included patients varies widely and thus the results are heterogenous and problematic to compare. In order to explore the hypothesis that low plasma glutamine concentration in critical illness represents a shortage that may motivate glutamine supplementation, glutamine kinetics in these patients needs to be characterized.

The first step was to establish and validate a bolus injection method to measure glutamine endogenous rate of appearance ( $\text{endoR}_a$ ) for studying endogenous glutamine production in the ICU setting. When this method was applied, a positive correlation was detected, where 35% of variability in plasma glutamine concentration could be related to  $\text{endoR}_a$  during critical illness.

The second part of the thesis consists of observational studies on plasma glutamine concentrations in connection to outcome and specific diagnoses. In the post ICU period, plasma glutamine concentration was within the reference range and was not related to mortality.

In liver failure, regardless of aetiology, severity and course of illness, a high plasma glutamine concentration was a common finding, although most frequent in patients with acute fulminant and acute-on-chronic liver failure. There was a positive correlation between the severity of liver failure and plasma glutamine concentration.

Admission hyperglutaminemia ( $\geq 930 \mu\text{mol/L}$ ) was an independent predictor for high mortality. A majority of the hyperglutaminemic patients had a liver condition, although hyperglutaminemia was also observed in patients without signs of liver affection.

The role of glutamine in critical illness is still not settled. Our observations give no indication that a high plasma glutamine per se is toxic. The finding that low plasma concentrations correlates with a lower endogenous production keeps the possibility open that there is a cohort of critically ill patients with too low glutamine availability, who would benefit from exogenous glutamine supplementation. The ultimate question if plasma glutamine concentration is just a biomarker or if it also gives signal of a deficit and/or an impaired handling of glutamine is still pending. Therefore, glutamine kinetics in critical illness needs to be further clarified.

## LIST OF SCIENTIFIC PAPERS

- I. **A Tracer Bolus Method for Investigating Glutamine Kinetics in Humans**  
Mori M, Smedberg M, Klaude M, Tjäder I, Norberg Å, Rooyackers O, Wernerman J  
Plos One (2014) 9(5):e96601. doi:10.1371/journal.pone.0096601
- II. **Plasma Glutamine Concentration After Intensive Care Unit Discharge: An Observational Study**  
Smedberg M, Nordmark Grass J, Pettersson L, Norberg Å, Rooyackers O, Wernerman J  
Critical Care (2014) 18:677 DOI 10.1186/s13054-014-0677-8
- III. **Plasma Glutamine Concentrations in Liver Failure**  
Helling G, Wahlin S, Smedberg M, Pettersson L, Tjäder I, Norberg Å, Rooyackers O, Wernerman J  
Plos One (2016) 11(3):e0150440.doi:10.1371/journal.pone.0150440
- IV. **Endogenous Production of Glutamine and Plasma Glutamine Concentration in Critically Ill Patients**  
Smedberg M, Rooyackers O, Norberg Å, Tjäder I, Wernerman J  
*Manuscript, submitted for publication*
- V. **Hyperglutaminemia at Intensive Care Unit Admission: An Observational Study**  
Smedberg M, Helleberg J, Tjäder I, Norberg Å, Rooyackers O, Wernerman J  
*Manuscript, submitted for publication*



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## LIST OF ABBREVIATIONS

ALT	Alanine Transaminase
APACHE	Acute Physiology And Chronic Health Evaluation
AST	Aspartate Transaminase
AUC	Area Under the Curve
BMI	Body Max Index
CRP	C-Reactive Protein
DNA	Deoxyribonucleic Acid
EndoR <sub>a</sub>	Endogenous Rate of appearance
GC-MS	Gas Chromatography-Mass Spectrometry
Gln	Glutamine
GLS	Glutaminase
GS	Glutamine Synthetase
ICU	Intensive Care Unit
IQR	Interquartile Range
MELD	Model For End-Stage Liver Disease
MPE	Molar Percent Excess
Phe	Phenylalanine
R <sub>a</sub>	Rate of appearance
R <sub>d</sub>	Rate of disappearance
RNA	Ribonucleic Acid
SAPS	Simplified Acute Physiology Score
SOFA	Sequential Organ Failure Assessment

# 1 INTRODUCTION

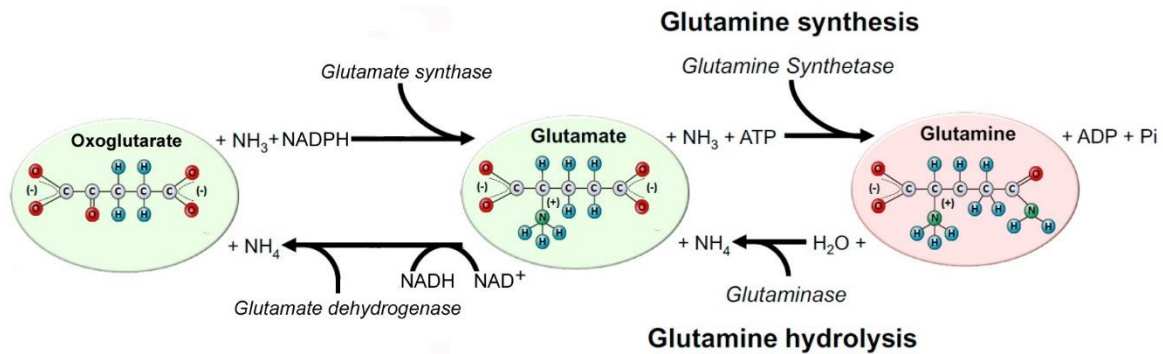
## 1.1 CRITICAL ILLNESS

Critical illness is a life-threatening failure in one or more vital organ; heart/cardiovascular system, lungs, brain, liver, haematological/coagulation system, or kidneys, resulting in the need of intensive care for survival. The patients in the intensive care unit (ICU) are a very heterogenous group, ranging from young to elderly patients with or without comorbidities and with single or multi organ failure. Most patients in the ICU have respiratory failure in need of mechanical ventilation, either solitary or in combination with other organ failures.

Before the 20th century, critical illness was hardly compatible with life. But with the evolvement of modern, highly advanced technique and medical treatments, there is now an increased capacity to keep patients alive. The intensive care supports vital functions and buys time, giving the body a chance to heal. Still, critical illness entails a high mortality; 20-30% within six months for ICU patients in Sweden (1). Critical illness exposes the body to an extreme situation, with a pathophysiology of its own and severely deviating physiological processes and metabolism. In the acute phase all systems are mobilized in a last attempt to survive, this results in high levels of inflammatory mediators and catecholamines, together with hemodynamic instability. With intensive care support, a majority of the patients recover during this phase. Those that do not, enter a prolonged catabolic phase, often accompanied by an increased metabolic rate (2, 3). These processes are sometimes highly maladaptive, especially for the patients with a prolonged ICU stay, where it can lead to cachexia, poor wound healing and immunosuppression with secondary morbidities and chronic infirmity as a result (2-4).

## 1.2 GLUTAMINE METABOLISM IN HEALTH

Glutamine ( $C_5H_{10}N_2O_3$ ) has a molecular weight of 146.14 g/mol and is the most abundant free amino acid in the human body, representing 20% of the free amino acid pool in plasma and up to 60% in specific tissues. A 70 kg adult has about 70-80 g of free glutamine distributed throughout the body (5). It is a non-essential amino acid, meaning that healthy individuals can produce all that the body requires. Endogenous glutamine production is estimated at a daily rate of the same quantity as the whole-body free glutamine content; 65-85 g/day in adults, corresponding to 4.5-5.8  $\mu\text{mol/kg/min}$  in a 70 kg subject, and can be derived both from de novo synthesis and protein breakdown (6). In addition, the protein in a normal diet contains 8-12% glutamine, accounting for an intake of about 10 g glutamine/day and, thus, a healthy person will have ready access to glutamine at any given point (7). This is fundamental, as this amino acid has several vital functions throughout the body (8).



**Figure 1.** Glutamine synthesis from, and hydrolysis to, glutamate and further hydrolysis to oxoglutarate, which in turn enters the citric acid cycle to form energy in the mitochondria. (Adapted with permission (5))

The interest in glutamine began with the finding that it is a crucial component for survival and proliferation in cell cultures (8). Glutamine can be utilized by several different metabolic pathways. It can easily be transformed to glutamate by the enzyme glutaminase (GLS), which removes NH<sub>3</sub>. The reaction is reversible through the endergonic reaction of adding NH<sub>3</sub>, by glutamine synthetase (GS). Nitrogen in the form of free ammonia is neurotoxic, but by attaching it to glutamate and form glutamine, safe transport between tissues is enabled. By reversing the reaction, the nitrogen can be disposed in liver and kidneys.

GS and GLS are expressed differently in tissues depending on if they are a main producer or consumer of glutamine. Skeletal muscle, liver, adipose tissue, and lungs have a high GS activity, whereas leukocytes, enterocytes and renal cells have a high GLS activity. Skeletal muscle cells are considered a key producer of glutamine as they have a high export. Thus, the muscles provide other tissues with glutamine and the main target is the splanchnic organs.

White blood cells and enterocytes utilize glutamine as a main energy fuel. In these cells, glutamine is converted to glutamate and then oxoglutarate by removal of both NH<sub>3</sub>-groups. It then enters the citric acid cycle in the mitochondria. Glutamine is also a precursor for nucleotides and nucleic acids in DNA and RNA synthesis. It is therefore a key substrate, supporting high mitotic activity in these tissues.

In the kidneys, the NH<sub>3</sub> from glutamine is exported to the collecting tubule and combined with H<sup>+</sup> to form NH<sub>4</sub><sup>+</sup> in the urine. Due to this possibility to dispose H<sup>+</sup>, the kidney can transform carbonic acid (H<sub>2</sub>CO<sub>3</sub>) into bicarbonate (HCO<sub>3</sub><sup>-</sup>), which is one of the major ways to regulate pH in the blood. Additionally, glutamine is a substrate for gluconeogenesis in both kidney and liver.

In the brain glutamine serves as a precursor for glutamate, which is one of the major excitatory neurotransmitters. Moreover, glutamine is a substrate for protein synthesis and a precursor to glutathione, an antioxidant which protects the body from free radicals, preventing oxidative stress and cell damage.

### **1.3 GLUTAMINE AND THE IMMUNE SYSTEM**

The function of glutamine in immune cells has been extensively studied *in vitro*, in both rodent and human cell lines. It has been found to be an important energy substrate, to activate intracellular signalling pathways, and to regulate gene expression of several genes. Thereby, glutamine has an essential role in cell proliferation and differentiation, tissue repair, and pathogen recognition (5) in immune cells. *In vitro* studies have also shown that glutamine can enhance a great variety of important functions such as T-cell proliferation, B-lymphocyte differentiation, macrophage phagocytosis, antigen presentation and cytokine production (8, 9). However, it is unclear how these findings translate to *in vivo* human immune cells. Firstly, the concentration of glutamine in cell cultures is 2-10 mM, which is far from the physiological normal plasma concentration of 0.4-0.7 mM. Secondly, a lot of the studies showing enhanced function are made on rodent cells and the same concentration-dependent enhancement has not always been replicable in human cell lines (10).

Nevertheless, it cannot be disregarded that a low plasma glutamine concentration may result in a partly impaired immune system. A considerable increase in metabolic activity, reflected as fractional protein synthesis rate, in circulating leukocytes and mononuclear cells has been reported as part of the acute phase response in both human endotoxemic sepsis models and ICU patients (11, 12). An activated immune system has a high cell proliferation, energy consumption, DNA translation and protein production, all of which consumes glutamine. Hence, it is likely that glutamine utilization increases with immune cell activity and that glutamine availability is vital.

### **1.4 GLUTAMINE AND THE INTESTINE**

The gut has a high glutamine uptake, both from intestinal lumen and from blood, indicating it as a key nutrient for this tissue. Even though the intestine is continuously provided with glutamine from the diet, it still utilizes about 30% of the total endogenously produced glutamine (13). Despite this very high influx of glutamine, the concentration of glutamine in the intestinal tissue is comparably low, though higher than in plasma. This is due to a high activity of glutaminase, which has a very high affinity for its substrate and therefore efficiently hydrolyses a large proportion of the available glutamine to glutamate (5). Hence, very little enterally provided glutamine reaches the blood stream (14, 15).

Glutamine serves many purposes in the gut aside from energy substrate. The enterocytes constitute one of our most important barriers, and its function is vital in both absorbing nutrients while at the same time shutting out unwanted substances and pathogens. As long as the microbiota is maintained in the intestinal lumen the symbiosis is beneficial. But, in a critically ill patient the barrier may fail, resulting in translocation of microbes and toxin (16, 17). This can cause a severe sepsis, which is particularly troublesome in patients colonized by antibiotic resistant bacteria. The barrier is regulated by a cooperation between the enterocytes, tight junction proteins, and submucosal immune cells. The integrity is highly dependent on

the constant renewal of the enterocytes, which have a lifespan of 3-5 days only. Glutamine is a main component for their proliferation, but also regulates apoptosis and cell survival and thus, helps maintaining the crucial balance of cell turnover (13, 18). The tight junction proteins seal the paracellular permeability. Their function is also dependent on a constant renewal, and presence of glutamine is important in gene expression for maintaining protein synthesis in the enterocytes (13). Moreover, inflammatory cytokines increase intestinal permeability and in vitro studies suggest that glutamine inhibits production of these cytokines (5, 13).

## **1.5 GLUTAMINE AND THE LIVER**

The liver is a key metabolic organ that serves to maintain homeostasis in many systems. The main functions of glutamine in the liver are as a gluconeogenesis substrate and a source of ammonia for urea synthesis. Glutamine is a regulator of the urea cycle, where higher glutamine concentrations increases urea production (5). Also, uptake of glutamine leads to cell swelling through osmosis. It has been suggested that this in turn stimulates anabolic processes in the hepatocytes and that, accordingly, reduced intracellular glutamine concentrations stimulates catabolic processes and insulin resistance (5, 19).

The hepatocytes positioned in the proximal sinusoids, where the blood enters from the portal vein, have a high uptake of glutamine, and expresses GLS, gluconeogenesis and urea synthesis enzymes. On the contrary, the hepatocytes in the distal sinusoids, right before the blood exits the liver, express GS instead. This enzyme has a high affinity for ammonia, and thereby these cells act as ammonia scavengers, making sure that very little free ammonia escapes to the general circulation. Consequently, the liver is both a consumer and a producer of glutamine and, although the glutamine synthesizing cells constitutes a much smaller proportion (5-7%), there is a net balance between consumption and production in a healthy liver (20).

Glutamine uptake in hepatocytes is mediated through a sodium-dependent transporter, which utilizes the Na/K electrochemical gradient across the plasma membrane to maintain an intracellular glutamine concentration of 3-7 mmol/L. The transporter has a pH-dependent activity, increasing with higher pH (20). Similarly, the activity of GLS increases fast with more alkalotic pH (19). Hence, this regulation favours glutamine synthesis in the liver during acidosis, which provides glutamine to the kidneys for  $\text{HCO}_3^-$  production (21). Moreover, it is important in patients with liver cirrhosis, where urea synthesis is maintained despite an impaired hepatocyte function, partly due to a habitual metabolic alkalosis. However, this makes the chronic liver disease patient vulnerable to any process resulting in acidosis, such as infections and hemodynamic instability, which in turn leads to hyperammonemia. Also, the cells expressing GS are reduced with up to 80% in cirrhosis, incapacitating the backup system that normally captures excess ammonia (19). Additionally, in patients with cirrhosis and/or portal vein thrombosis, blood from the intestine bypasses the liver and is therefore not

cleansed from ammonia, adding to increased ammonia concentration in the systemic circulation (22).

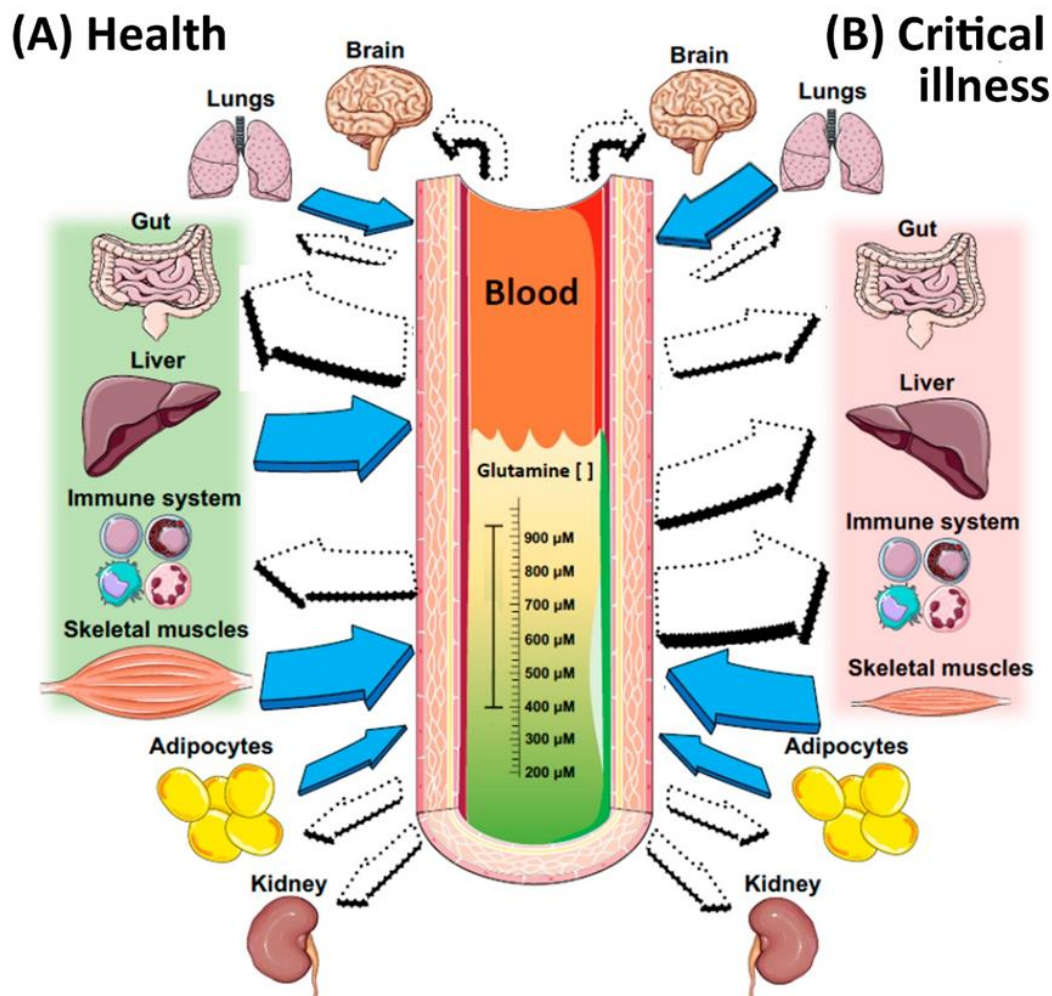
In patients with liver failure and hepatic encephalopathy, hyperammonemia is recognized as a part of the pathophysiology (23). Ammonia is toxic to nerve cells and the brain lacks an efficient urea cycle. Hence, glutamine synthesis in astrocytes is the only path for ammonia detoxication in the brain. The excess ammonia will lead to excessive glutamine synthesis in astrocytes, which in turn leads to osmotic cell swelling and is believed to contribute to brain edema and increased intracranial pressure (24).

## **1.6 GLUTAMINE METABOLISM IN CRITICAL ILLNESS**

When the human body is exposed to a major catabolic condition such as intense and prolonged exercise, cancer, major surgery, or critical illness, there is a presumed increased demand for glutamine (5). The stress of critical illness results in elevated cortisol levels, which in turn stimulates GS in skeletal muscle (25). Subsequently, there is an enhanced glutamine export from skeletal muscles, that remains constant over the duration of critical illness (26). Initially glutamine release from skeletal muscle exceeds the intracellular production, and as a result a general depletion of free glutamine in muscle is seen in critically ill patients (7). After a period of adaption, the endogenous production of glutamine can keep up with the demand, at the cost of substantial muscle protein wasting. Some patients admitted to the ICU have initial low plasma glutamine concentrations, which seems to be sustained over time (27-30). Although there are few reports on plasma glutamine concentrations over time in ICU patients (26-33). Because of the low concentrations in plasma as well as muscle, it has even been suggested that glutamine is “conditionally essential” in these patients (21).

The lungs are also reported to be an important glutamine provider in critical illness. Unlike skeletal muscle, the lungs do not have any glutamine storage, and in health they have a net balance of glutamine. But during sepsis and burn injury, GS activity is significantly increased, and pulmonary glutamine release is reported to account for about 20% of the total glutamine production (34, 35). However, these reports are based on measurements of blood flow and arteriovenous difference in glutamine which both have considerable limitations in accuracy. Hence, the significance of the lungs as a glutamine provider is still unclear.

There are several rationales for a higher glutamine demand in critical illness. Patients admitted to critical care often have severe infections and sepsis. This increases the activity of the immune system, consuming glutamine both for energy, protein synthesis and DNA synthesis as the cells multiply. Also, an activated immune system and inflammation will lead to more reactive oxygen species as it is part of the defence against pathogens. This leads to a high oxidative stress that increases the utilization of glutamine to produce glutathione and sustain redox balance. ICU patients have been demonstrated to have low total glutathione concentration in whole blood (36, 37).



**Figure 2.** Glutamine production and utilization in health (A) versus critical illness (B). Blue/filled arrows indicate tissues that have high glutamine synthetase (GS) activity and thus produce glutamine; white arrows indicate tissues with glutaminase (GLS) activity, which consume glutamine. In health there is an equilibrium in plasma and tissues, which is constantly maintained primarily by the liver and skeletal muscles. Cells of the immune system are dependent on glucose and glutamine provision in health, and even so more during severe illness. The gut is also a major site of glutamine consumption in health, whereas the acute phase of critical illness is associated with an impairment of enterocyte glutamine uptake. Additionally, the brain and the kidneys only express GLS, and hence mainly depend on plasma glutamine availability. The liver switches role from a major glutamine producer (A) to a major consumer (B) to sustain gluconeogenesis and increased protein synthesis. The lungs express both GS and GLS enzymes, and switch from a net glutamine balance to a possible glutamine producer in critical illness. Subsequently, the whole body relies mostly on the skeletal muscle to maintain glutamine concentration in critical illness, often accompanied by problematic muscle wasting. The adipose tissue expresses both GS and GLS enzymes, and hence can produce and consume glutamine in both situations. (Adapted with permission (5))



Moreover, it is common that the patients are acidotic, forcing the kidneys to produce more  $\text{HCO}_3^-$ . Acidosis leads to hypotension through vasodilation, impaired coagulation and declined enzyme function, so the ability to balance pH is crucial for these patients. Acidosis increases the uptake and catabolism of glutamine in the proximal tubule of the kidney (38). However, there are other pathways for pH regulation and hypoglutaminemia has not been related to acidosis.

Furthermore, the liver also exhibits an elevated glutamine uptake during critical illness in order to support the increased protein and glutathione synthesis and gluconeogenesis (19). In a rat endotoxemic sepsis model, the hepatic glutamine uptake increased nearly tenfold (39). On the contrary, ischemia, sepsis and severe inflammation seems to significantly reduce intestinal glutamine uptake from plasma in the acute phase (40-42).

### **1.6.1 Muscle wasting and glutamine depletion**

Most critically ill patients can meet an increased glutamine demand and sustain plasma glutamine concentrations. However, this entails a general depletion of protein and free glutamine in skeletal muscle. The intracellular free glutamine concentration in muscle is reduced by 70-80% in ICU patients (25, 43, 44). This profound drop seems to be part of the early acute phase response. Stress hormone induction of outward transport systems and/or electrical transmembrane potential changes have been suggested mechanisms that result in this rapid glutamine mobilization (7).

There is little correlation between plasma concentration and skeletal muscle concentration of glutamine (29, 45, 46). In elective post-op patients the glutamine concentration drops in muscle, whereas it remains stable in plasma (46). The intracellular glutamine depletion develops during the immediate postoperative period and the lowest concentration is seen in the first 2-4 days. The concentration is then restored slowly over 2-4 weeks (47). Parenteral glutamine supplementation in the postoperative period prevents this reduction in skeletal muscle, but if supplementation stops the reduction is only postponed (46).

In ICU patients the concentration in muscle drops extensively, while the plasma concentration is variable and has a less remarkable decrease (29, 45). This can be illustrated by the ratio of glutamine concentration in plasma compared to intracellular concentration in skeletal muscle. In healthy subjects this ratio is 1:30 whereas the critically ill have a ratio of 1:5-1:10 (45). This profound depletion takes a long time to restore and is not readily influenced by supplementation (29). One reason for that could be the net efflux of glutamine from muscle that might be hard to turn around as long as there is an increased demand in other, more prioritized tissues. Another theory is that muscle cells are constructed to be self-supporting in glutamine and do not have a system adapted to a large glutamine uptake, as only 25% of the intramuscular glutamine pool is derived from extracellular space normally (7).

The glutamine balance in skeletal muscle is complex and controlled by several different pathways. Elevated plasma levels of cortisol (48, 49) and tumour necrosis factor alpha, and low intracellular glutamine concentrations have been shown to stimulate GS activity (7). Also, protein breakdown in skeletal muscle, which results in release of free glutamine, is considerably increased during critical illness. On the other hand, catecholamines suppress GS so the proportions of stress hormones in the patient could affect glutamine balance (7).

In patients with severe burn injury, a distinct cohort with excessive hypermetabolism and severe muscle wasting over long time, a threefold increase in muscle glutamine synthesis is reported. However, the intracellular utilization was increased to the same extent and the export rate from muscle was increased, resulting in a net negative balance (25). Moreover, after two weeks in intensive care, glutamine de novo synthesis is reported to be reduced in burn patients resulting in a decreased glutamine availability (43).

The high efflux of glutamine from skeletal muscle has been shown to be sustained over at least the initial two weeks of critical illness (26). During the initial phase of critical illness, up to 10% of the muscle protein is wasted per week (50). In patients with a prolonged phase of critical illness this continuous protein breakdown leads to a general muscle depletion (28, 50). The muscle wasting results in a weakness and physical disability that can be sustained for years in ICU survivors (50).

It has been suggested that patients with an initial higher muscle mass can sustain glutamine export, and therefore are the selected survivors for long-term critical illness. It is known that patients with overweight and moderate obesity (BMI 25-40) have a lower mortality and a longer length of stay in the ICU than patients with normal weight (BMI 18.5-25) (51). The reason for this is unknown, but a possible explanation is that at least some of these patients have a higher muscle mass, which can sustain a high glutamine production and thereby contributes to their survival (52).

### **1.6.2 Hypoglutaminemia**

The prevalence of low plasma glutamine (<400-420  $\mu\text{mol/L}$ ) (36, 53) at ICU admission ranges from 31-65% (27, 36, 53-58). Especially, critically ill patients with multitrauma (27, 55, 57), major burn injury (59, 60) and severe sepsis (31, 57) have been linked to hypoglutaminemia. Low plasma glutamine concentration has been shown to be associated with a higher mortality, both at admission and during the course of illness (31, 36, 53).

According to several reports there is no significant correlation between hypoglutaminemia and severity of illness, expressed by conventional risk scoring Acute Physiology And Chronic Health Evaluation (APACHE II) (31, 36, 53). Also, adding low plasma glutamine concentration to risk scoring gives an improved mortality prediction. However, other studies with a very high prevalence of hypoglutaminemia (58% and 65%) have reported a correlation between low plasma glutamine and high risk scoring, in a mixed population of expected long-

stayers in South Africa (APACHE II) (57) and in mixed consecutive patients in Holland (APACHE IV) (56).

The flow of glutamine between different compartments varies and a low concentration in plasma is therefore not necessarily an indication of a general deficiency. Glutamine in plasma is only a small proportion of the whole-body free glutamine pool, as intracellular concentrations are much higher in several tissues (61-63). Also, the intracellular changes in glutamine concentration during critical illness follow a different pattern than plasma; skeletal muscle has a more general and profound drop than plasma, whereas liver and intestine tend to follow the same trend as plasma concentration (5, 64). Nevertheless, the plasma pool represents the glutamine immediately available for the immune system and other cells that rely upon glutamine supply from other tissues.

The theory that a low plasma glutamine concentration leads to an impaired immune system is appealing, but it is almost entirely based on cell culture studies and has little scientific support in vivo. Supranormal intracellular glutamine concentrations have been reported in polymorphonuclear neutrophils of trauma patients on day one. But from day five a persisting 30% decrease was found instead. Although 40% of the patients had hypoglutaminemia, the intracellular glutamine concentration in the neutrophils followed the same pattern in all included patients, regardless of plasma glutamine (58).

As hypoglutaminemia occurs in the acute phase of critical illness, when de novo synthesis and muscle release is accelerated, the low plasma concentration is probably a result of an acutely intensified utilization. However, ICU patients are often elderly and accompanied by comorbidities, chronic disease and malnutrition, all of which are also likely to decrease plasma glutamine concentrations (53, 65, 66).

Although an increased utilization is plausible, the pathophysiology resulting in hypoglutaminemia is largely unknown. Contributing factors include increased hepatic uptake, amino acid loss by urine in trauma patients, continuous renal replacement therapy (67), insufficient nutrition, blood loss and dilution secondary to resuscitation with crystalline fluids (68). A low plasma glutamine has been related to inflammation and active infection (56), correlating with elevated C-reactive protein (CRP) and interleukin 6 (IL6) levels (31, 54, 57, 69). Moreover, low plasma glutamine has been reported to be associated with low albumin (31, 53), which can be a result of both severe inflammation and malnutrition.

ICU long-stayers that develop chronic critical illness syndrome have elevated CRP and IL6 levels, immunosuppression and protein catabolism with cachexia (4), all of which can be connected to hypoglutaminemia.

A persisting insufficient glutamine availability may affect numerous metabolic pathways regulating cell survival and proliferation (18, 70), affecting wound healing, the intestinal barrier and the immune system (5). Also, based on in vitro studies, intracellular glutamine depletion promotes cell dehydration, which may further support the catabolic state and

contribute to insulin resistance (19, 71). A persistent low plasma glutamine during the course of critical illness has been reported to be associated with more infections and increased length of stay (27).

### **1.6.3 Hyperglutaminemia**

High plasma glutamine concentrations are less studied, but in available reports 5-15% of critically ill patients have hyperglutaminemia at ICU admission (36, 53, 54, 57, 72). The cut-off used is either  $>700 \mu\text{mol/L}$ , which is the upper limit for normal value in healthy individuals, or  $>930 \mu\text{mol/L}$ , which is the cut-off for increased mortality found in ICU patients (36). Hyperglutaminemia is also associated with an unfavourable outcome at admission (36), as well as during the course of illness (31). The underlying mechanism is unknown, but there is an overrepresentation of patients with liver failure in this group and higher plasma glutamine concentration is correlated with elevated alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin concentrations (31, 57). Hence, a decreased hepatic uptake of glutamine due to substantial liver damage may be a part of the pathophysiology. High plasma glutamine concentration is a characteristic of patients with acute fulminant liver failure (73), which is therefore considered a contraindication for glutamine substitution.

Plasma creatinine kinase, as an indicator of cell damage, has been reported to be higher in the hyperglutaminemia group at admission (53), but not after thirteen days in the ICU (31). In theory, considerable cell damage could give an acute raise in plasma glutamine concentration, as intracellular glutamine concentration is much higher than in plasma (61-63).

A proposed pathophysiology contributing to hyperglutaminemia in liver failure is an excessive utilization of the backup system for ammonia detoxication. When urea synthesis in the liver is impaired, increased ammonia concentration in the systemic circulation will stimulate GS, primarily in skeletal muscles. A possible fate for the resulting glutamine is oxidation in enterocytes, which in turn will produce two ammonia molecules leading to a vicious circle of increasing glutamine and ammonia production (74). Whereas high levels of ammonia lead to excess glutamine synthesis in the brain, which contributes to brain edema, isolated hyperglutaminemia does not seem to have the same effect.

## **1.7 GLUTAMINE SUPPLEMENTATION**

Proteins in enteral nutrition normally contain 7-8% glutamine, similar to a normal diet. Parenteral nutrition, however, does not, due to the instability of free glutamine in aqueous solution. It has, therefore, been suggested to supplement parenteral nutrition with a separate alanyl-glutamine or glycyl-glutamine dipeptide solution, in order to provide the patients with a normally composed nutrition.

As hypoglutaminemia at ICU admission is reported to be associated with a higher mortality in critical illness (36, 53), a hypothesis was raised that glutamine supplementation could normalize plasma glutamine concentration, thereby resulting in a beneficial effect. However, very few studies on glutamine supplementation have included plasma glutamine concentrations, and no study has shown a statistical connection between a change in plasma concentrations and the beneficial effect. Instead, all patients have been included and grouped by other criteria than glutamine concentration. As glutamine has been considered non-toxic, it has still been thought reasonable to provide supplementation to all intensive care unit patients, given the potential benefits.

An intravenous glutamine dose will have a more pronounced effect on plasma concentration as compared to the same dose given enterally (14, 75). The dose for parenteral glutamine supplementation used in intervention studies varies between 0.2-0.5 g/kg/24 , and has been shown to adequately restore plasma concentrations to the normal range (29, 76).

In a small group of ICU patients, 0.35 g glutamine/kg was given for four hours, corresponding to 2 g/kg/24 hours. The dipeptide was hydrolysed quickly, and plasma glutamine concentrations increased immediately to supranormal levels (1000-1200  $\mu\text{mol/L}$ ), but dropped back to baseline within eight hours after administration was stopped. No adverse effects were reported, despite the high dose and corresponding high plasma concentrations of glutamine (75). Similarly, a dose corresponding to 0.9 g/kg/24h given for four hours to cancer patients with normal plasma glutamine prior to surgery, also increased plasma glutamine  $>1000 \mu\text{mol/L}$  (14). When escalating doses of 0 (control), 0.28, 0.57 or 0.86 g of glutamine/kg/24h were given for five days to four groups of ICU patients, plasma concentrations increased in a dose-dependent way. The three groups receiving glutamine supplementation achieved normal plasma glutamine concentration within one day, and some patients in the group receiving the highest dose became hyperglutaminemic, whereas the control group stayed hypoglutaminemic (29).

Contrariwise, in trauma patients with high prevalence of hypoglutaminemia (60%), glutamine supplementation of 0.35 g/kg/24 hours for five days did not affect plasma concentration after the end of supplementation (27). However, as samples were obtained after supplementation was discontinued and plasma concentration drops back to baseline rapidly, these results are inconclusive.

If the dipeptide is given enterally it is hydrolysed similarly, but the effect on plasma glutamine concentration is modest (77). Considering the high use of glutamine in enterocytes, this is likely an effect of first-pass metabolism. As soon as the administration is discontinued the plasma concentration will drop back to preadministration levels, in the same way as for the intravenous substitution. The different routes of administration will clearly result in different distributions.

The Scandinavian Glutamine Trial, randomizing 418 ICU patients with full nutrition to either parenteral glutamine (0.28 g/kg/24h) or placebo, showed a 29% reduction in ICU mortality in

the intervention group (78). Several meta-analyses conclude that parenteral glutamine supplementation reduces infectious complications, mechanical ventilation duration, hospital length of stay and hospital mortality (79, 80). However, the effects of enteral administration of extra glutamine is not as convincing (81). Possibly, the different metabolic paths give rise to different effects, because only a limited extent of the glutamine from enteral administration reaches tissues where a possible beneficial effect emerges.

Previously, addition of parenteral glutamine was advised to all ICU patients on parenteral nutrition (82). However, current guidelines advice against parenteral supplementation of glutamine in the ICU and only recommend addition of enteral glutamine to patients with major burns or trauma (83). The reasons for this will be discussed in the next section.

## **1.8 IMMUNONUTRITION AND CONTROVERSY ON GLUTAMINE SUPPLEMENTATION**

Immunonutrition is based the concept that certain nutrients are considered to activate and/or modulate the immune system. Several nutrients are included such as glutamine, arginine, omega-3 fatty acids, nucleotides, selenium, and vitamins A, C and E. The reasoning for glutamine in the immunonutrition context is much the same as for glutamine supplementation in critical illness; positive effects on the immune system, the redox balance, tissue metabolism and cell survival (71, 84). The aim is to give high doses of these nutrients, not to supplement a deficit, but to induce pharmacological effects (85). This idea is to most part funded on cell culture and animal studies (86) and attempts to translate it into the clinical setting in critically ill patients have not had the results anticipated.

With the REDOX study came an alarming report that glutamine might be toxic (72). In this trial 1223 ICU patients were randomized to four groups; placebo, glutamine, antioxidants (selenium, zinc, beta carotene, vitamin C and E) or glutamine and antioxidants combined. The glutamine dose was high; 0.35g/kg intravenously plus 30 g enterally. For a 70 kg patient this means 54.5 g/day compared to about 21 g/day in the majority of earlier studies on glutamine supplementation. The enteral dose was administered even if the patient did not tolerate enteral nutrition, as it was separated from nutrition. The rationale behind this was that using both routes of administration would give positive results in the gastrointestinal system as well as systemically and that a higher dose was even more likely to give beneficial results. In a pilot pre-study, the dosage used gave the best effect on plasma concentrations without any reported adverse effects (87). However, the study result was the opposite, patients who received glutamine had a significantly higher six months mortality compared to the control group. They also had a longer length of stay, both in the ICU and in the hospital.

The studied patient group was severely ill with two or more organ failures and a majority was in shock, most previous studies have excluded these patients. Plasma glutamine concentrations were only measured in a small subgroup of patients and found to be in accord with earlier report of ICU patients, both at baseline and at day 4 and 7 of treatment. A

majority of patients were within the normal range and 30% were hypoglutaminemic. The patients that received glutamine showed a higher frequency of elevated urea levels. This could reflect a high metabolic demand on liver and kidneys, from the highly unbalanced supply of amino acids and possible nitrogen overload, as the given nutrition was hypocaloric, about 900 kcal/day, whereas the total nitrogen intake was high.

The SIGNET study, randomizing 502 ICU patients with at least 50% parenteral nutrition to get addition of glutamine (0.25-0.3 g/kg/24h), selenium, both or neither, showed no conclusive results (88). However, the patients were withdrawn from the study when enteral nutrition surpassed 50% and the intervention was limited to seven days, which meant that the number of patients in the study decreased considerably over time. Moreover, the glutamine was mixed with the parenteral nutrition, of which the administration was diminished over time and thus, the actual dose administered was not possible to deduce.

With enteral administration, the METAPLUS trial randomizing 301 ICU patients to get high protein enteral nutrition with or without immunonutrients (glutamine (0.28 g/kg/24h), omega-3 fatty acids, selenium, zinc and vitamins C and E), resulted in a higher six months mortality in medical patients in the immunonutrition group (30). This was, however, not related to glutamine concentrations, but rather to concentrations of the omega-3-fatty acids (89). Similarly, a study giving omega-3 fatty acids, gamma-linolenic acid and antioxidants (zinc, selenium, vitamins C and E, beta-carotene, taurine and carnitine) also resulted in a higher 60 days mortality in the treatment group, although glutamine was not included (90). It has been suggested that this combination of nutrients might block the possible beneficial effects of each other (91).

A study on the enteral nutrition Intestamin® randomized 55 ICU patients to either early enteral administration of Intestamin® (30 g glutamine, tributyrin, vitamins C and E, selenium, zinc) and Reconvan® (10 g glutamine, arginine, omega-3 fatty acids) or a control supplement and Fresubin® original. The calorie intake was the same, but the control nutrition had much less protein content (38 g versus 98 g). The study was originally planned to include 344 patients, but an interim analysis of the first 50 patients showed significant beneficial results and enrolment was, therefore suspended at this point, which limits the value of the study. The intervention group showed a faster improvement in organ dysfunction, represented by a significant reduction in delta SOFA score, which was the primary endpoint (92). It is possible that these results are attributable to the protein content, which is closer to current recommendations in the intervention group. However, the EAT-ICU trial with a similar difference in protein intake between intervention and control group, without glutamine, reported no difference in any outcome, including rate of organ failures (93).

Study	Patients	APACHE II Median (IQR) or Mean $\pm$ SD	SOFA Median (IQR) or Mean $\pm$ SD	Duration of intervention	Route of administration	Glutamine dose
Scandinavian glutamine trial (78)	Stabilized patients within 72 h of admission, receiving $\geq$ 80% of full nutrition	21 (18-26)	9 (7-10)	Throughout ICU stay, median 9 days	Parenteral	0.28 g/kg/24h
Redox (72)	Mechanical ventilation and $\geq$ 2 organ failures, within 24 h of admission	26.6 $\pm$ 7.6	8.5 $\pm$ 2.8	Throughout ICU stay, median 8 days	Parenteral + enteral	0.6-0.8 g/kg/24h
Signet (88)	Patients requiring $\geq$ 50% parenteral nutrition	20 (16-25)	5 (3-8)	Max 7 days, median 5 days	Parenteral	0.25-0.3 g/kg/24h*
Metaplus (30)	Mechanically ventilated, requiring enteral nutrition within 2 days after ICU admission and for >3 days	22 $\pm$ 8.5	8 (7-10)	Max 28 days, median 12 days	Enteral	0.28 g/kg/24h
Intestamin study (92)	Infection, $\geq$ 1 organ failure and expected ICU stay >3 days and enteral nutrition > 5 days	14.0 $\pm$ 5.5	7.3 $\pm$ 2.1	Max 10 days	Enteral	0.56 g/kg/24h

**Table 1.** This table summarizes the heterogeneity seen in conducted trials on glutamine supplementation. Patient cohorts and interventions varies greatly. Although only a few studies are presented here, this is representative of the collected studies made on glutamine supplementation, which makes them very hard to compare.

\*Intended dose, as the parenteral nutrition was diminished over time, the administered dose was lower.

The available studies on glutamine supplementation have different endpoints, routes of administration, doses, and purposes to give the supplementation (Table 1). In addition, plasma glutamine has most often not been measured and patients with a potential deficit are randomly mixed up among patients with normal or possibly high glutamine concentrations.



This will, naturally, give very variable results depending on the case mix of a certain study. In the end the original hypothesis has never been tested: Do some critically ill patients have a glutamine deficiency, and will this group benefit from supplementation?

Most importantly, though, the patients included vary decidedly, which makes the results hard to compare and generalize. Unstable patients in the acute phase with multiple organ failure are very different from patients tolerating full enteral nutrition, as those in the latter group are likely to be stabilized and less severely ill.

The acute phase response and the prolonged catabolic phase of ICU long-stayers are homogenous parts of critical illness. Still, it is unlikely that there is a “magic bullet” treatment that is applicable for the entire ICU population (91). The knowledge gained from these trials and other studies has highlighted a number of conditions where glutamine might not be advisable in the ICU; acute liver failure associated with deranged metabolism, acute kidney failure not receiving dialysis (94), hemodynamically unstable patients in shock, and early phase unstable patients that do not tolerate nutrition.

In conclusion, the effect of supraphysiological doses of glutamine has earlier only been described in cell cultures in vitro (9), it has not been explored in healthy subjects and seem to be unadvisable in critically ill. The possible role of glutamine to supplement a deficit in elect groups of critically ill patients is yet to be explored (64, 91, 95).



## 2 AIMS

There is a considerable heterogeneity of existing studies over glutamine supplementation in critical illness. It comes in the results reported, in the patient cohorts investigated, and in doses and routes of administration. To explain the heterogeneity in results, a better understanding of glutamine kinetic in critically ill patients is necessary. Existing evidence do not exclude the possibility of beneficial effects from glutamine supplementation in some subpopulations of critically ill patients. Glutamine kinetics in critical illness is not sufficiently characterized to enable us to design the proper studies to sort out the hypothesis that low plasma glutamine represents a shortage of glutamine, which may motivate a supplementation. The overall aim of this thesis is to help to fill this gap of knowledge.

The conventional method to study glutamine rate of appearance (endogenous glutamine production) with a constant infusion, is time-consuming. This constitutes a problem in the ICU setting, as there are frequent medical alterations that risks affecting the results when a measurement stretches over a longer time period. Hence, there is a need for an alternative method to study glutamine turnover in critically ill patients, in order to get a better understanding of the kinetics. One aim was, therefore, to establish and validate a bolus injection method for studying endogenous glutamine production, enabling shorter study periods (Study I).

In clinical practice it will be plasma glutamine concentration that is available. However, it is unclear what it represents and how it relates to glutamine availability for tissues with high utilisation. If a low plasma glutamine concentration is caused by a low production rate, there is a rationale for glutamine supplementation. Therefore, a second aim was to investigate the relation between plasma glutamine concentration and endogenous production during critical illness (Study IV).

A third aim of the thesis was to elucidate the relevance of plasma glutamine concentration to clinical conditions and outcomes in three observational studies. We explored this at discharge from the ICU and longitudinally in the post ICU period (Study II), in liver conditions (Study III) and when hyperglutaminemia was present at ICU admission (Study V).



## 3 STUDIES OVERVIEW

### 3.1 STUDY DESIGNS AND EXPERIMENTAL PROTOCOLS

#### I. A tracer bolus method for investigating glutamine kinetics in humans

The study is comprised of 4 substudies.

- (1) *Dose finding study*: Bolus injections of [ $1\text{-}^{13}\text{C}$ ] glutamine were given to healthy volunteers ( $n=6$ ) to find a dose that allowed accurate tracer determination in plasma with minimal effects on glutamine and insulin concentrations.
- (2) *Variation study*: Repeated measurements of endogenous rate of appearance ( $\text{endoR}_a$ ) were performed in four healthy volunteers ( $n=4$ ) 3-12 days apart.
- (3) *Alanyl-glutamine supplementation and parenteral feeding*: Healthy volunteers ( $n=17$ ) were given intravenous alanyl-glutamine supplementation (16.8 mg/kg/h glutamine, corresponding to a daily dose of 0.4 g/kg/24h) with and without parenteral nutrition, and the effect on the  $\text{endoR}_a$  of glutamine was determined.
- (4) *Alanine study*: Since glutamine supplementation is given in the form alanyl-glutamine, healthy volunteers ( $n=7$ ) were given intravenous alanine to determine whether it affects glutamine  $\text{endoR}_a$ .

#### II. Plasma glutamine concentration at ICU discharge: An observational study

Fully fed ICU patients intravenously supplemented with glutamine for  $>3$  days were studied at ICU discharge and post-ICU. Plasma glutamine concentration was followed on the day of discharge and every 5-7 day of the remaining hospital stay ( $n=63$ ), and plasma glutamine concentrations 24-72 hours after discharge was related to 12 months all-cause mortality ( $n=100$ ).

#### III. Plasma glutamine concentrations in liver failure

Four different groups of patients were studied; A) chronic liver failure ( $n = 40$ ), B) acute on chronic liver failure ( $n = 20$ ), C) acute fulminant liver failure ( $n = 20$ ), and D) post-hepatectomy liver failure ( $n = 20$ ). Child-Pugh and Model for End-stage Liver Disease (MELD) scores were assessed as indices of liver function. Group A, chronic liver failure, was only sampled and evaluated once in the out-patient clinic. The three other groups were sampled and evaluated first on the day of ICU admission (groups B and C) or at the recovery room (group D), and thereafter every 3rd day ( $\pm 1$  day) during their hospital stay. Outcomes were recorded up to 48 months after study inclusion.

#### IV. Endogenous production of glutamine and plasma glutamine concentration in critically ill patients

The tracer bolus method developed in study I was applied to determine  $\text{endoR}_a$  for glutamine in critically ill patients ( $n=20$ ) with varying glutamine concentration at ICU admission, as screened by a point-of-care device.

## **V. Hyperglutaminemia at intensive care unit admission: An observational study**

Consecutive admissions to a mixed general ICU were included (n=269). A blood sample to be analysed for glutamine concentration was obtained within one hour from admission. Conventional risk scoring SOFA (Simplified Acute Physiology Score) and SAPS III (Sequential Organ Failure Assessment) at admission, and mortality outcomes for up to 12 months were recorded for all included patients.

### **3.2 SUBJECTS**

In study I, all 32 subjects were healthy volunteers recruited in accordance with the ethical review board application.

Studies II, IV and V all include patients from the ICU of Karolinska University Hospital, Huddinge. The case mix includes both medical and surgical patients, where the surgical predominantly originate from transplant (liver and kidney) and major upper abdominal surgery (pancreas, ventricle, liver, and oesophagus). No traumatic injury, neuro or thoracic surgery patients are included. As the hospital is a liver transplant centre, patients with liver disease are overrepresented compared to other ICU populations in Sweden.

Study II recruited ICU patients who had received glutamine supplementation in the ICU for >3 days before they were discharged from the unit.

Study IV recruited patients in the ICU and study V included consecutive admissions to the ICU.

Study III recruited patients to four groups from different clinics in the hospital. The patients with chronic liver failure were recruited at a planned appointment with their doctor at an out-patient clinic. The two groups with acute or acute on chronic liver failure were recruited in the ICU. The post-operative patients were recruited in a high-dependency ward after surgery.

### **3.3 ETHICAL CONSIDERATIONS**

Observational and kinetic studies can help us understand the mechanism behind hypo- and hyperglutaminemia and thereby elucidate which patients could be targeted for substitution, an intervention that could possibly save lives in a patient group with high mortality. This should be considered in relation to the potential discomfort, risk or harm associated with the studies. All study protocols were approved by the Regional Ethical Review Board, Stockholm, Sweden and conformed to the ethical guidelines of the Declaration of Helsinki. All patient and volunteer data were encrypted and kept safe.

In study I, healthy volunteers received both arterial and venous catheters, associated with a minor discomfort. A total of 60 ml blood was collected, which is a negligible risk. A health questionnaire screening and health examination were performed by a trained physician, who also was available throughout the experimental protocol. Financial compensation was given to the subjects and they could withdraw from the study at any time.

In study II-IV, patients contributed with 9-30 mL of blood. The protocol was designed to minimize blood loss and this small volume is a negligible risk, also in the severely ill patients. The collection could be associated with a minor discomfort, when a separate venous puncture was necessary, but existing lines were used whenever possible.

In study IV, the tracer injection is not harmful. With the original sampling protocol, however, we encountered a problem with failing arterial lines. This was considered an unacceptable complication for the patient as the arterial line is needed in the ICU to continuously monitor blood pressure and obtain arterial blood gases. We, therefore, made alterations in the protocol to only include stable patients where arterial line is not as crucial and, thereafter, to change the sampling protocol from 61 to 17 samples in order to protect the arterial lines.

In study V, an extra vial of blood was collected along with routine samples at ICU admission. Informed consent to participate in the study was obtained retrospectively within one month, otherwise the sample was discarded.

Informed consent is often not possible to obtain from critically ill patients due to altered mental status and sedation, a close relative is then asked on behalf of the patient. Another ethical issue with studying ICU patients is that, due to the severity of the illness, both patients and relatives often are in an emotionally vulnerable state when asked for participation in the study. However, as the metabolic changes and potential benefits are specific for this patient group there are no alternative candidates.





## 4 METHODS

### 4.1 AMINO ACID ANALYSIS

Glutamine tracer enrichment (amount of labelled glutamine in relation to total glutamine) is analysed by a gas chromatography-mass spectrometry (GC-MS) method described previously (96).

We use two different methods to analyse glutamine concentration in the laboratory:

1. Free glutamine concentration in plasma is analysed by GC-MS by adding [U-<sup>13</sup>C<sub>6</sub>] glutamine as an internal standard. This technique is used in parallel when enrichments are analysed (as above).
2. Plasma glutamine concentration is analysed by high pressure liquid chromatography (HPLC) using an on-column derivatization with ortho-phthaldialdehyde/3-mercaptopropionic acid (OPA/3-MPA) and fluorescent detection as described earlier (97). This technique is used when glutamine concentration is needed without determination of enrichment.

Plasma glutamine concentration is measured in the ICU employing a point-of-care device (Nova Biomedical's BioProfile Analyser), where enzyme biosensors combine two membranes with enzymes and an electrochemical sensor to measure metabolites. Here accuracy is not on analytical level, but sufficient for clinical guidance and for characterization of patients for study inclusions (98).

### 4.2 DISTRIBUTION AND BODY COMPARTMENTS

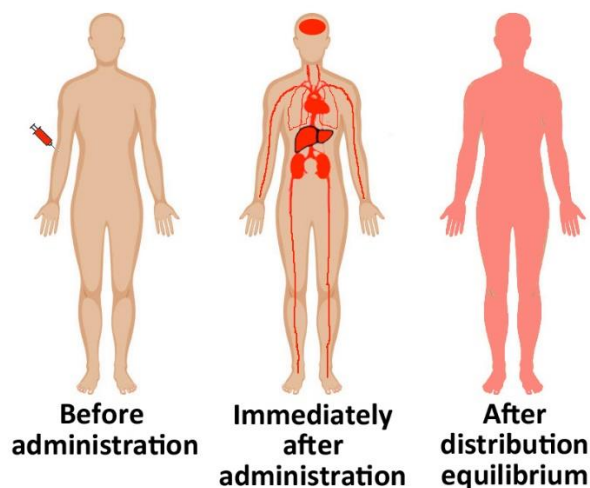
A main purpose of this work is to assess the turnover of glutamine, to improve understanding and interpretations of plasma concentrations of glutamine. Turnover can be studied by using a labelled form of the metabolite, an isotopic tracer.

The human body consists of a wide variety of tissue characteristics in terms of blood flow, water content, protein binding, and transport mechanisms over membranes related to size or electric charge of different metabolites. This will affect the distribution of different substances. For example, a lipophilic substance will accumulate in fat tissue and large molecules that do not move readily over capillary walls will stay in the plasma.

To describe the data that is achieved through different experiments there is a need of simplifications of the human physiology. One such method is to mathematically divide the body into different compartments or metabolic pools (99). A metabolic pool has a hypothetical volume of distribution, seldom matching any physiological volume, with a homogenous concentration of the studied metabolite. Kinetic models rely on metabolic pools to describe the exchange of metabolites between them. The plasma metabolic pool is a central compartment, in exchange with most other compartments, and readily available for sampling. Other examples could be the intracellular milieu of a certain tissue like skeletal muscle cells or enterocytes.

Glutamine is a small molecule that can easily move across cell membranes to be utilized by

tissues throughout the body. However, the intracellular concentrations are in general much higher than the plasma concentration (44, 61-63). Thus, the plasma concentration is just a small fraction of the total free glutamine pool, which has a large distribution volume, and it takes a very long time to reach equilibrium (6). Due to these characteristics, compartmental modelling for glutamine is complicated. Hence, we employ a non-compartment turnover model that due to the short measurement time mainly represents the flux of glutamine through plasma, the central compartment.



**Figure 3.** *Distribution of an injected substance in central compartment and subsequently in other compartments/tissues.*

### 4.3 TRACER METHODOLOGY TO ESTIMATE ENDOGENOUS PRODUCTION

A tracer is an isotope labelled version of the substance of interest (tracee) and can be used to study its metabolism. A perfect tracer should biochemically behave and be metabolized in the same way as the tracee, but still be detectable as different from the tracee by some analytical method. We use stable isotope labelled  $[1-^{13}\text{C}]$  glutamine and ring- $^2\text{H}_5$ -phenylalanine, which both have a higher molecular mass than the most common natural form and can be measured separately by mass spectrometry.

The concentration of a substance in plasma depends upon the rate of appearance ( $R_a$ ) and the rate of disappearance ( $R_d$ ), i.e. the flux into and out of plasma. In steady-state conditions, equilibrium prevails and  $R_a = R_d$ . The  $R_a$  is the sum of the production of the substrate (endogenous  $R_a$ ) and the dietary intake of the substrate (exogenous  $R_a$ ) that appears into plasma.  $R_d$  represents the substrate that leaves plasma and is comparable to clearance. To measure the  $R_a$  and  $R_d$ , the tracer can be introduced into the plasma pool either by a constant infusion or by a bolus injection (99). Most glutamine kinetic studies have used the constant infusion approach (100). During a constant infusion of the tracer, the concentration of the tracer in plasma will eventually reach a steady state. At that time, a single plasma

sample can give the ratio between the tracer and the tracee, and the percentage of the substance that is labelled can be calculated. This is called the enrichment and is often expressed as molar percent excess (MPE).

$$MPE = \frac{TTR}{TTR + 1} \times 100\%$$

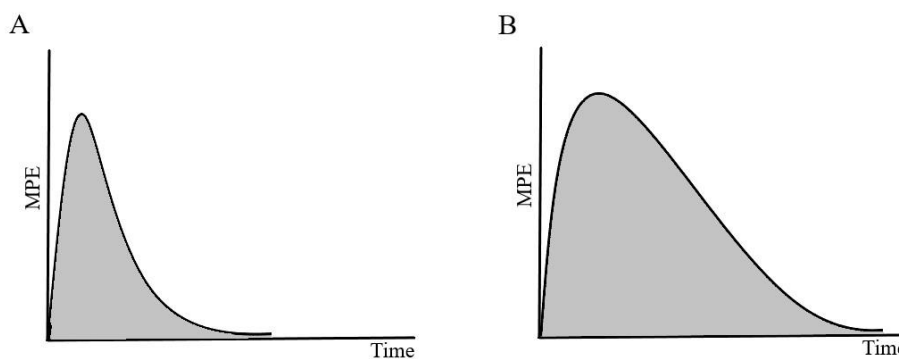
where TTR is the tracer/tracee ratio.

To avoid that the tracer affects the tracee metabolism, the enrichment is kept as low as possible, while still allowing detection. This can be supported by the absence of significantly elevated plasma concentrations of the tracee and affected hormones. Because the  $R_d$  for tracer and tracee is equal, the ratio depends on the  $R_a$ , as it dilutes the tracer in the plasma pool (99). Because of the complex and uneven distribution of glutamine it can be difficult to achieve a steady state. Therefore, it is important that the subject has an overall stable metabolic situation during the study time. The constant infusion method for glutamine requires an infusion of at least 4-6 hours. ICU patients are dynamic and have frequent medical and nutritional alterations, which makes a shorter protocol more relevant and feasible. Therefore, a tracer bolus injection method was established and used in this project.

A tracer bolus dose will give a quick peak in plasma followed by a decay curve of the enrichment. Again,  $R_d$  is assumed to be the same for tracer and tracee. Since the enrichment depends on the ratio, the slope of the decay curve depends upon the  $R_a$  that dilutes the tracer, as illustrated in Figure 4.

$R_a$  ( $\mu\text{mol/kg/min}$ ) is calculated by the formula:  $R_a = \frac{\text{Dose}}{\text{AUC}}$

where Dose is the amount of tracer injected in  $\mu\text{mol}$  per kg body weight and AUC is the Area Under the Curve of the MPE versus time plot.



**Figure 4.** Decay plots after a tracer bolus dose. A) A high rate of appearance ( $R_a$ ) will give a quicker dilution, a steep decay curve and a smaller area under the curve. B) A low  $R_a$  will give a slower dilution, a flatter decay curve and a larger area under the curve.

The glutamine entering plasma can derive from exogenous supply or endogenous release from cells. Enteral supply affects plasma concentrations only to a small extent due to splanchnic extraction and was therefore disregarded in study IV (14, 15). If the patient had parenteral supply, this was subtracted in the calculations.

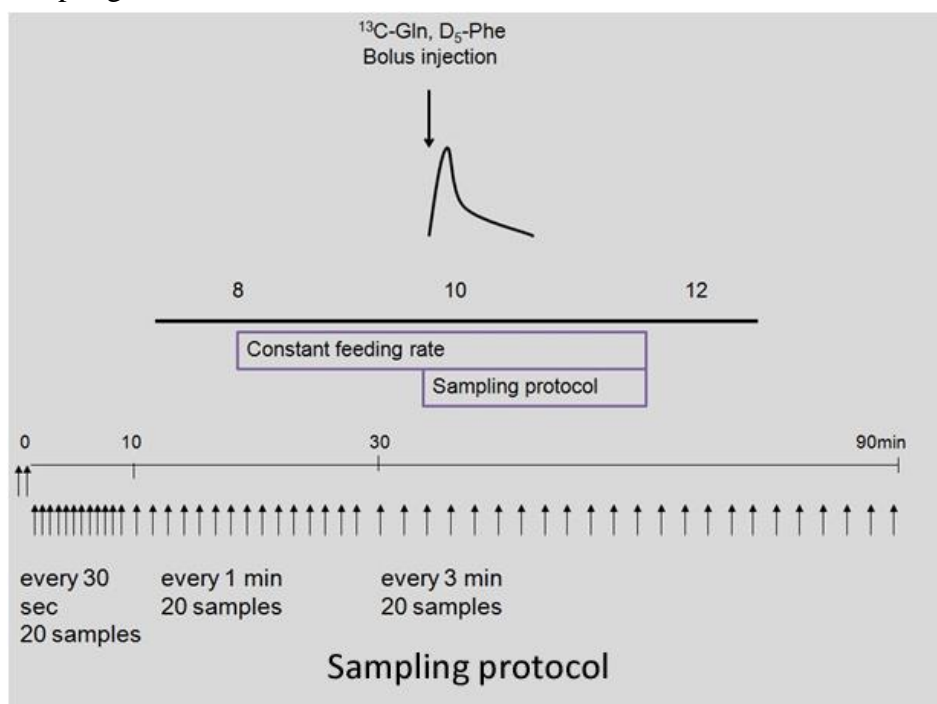
The endogenous  $R_a$  (endo $R_a$ ) of glutamine in plasma can originate either from de novo synthesis or protein break down in cells. To differentiate them, we use a phenylalanine tracer together with the glutamine tracer to estimate protein breakdown. The  $R_a$  of phenylalanine is calculated in the same way as for glutamine. However, as it is an essential amino acid that cannot be synthesized by humans, the  $R_a$  is assumed to come from protein breakdown only. By estimating the fraction of phenylalanine compared to glutamine from protein breakdown it is then possible to calculate glutamine  $R_a$  from protein breakdown and de novo synthesis respectively. A whole-body protein content of 7% glutamine and 4,2% phenylalanine was assumed for this calculation (101).

Clearance is the volume of plasma from which the substance is completely removed per unit time (L/min). Glutamine clearance was calculated by the formula:

$$Clearance = \frac{(\text{endo}R_a \times \text{body weight})}{\text{plasma concentration}}$$

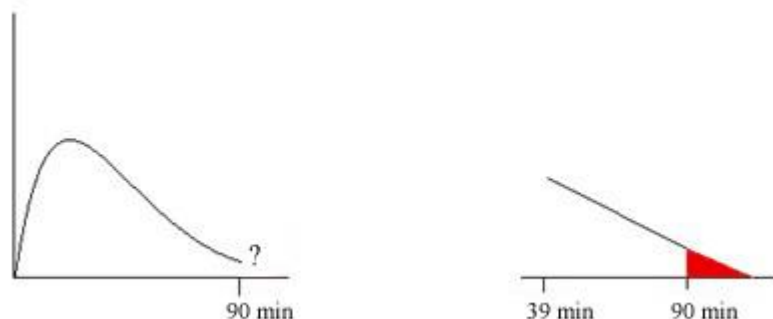
#### 4.4 BOLUS INJECTION METHOD

Subjects received an intravenous bolus injection of both glutamine and phenylalanine labelled with a stable isotope ( $1\text{-}^{13}\text{C}$ -glutamine 3 mg/kg and ring- $^2\text{H}_5$ -phenylalanine 0.3 mg/kg). A baseline sample was obtained prior to the bolus, next the bolus was given over 20 seconds, followed by 60 blood samples over 90 min to get a decay curve. A catheter in the radial artery is used for blood sampling, every 30 sec up to 10 min, then every minute up to 30 min and finally every 3 min up to 90 min (figure 5). Each blood sample is 0.5 mL resulting in a total sampling volume of 30 ml of blood.



**Figure 5.** An illustration of the sampling protocol.

Measurements stop at 90 minutes. However, the decay curve is not completely back to baseline at that time (figure 6). The final slope of the curve levels out and, therefore, the AUC after 90 minutes was extrapolated from the slope of the last measurements that appear to be on a straight line in the log domain of the curve ( $\lambda z$ ).



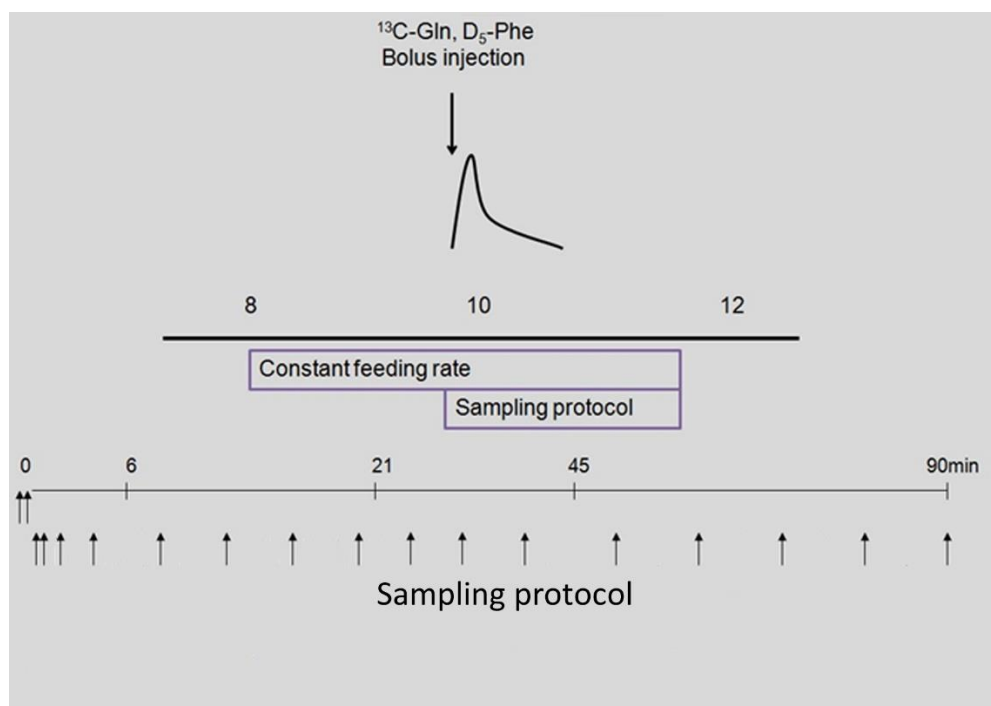
**Figure 6.** Illustration of the extrapolation of the decay curve after 90 minutes.

When the method was developed, this rather extensive sampling protocol was deemed necessary. Fewer initial samples could give significant differences from the default sampling protocol as the initial peak is not captured accurately, which tends to underestimate AUC,

resulting in a higher rate of appearance. Moreover, a shorter time period would have a significant effect on the calculated rate of appearance, as the extrapolation of the tail of the curve down to baseline becomes inaccurate as the curve has not levelled out enough yet. In summary, to achieve best accuracy both peak and tail needed to be well characterized.

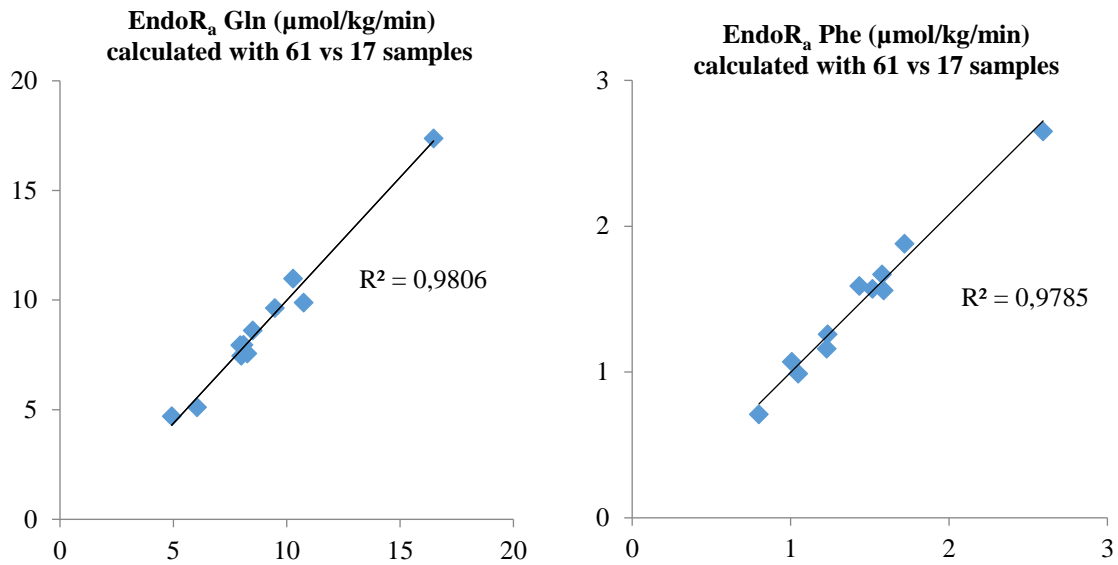
#### 4.4.1 Simplified sampling protocol

In study IV, a need for a simplified protocol was detected, as the frequent sampling jeopardized the arterial lines of the ICU patients. Hence, the protocol was substantially downsized from 61 to 17 samples. In the new protocol, a baseline sample was collected before the bolus injection, and after the bolus injection of tracer, blood was collected at 0.5, 1, 2, 4, 8, 12, 16, 20, 25, 30, 39, 51, 60, 69, 81 and 90 min.



*Figure 7. An illustration of the simplified sampling protocol.*

Before this alteration, calculations were made to ensure that the tracer kinetics could be defined with a similar accuracy from fewer samples. To capture the peak correctly, there is still more abundant sampling right after the bolus injection, then sparser towards the end, but the 90 minutes protocol is intact.



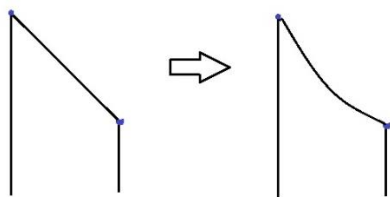
**Figure 8.** Calculations to validate a reduction of the number of samples needed to determine a correct AUC and ensuring that the tracer kinetics could be defined with a similar accuracy from fewer samples. The endoR<sub>a</sub> for both glutamine (Gln) and phenylalanine (Phe) were calculated with both 61 and 17 samples, using data from the first eleven patients of study IV.

To overcome the problem with overestimation of the AUC the linear-up log-down trapezoidal method was used instead of a linear only trapezoidal model.

When the AUC is calculated using the trapezoidal model, the difference between each time point makes up one piece, looking like this:



Then all the pieces are added together to give the total AUC. However, the actual slope is curved and when the line between the timepoints is straightened, it results in a too large AUC. Fewer time points make these lines longer and enhances this effect, which results in a larger overestimation of AUC. If the calculation is done in the log domain instead (linear-up log-down trapezoidal method), the result is a slightly curved line, which gives more accurate results.



## 4.5 STATISTICAL METHODS

In table 2, an overview of the statistical methods used is presented. For normally distributed continuous data Student's t-test was used, whereas Mann-Whitney u-test or Wilcoxon signed rank test was used when values were not normally distributed, according to Shapiro Wilk's normality test. Categorical data was analysed with Fisher's exact test.

In study I, substudies 1 and 3, were analysed with a paired t-test, as volunteers acted as their own controls. In substudy 2, the effect of alanyl-glutamine and parenteral feeding was analysed using analysis of variance (ANOVA), followed by Dunnett's post-hoc test.

In study II and V variables showing statistical significance ( $p < 0.05$ ) in univariate analysis were included in a forward stepwise multivariate regression or a multiple logistic regression to exclude confounders.

Spearman's rank correlation was used to compare plasma glutamine concentrations with liver function measured as MELD and Child-Pugh score (study III), clearance (study IV) and variable parameters (study V), whereas linear regression was used to compare plasma concentrations to rate of appearance (study IV).

In study III, high plasma glutamine values (cut-off 930  $\mu\text{mol/L}$ ) were related to Child-Pugh and MELD scores by receiver operator characteristics (ROC) curves.

In study V, Kaplan Meier and log rank test was used for survival analysis and comparison between the groups. Also, a ROC curve analysis of six-months mortality and plasma glutamine concentration was performed to identify a cut-off for predicting mortality.

	<b>Descriptive statistics</b>	<b>Statistical tests</b>
<b>Study I</b>	Mean, standard deviation	Paired t-test Analysis of variance (ANOVA), Dunnett's post-hoc test
<b>Study II</b>	Median, interquartile range	Mann-Whitney U-test, Wilcoxon signed rank test, Fisher's exact test, Univariate logistic regression, Stepwise forward multiple logistic regression
<b>Study III</b>	Median, range	Spearman's rank correlation, Receiver operating characteristic (ROC) curves
<b>Study IV</b>	Mean, standard deviation Median, range	Student's t-test, Mann-Whitney U-test, Linear regression, Spearman's rank correlation
<b>Study V</b>	Mean, standard deviation Median, interquartile range	Student's t-test, Mann-Whitney U-test, Fisher's exact test, Pearson's correlation, Spearman's rank correlation, Kaplan Meier survival analysis, Log rank test, Univariate logistic regression, Multiple logistic regression ROC curve

**Table 2.** Overview of statistical methods.



## 5 MAIN RESULTS AND DISCUSSION

### 5.1 STUDY I AND IV

A tracer dose of 3 mg/kg  $^{13}\text{C}$ -glutamine was chosen for the bolus injection method, as that gave a reliable decay curve and only resulted in a short peak in plasma glutamine concentration from 500  $\mu\text{mol/L}$  to 1200  $\mu\text{mol/L}$  over the first two minutes. Insulin concentration was unaffected.

To confirm reproducibility, four subjects were measured on two occasions. The coefficient of variation between two measurements was 5.5%, whereas the GC-MS analytical coefficient of variation was found to be 4.1%. Hence, the variation in rate of appearance was mostly due to the analytical variation.

Intravenous alanyl-glutamine supplementation increased plasma glutamine concentrations. Parenteral nutrition together with alanyl-glutamine gave an increase in glutamine  $\text{endoR}_a$ , whereas alanyl-glutamine supplementation only did not. Thus, the increased plasma glutamine concentration did not have a negative feedback mechanism on the glutamine  $\text{endoR}_a$ , indicating that glutamine production is not directly affected by the plasma concentration.

As an alanyl-glutamine dipeptide is used for glutamine supplementation, the effect of a pure alanine infusion was studied. Alanine did not have any detectable effect on glutamine  $\text{endoR}_a$  and is therefore probably not a significant stimulus for glutamine synthesis.

The bolus injection method is more convenient in an ICU patient group as the study protocol is only 90 minutes, which increases the chance that the patient is metabolically stable during the measurement period, compared to the constant infusion method that needs 4-6 hours of metabolic stability. For the study to interfere as little as possible with the general care of the ICU patients, it is also important that the method is valid during parenteral nutrition and glutamine supplementation.

The  $\text{endoR}_a$  measured by the bolus method in postabsorptive, healthy volunteers (study I) ranged between 4.5-7.5  $\mu\text{mol/kg/min}$ , mean  $5.7 \pm 0.7$ , which is comparable to previous reports from constant infusion studies, as depicted in Figure 9A (102, 103).

In study IV, glutamine  $\text{endoR}_a$  was measured in nineteen critically ill patients. Eight patients had a hypoglutaminemia ( $<400 \mu\text{mol/L}$ ) and eleven patients had plasma glutamine concentration within the reference range (400-930  $\mu\text{mol/L}$ ), mean concentration was 468 (range 199-830)  $\mu\text{mol/L}$ . Glutamine  $\text{endoR}_a$  was  $8.9 \pm 2.8 \mu\text{mol/kg/min}$ , of which  $7.0 \pm 2.7 \mu\text{mol/kg/min}$  was de novo synthesis. There was a significant correlation between plasma concentration and both glutamine  $\text{endoR}_a$  and de novo synthesis,  $R^2 0.35$  for both.

The  $\text{endoR}_a$  in the ICU patients included in this study corresponds to 1.9 g of glutamine/kg/day (1.5 g/kg/day de novo production), which is high compared to previous reports, see figures 9B and 9C. ICU patients fasted for 10-24 hours are reported to have

glutamine endoR<sub>a</sub> between  $4.9 \pm 0.3$  and  $5.7 \pm 0.3$   $\mu\text{mol/kg/min}$  ( $1.0\text{-}1.2$  g/kg/day) (33, 42, 104). A major difference is that most included patients in our study were in the fed state, whereas most previous studies on glutamine rate of appearance are performed on postabsorptive patients. However, the effect of nutrition on glutamine endoR<sub>a</sub> in ICU patients has been scarcely studied, and the few studies done give conflicting results. Addition of parenteral nutrition has been reported to have no effect on glutamine endoR<sub>a</sub> compared to the postabsorptive state (33). Likewise, a report on the effect of enteral nutrition on glutamine rate of appearance in healthy volunteers given high dose cortisol, showed no difference compared to fasted state, and a decrease in glutamine endoR<sub>a</sub> when extra glutamine was added to the nutrition (105). As cortisol stimulates glutamine synthesis, it is, however, possible that glutamine synthesis rates were already enhanced in these healthy subjects, before nutrition was provided (48, 49). In contrast, the results from study I show that parenteral nutrition increases rate of appearance in healthy subjects. Similarly, both parenteral and enteral nutrition have been reported to increase glutamine endoR<sub>a</sub> in healthy volunteers (106, 107). We hypothesize that a continuous provision of other amino acids results in a higher substrate availability for glutamine synthesis.

Another difference in our protocol is the use of <sup>13</sup>C glutamine tracer, where most other studies used <sup>15</sup>N glutamine, either on the amino group (2-<sup>15</sup>N) or on the amide group (5-<sup>15</sup>N). The carbon skeleton is more stable than the amino and amide groups that can both be easily lost by transamination. The <sup>13</sup>C tracer has been reported to show a 15-22% higher endoR<sub>a</sub> than the <sup>15</sup>N tracer (n=8) (6, 103). Possibly, this is the result of a recycling of the nitrogen label that may then re-enter the plasma pool attached to a new glutamine molecule (103). This would, in turn, result in a slower dilution of the tracer and thereby a lower rate of appearance. The amide group is hydrolysed in the first step and is therefore more likely to be recycled. Accordingly, the 5-<sup>15</sup>N tracer gives a lower endoR<sub>a</sub> than the 2-<sup>15</sup>N tracer (100).

The shorter study time in our protocol also affects the results in a way that will give a higher endoR<sub>a</sub>. Glutamine is a small molecule that moves back and forth over cell membranes. When the infusion is given over a long time, a considerable amount of the tracer will have entered the intracellular space, which increases the likelihood that a labelled glutamine molecule re-enters plasma. This will blur the picture and give a lower endoR<sub>a</sub>, together with <sup>15</sup>N recycling making “new” glutamine molecules look like tracers that will therefore not be detected. Also, the tracer distribution to other pools increases over time and thus, the endoR<sub>a</sub> will more and more represent the whole-body glutamine flux, which is lower than the exchange in the plasma pool. This is demonstrated when the infusion time is stretched to eleven hours, resulting in a lower endoR<sub>a</sub> (6). In comparison, our shorter protocol will be less affected by label recycling and can therefore be argued to be a truer reflection of the glutamine flux into plasma.

Other possible explanations for our higher glutamine endoR<sub>a</sub> are the inclusion of patients with a higher plasma glutamine concentration (mean 427 and 454  $\mu\text{mol/L}$ ) compared to other

measurements in ICU patients (mean 237, 340 and 357  $\mu\text{mol/L}$ ) (33, 42, 104), which according to our results would result in a higher rate of appearance. Moreover, the patient cohorts differ and may not be comparable. Two of the endoR<sub>a</sub> reports are based on postoperative patients in the acute phase after emergency abdominal surgery (33, 104), which are likely metabolically different from our stabilised patients with mostly respiratory failure. The metabolic status and level of inflammation and stress are likely to affect results. Burn patients fasted for eight hours had a glutamine endoR<sub>a</sub> of  $7.2 \pm 1.5 \mu\text{mol/kg/min}$  (108). These patients constitute a special cohort with high metabolism, but they were also studied during a stabilised phase.

Regardless whether the glutamine endoR<sub>a</sub> in study IV is overestimated or more correct compared to others, the assumptions made can be assumed to be equal for all patients and thus, the correlation to plasma concentration is still valid.

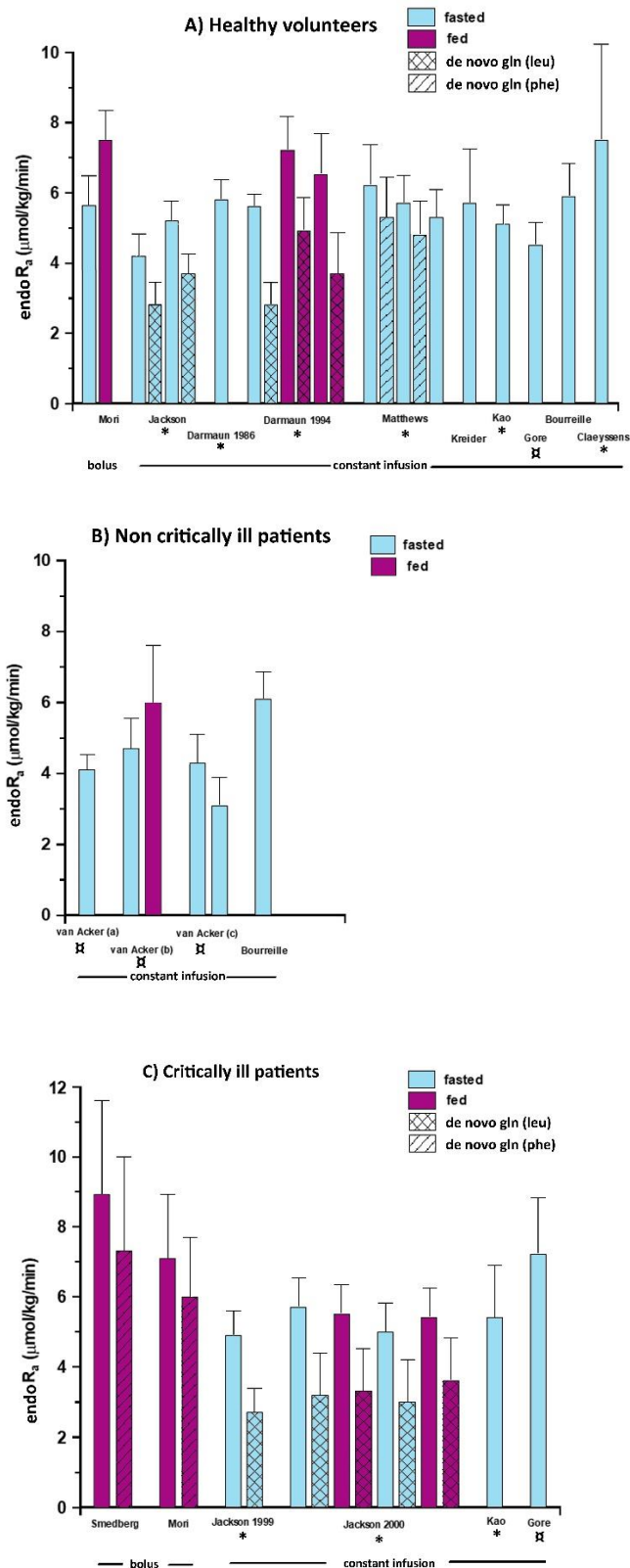
In addition, most previous studies use a leucine tracer instead of phenylalanine to estimate whole body protein break down. These two tracers have been reported to give similar values when used in parallel (6). However, in studies on healthy volunteers using phenylalanine the estimated protein break down is 15-21% of total endoR<sub>a</sub> (102) as opposed to 30-50% when leucine is used (104, 106). In our studies, the estimated proportion of glutamine deriving from protein break down is 15-18%, whereas others, using leucine, report this proportion to be 30-45% (33, 104) in ICU patients. Hence, it is possible that this difference is partly explained by the use of different amino acid tracers.

Due to these differences, the results of study IV are most closely comparable to the previous report from our working group, applying the same method on fully fed ICU patients, with similar glutamine endoR<sub>a</sub> (76).

Because of the non-steady state in other pools, the endoR<sub>a</sub> probably primarily represents the transport of nitrogen between tissues through the plasma pool, the glutamine-glutamate cycle, rather than the intracellular production (100). Glutamate is the substrate for GS and glutamate plasma concentration has been reported to positively correlate with the efflux of glutamine from skeletal muscle (26). In burn patients it has been reported that glutamine production and utilization within the muscle cells are both increased to a similar extent and that the glutamine that appears in plasma originates from an increased net export of free glutamine in muscle that is maintained despite the lower intracellular concentration (25). A complete equilibration with an isotopic steady state between different glutamine pools will not be possible, therefore an estimate of the flux through plasma may be the best possible reflection of glutamine production. However, as our aim is to have a better understanding of plasma concentrations, the flux into plasma is the relevant measure, not intracellular production that never enters plasma.

A limitation when studying the altered glutamine kinetics in the critically ill, is that the patients have a low plasma glutamine already at arrival. The profound muscle depletion is also present already at ICU admission, or soon after admission. These conditions are then

maintained throughout the ICU period; the patients with low admission plasma glutamine in general continue to stay low, and the intracellular muscle glutamine remains depleted. A new steady state of altered glutamine balance is created, that is quite different from that of a healthy person, especially considering concentration gradient across cell membranes. The conditions of this new steady state can be studied, but the transition between the two states is not accessible in clinical practice. Giving endotoxin to healthy volunteers is a possible way to simulate the alterations seen in early sepsis, however these changes will likely be less dramatic than the full scale acute response of life-threatening critical illness (97).



**Figure 9.** Overview of endoR<sub>a</sub> and de novo synthesis of glutamine from previous reports.

\* <sup>2-15</sup>N tracer    ⚡ <sup>5-15</sup>N tracer

A) Healthy volunteers, study I bolus method compared to previous constant infusion studies (42, 100, 102-106, 108, 109). B) Non-critically ill patients with abdominal diseases (6, 109-111). C) Critically ill patients, study IV compared to previous reports (33, 42, 76, 104, 108).

## 5.2 STUDY II, III AND V

Study II was designed after The Scandinavian Glutamine Trial, showing a reduced ICU mortality in the glutamine supplemented group, which was abolished six months after ICU discharge (78). Two questions appeared; (i) did the patients develop hypoglutaminemia when supplementation was discontinued, and (ii) would plasma glutamine concentrations post ICU, in a non-supplemented state, be predictive for six months mortality? A patient cohort similar to the treatment group was therefore studied, and the results were that (i) ICU survivors had normal plasma glutamine concentrations post-ICU, without supplementation, and (ii) the post-ICU plasma glutamine concentration was not a mortality predictor.

When supplementation was discontinued, plasma glutamine concentrations decreased, but remained within the reference range. Out of the 210 post-ICU samples collected, 89% were within the reference range for plasma glutamine concentration; only six were  $<400 \mu\text{mol/L}$  and sixteen  $>930 \mu\text{mol/L}$ . A majority (63%) of the high values were measured during readmission to the ICU, with glutamine supplementation reinstated.

In a post hoc analysis of the samples collected on the day of discharge, during ongoing supplementation, twelve-months survivors ( $n=40$ ) had lower plasma glutamine concentrations, and lower discharge SOFA scores than non-survivors ( $n=23$ ). A stepwise multiple regression analysis indicated that discharge plasma glutamine concentration added to the mortality predictive power of discharge SOFA score. A higher concentration during supplementation, still within the reference range, appeared to be a sign of unfavourable prognosis. However, the interpretation of this post hoc finding should be done with caution. Especially as the same patients after discontinuation of supplementation no longer had a plasma glutamine concentration associated with an increase in mortality.

Previous reports suggest that around one third of unselected patients admitted to a general ICU are hypoglutaminemic at admission and that this is associated with a higher mortality (36, 53). In the report from our working group, hyperglutaminemia was also associated with a higher mortality (36). In parallel, the results from the REDOX trial associate a high dose glutamine supplementation to a higher mortality (72). A third argument to investigate hyperglutaminemia is the well-known, but unclearly defined, association between high plasma glutamine concentrations and liver conditions (73). As earlier publications were indistinct to the type of liver condition, our first step was to design a study to explore plasma glutamine status in four different groups of liver insufficiency (study III). Thereafter, we launched a new study on ICU admission plasma glutamine concentrations including a more extensive clinical characterisation as compared to earlier studies (study V).

Four groups of patients with liver insufficiencies were studied. Patients with chronic liver failure (group A,  $n=40$ ) were sampled once at a routine visit to a hepatology outpatient clinic. In this group 78% had plasma glutamine concentrations within the reference range, and 20% had hyperglutaminemia ( $>930 \mu\text{mol/L}$ ). After twelve months, mortality was 48% and another 20% were liver transplanted.

Patients with acute-on-chronic liver failure (group B, n=20) and patients with acute fulminant liver failure (group C, n=20) were followed every third day from ICU admission. At admission 60-65% were hyperglutaminemic. After twelve months, patients in group B had a mortality of 85% and the remaining 15% were liver transplanted. Patients in group C had the lowest twelve-months mortality (15%), although 25% were liver transplanted.

Patients subjected to major hepatectomy (50-90% of the liver removed) were recruited in a high-dependency ward the day after surgery (group D, n=20). In this group 70% had plasma glutamine concentrations within the reference range and 20% had hyperglutaminemia. Among the hyperglutaminemic cases at inclusion (n=4), two died during the acute postoperative phase and two were still alive after twelve months.

To grade liver insufficiency, Child-Pugh and MELD scores were used. These scoring systems were developed to estimate prognosis in chronic liver failure and could therefore be assumed to indicate a more advanced liver disease (112, 113). Although not validated for acute liver failure or post hepatectomy liver failure, they are widely accepted as a reflection of the severity of liver insufficiency, and they were therefore used in all patient groups to enable comparison. When all four groups were combined (n =100) there was a positive correlation between plasma glutamine concentration and the degree of liver failure, reflected as Child-Pugh and MELD score. The four groups of subjects with liver conditions recruited in study III are case series, which makes comparisons between the groups and subjects in other studies difficult. The main result of the study was the identification of acute-on-chronic and acute fulminant liver insufficiency as the conditions most frequently associated with hyperglutaminemia, and the perspective that the survival prognosis in these groups was quite different.

In study V, plasma glutamine concentration was measured within one hour from admission in consecutive ICU admissions (n= 269). Admission hyperglutaminemia  $\geq 930$   $\mu\text{mol/L}$  (n=26) was associated with a higher six months mortality compared to admission plasma glutamine  $< 930$   $\mu\text{mol/L}$  (n=243); 46% versus 18%. In the group of patients with hyperglutaminemia, 85% had liver disease and/or signs of acute liver damage at ICU admission and the two major admission diagnoses were postoperative liver transplant and acute liver failure.

When the study protocol was designed, postoperative care of liver transplantation patients recruited from the waiting list were not identified as a subgroup that needed special attention. However, when the results were analysed it was obvious that a post hoc separation of these patients was motivated, as they had no six-months mortality, the admission criterion was relative, and the median ICU stay was less than 24 hours. Out of the 26 patients with hyperglutaminemia, seven were admitted postoperatively after liver transplantation, and if they were excluded six months mortality was 63% in the hyperglutaminemic group. When multiple regression was applied to predict six months mortality in all subjects (n=269), hyperglutaminemia, admission SOFA score and age were independent predictors. This finding became stronger when the postoperative liver transplantation patients were excluded

(n=226). Also, the previous finding of a cut-off for predicting six-months mortality of 930  $\mu\text{mol/L}$  was confirmed with a receiver operating characteristic curve, which strengthens hyperglutaminemia as a predictor of poor outcome (36). A unique feature of this study compared to other available studies on plasma glutamine concentrations at admission, is that all samples were collected within an hour after ICU admission, instead of within 24 hours, and that there are no missing values in the included patients.

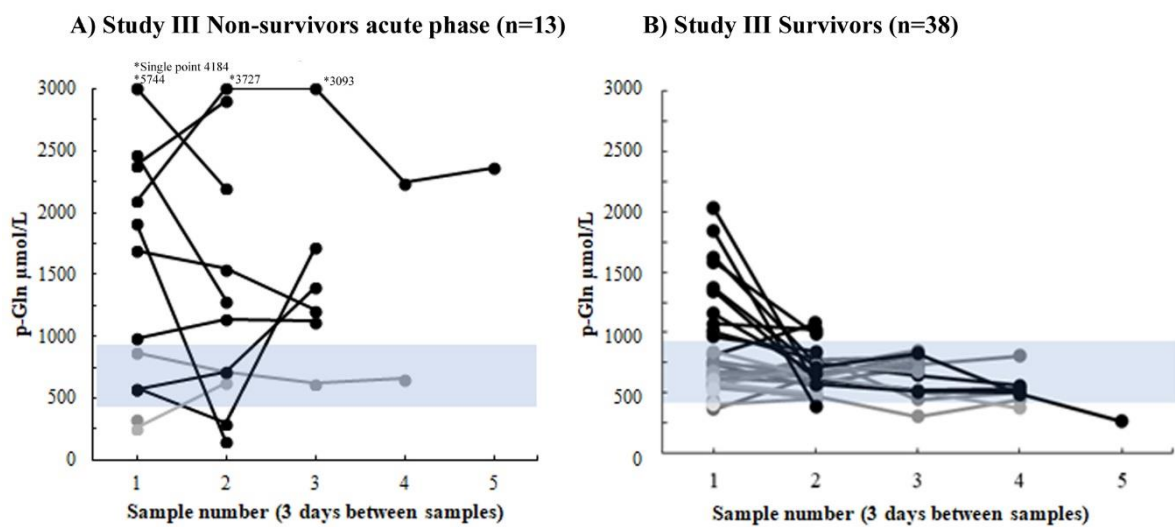
Study V is a report over a secondary aim of a study protocol that focused both on hypo- and hyperglutaminemia. However, the present patient cohort only contained 15% (40/269) hypoglutaminemic patients and there was no increased mortality in this group. As a result, the findings of the previous study from our working group was not reproduced (36). The most likely reason for this is that the exclusion criteria differed, which unintentionally resulted in a different case mix. In the present study several patients were excluded due to a do not resuscitate order at admission or because of an early death before informed consent could be obtained. The exclusion of these two groups constitutes the main difference between the cohorts included in the two studies. Although unknown, it is possible that these excluded patients had a high prevalence of hypoglutaminemic patients. This finding emphasises the problem to compare studies in the ICU with different inclusion criteria, which may result in completely different cohorts in this very heterogenous patient group, even when included in the same ICU.

The results of study III and V emphasises the role of the liver in glutamine metabolism. The positive correlation between hyperglutaminemia and bilirubin, ALT and AST in study V is consistent with previous reports (31, 57) and highlights the connection between liver disease and high plasma glutamine concentrations. Although liver failure has repeatedly been reported in connection with hyperglutaminemia before (31, 36, 57, 73), this is now clearer described by the link to acute liver decompensation and the finding that a more advanced liver disease is more likely associated with hyperglutaminemia.

The mechanism that connects a high plasma glutamine concentration to a higher mortality is unknown. However, the present results do not support that a high glutamine concentration per se is toxic. Rather, it is a biomarker that indicates a severe metabolic malfunction that is a highly unfavourable prognostic sign, if present at ICU admission together with organ failures. The connection between liver decompensation and hyperglutaminemia supports this theory. The finding that hyperglutaminemia had no connection to mortality in the patients that had already had a liver transplantation also suggests that the glutamine concentrations per se are not a problem, but rather the metabolic capacity, which has been restored in these patients. The patients in study III show a similar pattern (figure 10). The same trend can be seen in a report of plasma glutamine concentrations over time in select ICU long-stayers. The mortality of patients with a plasma glutamine concentration  $>830 \mu\text{mol/L}$  in a second sample was 100%. Likewise, the survivors among the hyperglutaminemic patients exhibited a dramatic decrease in plasma glutamine concentration. Also, 25% of non-survivors show a sudden,



dramatic increase to hyperglutaminemic levels, without the influence of glutamine supplementation (31). This trend can, to some extent, also be seen in the temporal pattern of post ICU plasma glutamine concentrations (study II), where the outliers that showed dramatic increases and pronounced instability in plasma concentrations died shortly afterwards. Hence, it is possible that hyperglutaminemia is a biomarker associated with a failing energy metabolism, incapable of keeping up intra/extracellular concentration gradients at the end of life. Still, it is reversible, and the patient outcome seems to be highly dependent on the ability to normalise plasma glutamine concentration, or the restoration of metabolic balance, that this possibly indicates. Thus, the specificity of hyperglutaminemia as a biomarker for unfavourable outcomes is lower than the sensitivity at ICU admission, particularly in the case of a single organ failure.



**Figure 10.** Plasma glutamine concentrations over time (measured 3 days apart) in patients with acute fulminant or acute-on-chronic liver failure from ICU admission, study III divided into A) acute phase non-survivors (died within one week after the last sample) and B) survivors. Light blue area indicates the reference range (400-930  $\mu\text{mol/L}$ ). Black lines indicate patients with at least one value  $>930 \mu\text{mol/L}$ .

## 6 CONCLUSIONS AND FUTURE PERSPECTIVES

The main conclusions are:

- A tracer bolus injection method was validated to measure glutamine endogenous production in humans with good reproducibility and small variation (study I).
- There was a positive correlation between plasma glutamine concentration and endogenous glutamine production in critically ill patients, where 35% of the variability in endogenous production could be attributed to variability in plasma glutamine concentration, or the other way around (study IV).
- In the post ICU period without exogenous glutamine supplementation, plasma glutamine concentrations were within the reference range and were not related to mortality (study II).
- In liver failure, regardless of aetiology, severity and course of illness, a high plasma glutamine concentration was a common finding, although most frequent in patients with acute fulminant and acute-on-chronic liver failure. There was a positive correlation between the severity of liver failure and plasma glutamine concentration (study III).
- Hyperglutaminemia ( $\geq 930 \mu\text{mol/L}$ ) at ICU admission was an independent predictor for high mortality. A majority of these patients had a liver condition, although hyperglutaminemia was also observed in patients without signs of liver affection (study V).

The role of glutamine in critical illness is still not settled. The finding that low plasma concentrations correlates with a lower endogenous production keeps the possibility open that there is a cohort of critically ill patients with too low glutamine availability who would benefit from exogenous glutamine supplementation (114). In addition, there is only one study on exogenous glutamine supplementation in critical illness that demonstrate harm (72). It is dissatisfying that the mechanism behind that harm has not been explored and explained. Our observations give no indication of toxic effects of glutamine, per se. Hyperglutaminemia was potentially reversible even in cases with very high plasma concentrations. Hence, glutamine kinetics in critical illness needs to be further clarified.

The simplified version of the tracer bolus injection method makes it possible to gain more information of glutamine endoR<sub>a</sub> in the ICU setting. In critically ill patients with glutamine plasma concentration in the low or normal range there is a relation to endoR<sub>a</sub>. Still, the

pathophysiology of hyperglutaminemia is so far poorly understood and needs to be studied further. Especially in the context of liver failure, is it to be regarded as a biomarker or as a causative factor to symptomatology? To study glutamine endoR<sub>a</sub> in hyperglutaminemic patients with and without liver failure would therefore be helpful.

The non-compartmental model employed in the tracer bolus method does not allow measurements of the total endogenous glutamine production. It reflects the influx into the central plasma compartment. With repeated measurements over time, it can give information of the endogenous glutamine production over the course of critical illness. The short-term measure of glutamine flux through plasma is probably the best estimate available for endogenous glutamine production in this context, and the most relevant for plasma concentrations of glutamine.

The effort to empirically link hyperglutaminemia, liver conditions, and mortality emphasizes the complexity of these relations. Hyperglutaminemia is more common in liver conditions, increasingly so with more severe liver disease and especially in combination with critical illness. Still, liver failure is not always accompanied by hyperglutaminemia, and the condition also exists in patients without signs of compromised liver function. The mortality risk in these patients is high, but the prediction is not absolute. As these patients are not common, our cohort was not big enough for this aspect of the study, but it served as hypothesis generating. Future observational studies should screen for hyperglutaminemia at admission and longitudinally follow plasma concentration and endoR<sub>a</sub>. A sustained high or unstable high plasma glutamine concentration may be associated with unfavourable outcomes, this is a hypothesis generating observation from our case series in studies II and III, that could be further explored.

A conclusion of this thesis is that hyperglutaminemia at ICU admission is associated with a higher mortality risk. This is not a new finding, but it emphasizes that plasma glutamine concentration is of importance for the critically ill, and as such deserves attention. Future studies with analyses of plasma glutamine concentration daily to follow the course longitudinally combined with measurements of glutamine endoR<sub>a</sub> will contribute to a better understanding. The ultimate question if plasma glutamine concentration is just a biomarker or if it also gives signal of a deficit and/or an impaired handling of glutamine is still pending.

## 7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kritisk sjukdom innebär en livshotande svikt i ett eller flera vitala organ, vilket medför en extrem stress där kroppen pressas till det yttersta för att försöka överleva. Det leder i sin tur till flera kroppsliga förändringar som är unika för detta tillstånd. Intensivvård kan stötta sviktande organsystem och hålla patienten vid liv medan kroppen får en chans att återhämta sig.

Syftet med denna avhandling är att kartlägga delar av omsättningen av glutamin hos kritiskt sjuka patienter. Glutamin är en aminosyra, de beståndsdelar som bildar proteiner. Det är den vanligast förekommande aminosyran i kroppen och varje dag produceras 65-85 gram, framförallt i muskelceller. Glutamin har flera viktiga funktioner i kroppen utöver att bygga proteiner. Det är den primära energikällan för vita blodkroppar och tarmceller. Den används för att bygga DNA, RNA, glukos och viktiga antioxidanter. Glutamin påverkar även processer för cellöverlevnad och celledelning, samt används för att transportera kväve/ammoniak mellan vävnader och för att producera bikarbonat för att reglera pH-balansen.

Studier har visat att både för hög och för låg glutaminkoncentration i plasma vid inskrivning till intensivvårdsavdelningen (IVA) är kopplat till en ökad dödlighet. Man vet också att muskelceller, som normalt innehåller mycket höga halter av glutamin, tömmer 70-80% av sin fria glutamindepå under den akuta fasen av kritisk sjukdom. Därefter fortsätter en omfattande nedbrytning av skelettmuskulaturen och dess proteiner. En av orsakerna tros vara att upprätthålla en hög utsöndring av glutamin till resten av kroppen. Kombinationen av att kroppens glutaminförråd i muskler utarmas och att patienter med låg plasma-glutaminkoncentration har en ökad dödlighet, har gett upphov till teorin att kritisk sjukdom kan leda till glutaminbrist. Omkring 30% av IVA-patienterna har låg plasma-glutaminkoncentration och hypotesen är att de kan gynnas av extra glutamintillförsel. Ett flertal studier har genomförts men de skiljer sig stort i vilka patienter som har inkluderats, vilken dos glutamin som har getts, om de tillförts intravenöst eller via tarmen, samt när och hur länge man givit glutamin. Resultaten är därmed spretiga och svåra att jämföra. Flera studier har visat att glutamintillförsel leder till minskad dödlighet, kortare sjukhusvistelse, snabbare sårhäkning och färre infektioner. Men en stor, randomiserad, klinisk studie där man gav väldigt hög dos glutamin (dubbelt jämfört med tidigare studier) till de allra sjukaste patienterna på IVA, i det akuta förloppet, visade istället på ökad dödlighet i gruppen som fick glutamin. Innan dessa resultat publicerades sågs glutamin som något helt ofarligt som kunde gynna vissa patienter och det ansågs därför rimligt att ge det till alla. Nu är ståndpunkten istället att om det kan vara skadligt för några så ska det inte ges till någon.

Tyvärr har ingen randomiserad studie på glutamintillförsel mätt plasma-glutaminkoncentration och selekterat patienter med låg koncentration. Därför har den

ursprungliga teorin, att det är dessa patienter som skulle gynnas, aldrig testats. För att få klarhet i om glutamintillförsel kan gynna somliga IVA-patienter behövs en större förståelse för vad som orsakar de avvikande glutaminkoncentrationerna. Dels för att förstå om en låg koncentration utgör en brist som behöver ersättas med extra tillskott, men även vilka patienter som utvecklar höga glutaminkoncentrationer och om detta i sig ger en ökad dödlighet.

Avhandlingen består av fem delarbeten.

Studie I och IV: För dessa studier används en isotopmärkt version av glutamin med en kolatom utbytt till  $^{13}\text{C}$  istället för  $^{12}\text{C}$ , vilket gör molekylen något tyngre och den kan då särskiljas i analys. Man mäter sedan hur proportionen av vanligt glutamin och  $^{13}\text{C}$ -glutamin i plasma förändras över tid. Eftersom de försvinner ur plasma i samma takt så beror den minskade proportionen av isotopmärkt glutamin på hur mycket kroppseget glutamin som kontinuerligt flödar in i plasma och späder ut det märkta. Man kan därigenom mäta hur mycket glutamin som produceras. Den tillgängliga metoden med kontinuerlig intravenös tillförsel av  $^{13}\text{C}$ -glutamin är ett tidskrävande protokoll som kräver att patienten är stabil och tillgänglig för studien under 4-6 timmar. Detta är svårt att uppnå hos kritiskt sjuka patienter, så i studie I utvecklades en ny metod för att studera omsättningen av glutamin. Istället för en kontinuerlig tillförsel över lång tid så gavs en hög stöddos (bolusdos) med  $^{13}\text{C}$ -glutamin och sedan tas upprepade blodprover för att mäta hur snabbt proportionen av  $^{13}\text{C}$ -glutamin avtar under 90 minuter.

Studie I består av fyra delstudier med friska försökspersoner:

1. Lämplig bolusdos bestämdes.
2. Reproducerbarhet kontrollerades, det vill säga att upprepade mätningar hos samma person gav likvärdiga resultat.
3. Effekten av intravenös näring- och glutamintillförsel kontrollerades. Att tillföra glutamin ökade plasmakoncentrationen, men påverkade inte den kroppsegna produktionen. Tillförsel av både glutamin och intravenös näringslösning ökade glutaminproduktionen, vilket antas bero på den ökade tillgängligheten av andra aminosyror som omvandlas till glutamin.
4. Intravenöst glutamin ges som en dipeptid; aminosyran alanin ihopkopplad med glutamin. Om alanin direkt omvandlas till glutamin skulle den ökade glutaminproduktionen bero på alanin-tillförseln. Detta skulle kunna maskera en eventuell negativ feedback-mekanism från det tillförda glutaminet. För att kontrollera detta tillfördes enbart alanin, vilket inte hade någon påverkan på glutaminproduktionen.

För att få en ökad förståelse av den förändrade omsättningen av glutamin vid kritisk sjukdom och klargöra varför vissa patienter får låg plasmakoncentration användes bolusmetoden för att mäta kroppsegen glutaminproduktion hos intensivvårdspatienter (studie IV). Produktionen jämfördes sedan mot plasma-glutaminkoncentrationen. Det fanns ett signifikant samband, patienter med lägre glutaminkoncentration hade en lägre produktion. Därmed kan det vara så att vissa patienter har en otillräcklig egen produktion och dessa kan gynnas av extra tillförsel av glutamin.

Studie II: I en tidigare, stor skandinavisk glutaminstudie randomiserades intensivvårdspatienter till att få intravenöst glutamintillskott eller placebo under hela IVA-vistelsen. De som fick glutamin hade en minskad dödlighet på IVA, men efter sex månader såg man inte längre någon skillnad mellan grupperna. Två frågor uppstod; (i) utvecklade patienterna låg glutaminkoncentration när de lämnade IVA och glutamintillförseln avbröts, och (ii) fanns det ett samband mellan plasma-glutaminkoncentration efter IVA och dödlighet? Patienter som fått glutamintillförsel på IVA studerades därför, och resultaten var att (i) IVA-överlevarna hade normala glutaminkoncentrationer, även utan tillskott, och (ii) glutaminkoncentrationen efter IVA hade inget samband med dödlighet.

Studie III och V: Dessa två studier fokuserade på hög plasma-glutaminkoncentration. Eftersom det fanns en känd, men relativt outforskad, koppling mellan höga glutaminkoncentrationer och leversvikt så studerades fyra typer av leversvikt (studie III); kronisk leversvikt, akut-på-kronisk leversvikt, akut leversvikt och leversvikt efter omfattande leverkirurgi. Alla grupper hade individer med hög glutaminkoncentration, men det var betydligt vanligare i de två grupperna med akut svikt. Det fanns också en signifikant koppling till graden av leversvikt, ju mer avancerad svikt, desto större förekomst av hög glutaminkoncentration.

I studie V mättes plasma-glutaminkoncentrationen vid ankomst till IVA på 269 patienter. Av dessa hade 26 hög glutaminkoncentration och i den gruppen dog 46% inom sex månader, jämfört med 18% för resterande patienter. Merparten av patienterna med hög glutaminkoncentration hade akut leverpåverkan och/eller känd leversjukdom, men det förekom även hos patienter utan leverpåverkan. För patienter som kom till IVA för eftervård efter en levertransplantation var överlevnaden efter sex månader emellertid 100% oavsett om de hade hög glutaminkoncentration eller inte. Sannolikt beror detta på att den nya levern snabbt kan återställa balansen hos dessa patienter. Om de levertransplanterade patienterna exkluderades var dödligheten efter sex månader 63% i gruppen med hög glutaminkoncentration. Dock är det ingenting som tyder på att de höga glutaminkoncentrationerna i sig är giftiga. De verkar snarare vara en markör för en kraftig påverkan av ämnesomsättningen, som vittnar om mycket avancerad sjukdom där förloppet är svårt att vända.

Glutaminets roll vid intensivvårdskrävande sjukdom är fortfarande inte klarlagd. Eftersom låga plasmakoncentrationer uppträder tillsammans med en lägre kroppsegen produktion är det möjligt att det finns en grupp kritiskt sjuka patienter med en relativ glutaminbrist. Dessa skulle kunna dra nytta av extra glutamintillskott. Dessutom finns det bara en studie där glutamintillskott till intensivvårdspatienter visar negativa effekter. Det är otillfredsställande att mekanismen bakom detta resultat inte har kunnat förklaras. Därför är det viktigt att glutaminomsättningen i samband med kritisk sjukdom klarläggs ytterligare.

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