Pig interorgan balance studies in health and disease

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Colophon

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Pig interorgan balance studies in health and disease

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Aan mijn moeder,

mijn echtgenoot André,

mijn kinderen Eva en Mats,

Ter nagedachtenis aan mijn vader
## Table of contents

8  
**Chapter 1**  
Short introduction

16  
**Chapter 2**  
Absorption kinetics of amino acids, peptides and intact proteins

34  
**Chapter 3**  
Simultaneous measurement of metabolic flux in portally-drained viscera, liver, spleen, kidney and hindquarter in the conscious pig

52  
**Chapter 4**  
Absence of post-prandial gut anabolism after intake of a low quality protein meal

76  
**Chapter 5**  
Enhanced lacto-tri-peptide bio-availability by co-Ingestion of macronutrients

106  
**Chapter 6**  
Characteristics of a *Pseudomonas aeruginosa* induced porcine sepsis model for multi-organ metabolic flux measurements

132  
**Chapter 7**  
Phenylalanine isotope pulse method to measure effect of sepsis on protein breakdown and membrane transport in the pig
Interorgan balance studies in disease models

Chapter 8
Arginine de novo and nitric oxide production in disease states

Chapter 9
Nitric oxide and L-arginine metabolism in a devascularized porcine model of acute liver failure

Chapter 10
Inhibited jejunal protein synthesis and breakdown in Pseudomonas aeruginosa induced severe sepsis pig model

Chapter 11
Altered transorgan protein kinetics during a Pseudomonas aeruginosa induced severe sepsis in the pig

Summary and conclusions

Chapter 12
Summary and future perspectives

Chapter 13
Samenvatting

Chapter 14
Valorization

Biography

Publications

Dankwoord / Thank you
Short introduction

A healthy human body needs nutrients to be kept in metabolic balance. To keep balance, complex interactions between multiple specialized organs for transportation, digestion, absorption, redistribution, utilization, conversion, breakdown of nutritional substrates during post-prandial and/or post-absorptive stages must occur. These interactions are highly dynamic and are disturbed in many pathophysiological conditions. For instance, amino acid substrate wasting in critical illness and other diseases in humans result in severe muscle wasting and is a major problem for their recovery/rehabilitation (1-7). Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection in critical illness, has symptoms like gut atrophy and stimulation of liver acute phase response, indicating complex interactions between organs, in addition to muscle wasting (8-10).

In another example, when an important metabolic organ like the liver is failing, metabolic routes between organs are disturbed and are the cause of progressive (but reversible) multi-organ failures (11-13). Therefore, it is critical to gain more knowledge about inter/trans-organ fluxes of nutritional substrates in health and disease to ensure that improved nutritional strategies or other interventions can be developed for a variety of pathophysiological conditions (6, 7).

Measuring concentrations by collecting blood sample of substrates in plasma is relatively easy in humans. However, to measure complex fluxes or trafficking (amounts in time) of substrates over and in organs is extremely difficult in humans due to the multitude of ethical issues and the limitations of invasive procedures. In pathophysiological conditions, like sepsis, it is especially difficult to obtain transorgan measurements as these patients are very unstable and have coagulation abnormality in addition to ethical problems (1, 4). Therefore, substrate studies in humans usually are done by measuring substrate flux on a whole body level with the use of stable tracers (14-19) or performing arterial-venous balance studies across the arm or leg as proxy for a muscle compartment (20). Additionally, by adding stable tracers to food, it is possible to get flux information across the splanchnic area (21-23). We also measured fluxes across individual organs, during surgery or catheterization for diagnostic purposes (24, 25).

Therefore, clinically relevant animal models are essential to study (patho)physiological interorgan metabolism in a controlled, repeatable way. Animal models also allow for the use of invasive techniques, providing multiple measurements and mechanistic data within the same animal in several nutritional/pathological stages. Due to the fact that the pig is an omnivorous mammal and have remarkable
similarity to humans in gastrointestinal tract anatomy, physiology, biochemistry (and even pathology), this species is often used in the biomedical research and more generally in the field of nutrition and associated metabolic disease states (26).

In this dissertation, I present different aspects of interorgan research studies in the pig. The dissertation describes interorgan/transorgan measurement of mainly protein/amino acid metabolism, their metabolites, and amino acid interaction with other macronutrients using advanced stable isotope tracer methods of measurement in health and disease models with multiorgan disturbed metabolism. The main focus is on a conscious disease model with acute severe sepsis induced by *Pseudomonas aeruginosa* bacteremia. In addition, I show a study in an unconscious devascularized acute liver failure model.

**Thesis outline**

Section 1 interorgan balance studies during feeding

*Chapter 2 and 3* are an extension of the introduction of the present dissertation. *Chapter 2* is an introductory review on the several kinetic aspects of the absorption of amino acids, peptides and intact proteins in the small intestines (27). The small intestine acts as an interface and regulator between the gut lumen and the rest of the body. The small intestine also controls the degree and rate of transport of amino acids coming from dietary protein via the portal vein to the liver and the systemic circulation. In this review, we discuss the factors that lead to enhanced, reduced, or modified absorption kinetics. To study these factors, a sophisticated model in multi catheterized pigs along with the use of isotopes that can calculate absorption and gut metabolism is necessary. In *chapter 3*, we describe the technical part of the multi catheterized pig model that is used throughout this dissertation (28).

*Chapter 4 and 5* describe in more detail interorgan balance studies to determine transorgan bioavailability of amino acids/peptides for potential anabolic/bioactive response. In *chapter 4*, we state that intake of low quality food plays a key role in e.g. environmental enteropathy, a subclinical chronic situation present in developing countries. However, limited data were available on acute effects of low quality foods. Therefore, we determined the acute effects of a low quality food on gut metabolism and the consequences for liver metabolism (29). In the last chapter in this section, *chapter 5*, we hypothesize that potential benefits on health of food-derived bioactive peptides depends on the availability on the site of action. Therefore, we determined transorgan availability/kinetics of food-derived peptides and the influence of other macronutrients in the food (30).

Section 2: Use of isotopes in pig sepsis models

In *chapter 6*, we describe a newly developed clinically relevant disease model of severe sepsis induced by intravenous administration of *Pseudomonas aeruginosa*.
This is a pathophysiological condition in which severe amino acid substrate wasting is related to high mortality (32). With this model, in chapter 7, we describe an example of the use of stable isotopes tracers in metabolic research. We compared two different stable isotope methods/models to determine whole body protein breakdown, on their sensitivity to detect physiological differences, their accuracy, precision, practicality in animal models and discuss their potency for human studies (33).

Section 3: Interorgan balance studies in disease models
Amino acid metabolism is not only involved in protein synthesis and breakdown, but also involved in many other metabolic routes. A multiorgan research approach is especially needed for advancement in studying disturbances in those routes with interorgan substrate trafficking. For instance, arginine, derived from dietary protein intake, body protein breakdown, or endogenous de novo arginine production, may be linked to the availability of citrulline, which is the immediate precursor of arginine and the limiting factor for de novo arginine production. Arginine metabolism is highly compartmentalized due to the expression of the enzymes involved in arginine metabolism in various organs. A small fraction of arginine enters the NO synthase (NOS) pathway. In chapter 8, we review the existing literature concerning the ARG-NO pathway in disease states (34). And in chapter 9, we studied this pathway in an unconscious multiorgan catheterized pig model with an acute liver failure. The aim of the study was to evaluate the evolution of disturbances in NO metabolism in relation to its regulators, L-arginine, asymmetric dimethylarginine, and arginase, in the first 6h of acute liver failure using stable isotope technology (35).

In chapter 10 and 11, multi-tissue and organ protein metabolism in Pseudomonas induced severe sepsis, are described in more detail and using different isotope models. Chapter 10 is focused on alterations of fractional synthesis and breakdown rates in splanchnic tissues in comparison with muscle and lung tissue. In chapter 11, we try to unravel/explain protein turnover alterations on whole body level with changes of net balances and protein synthesis/breakdown on organ level using multiple protein turnover related isotope tracers.

Section 4: Summary and conclusions
In chapter 12 we summarize the findings of the present dissertation with some key data figures and discuss future perspectives in this type of research. In chapter 13 we made a short summary in Dutch. Finally in chapter 14 we valorized certain findings of the present dissertation.


18. Mason A, Engelen MP, Ivanov I, Toffolo GM, Deutz NE. A Four Compartments Compartmental Model to Assess Net Whole Body Protein Breakdown Using a Pulse of


SECTION 1
Interorgan balance studies during feeding
Absorption kinetics of amino acids, peptides and intact proteins

Gabriella A.M. Ten Have, Marielle P.K.J. Engelen, Yvette C. Luiking, Nicolaas E.P. Deutz

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Abstract

The small intestine acts as interface and regulator between the gut lumen and the rest of the body and controls the degree and rate of transport of amino acids coming from dietary protein via the portal vein to the liver and the systemic circulation. To measure protein absorption, kinetics multi-catheter animal (pig) models in combination with amino acid tracer technology are available. Dietary factors influence the absorption rates from the lumen to the gut, metabolism of dietary component in the gut, and the release of amino acids to the portal circulation from digested protein. In a balanced-protein meal, the gut dietary amino acid utilization (30–50%) for gut protein synthesis will result in a labile protein pool in the gut that can be beneficial during the post absorptive state. To enhance gut retention, amount and quality of protein and the presence of carbohydrate are major factors. Besides this the use of a slowly digestible protein or the presence of fibre in the meal can increase retention further. During the absorption of low quality protein meals, fewer amino acids are utilized by the gut, resulting in higher amounts of amino acid release to the portal circulation. Malnutrition or starvation, protein depletion, deficiencies of specific nutrients, or illness such as sepsis all inhibit the growth and change protein turnover of the intestinal mucosa and therefore affect absorption kinetics. Therefore, the kind of protein meal that has the most optimal absorption kinetics (the most beneficial) for gut and for the rest of the body depends on these (patho) physiological circumstances. Despite the absence of different absorption kinetics between protein, peptides, and amino acids, they could be beneficial in specific circumstances.
**Introduction**

The gastrointestinal (GI) tract is the primary organ in the control of food digestion, absorption of food components, and release of food components to the rest of the body. Furthermore, the gut has a barrier function that protects the body against invasion (translocation) of endogenous luminary microorganisms and/or toxins (1).

To cover this variety of functions, the structure of the small intestine is characterized by a large luminal surface area (mucosa), through mucosal folding with the presence of villi and microvilli on top and crypts at the bottom. The intestinal mucosal layer is one of the most rapidly replicating tissues in the body, with a continuous process of differentiation of stem cells into specialized cells for absorption (enterocytes or epithelial cells), excretion (enteroendocrine cells), and mucus (water and glycoproteins) secretion (Goblet cells) (2).

Digestion of dietary protein begins in the stomach, but occurs mainly in the small intestine. While approx. 95% of dietary protein is absorbed in the small intestine (3), the remaining amino acids, undigested proteins and unabsorbed peptides, of either dietary or endogenous origin, can enter the large intestine. In the large intestine these components are subject to digestion and metabolism by the intestinal microflora (4).

The small intestine itself is the absorption organ for amino acids coming from dietary protein and is the first pass for amino acids to the circulation. Therefore, the small intestine acts as interface and regulator between the gut lumen and the rest of the body and controls the degree and rate of transport of amino acids coming from dietary protein via the portal vein to the liver and the systemic circulation. However, not all amino acids that pass the gut enter the circulation, since a part of the amino acids is used for local metabolism (e.g. oxidation, protein synthesis). The present review will focus on the influence of several dietary factors on gut amino acid absorption, metabolism and release to the portal system.

**Gut as a metabolic active organ**

Besides the organ that has direct contact with ingested food, the gut is also a very metabolic active organ (5). Supply of amino acids to the gut cells does not rely solely on luminal supply during feeding. There is also a substantial use from arterial supply and as such the gut is “competing” with other tissues for amino acid utilization (figure 1). The high level of metabolic activity in the gut is also demonstrated by the fact that rates of protein synthesis in the intestinal mucosa are among the highest in the body (6).
During feeding

Luminal contact with nutrients is very important in the regulation of intestinal protein metabolism (7-9). Feeding is known to rapidly stimulate protein synthesis in the gut. Approximately 50% of dietary amino acid intake is used by the Portal Drained Viscera (PDV), but this percentage varies between different amino acids (10, 11). It is known that mucosal protein turnover is high and that amino acids with a different origin, e.g. lumen and circulation contribute to protein synthesis at a different level during feeding (11).
However, mucosal protein synthesis rate is not affected by feeding and is less sensitive to short-term variations in nutritional status (12). In neonatal pigs, less than 20% of intestinal amino acid utilization is used for constitutive gut growth by the intestinal mucosa (13). It appears that an enhanced secretory (glyco) protein synthesis rate in these neonatal pigs contributes to the increased gut protein synthesis during feeding (11). Approximately 26% of the dietary protein intake reappeared in the portal vein by way of recycling of digested (glyco)-proteins. Therefore, intestinal recycling of amino acids apparently contributes to their overall systemic availability. The protein synthesizing capacity, however, depends on the dietary protein source used, as will be discussed later.

**During fasting**

Malnutrition or starvation, protein depletion or deficiencies of specific nutrients all inhibit growth and turnover of the intestinal mucosa (1, 14, 15). Interestingly during fasting, gut protein is considered a (labile) pool (16). After a meal there is a net accumulation of protein in the gut whereas in the post-absorptive state a net loss of protein takes place. The hypothesis is that net retention of amino acids as protein in the gut serves to ‘buffer’ a bolus meal containing protein. When a protein would be rapidly digested, absorbed and directly released to the portal system, the large flux of highly concentrated amino acids in the portal vein would give rise to a high rate of urea production, gluconeogenesis and amino acid oxidation (17). A more gradual release of amino acids from the gut would ensure a more prolonged supply of amino acids in the portal vein, resulting in lower plasma concentrations in the portal vein and a lower urea production and potentially more muscle anabolism (16). During prolonged fasting, the intestine is taking up amino acids from the arterial side. Especially glutamine coming from the muscle compartment is utilized as a major energy substrate by intestinal mucosal and immune cells (14, 18).

**Measurement of gut amino acid absorption kinetics in vivo**

The in- and outflow (flux) of amino acids, protein, and nitrogen in the gut exhibits a complicated pattern (19). The digestion of many dietary proteins by the gut is incomplete. Besides dietary supply, there is also a continuous (but variable) entry into the intestinal lumen of endogenous protein and amino acid nitrogen that is subject to digestion. To get insight in the absorption of a meal, methods are used that examine the digestion characteristics of a protein meal in the gut lumen, while other techniques are based on amino acid oxidation and the use of stable isotope techniques. In order to measure absorption and fluxes of amino acids across the gut, a multi-catheter technique is needed (20). Combination of catherization of
multiple organs and stable isotopes techniques enables measurement of *in vivo* gut metabolism and the interaction with other organs (10).

**In humans**

Multi-organ catheter techniques are often not applicable in humans. In humans, measurements across the portal drained viscera (PDV: the GI tract, pancreas, spleen, and associated adipose tissue) are only possible during surgery (21-23). Therefore, in studies in healthy conscious subjects, mostly measurement of splanchnic extraction of alimentary amino acids, which represents the sum of PDV and liver amino acid uptake and utilization, is done (24-31). Introduction of this doubly labeled stable isotope technique has enabled a more detailed study of splanchnic conversions and amino acid extraction (32, 33) in which two tracers of the same amino acid but with a different label are used. Simultaneously, one tracer is infused intra-gastric or orally, and the other intravenously. A multi-compartmental computing simulation model has been developed to describe dietary nitrogen postprandial distribution and metabolism in humans. The model is using experimental data on dietary nitrogen kinetics in certain accessible pools of the intestine, blood, and urine in healthy adults fed a [15N]-labeled protein meal (34, 35). However, in this model specialized information on the absorption/utilization of dietary amino acids in the gut is limited.

**Large and small animal models**

Small animals like rodents are considered suitable for investigation of the mechanisms of absorption and bio-availability (14), whereas larger animals are generally used to assess absorption kinetics (19, 20). Pigs are often used in metabolic research as pigs are very similar to humans with respect to renal, cardiovascular and digestive anatomy and physiology, (36), (37-39). Furthermore, larger species like the pig are also useful when multiple blood samples have to be taken in time.

**Multi-compartmental modeling**

Studying amino acid absorption and utilization by the gut is difficult. The complicating factor is that the intestinal mucosa receives nutrients from 2 sources, the diet (brush border membrane) and the systemic circulation (basolateral membrane). Flux of amino acids from the lumen to the portal system is mainly studied in animal models using multi-catheter techniques. This enables the sampling of blood entering (i.e. afferent blood vessel) and leaving the organ (i.e. efferent blood vessel) for measurement of concentration differences across the organ with simultaneous measurement of organ blood flow (20) (see figure 1). Net balance studies of amino acids across an organ, however, only provide information on the net uptake (net anabolism) or release (net catabolism) and dynamic processes (eg disposal and production) cannot be quantified.
Extension of substrate (tracee) studies by the use of isotopes (tracers) enables measurements of amino acid disposal and production across the organ. The principle is based on the dilution principle of Fick. During primed-continuous intravenous tracer infusion any change in the enrichment over time occurs as a consequence of dilution from the unlabeled tracee. The rate of appearance (Ra) of an amino acid is determined by measuring the change of the tracer/tracee ratio (TTR) in relation to the isotope infusion rate (40).

Figure 2. Model to measure amino acid uptake, metabolism, and release into the circulation after a protein–peptide–amino acid meal by using isotopes of amino acids (AA) that are present in protein.
In the gut, phenylalanine disposal and production (turnover) are related to protein synthesis and protein breakdown and are measured by calculating tracer disappearance rate in that organ and the net balance of tracer. To assess phenylalanine measured protein turnover, TTR measurements on the arterial and portal side are needed (figure 2). This is a so-called “two-compartment” model, with plasma and interstitial fluid representing the two compartments. A “three-compartment” model can also be applied when tissues or biopsies are available (third compartment represents the free tissue pool). Combination of several tracers makes it possible to get quantitative information of multiple metabolic pathways in the gut.

Flux of a single amino acid across the gut coming from a protein meal represents absorption, utilization and release to the portal system. To get more insight in the absorption rate in time of a single amino acid, two tracers of this amino acid are used (figure 2). One is done intravenously via a primed-continuous infusion protocol and the other is given orally, added to a protein meal.

**Dietary factors that affect gut amino acid absorption rate, metabolism and release**

The amount of protein, the protein source, and the presence of other macro nutrients in the meal influence the absorption rate and metabolism of amino acids in the gut and affect the release rate of amino acids to the portal system. All these factors determine the anabolic capacity of a meal.

**Protein amount**

The amount of protein in a diet has a major effect on the magnitude of change of protein metabolism in the gut and in the rest of the body. With a balanced meal approximately 90% of the dietary amino acids are absorbed by the gut. About 30-50% will be utilized by the intestine itself and the remainder released to the portal system. An excessive amount of protein intake potentially could lead to limitation of gut absorption, and thus to a reduction of the percentage protein absorption. However, it is more likely that the maximum that the gut cells can utilize for own metabolism is reached faster and that consequently higher protein intake will reduce the percentage of protein extracted. The amino acids that are not absorbed and undigested proteins will flow into the colon. During low protein intake, the percentage protein extracted by the gut will be higher, although the intestine can adapt to reduced protein intake by reducing its amino acid oxidation (41) (see Table 1).
### Table 1.

<table>
<thead>
<tr>
<th>Dietary factor</th>
<th>Effect on percentage gut retention</th>
<th>Effect on percentage portal appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increasing amount of Protein</td>
<td>Decrease</td>
<td>Increase</td>
</tr>
<tr>
<td>Excessive amount of Protein</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
<tr>
<td>Lowering amount of Protein</td>
<td>Minor increase</td>
<td>Minor decrease</td>
</tr>
<tr>
<td>Improving the quality of Protein</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Slowing of the rate of protein digestion</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Adding Carbohydrates to the meal</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Adding Fats to the meal</td>
<td>Minor increase</td>
<td>Minor decrease</td>
</tr>
<tr>
<td>Adding soluble Fiber to the meal (prolonged)</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Partially hydrolyze protein (&quot;peptides&quot;)</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Fully hydrolyse protein (&quot;free amino acids&quot;)</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

Table 1. Summary of the effects of dietary factors on percentage gut retention and portal appearance after bolus feedings of proteins, peptides, and amino acids under healthy conditions.

### Protein quality

The quality (nutritional value) of a dietary protein is related to both the bio-availability of ingested nitrogen and amino acids and the efficiency of their metabolic utilization to meet nitrogen and amino acid requirements for growth and renewal of body proteins (42-45). It is postulated that a high quality dietary protein source stimulates amino acid utilization in the gut and is therefore of benefit for the gut and for the rest of the body (11, 16). We compared in a pig study, soy (low quality) and casein (high quality) protein and observed that liver urea production and net release of essential amino acids by the gut was higher with soy (10). This was confirmed in a study in healthy subjects, demonstrating a lower net protein synthesis and higher ureagenesis after a soy containing meal (25). This suggests that amino acid retention across the PDV is higher after a casein protein meal. Possible explanations are the fact that soy is deficient in the essential amino acids methionine and lysine and contains less branched-chain amino acids and the difference in the digestion rate between both proteins (46). In addition, the high biological value of casein protein potentially is related to the release of peptides that have a local trophic effect on the gut and that can increases mucine production (47).

Metabolic utilization of amino acids in the gut thus depends on the composition of the meal with respect to the presence or absence of (in) dispensable essential amino acids. Complete lack of an amino acid in a protein meal makes the protein of inferior quality. Previous studies in pigs showed that ingestion of an isoleucine-lacking...
blood protein meal resulted in an elevated urea production. Concomitant intravenous isoleucine infusion lowered the increase of urea and promoted amino acid retention in the gut (17) (see figure 3). In conclusion, high quality proteins stimulate amino acid utilization in the gut and therefore induce more gut amino acid retention (see table 1).

Slow versus fast proteins

A difference in the speed of protein digestion and absorption in the gut, the so called slow vs. fast protein concept, also affects the quality of a protein (48). Postprandial plasma amino acid profile shows a rapid high increase with whey protein and a prolonged plateau of moderate hyperaminoacidemia with casein protein (49), resulting in a more prolonged positive net protein balance after casein than after whey intake in young healthy males (48, 49) (figure 3). The discussion whether a slow digestible protein (e.g. casein) is better than a fast digestible protein (e.g. whey) depends on the (patho) physiological situation. The ‘labile’ gut protein pool hypothesis suggests that prolonged amino acid release from the gut is better for the body in the long term. Recent evidence indicates that the optimal dietary protein source and feeding pattern changes with age. Stimulation of muscle protein synthesis in the elderly necessary to counteract sarcopenia is higher with fast proteins like whey, with protein hydrolyzates or with a protein feeding pulse pattern (50). Therefore factors like amino acid profile, digestibility and absorption rate of a protein can all influence gut retention of that protein.

Figure 3. Pattern of PDV amino acid (AA) efflux during bolus feeding with proteins.
Role of adding carbohydrates to the meal

In a multi-catheterized pig study, pigs were given a bolus meal consisting of high quality proteins with and without carbohydrates (CHO). Addition of carbohydrates to a protein meal resulted in an increased intestinal amino acid retention (Figure 3), lower urea production and increased gut GLN uptake and ALA release, indicating stimulated gut metabolism (3). This suggests that addition of carbohydrate to a protein meal improves the anabolic quality of a protein meal in the gut. Recent studies showed that adding high quality protein to a carbohydrate meal will have an insulinotropic effect (51). Intake of free leucine, phenylalanine and arginine further augmented the insulin response. More recent studies show that co ingestion of a protein hydrolysate with additional free leucine will maximize the insulinotropic response (in both type 2 diabetes patients as well as in normoglycemic controls (52, 53). Simultaneous ingestion of these amino acids and carbohydrates resulted in a 100% higher insulin response than with carbohydrate only (51).

Role of adding fat to the meal

Until now, the effect of fat intake on amino acid retention in the gut has not been studied directly. Studying splanchnic nitrogen retention after adding fat vs CHO to a protein meal using a simulation model revealed that fat enhances splanchnic dietary N anabolism only transiently, without significantly affecting the global kinetics of splanchnic retention and peripheral uptake (54). This suggests that after fat co-intake the absorption rate can be reduced in the lumen side but that no major gut metabolic changes are expected.

Role of adding fiber to the meal

Fiber is a well-known dietary ingredient that stimulates long-term mucosal growth, especially in the colon. The magnitude of growth depends on the type of fiber ingested (2). It is expected that, if the structural changes only take place in the colon, it will not influence the amino acid absorption in the small intestine. However, extra food retention due to the higher viscosity of a fiber containing meal can lead to more protein digestion retention in the intestinal lumen. Recent studies showed that prolonged ingestion of soluble dietary fiber like Pectin leads to structural changes in the small intestine (55). The total protein amount of the intestine was enhanced, which resulted in higher protein turnover, and consequently in higher energy and amino acid requirements for the digestive tract. It is expected that the enhanced mucosa amount will result in an increased absorption capacity and rate.

Absorption kinetics of peptides

Small peptides can be actively absorbed by the enterocytes via specific transporters. The peptides in the enterocyte are converted to single amino acids which
are released to the portal system or utilized by the gut itself. Another possibility is that the peptides are directly released to the portal system. In a study with healthy young pigs, the gut absorption kinetics of intact proteins, hydrolyzates and free amino acids mixture were found to be comparable (56). In this study the selected protein was a fast protein, with no digestion limitation in the gut lumen itself. This suggests that amino acid absorption coming from hydrolyzates can only be higher than that of intact protein, if these hydrolyzates come from a slow protein or are in a mixed meal that slowed digestion. This hypothesis is confirmed in a study with young man in which hydrolyzates of a fast and slow protein were compared with the intact protein (57).

Modification of the structure of dietary proteins by hydrolysis has been reported to improve the nutritional value of a meal in several studies under specific (patho) physiological circumstances. For instance in a post-exercise situation where hydrolyzate works insulinotropic (58). In another study it is suggested that the fact that milk peptide hydrolyzate solutions elicit greater release of glucose-dependent insulino tropic polypeptide (GLP) than the complete protein from which they are derived, might be beneficial in clinical situations where there is insulin resistance and glucose intolerance (57). Also in conditions of reduced digestibility as in critically ill patients, a meal with hydrolyzed protein or peptides might be beneficial (59).

It has been suggested that some proteins also contain bioactive sequences which may affect gut absorption and/or metabolism. Oligopeptides derived from casein for example stimulate mucin release from the Globlet cells in an isolated vascularly perfused rate jejunum (47).

Absorption kinetics of amino acids

As mentioned in the previous paragraph, the gut absorption rate of free amino acids and intact proteins coming from the same native protein are identical (56). This does not mean that the flux rate of all amino acids are identical. The flux rate depends whether or not these amino acids can be used by the gut itself (protein synthesis, oxidation) or converted in the gut to other amino acids that can be beneficial to the rest of the body (figure 1). The quantitative and qualitative utilization of amino acids by the gut is dependent on the amino acid profile of the dietary protein, the added macro-nutrients and the interaction with other organs.

How is absorption influenced by gut amino acid metabolism?

Involvement of several amino acids in metabolic pathways in the gut is reflected by their large intestinal extraction during enteral feeding: glutamate (96%), glutamine (64%), threonine (57%), arginine (65%), cysteine (44%), BCAA (50-60%) (10, 11,
Recently it was observed that the gut also uses threonine, sulfur amino acids (cysteine, methionine) and branched-chain amino acids (leucine, isoleucine and valine) to a higher extent than anticipated from the requirements for protein synthesis in the gut. Apparently gut extracted amino acids are involved in different kinds of pathways. Limiting these amino acids in a protein meal (low quality protein meal) will lead to a less than optimal amino acid profile for the gut. Because of this, amino acid flux can be changed into more release to the portal system (17). A single amino acid deficient in a protein meal can be compensated by extra intravenous infusion of this amino acid, which normalized the gut metabolism (17). Threonine is important for the structural protein mucus layer and that probably is the reason that in neonatal piglets, more than two thirds of the enteral threonine intake was used by the PDV. By this threonine metabolism can influence the absorption rate (11).

How is absorption influenced by illness?

Recently, pigs are studied pigs that were made septic by 24h infusion of endotoxins (18, 64). When these pigs were fed a meal with high quality proteins, the appearance of amino acids in the portal circulation was increased (figure 4), indicating that amino acid utilization of the gut was reduced in the septic pigs. Tracer studies revealed that protein synthesis was not changed but protein breakdown was enhanced in the septic pigs. Gut metabolism was clearly affected in the septic pigs as gut glutamine consumption was greatly reduced. So an altered gut metabolism caused by illness can change the amount of gut amino acid absorption and release to the circulation but also the profile of amino acid released to the gut.

The amount of protein received by continuous infusion was comparable between the groups. FedpostS= after 30h of starvation; FedpostS+LPS= after 30h of starvation

![Figure 4. The effect of sepsis on the portal appearance of amino acids in the pig (net PDV flux).](image)
and 24h of endotoxin (endotoxin) infusion. No change in Protein Synthesis was observed, while Protein Breakdown was increased. Percentage absorption was not different. PDV indicates portal-drained viscera.

**Conclusion**

In this review, we have discussed the factors that lead to enhanced, reduced or modified absorption kinetics. To study these factors, a sophisticated model in multicatheterized pigs that with the use of isotopes can calculate absorption and gut metabolism, is necessary.

**References**

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Simultaneous measurement of metabolic flux in portally-drained viscera, liver, spleen, kidney and hindquarter in the conscious pig


Laboratory Animals (1996) 30, 347-358

Abstract

A method was developed to measure metabolic fluxes simultaneously across the portally drained viscera (PDV), liver, spleen, kidney and hindquarter (HQ) in the conscious pig (20-25 kg). For this purpose, sampling catheters were implanted in the abdominal artery, portal vein, hepatic vein, splenic vein, renal vein and caval vein. Further, two extra infusion catheters were implanted in the splenic vein and abdominal artery. These allow continuous infusion of para-aminohippuric acid (PAH), providing a method for estimating the plasma flow of the liver, PDV, spleen, kidney and HQ.

To minimize the postoperative recovery period of the pigs, great attention was paid to the housing conditions. After a recovery period of seven days, pigs were used for experiments twice a week. During the three weeks experimental period, food intake, body temperature, weight gain, blood gas data and plasma flow were monitored. Mean plasma flow was: liver 52 ± 6, PDV 40 ± 5, HQ 20 ± 2, spleen 4 ± 1 and kidneys 15 ± 2 ml/kg body weight/min. These data were characteristic for a pig in a conscious normal resting and unstressed state. The long term patency rate of the sampling catheters was very high (ranging from 75% to 100%). This was probably due to the prevention of catheter-related infections using a gentamicin (20 mg/ml), a-chymotrypsin (225 U/ml) solution as catheter filling. We conclude that this model enables simultaneously liver, PDV, intestine, spleen, liver, kidney and HQ flux measurement of many metabolic substances in the conscious pig.
**Introduction**

The pig has become an accepted animal model in the research of human disease (1-10). Further, the pig allows a variety of surgical interventions that cannot be contemplated in humans or are not feasible in smaller animals like the rat. Finally, the large size of the animal enables multiple sampling of relatively large volumes enabling many parameters to be analysed in blood simultaneously and over a prolonged period.

Measurement of arterial-venous differences, repeatedly and over time is only possible when catheters are present in the blood vessels supplying and draining an organ. To calculate the production or consumption across an organ, it is necessary to measure the plasma flow. For flow measurement, it is preferable to use a method with minor extra techniques involved and which is capable of measuring plasma flow over a prolonged period. For this purpose, an indicator-dilution technique with para-aminohippuric acid (PAH) may be used which is well-known in animal research (11-16). Also, PAH can be used for the measurement of kidney flow, using PAH extraction by the kidney (17, 18). In our model, we used these techniques for plasma flow measurement of the splanchnic area, portally-drained viscera (PDV), spleen and hindquarter (HQ) and the renal flow measurement.

For our research, several organ fluxes have to be measured in the same animal. Although several catheter implantation techniques in large animals have been described (3, 5, 10, 19-21), no reports are available in which all these techniques have been combined in the same animal. Therefore, we have developed a technique to measure plasma flow and arterial-venous differences of several substances simultaneously for portally drained viscera, spleen, liver, kidney and hindquarter (HQ) in the conscious pig.

**Materials and Methods**

**Animals**

Female crossbred piglets (Dutch land-race x Yorkshire, 20-25 kg, age 8-12 weeks) were obtained from a commercial breeder. At least one week before surgery, the animals were allowed to adapt to individual housing in galvanized bar runs (2 x 3 m), equipped with drinking nipples and isolated floors. Environmental temperature was held at 22-25°C (22), humidity level was 55%, light was on for 12h per day (from 07:00 to 19:00 h). A radio set was switched on during the light period for them to get accustomed to human voices and noise. Light and music were switched on and off gradually. A ball was provided for the piglets to play with.
The piglets were fed maintenance pellets for adult sows (Landbouwbelang, Roermond, The Netherlands; 16% raw protein). Pigs used in laboratories should be fed balanced diets formulated for research-oriented maintenance rather than market production of meat, hence food intake was restricted to a maximum of 1 kg per day (2, 22). Water was available ad libitum.

Operation procedure

Before operation, the animals were fasted for 16 h. About 45 min after premedication with azoperone (8 mg/kg bw, i.m., Stresnil®, Janssen Pharmaceutica, Tilburg, The Netherlands), anaesthesia was induced with N20/02 (2:1) and halothane (4%, Trofield Surgicals AG, Zug, Switzerland) mixture by a face mask. Anaesthesia was maintained after oral endotracheal intubation (tube diameter 7mm) with N20/02 and halothane (0.8%) mixture. Immediately before surgery, lincomycin and spectinomycin (6.25 mg/kg bw and 12.5 mg/kg bw respectively, 2.5 ml dissolved in 100 ml saline (0.9% NaCl w/v); Lincospectin®, Upjohn, Ede, The Netherlands) were given as antimicrobial prophylaxis via the ear vein. Also, flunixine-meglumine (2.5 mg/kg bw, i.v., Finadyne®, Mycofarm Nederland, De Blitt, The Netherlands), a prostaglandinesterase inhibitor, was given as anticoagulant to avoid activation of the haemostatic system and for postoperative analgesia. During surgery, Ringer’s lactate (500 to 100 ml i.v., Lactetrol®, Aesculaap nv/sa, Boxtel, The Netherlands) was given via the same ear vein.

Through a midline abdominal incision, several 80 cm long Tygon® catheters (ID 0.040», OD 0.070», Norton Co. Akron, OH 44305, USA) were implanted.

1. Catheters were inserted via the left and right iliac circumflex profunda artery into the abdominal artery with the tip approximately 5 cm above the bifurcation (A1: Figure 1) and above the level of the right renal artery (A2: Figure 1), respectively.

2. Catheters were inserted via the left and right iliac circumflex profunda vein into the inferior caval vein with the tip approximately 5 cm above the bifurcation (V1: Figure 1) and above the level of the right renal vein (V2: Figure 1), respectively.

3. A catheter (R: Figure 1) was placed through a purse string suture into the kidney hilus of the left renal vein by puncturing the renal vein with a hollow needle containing the catheter. The catheter tip was placed approximately 1.5 cm before the entrance of the inferior caval vein. Finally, the catheter was fixated with two ligatures on the left abdominal wall. This procedure was found to ensure stable position of the renal catheter.
4. Two catheters were placed via the two branches of the splenogastric vein: one with its tip about 5 cm into the distal splenic part of the splenic vein (S1: Figure 2), and the portal vein catheter via the splenic vein with its tip positioned in the free liver hilum (P: Figure 2). Although the Tygon catheter tends to enter the cranial mesenteric vein rather than the portal vein, by manipulating the catheter in the portal vein gently, the tip position is easily located in the liver hilum.

5. A splenic vein catheter was placed through a purse string suture in the vein by puncturing the splenic vein with a hollow needle containing the catheter. Although the diameter of the splenic vein at this level is small, it is relatively easy to do. The catheter tip was positioned in the proximal part of the splenic vein approximately 7 cm away from the spleen (S2: Figure 2). Several
small branches of the splenic veins from the proximal part of the spleen were ligated to direct all venous blood via the large splenic vein. Normal arterial blood flow remained unaltered.

6. In the pig many small and large hepatic veins drain into the inferior cava vein. A large hepatic vein in the pig is the left common hepatic vein, draining the left lateral and central hepatic lobes. This vein can be felt as a groove in the left liver lobe. To puncture this vein, the liver was penetrated into this groove with a hollow needle containing the catheter as described by Imamura and Clowes (1975) (3). After successful puncturing, the needle was then withdrawn, leaving the catheter in place. Its tip was positioned 2 cm from the entrance of the hepatic vein in the inferior cava vein. The catheter entry was fixated with two ligatures in the liver lobe (H: Figure 2).

Figure 2. Location of catheters in the splanchnic area
The portal catheter (P), long splenic (S2) and the hepatic catheter (H) are used for sampling blood. The short splenic catheter (S1) is an infusing catheter.
All the catheters were filled with heparinized saline solution (50 U/ml Heparin Leo® Pharmaceutical Products BV, Weesp, The Netherlands, 0.9% NaCl w/v). The position of the catheter tips was checked during operation using fluoroscopy and iodine-containing contrast solution (Urografin® 76%, Shering AG, Germany). Finally, all catheters were secured in place by sutures and alpha-cyanoacrylate glue (Cyanolit® 201, Rubberhuis, Maastricht, The Netherlands). Catheters were tunneled through the left abdominal wall and subcutaneously. The exteriorized catheters were connected to two-way stopcocks (Baxter Healthcare Corporation, Uden, Holland). The abdominal incision was closed in three layers with a continuous stitch using Mersilene®, catgut and finally Vicryl® (Ethicon, Norderstedt, Germany). The skin wound is decontaminated with Aureomycin® spray (chlorotetracycline 2%, gentian violet 0.5%, Cyanamid animal health, Etten-Leur, The Netherlands). Total operation time was approximately six hours. At the end of the operation, the catheters were flushed with saline solution to remove the heparin and filled with a solution containing a mixture of gentamicin (20 mg/ml, Gentamicine 5%, Cuyk, The Netherlands) and a-chymotrypsin (225 U/ml, Merck, Darmstadt, Germany) (23). The pigs were fitted with a canvas harness to protect the catheters and to allow easy handling of the animals. After the operation, 2.5 ml Lincospectin® was given i.m. and the animal was given 100 g of feed.

**Postoperative care**

On the first two days after the operation the animals were given 100-200 g feed twice daily for reactivation of intestinal activity. This was usually accepted. Also, 2.5 ml Lincospectin® dissolved in 20 ml saline was given i.v. twice a day during the first two days. In the first week the animals were housed individually in a galvanized pen with straw bedding. Daily, the catheters were flushed with saline solution, the patency checked and again filled with the gentamicin-chymotrypsin solution. In order to minimize any risk of systemic toxicity of gentamicin, meticulous attention was paid to filling the catheter lumen only. Before flushing or one day before an experiment the contents were withdrawn. After the first week, the catheters were flushed twice a week and the animals were housed again in the galvanized bar runs.

**Experiments**

Experiments were started after seven days recovery. In this period, food and water intake had returned to preoperative levels. The animals were trained to become accustomed to a small movable cage (0.9 x 0.5 x 0.3 m). By this, the experiments could be done in unrestrained, conditioned, conscious animals, standing or lying in the small movable cage. During the experiment, the catheters were filled with a heparinized (50 U/mL) saline solution. After the experiment the catheters were filled again with the gentamicin-chymotrypsin solution.
Blood flows were determined by infusion of a measurable non-toxic indicator, paraaminohippuric acid (PAH, 25 mM, Sigma Chemicals Co, St Louis, USA). The PAH solution was made iso-osmolar (300 mOsm) by addition of NaCl and adjusted to pH 7.4 by titration of NaOH (1 N). The solution was sterilized before the experiment by filtration through a 0.22 pin filter (Millipore, Molsheim, France). The evening before an experiment, food was withheld. On the morning of an experiment (08:00h), a bolus of 5 ml PAH solution was given. This was followed by continuous infusion of PAH at a rate of 30 mL/h/catheter via the catheters in the short splenic vein (S1) and in the abdominal aorta (A1) during the experimental period. After one hour, steady state PAH concentrations were obtained (not shown).

Hereafter, blood samples (approximately 2 ml) were taken from the arterial catheter (A2), portal (P) and hepatic (H), splenic (S2), renal (R) catheter and from the catheter in the caval vein (V1) for the determinations of amino acids, ammonia, urea, glucose, lactate, PAH and blood gas analysis.

Experiments were done twice a week for a period of 21 days. After this period the animals were anaesthetized with 10-20 mg/kg sodium thiopental (Nesdonal®, Rhone Merieux, Lyon, France). The position of all catheter tips was checked using fluoroscopy and iodine containing contrast solution. Finally, the animals were euthanized with 200 mg/kg pentobarbital (Euthanasate®, Apharmo, Arnhem, The Netherlands) i.v.

**Analytical procedures**

During all procedures, accomplished promptly after collection of blood, the samples were heparinized and kept on ice. Hematocrit was measured by centrifugation in a micro centrifuge. All centrifugations were done at 4°C for 5 min at 8500 g in an Eppendorf 5413 centrifuge. Blood gas analysis was done immediately after sampling in a blood gas analyzer (Acid Base Laboratory (ABL3), Radiometer, Copenhagen, Denmark), after correction for temperature.

For PAH determinations, 300µl of whole blood was added to 600µl of 0.73 M trichloroacetic acid (TCA) solution, thoroughly mixed for deproteinization and centrifuged after which the supernatant was collected. To obtain plasma, blood was centrifuged. 500µl plasma was collected and deproteinized with 50µl of 2.5 M TCA solution, thoroughly mixed for ammonia, urea, lactate and glucose determination. For amino acid analysis, 100µl of plasma was deproteinized with 4 mg sulfosalicylic acid. After these preparations samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

Plasma ammonia, urea, glucose and lactate were determined spectrophotometricaly by standard enzymatic methods on an analyzer system (Cobas Mira, Hoffmann-La
Roche, Basel, Switzerland) using commercial kits (Ureum kit UV-800 of Bio Merieux, Mary L’Etoile, France; Ammonia Reagent kit of Raichem, San Diego, CA 92111, USA; glucose HK Unimate 5 of Hoffman-La Roche, Basel, Switzerland). Urea values were corrected for ammonia Plasma lactate was determined by a standard enzymatic method according to Pesce et al. (1975), Westgard et al. (1972) (24, 25). For PAH determination the method according to Brun (1951) was adapted for small volumes (26). Because the liver and kidney acetylate PAH to a certain content, PAH supernatant was deacetylated by heating for 45 min at 100°C. By this, the determined PAH is total PAH, and the flow measurement is independent of the acetylated grade. Hereafter, the PAH supernatant was measured at 465 nm on a Cobas Mira, using Ehrlich’s reagent (1 g p-dimethylaminobenzaldehyde (Merck, Darmstadt, Germany), 35 ml alcohol 96%, 4 ml TCA 2N, 61 ml aqua bidest) and a solution of 35% alcohol. After precolumn derivatization with α-phthalaldehyde, plasma amino acids were determined with a fully automated HPLC system (27, 28).

**Calculations and statistics**

All calculations were performed using plasma flows and plasma concentrations. For plasma flow determination we used PAH in two ways. First (Figure 3, top), by calculating the dilution of PAH, plasma flow is measured. In this situation, the amount of PAH entering the vessel by the infusion and by blood equals the amount leaving the vessel:

**Equation (1)** \[ \text{IN} = \text{OUT} \]

**Equation (2)** \[ \text{IN} = (\text{Infusion rate} \times [\text{PAH}]_{\text{infused}}) + (\text{Flow} \times [\text{PAH}]_{\text{pre}}) \]

**Equation (3)** \[ \text{OUT} = \text{Flow} \times [\text{PAH}]_{\text{post}} \]

Using the formulae (1), (2) and (3), calculation of plasma flows is easily made.

**Equation (4)** \[ \text{Flow} = (\text{Infusion rate} \times [\text{PAH}]_{\text{infused}}) / (\text{PAH}_{\text{post}} - [\text{PAH}]_{\text{pre}}) \]

[PAH]_{\text{infused}} is the concentration of PAH in the infused solution, [PAH]_{\text{post}} is the plasma concentration of PAH after the organ and [PAH]_{\text{pre}} before.

This model is used in the measurement of splenic, PDV and liver plasma flow (11, 13, 29). The catheter in the splenic vein (S1: Figure 2) was used for PAH infusion and by measuring the PAH concentration in the S2 catheter (first dilution), portal vein (second dilution) and liver vein (third dilution), plasma flow can be calculated. Also, measurement of HQ was by dilution of PAH infused in the abdominal aorta (A1: Figure 1) and measurement of the PAH dilution in the caval vein (V1).
Second (Figure 3, bottom), by measuring the extraction of PAH by the kidney, kidney flow can be calculated. In this situation, PAH is extracted by the kidney. The amount extracted equals the amount infused as the system is in steady state:

Equation (5) \[ \text{PAH extracted} = \text{Infusion rate} \times [\text{PAH}]_{\text{infused}} \]

Further, the amount extracted plus the amount leaving the kidney by the renal vein equals the amount entering the kidney:

Equation (6) \[ \text{IN} = \text{OUT} \]

Equation (7) \[ \text{IN} = \text{Flow} \times [\text{PAH}]_{\text{pre}} \]

Equation (8) \[ \text{OUT} = \text{PAH extracted} + (\text{Flow} \times [\text{PAH}]_{\text{post}}) \]

Using the formulae (5), (6), (7) and (8), the formula for calculating the kidney flow is easily made.

Equation (9) \[ \text{Flow} = (\text{Infusion rate} \times [\text{PAH}]_{\text{infused}}) / [\text{PAH}]_{\text{arterial}} - [\text{PAH}]_{\text{renal}} \]

Plasma flow is expressed as ml/kg bw/min.

Figure 3. Mechanistic model of infused PAH transport

[PAH] is the para-aminohippuric acid concentration in µM. Top: dilution principle used for the calculation of splenic, portal drained viscera, liver and HQ plasma flows. Bottom: uptake principle used for the calculation of kidney plasma flow.
Portally drained viscera (PDV) flux was calculated by multiplication of the portal-arterial difference with the portal plasma flow. The calculation for the liver flux is more complex, because of the portal and arterial routes of blood delivery. However, the total flux across the splanchnic region (liver+PDV) is easy to calculate as hepatic-arterial difference times splanchnic plasma flow (splanchnic plasma flow=liver plasma flow). Therefore, liver flux was calculated by subtracting PDV flux from the splanchnic flux. Splenic flux was calculated by multiplication of the splenic plasma flow and the splenic-arterial difference. Intestinal flux was calculated by subtracting splenic flux from the PDV flux. HQ plasma differences mainly represent plasma differences across muscle tissue. HQ flux was calculated by multiplication of the HQ plasma flow with the caval vein-arterial difference. Renal flux for two kidneys was calculated by multiplication of the renal plasma flow with the renal-arterial difference.

The Wilcoxon test was used to test significance from zero (P<0.05). For clarity reasons results are presented as means±SEM.

**Results**

Seventeen animals were operated: two animals died in the recovery phase of a splenic bleeding and an intestinal obstruction respectively. Fifteen animals were used for the experiments. The catheters A1, A2, V1, V2, S1, P and H were implanted in all animals, and in at least four animals catheters S2 and R were also implanted. Control data of two experiments per animal were used for the results of this article. Experiments were done randomly during the experimental period of 21 days. The number of observations are variable because samples were not taken every time from all catheters.

The first two days after the operation food was restricted to 200 g twice daily. After the third day the food intake was increased gradually until day eight, when the food intake was normalized (more than 900 g and a maximum of 1 kg a day). There was no weight gain, but also no weight loss during the recovery phase in the first week (0.08 ± 0.17 kg/week). After recovery, weight gain was 2.15 ± 0.21 kg/week with the restricted food intake of 1 kg. Body temperature during the experimental period was 38.2 ± 0.1 °C. No signs of infection were observed like catheter-related infections, wound infections, intestinal infections as diarrhea or unexplained high body temperature. Characteristic patterns in pCO₂, pO₂ and O₂ saturation of arterial, portal vein, hepatic vein, inferior caval vein, splenic vein and renal vein blood were observed (Table 1). For example, in the splanchnic region, percentage oxygen extraction (37%) is relatively high compared to other organs or regions. On the other hand, oxygen consumption of the spleen is very low (9%).
<table>
<thead>
<tr>
<th></th>
<th>Artery</th>
<th>Portal vein</th>
<th>Hepatic vein</th>
<th>Inf. caval vein</th>
<th>Splenic vein</th>
<th>Renal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct</td>
<td>29.3(0.7)</td>
<td>29.6(0.6)</td>
<td>30.1(0.6)</td>
<td>29.1(0.7)</td>
<td>29.1(1.1)</td>
<td>28.3(0.8)</td>
</tr>
<tr>
<td>pH</td>
<td>7.46(0.01)</td>
<td>7.44(0.03)</td>
<td>7.41(0.01)</td>
<td>7.42(0.01)</td>
<td>7.44(0.01)</td>
<td>7.42(0.01)</td>
</tr>
<tr>
<td>pCO2</td>
<td>5.42(0.11)</td>
<td>6.07(0.38)</td>
<td>6.51(0.15)</td>
<td>6.13(0.15)</td>
<td>5.68(0.17)</td>
<td>6.23(0.08)</td>
</tr>
<tr>
<td>pO2</td>
<td>12.33(0.3)</td>
<td>5.49(0.34)</td>
<td>4.47(0.24)</td>
<td>5.38(0.18)</td>
<td>7.65(0.35)</td>
<td>7.36(0.17)</td>
</tr>
<tr>
<td>HCO3</td>
<td>28.6(0.6)</td>
<td>30(0.6)</td>
<td>30.3(0.7)</td>
<td>29(0.8)</td>
<td>28.5(0.9)</td>
<td>29.2(0.5)</td>
</tr>
<tr>
<td>SAT</td>
<td>97(0.1)</td>
<td>74(2)</td>
<td>60(3)</td>
<td>71(2)</td>
<td>88(2)</td>
<td>87(3)</td>
</tr>
</tbody>
</table>

HCO₃ in mmol/l, pO₂ and pCO₂ in kPa and O₂ saturation (Sat) in %

The percentage of patent sampling catheters 28 days after the implantation is plotted in Figure 4. A patency rate less than 100% was only caused by a dislocation within the vessel of the catheter tip as a result of the increased size of the animals.

Plasma flows after the recovery time and under post-absorptive conditions are calculated and plotted in Table 2. Total plasma flow of the splanchnic area is 52.5 ± 5.9 ml/kg bw/min. By subtracting the portal plasma flow from the liver plasma flow, we found a hepatic artery plasma flow of 13.0 ml/kg bw/min. Variance was comparable for the liver, portal, HQ and renal plasma flow, but higher for the splenic plasma flow.

Figure 4. Patency rates
Patency rates 28 days after operation (in %) of the long arterial (A₂, n=15), portal (P, n=14), hepatic (H, n=14), short caval vein (V₁, n=15), long splenic (S₂, n=5) and renal (R, n=4) sampling catheters.
Table 2. Post absorptive plasma flows

<table>
<thead>
<tr>
<th>Plasma flow (ml/kg bw/min)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portally-drained viscera</td>
<td>39.5(5.0)</td>
</tr>
<tr>
<td>Liver</td>
<td>52.5(5.9)</td>
</tr>
<tr>
<td>Hindquarter</td>
<td>19.7(1.8)</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.4(1.3)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>15.2(1.6)</td>
</tr>
</tbody>
</table>

Values are mean(SEM)

Three examples of plasma concentration differences across organs are given in Figure 5. In addition, the fluxes are given in Figure 6. For ammonia, the PDV produces ammonia (Figures 5A and 6A) while the liver takes it up. Calculation of the splanchnic ammonia flux shows that ammonia does not escape from the splanchnic region (Figures 5A and 6A). Also, renal production was observed. However, neither production nor consumption was observed in the HQ. Glutamine (Figures 5B and 6B) is a non-essential amino acid, consumed by the intestine and produced by the spleen and HQ. Branched-chain amino acids (BCAA, Figures 5C and 6C) are calculated as the sum of the essential amino acids valine, leucine and isoleucine. These are neither produced nor consumed by the intestine, liver and spleen during postabsorptive conditions. However, there is a consumption of BCAA by the HQ and the spleen and production of BCAA by the kidneys.

Discussion

To study combined portally-drained viscera, liver, spleen, kidney and hindquarter flux measurement in the conscious pig, it is important that the recovery period be as short as possible to minimize the influence of the catheter implantation operation on subsequent experiments. Thus, a meticulous surgical technique and careful postoperative care is mandatory. First, the housing conditions were kept stable. Secondly, straw bedding was used in the bar runs, so that the pig can adapt her own body temperature changes to her environment during the recovery period. Straw bedding also stimulates the normal rooting behavior. Further, gradual reactivating of the intestinal functions by feeding small portions postoperatively during the first two days prevents intestinal complications like ileus or stasis. The operation and the reduced food intake in the first seven postoperative days were probably the reasons for the observed unchanged body weight. After the first week, when food intake was normalized (1 kg/day), a weight gain of about 2 kg/week was obtained. This is the normal weight gain, which might be expected in pigs with food restriction to about 5% of the body weight for growing pigs with a body weight of 20 kg (22).
Figure 5. Plasma concentration differences across organs post-absorptive ammonia (Fig 5A), glutamine (Fig 5B) and branched chain amino acids (BCAA, Fig 5C) plasma concentrations differences across the portally drained viscera (PDV, n=25), splanchnic region (SPL, n=26), hindquarter (HQ, n=17), spleen (n=8), kidneys (n=6). In µM values are mean ± SEM; *) is significant compared to zero (p<0.05, Wilcoxon).

Figure 6. Metabolic fluxes of different organs post-absorptive ammonia (Fig 6A), glutamine (Fig 6B) and branched chain amino acids (BCAA, Fig 6C) fluxes of the portally drained viscera (PDV, n=25), splanchnic region (SPL, n=26), hindquarter (HQ, n=17), spleen (n=8), kidneys (n=6), liver (n=23), intestine (INT, n=8). Values are in µmol/kg body weight/min and mean ± SEM; *) is significant compared to zero (P<0.05, Wilcoxon).
Mean arterial pressure (Table 1) is comparable with literature values (2, 30-32). Also, the oxygen saturation values in the portal, hepatic and caval vein are comparable with literature values (30, 33).

We observed very high patency rates (Figure 4), compared to previously reported results (10, 20, 21). There are several reasons for this high patency rate. First, it is important to use a catheter with a very smooth and inert inner and outer wall, such as Tygon®. Secondly, at the beginning of the operation, flunixin, a prostaglandin-esterase inhibitor, is given to lower platelet (thrombocyte) adhesion to minimize activation of the haemostatic system. Thirdly, regular catheter flushing, using strictly aseptic procedures, especially during the first week, might have prevented catheter-related infections and subsequent clotting at the catheter tips. In our opinion, a very important factor influencing catheter patency is the use of the gentamicin chymotrypsin solution as catheter-filling. This solution was found to be very successful in dogs (23), but experience was lacking in pigs. Before 1992, our average patency rates at day 28 after the operation were about 60% (not published), with the use of a saline solution with 50 U/ml lithium heparin as catheter filling. Also, the patency values in the literature for implanted catheters in the pig are relatively low (10, 20, 21). However, after the introduction of the gentamicin—chymotrypsin solution, average patency rates increased by 27% to nearly 100%. Catheter tip infections are probably the most important cause of occlusion.

Although transport of metabolic substances across the erythrocyte was shown to be slow (29), several other factors could influence this transport in vivo (e.g. duration of capillary contact, flow velocity). These make a valid estimation of the contribution of metabolic substance exchange via the erythrocyte impossible. Therefore, we assumed plasma exchange only to be an indicator of whole-blood exchange and all calculations were performed using plasma flow and plasma concentrations (13).

Plasma flow measurement is needed for determining the flux of different substances across an organ or body region. For measuring plasma flow, it is important that the experimental conditions are as constant as possible. It is known that the plasma flow in an organ or body region is dependent on the cardiac output (CO). For example, CO is related to the body posture: in humans, a standing position increases the CO by 20% and exercise increases CO by 200% (34). Thus, conditions and procedures during the experiment have to be stable and the conscious pig must not be stressed. Therefore, the pigs were studied while they were resting. In fact, the trained animals were normally sleeping while we were taking blood samples. Another factor for a reliable plasma flow measurement is the use of a technique which does not interfere with the physiological functioning of the vessels. Therefore, the position of the catheter insertion site has to be a safe distance from
the catheter tip position, to minimize interference with the normal contraction and relaxation pattern of the vessel.

The blood flow measurement in the splanchnic area and HQ was based on the general principle applied in physiology by Adolph Fick more than a century ago. The principle is, in effect, a restatement of the law of conservation of mass for an indicator. The indicator may be an endogenous substance like oxygen or an exogenous one introduced by the investigator. The Fick dilution method is one of the oldest yet most accurate ways of measuring cardiac output (34). The Fick dilution principle is also the basis of many procedures for determining regional blood flow using an exogenous indicator like PAH or indocyanine green.

Various lines of evidence suggest that the error in the indicator-dilution methods is probably about 5% (34). However, this may be overestimated, because its reproducibility in successive determinations can rarely be made better than approximately 7%, even with the most careful attention to technical details (3, 15, 22, 30, 34, 35). We observed an average variation of plasma flow across several organs (excluding splenic flow) of about 11%, and is therefore acceptable to us.

It is very difficult to compare absolute plasma flow values with literature values, because the procedures and conditions of the experiments are very variable or unknown. For this purpose CO-distribution can be used. For comparing our plasma flows with the literature we have calculated the splanchnic, HQ and renal plasma flow, in percentages of normal resting plasma CO in piglets of approximately 20 kg (105 ml/min/kg) (36). Splanchnic, HQ and renal percentages of plasma CO (50.0 ± 5.6%, 18.8 ± 1.7% and 14.5 ± 1.5%, respectively) are characteristic for pigs in a resting condition (3, 36) and comparable with humans in a resting condition (34). Also the ratio of portal/splanchnic flow is comparable with the literature (3).

Figures 5 and 6 are examples of measurement of metabolic substances in our piglet model. It illustrates a few of the many possibilities of this model. For example, the low oxygen consumption (Table 1) and a small glutamine production in the spleen suggests a non-activated immune system, because an activated immune system, which is a normal physiological response during the first three postoperative days, causes an enhanced utilization of glucose and glutamine (13). The advantage of this model is the possibility of studying metabolic substances in different areas of the body in a conscious animal.

In conclusion, multiple catheterization of pigs enables simultaneous measurement of liver, PDV, spleen, kidney and HQ flux in a conscious, unstressed animal over a prolonged time. The refinement of this model by optimizing housing conditions, the
use of a postoperative feeding schedule and meticulous attention to catheter care reduces the number of animals required for a certain experiment.

**Acknowledgements**

The authors wish to express many thanks to Mr H. M. H. van Eijk, BSc and Mr D. R. Rooyakkers, BSc for their accurate amino acid determinations and C. H. C. Dejong, MD, PhD for helping with the development of the surgical technique for the kidney catheter.

**References**


CHAPTER 4
Absence of post-prandial gut anabolism after intake of a low quality protein meal

Gabriella A.M. Ten Have, Mariëlle P.K.J. Engelen, Peter B. Soeters, Nicolaas E.P. Deutz


Abstract

Background & aims: Gut health relates to a diet with a high digestibility and quality. Limited data are available on the acute effects of low quality foods on gut metabolism and the consequences for liver metabolism.

Methods: A meal with the low quality protein gelatin (tryptophan deficient and low amount of essential amino acids) was compared to a meal with the high quality protein Whey and a tryptophan supplemented gelatin meal (Gel+TRP) in healthy pigs with chronic implanted catheters. In a conscious state, amino acid, ammonia, urea, glucose and lactate fluxes across the portal drained viscera (PDV) and liver were studied for 6 hours after administration of the protein meal.

Results: The average net portal appearance of amino acids was 99.8±14.6% of the intake in the Gel group as compared to 61.4±9.0% (p=0.022) in the Whey group. In addition, a net portal appearance of tryptophan was observed in the Gel group (p=0.005) of about 42% of tryptophan released in the Whey group. Intestinal energy metabolism and citrulline production was not affected. Adding tryptophan to the Gel meal diminished net portal AA appearance to 41.6±24.0% of the intake (p=0.012), but did not reduce the stimulated liver urea production.

Conclusions: In the postprandial phase after intake of a low protein quality meal, net anabolism in the healthy intestine is absent. It is likely that the intestine responds with a net breakdown of endogenous (labile) proteins to secure amino acid availability for the body. Addition of the first limiting essential amino acid to this meal improved protein anabolism in the intestine. Protein quality of a meal is related to the anabolic response of the intestine during the meal.
Introduction

A healthy gut is important for overall body health. The gut controls digestion and absorption of dietary protein and amino acids (AA), and therefore regulates the availability of substrates to the liver, muscle and other organs. Although the intestine in the prandial phase receives AA both from the small intestinal luminal side and via the systemic circulation, it has been observed that the intestine preferentially utilizes AA from the diet for its own metabolism. Luminal contact with nutrients is very important in the regulation of intestinal protein metabolism. Feeding is known to rapidly stimulate protein synthesis in the intestine (1, 2) as reflected by the large intestinal extraction during enteral feeding. Approximately 50% of dietary amino acid intake is used by the portal-drained viscera, but this percentage varies between different amino acids and diets (3-9).

Stimulation of overall protein anabolism by protein meals is reflected by the rapid stimulation of intestinal protein synthesis (6, 10). This means that the protein synthesizing capacity of the intestine depends on the same factors as whole body anabolism like the quality of the dietary protein source and the presence of carbohydrates in the protein meal (4, 9). We postulated that a high-quality dietary protein source stimulates amino acid utilization in the intestine for protein synthesis (11). After temporary retention of dietary protein as newly synthesized protein in the intestine, a tapered release of AA after the meal will ensure a more prolonged supply of AA to other tissues of the body. In this way, the intestine can act as a “buffer” or what is called, the labile protein pool (11).

A low quality protein meal is defined as a meal containing a normal amount of macronutrients and a protein source with low digestibility or a deficient/low amount of essential (indispensable) amino acids (EAA). In disease states, a meal with a low amount of functional and/or conditional EAA is also considered as low quality (5, 12, 13). It is hypothesized that intake of low quality food plays a key role in e.g. environmental enteropathy, a subclinical chronic situation often present in developing countries (14-17).

Previous studies examining the effects of low quality food mainly focused on whole body growth (18-20). Limited data are available of the direct effects of low quality foods on gut metabolism and the consequences for liver metabolism. Previously, we showed that intake of soy protein, characterized by low levels of essential amino acids, resulted in a higher net release of essential amino acids into the portal system, higher levels of liver urea production and lower values for net protein synthesis than high quality casein protein (10, 21). The different digestion and absorption rate of the two protein meals could partly explain this observation. The splanchnic tissues like gut and liver potentially can act as a gate keeper to dietary amino acid delivery to peripheral tissues. Furthermore, we (3) observed that intake of a pure blood protein
mixture, deficient of the essential amino acid isoleucine, resulted in increased net portal appearance of AA. Subsequent intravenous infusion of isoleucine was able to normalize the portal appearance of AA.

Information is still lacking whether the relative portal release of AA is increased in low quality protein meals with comparable digestion and absorption rates, and if low quality protein meals indeed will not stimulate intestinal net protein synthesis. Furthermore, it is unknown whether adding of the deficient AA to the meal will normalize the intestinal response to the meal.

In the present study, we examined the metabolic responses in the portal drained viscera (PDV) and liver to a gelatin protein meal (with low levels of EAA and TRP deficient) as compared to a high quality whey protein meal. In addition, we studied the effects of TRP supplementation to the gelatin protein meal. We hypothesize that adding tryptophan to a gelatin meal will mainly improve intestinal metabolism. The experiments were performed in healthy pigs with chronic implanted catheters, as models in pigs are viewed to be an excellent model for human gastro-intestinal tract and nutrition research in relation to gut and liver metabolism (4, 22-24).

Materials and Methods

Animals

Ten female crossbred (Yorkshire x Dutch Landrace: 20-22 kg) piglets were used. They were housed individually inside galvanized bar runs (2 by 3 m) equipped with an automated watering device and PVC-coated floors. During the whole experimental period, the piglets were fed sow feed (Landbouwbelang, Roermond, the Netherlands; 16% protein) on which the pigs grow at a rate of about 2 kg/week. This study was approved by the Animal Ethics Committee of the Maastricht University.

Surgical procedure

To perform cross-organ balances, several catheters are implanted under anesthesia as previously described (13, 22). Catheters for blood sampling were placed in the abdominal aorta, the portal vein and the hepatic vein. Catheters for the infusions of post-operative medication or para-aminohippuric acid solution (PAH) for flow measurement were placed in the central vein and splenic vein. Finally, a gastric catheter was placed.

Postoperative care

Postoperative care was standardized as previously described (13, 22) and the pigs remained healthy without signs of infection. During the recovery period the animals...
became accustomed to a small movable cage (0.9x0.5x0.3m). After a recovery period of 7-10 days, this cage training ensures that the testing of the experimental meals was done in conditioned and conscious animals, standing or recumbent in the small cage.

**Experimental protocol**

After a recovery period, four different experimental diets were tested in each pig in random order (Table 1 and 2). The period between the tests was 2-3 days. The following meals were given: an high quality protein meal with a relative high amount of EAA, high digestion and fast absorption rate: Whey protein meal (Whey). Secondly, a low quality protein meal with relative low EAA’s, a deficient EAA (TRP) but comparable digestion and absorption rates as the Whey meal: gelatin protein meal (Gel). Third, a gelatin protein meal to which the limiting amino acid tryptophan (Gel+TRP) was added in a sufficient amount to make tryptophan not the limiting EAA (25). All the protein meals were iso-nitrogen, iso-ionic (for sodium, chloride, potassium and calcium), iso-tonic and contained a similar amount of maltodextrins. The amount of protein and carbohydrates in the test meal represents about 30% of the normal daily intake of the pig of this age. The control test mixture did not contain any macronutrients and was used as control for volume, osmolarity and ion intake. This test mixture was either a solution of electrolytes, iso-osmolar to the protein meals (ContOsm) or an iso-ionic solution for sodium, potassium and calcium ions (Control). Each control mixture was given in 5 animals.

Food was withdrawn 16 hours prior to each test day. On the morning of a test day, a primed-continuous infusion of para-aminohippuric acid via the splenic catheter (PAH 25mM, iso-tonic, Sigma Chemicals CO, St. Louis, U.S.A.) at a rate of 60 ml/h after an initial bolus of 5 ml PAH solution was given. After taking initial blood samples from the arterial, portal and hepatic vein catheter, the liquid test meal (20 ml/kg bw) was given though the gastric catheter within 5 minutes. Blood samples were taken at the time intervals as indicated in the figures (Figure 1, 2 and 3), and used for amino acid and PAH concentrations analysis.

**Sample processing**

Immediately after collection, the blood samples were placed on ice and hematocrit (Ht) was measured. All samples were centrifuged at 4°C for 5 min at 8500 g in an Eppendorf 5413 centrifuge to obtain plasma. For glucose and lactate determinations, 300 µl of whole blood was centrifuged in sodium fluoride containing tubes (Microvette FH, Sarstedt, Nümbrecht, Germany). For PAH determinations, 300 µl of whole blood was added to 600 µl of 12% Tri-chloroacetic acid solution, thoroughly mixed and centrifuged. For ammonia and urea determination 200 µl plasma was collected, and. for amino acid analysis, 100 µl of plasma was deproteinized...
Table 1. Composition of experimental test meals**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Unit</th>
<th>Control</th>
<th>ContOsm</th>
<th>Whey†</th>
<th>Gel‡</th>
<th>Gel+TRP§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>g/kg bw</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1875</td>
<td>0.1875</td>
<td>0.1875</td>
</tr>
<tr>
<td>Protein</td>
<td>g/kg bw</td>
<td>0.0</td>
<td>0.0</td>
<td>1.44</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>mg/kg bw</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>8.83</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>g/kg bw</td>
<td>0.0</td>
<td>0.0</td>
<td>4.69</td>
<td>4.69</td>
<td>4.69</td>
</tr>
<tr>
<td>Sodium</td>
<td>mM</td>
<td>42.5</td>
<td>148.4</td>
<td>42.5</td>
<td>42.5</td>
<td>42.5</td>
</tr>
<tr>
<td>Chloride</td>
<td>mM</td>
<td>61.1</td>
<td>167.0</td>
<td>45.0</td>
<td>45.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Potassium</td>
<td>mM</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>Calcium</td>
<td>mM</td>
<td>8.92</td>
<td>8.92</td>
<td>8.92</td>
<td>8.92</td>
<td>8.92</td>
</tr>
<tr>
<td>Osmolariteit</td>
<td>mOsm</td>
<td>113.25</td>
<td>325.05</td>
<td>300.66</td>
<td>325.04</td>
<td>325.04</td>
</tr>
</tbody>
</table>

*) The control test meal (Control) was either a solution of electrolytes iso-osmolair to the other test meals (ContOsm) or a solution with similar amount of sodium, potassium and calcium ions (Contion).

†) Whey protein meal (Whey): Whey Protein Isolate, 90% w/w protein, supplied by DMV International, Veghel, The Netherlands.

‡) Gelatin protein meal (Gel): Gelatin, 97% w/w protein, (SoluGel P®) supplied by PB Gelatines, Tessenderlo, Belgium.

§) Gelatin protein meal with added amino acid tryptophan (Gel+TRP). Total amount of tryptophan added (T 0254, Sigma Chemicals CO, St. Louis, U.S.A.: 8.83 mg= 43.2 µmol) is sufficient to make tryptophan not the limiting amino acid (Cortamira et al 1991).

**) Total nitrogen of the protein was determined by an elementary analyzer (CHN O RAPID, Heraeus, Hanau, F.R.G.). All the protein meals were iso-nitrogen, iso-ionic (for sodium, chloride, potassium and calcium) and iso-tonic. They contained a similar amount of maltodextrins: Maldex 20, supplied by Amylum NV, Aalst, Belgium, is 28 mmol glucose/kg bw. Test meal ingredients were dissolved in trice distillate water (MilliQ) and electrolytes (NaCl, KCl and CaCl2) were added to the indicated concentrations. The test meals were prepared the day before the experiment, kept over-night at 4°C and heated up in a warm water bath to 37°C just before the intra-gastric administration. Test meal volume was 20 ml/kg bw.

Biochemical analysis

Plasma ammonia, urea, glucose and lactate were determined spectrophotometrically by standard enzymatic methods as described previously (4, 26, 27) with commercial available kits on a centrifugal analyzer system (Cobas Bio, Hoffmann-La Roche, Basel, Switzerland). Urea values were corrected for ammonia. PAH concentrations were determined spectrophotometrically after deacetylation of the supernatant at 100°C for 45 min as previously described (4, 22, 26). Plasma AA were
Table 2. The amino acid content of the Gelatine and Whey protein isolate

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Gelatin* μmol/kg bw given in the meal</th>
<th>Whey** μmol/kg bw given in the meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>180</td>
<td>573</td>
</tr>
<tr>
<td>ASN</td>
<td>253</td>
<td>363</td>
</tr>
<tr>
<td>GLU</td>
<td>431</td>
<td>1035</td>
</tr>
<tr>
<td>GLN</td>
<td>320</td>
<td>713</td>
</tr>
<tr>
<td>SER</td>
<td>178</td>
<td>535</td>
</tr>
<tr>
<td>GLY</td>
<td>3168</td>
<td>250</td>
</tr>
<tr>
<td>THR (essential)</td>
<td>226</td>
<td>688</td>
</tr>
<tr>
<td>HIS (essential)</td>
<td>83</td>
<td>106</td>
</tr>
<tr>
<td>ALA</td>
<td>1249</td>
<td>738</td>
</tr>
<tr>
<td>ARG</td>
<td>552</td>
<td>195</td>
</tr>
<tr>
<td>TYR</td>
<td>52</td>
<td>207</td>
</tr>
<tr>
<td>VAL (essential)</td>
<td>280</td>
<td>640</td>
</tr>
<tr>
<td>MET (essential)</td>
<td>45</td>
<td>213</td>
</tr>
<tr>
<td>ILE (essential)</td>
<td>179</td>
<td>616</td>
</tr>
<tr>
<td>PHE (essential)</td>
<td>155</td>
<td>227</td>
</tr>
<tr>
<td>TRP (essential)</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td>LEU (essential)</td>
<td>268</td>
<td>1044</td>
</tr>
<tr>
<td>LYS (essential)</td>
<td>305</td>
<td>770</td>
</tr>
<tr>
<td>Sum amino acids</td>
<td>7924</td>
<td>9007</td>
</tr>
<tr>
<td>Sum N of amino acids</td>
<td>9767</td>
<td>10774</td>
</tr>
<tr>
<td>Sum N of non-ess amino acids</td>
<td>8105</td>
<td>6286</td>
</tr>
<tr>
<td>Sum N of ess amino acids</td>
<td>1662</td>
<td>4488</td>
</tr>
<tr>
<td>CYS§</td>
<td>28</td>
<td>233</td>
</tr>
<tr>
<td>PRO</td>
<td>1150</td>
<td>753</td>
</tr>
<tr>
<td>HydroxyPRO</td>
<td>1090</td>
<td>0</td>
</tr>
<tr>
<td>HydroxyLYS</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

The amino acid content of the protein is determined in triplicate with methods, suggested by the FAO/WHO (FAO/WHO 1991) and were determined by the manufacturer.

*) The content of glutamine and asparagines in the Gelatine are calculated from amino acid compositions (from protein sequencing) given by Genome Net (Kyoto University Bioinformatics Center, Japan), assuming that the Gelatine used contains mainly collagen, type V, alpha 2 [Sus scrofa] (code AB191304.1, National Center for biotechnology Information, USA).

**) The content of glutamine and asparagines in the Whey protein isolate are calculated from amino acid compositions (from protein sequencing) given in Table A-6 from Walstra and Jennes (Walstar and Jennes 1984) for individual milk proteins, assuming that the (bovine)
determined with a fully automated HPLC system after pre-column derivatization with o-phthaldialdehyde (28).

**Calculations**

Plasma flow was calculated by means of an indicator-dilution technique as described (4, 13, 22).

\[
\text{Plasma flow} = \frac{\text{infusion rate} \times [\text{PAH-infused}]}{[\text{PAH-post}] - [\text{PAH-pre}]}
\]

[PAH-infused] is the concentration of PAH in the infused solution, [PAH-post] is the concentration in plasma after the organ and [PAH-pre] in plasma before the organ. Plasma PAH concentrations were used and calculated as [blood PAH]* (100/ (100-Hematocrit)). Plasma flow was expressed as ml×kg BW-1×min-1. Organ plasma flow is highly dynamic and depends on the high variance in cardiac output in conscious subjects (29). This means that measuring individual plasma flow can have a high variance. Therefore, multiple measurements were done in time and transorgan fluxes were calculated with the mean plasma flow for each time point of each experimental group. PDV flux was calculated by multiplication of the portal-arterial concentration difference with the portal plasma flow. Liver flux was calculated by the subtracting PDV flux from the splanchnic flux (hepatic-arterial concentration difference* liver plasma flow). Total net balance (net release or net uptake) of an organ was calculated by the area under the curve (AUC) of the respective flux time course from t=0h to the time, indicated. A positive value of flux or net balance means net release. A negative value means net uptake.

In the present study, comparisons of groups of amino acids with their nitrogen contents were done between protein meals of a different quality and quantity of amino acid but comparable amounts of nitrogen. Amino acid nitrogen (AA_N) was calculated as the sum of N of all AA that were detected in the amino acid analyses and therefore cysteine, proline, hydroxyproline and hydroxylysine were not included (Table 2). The essential amino acid nitrogen (EAA_N) and the non-essential amino acid nitrogen (non-EAA_N) were calculated as the sum of N of EAA and non-essential amino acids (non-EAA), respectively.

**Statistics**

Results are presented as means ± SEM. Levels of significance were set at P<0.05 unless stated otherwise. For statistical analysis of variance, the two-way ANOVA
POST-PRA NDIAL GUT CATABOLISM

CHAPTER 4

60

test was used to compare time courses of plasma flows, arterial concentrations and the organ fluxes of the test groups. Student’s t-test was used to compare the total amount of net release or net uptake of metabolites over the 6h experimental period (= total net balances). One sample t-test was used to determine if data are different from zero or from 100%. We used Prism 5.04 (Graphpad.com) for statistical analysis.

Results

Main

Overall, catheters remained patent. Incidentally, some minor technical problems were present with the hepatic catheter, resulting in a reduced number of liver data points (especially in the Gel+TRP group). In this case, hepatic observations were under-powered. As the results of the ContOsm group and Control group were not significantly different, the results of these groups were combined and discussed below as Control group.

Plasma flow

PDV plasma flow in time (not shown) after administration of the protein meals Gel and Whey was higher compared to the post-absorptive (Control) group (resp. p=0.005 and p=0.014). There were no differences in liver plasma flow. Mean plasma flows over the 6h experimental period per group are given in table 3.

Table 3. Mean plasma flow during the experimental period (6h)

<table>
<thead>
<tr>
<th>Organ plasmaflow</th>
<th>Control</th>
<th>Whey</th>
<th>Gel</th>
<th>Gel+TRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Portal drained viscera</td>
<td>8</td>
<td>38(9)</td>
<td>7</td>
<td>44(9)</td>
</tr>
<tr>
<td>Liver</td>
<td>8</td>
<td>58(12)</td>
<td>9</td>
<td>55(9)</td>
</tr>
</tbody>
</table>

Mean plasma flow during the experimental period (6h) was calculated by integration of the respective plasma flow time courses from t=0h to t=6h (area under the curve divided by time). Values are mean ± SEM. Significance tested with Student t-test: no significance differences were observed.

Nitrogen balances

Amino acids

In the present study, nitrogen balances provided overall information about the effects of protein meals with a different quality and quantity of amino acid contents but comparable amounts of nitrogen. Arterial AA_N (Figure 1A) increased from approx. 3.7 mM at t=0 to a maximum of 6.2 mM at t=1 hour after Whey feeding, and to a maximum of 5.5 mM in both Gel-groups at t=2 hour. The levels of the Gel-groups did not return to baseline values at the end of the experiment (Gel: p=0.002,
The total amount of measured AA_N in the Whey and Gel meals were slightly different. Therefore PDV AA_N net balances were expressed as percentage of AA_N intake in Figure 4. Thirty-nine percent (p=0.022) of the AA_N intake in the Whey-group was not released to the portal system. This is in contrast to the Gel-group where 99.8 ± 14.6% of the intake was released into the portal system. The Gel+TRP-group and Whey-group showed a comparable release of AA_N.

Essential amino acids

The total net amounts of EAA_N (Figure 3B) released by the PDV after a test meal were different in all groups except between Gel and Gel+TRP group where only a tendency (p=0.097) was observed. Seventeen percent of the intake (Figure 4, p=0.033) in the Whey-group was not released to the portal system. The released amount of EAA_N as % of intake was in the Gel group higher (p=0.046) than in the Whey group. A tendency was observed that it was higher (124±20%, p=0.12) than the intake. The released amount as % of intake in the Gel+TRP group was not significantly different as compared to the Whey-group. The arterial EAA_N time courses (Figure 1B) in the Gel-groups were not different from the level in the post-absorptive state, although the effect on the arterial concentrations in time where significantly different (ANOVA Interaction: Gel p=0.001, Gel+TRP p<0.0001). In the first two hours the arterial EAA_N concentrations in the Gel-group were higher than in the Control-group, but in the last 3 hours of the experiment the concentrations were below the Control-group (post-absorptive) time course (Gel:p=0.003, Gel+TRP:p<0.0001).

Non-Essential amino acids (Figure 1C, 2C, 3C and 4)

As percentage of intake (Figure 4), different amounts of non-essential amino acid nitrogen (nonEAA_N) were released by the PDV over the 6 hours feeding period. The Whey-group showed that the equivalent of 54% of the intake (p<0.0006) of nonEAA_N was not released to the portal system. This percentage was significantly lower in the Gel-group (5% of the intake, p<0.012). The release of nonEAA_N in the Gel+TRP group was comparable to that of the Whey-group. Although the total absolute splanchnic release (Figure 3C) was comparable between all protein meals groups, arterial nonEAA_N concentration time courses (Figure 1C) of the Gel groups were significantly higher (p<0.0001), than the Whey-group and did not reach baseline values at the end of the experiment (Gel:p=0.003, Gel+TRP=0.029).
Figure 1. Arterial amino acid nitrogen (AA_N)(A), essential amino acid nitrogen (EAA_N) (B) and amino acid nitrogen (NonEAA_N)(C) concentrations, in µM, in post-absorptive state (Control, n=9) and during 6 hours after administration of Whey protein (Whey, n = 8), gelatin (Gel, n = 9) or gelatin + tryptophan (Gel+TRP, n = 9) meals. Values are mean ± SEM. ANOVA tested, significant meal effect (p < 0.05): (A) protein meals vs. Control; (B) Whey vs. all other test meals; (C) proteins vs. control, Whey vs. Gel groups. Significant interaction (p<0.05): (A) Control vs. protein meals, Gel vs. Whey;(B) Control vs. all protein meals, Whey vs. all test meals; (C) Control vs. Gel groups, Gel vs. Whey.
Figure 2. PDV fluxes of amino acid nitrogen (AA_N)(A), essential amino acid nitrogen (EAA_N) (B) and amino acid nitrogen (NonEAA_N)(C) in µmol/kg bw/min, in post-absorptive state (Control, n=8) and during 6 hours after administration of Whey protein (Whey, n=7), gelatin (Gel, n=9) or gelatin + tryptophan (Gel+TRP, n=6) meals. Values are mean ± SEM. ANOVA tested, significant meal effect (p<0.05): (A) Control vs. all protein meals; Gel vs. Gel+TRP; (B) Control vs. all protein meals; Whey vs. Gel meals; Gel vs. Gel+TRP; (C) Control vs. all protein meals; Whey vs. Gel; Gel vs. Gel+TRP. Significant interaction (p<0.05): (A) Control vs. Gel; Control vs. Whey; (B) Whey vs. all other meals; (C) Control vs. Gel. Positive values = release to portal system. Negative values = uptake from portal system.
Figure 3. Total net balances across organs comparing with intake amounts of amino acid nitrogen (AA_N)(A), essential amino acid nitrogen (EAA_N)(B) and amino acid nitrogen (nonEAA_N)(C), in µmol/kg bodyweight, over 6 hours after administration of Whey protein (Whey, n = 8), gelatin (Gel, n = 9) or gelatin + tryptophan (Gel+TRP, n = 9) meals. Organs showed are portal drained viscera (PDV), liver and total splanchnic area (SPL = PDV + Liver). Values are mean ± SEM. Significance with Student t-test: P < 0.05, indicated with hooks. Positive values = release. Negative values = uptake.
Ammonia
Ammonia (NH$_3$) (Figure 5A) is a nitrogen-carrier mainly coming from protein breakdown and production by the intestinal lumen bacteria followed by NH$_3$ uptake of the gut on the lumen side and release into the portal system. Arterial NH$_3$ was increased after meal intake, but no significant differences were observed between the different meals. At organ level, an increased release of NH$_3$ was seen by the PDV after administration of a protein meal and NH$_3$ uptake by the liver was increased (Table 4). As a consequence no net splanchnic release of NH$_3$ was observed over the 6 hour experimental period.

Urea
Urea (Figure 5B, Table 4) is the carrier of nitrogen coming from amino acid breakdown and ammonia absorption, is mainly produced by the liver. Arterial time course of urea concentration and net urea production by the splanchnic area was enhanced in the Gel groups in comparing to the Control group (Arterial time course: Gel: p=0.030, Gel+TRP: p=0.010) during the total experimental period (6 hours), which was mainly due to the increased liver urea production.

The deficient essential amino acid: Tryptophan
TRP (Figure 6) was deficient in the Gel group but was added to the meal in the amount of 43.2 µmol TRP per kg bodyweight in the Gel+TRP group. The TRP PDV flux in the Gel group was biphasic: in the first hour it was not different from the
control group. But in the last four hours of the experiment it was significant higher than the control group (p<0.0001) and comparable with the Whey and Gel+TRP group. In the Gel+TRP group, the PDV flux in the first hour was higher than the Gel group (p=0.033) but not different from the Whey group.

Remarkably this phenomenon resulted in a net PDV release (Different from zero: p=0.005) of TRP (Figure 6C) in the TRP-deficient Gel group. The amount was 42% of the release in the Whey group. Due to the biphasic shape of the PDV flux time course in the Gel+Trp group, the PDV net balance was not different from zero (p=0.551). Splanchnic flux time courses showed the same patterns (data not shown).

As in the PDV and splanchnic fluxes, the group differences in arterial TRP time courses (Figure 6) were comparable in the first hour. But in the last four arterial time courses didn’t reflect the PDV fluxes: the time course of the Gel-group was significant lower (p<0.0001) than the time course of the control group. Gel +TRP group was not different with the Whey group.

Figure 5. Arterial ammonia(A), urea(B), glucose(C), lactate(D), in post-absorptive state (Control, n=9) and during 6 hours after administration of Whey protein (Whey, n = 8), gelatin (Gel, n = 9) or gelatin + tryptophan (Gel+TRP, n = 9) meals. Values are mean ± SEM. ANOVA tested, significant meal effect (p < 0.05): (A)(C)(D)protein meals vs. Control (A) Whey vs. Gel + TRP; (B) Whey vs Gel meals; (C) Gel vs. Gel+TRP; (D) Gel vs. Whey only on T=2h. No time courses were different.
Total net balance (production or uptake) across the organ was calculated by integration of the respective flux time courses (area under the curve) from t=0h to t=6h. Values are mean ± SEM. Positive values mean net release. Negative values mean net uptake. Significance with Student t-test: a) P < 0.05 vs. Control. b) P < 0.05 versus Whey.

<table>
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<th>Substance (mmol/kg bw)</th>
<th>Portal drained viscera</th>
<th>Liver</th>
<th>Splanchnic area (mmol/kg bw)</th>
<th>Portal drained viscera</th>
<th>Liver</th>
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<td>297 (52)</td>
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<td>4 (79)</td>
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<td>1 (14)</td>
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<td>Urea (mmol/kg bw)</td>
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<td>6 (97)</td>
<td>6 (201)</td>
<td>227 (17)</td>
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<tr>
<td>Ammonia (mmol/kg bw)</td>
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<td>227 (26)</td>
<td>6 (97)</td>
<td>6 (201)</td>
<td>227 (17)</td>
</tr>
</tbody>
</table>

Table 4. Total net balance during the experimental period (6h)
Figure 6. Arterial tryptophan concentrations (A), gut fluxes of tryptophan (B) and total net balances (C), in post-absorptive state (Control) and during 6 hours after administration of Whey protein (Whey), gelatin (Gel) or gelatin + tryptophan (Gel+TRP) meals. Values are mean ± SEM. (A) and (B) time courses are tested with ANOVA. Significant meal effect (p < 0.05): (A) All time courses were different (B) Control vs. all meals; Whey vs. all meals. (C): Significance with Student t-test; p < 0.05, indicated with hooks.
Energy parameters

Glucose

Despite equal amounts provided, the arterial glucose concentration time course of the Gel group was significant higher comparing to the Gel+TRP group (p=0.003) with a maximum of 9.9 mM at T=1h (Figure 5C). Furthermore in all protein test meals, a similar net PDV release of glucose, net uptake by the liver, resulting in net splanchnic release was seen (Table 4). The mean percentage of glucose released by the PDV was 82% of the glucose intake during the 6 hour feeding period.

Lactate

In a post-absorptive state, the systemic levels of lactate (Figure 5D) were around 0.5 mM in the control group. The PDV was releasing lactate and the liver was taking up lactate, with net uptake across the splanchnic region (Table 4). Lactate in the Gel group stayed longer at the maximum level then in the Whey and Gel+TRP group, and returned to baseline values at T= 4 hours (ANOVA between 1 and 4h: resp. p=0.003 and p=0.034). There were no significant differences in lactate production by the PDV between the test meals, although the uptake of lactate by the liver was lower in the Whey group in comparison with the Gel group (p=0.015).

Intestinal function parameter: Citrulline

The PDV production of Citrulline is related to intestinal function (30, 31). The PDV net production (Table 4) was comparable between the post-prandial period and the post-absorptive state. Also on the splanchnic level there were no net differences in CIT release between the groups.

Discussion

In the present study, we examined the effects of a low quality protein meal (gelatin protein in a protein-carbohydrate meal) on intestinal metabolism, and whether improving the biological value of this meal (by adding tryptophan) would affect intestinal, hepatic metabolism and systemic availability of nutrients. PDV metabolism was studied and assumed to be representative of intestinal metabolism. We observed that after intake of a low protein quality meal (Gel), the healthy intestine released into the portal vein an equivalent amount of amino acids that was given with the meal together with a substantial amount of the deficient TRP. We also observed a tendency that more EAA's were released to the portal system then consumed. It is likely that the intestine responds to such a meal by net breakdown of endogenous (labile) gut protein. Moreover at whole body level, post-prandial systemic EAA levels were below post-absorptive levels, suggesting that net peripheral protein synthesis is stimulated by meal intake itself. Finally, adding the deficient amino acid TRP to the meal resulted in normalized intestinal net balance but not the systemic
EAA and urea levels, suggesting improved intestinal metabolism without improved whole body response.

Quality of the model used

The studies were done in a normal physiological condition. We found that plasma portal flow values were not different between the protein meals suggesting that the differences in portal flow will not play a role in the observed differences in net balance. Net balance data of amino acids across an organ only provide information on the net uptake (net anabolism) or net release (net catabolism) of those amino acids, and reflects the net result of dynamic processes like (arterial) disposal, production, recycling of endogenous secretions, synthesis, breakdown etc. These dynamic processes cannot be quantified without amino acid isotope tracer technology. In a post-prandial condition, net release of EAA to the portal system (PDV net balance) does not reflect net catabolism as the PDV absorbs amino acids from the luminal side. The PDV is in a net catabolic state when release of EAA is more than 100% of its intake.

The percentage of protein digestion by the intestinal lumen and absorption of AA by the gut tissue itself was not measured in the present study. Based on the literature we assumed that in healthy intestine, the percentage of protein digestion and amino acid absorption is nearly the same (around 95%) in the two test meals (32). This is supported by the fact that gut ammonia production was comparable between the test meals, suggesting that the amount of amino acids available for intra luminal bacteria was comparable. Also, t50 of total net portal appearance was not different between the gelatin and the whey protein meal (data not shown), suggesting that the digestion rates of gelatin and whey protein are comparable. Therefore, gelatin protein is the most optimal candidate to study the effects of a low quality protein meal on gut and liver metabolism. As the changes in PDV metabolism were mainly representing changes in intestinal metabolism, we hypothesize that the difference between net intake and observed net portal release of AA is the net fraction of AA that was utilized/oxidized by the intestine itself.

Nitrogen metabolism

The absence of Portal Drained Viscera anabolic state after a low quality Gel meal

The observed fraction of intestinal amino acid utilization/oxidation after intake of the high quality Whey protein meal is in line with previous studies (6, 10). Our present data show the absence of AA utilization/oxidation after a low quality meal. In addition, the substantial release of TRP by the intestine in the TRP deficient test group and the tendency to release more EAA than was ingested (124% of the intake) indicate that a single bolus meal of a very low-quality meal with gelatin protein can
induce net catabolism in the intestine. Further studies with tracer amino acids are needed to confirm this hypothesis.

Remarkably, after 6 hours the intestine was still releasing AA in the Gel-group in contrast to the high quality meal and the Gel+TRP-group, suggesting that a catabolic state can be anticipated in a more extended experimental period. Adding the deficient amino acid TRP to the Gel-meal was enough to prevent the non-anabolic state of the intestine. This suggests that in order to induce intestinal anabolism, a protein meal should contain a complete essential amino acid profile or even maybe need a complete 20 AA’s profile (20). TRP is for the intestine beside an EAA for protein synthesis also a functional amino acid as it is a precursor for melatonin and serotonin production (33, 34). It is therefore unlikely that non-anabolic responsiveness of the intestine is exclusively a TRP-phenomenon. Previously we showed (3) that a protein mixture without the essential amino acid isoleucine was less utilized/oxidized by the intestine and therefore is of less or no benefit to the intestine.

Enhanced urea production by the in low quality meals
AA and ammonia released into the portal system are known to stimulate urea production in the liver. Intestinal ammonia production was comparable between all protein meals and therefore cannot explain the enhanced splanchnic urea production during both Gel meals, resulting in enhanced systemic urea concentrations. The total net intestinal amino acid nitrogen release was elevated only in the Gel meal but not in the TRP supplemented Gel-meal. Although the time courses of the systemic AA_N concentration were different between the high and low quality meals, these observations do not explain the enhanced urea production. Interestingly, the difference between the systemic post-prandial increase in nonEAA_N and EAA_N in the low quality meals compared to the high quality meals was completely different. The ratio nonEAA_N/EAA_N of the post-prandial increase in the low quality meals was 9.5±1.7 (Gel) and 8.0±1.7 (Gel+TRP) versus 0.7±0.2 in the high quality meal, one hour after the meal administration. This was not reflected by the ratio in the meals itself: ratio nonEAA_N/EAA_N in low quality meal was 4.8(Gel: p=0.029, Gel+TRP: p=0.001) versus 1.3 (p=0.029) in the high quality meal. Therefore, it is more likely that the enhanced systemic Non EAA_N concentration has driven the urea production. The ratio was still very high and did not return to baseline values at the end of the experiment. Furthermore, this hypothesis is supported by the shape of the systemic urea time course. This time course was still increasing at the end of the experimental period in the low quality experimental groups.

Effects of splanchnic release of limited EAA_N on post-prandial peripheral metabolism
Systemic (arterial) post-prandial amino acid concentrations are the result of net splanchnic release of AA and whole body use of AA for net protein synthesis.
Post-prandial whole body protein synthesis is stimulated by AA appearing into the systemic circulation but also via the hormonal action of insulin (4, 35). Splanchnic extraction of AA coming from a meal is a major factor determining systemic availability of AA for protein synthesis in the periphery. The systemic AA_N, EAA_N and nonEAA_N time courses were not different between the low quality meals. Remarkably, the EAA_N time courses were below baseline after T= 2h (ANOVA T= 2-6h. Gel:p=0.011, Gel+TRP:p=0.005) . This suggests that, although the intestinal amino acid metabolism was normalized by the TRP supplementation and systemic TRP concentration were elevated, other EAA_N amino acids in the Gel protein meals became limited for the stimulated whole body net protein synthesis, due to the fact that the ratio nonEAA_N/EAA_N was still not optimal in these meals. The below baseline values of the EAA_N, suggest that the demand for EAA_N is higher than the splanchnic area can deliver.

**Energy metabolism**

The comparable responses in post-prandial splanchnic glucose release indicate that net glucose metabolism in the splanchnic region was not influenced by the quality of the protein but that other processes in the periphery are responsible like an enhanced insulin response in the TRP supplemented meal (25).

In addition, net PDV production of lactate and alanine (products of the post-prandial glucose glycolysis) (36) was not different between the test meals, and differences in alanine content between the test meals had no effect on the net intestine production (data not shown). Therefore, we conclude that post-prandial intestinal glucose glycolysis was not changed.

The amount of lactate taken up by the liver during a normal high-quality meal was reduced, most likely due to inhibition of gluconeogenesis by the increase of insulin. However lactate (substrate for the gluconeogenesis) uptake in the low quality meal was much higher and reached comparable levels as in the post-absorptive state. This observation and the fact that a higher systemic concentration of lactate was found indicates that more lactate is produced in the periphery after intake of the low quality meal, resulting in enhanced turnover in the Cori-cycle. Elevated systemic lactate levels can stimulate gluconeogenesis (an energy consuming process) despite the portal net glucose appearance. It also indicates that a low quality meal can compromise metabolism in the periphery (muscle) and result in higher post-prandial energy expenditure. Metabolic studies on a muscle compartment are necessary to confirm this hypothesis. Interesting, adding the deficient amino acid TRP to this meal resulted in normalization of lactate metabolism.
Gut health after a single low quality protein meal

CIT production of the intestine can be a marker for gut function/health (30, 31). In the present study, PDV CIT production was not influenced by either the quality of the meal or the available amount of potential precursors in the meal (GLN, ARG or PRO), which suggests that the healthy gut is able to compensate to keep its major functions like CIT production when a low quality meal is given. It could well be that in this situation the intestine is breaking down endogenous proteins to secure continuity of protein synthesis of other endogenous proteins which is necessary for the most important intestinal function; absorption and barrier function. It is also possible that the intestine is compensating for the higher demand for AA’s for peripheral protein synthesis that is stimulated by the meal itself (insulin). This supports the hypothesis that the intestine can act as buffer/interface for systemic AA bio-availability for the rest of the body in non-optimum food situations (11). Further studies with stable isotopes of AA are necessary to confirm this.

These data also support the hypothesis that the total amino acid profile of a protein meal determines the biological value of the meal for the intestine and is independent of the sources of the proteins (20, 37). Therefore, it is important particularly in vegetarian diets that a meal contains different protein sources to prevent that a complete meal becomes of very low of quality because of one deficient EAA_N.

Conclusions

After a low quality protein meal, the healthy gut is not able to generate an anabolic response. Our data suggest that in this situation, the intestine breaks down its own labile protein pool to fulfill the increased systemic amino acid demand. Supplementation of the deficient essential amino acid does improve intestinal metabolism, but the next limiting amino acid will determine the quality of the ingested protein for the remainder of the body. It is likely that chronic intake of low quality proteins can lead to exhaustion of the labile intestinal protein pool, possibly resulting in reduced function of the gut and susceptibility to intestinal and other disease states.

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References


Enhanced lacto-tri-peptide bioavailability by co-ingestion of macronutrients

Gabriella A.M. Ten Have, Pieter C. van der Pijl, Arie K. Kies and Nicolaas E.P. Deutz


Abstract

Some food-derived peptides possess bioactive properties, and may affect health positively. For example, the C-terminal lacto-tri-peptides Ile-Pro-Pro (IPP), Leu-Pro-Pro (LPP) and Val-Pro-Pro (VPP) (together named here XPP) are described to lower blood pressure. The bioactivity depends on their availability at the site of action. Quantitative trans-organ availability/kinetic measurements will provide more insight in C-terminal tri-peptides behavior in the body. We hypothesize that the composition of the meal will modify their systemic availability.

We studied trans-organ XPP fluxes in catheterized pigs (25 kg; n=10) to determine systemic and portal availability, as well as renal and hepatic uptake of a water-based single dose of synthetic XPP and a XPP containing protein matrix (casein hydrolyte, CasH). In a second experiment (n=10), we compared the CasH-containing protein matrix with a CasH-containing meal matrix and the modifying effects of macronutrients in a meal on the availability (high carbohydrates, low quality protein, high fat, and fiber).

Portal availability of synthetic XPP was 0.08 ± 0.01% of intake and increased when a protein matrix was present (respectively 3.1, 1.8 and 83 times for IPP, LPP and VPP). Difference between individual XPP was probably due to release from longer peptides. CasH prolonged portal bioavailability with 18 min (absorption half-life, synthetic XPP: 15 ± 2 min, CasH: 33 ± 3 min, p<0.0001) and increased systemic elimination with 20 min (synthetic XPP: 12 ± 2 min; CasH: 32 ± 3 min, p<0.0001). Subsequent renal and hepatic uptake is about 75% of the portal release. A meal containing CasH, increased portal 1.8 and systemic bioavailability 1.2 times. Low protein quality and fiber increased XPP systemic bioavailability further (respectively 1.5 and 1.4 times).

We conclude that the amount and quality of the protein, and the presence of fiber in a meal, are the main factors that increase the systemic bioavailability of food-derived XPP.
Introduction

In the recent years our understanding of potential food-derived bioactive peptides and their potential health benefits advanced (1, 2), mainly on the role of bioactive peptides from milk. For instance, the effects on blood pressure of the lacto-tri-peptides Isoleucine-Proline-Proline (IPP) and Valine-Proline-Proline (VPP) is described in animal (3) and human studies (4-12). The hypothesized working mechanism of these peptides is inhibition of the angiotensin I converting (ACE) enzyme (4) and is related to the presence of a proline residue on the C-terminal of the lacto-tri-peptides (13). Animal and human studies also suggest that IPP and VPP reduce arterial stiffness and improve endothelial activity (14, 15).

The beneficial effects of food-derived bioactive peptides depend on availability on the site of action. For instance, to exert a potential ACE inhibition effect after oral intake of the lacto-tri-peptides, the peptides have to reach the cardiovascular system in an active form (16). Metabolic active organs like the intestine, liver, and kidney play a major role in the availability. Fluxes of these peptides through those organs are likely to be dependent on the fluxes of other macronutrients, especially proteins and amino acids (17-22). No fundamental knowledge of quantitative organ flux data are available for food-derived bioactive lacto-tri-peptides to get more insight in the behavior of those peptides in the body (23). Therefore, the main aim of the present study is to collect quantitative data about how much food-derived bioactive lacto-tri-peptides is absorbed and released to the portal system, the role of kidney and liver and their effect on the systemic availability under different nutritional conditions. We studied IPP, VPP and LPP (Leucine-Proline-Proline), named together as XPP as model for C-terminal proline containing food-derived peptides in general, because they are relatively resistant against breakdown in the gastrointestinal tract and therefore expected to be similar in their kinetics (3, 24).

Previously, we found that the systemic bioavailability of a single dose of synthetic XPP, when given orally as a single dose, was less than 0.1% (25) with a half-life maximum at 15 min. In the present study, we hypothesize that dietary composition modifications improve absorption kinetics of XPPs and thus their bioavailability.

Therefore, we studied trans-organ XPP fluxes in catheterized pigs to determine the effect of a protein matrix on the XPP systemic and portal availability, renal and hepatic uptake using a XPP containing casein hydrolyte (CasH). Casein is the main lacto-protein that is rich of encrypted XPP’s (www.genome.jp). This, in contrast with the lacto-protein whey or a non-milk protein soy with no encrypted XPP’s. The studied CasH is rich of liberated IPP and LPP, produced by using enzymes including a specific prolyl-endoprotease (26). This CasH is proven to have ACE inhibitory potential (12) and has a faster digestion and absorption rate (27) than casein, which potentially relates to the maximally available systemic XPP concentrations.
In a second experiment, we studied the potential effect on XPP availability when the XPP containing CasH was added to a meal and studied the potential modifying effects of macro-nutrients (high carbohydrates, low quality protein, high fat, and fiber) in the meal. Quantification of intestinal absorption kinetics of XPP and the role of liver and kidney is not possible in humans, therefore we measured the transorgan XPP fluxes in conscious multi-catheterized pigs (28, 29) as the gastro-intestinal tract of the pig is comparable with that of humans (30-32).

The quantitative results of the present study are important as crucial fundamental information for the development of therapies with bioactive peptides.

Materials and Methods

Animals

For each study, we used 10 pathogen-free, female piglets (Dutch landrace x Yorkshire; 8-12 weeks of age; 25.2 ± 1 kg body weight) that were adapted to individual housing in straw containing pens (2 x 3 m) with 12h day-night cycle and an environmental temperature of 23°C one week before surgery. Catheters for blood sampling were placed during surgery (28) under isoflurane (2% mixture with oxygen) anesthesia and flunixin meglumine (50 mg/25 kg bw) analgesia, in the abdominal aorta, the portal vein, the hepatic vein, and the renal vein. Catheters for infusions of post-operative medication or para-aminohippuric acid solution (PAH; flow measurements) were placed in the central vein and splenic vein and for gastric administration a gastric catheter. We checked the position of the catheter tips with X-ray during surgery using fluoroscopy and an iodine containing contrast solution.

During the recovery period (7-10 days), twice daily, we and the assigned veterinarian of the animal care facility monitored the animals for general behavior, body temperature, food intake, body weight and catheter patency and administered buprenorphine (0.03mg/kg) analgesia, intra-venous, twice daily on day 1-4 and when needed. Animals were accustomed to a small movable cage (0.9 x 0.5 x 0.3 m) to perform the experiments. At all times, animals would be euthanized with 0.5 ml/kg bw Euthanasol® (390 mg pentobarbital sodium + 50 mg phenytoin sulfate/ml), intra-venous, if imminent death is expected.

Ethics Statement

The animal ethics committee of Maastricht University, The Netherlands approved the studies: 2004-101. We performed the surgery under isoflurane anesthesia, and all efforts were made to minimize suffering. The researchers and the assigned veterinarian of the animal care facility monitored the anesthesia and recovery closely.
**Experimental protocol**

**Study 1**

After a postoperative recovery period of 7-10 days, we conducted five test days with XPP mixtures according to a Williams cross-over design (33) with a minimum of 2 days (washout) between the test days. One intra-venous administered IPP, LPP and VPP mixture (data previously published (25)) and four intra-gastric administered XPP mixtures were studied (Table 1). We studied a control solution (Control-group) without XPP, but iso-ionogenic to the other mixtures for sodium, potassium and calcium ions, to determine potential occurring endogenous XPP fluxes. We gave a water-based matrix with comparable amounts of IPP, LPP and VPP (XPP-group) for comparison with a protein based XPP mixture (casein hydrolyte, CasH-group). The CasH contained known amounts of free XPP with a C-terminal proline. We gave a XPP spiked protein matrix (CasH+XPP group) to evaluate the influence of a protein matrix on individual XPP’s. In the present study, we only will discuss the data of the four intra-gastric administered XPP. We administered these mixtures as a single bolus with an end volume of 14 ml/kg body weight and temperature of 37ºC.

<table>
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<td>81.6</td>
</tr>
<tr>
<td>Protein</td>
<td>mg/kg bw</td>
<td>0.0</td>
<td>0.0</td>
<td>648</td>
<td>648</td>
</tr>
<tr>
<td>Sodium</td>
<td>mM</td>
<td>3.01</td>
<td>3.01</td>
<td>3.01</td>
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<tr>
<td>Potassium</td>
<td>mM</td>
<td>71.14</td>
<td>71.14</td>
<td>71.14</td>
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<tr>
<td>Calcium</td>
<td>mM</td>
<td>1.96</td>
<td>1.96</td>
<td>1.96</td>
<td>1.96</td>
</tr>
<tr>
<td>Phosphate</td>
<td>mM</td>
<td>71.1</td>
<td>71.1</td>
<td>102.9</td>
<td>102.9</td>
</tr>
</tbody>
</table>

1 Chemical purities of the IPP, LPP, and VPP synthetic products were 93.4, 95.0 and 98.7%, respectively (Bachem, Weil am Rhein, Switzerland).

2 The given amounts of tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in the casein hydrolysate (CasH, Casimax®, DSM Food Specialties, Delft, The Netherlands). The casein hydrolysate contained 57% protein with 5.4, 16.5 and 0.3 mg/g protein of LPP, LPP and VPP, respectively.

3 Total XPP = total amount of IPP, LPP and VPP.

**Study 2**

After a postoperative recovery period of 7-10 days, we conducted six test days with CasH interventions according to a Williams cross-over design (33) with a minimum of 2 days (washout) between the test days. We compared a CasH supplemented meal matrix (Basal-group) to a CasH only protein matrix (CasH2-group). We also compared four other CasH supplemented meals with low protein quality (LQprot-group),...
high carbohydrate (hCHO-group), high fat (hFat-group) and fiber containing Basal meal (Fiber-group) to Basal to evaluate the influence of macronutrients (Table 2). The Basal meal contains a ratio of 25:50:25 En% of the macronutrients protein, carbohydrates and fat. All meals were iso-caloric (30% of daily intake: 37.2 kcal/kg bodyweight) and composed by Research Diet Services (Wijk bij Duurstede, The Netherlands). The protein fraction in the meals was a high quality whey protein isolate, while in the low quality protein diet a soy protein isolate that had 24% less essential amino acids. We gave all meals intra-gastric as a single bolus with an end volume of 24 ml/kg bodyweight and 37°C and observed for 360 min.

Table 2. Composition of test meals - Study 2

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Units</th>
<th>Cash2</th>
<th>Basal</th>
<th>LQprot</th>
<th>hCHO</th>
<th>hFat</th>
<th>Fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey protein</td>
<td>Energy %</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Soy protein</td>
<td>Energy %</td>
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<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CHO</td>
<td>Energy %</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>65</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Fat</td>
<td>Energy %</td>
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<td>10</td>
<td>50</td>
<td>25</td>
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<tr>
<td>Total energy intake</td>
<td>kcal/kg bw</td>
<td>0</td>
<td>37.2</td>
<td>37.2</td>
<td>37.2</td>
<td>37.2</td>
<td>37.2</td>
</tr>
<tr>
<td>Whey protein isolate$^2$</td>
<td>g/kg bw</td>
<td>0</td>
<td>2.32</td>
<td>0</td>
<td>2.32</td>
<td>2.32</td>
<td>2.32</td>
</tr>
<tr>
<td>Soy protein isolate$^2$</td>
<td>g/kg bw</td>
<td>0</td>
<td>0</td>
<td>2.32</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CHO§</td>
<td>g/kg bw</td>
<td>0</td>
<td>4.64</td>
<td>4.64</td>
<td>6.04</td>
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<tr>
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<td>1.04</td>
<td>0.42</td>
<td>2.07</td>
<td>1.04</td>
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<td>Supplement</td>
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<td></td>
</tr>
<tr>
<td>Casein hydrolysate†</td>
<td>g/kg bw</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Fiber††</td>
<td>g/kg bw</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>Tri-peptides$^3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPP</td>
<td>µmol/kg bw</td>
<td>12.1</td>
<td>12.1</td>
<td>12.1</td>
<td>12.1</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>LPP</td>
<td>µmol/kg bw</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
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</tr>
<tr>
<td>VPP</td>
<td>µmol/kg bw</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
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<tr>
<td>Gross weight</td>
<td>g/kg bw</td>
<td>1.28</td>
<td>9.80</td>
<td>9.94</td>
<td>10.7</td>
<td>8.32</td>
<td>9.99</td>
</tr>
<tr>
<td>End Volume</td>
<td>ml/kg bw</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

1 Indicated is the amount of protein supplied by the isolates.

2 CHO: maize starch, sucrose and glucose in the weight ratio 2:1:1.

3 Fat: soybean oil and sunflower oil in the weight ratio 4:1.

4 The casein hydrolysate product (Casimax®, DSM Food Specialties, Delft, The Netherlands) contained 57% protein with 5.4, 16.5 and 0.3 mg/g protein LPP, LPP and VPP, respectively.

5 Fiber: modified citrus pectin.

Sixteen hours before each test day, we removed food. Plasma flow determination started 1h before feeding (t=0) and continued throughout the experiment by a primed-continuous infusion of para aminohippuric acid through the splenic infusion.
catheter. At steady state concentrations of PAH (1 hour (28)), we took blood samples from the arterial, portal, hepatic, and renal vein. We gave the liquid test mixture or meal via the gastric catheter within 5 min. Subsequently, we took blood samples (1.5 ml/sample) in study 1 from the arterial, portal, hepatic and renal catheter at T=1, 3, 5, 7, 9, 12.5, 17.5, 25, 30, 40, 50, 60 and 90 min; in study 2 from the arterial and portal catheter at T=10, 20, 40, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min.

**Sample processing**

Directly after collection in Lithium-Heparin tubes, we placed the blood samples on ice. We processed all blood samples within 1 hour after collection to ensure stability of the substances. We centrifuged at 4°C for 15 min at 3000 g to obtain plasma. For PAH concentration determinations we added 40 µl of 50% w/v Tri-chloroacetic acid solution (TCA) to 400 µl of plasma and mixed. For XPP analyses, we added 10 µl of 10% w/v tri-fluoroacetic acid (TFA) to 500 µl plasma and mixed. We froze the samples in liquid nitrogen and stored at 80°C until further analysis. We took samples from the PAH infusion solutions and the test mixtures.

**Biochemical analyses**

We analyzed the plasma samples and test mixtures for the peptides IPP, LPP and VPP (XPP) content (25, 34). We added 50 µl internal standard solution, containing U-13C-IPP, U-13C-VPP, and U-13C-LPP to homogenized plasma (20 µl) and 480 µl water. After mixing, we acidified this aliquot with tri-fluoroacetic acid to pH <3. We removed proteins by heating the aliquot at 95 °C for 2 min, followed by centrifugation at 22,000 g for 30 min at 15°C. We quantified the XPP’s in the supernatant with LC-MS (Quattro Ultima, Waters, Milford, MA, USA). The detection limit of quantification of this procedure was 0.28, 0.28, and 0.71 nM for IPP, LPP, and VPP respectively.

We tested samples of test mixtures by mixing 100 µl of the sample with 100 µl of a standard solution of universally 13C- labeled IPP [U-13C-IPP] and VPP [U-13C-VPP] (Biopeptide Co., San Diego, CA, USA). Then we mixed for 1 min, followed by centrifugation for 20 min at 16,000 x g at room temperature. This procedure allows determination of XPPs in the test mixtures between 1.9 and 123 µM.

We analyzed in a pilot set of plasma samples from Study 1, the occurrence of di-peptides with proline (Proline-Proline (PP), leucine (LP), valine (VP), isoleucine (IP)). We determined di-peptide concentrations with an automated LC-ESI-MS system (QTrap 5500 MS (AB Sciex, Foster City, CA, USA) with ExpressHT Ultra LC (Eksigent Div., AB Sciex, Foster City, CA, USA). We added supernatant (20 µl) of centrifuged TCA deproteinized plasma to a 0.1 N hydrochloric acid containing internal standard (20 µl) of a stable isotopomer [D8-valine, D5-proline]. We derivatized internal standard containing samples and di-peptide external standards with 9-Fluorenylmethoxycarbonyl (Fmoc), neutralized and injected 160 nL of the solution.
on a micro LC column 0.5 x 100 mm HALO C18, 2.7 µm, 90A pores at 35°C. We eluted the analytes with a segmentally linear gradient from 35% to 85% acetonitrile in water supplemented with ammonium acetate to 10 µM and 5% isopropanol. We detected by electrospray triple quadrupole tandem mass spectrometry in multiple reactions monitoring mode. For concentration calculations of di-peptides, we normalized MS signals of the samples and the external standards with their internal standard. We determined di-peptide concentrations with the calibration curve of the external standards.

We determined plasma PAH concentrations with a standard enzymatic method on an automatic spectrophotometric analyzer (Cobas Mira, Hoffmann-La Roche, Basel, Switzerland) as described (22, 30-32). In brief, we measured plasma PAH concentration at 465 nm using Ehrlich’s reagent: 1 g p-dimethylaminobenzaldehyde (Merck, Darmstadt, Germany), 35 ml alcohol 96%, 4 ml TCA 2N, 61 ml water (bi-distilled) and a solution of 35% alcohol.

Calculations

Systemic pharmacokinetics

Using a 1-compartment model (25) we determined in study 1, absorption half-life (t1/2,a;min), time to maximum plasma concentration (tmax;min), maximum plasma concentration (Cmax;nM), elimination half-life (t1/2,e;min) and systemic absolute bioavailability (fAbs;%). For study 2, we used a non-compartmental analysis calculate AUCs of the arterial XPP time curves from 0 to 360 min (nmol/l*min). We calculated the systemic bioavailability relative to regular, basal meal using the following equation:

\[ f_{Rel} = \frac{(AUC_{Basal} \times D_{test})}{(AUC_{Test} \times D_{Basal})} \times 100\% \]

where \( f_{Rel} \) = the bioavailability for an administered XPP from a meal relative to Basal (%), \( AUC_{Basal} \) = the AUC for Basal (nmol/l*min), \( D_{test} \) = the dose of an XPP in the meal (nmol), \( AUC_{Test} \) = the AUC for the meal (nmol/l*min), \( D_{Basal} \) = the XPP dose of Basal (nmol).

XPP organ fluxes

We measured plasma flow, necessary to calculate amino acid fluxes across organs. We calculated PDV fluxes of IPP, LPP and VPP by portal arterial XPP concentration difference times portal plasma flow, liver fluxes by subtracting PDV flux from splanchnic flux (hepatic arterial difference* liver plasma flow) and renal flux by renal plasma flow times renal-arterial XPP concentration difference (22, 28, 31, 32, 35). We expressed organ flux as pmol/kg body weight/min and a positive flux is net release of XPP and negative flux net uptake.
We calculated total net release or uptake (=organ net balance) of XPP across an organ from the area under curve of the respective organ flux time course. We expressed total net balance as pmol/kg bodyweight. For the PDV, the portal bioavailability is the total net balance as fraction dose of IPP, LPP or VPP in the test mixtures/meals. We calculated the free available XPP in the test mixtures of study 1 or test meals in study 2 from information, made available by the manufacturer.

Statistical procedures
We used Prism 6 (Graphpad Software, La Jolla, CA, USA) for statistics, expressed the results as means ± SEM and set the level of significance on p<0.05 and tendencies are defined as p<0.01. We tested the data for normality and used the Wilcoxon test to determine if means are different from zero.

Comparisons between matrixes and mean XPP responses where done with two-way ANOVA. When appropriate, followed by post-hoc test (Sidak’s, Dunnett’s, Fisher’s LSD or Wilcoxon Signed Rank test as indicated in tables and figures) for planned physiological relevant multiple comparison.

We compared the means of two groups with unpaired t-test or Wilcoxon matched pairs signed rank test, means of more than three groups with One-way repeated measures ANOVA. When appropriate, followed by a post-hoc test (Dunnett’s) for planned physiological relevant multiple comparison.

We compared means of groups in the time courses with two-way ANOVA test, mixed model: repeated measurements for time (fixed effect) but not for test mixtures, for planned physiological relevant comparisons.

Results

Effect of a protein matrix on systemic bioavailability and pharmacokinetics of XPP peptides.

Systemic (=arterial) concentrations of XPP in time (Figure 1) showed no measurable baseline/post-absorptive endogenous XPP (Control group, tested with Wilcoxon). The XPP concentrations in the CasH groups were not back to baseline at 90 min post-prandial (one-way ANOVA with Dunnett’s post-hoc test: IPP p<0.002; LPP p<0.001; VPP p<0.05). The calculated systemic absolute bioavailability (f_{abs} (%) = fraction dose absorbed, Table 3) was increased in the protein based CasH group (IPP: 4.7 times, p<0.0004; LPP: 2.5 times, p<0.002; VPP: 121 times, p<0.0001). The spiked CasH group (CasH+XPP) showed lower systemic bioavailability for VPP (65 times, p<0.0001) in comparison to CasH. There was a delay in the time to maximum
Figure 1. Systemic levels of XPP peptides – Effect of protein matrix

Post-prandial arterial concentrations after intra-gastric administration of control salt solution (Control), synthetic XPP’s (XPP), casein hydrolysate rich in XPP (CasH) and spiked CasH (CasH + XPP). A: Isoleucine-proline-proline (IPP). B: Leucine-proline-proline (LPP). C: Valine-proline-proline (VPP). Respective number of observations for Control, XPP, CasH and CasH + XPP are for graph A: n = 9, 10, 9 and 10; graph B: n = 10, 10, 9 and 10; graph C: n = 8, 10, 9 and 10. Values are mean ± SEM. Statistics: repeated measures two-way ANOVA, mixed model, planned comparisons. All curves are significantly different from the XPP mixture: effect test mixture p<0.001; effect time p<0.001; interaction p<0.001.
### Table 3. Systemic bioavailability and pharmacokinetics of XPP peptides – Effect of a protein matrix

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Matrix</th>
<th>Group</th>
<th>IPP</th>
<th>LPP</th>
<th>VPP</th>
<th>p-value²</th>
<th>p-value²</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Matrix/spike effect</td>
<td>XPP effect</td>
<td>Interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f_{abs} (%)</td>
<td>Water-based</td>
<td>XPP¹</td>
<td>0.08 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>CasH</td>
<td>0.38 ± 0.05*</td>
<td>0.15 ± 0.03*</td>
<td>8.49 ± 0.69*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CasH+XPP</td>
<td>0.26 ± 0.06⁶</td>
<td>0.16 ± 0.04</td>
<td>0.13 ± 0.78⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t_{1/2,a} (min)</td>
<td>Water-based</td>
<td>XPP¹</td>
<td>3.3 ± 0.5</td>
<td>2.0 ± 0.5</td>
<td>4.6 ± 1.1</td>
<td>0.003</td>
<td>0.687</td>
<td>0.581</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>CasH</td>
<td>11.2 ± 3.3</td>
<td>7.8 ± 2.3</td>
<td>8.5 ± 2.5</td>
<td>0.978</td>
<td>0.789</td>
<td>0.697</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CasH+XPP</td>
<td>7.8 ± 3.6</td>
<td>8.0 ± 2.6</td>
<td>10.5 ± 2.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t_{max} (min)</td>
<td>Water-based</td>
<td>XPP¹</td>
<td>8.6 ± 0.6</td>
<td>7.1 ± 0.7</td>
<td>8.9 ± 0.6</td>
<td>&lt;0.0001</td>
<td>0.628</td>
<td>0.805</td>
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<tr>
<td></td>
<td>Protein</td>
<td>CasH</td>
<td>22 ± 5</td>
<td>19 ± 2</td>
<td>19 ± 2</td>
<td>0.775</td>
<td>0.771</td>
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<td>CasH+XPP</td>
<td>19 ± 5</td>
<td>19 ± 3</td>
<td>20 ± 3</td>
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<td></td>
</tr>
<tr>
<td>C_{max} (nM)</td>
<td>Water-based</td>
<td>XPP¹</td>
<td>12 ± 3</td>
<td>11 ± 3</td>
<td>9 ± 2</td>
<td>0.026</td>
<td>0.823</td>
<td>0.762</td>
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<td>Protein</td>
<td>CasH</td>
<td>16 ± 3</td>
<td>17 ± 3</td>
<td>16 ± 3</td>
<td>0.022</td>
<td>0.905</td>
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<td></td>
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<td>CasH+XPP</td>
<td>25 ± 3</td>
<td>21 ± 3</td>
<td>21 ± 3</td>
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<td></td>
</tr>
<tr>
<td>t_{1/2,e} (min)</td>
<td>Water-based</td>
<td>XPP¹</td>
<td>9.3 ± 1.1</td>
<td>15 ± 4</td>
<td>12 ± 6</td>
<td>&lt;0.0001</td>
<td>0.590</td>
<td>0.192</td>
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<tr>
<td></td>
<td>Protein</td>
<td>CasH</td>
<td>39 ± 5</td>
<td>31 ± 11</td>
<td>27 ± 4</td>
<td>0.970</td>
<td>0.236</td>
<td>0.462</td>
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<td>CasH+XPP</td>
<td>36 ± 6</td>
<td>42 ± 12</td>
<td>23 ± 4</td>
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</table>
XPP plasma concentration ($t_{\text{max}}$) with the CasH groups compared to the XPP test mixture (12 min, $p<0.0001$, Wilcoxon matched pairs signed rank test). The calculated absorption half-life ($t_{\frac{1}{2},a}$) and elimination half-life ($t_{\frac{1}{2},e}$) was delayed significantly in CasH groups (resp. 6 min and 20 min, $p<0.0001$, Wilcoxon matched pairs signed rank test). Plasma concentrations of the dipeptides LP, IP, VP and PP in the post-prandial period, measured in a limited set of samples, were in the range of 1-7 µM, compared to 1-30 nM for XPP’s in this sample set (Supplemental Table 1. Dipeptide/tripeptide ratio - Study 1).

Effect of meal matrix on portal availability of XPP peptides

PDV fluxes showed a net release of all XPP after intra gastric administration of XPP in different matrixes (Figure 2). We observed no endogenous production of XPP by the PDV. The patterns of the curves were comparable for IPP, LPP and VPP. No differences in PDV plasma flows were observed (Supplemental Table 2. Plasma flows - Study 1). The single dose water-based synthetic XPP matrix, showed comparable portal availability for IPP, LPP and VPP, with an average of 0.08 ± 0.01 % of the intake (Table 4). The portal availability was higher in a protein (CasH) matrix than in water-based XPP matrix, but different between IPP, LPP and VPP (IPP 3.1 times, $p=0.026$; LPP 1.8 times, $p=0.23$; VPP 83 times, $p<0.0001$). For VPP, the spiked CasH showed a different portal availability in comparing to the non-spiked CasH matrix (0.05 times of CasH, $p<0.0001$). To understand the significant XPP effect and spike effect, data were further analyzed, by expressing the data as % of theoretically intake of XPP (Supplemental Table 3. Theoretical XPP intake and their portal availability - Study 1), considering the tri-peptide sequences in the source of the CasH: amino acid sequence of bovine $\kappa$ and $\beta$ casein (www.genome.jp; CASB-BOVIN, CASK-BOVIN).

No spike effect of VPP was observed when corrected of potential non free available

**Table 3 (left). Systemic bioavailability and pharmacokinetics of XPP peptides – Effect of a protein matrix**

Pharmacokinetic parameters derived from the systemic response of an intragastric bolus administrated of the tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in water-based matrix (synthetic XPP) or in a protein matrix (casein hydrolysate rich in XPP: CasH). $f_{\text{abs}}$ (%): systemic absolute bioavailability, $t_{\frac{1}{2},a}$: absorption half-life, $t_{\text{max}}$: time to maximum plasma concentration, $C_{\text{max}}$: maximum plasma concentration, $t_{\frac{1}{2},e}$: elimination half-life. Parameters calculated with a 1-compartment model. Values are means ± SEM. For $f_{\text{abs}}$ n=8. For other parameters XPP: n=10; CasH: n=10; CasH+XPP: n=9. Significance: $p<0.05$.

1): Data XPP group from the present study 1, are published in van der Pijl et al. (25).

2): Significance for comparison of water-based matrix (XPP) with protein matrix (CasH). Or significance for comparison between XPP spiked (CasH+XPP) and non-spiked protein matrix (CasH): Two-way ANOVA. When appropriate, a post-hoc unpaired t-test is done:

$: p<0.05$ significance for comparison IPP, LPP or VPP of XPP relative to CasH.

$: p<0.05$ significance for comparison IPP, LPP or VPP of CasH relative to CasH+XPP.
Figure 2. Portal Drained Viscera fluxes of XPP – Effect of a protein matrix
Post-prandial portal drained viscera (PDV) fluxes after intra-gastric administration of tri-peptide (XPP) mixtures: control salt solution (Control), synthetic XPP’s (XPP), casein hydrolyte rich in XPP (CasH) or spiked CasH (CasH + XPP). A: Isoleucine-proline-proline (IPP). B: Leucine-proline-proline (LPP). C: Valine-proline-proline (VPP). Respective number of observations for Control, XPP, CasH and CasH + XPP are for graph A: n = 6, 9, 8 and 9; graph B: n = 6, 10, 9 and 9; graph C: n = 5, 9, 9 and 9. Values are mean ± SEM. Positive values is net release, negative values is net uptake. Statistics: repeated measures two-way ANOVA, mixed model, planned comparisons. All curves are significantly different from the XPP mixture: effect test mixture p<0.01; effect time p<0.01; interaction p<0.01.
### Table 4. Portal bioavailability and release half-life of XPP peptides – Effect of a protein matrix

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Matrix/Spike effect</th>
<th>XPP effect</th>
<th>Portal bioavailability</th>
<th>Release half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPP</td>
<td>Protein (CasH)</td>
<td>0.09 ± 0.03 (0.07 ± 0.02)</td>
<td>&lt;0.0001</td>
<td>0.25 ± 0.04 (0.17 ± 0.02)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
<tr>
<td>LPP</td>
<td>Protein (CasH)</td>
<td>0.17 ± 0.03 (0.16 ± 0.02)</td>
<td>&lt;0.0001</td>
<td>0.21 ± 0.03 (0.19 ± 0.02)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
<tr>
<td>VPP</td>
<td>Protein (CasH)</td>
<td>0.28 ± 0.05 (0.26 ± 0.04)</td>
<td>&lt;0.0001</td>
<td>0.30 ± 0.04 (0.29 ± 0.03)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
<tr>
<td>IPP</td>
<td>Water-based XPP</td>
<td>0.06 ± 0.03 (0.03 ± 0.01)</td>
<td>&lt;0.0001</td>
<td>0.12 ± 0.03 (0.10 ± 0.02)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
<tr>
<td>LPP</td>
<td>Water-based XPP</td>
<td>0.18 ± 0.03 (0.16 ± 0.02)</td>
<td>&lt;0.0001</td>
<td>0.22 ± 0.04 (0.20 ± 0.03)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
<tr>
<td>VPP</td>
<td>Water-based XPP</td>
<td>0.32 ± 0.05 (0.30 ± 0.04)</td>
<td>&lt;0.0001</td>
<td>0.34 ± 0.04 (0.33 ± 0.03)</td>
<td>0.0010 ± 0.0003 (0.0010 ± 0.0002)</td>
</tr>
</tbody>
</table>

*Significance: p<0.05 significance for comparison of protein (CasH) with water-based XPP (n=5); CasH: n=7; CasH+XPP: n=6. When appropriate post-hoc LSD is done: §: p<0.05 significance for comparison of protein (CasH) with water-based XPP (n=5); CasH: n=7; CasH+XPP: n=6.*
VPP sequences in the CasH. With this correction we determined that on average the portal availability was 1.8 times higher in the protein matrix (XPP 0.08 ± 0.01 vs CasH 0.14 ± 0.02, two-way ANOVA, matrix effect, p=0.004). Further analyses of the PDV flux curves showed an increase of 2.1 times of the portal release half-life (Table 4) in the protein matrix in comparison to the water-based matrix (XPP: 15 ± 2 min vs CasH: 33 ± 3 min, two-way ANOVA, matrix effect <0.0001).

Multiple organ interactions of XPP peptides

In Study 1, we determined the XPP fluxes across multiple organs, to understand more about the influence of different organs on the systemic availability. We found no effect of the matrixes on the plasma flows of PDV, liver and kidneys (Supplemental Table 4). In the post-absorptive Control group, total net balances of XPP were not different from zero (Figure 3). Post-prandial balances showed a net release of XPP by the PDV, followed by a partial uptake by the liver, resulting in net release of XPP by the splanchnic area. Systemic available XPP are in part, taken up by the kidneys.

Effect of meal matrix on the systemic availability of XPP peptides

In study 2, we compared XPP responses between a protein and a meal matrix and the potential effects of different macro-nutrients in a meal matrix. With the Basal meal matrix, the systemic availabilities of XPP’s were 1.2 times (p=0.019, Wilcoxon rank test) increased over a post-prandial period of six hours in comparison to the protein matrix CasH2 (Table 5). The low quality protein and fiber were the macro-nutrients that increased the XPP systemic availability in a meal further with resp. 1.5 and 1.4 times in comparison to the Basal meal. (Two-way ANOVA with post-hoc Dunnett’s test; macronutrient effect: resp. p=0.0003 and p=0.0009).

Effect of a meal matrix on portal availability of XPP peptides

Expressed as the fraction dose of total PDV net balance over the experimental period of six hours, the portal availability was increased with an average 1.8 times (56±7 % of Basal, two-way ANOVA, matrix effect, <0.0001) if the XPP containing CasH was given with a complete meal (Table 6). In contrast with the systemic bioavailability, we did not observe differences between the basal and the other meals and in PDV plasma flows (Supplemental Table 4. PDV Plasma flows - Study 2.). Further analysis of the PDV flux curves showed an increase of 2.6 times of the portal release half-life (Table 7) in the meal matrix in comparison to the protein matrix (CasH2: 47 ± 5 min; Basal: 122 ± 11 min; two-way ANOVA, matrix effect <0.0001). Also, the portal release half-life was reduced in a low quality protein meal 1.5 times, and increased 1.3 times in a high fat containing meal (LQprot: 80 ± 7; hFat: 163±10; two-way ANOVA with post-hoc Dunnett’s multiple comparison, macronutrient effect resp. p=0.009 and p=0.014).
Figure 3. Organ Total Net balances of XPP peptides
Study 1. Post-prandial total net balances across organs over 90 min experimental period after intra-gastric administration of tri-peptide (XPP) mixtures: a synthetic dose of XPP (XPP), XPP containing casein hydrolyte (CasH) and spiked CasH (CasH + XPP). Organs: portal drained viscera (PDV), Liver, Splanchnic area (SPL), Kidneys. Positive balance is net release by an organ. Negative balance is net uptake by an organ. Values are mean ± SEM. Statistics: un-paired t-test for comparison of net balances of natural occurring XPP vs administrated XPP (Control vs XPP), water-based vs protein matrix (XPP vs CasH), and for comparison protein matrixes with vs without XPP spike (CasH vs CasH+XPP). Hooks: significance p<0.05. dotted hooks: tendency p<0.10.
### Table 5. Systemic bioavailability of XPP peptides – Effect of a meal matrix

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Group</th>
<th>IPP(%)</th>
<th>LPP(%)</th>
<th>VPP(%)</th>
<th>Statistics</th>
<th>Statistics</th>
<th>Statistics</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matrix/diet effect</td>
<td>XPP effect</td>
<td>Interaction</td>
<td>Macronutrient effect</td>
</tr>
<tr>
<td>protein</td>
<td>CasH2</td>
<td>84 ± 11</td>
<td>86 ± 8</td>
<td>84 ± 14</td>
<td>p^1</td>
<td>0.010</td>
<td>0.986</td>
<td>0.986</td>
</tr>
<tr>
<td>meal</td>
<td>Basal</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>p^2</td>
<td>0.0001</td>
<td>0.832</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>LQprot</td>
<td>160 ± 22</td>
<td>152 ± 15</td>
<td>143 ± 19</td>
<td></td>
<td></td>
<td>p^3</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>hCHO</td>
<td>105 ± 8</td>
<td>109 ± 11</td>
<td>100 ± 11</td>
<td></td>
<td></td>
<td>p^3</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>hFat</td>
<td>112 ± 14</td>
<td>115 ± 16</td>
<td>116 ± 18</td>
<td></td>
<td></td>
<td>p^3</td>
<td>0.628</td>
</tr>
<tr>
<td></td>
<td>Fiber</td>
<td>126 ± 18</td>
<td>152 ± 29</td>
<td>140 ± 23</td>
<td></td>
<td></td>
<td>p^3</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Systemic absolute bioavailability derived from the systemic response after an intra gastric bolus administrated of the tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in a protein matrix (casein hydrolysate rich in XPP: CasH2) or in CasH containing meal matrix (Basal). Or in meals with different amount of macronutrients: low quality protein (LQprot), high amount of carbohydrates (hCHO + CasH), high amount of fat (hFat) or with fiber (Fiber). Systemic bioavailability is expressed relative to Basal in %. Values are mean ± SEM. Basal, hCHO: n=10; hFat, LQprot: n=9; CasH2: n=8. Significance: p<0.05.

p^1: significance for comparison of protein matrix with meal matrix: Two-way ANOVA.

p^2: significance for comparison meal matrices: Two-way ANOVA.

p^3: significance for macronutrient effect (LQprot, hCHO, hFat, or Fiber meal vs Basal): post-hoc Dunnett’s multiple comparison test.
Portal bioavailability is derived from the systemic response after an intra gastric bolus administration of the tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in a protein matrix (casein hydrolysate rich in XPP: CasH2) or in meal matrixes (Basal, LQprot, hFat, hCHO, Fiber). Portal bioavailability is expressed relative to Basal in %. Values are mean ± SEM. Basal = 100% for all experimental groups. Significant: p<0.05. Significant comparison of protein matrix with meal matrix (Basal): Two - way ANOVA; *): p<0.05. Significant: p<0.05. Significant: p<0.05.

### Table 6. Portal bioavailability of XPP peptides – Effect of a meal matrix

<table>
<thead>
<tr>
<th>Matrix/group</th>
<th>Basal</th>
<th>LQprot</th>
<th>hFat</th>
<th>hCHO</th>
<th>Fiber</th>
<th>0.899</th>
<th>0.999</th>
<th>0.001</th>
<th>0.948</th>
<th>0.999</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPP(%):</td>
<td></td>
<td></td>
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<td>106</td>
<td>99</td>
<td>106</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>VPP(%):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>106</td>
<td>99</td>
<td>106</td>
<td>100</td>
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<tr>
<td>XPP effect</td>
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<td></td>
<td></td>
<td></td>
<td>106</td>
<td>99</td>
<td>106</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>106</td>
<td>99</td>
<td>106</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Statistics**

- p: Significance for comparison of protein matrix with meal matrix. Two-way ANOVA.
- *: p<0.05 significance for comparison of IPP, LPP or VPP portal availability of CasH2 relative to Basal with post-hoc Wilcoxon Signed Rank test.
- p: Significance for comparison of protein matrix with meal matrix. Two-way ANOVA.
- p: Significance for comparison of protein matrix with meal matrix. Two-way ANOVA.
### Table 7. Portal release half-life of XPP peptides – Effect of a meal matrix

<table>
<thead>
<tr>
<th>Matrix group</th>
<th>IPP (min)</th>
<th>LPP (min)</th>
<th>VPP (min)</th>
<th>Matrix/diet effect</th>
<th>XPP effect</th>
<th>Interaction</th>
<th>Macronutrient effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CasH2</td>
<td>47 ± 7*</td>
<td>49 ± 9*</td>
<td>45 ± 7*</td>
<td>p^1 &lt;0.0001</td>
<td>0.977</td>
<td>0.916</td>
<td></td>
</tr>
<tr>
<td>meal</td>
<td>125 ± 19</td>
<td>116 ± 21</td>
<td>125 ± 21</td>
<td>p^2 &lt;0.0001</td>
<td>0.797</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td>LQprot</td>
<td>85 ± 12</td>
<td>76 ± 13</td>
<td>78 ± 13</td>
<td>p^3 0.009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCHO</td>
<td>112 ± 16</td>
<td>113 ± 21</td>
<td>122 ± 19</td>
<td>p^3 0.970</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hFat</td>
<td>166 ± 17</td>
<td>168 ± 15</td>
<td>155 ± 22</td>
<td>p^3 0.014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber</td>
<td>136 ± 10</td>
<td>114 ± 19</td>
<td>129 ± 18</td>
<td>p^3 0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time after administration when 50% of total portal tri-peptide release (net balance) has occurred (t½,r in min). Values are mean ± SEM. (Basal, LQprot, hFat: n=10; CasH2, hCHO: n=9; hCHO: n=7) Significance: p<0.05.

p^1: significance for comparison of comparing protein matrix with meal matrix: Two-way ANOVA.

^*: p<0.05 significance for comparison IPP, LPP or VPP portal release half-life of CasH2 relative to Basal with post-hoc Sidak’s multiple comparison test.

p^2: significance for comparison meal matrices: Two-way ANOVA.

p^3: significance for macronutrient effect (LQprot, hCHO, hFat, or Fiber meal vs Basal) with post-hoc Dunnett’s multiple comparison test.
Discussion

Systemic bioavailability of orally ingested lacto-tri-peptides (XPP) may affect their potential bioactive impact. We hypothesized that macronutrients, especially protein, are able to change systemic availability and that differences in absorption kinetics of the XPP in the splanchnic region cause these changes.

Does a protein matrix improve bioavailability? In a post-absorptive state, we do not detect XPP in plasma above the limit of detection and thus any measurable XPP most likely originates from the meal (Figure 1). The calculated systemic bioavailability of XPP in the presence of a protein matrix (casein hydrolysate) does improve for all XPP, but not in the same proportion (Table 3, IPP: 4.7 times; LPP: 2.5 times; VPP: 121 times). The calculated systemic bioavailability pattern reflects the pattern of the measured portal bioavailability (Table 4), albeit underestimated because of delayed and prolonged systemic bioavailability after 90 min. This indicates that the systemic availability mainly relates to the portal availability of XPP. A protein matrix improves the portal bioavailability of all XPP by prolonging gut absorption (Figure 2, Table 4), but also not in the same proportion. The spiked protein matrix indicates that the relative large improvement of VPP bioavailability was not matrix related.

A point to consider is whether the presence of not-freely-available XPP’s (XPP’s bound in longer peptides (“encrypted”) in the casein hydrolysate affected our results. The portal bioavailability of XPP in a water-based matrix is comparable between the XPP. This in contrast with XPP in the protein matrix. We assume that this is related to encrypted VPP in the protein source of CasH. The source of CasH is ß- and ß-casein and these proteins have lacto-tri-peptides encrypted (36), that are liberated during an enzymatic hydrolysis process by the manufacturer. It is likely that still encrypted XPP’s in the larger peptides of the hydrolysate are liberated during the digestive process in the pig (1). These XPP also will contribute to the intestinal net balance. More in detail, the current enzymatic hydrolysis process (information of the manufacturer) liberates mainly VVVP-peptides and only a small amount of VPP. Therefore, we conclude by theoretically calculations (Supplemental Table 3. Theoretical XPP intake and their portal availability - Study 1.) that the high fraction dose of VPP release in comparison to IPP and LPP relates to this mechanism. We found that the average improvement of XPP portal bioavailability by a protein matrix is 1.8 times, albeit underestimated because of prolonged portal bioavailability after 90 min.

The prolonged XPP net absorption from the protein meal could be the result of processes such as stomach emptying, digestion and competition between the absorption of XPP and amino acids on the lumen side. Some of the digested peptides in the dietary proteins are resistant to further digestion because of the type of amino acids bonds to other amino acids like for proline-containing peptides (2,
37). Trans-epithelial transport in the intestinal tract to the portal system of XPP can occur in three different ways: para-cellular, trans-cytotic or via peptide transport system via the proton-dependent transporter, PepT1 (24, 38). In vitro measurements of trans-epithelial flux of VPP across a Caco-2 cell monolayer (38) indicated that the para-cellular route is the most likely route for intact VPP. Because absorption of XPP in hydrolysates in healthy pigs is not more limited than the same amount of single amino acids (39, 40), we think that delayed stomach emptying caused the prolonged absorption.

The systemic pharmacokinetics suggests that the improved bioavailability mainly is caused by delay in absorption and prolonged elimination. Systemic bioavailability is not only dependent on the portal availability but also depending on the elimination from systemic plasma to other organs. We found hepatic and renal XPP uptake (Figure 3), but uptake is approx. 75% of total PDV release. This means that other, not measured organs, also take up XPP (3). Uptake means that XPP is available for potential ACE-inhibition targets in organs like the vascular endothelial cells or absorptive epithelial cells (kidney) (3, 4), but also that XPP peptides are eliminated from the systemic by intracellular breakdown to amino acids by peptidases, or excretion to urine in the kidney. Further research in intra-organ kinetics is needed to quantify those processes.

Foltz et al (41) determined in vitro that also di-peptides with a C-terminal proline residue has ACE inhibitory effects. In a pilot analysis, we found that the plasma concentration of related di-peptides is 20-400 times higher than that of XPP (Supplemental Table 1. Dipeptide/tripeptide ratio - Study 1). This suggest that food-derived lacto-di-peptides have a potential synergetic role in the bioactivity, but that need to be determined in another experimental design.

Does a meal improve XPP systemic availability by improved portal availability? Systemic availability increased by a meal matrix but only by 20% (Table 5) in comparison to a protein matrix. Portal availability increased 80% by a meal matrix (Table 6). Higher portal release half-life (Table 7) indicates prolonged absorption and release to the portal system by the gut in a meal matrix.

Why does a complete meal matrix improve portal XPP availability? First, it is likely that the speed of absorption of regular meals is slower than a single dose of CasH and that this prolongs absorption of peptides. Fat is known to delays gastric emptying (42). In contrast, we did not observe any improvement of the portal bioavailability with the meals that contain a high amount of fat. Secondly, the small intestine is the interface between the gut lumen and the rest of the body and therefore controls the degree and rate of transport of amino acids coming from the meal via the portal vein (29). It could be that competition of amino acids trans-epithelial transport plays
a role, because the amount of amino acids from digested proteins (3.05 g/kg body-weight, Whey and Soy isolate together with CasH) is higher in the complete meals as compared to a single dose of CasH (0.73 g/kg bodyweight). We think however that this is also unlikely as the absolute amount of protein given in the test is still relative low (30% of restricted daily intake), considering the potential absorption capacity of the gut of a pig (43). Therefore, we need to consider stimulated gut metabolism after the meal in comparison to a protein mixture alone (19). We found that adding carbohydrates leads to higher insulin levels, it stimulates increased protein synthesis and reduced protein breakdown, leading to more gut tissue anabolism (44, 45). Therefore, we think that reduced intracellular intestinal protein breakdown is related to the reduced XPP breakdown and thus higher transport to the portal vein. We therefore think that the gut anabolic response in the meals is the most likely mechanism to a higher portal bioavailability. Further research is needed to confirm our hypothesis.

What is the influence of the macronutrients on the systemic availability? Although total portal release of XPP is comparable during the experimental period (Table 6), systemic availability is higher with the low quality protein meal and high fiber meal (Table 5). Additionally in contrast, low protein decreases and high fat increases the portal release half-life of XPP (Table 7), suggesting that increased systemic availability can not be related to differences in intestinal XPP absorption, but suggests that other organs/metabolism are involved. For instance, we observed that soy protein leads to less anabolism (17, 46). This means that organs take up less amino acids and probably also less XPP from the systemic circulation for protein anabolism, resulting in higher systemic availability of XPP. We have no good explanation for the observation of the improved XPP systemic availability by high fiber meal. Fiber has multiple health benefits but limited info is available about a direct relation between fiber and peptide/protein metabolism (47).

**Conclusion**

Quantitative measurements of trans-organ availability/kinetics provide more insight of the behavior of food-derived C-terminal lacto-tri-peptides like XPP. The present study showed that XPP in a protein matrix have a prolonged portal bioavailability. In a meal with all macronutrients present, XPP are more available albeit at a low percentage (0.2-0.3%). The digestion rate of the meal, the quality of protein, and fiber contents, mainly determine systemic XPP bioavailability after a meal.
Acknowledgements

We thank Ayhan Şik, MSc and Ruud M Ramakers, BSc for skilled technical assistance in carrying out the animal experiments and Ed Rosing, PhD and Martijn Brandt, BSc for accurate XPP analyses and John J. Thaden, PhD for help with the measurements of the XPP. Victor Wilson, PhD, statistician for help with the statistical analysis and Guus S.M.J.E. Duchateau, PhD for continuous support.

References

hydrolysate containing the antihypertensive tripeptides Val-Pro-Pro and Ile-Pro-Pro improves vascular endothelial function independent of blood pressure-lowering effects: contribution of the inhibitory action of angiotensin-converting enzyme. Hypertension research : official journal of the Japanese Society of Hypertension. 2007;30(6):489-96.


## Supplemental Information

### Supplemental Table 1. Dipeptide/tripeptide ratio - Study 1

<table>
<thead>
<tr>
<th>Dipeptide/tripeptide</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP/IPP</td>
<td>204 ± 114</td>
</tr>
<tr>
<td>LP/LPP</td>
<td>36 ± 15</td>
</tr>
<tr>
<td>VP/VPP</td>
<td>354 ± 153</td>
</tr>
<tr>
<td>PP/XPP</td>
<td>28 ± 12</td>
</tr>
<tr>
<td>XP/XPP</td>
<td>193 ± 119</td>
</tr>
</tbody>
</table>

Ratio’s between the dipeptide and tripeptide plasma concentrations. Pilot data from 2 pigs with tripeptide concentrations above baseline in the range of 1–30 nM. Corresponding dipeptide concentration range: 0.5 – 7 µM (n=11). Values are means ± SEM. Ratio’s were higher than 1 (Wilcoxon Rank test, p<0.01). The VP/VPP ratio was significant different from LP/LPP and PP/XPP (Mann-Whitney, p<0.05).

### Supplemental Table 2. Plasma flows - Study 1

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Group</th>
<th>PDV</th>
<th>Liver</th>
<th>Kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>water-based</td>
<td>Control</td>
<td>47.5 ± 7.5</td>
<td>71.5 ± 10.0</td>
<td>43.6 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>XPP</td>
<td>45.9 ± 4.2</td>
<td>68.6 ± 6.4</td>
<td>35.6 ± 4.0</td>
</tr>
<tr>
<td>protein</td>
<td>CasH</td>
<td>54.4 ± 5.9</td>
<td>77.6 ± 8.2</td>
<td>31.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>CasH + XPP</td>
<td>51.7 ± 2.4</td>
<td>74.9 ± 5.8</td>
<td>33.3 ± 5.5</td>
</tr>
</tbody>
</table>

Mean plasma flows in different organs of pigs after intra-gastric administration of control salt solution (Control), a synthetic dose of XPP (XPP), casein hydrolysate rich in XPP (CasH) or spiked CasH (CasH + XPP). Data are expressed as mean ± SEM in ml/kg bodyweight/min. No differences were observed between means of the XPP containing groups in each organ and Control (one-way ANOVA).

### Supplemental Table 4. PDV Plasma flows - Study 2

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Group</th>
<th>PDV plasma flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
<td>CasH2</td>
<td>57.3 ± 8.6</td>
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<td></td>
<td>Basal</td>
<td>52.2 ± 7.2</td>
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<tr>
<td></td>
<td>LQprot</td>
<td>58.9 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>hCHO</td>
<td>57.1 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>hFat</td>
<td>58.1 ± 6.7</td>
</tr>
<tr>
<td>meal</td>
<td>Fiber</td>
<td>50.7 ± 4.3</td>
</tr>
</tbody>
</table>

Mean plasma flows of portal drained viscera (PDV) in pigs after administration of casein hydrolysate (CasH2) supplement, casein hydrolysate suppleted iso-caloric meal (Basal). Or meals with different amount of macronutrients: low quality protein (LQprot), high amount of carbohydrates (hCHO + CasH), high amount of fat (hFat) or with fiber (Fiber). Data are expressed as mean ± SEM in ml/kg bodyweight/min. No differences were observed between means of different matrixes: CasH2 vs Basal, unpaired t-test. Or between means of different meals (one-way ANOVA).
Supplemental Table 3. Theoretical XPP intake and their portal availability - Study 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Matrix</th>
<th>Group</th>
<th>IPP</th>
<th>LPP</th>
<th>VPP</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured</td>
<td>Water-based</td>
<td>XPP</td>
<td>13.7</td>
<td>15.8</td>
<td>14.1</td>
<td>Matrix/Spike effect</td>
</tr>
<tr>
<td>Intake (µmol/kg)</td>
<td>Protein</td>
<td>CasH</td>
<td>10.3</td>
<td>28.0</td>
<td>0.5</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cash+XPP</td>
<td>23.1</td>
<td>42.6</td>
<td>13.3</td>
<td>0.480</td>
</tr>
<tr>
<td>Theoretical</td>
<td>Protein</td>
<td>CasH</td>
<td>25.9</td>
<td>32.8</td>
<td>15.9</td>
<td>0.004</td>
</tr>
<tr>
<td>Intake (µmol/kg)</td>
<td></td>
<td>Cash+XPP</td>
<td>38.2</td>
<td>67.4</td>
<td>21.2</td>
<td>0.480</td>
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<tr>
<td>PDV total net balance</td>
<td>Water-based</td>
<td>XPP</td>
<td>0.08</td>
<td>0.09</td>
<td>0.07</td>
<td>0.004</td>
</tr>
<tr>
<td>(% of theoretical intake)</td>
<td>Protein</td>
<td>CasH</td>
<td>0.10</td>
<td>0.14</td>
<td>0.19</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cash+XPP</td>
<td>0.12</td>
<td>0.10</td>
<td>0.18</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Intake of the tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in a water-based matrix (synthetic XPP) or in a protein matrix (casein hydrolysate rich in XPP: CasH). Values are means ± SEM; XPP: n=9; CasH: n=8; Cash+XPP: n=10. ¹) Intake is average (measured) amount of free available XPP in each test mixture. ²) Intake is theoretically intake, considering the tri-peptide sequences in the source of the CasH: amino acid sequence of bovine κ and β casein (www.genome.jp; CASB-BOVIN, CASK-BOVIN).

Portal bioavailability measured as post-prandial total net release to the portal system (PDV total net balance) after an intra gastric bolus administrated of the tri-peptides isoleucine-proline-proline (IPP), leucine-proline-proline (LPP), valine-proline-proline (VPP) in a water-based matrix (XPP) or in a protein matrix (XPP containing casein hydrolysate: Cash). Values are means ± SEM; XPP: n=8; Cash: n=8; Cash+XPP: n=9. Significance: p<0.05. Tendency: p<0.10. p₁: significance for comparison of water-based matrix (XPP) with protein matrix (Cash): Two-way ANOVA p²: significance for comparison between spiked (Cash+XPP) and non-spiked protein matrix (Cash): Two-way ANOVA. When appropriate post-hoc unpaired t-test is done: *: p<0.05 significance for comparison IPP, LPP or VPP of water-based matrix (XPP) relative to protein matrix (Cash).
SECTION 2

Use of isotopes in pig sepsis models
CHAPTER 6
Characteristics of a *Pseudomonas aeruginosa* induced porcine sepsis model for multi-organ metabolic flux measurements

Gabriella A.M. ten Have, Renske C.I. Deutz, Mariëlle P.K.J. Engelen, Robert R. Wolfe, Nicolaas E.P. Deutz


**Abstract**

Survival in sepsis is related to loss of muscle mass. Therefore, it is imperative to further define and understand the basic alterations in nutrient metabolism to improve targeted sepsis nutritional therapies. We developed and evaluated a controlled hyperdynamic severe sepsis pig model that can be used for *in vivo* multi-organ metabolic studies in a conscious state. In this catheterized pig model, bacteremia was induced IV with $10^9$ CFU/hour *Pseudomonas aeruginosa* (PA) in 13 pigs for 18 hours. Both the PA and control (nine) animals received fluid resuscitation and were continuously monitored. We examined in detail the hemodynamics, blood gases, clinical chemistry, inflammation, histopathology and organ plasma flows. The systemic inflammatory response (SIRS) diagnostic scoring system was used to determine the clinical septic state. Within six hours from the start of PA infusion, a septic state developed as reflected by hyperthermia and cardiovascular changes. After 12 hours of PA infusion, severe sepsis was diagnosed. A disturbed cardiovascular function, a decreased portal drained viscera plasma flow (Control: 37.6±4.6 ml/kg bw/min; PA 20.3±2.6 ml/kg bw/min, p<0.001) as well moderate villous injury in the small intestines were observed. No lung, kidney or liver failure was observed. Acute phase protein CRP and Il-6 levels did not change in the PA group. However, significant metabolic changes such as enhanced protein breakdown, hypocalcemia and hypocholesterolemia were found. In conclusion, the PA induced bacteremia in the catheterized pig is a clinically relevant model for acute severe sepsis and enables study of complex multi-organ metabolism.
Introduction

Sepsis is a complex bacteremia induced pathophysiological state characterized by both cardiovascular and metabolic responses (1-4). Its dynamic character, the variability in responses, and the complicated clinical logistics make it difficult to study the metabolic interaction between different organs in septic patients in a controlled setting (5, 6).

One characteristic metabolic alteration in sepsis is loss of muscle mass. Loss of more than 25% of muscle mass is incompatible with survival (7, 8). Less severe muscle loss contributes to morbidity and mortality (9), and hinders rehabilitation to normal health and function after recovery. Moreover, intestinal mucosal atrophy mainly due to reduced protein synthesis, is often present in sepsis, and contributes to increased translocation of bacteria from the gut into the bloodstream (10, 11). Increased translocation of gut bacteria perpetuates the septic state. Also, a severe decrease of plasma albumin is associated with higher mortality in sepsis (12). Albumin is the principal oncotic protein in plasma, and a decrease in concentration leads to loss of fluid from the vascular space. Finally, the physiological significance of the acute phase response has been debated (13), but clearly represents a metabolic priority in sepsis. More knowledge about the metabolic changes in sepsis should contribute to more effective potential nutritional support (9, 14).

Although the debate over the relevance and validity of various animal models of sepsis is still ongoing (3, 15, 16), the high morbidity and mortality resulting from sepsis illustrate the scientific and clinical need to further define and understand the basic nutrient metabolism alterations to improve existing targeted sepsis nutritional therapies. For nutritional therapies in sepsis, an optimal animal model to study metabolic responses in the hyperdynamic septic state should be clinically relevant, controlled and performed in the conscious state. It should also have a potential therapeutic window, with the possibility to study multi-organ metabolism. For this model, the first consideration is that the septic state in the animal model should not exceed severe sepsis. Therefore, the septic shock models without potential therapeutic windows would not be valid. Secondly, induced sepsis should start with invasion of the blood stream by micro-organism, usually bacteria. It has been indicated that animal models using live bacteria are the most clinically relevant models (15, 17). Thirdly, pig models are preferable as they are omnivores like humans and the pig has a similar renal, cardiovascular, and gastrointestinal anatomy, physiology and metabolism. Therefore, studying therapeutic nutritional support in a pig model is viewed as a highly translational research model (18, 19). Fourthly, the metabolic measurements should preferably be done in a conscious state, because it is more clinical relevant/translational. In addition anesthetic management in sepsis is challenging (20), and interferes with metabolism, especially splanchnic metabolism (e.g. down regulation of the gut movements, detoxification activity of anesthetics by the
Sepsis is associated with clinical symptoms of systemic (body wide) illness such as fever, chills, malaise and general feeling of sickness. However as we know from clinical situations, pain is not a primary symptom. Therefore, it is acceptable to measure metabolism in conscious state as long as the animal is not in shock, which is characterized by multiple organ failures and the necessity of ventilation with sedation (21, 22). Finally, the animal should be instrumented to accommodate painless blood sampling and infusions during multi-organ metabolism study (23).

Currently, there are no animal models available with these characteristics for relevant metabolic and future nutritional therapies in sepsis. Previously, we studied multi-organ metabolism in a 24h endotoxin induced catheterized sepsis pig model (24-26). Although endotoxin is the major virulent factor from a gram-negative bacterium in sepsis, it is possible that important components of the metabolic dynamics are missing in this which are present when sepsis is induced by a live bacterium (15, 17).

The aim of the present study was to characterize in detail the response in hemodynamics, blood gases, clinical chemistry, inflammation and organ plasma flows in a new *Pseudomonas aeruginosa* (PA)-induced septic instrumented porcine model. The model will be used for studying clinical relevant, multi-organ metabolism in sepsis. We modified a widely used, high dose bacteremia porcine septic shock model (16, 27-30) to obtain a controlled hyperdynamic conscious sepsis model by administrating the bacteria *intravenously* (IV) at a low dose via continuous infusion, and simultaneously preventing low blood pressure by fluid resuscitation. PA was used as it is one of the most frequently isolated bacteria in sepsis in humans in hospital intensive care units, apart from lipopolysaccharide, and other virulence factors like flagella and pili, which inject a set of toxins directly into host cells (31). The pigs were surgically catheterized and subsequently acclimatized 10 days before the induction of sepsis. This was done to better control the sepsis induction and subsequent hyperdynamic state, and to perform multi-organ metabolic flux measurements over time in the conscious state (23, 24, 32).

**Materials and Methods**

**Animals**

Twenty seven female Yorkshire Crossbred pigs (from multiple litters, Metz Farms, Russellville, AR, USA) with an average body weight of 20-25 kg were used in the present study. General health status was determined by assigned veterinarian of the animal research facility (Arkansas Children’s Hospital Research Institute, Little Rock, AR, USA, AALAC certified). The pigs were individually housed in steel pens.
(2 x 3 m) in a controlled housing, biosafety level two facility: large animal cubicle, plastic coated mesh-style grid floor, room temperature 22-24°C, 12-hour light-dark cycle, and standardized food intake (1kg/day) (Harlan Teklad Vegetarian Pig/Sow Grower). The pens were enriched with toys to stimulate natural pig rooting behavior and to prevent stereotypic behavior. A heating pad was added to the pen to ensure natural body temperature control post-surgery. Water was provided ad libitum. Clinical examination was carried out at arrival, before surgery (7-10 days after arrival) and frequently post-surgery (twice daily on the first three/four days post-surgery and then every two/three days until the sepsis study). The present animal study including its justification for number, breed and single sex was approved by the animal experiment ethics committee (IACUC) of University of Arkansas Medical Sciences (Little Rock, AR, USA).

**Surgical procedure**

To order to perform multi-organ metabolism studies in the septic pig, we implanted catheters in blood vessels with a surgical procedure as described in detail previously (23, 32). After an acclimatization period of seven to ten days, a pre-surgery clinical exam and anesthesia was performed by assigned veterinarian. Anesthesia was induced with intramuscular (I.M.) injection of mixture of telazol, ketamine and xylazine (PigMix®, dose 0.4 ml/kg bw). With the subsequently intubation of the animal, general anesthesia was started and maintained with isoflurane (2% mixture with oxygen). Via an ear vein cannula flunixin meglumine (Flunixamine®, 2 mg/kg bw) was administered as analgeticum and as an antibioticum, lincomycin-spectinomycin mixture (Linco-Spectin®, lincomycin 5 mg/kg bw, spectinomycin 10 mg/kg bw) was administered as prophylaxis. The animal received 500-1000 ml Ringers-Lactate during the surgery to keep up the blood pressure and fluid balance. ECG, CO2 and oxygen saturation were monitored continuously. In brief, during midline laparotomy, a catheter for blood sampling over time and monitor mean arterial pressure (MAP) was placed in the abdominal aorta. An inferior caval vein catheter was placed, allowing administration of post-surgery medication and experiment related infusions. A second arterial catheter, just before the bifurcation, and a splenic vein catheter were placed allowing infusion of para-aminohippuric acid (PAH) for plasma flow measurements. A swan ganz catheter (5 Fr, #132F5, Edwards life sciences, Irvine, CA, USA) was inserted via the right jugular vein to monitor mean pulmonary arterial pressure (MPAP). The position of the tip of the Swan Ganz catheter was determined following the pressure readings when the catheter was pushed through the right atrium and ventricle. Additionally, an extra caval vein, a portal vein and hepatic vein catheter were placed for multi-organ blood sampling. The duration of the surgery was approximately five hours. Postoperative care was standardized as described in detail previously (23, 32) and monitored by assigned veterinarian. After surgery, we kept the animal hooked up to the ECG, pulse ox, and ventilator until the animal responded with multiple swallow reflexes, eye blinking, fighting the
ventilator, and trying to rise. A second dose of lincomycin-spectinomycin was given I.M. The animal was observed (breathing and heart rate) further until it sits sternely. During the first four days post-surgery twice daily, the animals were clinically examined (body temperature, appearance and behavior), buprenorphine (0.03 mg/kg bw) and lincomycin-spectinomycin were administered via the implanted central vein catheter. We fed the animals half of normal ration of food during the first two days. Catheters were kept patent by checking daily during the first three days, and by using antimicrobial and anticoagulant catheter filling (20 mg/ml gentamycin and 225U/ml alfa-chymotrypsin) as described previously (23). Subsequently, every two/three days catheters were checked for patency and the animals were clinically examined. Before and after surgery, during the recovery period (seven to ten days) animals were accustomed to a small movable cage (0.9x0.5x0.3m) to ensure that the PA experiments could be performed in the cage in conscious animals in free standing, sitting or lying positions. Until the day of the sepsis experiment, the pig spent at least ten times in this movable cage.

**Experimental setup of the sepsis model**

The design to characterize the septic response of the PA bacteremia is shown in Figure 1. The experiment started after a recovery period 7-10 days post-surgery. These animals were allocated for the PA group or the Control group in a randomized fashion. Basal blood pressures, plasmaflow, blood gas, clinical chemistry and inflammatory data (pre-septic period; t=−2h to 0h) measurements were started 4-6h after the last food intake (half of the daily amount: 0.5 kg). No food was administered during the rest of the experimental period (18h). At T=0h bacteremia was induced by the start of IV continuous infusion of PA (IRS 12-4-4, Shriners burns institute, University of Texas Medical Branch, Galveston; original from a burn patient at Brook

![Figure 1. Design induction of sepsis with *Pseudomonas aeruginosa* bacteremia](image)

MAP: mean arterial pressure; MPAP: mean pulmonary arterial pressure; PAH: para-aminohippuric acid infusion for plasma flow measurements. IV: intravenous; bw: bodyweight.
Army Medical Center in San Antonio, TX.; \(10^9\) CFU/hour in 1 ml 0.9% NaCl solution). PA dose initially obtained from the method of Rimmele et al (16) was tested for virulence and adjusted in a pilot (three animals). The Control group received 0.9% NaCl solution in the same volume. Fluid resuscitation (30 ml/kg bw/hour 0.9% NaCl, IV) was started at the same time as the PA infusion. General appearance (alertness, breathing) was monitored and recorded continuously. Multiple blood samples were taken over time (see details following section). At T=18 hours animals were euthanized with 125 mg/kg pentobarbital sodium and 16 mg/kg phenytoin sodium (Euthanasol®) administered via the central vein catheter.

**Hemodynamics**

Hemodynamics were monitored continuously to ensure that the hyperdynamic state was kept in the expected ranges for severe sepsis (not shock): maximal body temperature (normal range: 37-39.6 °C (33)) increase or decrease of two to three degrees Celsius, maximal MPAP (normal range: 11-24 mmHg (33)) of 35 mmHg, heart rate (normal range: 90-107 BPM (33)) of 200 beats per minute (BPM) and MAP (normal range: 86-123 mmHg (33)) higher than 60 mmHg, using the arterial and Swan Ganz catheter for invasive blood pressure measurement (monitor: Propaq Encore, Welch Allyn, Skaneateles Falls, NY, USA; transducer: TruWave, Edwards Lifesciences, Irvine CA, USA). If the heart rate became >200 bpm and/or MPAP >35 mmHg, the PA infusion was stopped temporarily to limit the increase of the right ventricular afterload (16) until heart rate and MPAP were below 200 bpm and 35 mm Hg, respectively.

**Clinical chemistry**

At T=0, 12 and 18h, 500 µl serum was collected and stored at -80°C for a standard clinical chemistry diagnostic panel (Supplemental Table 2, Veterinary Diagnostic Laboratory, Little Rock AR, USA).

**Blood gas**

Arterial and central venous blood samples were collected for blood gas levels, every 6 hours, using an on-site blood gas analyzer (i-STAT, cartridge CD4+, Abbott Laboratories Inc., Abbott Park, IL). We compared and interpreted blood gas data using the alpha-stat scientific model with no temperature correction (34). Fractional oxygen extraction rate from the capillary bed were calculated: \(\text{SaO}_2-\text{SvO}_2/\text{SaO}_2\) (Normal range: 22-32%, \(\text{SaO}_2=\)arterial oxygen saturation, \(\text{SvO}_2=\)venous oxygen saturation). Pulmonary oxygen exchange efficiency was calculated by dividing arterial oxygen pressure (\(\text{PaO}_2\)) with the fraction of inspired oxygen (\(\text{FiO}_2=0.21\); Normal range: >300).
Plasma flow measurements

We measured organ plasma flow by using PAH dilution model as described previously (35). In brief: plasmalflow was measured with a primed-continuous infusion of PAH (25mM), via a splenic and one of the arterial catheters (75 ml/h). Arterial, portal, hepatic and caval vein lithium-heparinized blood samples (0.5ml/port) were collected at T= -1, -0.5, 0, 12, 14, 15, 16, 17 and 18h for portal drained viscera (PDV), splanchnic (SPL) and hindquarter (HQ) plasma flow determination. These samples were directly cooled (four degrees Celsius), centrifuged (8000 x g, five min, four degrees Celsius) and plasma was deproteinized with trichloroacetic acid (50 mg per one ml plasma) within one hour after collection. Samples were stored at -80°C until further analysis. For the measurement of the plasma flow, PAH concentrations in trichloroacetic acid deproteinized plasma samples were compared with PAH standards and read out with a Microplate spectrophotometer (ELx 808 Ultra microplate reader, Bio-Tek Instruments Inc, Winooski, VT, USA) (36). Plasma flow was calculated using the dilution of PAH across the organs (23).

Histology

At T=18h, immediately after euthanasia, small fragments (approximately 200 mg) of muscle (M. gastrocnemius, right hind leg, middle part), jejunum, ileum, liver, and lung were obtained from seven control and six PA animals. Tissues were rinsed with saline and fixation was done in 10% buffered formalin for 24h. Where after the tissues were transferred to 70% alcohol and stored at room temperature until further routinely processed and stained with hematoxylin and eosin (Texas A&M University, Department of Veterinary Pathobiology, TX, USA). We examined the tissues blinded for sepsis related non-specific inflammation and/or organ specific histopathological lesions (37): in general the influx of inflammatory cells; in muscle the initial signs of atrophy (38); in jejunum/ileum mucosal villous injury using a previously described scoring system (39) and subepithelial spacing in the crypt regions. In addition, in the liver we looked for signs of the fatty change and finally in the lung the signs of diffuse alveolar damage (DAD).

Inflammation parameters

Acute phase cytokine Interleukin-6 (IL-6) and C-reactive protein (CRP) in arterial plasma were measured at time points -2,-1, 0, 12, 14, 16, 17, 17.5, and 18 hour and analyzed using commercially available ELISA following manufacturers instruction (Porcine IL-6 DuoSet, Porcine CRP Duoset ELISA, RnD systems, Minneapolis, MN, USA). Plasma IL-6 and CRP amounts will be expressed per liter plasma and mg plasma protein (30). The dynamics of IL-6 and CRP plasma concentrations over time (Supplemental Figure 1), showed non-physiological relevant changes between 12 and 18h in a pilot amount of animals (IL-6: Control N=2; PA N=4; CRP: Control N=2; PA N=2). Therefore, only the pre (T=-2h) and post PA (T=17h) data were presented.
Statistical analyses

Data are expressed as mean ± SE. Graphpad Prism (version 6) was used for statistics. Levels of significance was set on p<0.05. Hemodynamics, organ plasma flows, histological villous scoring, blood gas data, clinical chemistry data and acute phase protein were compared with Two-way ANOVA, when appropriated with repeated measures. Also when appropriate a post-hoc Bonferroni, Tukey or uncorrected Fisher’s LSD multiple comparison test was done as indicated in figures and table legend.

Results

From the 27 animals, five animals were lost due to intestinal complications (ileus, ascites or peritonitis) in the recovery phase of the catheter implantation surgery. However, no animals were lost during surgery and the PA experiment. General appearance was monitored: PA animals started shivering between one and two hours after start of PA infusion, deeper breathing (less frequent) started between four and six hours, animals were in general calm but still alert. When needed, PA infusion was stopped between seven and 18 h (average 14.5 hours) after start of PA to prevent the septic response from developing further into an acute septic shock. Thirteen animals with PA (PA group) and nine animals without PA (Control group) completed the study.

Hemodynamics and body temperature

The initial increased peak in heart rate was parallel to that of the MPAP up to six hours (Figure 2). The average temporary stop of PA to limit the increase of the right ventricular afterload (16) infusion lasted 18.5 min. MPAP went slowly down between 2 and 3h after start of the PA infusion. The heart rate showed a biphasic pattern: an initial peak in the first five hours and subsequently increase that showed a stable tachycardia after ten hours. The MAP data revealed an initial hypertension phase in the first few hours, followed by a decrease after five hours. We managed to keep the MAP data stable after ten hours between 75 and 82 mmHg (hypotension) compared with between 102 and 113 mmHg for the controls. Body temperature increased within a few hours after start of the PA infusion and reached a maximum after 4h, followed by a gradual decrease. No hypothermia was observed throughout the study.

Blood gas characteristics

Arterial and venous oxygen saturations were kept within normal ranges (Supplemental Table 1). The fractional uptake of oxygen from the capillary bed, did not change (O2ER). The efficiency of pulmonary gas exchange (average PaO2/FiO2 > 300) was comparable between the groups. The tissue perfusion variable lactate
increased over time (>1 mM); however, it does not exceed the upper limit of the normal range. The venous pCO₂ and HCO₃ was lower than normal in the sepsis group, the resulting pH was expectedly higher; however, both still within the normal range at T=18h.

**Blood flow characteristics**

Baseline plasma flow was not different between the Control and PA groups (Figure 3). PA infusion induced a decrease in plasma flow in the PDV area (p<0.001), but not in the whole splanchnic area. In the HQ, an increase of plasma flow was observed in both the PA and control groups.
Figure 3. Organ Plasma flow

Organ plasma flows changes before and after Pseudomonas aeruginosa (PA) induced bacteremia in the pig. “Pre” is average plasma flow one hour before induction bacteremia. “Post” is average plasma flow between 14 and 18 hours after start induction bacteremia. Control N=9; Sepsis N=12. Values are mean ± S.E.M.. Statistics: Two-Way ANOVA repeated measures for time, when appropriate a post-hoc Tukey multiple comparison test; hook: significance p<0.05.

A. Portal drained viscera: Interaction p=0.0008, Time effect p=0.5682, PA effect p=0.1297
B. Splanchnic area: Interaction p=0.7225, Time effect p=0.2849, PA effect p=0.2992
C. Hindquarter: Interaction p=0.6024, Time effect p<0.0001, PA effect p=0.2992
Clinical chemistry

Metabolic parameters i.e. total protein, albumin, and globulin concentrations, cholesterol, as well as calcium (important for ion-homeostasis) decreased in the PA group (Supplemental Table 2.) Creatinine phosphokinase (CPK), indirect marker of muscle degradation, and lactate concentrations (Supplemental Table 1) were increased. The energy parameter glucose reached its lowest value at T=12h. Urea (marker of protein metabolism and kidney function) and creatinine levels as well as urea/creatinine ratio were elevated. The liver degradation parameters alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) were not altered but aspartate aminotransferase (AST) and alkaline phosphatase were increased.

Histology

The muscle (*M. gastrocnemius*) showed a mild influx of inflammatory cells between the muscle fibers in all sepsis animals, but also in two of the control animals. No initial signs of muscle atrophy were observed. No increase in inflammatory cells were found in the jejunum and ileum, although in the jejunum, villous damage was found in both the control and in the PA animals. The vacuolization and irregular/blunted villi in jejunum were more commonly present and severe in the PA animals (Figure 4). Subepithelial space in jejunal mucosa crypts was observed in five PA and two Control animals. Moderate overall flattening of ileum villi occurred in the PA animals. In the liver, we observed fatty change and mild influx of inflammatory cells around the central vein in PM animals. The lung showed no indication of DAD.

![Figure 4. Villous injury score](image)

**Figure 4. Villous injury score**

Histological scoring of villous damage in jejunum and ileum after *Pseudomonas aeruginosa* (PA) induced bacteremia in de pig. Control N=7, Sepsis N=6. Values are mean ± S.E.M.. Statistics: Two-Way ANOVA comparison; Significance p<0.05; PA effect: p=0.022; Tissue effect: p=0.032; Interaction: p=0.572). When appropriate a post-hoc uncorrected Fisher’s LSD was used to test PA effect per tissue; hook: significance p<0.05).
### Table 1. Interleukin-6 (IL-6) and C-reactive protein (CRP) plasma concentrations

| Parameter | Control | | | | PA | | | | | | Statistics |
|-----------|---------|---|---|---|---|---|---|---|---|---|
|           | Pre     | Post | Pre | Post | Time effect | PA effect | Interaction |
| IL-6 (ng/L) | 323 ± 138 | 334 ± 148 | 480 ± 263 | 371 ± 131 | 0.570 | 0.718 | 0.483 |
| IL-6 (ng/gr protein) | 6.2 ± 2.7 | 6.3 ± 2.8 | 8.7 ± 4.4 | 7.0 ± 2.2 | 0.578 | 0.728 | 0.534 |
| CRP (mg/L) | 33 ± 5.9 | 21 ± 3.3 | 45 ± 5.8 | 25.8 ± 2.1 | 0.002 | 0.075 | 0.414 |
| CRP (mg/gr protein) | 0.60 ± 0.10 | 0.40 ± 0.06 | 0.87 ± 0.10* | 0.51 ± 0.04* | 0.001 | 0.029 | 0.347 |

IL-6 and CRP changes before and after *Pseudomonas aeruginosa* (PA) induced bacteraemia in the pig. “Pre” is one hour before induction bacteraemia. “Post” is 17 hours after start induction bacteraemia. Values are means ± S.E.M.; Control: N=9; PA: N=13; Statistics: Two-way ANOVA, data of effects are p-values; significance p<0.05. When appropriate post-hoc uncorrected Fisher’s LSD is done: *) significance between pre and post; #) significance between Control and PA.
Cytokines and acute phase proteins

IL-6 data did not change during PA administration (Table 1). CRP values were lower after 17h of PA administration even after compensation for the decrease in total protein in plasma (expressed as mg/mg protein). Both IL-6 and CRP initial concentration data (resp. 419 ± 163 ng/L and 40.0 ± 4.3 mg/L) showed a high coefficient of variability (CV): 183 and 50.0 %CV, respectively.

Discussion

We developed a controlled hyperdynamic porcine model of sepsis with cardiovascular, inflammatory, metabolic characteristics as present in the human septic state. This model enables us to conduct studies unraveling the disturbances in organ metabolic pathophysiology in the early hyperdynamic septic state.

Diagnose of the septic state

Sepsis is the clinical syndrome of systemic inflammatory response (SIRS) that complicates severe infection. Diagnostic criteria include a documented pathogen plus two or more SIRS abnormalities (1, 3, 4). The absolute values of clinical parameters can be slightly different in the pig in comparison with humans. Therefore in the present study, we compared the changes of these parameters with normal pig values. Physiological and pathophysiological responses of the pig are highly comparable with humans (18, 19), therefore described responses (equating to change in absolute values) of human SIRS criteria can also be used for pigs. With these main diagnostic criteria of SIRS (body temperature: increase of more than two degrees Celcius; heart rate: >90 beats/min; PaCO₂: <32 mmHg), we concluded that the present infection has successfully induced sepsis in pigs (Figure 2, Supplemental Table 1). This means that the acute septic response of the chosen rate of PA infusion could be controlled by monitoring the hemodynamics and general appearance (alertness, breathing) of the animal during the PA infusion. Sepsis was induced within 6 hours after the start of PA infusion. In this initial phase of sepsis, we observed that it is necessary to carefully monitor MPAP and heart rate in the first two hours to prevent a deep, unrecoverable septic shock. We found that stopping the PA infusion for a few minutes was sufficient to stabilize the animal. The increase in the right ventricular afterload is well described in the medical literature and is likely related to the release of proinflammatory mediators such as TNFα and immunoreactive endothelin-1 which are strong vasoconstrictors on pulmonary vessels (16).

Organ dysfunction

Severe sepsis state is characterized by one or more organ dysfunctions (1, 3, 4). We found that despite fluid resuscitation pronounced hypotension, tachycardia and a decreased PDV plasma flow were present, indicating a disturbed cardiovascular
function (Figure 1 and 2). The decreased PDV flow could play a role in the occurrence of the injured jejunal and ileum mucosa tissues (40). A potentially compromised PDV microcirculation (41) can especially result in a decreased absorption capacity/gut metabolism due to mild/moderate damaged villi (Figure 4). In jejunum of the PA group, it is unclear why subepithelial spacing was not only observed in the villi but also in the crypts. It is likely that the combination of the abnormal translocation of principal oncotic protein in plasma albumin to the intestines that leads to loss of fluid from the vascular space and the fluid resuscitation, could play a role (12) (42). The respiratory system showed no arterial hypoxemia (Supplemental Table 1); therefore, no mechanical ventilation was needed as confirmed by the absence of DAD occurrence in lung tissue. The tissue perfusion parameter lactate revealed hyperlactatemia (>1mM) according to human criteria (1), but for a pig it was still in the normal physiological range (33). Therefore, we conclude that tissue perfusion is changing in the PA group, however the change is not physiological meaningful.

Acute kidney failure, as reflected by a substantial increase in the creatinine systemic plasma level, was not observed in the present model (Supplemental Table 2) (1, 4). Doi et al (43) indicated that creatinine levels can be inconclusive due to a potential decrease in the creatinine production in sepsis. The fact that we did not find any change in the arterial PAH concentrations, used for organ plasma flow calculations, confirms that renal function was not compromised in the present sepsis model. Although ALT and AST are both biomarkers for liver injury, ALT is more liver specific. Increased AST alone, indicates that there is extrahepatic injury (for instance in skeletal muscle) and fits with the increase of CPK (muscle degradation marker). We conclude that there are no indications of acute hepatic dysfunction (Supplemental Table 2), although liver histology showed mild infection related abnormalities (37). Therefore, we conclude we have developed a sepsis model characterized by severely compromised function of the cardiovascular system, and a mildly compromised function of the PDV by infusing PA. Severe sepsis was established between 12-18 h after starting the PA infusion. Furthermore, we observed several changes in clinical parameters independent of the presence of sepsis, potentially induced by fluid resuscitation (37), indicating that the control group was crucial to discriminate between sepsis and non-sepsis related changes.

**Systemic Inflammation symptoms**

The steep increase in body temperature indicated the onset of a PA induced inflammatory response (Figure 2). Additionally, in a follow-up study we conducted a white blood cell (WBC) count (total WBC, lymphocytes, granulocytes and platelets) in a pilot amount of animals (four) in the first six hours after start of PA (Supplemental Figure 2). The observed disappearance of lymphocytes from the systemic blood to the peripheral tissues is expected as early response on the trapped PA bacteria in tissue (44, 45). This results in an initial decrease of total WBC, but is quickly
attenuated by stimulated increase in availability of granulocytes (46). Therefore, we concluded that we observed a clinical expected systemic WBC response on PA. However, this was not accompanied by a clear cytokine (IL-6) and acute phase protein (CRP) response between 12-18 hours (Table 1).

Although, a general cytokine response is expected, many factors could be involved, which may explain why we did not see a response. IL-6 and CRP response on PA in the pig over time is a result of amount/strain/route of administration of PA and subsequent complex interaction of multiple host and microbial factors. Therefore, can be highly variable between different studies (47, 48) and between animals (49).

The following explanations need to be considered. First, the uncontrolled microbial status of the farm pigs may likely be the cause of high variability of the IL-6 and CRP values before the bacteria were infused (50). However, supplemental Figure 1 illustrates that the initial IL-6 or CRP variability is not related to the non-response in the period between 12 and 18 h. Therefore, we conclude that it is highly unlikely that the differences in initial cytokine status has an influence on PA cytokine response. Secondly, serum amyloid A (SAA) could potentially be a more sensitive alternative for CRP, but was not measured in the present study (51). However acute phase protein response is considered to be initiated by interleukins like IL-6, also SAA. Therefore, the absence of the CRP response is potentially caused by an absence of an IL-6 response. Thirdly, we have to consider that the absence of a clear IL-6 and CRP response is a reflection of an inefficient or delayed inflammatory response. This phenomenon in humans is associated with higher mortality in sepsis (50, 52). Finally, we need to consider time of measurement. Rimmele et al presented a very early (in the first six hours after administration of PA) but mild response on PA infusion in the pig (16). And is in line with observations in humans with experimental endotoxemia (46). We didn’t measure IL-6 in this time window in the present study. Therefore, in a follow-up pilot study using this model (four animals), we could confirm a small IL-6 response in the first six hours (Supplemental Figure 3). Therefore, we can conclude that the cytokine response of IL-6 in the current model occurred in a very early stage, but didn’t continue in the severe septic stage. On the other hand, measurement was maybe too early. For instance, Benes et al found in a ventilated pig model an IL-6 response on comparable PA infusion was started after 18 hours (30). However, the IL-6 response was still mild in comparison with a peritonitis induced sepsis model in the pig. Overall, we can conclude that the model described in the present study showed a clinically relevant inflammatory response in the first six hours.

**Potential value for nutritional research in septic state**

Nutritional intervention can be supportive and of high clinical value in severe sepsis (9), in contrast to the septic shock state (53). Sepsis related metabolic changes
Like protein breakdown (5, 24), hypocalcemia (54) and hypocholesterolemia (55) were observed in the current model (Supplemental Table 2). Therefore, we concluded that with these metabolic characteristics, the presented PA model with implanted catheters to ensure the possibility of measuring metabolism across organs (23, 24, 26), could be of high value for preclinical nutritional research in severe sepsis. Though other PA infused pig models are available, but they are septic shock models in an unconscious ventilated pig (16, 30), and therefore not suitable for nutritional research.

**Limitation of the model**

First, though PA is a clinically relevant bacteria in sepsis, it is not the only bacteria that can cause sepsis. When comparing other live bacteria used to induce severe sepsis in pigs, the pathophysiology of a single dose of gram positive *Staphylococcus aureus* (22) in a conscious pig model showed slightly different patterns in the hyperdynamics, and a late (after 18 hour) cytokine response. A similar late cytokine response was observed in a mixed bacterium model (peritonitis created by intraperitoneal autologous fecal inoculum) (19). Therefore, we conclude that although metabolic data are lacking from these models, it is highly likely that metabolic results coming from the present PA severe sepsis model can be translational to general severe sepsis, independent of the bacteria that caused the septic state. Secondly, we need to consider that the relative young age of the studied pigs cannot represent metabolic implications seen in adults. We made these concessions for practical reasons (handling, catheter protection with the used harness), but with the knowledge that the development stage of gastro intestinal tract in these animals are already mature (56). Therefore, we can still make the translation of metabolic findings to broader age range. Finally, only the initial phase of severe sepsis has been represented. This means that results cannot be easily translated to a recovery phase in sepsis (for instance after antibiotic treatment). Therefore further development of the sepsis model is needed for studying metabolism in the recovery phase.

**Conclusion**

The present acute sepsis model has the characteristics to be hyperdynamic and bi-phasic with a severe septic phase present between 12-18h after PA infusion. The present PA induced porcine sepsis model is a clinically relevant model for acute severe sepsis. Implanted catheters can accommodate an in-depth study of multi-organ metabolism and nutrient metabolism in sepsis.
Acknowledgements

We thank the assigned veterinarians and animal caretakers of the Arkansas Children’s Hospital Research Institute for their dedication in providing optimal care for the pigs in our experiment. We thank Joshua Spore, Bea Zoer, Eva M.C. Oosterlaken and Sonya R. Mehta for skilled technical assistance in carrying out the animal experiments and/or sample processing.

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References


48. Tayabali AF, Coleman G, Nguyen KC. Virulence Attributes and Host Response


Supplemental information

Supplemental Table 1 (right). Arterial and Venous blood gas values before and after 6, 12 and 18 hours of Pseudomonas aeruginosa (PA) infusion

Data are means ± S.E.M.; Control N=8; Sepsis N=11. CaO2: content of oxygen in arterial blood; ERO2: extraction ratio of oxygen; FiO2: fraction of inspired oxygen; PaO2/FiO2: oxygenation index. Statistics: Two-way ANOVA; data of effects are p-values; significance p<0.05. References: *) (57); **) (33); ***) (58); ****) (59).
Supplemental Table 1. Arterial and Venous blood gas values before and after 6, 12 and 18 hours of Pseudomonas aeruginosa (PA) infection.

<table>
<thead>
<tr>
<th>Parameter Unit</th>
<th>Time Before Infection</th>
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<th>12h</th>
<th>18h</th>
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<th>12h</th>
<th>18h</th>
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<td>PaCO₂ (mmHg)</td>
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Note: Data are reported as mean ± SD.
Supplemental table 2. Arterial clinical chemistry values before and after 12 and 18 h of *Pseudomonas aeruginosa* (PA) infusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Normal Range</th>
<th>0h Control</th>
<th>12h PA</th>
<th>18h Control</th>
<th>18h PA</th>
<th>Statistics</th>
</tr>
</thead>
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<tr>
<td>Sodium</td>
<td>mM</td>
<td>135 - 150</td>
<td>141 ± 0.8</td>
<td>140 ± 1.1</td>
<td>145 ± 1</td>
<td>148 ± 1.5</td>
<td>146 ± 1.1</td>
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<tr>
<td>Potassium</td>
<td>mM</td>
<td>4.4 - 6.7</td>
<td>4.1 ± 0.2</td>
<td>4 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>3.2 ± 0.1</td>
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<tr>
<td>Chloride</td>
<td>mM</td>
<td>100 - 105</td>
<td>100 ± 0.8</td>
<td>101 ± 1.7</td>
<td>109 ± 1</td>
<td>111 ± 1.5</td>
<td>111 ± 0.7</td>
</tr>
<tr>
<td>TCO2</td>
<td>mM</td>
<td>18 - 27</td>
<td>23 ± 0.8</td>
<td>21 ± 0.8</td>
<td>19 ± 0.8</td>
<td>18 ± 1.2</td>
<td>21 ± 0.6</td>
</tr>
<tr>
<td>Anion Gap</td>
<td>mM</td>
<td>-</td>
<td>22 ± 0.7</td>
<td>21 ± 0.8</td>
<td>22 ± 0.3</td>
<td>22 ± 0.8</td>
<td>17 ± 0.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>mM</td>
<td>3.6-6.5</td>
<td>3.4 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>4 ± 0.2</td>
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<td>Urea</td>
<td>mM</td>
<td>2.8-8.6</td>
<td>3.3 ± 0.3</td>
<td>2.6 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mM</td>
<td>0.09-0.24</td>
<td>0.07 ± 0.004</td>
<td>0.07 ± 0.003</td>
<td>0.05 ± 0.003</td>
<td>0.07 ± 0.002</td>
<td>0.05 ± 0.003</td>
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<td>Urea/creatinine</td>
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<td>47 ± 3.7</td>
<td>39 ± 3.3</td>
<td>27 ± 1.6</td>
<td>43 ± 2.6</td>
<td>23 ± 0.9</td>
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<tr>
<td>Calcium</td>
<td>mM</td>
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<td>mM</td>
<td>1.8-3.0*</td>
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<tr>
<td>Cholesterol</td>
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<td>2.1-3.5*</td>
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<td>1.3 ± 0.1</td>
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<tr>
<td>Amylase</td>
<td>IU/L</td>
<td>271 - 1198</td>
<td>2247 ± 138</td>
<td>2110 ± 154</td>
<td>1812 ± 125</td>
<td>1585 ± 141</td>
<td>1811 ± 147</td>
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<tr>
<td>Total Protein</td>
<td>g/L</td>
<td>58.3-83.2*</td>
<td>53 ± 1.5</td>
<td>51 ± 1.6</td>
<td>46 ± 1.6</td>
<td>36 ± 2.6</td>
<td>47 ± 2.3</td>
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<tr>
<td>Albumin</td>
<td>g/L</td>
<td>22.6-40.4*</td>
<td>27 ± 1.1</td>
<td>27 ± 1</td>
<td>23 ± 0.9</td>
<td>20 ± 1.3</td>
<td>23 ± 0.9</td>
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<tr>
<td>Globulin</td>
<td>g/L</td>
<td>39.5-60.0*</td>
<td>26 ± 1.5</td>
<td>24 ± 1.3</td>
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<td>23 ± 2.1</td>
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<tr>
<td>Albumin / Globulin</td>
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<td>.42 - .76</td>
<td>1.09 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>Total Bilirubin</td>
<td>mg/dL</td>
<td>0 - .2</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
<td>0.2 ± 0</td>
<td>0.1 ± 0</td>
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<tr>
<td>ALT</td>
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<td>54 ± 3</td>
<td>52 ± 3.6</td>
<td>47 ± 3</td>
<td>44 ± 5.1</td>
<td>45 ± 2.5</td>
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<tr>
<td>AST</td>
<td>IU/L</td>
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<td>32 ± 3</td>
<td>34 ± 6.4</td>
<td>30 ± 3.9</td>
<td>143 ± 20.8</td>
<td>30 ± 4.4</td>
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<tr>
<td>GGT</td>
<td>IU/L</td>
<td>31.0-52.0*</td>
<td>36 ± 3.3</td>
<td>34 ± 2.4</td>
<td>28 ± 1.6</td>
<td>28 ± 2.7</td>
<td>23 ± 1.6</td>
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<tr>
<td>Alkaline Phosphatase</td>
<td>IU/L</td>
<td>41.0-176.1*</td>
<td>82 ± 7.7</td>
<td>86 ± 10.2</td>
<td>76 ± 6.6</td>
<td>103 ± 7.2</td>
<td>73 ± 6</td>
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<tr>
<td>CPK</td>
<td>IU/L</td>
<td>65.7-489.4*</td>
<td>221 ± 26</td>
<td>330 ± 100</td>
<td>230 ± 18</td>
<td>999 ± 151</td>
<td>324 ± 38</td>
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<tr>
<td>Osmolality</td>
<td>mmol/kg</td>
<td></td>
<td>277 ± 1.9</td>
<td>275 ± 2.2</td>
<td>283 ± 1.8</td>
<td>287 ± 2.6</td>
<td>286 ± 2</td>
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</tbody>
</table>
Supplemental table 2 (left). Arterial clinical chemistry values before and after 12 and 18 h of *Pseudomonas aeruginosa* (PA) infusion.

Data are means ± S.E.M; Control N=9; Sepsis N=13; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; CPK: creatinine phosphokinase. Statistics: Two-way ANOVA; data of effects are p-values; significance p<0.05. References normal ranges: *') (58).

Supplemental Figure 1. Individual time courses of arterial Interleukin-6 (Il-6) and C-reactive protein (CRP) values, pilot.

Dash line are control animals; Straight line are *Pseudomonas aeruginosa* (PA) animals. Il-6: Control N=2; PA N=4; CRP: Control N=2; PA N=2. Left graphs expressed per liter; Right graphs expressed per gram plasma protein. Regression slope of time courses (-2-0; 12-18h) not significant from zero for IL-6 and minor positive and negative slopes in the CRP time courses.
Supplemental Figure 2. White blood cell count (WBC)
In pilot amount of pigs (four) from a follow-up study. During the first six hours of Pseudomonas aeruginosa induced development of sepsis.

Supplemental Figure 3. Interleukin (IL-6)
In pilot amount of pigs (four) from a follow-up study. During the first six hours of Pseudomonas aeruginosa induced development of sepsis.
Phenylalanine isotope pulse method
to measure effect of sepsis on
protein breakdown and membrane
transport in the pig.


Abstract
The primed-continuous (PC) phenylalanine (Phe) stable isotope infusion methodology is often used as a proxy for measuring whole body protein breakdown (WbPB) in sepsis. It is unclear, if WbPB data obtained by an easy-to-use single IV Phe isotope pulse administration (PULSE) are comparable to those by PC. Compartmental modeling with PULSE could provide us more insight in WbPB in sepsis. Therefore, in the present study, we compared PULSE with PC as proxy for WbPB in an instrumented pig model with *Pseudomonas aeruginosa* induced severe sepsis (Healthy: n=9; Sepsis: n=13). Seventeen hours after sepsis induction, we compared the Wb rate of appearance (WbRa) of Phe obtained by PC(L-[ring-13C6]-Phe) and PULSE(L-[15N]-Phe) in arterial plasma using LC-MS/MS and (non)compartmental modeling.

PULSE-WbRa was highly correlated with PC-WbRa (r=0.732, p<0.0001) and WbPB (r=0.897, p<0.0001) independent of the septic state. PULSE-WbRa was 1.6 times higher than PC-WbRa (p<0.001). Compartmental and non-compartmental PULSE modeling provides comparable WbRₘ values, although compartmental modeling was more sensitive. WbPB was elevated in sepsis (Healthy: 3378±103; Sepsis: 4333±160 nmol/kg BW/min, p=0.0002). With PULSE, sepsis was characterized by an increase of the metabolic shunting (Healthy: 3021±347; Sepsis: 4233±344 nmol/kg BW/min, p=0.026). Membrane transport capacity was the same.

Both PC and PULSE-methods are able to assess changes in WbRₘ of plasma Phe reflecting WbPB changes with high sensitivity, independent of the (patho-)physiological state. The easy-to-use (non-)compartmental PULSE reflects better the real WbPB than PC. With PULSE compartmental analysis, we conclude that the membrane transport capacity for amino acids is not compromised in severe sepsis.
Introduction

The primed-constant and continuous infusion methodology (PC) is often used for measuring whole body rate of appearance (WbRa) of amino acids in humans and animals (1-3). The principle is, that a single pool model (Figure 1) in which amino acids (e.g. from whole body protein breakdown, WbPB) are appearing, is the same pool in which the stable amino acid isotopes are infused. It is assumed that the pool represents mainly the extracellular compartment. In this model, WbRa is calculated by dividing the tracer (stable isotope) infusion rate by the tracer/tracee ratio (TTR) in the extracellular pool at steady state, and in which the tracee is the natural occurring amino acid. Arterial blood is mostly used to represent the extracellular pool. The main advantage of measuring WbRa using this method is that when the TTR is low, the effects on tracee metabolism are minimal. Furthermore, when steady state is obtained, only two blood samples are needed (background and at steady state). The main disadvantage is that it might take several hours to reach a tracer steady state, although this can be overcome by correctly priming the tracer pool. The estimation of the pool size for stable tracer studies depends on the size of the plasma and tissue pool of the tracee. In studies in diseased animals or humans, the individual variation of the pool size affects the probability to correctly prime the pool. In sepsis for example, plasma phenylalanine (Phe) concentrations can be increased 2 fold, while other amino acid concentrations also can be increased many fold up or down (4). Furthermore, it is very difficult to correctly prime the pools of urea, glutamine, taurine, and 3-methylhistidine (5, 6). Moreover, the PC method is highly dependent on the accuracy of the infusion pump. Although the calculation is very simple, WbRa is the only information that can be obtained from the PC method as no further metabolic modeling is possible.

To overcome these disadvantages, we explored the use of a single pulse of tracer (PULSE) into the extracellular pool (Figure 1) to measure WbRa (1). In this approach, the shape of the TTR decay curve depends on how many pools are connected to the extracellular pool. For most substrates like amino acids, the intracellular pool is the main secondary pool and the amino acid decay curve therefore reflects two compartments. This TTR decay curve can be used to perform both non-compartmental and compartmental modeling (7-9). Compartmental modeling enables the calculation of the Ra into the extracellular and the intracellular pools, as well as the fluxes between the pools. The appearance of an essential amino acid (e.g. Phe) into the intracellular pool represents protein breakdown (PB). Advantages of the PULSE method are that only one single tracer dose needs to be administered, no pumps are required, low pharmacy costs, and there is no need to prime. A major disadvantage however is that multiple blood samples are needed to describe the enrichment decay curve accurately. Although the calculations are not as simple as with the PC model, modern modeling/fitting programs are more available and user-friendly. For routine Ra measurements, the non-compartmental WbRa calculation
Figure 1: Calculation models for whole body protein breakdown

Two calculation models for whole body protein breakdown (WbPB) by determination of whole body rate of appearance (WbRa) of phenylalanine (Phe). (A) Non-compartmental model using a primed-continuous infusion of L-[ring-13C6]-Phe (PC). WbRa is the tracer infusion rate divided by the fraction of tracer found in plasma/extra cellular pool (TTR=tracer/tracee ratio) in a tracer steady state. (B) Compartmental model using bolus infusion, L-[15N]-Phe (PULSE) and SAAMII computer modeling. Computer parameters: Q= pool sizes of the compartments; k21= rate parameter to pool 2, from pool 1; k02 rate parameter of irreversible loss from pool 2; U2: rate of appearance of Phe in pool 2. (C) Physiological assignment of SAAMII computer model: Flux (F12) of Phe from the intra-cellular pool to the extra cellular pool represents the k21 times Q1, and is equal to F21 in physiological steady state; U2 (=F20) represents WbPB; Flux of Phe from the intra-cellular pool to hydroxylation and protein synthesis represents the irreversible loss (F02 = k02Q2); WbR0 of Phe is the fraction of Phe from protein breakdown that appears in Q1 that is not irreversible lost; The amount of Phe flux between Q1 and Q2 that is not irreversible lost is shunt back to Q1 (F21-WbRa).

In the PULSE method using the fitting of the decay curve with e.g. the software program GraphPad Prism or add-ins of EXCEL, and additional relative simple calculations of WbR0 can be attractive (1). Previously, the computer modeling program SAAM has been used in the compartmental analysis to measure 3-methylhistidine appearance (as indirect marker of myofibrillar muscle PB) (10) or citrulline production.
Compartmental modeling can be extended to measure the flux between the different metabolic routes (8, 12). PULSE methods with a small amount of tracer are also used to measure tissue specific fractional protein synthesis (FSR) and breakdown (FBR) (13-16), but these methods require additional sampling from the tissue compartment.

In the past, comparisons of the tracer PULSE and PC infusion methods were done with theoretically modeling (17), in healthy subjects and animals (9, 10, 13), but not in disease states. In disease conditions with large changes in the tracee pools and in situation in which there is a large effect of the disease on e.g. whole body protein breakdown, it is unclear if the PULSE method is providing the same clinical information as the more generally used PC method. Specifically in disease states, the additional information coming from the PULSE compartmental modeling can increase the understanding of the clinical situation. Therefore, in the present study, we measured whole body rate of appearance (WbRa) of Phe as proxy for WbPB using the PC protocol in comparison to a single-dose PULSE tracer protocol using Phe stable isotopes during Pseudomonas aeruginosa (PM) induced severe sepsis in the pig. The severe septic state is used as a disease model in which large changes in tracee pools and substantial effects on WbPB can be expected (18). We hypothesize that both the PC and the PULSE method are able to provide the expected enhanced WbPB information in severe sepsis. We compared the more practical non-compartmental modeling with the PULSE method with the compartmental modeling to obtain this information for a routine setting. Finally, we explored with the compartmental modeling the extra intracellular information (e.g. membrane transport capacity).

Material and Methods

Animals
We used Female Yorkshire cross/domestic pigs (20-25 kg BW) in our experimental studies. We housed the pigs in steel pens (2m x 3m) in a controlled housing facility (large animal cubicle, room temperature 22-24°C, 12 hours light-dark cycle, standardized food 1kg/day (Harlan Teklad Vegetarian Pig/Sow Grower) and provided water ad libitum.

Surgical procedure
Animals received catheters and a jejunal stoma during a surgical procedure as described in detail previously (19, 20). In brief, during midline laparotomy, we placed catheters for blood sampling into the abdominal aorta and in the caval vein for administering post-surgery medication and experiment related tracer infusions. We
placed a second arterial catheter to monitor mean arterial blood pressure (MAP) and a Swan ganz catheter (5 Fr, #132F5, Edwards life sciences, Irvine, CA, USA) via the right jugular vein to monitor mean pulmonary blood pressure (MPAP). We standardized preoperative and postoperative care (19, 20). During the recovery period (7-10 days) animals were accustomed to a small movable cage (0.9x0.5x0.3m) and performed the experiments in this cage in awake animals. This study was approved by the animal experiment ethics committee of University of Arkansas Medical Sciences.

**Experimental Design (Figure 2)**

The experiment started after a recovery period of 7-10 days. Four hours after the last food intake (half of the daily amount: 0.5 kg), we selected animals for the Sepsis group and the Healthy group in a randomized fashion (Healthy n=9, Sepsis n=13). We monitored basal monitoring blood pressures in the pre-septic period (T= -2 h - 0 h). At T=0h, we induced sepsis by continuous infusion of *Pseudomonas aeruginosa* (PM, $10^9$ CFU/ml/hour), while the Healthy group received an equal volume of 0.9% NaCl solution. We started fluid resuscitation (30 ml/kg bw/hour) also at T=0h and monitored hemodynamics continuously. For 1 hour, between 17 and 18 hours after the start of PM, we compared the PC tracer protocol with the PULSE tracer protocol and at t=18h, we euthanized the pigs with 125 mg/kg pentobarbital sodium and 16 mg/kg phenytoin sodium (Euthanasol®) administered via the central vein catheter.

*Pseudomonas aeruginosa*

For the induction of sepsis, we used a live PM human strain (IRS 12-4-4, Shriners burns institute, University of Texas Medical Branch, Galveston). Originally this PM

![Figure 2: Protocol](image_url)

PC: primed-continuous infusion: L-[ring-$^{13}$C$_6$]-Phenylalanine (Phe). PULSE: bolus infusion: L-$^{15}$N-Phe. Abbreviations: MPAP = mean pulmonary artery pressure, MAP = mean arterial pressure.
strain was isolated from a burn patient at Brook Army Medical Center in San Antonio, TX. Based on pilot virulence experiment, 10⁹ Colony Forming Units per hour (CFU/hour) in a volume of 1 ml 0.9% NaCl solution was needed to obtain similar cardiovascular responses and hemodynamic variables with characteristics of severe hyperdynamic sepsis. We monitored hemodynamics continuously to ensure that the hyperdynamic state was kept in the expected ranges for severe sepsis (body temp increase of 2-3 °C, respiratory rate increased, MPAP increased but <35 mmHg, heart rate increased but <200 BPM).

**Infusion and sampling protocol**

**Stable isotopes**

We used two stable isotopes of Phe: L-[ring-13C₆]-Phe and L-[15N]-Phe (Cambridge Isotopes, Andover, MA) as tracers to study WbRa of Phe with two different tracer infusion protocols. We previously used Phe to determine WbPB (1, 20, 21). We based the prime amount and tracer infusion rates on our previous studies (21). For the PC infusion protocol, we used L-[ring-13C₆]-Phe. The prime (1.58 µmol/kg bw) and infusion (4.32 µmol/kg bw/hour) was given respectively in a volume of 2 ml/kg bw and 2 ml/kg bw/hour, and started 12 hours after the start of PM, 5 hours before the PULSE protocol in which we used L-[15N]-Phe (26.3 µmol/ kg bw in a volume 0.5 ml/kg bw). We gave all tracers via the central caval vein catheter.

**Blood sampling and sample processing**

We took blood samples and directly cooled them on ice. We processed the blood samples within one hour (19, 21, 22). In brief: for amino acid concentration and enrichment analysis, we centrifuged heparinized blood at 4°C, 5 minutes, 8000 G and deproteinized 250 µL plasma with 25 µL tri-chloroacetic acid solution (TCA, 50% w/v) and finally snap freeze in liquid nitrogen and store at -80°C.

**Amino acid concentration and enrichments**

We determined amino acid isotope concentrations and amino acid enrichments (tracer-to-tracee ratios, TTR) on a fully automated LC-ESI-MS system (QTrap 5500 MS (AB Sciex, Foster City, CA, USA) with ExpressHT Ultra LC (Eksigent Div., AB Sciex, Foster City, CA, USA). For concentration and TTR measurements we added 20 µl of TCA deproteinized plasma supernatant to 20 µl L-[U-13C₉]-Phe internal standard. Within 3 days before the LC-ESI-MS analysis, we derivatized the samples together with external standards at concentrations within the physiological range (calibration curve of concentration) and enriched external standards in the range of expected TTRs (calibration curve for TTRs) with 9-fluorenylmethoxycarbonyl (Fmoc). After neutralization, we injected 160 nL of the solution onto a micro LC column 0.5 x 100 mm HALO C18, 2.7 um, 90A pores (ABsciex, Foster City, CA, USA), kept at 35°C. We eluted analytes with a segmentally linear gradient from 35% to 85% acetonitrile in water supplemented with ammonium acetate to 10 µM and 5% isopropanol. We
used electrospray triple quadrupole tandem mass spectrometry in multiple reactions monitoring mode for detection. We fragmented the Fmoc amino acid derivatives in the collision cell for detection of either free aminoacyl anions or a fragment larger by 26 atom mass units (coming from the Fmoc derivative) to have the highest sensitivity. We simultaneously did mass analyses for Phe, its tracers and internal standards. We calculated the mass signal areas to enable TTR or tracee concentrations calculations.

**Calculations**

**Calculation of Phe tracee concentration and TTR from LCMS obtained signals**

For concentration calculations, we normalized the tracee signals of the samples and the external standards with their internal L-[13C₉]-Phe standard. We determined the plasma tracee concentration with the calibration curve external Phe standards. Plasma enrichment (TTR) was determined using the peak area ratio of the tracee and tracer signal and a calibration curve of enriched external standards. For the tracer concentration in the infusions, we used a calibration curve of tracer containing external standards.

**Calculation WbRₐ with PC and PULSE method (Figure 1)**

In the present study we compare the calculations of WbRₐ of Phe into the circulation in a post-absorptive (patho-)physiological state, using non-compartmental model in the PC group versus a compartmental model made up by two compartments in the PULSE group (1).

PC: We derived WbRₐ from equation 1 by using the L-[ring-13C₆]-Phe isotope. The tracer infusion rate (I) is divided by TTRA. TTRA is the tracer to tracee ratio in arterial plasma.

Equation (1) \[ WbRₐ = \frac{I}{TTR} \]

PULSE: We used non-compartmental analysis in GraphPad Prism (version 6) to perform curve fitting of the exponential decay curve. We found that all data fitted best (R² and observation) with a two-exponential fit:

Equation (2) \[ \text{Enrichment}(t) = A₁e^{-B₁t} + A₂e^{-B₂t} \]

The non-compartmental WbRₐ equation 1 is generally applicable to multiple exponential decays. This equation can be written in terms of the parameters of the curve fits as follows:

Equation (3) \[ WBRₐ = \frac{I}{(A₁/B₁ + A₂/B₂)} \]
The two-exponential fit is related to a two compartmental model in line with what is expected for essential amino acids (1). The two compartmental analysis of the tracer decay curve follows the Michaelis-Menten elimination kinetics (23). The assumption that the rate of change of the amount of tracer in a pool is equal to the rate that tracer enters the pool minus the rate exits the pool can be stated mathematically, as we described previously (1):

Equation (4) \[ \frac{dq_1(t)}{dt} = k_{12} \cdot q_2(t) - k_{21} \cdot q_1(t) \]

Equation (5) \[ \frac{dq_2(t)}{dt} = k_{21} \cdot q_1(t) - k_{12} \cdot q_2(t) - k_{02} \cdot q_2(t) \]

Equation (6) \[ TTR(t) = \frac{q_1(t)}{Q_1} \]

Where \( dq_1(t)/dt \) and \( dq_2(t)/dt \) are the rates of change of the tracer pool size in pools 1 and 2 respectively, and \( Q_1 \) the tracee pool size in pool 1. These equations/model (Figure 1), the measured enrichment data, and the size of the administered tracer pulse are entered into the SAAM II software that provides the calculated values of \( k_{21}, k_{21}, k_{02} \) and \( Q_1 \). We converted the \( k \) values to whole-body rate of appearance (WbRa) or intracellular production as described previously (1, 11). We estimated the transfer rates as fractional rates between pools (\( k_{12}, k_{21}, k_{02} \)) and the extracellular pool size (\( Q_1 \)) in all subjects. Assuming that the measurements were done in a physiological steady state, meaning no net loss or production of Phe tracee in \( Q_1 \) during the experimental period, we calculated the flux (\( F \)) from \( Q_2 \) to \( Q_1 (F_{12}) \) with the fractional rate of intracellular uptake (\( k_{21} \)) from \( Q_1 \):

Equation (7) \[ Flux = F_{12} = F_{21} = k_{21}Q_1 \]

The size of the intracellular pool (\( Q_2 \)) can be calculated with the fractional rate of intracellular release (\( k_{12} \)):

Equation (8) \[ Intracellular \ pool = Q_2 = F_{12}/k_{12} \]

The irreversible loss from the intracellular pool (\( F_{02} \)) calculated with the fractional rate of irreversible loss (\( k_{02} \)):

Equation (9) \[ Irreversible \ loss = F_{02} = k_{02}Q_2 \]

In a physiological steady state, irreversible loss is equal to appearance of Phe in the intracellular pool. Assuming that the appearance of Phe in the intracellular pool is coming from PB, and PB is occurring intracellular in post-absorptive state, we thus determine WbPB:
Equation (10) \[ WbPB = U_2 = F_{20} = F_{02} \]

The fraction of the amount of Phe coming from PB that will appear in the extracellular pool (Q₁) is the amount that is not irreversibly lost:

Equation (11) \[ WbRa = F_{20} \times (1 - F_{02}/(F_{20} + F_{21})) \]

The amount of Phe flux between Q₁ and Q₂ that is not irreversibly lost is shunt back to Q₁:

Equation (12) \[ \text{Shunting} = F_{12} \cdot WbRa \]

**Statistical analyses**

Results are presented as means ± SEM. Graphpad Prism (version 6) was used for statistics. Level of significance was set on p<0.05. To determine physiological (tracee) or tracer Phe steady state during the experimental period, linear regression was used to determine if the slope of the best fitted line was different from zero. No difference from zero is steady state. To compare data between the Healthy and Sepsis group an unpaired t-test was used. To compare physiologically relevant models/parameters a Pearson correlation test was used. Best-fitted line that describes the relation between both models/parameters was done with linear regression. A shared fitted line was determined when no significant differences were observed between the Healthy and the Sepsis fitted line. Additionally, a Bland-Altman plot was made to determine potential discrepancy between methods (24). For the limits of agreement 2 times the standard deviation of the average discrepancy was used. Post-hoc Wilcoxon Signed Rank test and D’Agostino&Pearson normality test was used to characterize a discrepancy.

**Results**

**Characteristics/Validation of tracer models**

Plasma tracer/tracee ratios of both tracer methods.

Figure 3 characterizes the shape of the enrichment (TTR) curve after a PULSE of L-[¹⁵N]-Phe and the PC infusion enrichment of L-[¹³C₆]-Phe in a septic and healthy animal. The exponential decay curves of all individual animals are shown in Supplemental Figures 1 and 2. We evaluated the residual errors by visual inspection. We determined goodness of fit with the coefficient of determination (R square): 0.9991±0.0002 (Healthy) and 0.9991±0.002 (Sepsis). We determined, using linear regression, that L-[¹³C₆]-Phe tracer steady state (horizontal lines in time during the experimental period) was present. In all animals the TTR was in steady state.
Tracee concentrations
We found that the arterial tracee Phe concentrations during the experimental period (Figure 4) were stable in the Healthy group but slightly increasing in the Sepsis group (regression slope was different from zero: p=0.0211). The plasma Phe concentration was increased in the sepsis animals (median over the experimental period, Healthy: 64.6±4.0 µM; Sepsis 113±8.3 µM; p=0.0002).

Precision of model parameters with PULSE method
In Table 1 we show the results of the fitting of the decay curves of Phe enrichments performed with Graphpad Prism. For non-compartmental analysis with the PULSE method, the correlation between the fit and the data ($R^2$) should be greater than 0.95 (1). We found that for all fitted curves, the $R^2$ was higher than 0.95. The correlation between the various parameters (dependency) indicates that the use of a two-exponential equation is appropriate.

The coefficient of variation of each compartmental model parameter should be reasonable and models with coefficients above 100% should be rejected (1). We found for all animals that the parameter estimations performed with SAAM II (Table 2) had all coefficients of variation below 100%.

Figure 3: Example plasma tracer/tracee ratio’s
Example of plasma tracer/tracee time courses of arterial phenylalanine (Phe) in a Healthy and a Sepsis animal. PC: Steady state curve, 5 h after the start of a primed-continuous infusion of L-[ring-$^{13}$C$_6$]-Phe tracer; PULSE: A two-exponential decay curve after a bolus infusion tracer is L-[$^{15}$N]-Phe. TTR is tracer/tracee ratio.
**Table 1: Precision of decay curves of Phe enrichment**

| Parameter | Healthy | | | Sepsis | | |
|-----------|---------|-----------------|-----------------|-----------------|-----------------|
|           | Mean    | Mean SD         | Mean CV%        | Mean Value      | Mean SD         | Mean CV%        |
| $A_1$     | 0.371   | 0.025           | 6.7             | 0.932           | 0.084           | 15              | 0.910           |
| $A_2$     | 0.231   | 0.020           | 8.6             | 0.988           | 0.016           | 8.4             | 0.982           |
| $B_1$     | 0.153   | 0.018           | 11              | 0.984           | 0.042           | 18              | 0.981           |
| $B_2$     | 0.021   | 0.002           | 8.5             | 0.956           | 0.020           | 10              | 0.940           |
| $R^2$     | 0.9998  |                 | 0.9991          |                 |                 |                 |

Mean variations of data of two-exponential fit of individual decay curves of Phe enrichment
Equation: $\text{Enrichment}(t) = A_1e^{-B_1t} + A_2e^{-B_2t}$. Fitted with Graphpad Prism. Used fitting constrains: $B_1 < 0.4$, $B_2 > 0$, plateau = 0. $R^2$ is the correlation between the fit and the data. Healthy $n=9$; Sepsis $n=13$.

**Figure 4. Phenylalanine (Phe,tracee) concentrations**

A: Arterial plasma Phe tracee (non-tracer) concentrations after administration of L-[15N]-Phe pulse. Between 17-18h after start administration of *Pseudomonas aeruginosa*. Data expressed as mean ± SD. Healthy $n=9$, Sepsis $n=13$. Statistics for physiological steady state: linear regression, significance $p<0.05$ if slope was different from zero. Slope in Sepsis group was different from zero ($p=0.0211$). B: Average tissue Phe concentration from muscle, jejunum, ileum, liver, lung in µmol/kg ww. At necropsy, 18h after start administration of *Pseudomonas aeruginosa*. Data expressed as mean ± SEM. Healthy $n=9$, Sepsis $n=13$. Statistics: sepsis compared to Healthy, unpaired t-test. *) significance $p<0.05$. 
Table 2 Precision of compartmental model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy</th>
<th>Sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Value</td>
<td>Mean SD</td>
</tr>
<tr>
<td>$Q_1$ (µmol)</td>
<td>1121</td>
<td>49</td>
</tr>
<tr>
<td>$k_{02}$ (min⁻¹)</td>
<td>0.033</td>
<td>0.005</td>
</tr>
<tr>
<td>$k_{21}$ (min⁻¹)</td>
<td>0.051</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Mean variations of parameters generated from the two compartmental analysis of individual decay curves of Phe enrichment with computer software SAAM II

$Q_1$ is plasma pool size; $k_{02}$, $k_{12}$, $k_{21}$ are proportionality constants. Healthy $n=9$; Sepsis $n=13$.

Formulas used:

\[
\begin{align*}
\frac{dq_1(t)}{dt} &= k_{12} \cdot q_2(t) - k_{21} \cdot q_1(t) \\
\frac{dq_2(t)}{dt} &= k_{21} \cdot q_1(t) - k_{12} \cdot q_2(t) - k_{02} \cdot q_2(t) \\
TTR(t) &= \frac{q_1(t)}{Q_1}
\end{align*}
\]

Where $\frac{dq_1(t)}{dt}$ and $\frac{dq_2(t)}{dt}$ are the rates of change of the tracer pool size in pools 1 and 2 respectively.

Whole body rate of appearance (WbRₐ) comparisons

We compared healthy and septic animals to determine if both PC and PULSE can be used to observe changes in WbPB (Figure 5). We found that WbRₐ of Phe was higher in the sepsis group with both methods (PC: $p=0.003$; PULSE: $p=0.0001$), albeit that WbRₐ was higher with the PULSE method in both groups (Figure 5A, 5B). Data from the non-compartmental WbRₐ-PULSE analysis were not different (Healthy: $p=0.788$; Sepsis: $p=0.841$) from those from the compartmental WbRₐ-PULSE analysis (Figure 5B). Although the PC and PULSE WbRₐ data correlated very well ($r=0.732$, $p<0.0001$) (Figure 5C), a systemic difference between the methods was present as reflected by the 1.6 times higher WbRₐ with the PULSE method, independent of the septic state (Figure 5D). Although the WbRₐ compartmental versus non-compartmental data with the PULSE method correlated highly ($r=0.969$, $p<0.0001$) (Figure 5E), the discrepancy didn’t pass the D’Agostino&Pearson normality test due to higher variability of the data obtained by the non-compartmental PULSE analysis.

Additional metabolic information with the PULSE tracer model

WbRₐ in comparison with WbPB

To determine if the WbRₐ of Phe in the extra cellular pool is representing WbPB, we compared it with the WbPB (Figure 6). While the WbPB was higher ($p=0.002$) in the sepsis group, the correlation with WbRₐ-PULSE was excellent ($r=0.897$: $p<0.0001$) and was 1.7 times higher than the WbRₐ-PULSE independent of the septic state.

Comparison whole body Phe pools

Both extracellular and intracellular Phe pool are increased in sepsis (Table 3) and are highly correlated ($r=0.802$: $p<0.001$) and the intracellular pool is 2.2 times larger
Figure 5. Effect of sepsis on whole body rate of appearance (WbRa) of phenylalanine (Phe) using two different tracer methods

WbRa as proxy for whole body protein breakdown in sepsis and healthy pigs. Calculated with

A. PC tracer method: primed-continuous infusion of L-[ring-13C6]-Phe. Or B. with the PULSE tracer method: bolus infusion of L-[15N]-Phe. Compartmental and non-compartmental data analyses. Data expressed as mean ± SEM. Healthy n=9, Sepsis n=13. Statistics: sepsis compared to Healthy, unpaired t-test. *) significance p<0.05. C: Correlation between WbRa-PULSE (compartmental) vs WbRa-PC. Statistics for correlation: Pearson correlation (r=0.732), with a likelihood for real correlation (p<0.0001). Linear regression is used for the prediction of best line (r²=0.507, slope 1.61). D. Bland-Altman plot. Ratio vs the average of the two different tracer
methods. The discrepancy (mean ± SD) is 1.628 ± 0.2279. Wilcoxon Signed Rank test was used to determine the discrepancy was different from one: p<0.0001. Discrepancy passed D’Agostino&Pearson normality test. E. Correlation between WbRα-PULSE (compartmental) vs WbRα-PULSE (non-compartmental). Statistics for correlation: Pearson correlation (r=0.969), with a likelihood for real correlation (p<0.0001). Linear regression is used for the prediction of best line (r²=0.9386, slope = 1.005). F. Bland-Altman plot. Difference vs the average of the two different PULSE data analysis methods. The discrepancy (mean ± SD) is -26.51 ± 108.1. Wilcoxon Signed Rank test was used to determine the discrepancy was different from zero: No significance. Discrepancy didn’t passed D’Agostino&Pearson normality test.

Figure 6. Effect of sepsis on whole body protein breakdown (WbPB)
WbPB data obtained with compartmental modeling (PULSE method). A: WbPB. Data expressed as mean ± SEM. Healthy n=9, Sepsis n=13. Statistics: Sepsis compared to Healthy, unpaired t-test, *) significance p<0.0002. B: correlation between WbPB and whole body rate of appearance (WbRa). Statistics: Pearson correlation (r=0.897), with a likelihood for real correlation (p<0.0001). Linear regression is used for the prediction of best line (r²=0.803, slope=0.60).

than the extracellular pool. A significant relationship is also present between the extracellular Phe pool size and plasma Phe concentration (r=0.613; p=0.002).

Other metabolic parameters
Beside the increase in Phe pools, we also observed an increase in Phe flux between the extra- and intracellular pools, an increase in the amount of shunting (Table 3), and an increase in irreversible loss. No changes were found in the fractional release, uptake and irreversible loss of Phe in the intracellular pool.
Table 3. Whole body metabolic information obtained with PULSE model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Healthy</th>
<th>Sepsis</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular pool (Q₁)</td>
<td>µmol/kg BW</td>
<td>44(2.2)</td>
<td>68(5.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Intracellular pool (Q₂)</td>
<td>µmol/kg BW</td>
<td>105(8.0)</td>
<td>157(8.2)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Flux (F₁₂=F₂₁)</td>
<td>µmol/kg BW/min</td>
<td>5.02(0.40)</td>
<td>6.86(0.42)</td>
<td>0.006</td>
</tr>
<tr>
<td>Shunting (F₁₂-WbRₐ)</td>
<td>µmol/kg BW/min</td>
<td>3.02(0.35)</td>
<td>4.23(0.34)</td>
<td>0.026</td>
</tr>
<tr>
<td>Irreversible loss(F₀₂)</td>
<td>µmol/kg BW/min</td>
<td>3.38(0.10)</td>
<td>4.33(0.16)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fractional rate of irreversible loss(k₀₂)</td>
<td>%/min</td>
<td>3.33(0.21)</td>
<td>2.83(0.15)</td>
<td>0.065</td>
</tr>
<tr>
<td>Fractional rate of intracellular release (k₁₂)</td>
<td>%/min</td>
<td>5.09(0.69)</td>
<td>4.43(0.25)</td>
<td>0.316</td>
</tr>
<tr>
<td>Fractional rate of intracellular uptake (k₂₁)</td>
<td>%/min</td>
<td>11.5(0.71)</td>
<td>10.6(0.76)</td>
<td>0.423</td>
</tr>
</tbody>
</table>

Mean values of parameters obtained and calculated from the fitting of individual decay curves of Phe enrichment. Expressed as mean ± SEM. Healthy n=9; Sepsis n=13. Statistics: unpaired t-test.

Discussion

The present study shows that the WbRₐ obtained by the PULSE method is highly correlated with that of the PC method, albeit the absolute values were higher for the PULSE method but independent of the presence of sepsis. Furthermore, the PULSE method is able to measure protein breakdown using a compartmental model made up of two compartments, and provides additional valuable information i.e. membrane transport characteristics (fractional release, uptake, irreversible loss). The Phe pools and fluxes between pools are increased in the sepsis animals, however the transport characteristics between the pools are not changed.

Validation models

Choice of tracers

The stable isotope tracers of Phe that can be used for both the PULSE as the PC method are dependent on the choice of analytical method (e.g. LCMS, GCMS or IRMS), precision of this method (e.g. higher masses can give more sensitivity, possible use of neutral loss approach). Additionally, in the present study we considered potential chemical interference during the analytical procedures. For instance, if masses of two tracers are too close to each other, contribution of the lower mass (natural abundance) to the higher mass can be expected. Although biological interference (unwanted modifications of tracers in the body) like the potential disappearance of ¹⁵N-tracer by transamination can occur (25), for the PULSE method it will not interfere with the WbRₐ (part of irreversible loss). Also, the transamination of phenylalanine in the body and back can be considered very low, because for instance the use of ¹⁵N-Phe and 1-¹³C-Phe gives comparable TTR’s (26). This is another reason why phenylalanine is a preferred amino acid in our type of measurements. Any
stable isotope tracer of phenylalanine can be used for WbR\textsubscript{a}, as long as it is possible to measure both high and low enrichments with sufficient precision.

**Physiological steady state**

During the 1h testing period, our animals were all in a physiological steady state and tracer steady state for the PC method. We found that the Phe tracee curves were not significantly affected by the amount of the Phe isotope in the PULSE, although an increasing tracee concentration was found in the septic animals, likely related to the septic condition. Because the increase was minor and the tracer enrichment was in a steady state for the PC method, we didn’t adjust the PC-WbR\textsubscript{a} formula with e.g. Steele equation correction for non-steady state measurements (27), which for Phe only has a minor effect on the final results. We suggest that future studies should use small amounts of tracers to reduce the effect on metabolism further.

**Fitting**

Due to time limitations and experimental design constraints, we did not take more than 6 samples to describe the PULSE decay curve in the present study. However, the Goodness of fit and CV\% of each parameter with as well Graphpad Prism (for non-compartmental data analysis) as SAAM II software (for compartmental data analysis) shows high reliable decay curves thanks to very accurate LCMS TTR measurements (CV\%<2\%). For the chosen compartmental model (two compartments) (11, 13, 23, 28) and to determine changes in sepsis, this number of samples was sufficient in the present study. In case of more complex compartmental models (three or more compartments) or less accurate analytical methods, we think that more sample points are necessary.

**Identifiability**

The general idea is to use compartments to model various components of the physiological metabolic system. The structure of the model is developed from evaluation of the relationships between the exponential functions of the decay curve and an understanding of the metabolic system to be modeled. Usually the number of exponential terms is directly related to the number of compartments in the model. The minimal number of detectable compartments in the current study is two based on the goodness of fit. The physiological identifiability of the two compartments PULSE model is not necessary univocal, but this interpretation fits with a general used two-pool model for amino acid metabolism (1) where Q\textsubscript{1} is considered the extracellular pool and Q\textsubscript{2} the intracellular pool. If we compare Q\textsubscript{1} Phe pool with the plasma Phe pool (plasma Phe concentration times estimated plasma volume per kg: 80 ml/kg blood × plasma fraction) we concluded that the plasma pool is only 9\% of the Q\textsubscript{1} Phe pool (healthy: 9.0±2.0\%; sepsis 9.4±2.5\%). Assuming that the extracellular concentration is 20\% higher in healthy subjects then the arterial plasma concentration (29), we determined that the extra vascular Phe pool is about
58% of the Q₁ Phe pool (healthy: 53±4; sepsis 61±8, weight gain by resuscitation included). Although we assume that Q₁ is the extracellular Phe compartment, these calculated pools combined represents only 70% of Q₁ Phe pool. Q₂ is the space where Phe is entering in a post-absorptive state from PB and can be used directly for protein synthesis (=recycling) or hydroxylation that takes place intracellular. If we compare the Q₂ Phe pool with the intracellular pool (calculated with an average Phe concentration in tissue, data in Figure 6), the intracellular pool represents 61% of the Q₂ pool (healthy: 66±4%; sepsis 58±3%). At the moment, we do not have a good explanation for these discrepancies (1, 23, 28). It could well be that the above general assumptions (not observations) for the physiological pools are not realistic enough to compare it with the numeric Q₁ and Q₂ pools in animals that are in resuscitated physiological condition. It could well be that the numeric fast exchanging Q₁ Phe pool is partly also intracellular and the slow exchanging Q₂ pool is partly also located extracellular. So the numerical Q₁ and Q₂ pools are likely not strict physiological separated. Further research is needed to determine the exact translation of Q₁ and Q₂ pools to physiological pools.

**WbRₐ PULSE in relation to WbRₐ PC**

In the present study, we measured WbRₐ of Phe as an index/proxy for WbPB. We showed that both WbRₐ-PULSE and PC are related to total intracellular rate of appearance of Phe from PB in the healthy and disease state. However, the absolute value of WbRₐ was 1.6 times lower with the PC method.

For both methods, sampling was done in the same arterial plasma pool and TTR was analyzed in the same blood sample on the LC-MS/MS and calibrated with enriched standard curves. Although, the exact amount of the tracer administered in the PC model, is more error sensitive (e.g. prime is estimated, performance infusion pump, temporary pump errors) than with the PULSE model (single dose with syringe), we think it is unlikely that this can explain the discrepancy in WbRₐ between the models.

Rakotoambinina et al (9) also compared the PULSE approach with the PC for the non-essential amino acid taurine and found also 1.8 times higher WbRₐ with the PULSE approach. One of their explanations was that the area under the curve (AUC) under the decay curve could not well be determined because of the detection level of the used GC-MS. The AUC is related to the final absolute WbRₐ. In the present study we described the decay curve with 6 time points in a one-hour time window. The reliability of the curves was very high due to accurate LC-MS/MS measurements (CV% <2%). In practice, the calculation of non-compartmental WbRₐ with the PULSE method is strongly influenced by the tail of the decay curve because it can influence the area under the curve substantially. The calculation of the pool size Q₁ is strongly influenced by the enrichment close to zero. Q₁ is used to calculate the compartmental WbRₐ. Since the non-compartmental and compartmental PULSE
approaches give comparable WbR$_a$’s, we are able to conclude that although the decay curves were fitted with only six sample points, these samples were collected on the right time and provided valid data. Therefore, in the present study a potential inaccuracy of the decay curve fitting cannot explain the WbR$_a$ difference between the PULSE and PC method.

The major difference between the PC and PULSE approach is the level of simplicity that is chosen to describe PB by determining WbR$_a$ of Phe. The WbR$_a$-PC provides an underestimation of the intracellular appearance (1, 30). This underestimation is related to the fact that the WbR$_a$-PC is greatly limited by the necessary assumption that production appears in the sample compartment (i.e. plasma) in which the tracer is also administered. Physiologically only a few substrates appear directly in plasma, such as glucose and urea. In post-absorptive state that we studied, the essential amino acid Phe appears intracellularly. The WbR$_a$-PULSE will better represent the dilution both in plasma as well as in tissue. This is related to the fact that the decay curve of the PULSE method has two exponentials that in the compartmental analysis represents most likely the extracellular and intracellular pools. However when we measure changes in PB both WbR$_a$ methods will identify these changes, albeit at different turnover rates. We therefore conclude that WbR$_a$-PULSE better reflects the actual absolute PB rate. In case the total amount of PB is of interest in a scientific question, the compartmental PB analysis with the PULSE method will be the only option that is appropriate, because WbR$_a$-PC will not account for the intracellular irreversible loss.

Protein kinetic changes in severe sepsis animals obtained with PULSE method

WbR$_a$ of Phe is related to WbPB, and is considered as important information to compare different physiological conditions. In the present study, both PC as PULSE WbR$_a$ provide us the same information: WbPB is enhanced in sepsis. The PULSE method provides us more additional information. For instance, how Phe compartments relate to each other (1, 7, 13), how WbR$_a$ relates to WbPB (ratio). A fraction of the difference of those two is the total amount of Phe that is directly reused (= recycling) for protein synthesis. The other fraction is hydroxylated to tyrosine. An extra tyrosine pulse tracer and the enrichment of the tyrosine metabolite, coming from the chosen Phe-tracer- PULSE is needed to determine the exact fraction of hydroxylation and subsequently the fraction of Phe recycling and whole body protein synthesis. In this case, it is not preferred to use a $^{15}$N-Phe tracer due to potential (but low) interference of transamination (25, 26).

Also with the PULSE method we could determine that the change in WbR$_a$ in the current sepsis model, is caused by differences in Phe intracellular appearance and not the membrane transport capacity between the pools. We found that membrane
transport of Phe in severe sepsis is not compromised, but that only WbPB in sepsis is increased (18, 31) and supports the notion that PULSE isotope methods provide us with additional physiologically important information about membrane transport.

**Conclusion**

The increased WbRa found by both the PC method and PULSE method reflects an enhanced WbPB in sepsis. In contrast to the PC method, the easy-to-use PULSE method provides additional information about intra- and extracellular fluxes, cell membrane transport capacity and WbPB. WbRa-PULSE therefore better reflects the actual absolute PB. Additionally, the non-compartmental PULSE data analysis can be very practical in routine measurements and circumvent the problems of pool priming seen with PC methods but is a little less sensitive than the compartmental PULSE data analysis. In severe sepsis, on whole body level, membrane transport capacity of amino acids is not compromised and is therefore not a rate limited factor for protein synthesis.

**References**


28. Carson ERC, C.; Finkelstein, L. The mathematical modeling of metabolic and


Supplemental Information

Supplemental Figure 1. Individual decay curves of Healthy Animals (PULSE)
Plasma tracer/tracee (TTR) time courses of arterial Phe in healthy animals. PULSE: A two-exponential decay curve after a bolus infusion tracer with L-[15N]-Phe.
Supplemental Figure 2. Individual decay curves of Sepsis Animals (PULSE)
Plasma tracer/tracer (TTR) time courses of arterial Phe in sepsis animals. PULSE: A two-
exponential decay curve after a bolus infusion tracer with L-[15N]-Phe.

Supplemental Table 1: Phenylalanine (Phe) concentration in tissues

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<th>Healthy</th>
<th>Sepsis</th>
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<td></td>
<td>114 ± 7.2</td>
<td>161 ± 10.8</td>
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Average tissue Phe concentration from muscle, jejunum, ileum, liver, lung in µmol/kg wet weight
SECTION 3

Interorgan balance studies in disease models
Arginine de novo and nitric oxide production in disease states

Yvette C. Luiking, Gabriella A.M. Ten Have, Robert R. Wolfe, Nicolaas E.P. Deutz


Abstract

Arginine is derived from dietary protein intake, body protein breakdown or endogenous de novo arginine production. The latter may be linked to the availability of citrulline, which is the immediate precursor of arginine and limiting factor for de novo arginine production. Arginine metabolism is highly compartmentalized due to the expression of the enzymes involved in arginine metabolism in various organs. A small fraction of arginine enters the NO synthase (NOS) pathway. Tetrahydrobiopterin (BH4) is an essential and rate-limiting cofactor for the production of NO. Depletion of BH4 in oxidative stressed endothelial cells can result in so-called NOS3 ‘uncoupling’, resulting in production of superoxide instead of NO. Moreover, distribution of arginine between intracellular transporters and arginine-converting enzymes, as well as between the arginine-converting and arginine-synthesizing enzymes, determines the metabolic fate of arginine. Alternatively, NO can be derived from conversion of nitrite.

Reduced arginine availability stemming from reduced de novo production and elevated arginase activity have been reported in various conditions of acute and chronic stress, which are often characterized by increased NOS2 and reduced NOS3 activity. Cardiovascular and pulmonary disorders such as atherosclerosis, diabetes, hypercholesterolemia, ischemic heart disease and hypertension are characterized by NOS3 uncoupling. Therapeutic applications to influence (de novo) arginine and NO metabolism aim at increasing substrate availability or at influencing the metabolic fate of specific pathways related to NO bioavailability and prevention of NOS3 uncoupling. These include supplementation of arginine or citrulline, provision of NO donors including inhaled NO and nitrite (sources), NOS3 modulating agents, or the targeting of endogenous NOS inhibitors like asymmetric dimethylarginine.
Introduction

The purpose of this review is to give an overview of and highlight recent developments on arginine metabolism and nitric oxide (NO) production in health and disease. The endogenous de novo production of arginine will be a special focus, and alterations in this pathway in disease and its relevance will be discussed. Finally, therapeutic applications to influence (de novo) arginine and NO metabolism will be summarized.

Physiology of arginine, and arginine de novo and NO metabolism

Arginine is a conditionally essential amino acid in healthy adults and therefore there is no specific nutritional requirement (1). However, dietary arginine is required in neonates, infants and in certain conditions and diseases (2). In the 1980s, L-arginine was identified as the precursor for NO (3). While first discovered in endothelial cells (4), NO appeared to be an ubiquitous molecule present in a variety of cells, including cells from the cardiovascular and nervous system and also inflammatory cells. As such, NO has many physiological functions, and the relationship between arginine availability and NO production emphasizes the functional relevance of arginine.

Metabolic pathways

Arginine is derived from dietary intake, body protein breakdown or endogenous de novo arginine production (Figure 1). In the post-absorptive state, whole body arginine flux in healthy adults is about 70-90 µmol/kg.h, which equals 15-20 g/day (5, 6) for review), while daily dietary arginine intake is about 4-6 grams (8, 9). De novo arginine production, which contributes about 10-15% to whole body arginine production under normal conditions (6, 10), involves the conversion of citrulline to arginine and is catalyzed by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (11, 12). This conversion is part of the so-called intestinal-renal axis, with intestinal production of citrulline and renal synthesis of arginine (13-17). Citrulline availability is a limiting factor in this conversion (12).

Arginine is a constituent for body protein synthesis, 80% of which is derived from recycling amino acids originated from protein breakdown. Moreover, arginine plays a key role in several other metabolic pathways catalyzed by various enzyme systems (see Refs (2, 18, 19) for recent reviews) (Figure 2). Arginine metabolism is highly compartmentalized within the body since the enzymes involved in arginine metabolism are expressed in various organs, although to a different extent. The only exceptional organ is the liver, which contains the complete urea cycle and its related enzymes. However in healthy conditions, arginine produced in the liver urea cycle is not exchanged to plasma (20). Due to compartmentalization, arginine metabolism and recycling is only partly in balance with plasma arginine concentration. This so-called ‘arginine paradox’ explains that acute exogenous arginine provision
Arginine flux as measured with an intravenously-infused stable isotope and subsequent dilution of this isotope in the plasma compartment reflects the whole body appearance of arginine in plasma. This plasma arginine flux does not account for hidden compartments (such as liver cells) in which arginine is produced without first being released into plasma. Of the plasma arginine flux, 15% enters the (extrahepatic) arginase pathway (6) that degrades arginine to ornithine and urea.

There are two isoforms of the enzyme arginase. Type I (cytosolic) arginase is predominantly expressed in the liver, as part of the urea cycle, but was also demonstrated at lower levels in various extrahepatic organs in rodents with a main role in production of ornithine for polyamines biosynthesis (23). Type II (mitochondrial) arginase is expressed in low levels in extrahepatic tissues and cells (such as brain, kidney, small intestine, red blood cells, and immune cells) and is mainly involved in the synthesis of ornithine, proline and glutamate (24, 25). Based on the variability of
arginase I and II among organs in rodents, organ-specific roles of arginase isoforms have been suggested (26). Under normal conditions, about 40% of dietary arginine is extracted in the splanchnic area (27), which is likely due to the relatively high arginase activity in the intestinal mucosa. Arginine is a substrate for citrulline synthesis in the intestine through conversion by arginase II and ornithine transcarbamylase (OTC) metabolic pathways with interorgan exchange of ornithine (28, 29).

Arginine is substrate for creatine synthesis, which also requires glycine and methionine. Creatine synthesis consumes some 20-30% of arginine’s amidino groups, whether provided in the diet or synthesized within the body, and therefore imposes an appreciable burden on the metabolism of arginine. Creatine is excreted from the body as urinary creatinine. This is a non-enzymatic and unregulated breakdown process, that occurs at a rate of approximately 1.7% of total body creatine and creatine phosphate per day ((30) for review).

About 1.5% of arginine flux enters the NO synthase (NOS) pathway (6) that converts arginine to NO and citrulline by either of three isoforms of the NOS enzyme (31, 32). NOS1 (neuronal NOS) and NOS3 (endothelial NOS) are constitutive enzymes that are controlled by intracellular Ca$^{2+}$/calmodulin. NOS2 is inducible at the level of gene transcription, Ca$^{2+}$ independent, and expressed by macrophages and other tissues in response to (pro)-inflammatory mediators. A mitochondrial NOS isoform (mtNOS) for production of NO in mitochondria has been proposed but several studies have challenged the existence of a mitochondrial isoform (33). Several
co-factors are known for NOS of which tetrahydrobiopterin (BH4) is essential and rate-limiting, and is synthesized from guanosine triphosphate (GTP) via the GTP-cyclohydrolase-I (GTP-CH) pathway (34) for recent review. Other known cofactors are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and heme (reviewed by (35)). Finally, arginine can be decarboxylated to agmatine, which acts as a cell-signaling molecule (18).

NO can also be derived from conversion of nitrite. The conversion of nitrite to NO can occur via simple non-enzymatic nitrite reduction under acidic conditions \((e^- + 2H^+ + NO_2^- \rightarrow NO^- + H_2O)\), resulting in NO production in the stomach (36). This NOS-independent NO production is controlled by oxygen tension, pH, reducing substrates and nitrite levels (37). Production of NO from nitrite was first observed in heart tissue under conditions of ischemia with intracellular acidosis (38) and occurs primarily in tissues and not in blood (39). The level of nitrite-derived NO under ischemic conditions with acidosis is comparable to maximum constitutive NOS production, which makes this NOS-independent route a practical alternative pathway under ischemic conditions where NO production from NOS is compromised (37). Dietary nitrate, mainly from vegetables, is reduced to bioactive NO\(_2^\) by facultative anaerobic bacteria in the saliva, and as such can serve as NO source. Arginine metabolic pathways and enzymes are summarized in Figure 3.

![Figure 3. Arginine metabolic pathways in healthy humans](image-url)

ARG, arginine; ORN, ornithine; PRO, proline; GLU, glutamate; CIT, citrulline; ASP, aspartate; GLN, glutamine; NO, nitric oxide; NOS, nitric oxide synthase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; ADMA, asymmetric dimethylarginine; L-NMMA, NG-methyl-L-arginine; BH4, tetrahydrobiopterin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.
Role of arginine and NO in normal physiology

Arginine is a constituent of body proteins and is an intermediate in the urea cycle in the liver. The urea cycle is a series of five reactions in which urea synthesis is the final step in the detoxification of ammonia. Moreover, the urea cycle has been considered as a major pathway for the removal of metabolically generated bicarbonate, and as such a role for the liver in pH homeostasis (40). Other roles of arginine are related to arginine-derived metabolites. These include, among others that we will not further specify here, ornithine and derived polyamines (putrescine, spermine, and spermidine), which are important for cell growth and differentiation. Proline, which is hydroxylated to hydroxyproline post-translationally, can also be derived from arginine and has a role in collagen formation, tissue repair and wound healing. Creatine is also derived from arginine and plays a role in energy metabolism in muscle and neurons (see Ref. (20) for review). Apart from actions via its metabolites, arginine directly activates p70 S6 kinase and phosphorylation of 4E-BP1 through the mTOR signaling pathway (41) with stimulation of protein synthesis in a NO-independent way (42). NO has various roles in normal physiology, but we will not review all roles in full detail. NO derived from NOS1 and NOS3 acts as a neurotransmitter and a vasodilator, respectively (32). NO in the brain regulates many physiological processes affecting behavior and cognitive function, including synaptic plasticity. In addition, NO controls brain blood flow, promotes angiogenesis, maintains cellular redox state, cell immunity and neuronal survival. However, despite the many diverse roles of NO, regulation of the amount produced is important, as over-production of NO may lead to neurodegeneration (43). NO is synthesized at high levels by NOS2 when activated during inflammatory processes by elevated circulating cytokine concentrations (mainly TNF-α, and IL-1, IL-6, and IL-8) and/or microbial products like LPS (31, 32, 44, 45). This NO has immune regulatory functions, such as control or killing of infectious pathogens, modulation of cytokine production and T-helper cell development. Moreover, NO can act as a free radical scavenger (46). Local NO responses are concentration and exposure time dependent (47). In general at low concentrations, NO promotes cell survival and proliferation and at high concentrations promotes cell cycle arrest, apoptosis and senescence. As such, arginine has an indirect role in NO-mediated functions, including immune modulation (48, 49) or acts immune response-enhancing during immunological challenge (50).

Factors that mediate arginine and NO metabolism and availability

The level of dietary arginine intake and endogenous production on the one hand, and the extent of utilization or clearance on the other hand influence arginine metabolism and availability (Figure 4).
Systemic and intracellular arginine availability

Transporters for arginine uptake in the cell are often co-localized with arginine-converting enzymes and as such can modulate cellular arginine metabolism (20). For example, cationic amino acid transporter (CAT)-1 and NOS3 are co-localized in plasma membrane caveolae (51). This facilitates specific channelling of arginine to endothelial NO production without mixing with the total intracellular pool. This is in line with observations in vitro of extracellular rather than intracellular arginine being the major determinant for NO production in endothelial cells (22). Another example of relation between arginine metabolism and its transporter is the up-regulation by inflammatory cytokines of CAT-2 and down-regulation of CAT-1 arginine transporters (52, 53), that results in increased availability of arginine to NOS2 and decreased availability to NOS3. Competition with lysine, ornithine, glutamine and certain endogenous NOS-inhibitors that use the same transporter as arginine may compromise intracellular arginine transport in conditions of low arginine (54).

In addition to the link between arginine transport and intracellular arginine availability, the coupling between arginine-synthesizing and converting enzymes or the competition between enzymes for arginine as a substrate determine its metabolic fate. For example, coupling between de novo arginine synthesis and NO production is supported by co-localization in endothelial cells of NOS3 and ASS/ASL (21). On the other hand, substrate competition between arginase and NOS reciprocally regulates NO levels in endothelial cells (20, 55, 56).

De novo arginine production from citrulline can be impaired by renal failure (57-59), but citrulline delivery to the kidney is the rate limiting determinant of renal arginine production (12, 59). Impaired intestinal function is a major underlying reason for reduced citrulline availability (see Ref. (60) for review)(61), supported by the observations that 80 to 90% of the citrulline is derived from conversion of glutamine to citrulline in the intestine (16, 62-65).

Dietary arginine availability influences its own catabolism and that of other amino acids by controlling ureagenesis. Arginine is not only a substrate for ureagenesis, but also an activator of N-acetyl glutamate synthetase, which is a key ureagenic enzyme (66). On a low-arginine diet, arginine catabolism (i.e. arginine hydroxylation with conversion to ornithine) is reduced with maintained de novo arginine production and reduced plasma arginine (67-69). This also applies when a low protein (nitrogen) diet is fed and amino acids are more efficiently used for other processes than oxidation, and subsequently less ammonia is present for detoxification in the liver urea cycle. However, a concept presented by the Cynober group states that, under the condition of low dietary protein intake, the intestinal conversion of arginine into citrulline by intestinal arginase and OTC is activated, . The newly formed citrulline then bypasses liver metabolism and is converted back to arginine in the
<table>
<thead>
<tr>
<th>Mediation system</th>
<th>Examples</th>
<th>Effect in Healthy state</th>
<th>Reference</th>
<th>Pathological condition</th>
<th>Name</th>
<th>(potential) effect</th>
<th>Reference</th>
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<tr>
<td>Co-localization</td>
<td>ARG transporter + NOS: CAT-1 + NOS3 CAT-2 + NOS2</td>
<td>local ARG availability ↑</td>
<td>(20, 51)</td>
<td>Inflammation</td>
<td>CAT-2 CAT-1</td>
<td>(52, 53)</td>
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<td></td>
<td>ARG de novo synthesis + NOS: ASS/ASL + NOS3</td>
<td>local ARG availability ↑</td>
<td>(21)</td>
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<td>Substrate</td>
<td>ARG transporter</td>
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<td>(54, 72, 73)</td>
<td>ARG deficiency</td>
<td>LYS, ORN, GLN, endogenous NOS inhibitors</td>
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<td>(54)</td>
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<td>competition</td>
<td>NOS</td>
<td>Endogenous NOS inhibitors (ADMA, L-NMMA)</td>
<td>(72)</td>
<td>Impaired liver function</td>
<td>Endogenous NOS inhibitor (ADMA)</td>
<td>ARG</td>
<td>(75, 76)</td>
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<td>Protein catabolism, impaired renal function</td>
<td>Endogenous NOS inhibitors (methylarginine)</td>
<td>ARG</td>
<td>(75, 76)</td>
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<td>de novo ARG</td>
<td>Impaired intestinal function</td>
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<td>de novo CIT</td>
<td>Dietary ARG, ARG catabolism</td>
<td>de novo CIT</td>
<td>(60)</td>
<td>de novo CIT ↓ → de novo ARG ↓ (61)</td>
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<td>Co-factor availability</td>
<td>BH4</td>
<td>BH4 and other co-factors (FAD, FMN, heme)</td>
<td>NO synthesis</td>
<td>(35)</td>
<td>BH4/BH2 ↓ → NO synthesis ↓ + super oxide ↑ (=NOS3 uncoupling) (34)</td>
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<td>Vit C, folate, other antioxidants</td>
<td>BH4 ↑</td>
<td>(78)</td>
<td>Oxidative stress</td>
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**Figure 4. Mediators of ARG-NO metabolism**

ARG, arginine; NOS, nitric oxide synthase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; CAT, cationic amino acid transporter; LYS, lysine; ORN, ornithine; GLN, glutamine; ADMA, asymmetric dimethylarginine; L-NMMA, NG-methyl-L-arginine; , in balance; , unbalanced; BH4, tetrahydrobiopterin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.
kidney (de novo arginine production), while at the same time ureagenesis is limited and arginine and other amino acids are spared for protein synthesis (70, 71).

**Endogenous NOS inhibitors and enzymatic cofactors.**
Methylarginines, such as a-symmetric dimethylarginine (ADMA) and N^G^-methyl-L-arginine (L-NMMA), are the most powerful endogenous and competitive non-specific NOS inhibitors that compete with L-arginine for the active site of NOS and for intracellular transport ((72) for review)(73). These methylarginines are derived from the catabolism of post-translational modified proteins that contain methylated arginine residues. Methylarginines are eliminated from the body by a combination of renal excretion and metabolism through enzymatic degradation by dimethylaminohydrolase (DDAH) to citrulline and methylamines (74). Increased protein catabolism and impaired renal function can thus contribute to elevated levels of methylarginine. High hepatic expression of DDAH and uptake of ADMA makes the liver important in the metabolism of ADMA, and hepatic dysfunction a prominent determinant of ADMA concentration (75, 76). Reference values (2.5th-97.5th percentile) for the L-arginine/ADMA ratio are between 74.3 and 225 (77).

Enzymatic cofactors for NO production, such as tetrahydrobiopterin (BH4), can be affected by several factors and can subsequently influence NO production and endothelial function. Factors that regulate BH4 activity are nutritional, therapeutic, and endothelium-derived factors. Vitamin C, folate, and other antioxidants enhance endothelial BH4 bioavailability through chemical stabilization or scavenging of reactive oxygen species (78). Depletion of BH4 by oxidation into 7,8-dihydrobiopterin (BH2) in oxidative stressed endothelial cells can result in so-called NOS3 'uncoupling', with production of superoxide instead of NO. It is now believed that the intracellular BH4 to BH2 ratio, rather than absolute concentrations of BH4, is the key determinant of this NOS3 uncoupling (34).

**Metabolic alterations related to age, sex, animal species and strain differences**
In neonates, de novo arginine production via the interorgan intestinal-renal axis is not yet developed (2), but conversion of citrulline to arginine occurs in the intestine. The precursor of intestinal citrulline in this condition is proline. In addition, the absence of arginase in the placenta of the mother and in the neonatal intestines suggests a metabolic strategy to maximize the availability of arginine in the systemic circulation from mother to fetus and from maternal milk to neonate. In addition, polyamines and NO are essential for growth and angiogenesis. Therefore, limited de novo arginine production capacity makes arginine an essential amino acid in early life. In neonatal piglets, dietary arginine is conserved in times of deficiency by decreasing arginine hydroxylation and increasing recycling (79). Also in a wide range of livestock reproduction processes, non-optimum availability of arginine results in suppression or
inhibition of arginine metabolism (2). NO bioavailability diminishes with aging and as such adds to the pathogenesis of disturbances in endothelium-dependent vasodilation related to aging (80). This diminished NO bioavailability has been related to a decrease in BH4 and uncoupling of NOS3 with increased oxidative stress in aging (81, 82), but also to increased arginase activity with subsequently reduced arginine availability for NOS ((83) for review). In the brain, the excitatory glutamate-NO-cGMP neurotransmission is normally in balance with the inhibitory GABA neurotransmission. In rats, an age-related regional imbalance of the glutamate/GABA balance was observed, caused by decreased glutamate levels. This is correlated with changes in levels of L-arginine and its metabolites in many brain regions (84).

In mice, differences in arginine metabolism are described between sex and strains, such as between C57BL6/J and FVB mice, as well as between B6 and ICR mice (85, 86). In female mice, plasma arginine was reported to be higher, and strain differences with regard to whole body de novo arginine production and portal-drained visceral (PDV) arginine metabolism were found (85).

Alterations of arginine and NO metabolism in disease

Arginine metabolism is altered in disease states both with regard to its synthesis and catabolism. This can result in a disruption of the normal homeostasis between metabolic pathways and the fasted blood arginine level. Tang et al (87) proposed the global arginine bioavailability ratio (GABR), defined as plasma arginine divided by the sum of ornithine and citrulline, to account for arginine catabolic metabolites. This ratio was derived as a better index of reduced NO synthetic capacity than systemic arginine levels alone. Impaired intestinal absorption (88), impaired organ function such as intestinal dysfunction (89) or renal dysfunction (90), enzyme competition and impaired cellular uptake may further compromise (de novo) arginine metabolism and (cellular) arginine availability. Subsequent functional consequences of altered arginine metabolism, such as altered endothelial function with hemodynamic changes on systemic (especially hypertension) and (micro) circulatory level, as well as immune alterations, are well known.

Metabolic alterations of arginine, arginine de novo and NO under stress conditions

Normal fasted plasma arginine ranges between 80-100 µmol/L (162) while recent reference values (2.5th-97.5th percentiles) from relatively healthy subjects from the Framingham Offspring Cohort were reported to be lower between 41-114 µmol/L (77). This could well be related to the fact that in this study, the special treatment of plasma samples for arginine (immediately cooling and cooled spinning down
arginine and no metabolism

170

within 30 min) was not done (91). When taking care of proper treatment of blood samples, under stressed conditions plasma arginine is decreased (92), to levels as low as 50 µmol/L in patients with sepsis (93-95). The intestinal-renal pathway resulting in de novo arginine synthesis from citrulline is impaired in sepsis (95), which may be the results of limited citrulline availability due to intestinal failure (95, 96) and impaired glutamine to citrulline conversion (97), or limited arginine production due to renal failure (12, 98). Higher level proteolysis can mask the decline in de novo synthesis of arginine, and as a consequence total arginine availability may be maintained (95, 99).

Increased utilization of arginine for the synthesis of proteins associated with the stress response, such as the acute phase proteins, will reduce arginine availability in these conditions and may result in alterations in (isoform-specific) NOS enzyme activity. Increased NOS2 activity during sepsis coincides with the down-regulation of the activity of other NOS isoforms (100-102). This reduces NO production enzyme-specifically in conditions of overall NO production being either lowered or not different from healthy controls (95, 103). Increased plasma arginine clearance (103) due to enhanced arginase activity also reduces arginine availability for other catabolic pathways. Moreover, increased arginine oxidation is observed during sepsis in paediatric patients (99). The nutritional status also affects the metabolic response to endotoxemia, as demonstrated in a pig model of sepsis. A well-nourished condition before prolonged endotoxemia in this model resulted in a better ability to adapt to endotoxin-induced metabolic deterioration of arginine-NO metabolism compared with reduced caloric intake before endotoxemia (104).

Acute conditions, such as trauma, sepsis and acute liver failure

In sepsis, a reduced plasma arginine concentration was related to worse survival (93). Increased mortality in critically ill patients has also been related to elevated ADMA levels, which by substrate competition may be a causative factor in the development of multiple organ dysfunction (105). Sepsis, in particular septic shock, is characterized by elevated cardiac output and hypotension caused by vasodilatation that are associated with misdistribution of blood flow and low peripheral vascular resistance. These characteristics of sepsis have been attributed to an increased NO production by NOS2 (44, 106). However, simultaneously decreased NOS3 expression may be related to microcirculatory shutdown and shunting, contributing to the reduced microvascular blood flow and impaired tissue oxygenation (107-111). Others suggested that an elevated NO production in critically ill patients impairs substrate and oxygen utilization by enhanced protein nitrosylation and inhibition of mitochondrial respiration (112, 113).

Increased NO production is likely also responsible for the hyperdynamic circulation found in patients with liver cirrhosis, and may be an important mediator of
the exaggerated circulatory abnormalities during acute systemic inflammation in so-called acute-on-chronic liver failure with increased cerebral blood flow (114). Up-regulation of the NOS2 pathway in the endothelium with simultaneous down-regulation of NOS3 mediated NO production, was observed in critically ill cirrhotic patients after transjugular intrahepatic stent-shunt (TIPPS) placement through exacerbation of existing endotoxemia (115). Recently, in a pig model with early phase of acute liver failure, arginine deficiency and increased ADMA did not limit whole body NO production. Arginine deficiency was caused by arginase-related arginine clearance. The stimulated intestinal-renal axis was insufficient to compensate the arginine deficiency (116).

Chronic conditions, such as obesity, diabetes and cardiovascular diseases

Abnormalities in NO production and transport in vascular systems result in various cardiovascular pathologies including hypertension, atherosclerosis and angiogenesis-associated disorders (for recent review on the role of NO in the vasculature see (117)). Reduced basal, NOS3-mediated, NO synthesis, or action leads to vasoconstriction, elevated blood pressure or thrombus formation. On the other hand, NO overproduction by NOS2 leads to vasodilation, hypotension, vascular leakage, disruption of cell metabolism and atherosclerosis, either directly or indirect via the formation of NO adducts such as peroxynitrite (43). Cardiovascular and pulmonary disorders such as atherosclerosis, diabetes, hypercholesterolaemia, ischemic heart disease and hypertension are characterized by NOS3 uncoupling with formation of superoxide instead of NO ((34, 118) for recent reviews). The lower NO production in these conditions due to a deficiency of BH4 also underlies the impaired action of insulin in the vasculature of obese and diabetic subjects (119). Diet-induced oxidative scavenging of NO and reduced NO bioavailability was also shown to accompany early diet-induced insulin resistance (120). Oxidative stress causing S-glutathionylation of NOS3 in endothelial cells with loss of NO and gain of superoxide is increased in hypertensive vessels (121). By inhibiting NO production, elevated cellular levels of endogenous methylarginines can impair vascular relaxation and are mediators of vascular dysfunction in disease. Elevated ADMA levels have been reported in hypercholesterolemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus and chronic renal failure (73, 122). L-arginine: ADMA ratio was positively associated with the estimated glomerular filtration rate and diastolic blood pressure in a large cohort study (77), and may act as a clinical diagnostic tool for improved cardiovascular risk assessment (122). ADMA was also identified as an independent risk marker for mortality in ambulatory patients with peripheral arterial disease (123).
Neurological diseases

In the brain, arginine as a precursor for NO is necessary for cerebrovascular homeostasis (NOS3) and is involved in learning and memory capacities via the glutamate-NO-cGMP pathway (NOS1). In neurological diseases, one or both of these routes are impaired. Also, high amounts of induced NO production by inflammatory factors (NOS2) contribute to oxygen stress and therefore can play a role in the severity of the diseases. In the onset of brain stroke, cerebrovascular disease and Alzheimer disease, hypoperfusion as underlying cause of oxygen stress is one of the present avenues to understand the initiation of these diseases ([124] for review). A chronic imbalance of NOS in the brain is believed to be a key element.

In hepatic encephalopathy (HE), a neurocognitive disorder in which brain function is impaired and is associated with both acute and chronic liver dysfunction, hyperammonemia plays an important role in the pathophysiology. Alterations in glutamate-NO-cGMP pathway are described, especially in acute HE and in relation with excessive glutamine production (ammonia detoxification by conversion of glutamate into glutamine) in the brain (125). Besides a direct effect on the glutamate neurotransmission cycle, glutamine can also limit the transport of arginine into the neurons and astrocytes, because it competes with the glutamine transport (126). The implications are not clear yet and may differ in different stages of the disease.

Cancer

Humans with cancer have a decreased systemic availability of arginine independent of the type of cancer, age, sex or cachectic state (127). In mice, cancer affects de novo arginine production probably through diminished intestinal citrulline production (128). In addition, high arginase activity is observed via the myeloid suppressor cells in the microenvironment of tumors (129). Other research is focused on tumor growth and the arginine dependence of certain tumors that do not express ASS. Such tumors, such as melanoma and hepatocellular carcinoma are sensitive to arginine depletion by arginine degrading enzymes such as arginine deiminase (see Ref. (130) for review) or a recombinant form of human arginase I (131). A disturbed arginine metabolism could be a factor that is causing relative poor clinical outcome. NO is not only required for an adequate immune reaction during a surgical trauma after a tumor extraction, but also contributes to cytotoxic induced antitumor processes (132, 133).

Therapeutic approaches to influence arginine and NO metabolism

Reduced arginine intake in disease or malnutrition as well as increased metabolic needs can result in arginine deficiency or its increased requirement. Therapeutic
Arginine supplementation can aim at increasing substrate availability by supplementation, or at influencing the metabolic fate of specific pathways related to NO bioavailability and prevention of NOS3 uncoupling.

**Arginine supplementation**

Arginine supplementation varying between 3g/day up to over 100g/day has been used in clinical studies. Single doses of 3-8g appear to be safe and rarely provoke adverse events (134), but single doses exceeding 9g and especially when part of a dosing regimen of over 30g/day have been associated with gastrointestinal discomfort, nausea and (osmotic) diarrhea (135). Arginine has been used in supplemental nutrition for surgical patients, burn patients, and patients with sepsis and cancer to benefit regulation of blood pressure, wound healing, immunomodulation, or to serve as an anabolic stimulus. However, the benefits of arginine in these conditions are not uniformly proven and accepted.

Arginine supplementation in sepsis patients has been combined with a mixture of amino acids and other nutrients, referred to as immunonutrition (136-139). Several reviews and opinion papers on its use have been published (140-147), but conclusions regarding the benefits and potential use in sepsis are not uniform. Arginine treatment starting before endotoxemia in a pig model appeared beneficial by improved hepatosplanchnic perfusion and oxygenation during prolonged endotoxemia, without causing deleterious systemic side effects. This is probably mediated through enhanced NO synthesis (148). Arginine supplementation has inconsistent effects on intestinal ischemia-reperfusion injury, and a recent publication with long-term intra-duodenal arginine supplementation in a rat model of intestinal ischemia and reperfusion injury did not show observable benefits on intestinal morphology or on the inflammatory response (149). Remarkably, serum NOx was even decreased with arginine supplementation in this study, which suggests that arginine was probably not available for NO production due to substrate competition for arginine. Supplementing arginine in partial enteral feeding in neonatal pigs modestly increased intestinal mucosal growth through a NO-independent mechanism (150).

Beneficial effects of arginine supplementation were observed in patients with sickle cell disease suffering from pulmonary hypertension, as well as in the prevention of age-related glomerular injury, in reversing impaired vasodilatation in clinically asymptomatic hypercholesterolemic adults, and improving wound healing (151-155). A growing body of evidence indicates that arginine supplementation is beneficial in growth, health and disease and may provide novel and effective therapies for obesity, diabetes and metabolic syndrome (2). Malnourished head and neck cancer patients who received arginine-enriched nutrition peri-operatively demonstrated better 10-year survival (156). Moreover, arginine was proposed as a therapy in hypertension, to interrupt the vicious cycle that initiates and maintains low NO (157).
A recent meta-analysis concluded that short-term oral L-arginine supplementation is effective in improving the fasting vascular endothelial function, i.e. flow-mediated vasodilation (FMD), when the baseline FMD is low and thus endothelial dysfunction can be restored (158). FMD is an early pathophysiological feature of cardiovascular disease and reflects local bioavailability of NO under physiological stimulation. Whether long-term arginine supplementation is beneficial is debated, since exogenous arginine also increases arginase with subsequent diversion of arginine from NOS and subsequent NO production (159).

**Citrulline supplementation**

Citrulline supplementation as a single oral dose of 2, 5, 10 or 15 g is safe and well tolerated in healthy adults with no effect on plasma levels of insulin and growth hormone and urinary excretion of citrulline remaining low (<5%) even at high doses. Citrulline supplementation has proven to be an effective precursor for arginine and ornithine, but saturation of the renal conversion of citrulline into arginine probably occurred at the highest citrulline dose (15g) (160). Citrulline-malate is an alternative citrulline source that is also applied as anti-asthenia treatment and quickly lowers ammonia levels in hyperammonaemia (89).

Citrulline supplementation likely restores the optimal balance between arginine production and metabolism, as well as improving NO production and related functions. In an arginine-deprived *in vitro* model of macrophages, addition of citrulline restored NO production, while glutamine interfered with citrulline-mediated NO production (161). Therefore, in conditions of acute or chronic inflammation with arginine deficiency, citrulline supplementation is a potentially powerful approach to restoring NO production (71). In sickle cell disease, oral citrulline supplementation maintained elevated arginine levels and maintained nearly normal total leukocyte and neutrophil counts, and has therefore been suggested as a useful palliative therapy in this condition (162). Citrulline supplementation ameliorated the development of pulmonary hypertension and increased NO production in piglets exposed to chronic hypoxia (163); this suggests that neonates exposed to prolonged periods of hypoxia from cardiac or pulmonary causes may potentially benefit from citrulline supplementation. In middle-aged men, citrulline supplementation improved arterial stiffness, which is considered a powerful predictor of cardiovascular disease (164). Citrulline supplementation restored nitrogen balance and generated large amounts of arginine in rats with short bowel syndrome (165).

**NO donors and NOS3 modulating agents**

NO can also be derived from so-called NO donors, inhaled NO, and nitrite (sources). NO donors, such as nitroglycerin are well known and used as vasodilators to treat heart conditions such as angina and chronic heart failure. In septic patients, nitroglycerin increased sublingual microvascular flow, even though arterial and central
venous pressure dropped temporarily (166). The use of inhaled NO in the perioperative setting for the treatment of pulmonary hypertension in children is recommend ed (167). Short-term nitrite therapy reversed age-associated vascular endothelial dysfunction, large elastic artery stiffness, oxidative stress, and inflammation in old mice, by restoring NO bioavailability through a NOS-independent conversion. The authors therefore suggested that sodium nitrite may be a novel therapy for treating arterial aging in humans (168). Nitrite is also currently undergoing or planned for clinical trials as a vasodilator drug in patients with cardiovascular diseases such as ischemic stress, sickle cell disease, coronary artery disease, and pulmonary hypertension (37). Nitrate-rich vegetable juice acutely increased nitrite (within 2.5h), and reduced blood pressure as well as oxygen costs of moderate intensity exercise in normotensive subjects. These effects were sustained during continuous juice intake over 15 days (169).

Other novel pharmacological approaches under development to increase NO bioavailability are targeted at preventing NOS3 uncoupling or enhancing NOS3 expression ((34, 118, 170, 171) for recent reviews). Regarding the latter, BH4 or its synthetic versions may be a new therapeutic strategy to tackle myocardial and endothelial dysfunction (172). Other agents or therapies e.g. statins, intravenous ascorbic acid administration or exercise, act on preventing BH4 loss, on improving BH4 availability or on BH4 stability by scavenging superoxide; this functions by improved endothelial NOS coupling and vascular NO bioavailability (82, 171, 173). Local ascorbic acid infusion was demonstrated to improve NO-mediated muscle blood flow during exercise in elderly (174). While BH4 repletion only partly restored NOS activity and NO-dependent vasodilation, reversion of another redox-regulated mechanism controlling NOS function by thiol-specific reducing agents can restore vasodilation when NOS3 S-glutathionylation is increased (121). A pharmacological NOS3 enhancer (AVE3085) ameliorated endothelial dysfunction in db/db mice through increased NO bioavailability, which makes targeting NOS3 and NO a promising approach to combat diabetic vasculopathy (175).

**Targeting endogenous NO inhibitors**

An alternative approach to increase NO bioavailability is via targeting endogenous inhibitors of nitric oxide synthesis such as ADMA or arginase. Pharmacological modification of dimethylarginine dimethylaminohydrolase (DDAH) enzymes that metabolize ADMA (176) or treatment with the arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA) are options (177, 178). Nor-NOHA restored the microvascular coronary artery function in type 2 diabetic rats and caused cardioprotection against myocardial ischemia-reperfusion injury in rats by a mechanism with increased utilization of arginine by NOS and increased NO availability (177, 178). Recently published reference levels for L-arginine:ADMA ratio may be helpful for evaluation of
the effects of L-arginine supplementation in participants with an impaired L-arginine/ 
NO pathway (77).

**Therapies that influence NO-mediated effects**

Patients receiving IL-2 cytokine treatment for advanced malignancy demonstrate 
increase endogenous nitrate synthesis (132), while NOS3 knockout mice were re-
sistant to IL-2 induced hypotension and vascular leak. Methylene blue, by inhibiting 
guanylate cyclase and cGMP, could inhibit this NOS3 mediated vascular leak (179). 
Selective inhibition of p38 mitogen-activated protein kinase (MAPK), a mediator of 
vascular inflammation and activated by oxidized low-density lipoproteins, improved 
NO-mediated vasodilation in patients with hypercholesterolemia (180). The authors 
suggested that p38 MAPK could therefore be a novel target for patients with car-
diovascular disease.

**Summary, conclusion and future research**

While NO production is dependent on arginine availability as its precursor, the odd 
circumstance is that only a small percentage of arginine is used for NO synthe-
sis and that either too much or too little NO is detrimental. This suggests that the 
relation between arginine availability and NO production is not simply a case of 
precursor availability. Rather, it is more likely the combination of the availability of 
arginine, along with cofactors and rate-limiting enzymes that determine the rate of 
production of NO. The compartmentalization of arginine metabolism plays a role 
here, in what is also referred to as the ‘arginine paradox’.

During the past few years, it has become recognized that endothelial NOS uncou-
pling and NOS3 dependent superoxide generation, induced by stress, are key 
mediators in the pathogenesis of cardiovascular and pulmonary diseases. Local 
arginine deficiency, which can be the result of arginine catabolism via arginase or 
competition with methylarginines, results in endothelial NOS uncoupling. Modulating 
NOS uncoupling and targeting NOS3-dependent ROS formation are recent devel-
opments that require further clinical testing. Arginine seems to have a critical and 
dual role here, both as a substrate for NOS and as a radical scavenger. Since argi-
nine released from local protein breakdown may not be available for NOS, coupling 
of the enzymes for de novo arginine and NOS3 could make citrulline a good and 
maybe even better source for NO. The antioxidant action of citrulline could further 
contribute to prevent NOS uncoupling, but this is not yet known. Specific drugs that 
act on increasing local arginine availability for NO production, or those that mediate 
or provide co-factors for NO production are also considered useful.

In conclusion, the complex regulation of NO synthesis and intracellular availability of 
arginine as its precursor probably requires an approach beyond the primary provi-
sion of extra arginine. A multi-target approach addressing substrate competition,
precursor availability and cofactor availability may be useful, and future research could focus on developing such strategies that can optimize NO bioavailability. This can be applied to conditions of compromised or unbalanced NO production, such as those of endothelial dysfunction in various acute and chronic diseases.

Acknowledgement

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ARGININE AND NO METABOLISM

CHAPTER 8


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Nitric oxide and L-arginine metabolism in a devascularized porcine model of acute liver failure


Abstract

In acute liver failure (ALF), the hyperdynamic circulation is believed to be the result of overproduction of nitric oxide (NO) in the splanchnic circulation. However, it has been suggested that arginine concentrations (the substrate for NO) are believed to be decreased, limiting substrate availability for NO production. To characterize the metabolic fate of arginine in early-phase ALF, we systematically assessed its interorgan transport and metabolism and measured the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) in a porcine model of ALF. Female adult pigs (23–30 kg) were randomized to sham (N = 8) or hepatic devascularization ALF (N = 8) procedure for 6 h. We measured plasma arginine, citrulline, ornithine levels; arginase activity, NO, and ADMA. Whole body metabolic rates and interorgan flux measurements were calculated using stable isotope-labeled amino acids. Plasma arginine decreased >85% of the basal level at t = 6 h (p < 0.001), whereas citrulline and ornithine progressively increased in ALF (p < 0.001 and p < 0.001, vs. sham respectively). No difference was found between the groups in the whole body rate of appearance of arginine or NO. However, ALF showed a significant increase in de novo arginine synthesis (p < 0.05). Interorgan data showed citrulline net intestinal production and renal consumption that was related to net renal production of arginine and ornithine. Both plasma arginase activity and plasma ADMA levels significantly increased in ALF (p < 0.001). In this model of early-phase ALF, arginine deficiency or higher ADMA levels do not limit whole body NO production. Arginine deficiency is caused by arginase-related arginine clearance in which arginine production is stimulated de novo.
**Introduction**

Acute liver failure (ALF) is characterized by sudden and severe liver dysfunction with rapid progression to coagulopathy, encephalopathy, and multiorgan failure. Without liver transplantation, mortality from ALF is about 50% (1). A hallmark of ALF is the presentation of systemic hypotension and a hyperdynamic circulation (2). This is associated with increased guanylate cyclase (GC) activation by nitric oxide (NO) (3), which converts guanidine triphosphate to cyclic guanidine monophosphate and is thought to cause the vasodilation seen in ALF (4). Previously, we have reported the characteristics of the same devascularized porcine liver model of ALF, which was shown to have a hyperdynamic circulation as evidenced by a high cardiac output and low mean arterial pressure and systemic vascular resistance (5). However, in this hyperacute model of ALF, no significant changes in the metabolites of NO were observed. Indeed, the kinetics of NO in this model and whether it is involved in the initiation of the vasodilation of ALF remain unknown.

L-Arginine has several important biological functions (6-8), one of which is to be the nitrogen-donating substrate for endothelial NO synthase (eNOS) to produce NO and citrulline in stoichiometric quantities (9). The plasma concentration of arginine is believed to be the rate-limiting factor in its synthesis (10). In ALF, plasma concentrations of L-arginine have been measured, but the results are contradictory with some studies suggesting that the levels remain the same (11) and others showing a reduction (12) or an increase (13, 14).

Other regulators that may influence the amount of NO generated in ALF are the concentrations of the endogenous NOS inhibitor asymmetric dimethyl arginine (ADMA) (13) and levels of plasma arginase activity (15). In a previous study, we have shown that ADMA levels were higher in patients with ALF and that the increase was associated with worse outcome (16). Similarly, an increase in the level of plasma arginase, released during hepatic stresses (17), can reduce the amount of available arginine, preventing eNOS-mediated NO synthesis. Both these observations can relate to a reduced NO production.

It is still unclear as to the exact mechanism of the vascular derangement in the initiation of vasodilation in ALF. Our group has previously described the classical hyperdynamic circulation and reduced systemic vascular resistance, along with hepatic encephalopathy, high intracranial pressure, and coagulopathy in a devascularized porcine model of ALF, which is ideal to study the initiation of metabolic disturbances in relation to hemodynamic changes (18). The aim of the study was to evaluate the evolution of disturbances in NO metabolism in relation to its regulators, L-arginine, ADMA, and arginase, in the first 6 h of ALF using stable isotope technology.
Materials and Methods

Study outline

The Norwegian Experimental Animal Board approved the present study. Sixteen female Landrace pigs (23–30 kg) were randomly allocated into Sham-operated control or ALF groups. Study outline is shown in Figure 1. Blood and urine sampling was performed 30–45 min after creation of the portacaval shunt (PCS) or completion of Sham surgery (t = 0 h). ALF was induced by hepatic artery ligation (t = 0 h) immediately after completion of the sampling procedures. The experiments were terminated with an overdose of pentobarbital sodium and potassium chloride at t = 6 h.

Animal preparation and surgery

The pigs were kept in the animal department for at least 2 days before the experiments. Details regarding the animal room facilities, anesthesia, and surgical preparation have been previously reported (19, 20). Briefly, the pigs underwent a tracheotomy, were intubated, and ventilated on a volume-controlled respirator (Servo 900; Elema-Schnander, Stockholm, Sweden). Ventilation was not altered after t = 0 h. Core body temperature was maintained normothermic at 38.5 ± 1°C with a heating pad and blankets. All animals received 500 ml of 0.9% NaCl containing 625 mg of glucose as a preoperative load to prevent dehydration. During the experiment, 0.9% NaCl was infused at a rate of 3 ml/kg per h. 0.9% NaCl, 50% glucose, and 20% human albumin (Octapharm, Hurdal, Norway) was infused continuously at the rate of

![Figure 1. Study design for the porcine model of acute liver failure](image-url)

PAH, p-Aminohippuric acid; ALF, acute liver failure.
3 ml kg per h, 0.6048 ml/kg per h, and 0.66 ml/kg per h, respectively. Sham animals were given half the amount of glucose to make the glucose levels comparable between the groups. Anesthesia was stopped in the ALF group after the liver was devascularized although, if the degree of sedation became insufficient, small doses of fentanyl and midazolam were given as a bolus. Sham-operated animals received continuous anesthesia during the experimental period and received equal amounts of intravenous fluids. ALF was induced with an end-to-side PCS followed by ligation of the hepatic arteries. Details of the surgery, including the Sham-operation procedure, have been described elsewhere (18, 20).

**Positioning of catheters, flow probes, sampling, and analytical procedures**

Catheters, combining our previously described approach in mice (21) and pigs (22), were inserted in the abdominal aorta, renal vein, portal vein, and femoral vein for arterial and venous across-organ blood sampling. A 16-G central venous catheter (Secalon T; Ohmeda, Swindon, UK) was introduced into the left external jugular vein for administration of drugs and fluids. p-Amminohippuric acid (PAH; 25 mM; A1422; Sigma, St. Louis, MO) was infused at a rate of 30 ml/h through this catheter after an initial bolus of 6 ml (22). Portal and femoral blood flows were measured by the use of perivascular ultrasonic transit time flow probes (CardioMed Systems; Medistim A/S, Oslo, Norway). A 5-Fr Edwards Swan-Ganz catheter (Baxter Healthcare, Irvine, CA) was floated into the pulmonary artery via the right external jugular vein. The urine bladder was drained via a cystotomy. Blood and urine samples were collected on ice at the times for measurement of blood flow and processed as described previously (23). Tissue samples were freeze clamped with Wollenberger tongs cooled in liquid nitrogen (21) and frozen at -80°C. Ammonia, urea, and PAH concentrations were determined spectrophotometrically (23). Amino acids concentrations were determined using HPLC (24).

**The use of stable amino acid isotopes**

A venous blood sample was drawn for the measurement of the natural abundance of plasma amino acids in each subject, before the start of the stable isotope primed-continuous infusion protocol. Stable isotopes were administrated into the left external jugular vein, which included a priming dose followed by continuous infusion of a mixture of L-[guanido-15N2] arginine (N2-arginine prime: 1 mg/kg body wt per h) and L-[ureido-13C; 5,5-2H2] citrulline (C1D2-citrulline, prime: 0.1 mg/kg body wt, infusion: 0.1 mg/kg body wt per h; Cambridge Isotope Laboratories, Woburn, MA) (25). Plasma enrichments of amino acid isotopes were determined by measuring the ratio of the amino acid stable isotope/amino acid (tracer/tracer ratio) with a liquid chromatography mass spectrometry method (26).
Plasma ADMA was measured as described previously (27) using fragmentation-specific stable isotope dilution electrospray mass spectrometry-mass spectrometry. Samples were deproteinized with acetonitrile containing $^2$H$_6$-ADMA, chromatographed (acetonitrile:water, 1:1; with 0.025% formic acid) on a Teicoplanin guard column 10-mm X 2.1 mm inner diameter (Chirobiotic T; ASTEC, Congleton, UK), and analyzed using a SCIEX API4000 (Applied Biosystems, Warrington, UK) in positive-ion multiple-reaction-monitoring mode.

Calculations

Plasma flow rate (ml/kg body wt per min) of the kidneys was calculated using the formulae based on the method of indicator dilution and Fick’s principle (23, 28). The PAH-determined blood flow and data from the perivascular blood flow probes were converted to plasma flow using the hematocrit. Substrate fluxes across organs were calculated as the venous-arterial concentration difference multiplied by the plasma flow. Positive values reflect substrate release, and negative fluxes reflect substrate uptake. Kidney and hind leg data are multiplied by two to reflect both organs. The flux of amino acid across the leg represented flux across a muscle compartment with defined arterial and venous sampling. Plasma arginase activity was assessed by colorimetric assay (17). Whole body rate of appearances of amino acids were calculated by the continuous stable amino acid isotope infusion rate divided by the arterial plasma enrichment of that isotope (29).

Whole body rate of appearances of NO was determined with the flux of plasma L-arginine to L-citrulline-stable isotopes as described previously (25). Whole body rate of appearance of de novo arginine was determined with the flux of plasma L-citrulline to L-arginine-stable isotopes as described previously (30).

Whole body arginine clearance is defined as the amount of arginine that is cleared each minute from arginine and was calculated by whole body rate of appearance of arginine divided by the arterial arginine concentration (31).

Statistics

Data are expressed as means ± SE. Significance of difference between groups was tested with Student’s $t$-test or the Mann-Whitney comparisons test for nonparametric data as appropriate. Continuous data sets were compared using two-way ANOVA; $P < 0.05$ was taken to be statistically significant. Software used included Microsoft Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).
Results

Whole body rate of appearance of NO in ALF

The whole body rate of appearance of NO at the time of PCS or after ALF induction was not significantly different between the two groups. There was no significant change in the whole body rate of appearance of NO between the Sham-operated and ALF groups throughout the experimental period (Figure 2).

L-arginine, citrulline, and glutamine metabolism

Arterial arginine concentration was not significantly different from the basal level after the creation of the PCS and at induction of ALF but was reduced significantly (p < 0.001) at 2 h and continued to decrease until end of the experimental period compared with the Sham group, in which no significant change was observed (Figure 3A). Citrulline concentration was not significantly different between Sham and ALF pigs after creation of PCS; however, the level significantly increased (p < 0.01) after the induction of ALF, and it continued to increase throughout the experimental period (p < 0.001) compared with the Sham group, in which no significant changes were observed (Figure 3B). Glutamine concentration was not different from the Sham-operated group after induction of PCS (Sham 547 ± 45 µM, ALF 623 ± 57 µM at t = 0) but increased significantly after ALF induction (p < 0.05) and continued to increase significantly till the end of the experiment (p < 0.001), as shown and discussed in a previous paper by our group (32).

Interorgan metabolism of L-arginine and citrulline

The effect of PCS and ALF on muscle metabolism is shown in Table 1, which shows the net flux across the muscle. Glutamine was released from the leg muscle throughout the experimental period, increasing non-significantly at 2 h after the induction of ALF, which was not observed in the Sham group (32). There is no significant uptake or release of citrulline or arginine from the muscle.

Figure 2. Whole body rate of appearance of nitric oxide (NO) in Sham-operated and ALF pigs
Data shown are means ± SE.
Table 1. Net flux of L-arginine and citrulline across muscle

<table>
<thead>
<tr>
<th>Time</th>
<th>Sham</th>
<th>ALF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine (μmol/kg bw/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>-0.0072±0.018</td>
<td>-0.0048±0.017</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>0.0702±0.024</td>
<td>0.0361±0.0358</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>0.0721±0.019</td>
<td>-0.0082±0.030</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>0.0432±0.009</td>
<td>0.0033±0.025</td>
</tr>
<tr>
<td>Citrulline (μmol/kg bw/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>-0.0038±0.013</td>
<td>0.0015±0.011</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>0.0166±0.006</td>
<td>0.0037±0.017</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>-0.0118±0.008</td>
<td>0.0163±0.034</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>-0.0106±0.010</td>
<td>-0.0444±0.050</td>
</tr>
</tbody>
</table>

ALF, acute liver failure

Figure 3. Plasma arginine (A), citrulline (B) concentration in Sham-operated and ALF pigs with time (***P < 0.001, **P < 0.01, 2-way ANOVA). Data shown are means ± SE.
The effect of PCS and ALF on portal-derived viscera (PDV) metabolism was determined. Glutamine was taken up by the PDV throughout the experimental period in both the PCS and ALF groups (32). Citrulline was released from the PDV at the point of creation of the PCS, and the release significantly increased in the ALF animals compared with the Sham animals \(p < 0.05\). There were no significant differences in net fluxes of arginine in the PDV (Table 2).

**Table 2. Net flux of L-arginine and citrulline across the PDV**

<table>
<thead>
<tr>
<th>Time</th>
<th>Sham</th>
<th>ALF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arginine (µmol/kg bw/min)</td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>-0.0256±0.135</td>
<td>0.1387±0.061</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>0.1665±0.131</td>
<td>0.3117±0.056</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>0.1440±0.127</td>
<td>0.0941±0.079</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>-0.0444±0.084</td>
<td>0.0491±0.118</td>
</tr>
<tr>
<td></td>
<td>Citrulline (µmol/kg bw/min)</td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>0.1647±0.107</td>
<td>0.3768±0.092</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>0.4195±0.092</td>
<td>0.6975±0.069</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>0.3421±0.068</td>
<td>0.5882±0.094</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>0.2451±0.101</td>
<td>0.8322±0.572*</td>
</tr>
</tbody>
</table>

*\(p < 0.05\) Sham vs. ALF, 2-way ANOVA. PDV, portal-derived viscera.

The effect of PCS and ALF on renal metabolism was also determined. Citrulline was taken up by the kidney after the creation of the PCS, and the uptake increased significantly compared with the Sham-operated group \(p = 0.05\). Arginine was released from the kidney after the induction of ALF, and the release remained significantly higher compared with the Sham animals \(p < 0.0001\) for the remainder of the study period. There was no significant change in the metabolism of glutamine (32) (Table 3).

**Table 3. Net flux of L-arginine and citrulline across the kidneys**

<table>
<thead>
<tr>
<th>Time</th>
<th>Sham</th>
<th>ALF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arginine (µmol/kg bw/min)</td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>0.5448±0.083</td>
<td>0.6855±0.112</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>0.2924±0.123</td>
<td>0.5887±0.101</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>0.3604±0.135</td>
<td>0.5279±0.134</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>0.2439±0.112</td>
<td>0.4351±0.255†</td>
</tr>
<tr>
<td></td>
<td>Citrulline (µmol/kg bw/min)</td>
<td></td>
</tr>
<tr>
<td>T= 0 hrs</td>
<td>-1.246±0.159</td>
<td>-1.1107±0.175</td>
</tr>
<tr>
<td>T= 2 hrs</td>
<td>-0.8352±0.150</td>
<td>-1.3775±0.358</td>
</tr>
<tr>
<td>T= 4 hrs</td>
<td>-1.0228±0.160</td>
<td>-1.4413±0.305</td>
</tr>
<tr>
<td>T= 6 hrs</td>
<td>-0.8241±0.072</td>
<td>-1.4620±0.427*</td>
</tr>
</tbody>
</table>

*) \(p < 0.05\); †) \(p < 0.001\), Sham vs. ALF, 2-way ANOVA.
Figure 4. Whole body rate of appearance of arginine (WbRa, A), de novo arginine (B), and arginine clearance (C) of Sham-operated and ALF pigs with time

*) p < 0.05; ***) p < 0.001; 2-way ANOVA. Data shown are means ± SE.
Whole body rate of appearance of arginine and *de novo* arginine in ALF

The whole body rate of appearance of arginine showed no difference between the Sham and ALF groups at the point of creation of the PCS. There was also no significant difference observed between the Sham and ALF group in the whole body appearance of arginine throughout the experimental period (Figure 4A). The whole body rate of appearance of *de novo* arginine was non-significantly different between the two groups at the point of creation of the PCS. The rate of appearance of arginine increased non-significantly in the ALF group compared with the Sham-operated group through the period of experimentation although it showed a significant increase in production in the ALF group at the end of the experiment compared with the Sham group (p < 0.05, Figure 4B). There was a significant difference in the clearance of arginine between the Sham and the ALF pigs, wherein the

![Figure 5. Plasma ornithine (A) and plasma arginase activity (B) in Sham-operated and ALF pigs throughout the experimental period](image)

There is a significant increase in the plasma ornithine levels with a corresponding significant increase in the plasma arginase activity at the end of the experimental period. ***) p < 0.001; 2-way ANOVA. Data shown are means ± SE.
ALF pigs showed a significantly increased arginine clearance at the end of the experiment compared with the Sham and the levels of clearance remained constant throughout the experimental period (p < 0.001, Figure 4C).

**Plasma arginase activity**

Ornithine concentration was not different between Sham and ALF groups at the creation of PCS, but the arterial concentration of ornithine increased after induction of ALF and continued to increase throughout the duration of the experiment to a significant level (p < 0.001 at 6 h) compared with the Sham-operated animals (Figure 5A) in which no significant changes were observed. The plasma arginase activity was equal in the two groups at the point of PCS and the induction of ALF. The plasma arginase activity increased through the course of the experiment and, at the end of the experiment, was significantly increased (p < 0.001, Figure 5B) in the ALF group compared with the Sham-operated group.

**Plasma arterial ADMA**

The arterial ADMA concentration was not significantly different between the two groups after the creation of the PCS. The ADMA levels remained constant in the Sham group through the course of the experiment. However, after the induction of ALF, the arterial concentrations of ADMA were increased at 2 h and continued to be significantly elevated throughout the remainder of the study period (p < 0.001, Figure 6). The normal values of L-arginine to ADMA ratio is about 100:1 in healthy controls. However, in the setting of ALF, the L-arginine to ADMA ratio was markedly reduced (p < 0.001, Figure 7).

![Figure 6. Plasma asymmetric dimethyl arginine (ADMA) concentration in Sham-operated and ALF pigs with time](image)

There is a significant increase in the arterial ADMA concentration in the ALF pigs compared with the Sham-operated pigs at 2 h and continues to increase significantly until the end of experiment. ***) p < 0.001; 2-way ANOVA. Data shown are means ± SE.
Discussion

The most important finding of this study was the observation that the rate of NO production was not significantly different between animals with ALF and Sham controls, indicating that NO is unlikely to be involved in initiating the vasodilation and hypotension observed in early ALF. These results are unexpected and may in part be explained by substantially reduced plasma arginine levels through the action of arginase. However, we also show that there is an increase in de novo arginine synthesis, resulting in only a relative arginine deficiency, although there appears to be insufficient capacity in this mechanism to restore normal levels. In relation to ADMA, the significant elevation seen in ALF plasma is likely to reflect the impairment of hepatic ADMA metabolism and/or its increased generation. Given the devascularized liver in this model, the significance of higher arterial ADMA values compared with sham are likely to reflect the spillover of an increased capacity to compete with arginine at tissue level, in turn limiting the generation of NO. It is also possible that the site of NO production within the endothelial cells is unaffected by the reduction in plasma arginine. Furthermore, our findings of no significant change in rate of NO production are still compatible with other studies in ALF that have observed changes in activity of guanylate cyclase (3, 4). We and others have shown that the activation of guanylate cyclase can be independent of NO (33, 34) and that guanylate cyclase activation itself may be altered in the context of liver injury.

This experimental model of hyperacute ALF has been well characterized (18). The ability to infuse relevant amino acids with stable isotope labels to measure the
conversion of arginine to citrulline as a surrogate for NO production was employed in this model to evaluate the role of NO in initiating the hemodynamic disturbances, which is not possible to do in a clinical setting, as patients will invariably present late in the course of the condition. These findings lead us to speculate that there may be some other mechanism involved in the pathophysiology of the vascular abnormality seen in the onset of ALF as seen in patients (2) and also this model (5). This result is in contrast to other published articles where increases in the metabolites of NO were described (4). The human studies were performed when ALF was already manifest, and the condition is invariably associated with systemic inflammation. The present study focused on evaluating the role of NO in initiating hemodynamic disturbances and was founded on prior evaluation of this model where no clear evidence could be shown for systemic inflammation. Thus the changes observed can be considered to occur more on a background of metabolic dysfunction, as demonstrated by hyperammonemia, hyperlactatemia, and derangements in glycogenolysis previously described in this model (35).

The sole nitrogen-donating substrate for the synthesis of NO is L-arginine. Arginine is oxidized to NO and citrulline by the enzyme NOS. In the setting of ALF, plasma arginine levels have been found to be lower than those in healthy animals. This finding was described in the early description of this devascularized porcine model of acute liver failure, wherein they also found that the concentrations of citrulline and ornithine were increased (36). In septic patients, low arginine levels (37, 38) have also been observed, which is associated with poor outcome. Arginine clearance was found to progressively increase over the duration of the study (Figure 4C). Although the whole body rate of appearance of arginine was unchanged, the reduction in the plasma levels was significant. This increased clearance rate most likely provided stimulation for the observed de novo synthesis of arginine via the intestinal-renal axis; however, the amount generated during the experimental period was insufficient to restore normal plasma levels. The mechanism of the increased de novo synthesis of L-arginine is revealed from the interorgan experiments. The increased glutamine that was released from the muscle was taken up by the PDV (gut), where it was converted to citrulline. This citrulline was converted stoichiometrically to arginine in the kidney. It is important to acknowledge that there are limitations to this hyperacute model of ALF in that the experimental duration is over 6 h and occurs in a devascularized liver. The findings, while questioning the exclusive role of NO in modulating the hyperdynamic circulation in ALF, do need further testing in other ALF model systems.

A second important observation was the finding of increased plasma levels of ornithine. The increase in ornithine and the simultaneous reduction in arginine indicate that there is an increase in arginase activity, which was found to be markedly increased in the ALF animals. Arginase I is a cytosolic enzyme that forms a part
of the urea cycle and is predominantly present in the liver (39); arginase II is a mitochondrial protein found most abundantly in the kidney (40). Other studies have also found arginase to be present in the plasma following liver injury (41, 42). In this model of hepatic arterial devascularization, the hepatic vein, and hence venous drainage from the liver, remains intact. It is likely that the arginase that was found in the plasma is derived from the breakdown of and leakage from hepatocytes and enters the systemic circulation due to a small amount of residual collateral venous drainage from the liver post-PCS surgery and hepatic artery ligation. Although the major blood supply routes to the liver are removed, there still remains a small but significant contribution from collateral vessels above the section point of the portal vein (43). Although we propose that the majority of the measured arginase activity derives from the liver, the kidney and other vasculature may undertake a substantial role in the production and release of arginase. The mechanism of reduction in arginine may be related to this increase in arginase activity, which is known to regulate the bioavailability of arginine. This inference can be drawn from genetic studies wherein it has been shown that arginase I deficiency leads to hyperargininemia in both humans and mice (44, 45). Similarly, a deficiency of arginase II also leads to a twofold increase in the level of arginine (46). Apart from these mechanisms, the arginase-mediated depletion of arginine may also inhibit the expression of inducible NOS by repressing the translation and stability of the inducible NOS protein (47, 48).

Furthermore, the significantly increased ADMA levels also provide a possible mechanism for an impaired production of NO in this model of ALF. ADMA, an endogenous inhibitor of NOS, is synthesized by the action of protein arginine methyl transferase on arginine residues of nuclear proteins. ADMA is released following proteolysis, and most of the freely circulating ADMA is metabolized by dimethylarginine dimethylaminohydrolase, which occurs mostly in the liver (49) but can also occur in other organs such as the kidney (50). Data from this model study have previously been described and shown to have renal dysfunction and changes in vascular dynamics as part of the developing multiple organ dysfunction syndrome (5). Renal dysfunction, particularly in the context of reno-vascular hypertension, has been described as being associated with higher than normal range values of ADMA (51). Plasma ADMA levels have been shown to be markedly increased in ALF, and its levels correlate with severity of liver failure and the presence of added inflammation and are significantly higher than the levels described in renal disease, which would support the assertion that the ADMA levels in these models are primarily impacted on by hepatic impairment (16, 52). As ADMA is a competitive inhibitor of the NOS-arginine binding site, lowering the arginine to ADMA ratio may directly result in a reduction in NOS activity. In our study, an increase in plasma ADMA level and a reduction of the arginine to ADMA ratio were observed. This suggests that the lack of increase in NO production that was observed in the ALF group may be attributable to the reduction in eNOS activity in the presence of an elevated ADMA and low arginine
plasma levels. This is further compounded by the fact that high arginase also plays its part in reducing the eNOS activity and arginine levels.

In conclusion, this study shows for the first time that, during the initiation of ALF, NO may not play a significant role in mediating the hemodynamic disturbances. This lack of augmentation in the production of NO may be due to a relative reduction in L-arginine concentration despite increased \textit{de novo} production, through the action of arginase and a reduction in its effect on NOS by the relative increase in the levels of its competitive antagonist, ADMA.

References


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Inhibition of jejunal protein synthesis and breakdown in *Pseudomonas aeruginosa* induced severe sepsis pig model

Gabriella A.M. ten Have, Mariëlle P.K.J. Engelen, Robert R. Wolfe, Nicolaas E.P. Deutz

**Abstract**

Maintenance of gut integrity has long been recognized as crucial for survival in sepsis but alterations in protein metabolism have not previously been documented. Therefore in the present study, we measured in a *Pseudomonas aeruginosa* induced porcine sepsis model, fractional protein synthesis (FSR) and breakdown rates (FBR) in jejunal mucosa in a fasted conscious state. We determined FSR by the measurement of the incorporation of stable isotope labeled amino acid (primed continuous infusion, L-[ring-13C6]-phenylalanine) into tissue protein, and FBR using the relation between blood arterial enrichment and intracellular enrichment in consecutive mucosal biopsies after a pulse of L-[15N]-phenylalanine. Additionally, we determined the FSR of other splanchnic tissue (jejunum, ileum, liver) and compared them with muscle and lung tissue.

We found in this sham controlled acute severe sepsis pig model (Sham-healthy (H): N=9; Sepsis (S): N=13) that jejunal mucosal protein turnover is reduced with both decreased FSR (H: 3.29±0.22; S: 2.32±0.12 %/hour, p=0.0008) and FBR (H: 0.72±0.12; S: 0.34±0.04 %/hour; p=0.006). We also found that FSR was unchanged in ileum and muscle, while it was higher in the liver (H: 0.87±0.05; S: 1.05±0.06 %/hour; p=0.041) and tended to be higher in the lung (H: 0.57±0.04; S: 0.73±0.07; p=0.12).

In conclusion, our data, obtained with a highly translational acute severe sepsis model, suggest that jejunal mucosal protein metabolism is diminished in acute severe sepsis. Comparison with other tissues indicates that the most serious acute changes in severe sepsis occur in the gut rather than the muscle. Therefore, we hypothesize that preventing a compromised gut is critical to maintain gut function during sepsis.
Introduction

Sepsis is characterized by severe whole body protein breakdown (PB) and organ dysfunction (1). In addition to enhanced muscle breakdown (14), disturbed protein metabolism in splanchnic tissue could potentially play a role in the response to sepsis. It is known that gut atrophy causes gut dysfunction in sepsis (2, 3). Gut dysfunction is responsible for increased translocation of bacteria from the gut into the bloodstream and therefore contributes to the severity of the septic state. Furthermore, altered gut function could compromise nutrient absorption. Gut mucosa is a tissue with very high protein turnover rates (4, 5). Therefore, gut atrophy could reflect a reduced mucosal protein synthesis and/or increased mucosal PB. In contrast with a potential enhanced PB in the gut, the liver is expected to increase its protein synthesis (PS) due to initiation of immunological acute phase response (6, 7).

In humans, research on protein metabolism in sepsis has mainly focused on the occurrence of severe whole body PB and related muscle PB (8-10), in part because of the difficulty in obtaining gut tissue biopsies. Although preserving muscle is important for survival and quality of life after sepsis, the role of the gut is a critical consideration when developing/improving an appropriate nutritional interventions in septic humans. Therefore, we need to gain knowledge on gut and liver tissue protein metabolism in relation to other tissue protein metabolism in sepsis.

Studying protein synthesis and breakdown rates in the gut and liver in septic humans is very difficult (10-12) (13). It is only possible to measure gut PS and PB by taking mucosal biopsies through a stoma or during an endoscopic procedure and liver PS during diagnostic liver biopsy procedure. Therefore, we and others have developed large animal models in pigs (14, 15) to make this type of research possible. In clinically relevant large animal models of bacteremia induced acute severe sepsis, it is unknown how PS and PB in the gut is affected in an early stage of severe sepsis. We found in arterial-venous organ balance studies that when endotoxins are continuously infused in pigs, jejunal motility is affected (16) in relation to reduced gut protein synthesis (17). Recently, we found mild gut atrophy and disturbed portal drained viscera plasma flow in a well characterized live bacteria (Pseudomonas aeruginosa, PA) induced hyperdynamic severe sepsis catheterized pig model (18).

Therefore in the present study, we have measured jejunal mucosa protein synthesis (FSR) and breakdown rates (FBR) in a pig model of PA. In order to most appropriately simulate the human condition we have performed this study in conscious pigs. We determined protein FSR by the measurement of the incorporation of stable phenylalanine (Phe) tracer into tissue protein over a period of several hours (19) and measured protein FBR using the relation between blood arterial and intracellular enrichment after a pulse of a different stable Phe tracer (19). Additionally, for
comparison we determined the FSR of other splanchnic tissues (jejunum, ileum, liver) as well as muscle and lung tissue obtained during necropsy.

**Materials and Methods**

**Animals**
Twenty two female Yorkshire cross/domestic pigs, average bodyweight of 20-25 kg were used for the present study. The pigs were individually housed in steel pens (2m x 3m) in a controlled housing facility: room temperature 22-24°C, 12 hours light-dark cycle, standardized food 1kg/day (Harlan Teklad Vegetarian Pig/Sow Grower). Water was provided ad libitum. This study was approved by the animal experiment ethics committee (IACUC) of University of Arkansas Medical Sciences (Little Rock, AR, USA).

**Implantation catheters**
We implanted several catheters in blood vessels with a surgical procedure about 10 days for the induction of sepsis as described previously (18) (20, 21) to determine stable tracer enrichment in afferent blood of the tissues (precursor for tissue FSR) and arterial enrichment for jejunal mucosal FBR measurement. We created a jejunostomy for sampling jejunal mucosa biopsies. Preoperative and postoperative care was standardized as described in detail previously (18) (20, 21). During the recovery period (7-10 days) animals were familiarized to a small movable cage (0.9x0.5x0.3m) to ensure that the experiments could be performed in this cage in conscious animals.

**Experimental design**
The experiment (Figure 1) started after a recovery period of 7-10 days. Food was withheld for 6-8 hours before the start of the experiment. Animals were selected for the Sepsis group or the Healthy group in a randomized fashion. At T=0h sepsis was induced by the start of IV continuous infusion of *Pseudomonas aeruginosa* (PA, $10^9$ CFU/ml/hour) and monitored continuously as we described previously (18). The control group received 0.9% NaCl solution in the same volume. A primed continuous infusion of the stable amino acid tracer L-[ring$^{13}$C$_6$]-Phe (prime 1.58 µmol/kg bw; infusion 4.32 µmol/kg bw/hour) was administered *intravenously* in the pre-septic phase (-2 to 0h) and in the septic state (12-18h) via the central vein catheter. A pulse of the stable tracer L-[ring$^{15}$N]-Phe (26.3 µmol/ kg bw) was given at T=17h. We collected basal blood samples and mucosa samples to determine natural abundances of the Phe isotopes at T=-2h. For jejunal mucosal FSR, we took biopsies at T=12 and 18 for protein bound L-[ring$^{13}$C$_6$]-Phe enrichments. And at T=12, 14, 16, 17.08, 17.5, 18 hours to determine average intracellular precursor enrichment over the incorporation time.
For determination of jejunal mucosal FBR, we measured L-[^15N]-Phe enrichment in arterial plasma at T=17.08, 17.17, 17.33, 17.5, 17.75 and 18 hours. And intracellular jejunal mucosal enrichment at 17.08, 17.5 and 18.0 hours. For FSR in other tissues, we collected at T=18 hours after euthanized with 125 mg/kg pentobarbital sodium and 16 mg/kg phenytoin sodium (Euthanasol®) administered via the central vein catheter, tissue samples of jejunum, ileum, liver, muscle and lung within 10 min after the euthanization to obtain enrichments (L-[ring13C6]-Phe) in tissue bound proteins. Blood samples were taken to determine average plasma outflow enrichment of the precursor (Phe) of each tissue during incorporation time at T= -1, -0.5, 0, 14, 16, 17, 17.5, 18.0: portal vein plasma was taken for jejunum and ileum; hepatic vein plasma for liver; arterial plasma for lung and abdominal vein plasma outflow from the hindquarter for muscle.

**Blood and tissue sample processing**

We collected blood in lithium-heparinized tubes (microtube, Sarstedt, Newton, NC, USA) and immediately placed them on ice (4°C) and processed them within one hour as described previously (20, 22, 23). In brief: for plasma flow measurements, blood was centrifuged (4°C, 5 minutes, 8000 G), after which 250 µL plasma were deproteinized with 25 µL trichloroacetic acid solution (TCA, 50% w/v) and snap frozen in liquid nitrogen. We collected mucosa biopsies with a short endoscopic flexible biopsy needle via the jejunal stoma, and rinsed and cooled them directly in cold saline, blotted them on paper towel and transferred into a tube. The tube was snap frozen in liquid nitrogen. Samples of jejunum, ileum, liver, muscle and lung, were collected during necropsy (within 10 min after the euthanization) were treated in the same way. All samples were stored until further analyses at -80°C.

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**Figure 1. Experimental Design**

PA = *Pseudomonas aeruginosa* (1e⁹ CFU/ml/min); ^13C₆-L-Phe is intravenous primed continuous infusion of stable isotope tracer ^13C₆-L-phenylalanine; ^15N-Phe is intravenous pulse administration of ^15N-Phenylalanine.
Before the analyses, tissues were crunched under freezing conditions (-190°C). Tissue powder (30-80 mg) were transferred to a microtube (Sarstedt, #72.730, Newton, NC, USA) with 250µl 0.098M TCA (4°C) and 0.1 gr glass beads and subsequently homogenized with mini-bead beater (Biospec, Bartlesville, OK, USA) for 30 seconds on low speed. After centrifugation (4°C, 5 minutes, 8000 G), supernatant was used for intracellular amino acid concentration measurements with the LCMS. The protein pellet was washed three times with 0.3M TCA and transferred to larger volume (8 ml) glass tube. Hydrolysis of the protein in the air-tight capped glass tube was performed with 1 ml 6N HCL at 110°C for 24 hours. After hydrolysis 5 ml MilliQ water was added to dilute the strong acid, where after the tubes were dried down. Capped samples were stored dark and at room temperature until analysis.

**Stable isotope tracer enrichment measurement**

We determined amino acid enrichments (tracer-to-tracee ratios, TTR) on a fully automated LC-ESI-MS/MS system (QTrap 5500 MS, AB Sciex, Foster City, CA, USA) with ExpressHT Ultra LC (Eksigent Div., AB Sciex, Foster City, CA, USA). Within 3 days before the LC-ESI-MS/MS analysis, we derivatized the samples together with enriched external standards in the range of expected TTRs (calibration curve for TTRs) with 9-fluorenylmethoxycarbonyl (Fmoc). After neutralization, we injected 160 nL of the solution onto a micro LC column 0.5 x 100 mm HALO C18, 2.7 µm, 90A pores (ABSciex, Foster City, CA, USA), kept at 35°C. We eluted analytes with a segmentally linear gradient from 35% to 85% acetonitrile in water supplemented with ammonium acetate to 10 µM and 5% isopropanol. We used electrospray triple quadrupole tandem mass spectrometry in multiple reactions monitoring mode for detection. We fragmented the Fmoc amino acid derivatives in the collision cell for detection of either free aminoacyl anions or a fragment larger by 26 atom mass units (coming from the Fmoc derivative) to have the highest sensitivity. We simultaneously did mass analyses for Phe tracee, tracers and internal standards. We calculated the mass signal areas to enable TTR.

**Calculations**

**FSR**

FSR of the tissues was determined by measurement of the incorporation of the stable isotope labeled amino acid (=precursor) over a period of time in protein (=product) as described by Wolfe et al (19):

\[
\text{FSR} = \frac{(E_{\text{product}(T_2)} - E_{\text{product}(T_1)})}{(E_{\text{precursor}} \times \Delta \text{time})}
\]

E is the enrichment in unit Mole Percent Excess (MPE= TTR/(1+TTR)). Eproduct (T₁) is the natural abundance of mucosal protein at t=−2h. Eproduct (T₂) = enrichment of tissue protein at the time of tissue collection. Eprecursor = average plasma outflow enrichment of the precursor (Phe) of each tissue during incorporation time: portal.
vein plasma for jejunum and ileum; hepatic vein plasma for liver; arterial plasma for lung and abdominal vein plasma outflow from the hindquarter for muscle. Delta time: Incorporation time (-2-0h and 12-18h). For the FSR in mucosal biopsies we used the same formula, replaced Eproduct (T1) with the enrichment in mucosal protein at T=12h. Eproduct (T2). Eprecursor is the average intracellular mucosa enrichment of precursor and Delta time is 12-18h.

FBR
The rate of decay of the intracellular enrichment (TTR), after a pulse of stable amino acid tracer (\(15^N\)-phenylalanine) in plasma, is related to FBR. Arterial and intracellular decay curves in mucosa biopsies of this tracer are used for the calculations following the method of Wolfe et al (19). FBR is measured between t=17 and 18h.

Net protein synthesis
Net protein synthesis was calculated by subtracting the FBR from FSR.

Statistics
Results are presented as means ± SEM. Graphpad Prism (version 7) was used for statistics. Levels of significance was set on p<0.05. Tendency on p<0.1. To compare data between the Healthy and Sepsis group an unpaired t-test was used.

Results
To determine changes in protein synthesis in acute severe sepsis, we measured protein turnover in the gut and of several tissues of major organs in a post-absorptive state.

Jejunal mucosal protein turnover
In relation to the FSR determination (Figure 2), the enrichments of Phe in the intracellular precursor pool and protein pool were decreased in sepsis (respectively p=0.036, p<0.001) (Figure 4) (p=0.0008). For FBR determination (Figure 3), the arterial plasma \(15^N\)-Phe enrichment decay curve was lower (p<0.0001) in sepsis but the intracellular enrichment curve was not changed. Both FSR (p= 0.0008) and FBR (p=0.0065) were reduced in jejunal mucosa in septic animals (Figure 4). Mucosal FSR is on average 5.9 times higher than FBR. Net Protein synthesis and overall protein turnover (PS + PB) were decreased in sepsis (Figure 5, respectively p=0.0247, p=0.0096).
Figure 2. Enrichments of phenylalanine for mucosa FSR calculations
Expressed as mean ± SE in MPE (=mole percent excess). A. Intracellular (precursor) enrichment (average) of phenylalanine during the incorporation time. B. Protein enrichment of phenylalanine in jejunal mucosa at the start (T₁) and end (T₂) of the tracer incorporation time. Healthy N=9, Sepsis N=11. Statistics: A: Student t-test; B: Two-way ANOVA with post-hoc Uncorrected Fisher’s LSD test: Sepsis effect p<0.0001, Time effect p<0.0001, Interaction p<0.0001; Hook: p<0.05.

Figure 3. Enrichments of phenylalanine for mucosa FBR calculations
Expressed as mean ± SE in TTR (=tracer/tracee ratio). A: Arterial plasma 15N-phenylalanine enrichment after IV pulse administration of 15N-phenylalanine 17 hours after the induction of sepsis. B. Intracellular enrichment in jejunal mucosa of 15N-phenylalanine. Healthy N=9, Sepsis N=12. Statistics: Two way ANOVA, A: Time effect p<0.0001; Sepsis effect p<0.0001; Interaction p<0.0001. B: Time effect p<0.0001; Sepsis effect p=0.134; Interaction p=0.089. When appropriate a post-hoc Sidak’s multiple comparisons test was done; *) p<0.05.
Tissue FSR

The average enrichment of Phe of the different possible proxies for the precursor pools (Figure 6) are decreased in sepsis animals ($p<0.0001$). The enrichment of bound Phe in tissue protein was decreased in jejunum ($p=0.0017$) but not in other tissues. In a post-absorptive state (Figure 7), FSR in jejunum is about 10 times higher than in muscle. Jejunum FSR was decreased ($p=0.0328$) in acute severe sepsis, but no effect of sepsis was seen in the ileum and muscle. Liver FSR was increased and lung FSR tended to be increased.

Figure 4. Fractional Synthesis Rate (FSR) and fractional breakdown rate (FBR) of protein in mucosa
Expressed as mean ± SE in percent per hour. Post-absorptive state. FSR: Healthy N=9, Sepsis N=11. FBR: Healthy N=8, Sepsis N=9. Statistics: Student t-test; Hook: $p<0.05$.

Figure 5. Mucosa Net protein Synthesis (NetPS) and protein turnover
Expressed as mean ± SE. Post-absorptive state. Healthy: N=8; Sepsis: N=10. Statistics: Student t-test; Hook: $p<0.05$. 

Tissue FSR
Discussion

Disturbed net protein synthesis in the gut is the basis for gut atrophy. We studied protein synthesis and breakdown rates of gut tissue to observe how this relates with other potential PA affected tissues in the body. We found in this acute severe sepsis pig model that jejunal mucosal protein synthesis and breakdown was reduced while protein synthesis rate was higher in the liver but unchanged in ileum and muscle.
Measurement of protein synthesis

The absolute FSR depends on the chosen precursor pool, route of administration, tracer itself (4), and the chosen incorporation time.

Precursor pool
Aminoacyl-tRNA is the true precursor of protein synthesis, but to measure its enrichment is technically very difficult, and in small tissue pieces such as gut mucosal biopsies almost impossible. The amino acids charging the tRNA must come from either the interstitial fluid compartment (being picked up at cell membrane) or from the intracellular fluid compartment. For amino acids such as phenylalanine, there is only a small difference in the enrichments in the interstitial or intracellular fluid compartment and therefore reasonably reflect the tRNA enrichment (24). The intracellular precursor enrichment during necropsy does not represent the precursor enrichment during the whole incorporation time due to the dynamics of the model. Therefore, we choose to use the average enrichment of the precursor in the plasma venous outflow of the organs during the incorporation time as the precursor enrichments to calculate the different tissue protein FSR’s (19, 24).

Route of administration
In the gut, the amino acids utilized for mucosal protein synthesis originate from the luminal or basolateral side. The measured rate of mucosal protein synthesis can be affected by the route of administration of the stable tracers (25). Intravenous administered tracers will be preferentially incorporated in the crypt cells and lower villi. In post-absorptive condition, precursor amino acids are coming mainly from the basolateral side in jejunal mucosa and therefore we chose to enrich the mucosal intracellular precursor pool via the vascular side. This means that the observed altered post-absorptive mucosa FSR is mainly a change of PS in the crypt and lower villus cells.

Choice of tracer
Stable tracers of phenylalanine or leucine are chosen frequently to measure rate of incorporation in protein. In muscle, both tracers will provide the same absolute protein synthesis rate (24, 26). Also Bouteloup-Demange (4) found that the same absolute mucosal FSR is measured with both stable tracers when plasma enrichment is used as precursor pool, but not when intracellular enrichments are used. No explanation was provided for that observation. Therefore, absolute values of FSR should be taken with caution because these can depend on the chosen tracer and precursor pool, especially, if the absolute quantity of FSR is of interest. We used a sensitive and precise measurement of the FSR that is based on an isotope with a high mass (6 masses higher than tracee that has no measurable natural abundance background) and LC-MS/MS analyses. Our approach also enabled us to reduce the
chosen total incorporation time needed to be able to pick-up tracer incorporation in the protein, especially in slow turn-over tissues like the muscle.

**FSR and FBR in jejunal mucosa**

In the healthy animals, the measured mucosa FSR was comparable with the human gut mucosal PS rate (4). The present mucosa PS and PB data showed that the ratio between FSR and FBR is much larger in mucosa than in other tissues. Mucosa is a net protein producing tissue. The FSR of the endogenous secreted protein mucin, which is needed for an adequate intestinal defense is very high (130%/day) (24). Therefore, the substantial decrease of mucosal FSR in the sepsis animals will lead to compromised mucosal net protein synthesis and will affect processes including mucin production, proteins involved in intestinal protection/repair, proliferation epithelial cells and intestinal defense against bacterial translocation. The observed diminished FBR could attenuate but not prevent this effect. The observed inhibition of jejunal mucosal protein metabolism is in line with previously described histopathology of the small intestines (18), in which we observed mild jejunal mucosa injury. We believe that observed reduced portal drained viscera PDV flow (18) could play a role in the occurrence of the injured jejunal and ileum mucosa tissues (27) in relation to compromised microcirculation (28) and subsequent inhibition of jejunal mucosal protein metabolism.

**FSR in the liver**

In contrast to the gut, liver FSR was increased probably due to the initiation of the acute phase response (29). Although we did not found a substantial increase of IL-6 and CRP in our model (18), it remains to be established whether the increased liver FSR is related to the synthesis of other hepatic acute phase proteins like fibrinogen.

**FSR in other tissues.**

In the present severe sepsis model, baseline muscle FSR was not affected, potentially due to amino acid membrane transport being maintained, which ensures the availability of amino acids from plasma for charging the tRNA (30). This is in contrast to previous studies that found reduced muscle FSR in large and small animal studies, with endotoxin or bacteremia induced sepsis (6, 7, 31). However, the results in the present paper are more in line with what is observed in critically ill humans with muscle FSR still within normal physiological ranges (32, 33). These observations also support the notion that our sepsis model in the pig is a reasonable model for sepsis in critically-ill patients.

Very limited data are available that describe protein synthesis dynamics in the lungs. Breuille et al (7) described an increase of lung PS in the early phase in a chronic bacteremia induced septic rat model and speculated that influx of inflammatory cells could possibly be responsible for the overall increase of PS in the lung,
as reflected in the present model. However, in the time window we measured, lung function was maintained (18). Therefore, we conclude that with the lung function still intact, lung PS is potentially affected in early phase of severe sepsis, but it is unknown if lung PB also is affected.

**Nutritional implications for gut metabolism in sepsis**

We hypothesize that the decreased net PS in the jejunal mucosa will compromise the nutrient and barrier function of jejunal mucosa in severe sepsis (34). Considering the highly dynamic character of intestinal metabolism, it is unclear if this phenomenon is only temporarily present or will sustain longer in the recovery phase of severe sepsis. Although certain specific dietary components are considered to be potentially helpful for mucosal healing, clinical trials (35) or studies in clinical relevant animal models with mucosal injury are limited (36). Therefore, additional studies in sepsis recovery phase are needed to study these dynamics.

**Conclusion**

In conclusion, our data, obtained with a highly translational acute severe sepsis model, suggest that jejunal mucosal protein metabolism is diminished in acute severe sepsis, even if origin of the sepsis was not located in the gut. Therefore, goals of nutritional support in sepsis should not only focus on preventing muscle breakdown but also on stimulation of gut metabolism to prevent/heal the leaking/atrophic gut.

**Acknowledgements**

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Altered transorgan protein kinetics during a *Pseudomonas aeruginosa* induced severe sepsis in the pig

Gabriella A.M. ten Have, Merel P.J. Hommen, Mariëlle P.K.J. Engelen, Steven W.M. Olde Damink, Robert R. Wolfe, Nicolaas E.P. Deutz.

**Abstract**

Severe sepsis is characterized by multi-organ pathophysiology and tissue protein breakdown (PB). Unraveling the disturbed metabolic pathways in sepsis is therefore important to gain basic knowledge for potential improvement of clinical nutrition strategies in septic humans. Little work has been done to study inter/multi organ amino acid (AA) kinetics in sepsis.

Therefore, we studied the metabolic pathways that are involved in net catabolism in severe sepsis, by measuring AA substrate fluxes with multiple stable amino acid tracers on whole body (Wb) level by measuring rate of appearances (Ra) of AA, and AA net balances (NB), PB and protein synthesis (PS) across the portal drained viscera (PDV), splanchnic area (SPL), liver and hindquarter (HQ) in a clinically relevant *Pseudomonas aeruginosa* (*PA*) induced acute severe sepsis model in a catheterized pig in a fasted conscious state.

We measured baseline substrate metabolism (BL) and between 12 and 18 hours (POST) after start of intra venous *PA* infusion. Comparison between the experimental groups: Control (C, N=9) and Sepsis (S, N=13). POST results are expressed as difference POST-BL ± SE in nmol/kg bw/min. We could establish in this sham controlled acute severe sepsis pig model that enhanced WbPB (C:-154±31; S:107±39; p=0.0001), WbPS (C:-163±27; S:49±37; p=0.0004), Wb net catabolic state (C:2±4; S:58±11; p=0.001) and myofibrillar muscle breakdown (C:10±1; S:25±1; p<0.0001) occurs in sepsis. Across PDV, both PB (C:-28±29; S:-274±39; p=0.0002) and PS (C:15±49; S:-297±80; p=0.014) are decreased. The liver showed substantial increased uptake of amino acids (C:44±1716; S:10879±3401; p=0.0218) and severe increase of ureagenesis (C:-3532±641; S:2517±661; p<0.0001). Overall the SPL and HQ NB could not explain the enhanced whole body PB. Interacting Glu, Gin and Ala multiorgan pathways showed that severe systemic Glu decrease in sepsis (C:-38±5; S:-87±6; in µM, p<0.0001), in humans related to enhanced mortality, was associated
with decreased Glu release of the liver (C: 59±225; S: 1597±317; p=0.0026) to the systemic plasma pool that could not be compensated by interacting Gln and Ala multiorgan pathways.

We conclude that enhanced protein turnover in the muscle cannot fully explain changes in WB protein turnover. Further, the decreased protein turnover in the PDV is in contrast to the WB changes. Consequently, protein turnover must be increased in tissues and organs that we have not measured directly. On the basis of the present results we hypothesize that in the acute phase of severe sepsis, the overall metabolic downregulation of PDV and the compromised liver are previously unappreciated metabolic factors in sepsis.

### Abbreviations

AA: amino acid; Ala: alanine; Arg: arginine; Asp: asparagine; BCAA: branched chain amino acids; BL: Baseline T= -2 to 0h; EAA: essential amino acids; Cit: citrulline; FSR: fractional synthesis rate; Gln: glutamine; Glu: glutamate; His: histidine; HQ: hindquarter; Iso: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; mHis: 3-methylhistidine; MPAP: mean pulmonary arterial pressure; NB: Net balances; Orn: ornithine PA: *Pseudomonas aeruginosa*; PB: protein breakdown; PDV: portal drained viscera; Phe: phenylalanine; POST: period T=12 to 18h after start sepsis induction; Pro: proline; PS: protein synthesis; Ser: serine; SPL: splanchnic area; TAA: total amino acids (measured); Thr: threonine; Tryp: tryptophan; TTR: tracer-tracee ratio; Tyr: tyrosine; Val: valine; Wb: whole body; WbRa: whole body rate of appearance.

### Introduction

Severe sepsis is characterized by multi-organ pathophysiology and tissue protein breakdown (PB) (1-3). Muscle tissue PB is considered to contribute substantially to whole body breakdown (WbPB) (4). As loss of muscle is related to poor survival and quality of life after sepsis, most research in sepsis is focused on muscle protein kinetics (5-8).

Altered protein kinetics in sepsis are also related to observations like gut mucosal atrophy, stimulation of liver acute phase protein synthesis (Chapter 10) (9-11), low plasma protein levels like albumin (12), or disturbed amino acid (AA) metabolism (2, 13). In addition, muscle loss, low albumin and reduced glutamate plasma levels are related to high mortality in sepsis (13, 14). Unraveling the disturbed metabolic pathways in sepsis is therefore important to gain basic knowledge for potential...
improvement of clinical nutrition and metabolic strategies in septic humans. Little work has been done to study interorgan protein/AA kinetics in sepsis (7).

Although critical for the interpretation of the metabolic response during sepsis in humans, it is very difficult to study protein kinetics on an organ level (7, 15). Therefore, we developed a clinical relevant model in pigs with infusion of live bacteria (16). In the present study, we applied our multorgan approach in the new sepsis model. In this model, we previously reported mild atrophy and decreased protein turnover in jejunal mucosa during sepsis (16)(Chapter 10), suggesting changes in intestinal protein kinetics. Also, we observed an increased fractional protein synthesis rate (FSR) in liver tissue, indicating a stimulation of acute phase protein synthesis. The fraction of AA derived from enhanced WbPB that is derived from muscle protein breakdown that is “reused” for acute phase protein in the liver or is released from other organs like the intestines (7) is still unclear.

In the present paper, we present AA fluxes on whole body (Wb) level and AA net balances across the portal drained viscera (PDV, mainly intestines), splanchnic area (SPL, liver and PDV), liver and hindquarter (HQ, muscle compartment). With the use of stable isotopes we are able to obtain information on protein synthesis (PS) and (PB) in multiple organs (17) in severe sepsis. Data from the present study further unravel the disturbed metabolic pathways in sepsis and therefore provide basic knowledge for potential improvement of clinical nutrition strategies in septic humans.

Materials and Methods

Animals

We used Female Yorkshire cross/domestic pigs (20-25 kg BW) in our experimental studies. We housed the pigs in steel pens (2m x 3m) in a controlled housing facility (large animal cubicle, room temperature 22-24°C, 12 hours light-dark cycle, standardized food 1kg/day (Harlan Teklad Vegetarian Pig/Sow Grower) and provided water ad libitum.

Surgical procedure

Animals received catheters during a surgical procedure as described in detail previously (18) (16). In brief, during midline laparotomy, we placed catheters for blood sampling into the abdominal aorta, portal vein, hepatic vein, and in the caval vein. An extra central caval vein catheter was placed for administering post-surgery medication and experiment related tracer infusions. We placed a second arterial catheter to monitor mean arterial blood pressure and a Swan Ganz catheter (5 Fr, #132F5, Edwards life sciences, Irvine, CA, USA) via the right jugular vein to monitor mean
pulmonary arterial pressure (MPAP) and to obtain blood from the right ventricle. We standardized preoperative and postoperative care. During the recovery period (7-10 days) animals were accustomed to a small movable cage (0.9x0.5x0.3m) that was subsequently used to perform the experiments in conscious animals. This study was approved by the animal experiment ethics committee of University of Arkansas Medical Sciences.

**Experimental Design (Figure 1)**

The experiment started after a recovery period of 7-10 days. The experiments were started at 15:00h on the experimental day, six hours after the last food intake (half of the daily amount: 0.5 kg) and selection of animals for the Sepsis group and the Control group in a randomized fashion (Control n=9, Sepsis n=13). We obtained baseline blood pressures in the pre-septic period (T= -2 to 0h, Baseline: BL). At T=0h, sepsis was induced by continuous infusion of *Pseudomonas aeruginosa* (PA, \(10^9\) CFU/ml/hour), while the Control group received an equal volume of 0.9\% NaCl solution. Fluid resuscitation was provided (0.9\% NaCl solution, 30 ml/kg bw/hour) from T=0h and hemodynamics was monitored continuously.

Para-aminomhippuric acid was infused to determine plasma flow (25 mM; 60 ml/h into the splenic vein for SPL and PDV plasma flow; 60 ml/h into the femoral artery for HQ plasma flow) as described previously (18, 19). We described that in the timeframe of 12-18h the pigs showed a relative stable cardiovascular period (16). Therefore, in the BL period and T=12 to 18h (POST) after the start of PA, we measured systemic (arterial) AA levels, Wb protein metabolism and across organ net balances (NB) of

![Figure 1. Experimental Design](image)

IV infusions and blood sampling. PA is *Pseudomonas aeruginosa* infusion (\(10^9\) CFU/ml/hour). Fluid resuscitation is 0.9\% NaCl, 30 ml/kg bw/hour. Tracer A: primed-continuous infusion: L-[ring-\(^{13}\)C\(_6\)]-phenylalanine, L-[3,3-\(^2\)H\(_2\)]-tyrosine, L-[\(^{13}\)C]-L-leucine (2 ml/kg bw/hour). Tracer B: primed-continuous infusion: L-[guanido-\(^{15}\)N\(_2\)]-arginine, L-[\(^3\)H\(_3\)]-methyl-histidine, \([^{13}\)C\] urea, (2 ml/kg bw/hour). Arrow is multiple transorgan blood sampling from arterial, portal, hepatic, caval vein.
splanchnic (SPL), Portal-Drained Viscera (PDV) and Hindquarter (HQ). With the use of AA tracers, we also measured Protein Synthesis (PS) and Protein Breakdown (PB) of SPL, PDV and HQ. At T=18, we euthanized the pigs with 125 mg/kg pentobarbital sodium and 16 mg/kg phenytoin sodium (Euthanasol®) administered via the central vein catheter.

*Pseudomonas aeruginosa*

For the induction of sepsis, we used a live PA human strain (IRS 12-4-4, Shriners burns institute, University of Texas Medical Branch, Galveston). Based on pilot virulence experiment, $10^9$ (CFU/hour) in a volume of 1 ml 0.9% NaCl solution was needed to obtain similar cardiovascular responses and hemodynamic variables with characteristics of severe hyperdynamic sepsis. We monitored hemodynamics continuously to ensure that the hyperdynamic state was kept in the expected ranges for severe sepsis (body temp increase of 2-3°C, respiratory rate increased, MPAP increased but <35 mmHg, heart rate increased but <200 BPM).

**Infusion and sampling protocol**

**Stable isotopes**

Primed continuous infusions of the following stable isotopes were given via the central caval catheter: L-[ring-$^{13}$C$_6$]-Phe, L-[3,3-$^2$H$_2$]-Tyr, L-[1-$^{13}$C]-Leu, L-[$^2$H$_3$]-mHis, [$^{13}$C]-Urea and L-[guanidino-$^{15}$N$_2$]-Arg (Cambridge Isotopes, Andover, MA) to determine whole body or across organ PB or PS. Additionally, myofibrillar muscle PB was determined by the whole body rate of appearance (WbRa) of methyl histidine (20, 21), and urea metabolism by studying total and extra hepatic ureagenesis (Table 1). The prime and infusion was given respectively in a volume of 2 ml/kg bw and 2 ml/kg bw/hour in the time periods indicated in Figure 1.

**Table 1. Stable isotope tracers**

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Prime (μmol/kg bw/min)</th>
<th>Infusion rate (μmol/kg bw/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[ring-$^{13}$C$_6$]-phenylalanine</td>
<td>1.6</td>
<td>4.3</td>
</tr>
<tr>
<td>L-[3,3-$^2$H$_2$]-tyrosine</td>
<td>2.5</td>
<td>5.4</td>
</tr>
<tr>
<td>L-[1-$^{13}$C]-leucine</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>L-[guanido-$^{15}$N$_2$]-arginine</td>
<td>4.6</td>
<td>8.4</td>
</tr>
<tr>
<td>L-[{$^2$H$_3$}]-methyl-histidine</td>
<td>0.38</td>
<td>0.74</td>
</tr>
<tr>
<td>[$^{13}$C]-urea</td>
<td>73</td>
<td>15</td>
</tr>
</tbody>
</table>

**Blood sampling and Sample processing**

Blood samples were collected on ice and immediately processed within one hour as described previously (16, 19) In brief: for AA concentration and enrichment analysis, we centrifuged heparinized blood at 4°C, 5 minutes, 8000 G and deproteinized
250 µL plasma with 25 µL tri-chloroacetic acid solution (TCA, 50% w/v) and finally snap frozen in liquid nitrogen and store at -80°C.

**AA concentration and enrichments**

We determined AA isotope concentrations and AA enrichments (TTR) on a fully automated LC-MS/MS system (QTrap 5500 MS, AB Sciex, Foster City, CA, USA) with Express HT Ultra LC (Eksigent Div., AB Sciex, Foster City, CA, USA). For concentration and TTR measurements we added 20 µl of TCA deproteinized plasma supernatant to 20 µl AA (high mass stable isotopes) internal standard. Within 3 days before the LC-MS/MS analysis, we derivatized the samples together with external standards at concentrations within the physiological range (calibration curve of concentration) and enriched external standards in the range of expected TTRs (calibration curve for TTRs) with 9-fluorenylmethoxycarbonyl (Fmoc). After neutralization, we injected 160 nL of the solution onto a micro LC column 0.5 x 100 mm HALO C18, 2.7 μm, 90A pores (ABSciex, Foster City, CA, USA), kept at 35°C. We eluted analytes with a segmentally linear gradient from 35% to 85% acetonitrile in water supplemented with ammonium acetate to 10 μM and 5% isopropanol. We used electrospray triple quadrupole tandem mass spectrometry in multiple reactions monitoring mode for detection. We fragmented the Fmoc AA derivatives in the collision cell for detection of either free aminoacyl anions or a fragment larger by 26 atom mass units (coming from the Fmoc derivative) to have the highest sensitivity. We simultaneously did mass analyses for AA tracee, its tracers and internal standards. We calculated the mass signal areas to enable TTR or tracee concentrations calculations.

**Calculations**

**Calculation tracee concentration and TTR from LC-MS/MS obtained signals**

For concentration calculations, we normalized the tracee signals of the samples and the external standards with their internal AA standard. We determined the plasma tracee concentration with the calibration curve of external AA standards. Plasma enrichment (TTR) was determined using the peak area ratio of the tracee and tracer signal and a calibration curve of enriched external standards. For the tracer concentration in the infusions, we used a calibration curve of tracer containing external standards.

**Calculation Wb fluxes**

Wb protein metabolism fluxes on post-absorptive state were determined by measuring WbRa of protein turnover related AA’s as described previously (18, 22-24):

\[
\text{Equation (1)} \quad \text{WbRa} = \frac{I}{\text{TTR}}
\]

Where “I” is infusion rate of the tracers and “TTR” the arterial steady state TTR. Proxies for WbPB are the WbRa of Phe or Leu:
Equation (2) \[ WbRa_{\text{Phe}} = \frac{I}{\text{TTR}_{[\text{ring-}^{13}\text{C}_6]}-\text{Phe}} \]

Or

Equation (3) \[ WbRa_{\text{Leu}} = \frac{I}{\text{TTR}_{[^{1-13}\text{C}]-\text{Leu}}} \]

The rate \((Q)\) of Phe that is hydroxylated to Tyr is:

Equation (4) \[ Q_{\text{phe}>\text{tyr}} = \frac{WbRa_{\text{Tyr}} \times \text{TTR}_{[\text{ring-}^{13}\text{C}_6]}-\text{Tyr}}{\text{TTR}_{[\text{ring-}^{13}\text{C}_6]}-\text{Phe}} \]

Equation (5) \[ WbPS = WbRa_{\text{Phe}} - Q_{\text{Phe}>\text{Tyr}} \]

Equation (6) \[ \text{Urea production} = WbRa_{\text{urea}} = \frac{I}{\text{TTR}_{[{^{13}\text{C}]}-\text{urea}}} \]

Equation (7) \[ \text{Extra hepatic arginase activity} = Q_{\text{arg}>\text{urea}} = \frac{WbRa_{\text{urea}} \times \text{TTR}_{[^{15}\text{N}_2]}-\text{urea}}{\text{TTR}_{[\text{guanido-}^{15}\text{N}_2]}-\text{arginine}} \]

A proxy for skeletal muscle breakdown is the WbRa of the post-translationally modified AA mHis.

Equation (8) \[ \text{Myofibrillar protein breakdown} = WbRa_{\text{mHis}} = \frac{I}{\text{TTR}_{[{^2\text{H}_3}]}-\text{mHis}} \]

**Calculation transorgan net arterial-venous balances (NB)**

Transorgan or organ NB of AA are calculated as described previously (18, 22, 24). In principle the tracee NB across an organ is the multiplication of arterial-venous AA concentration difference with the plasma flow:

Equation (9) \[ \text{Transorgan net balance} = \text{NB} = \text{Flowplasma} \times ([A] - [V]) \]

A positive value represents net uptake and a negative value represents net release. To calculated PDV, SPL and HQ AA NB’s, we used the previously reported plasma flow of the present used cohort of animals (16).

**Calculation organ disposal and production**

Organ disposal and production of AAs which are only used for PS and come from only PB can be used as a proxy for organ PS and PB and were calculated as described previously using a two pool model (22, 24). Therefore for the HQ, we used the Phe/Leu/Tyr tracers for PB and Phe/Tyr for PS due to conversion of Leu to α-ketoisocaprate (KIC) in the HQ. For the total SPL area, we used Phe for PB as hydroxylation of Phe to Tyr takes place in the liver and Leu is converted to KIC in
PDV, invalidating Phe and Leu to be used for PS. Additionally conversion of KIC to Leu in the liver invalidates the use of Leu for PB. In the PDV, Phe/Leu/Tyr tracers can be used for PB and Phe/Tyr for PS.

Equation (10) \[ \text{Organ tracer net balance} = nb = \text{Flow} \times ([A]^{\text{TTR}_{\text{art}}} - [V]^{\text{TTR}_{\text{venous}}}) \]

AAs that are used for organ PS:

Equation (11) \[ \text{Disposal} = \frac{nb}{TTR_{\text{venous}}} \]

AAs that comes from organ PB:

Equation (12) \[ \text{Production} = \text{Disposal} - \text{NB} \]

Statistical analyses

Results are presented as means ± SEM. For the baseline period (BL) absolute values were given. In the POST period absolute differences (difference POST-BL) are described. Graphpad Prism (version 7) was used for statistics. Levels of significance was set on \( p<0.05 \). To compare data between the Control and Sepsis group in the POST period an unpaired t-test was used. Wilcoxon Signed Rank Test was used to determine if fluxes, NB, disposal, production and difference POST-BL are different from zero. Average AA concentrations in BL and POST were determined with horizontal line fitting. Steady state TTR values in these same periods were estimated with plateau values obtained from one-phase association fitting of the individual TTR curves (Graphpad Prism, version 7).

Results

Clinical characteristics including hemodynamics, clinical chemistry, inflammation and plasma flow of the cohort of animals described in the present study are described extensively in Chapter 6 (16). In brief: of the 27 animals, we lost five animals in the recovery phase of the catheter implantation surgery due to intestinal complications (ileus, ascites or peritonitis). However, no animals were lost during surgery and the PA experiment. With main diagnostic criteria of SIRS (body temperature: increase of more than two degrees Celsius; heart rate: \ (>90 \text{ beats/min}; \text{PaCO}_2: <32 \text{ mmHg})\), we concluded that the present PA bacteremia has successfully induced sepsis in pigs. We found that despite fluid resuscitation pronounced hypotension, tachycardia and a decreased PDV plasma flow were present, indicating a disturbed cardiovascular function. Severe sepsis was established between 12-18 h after starting the PA infusion.
Whole body

Systemic AA concentrations (Table 2)
Most of the AA concentrations were decreased in both experimental groups in the POST period in comparison with the baseline (Figure 2, 4, 5). Only Glu concentrations

Figure 2. Total amino acid concentrations and organ net balances (NB)
Baseline and post infusion of Pseudomonas aeruginosa. HQ is hindquarter (muscle compartment), PDV is Portal drained viscera, SPL is splanchnic area. Control n=9, Sepsis n=13. Statistics: unpaired Student t-test, Significance p<0.05 (hook).
decreased more severely in the Sepsis group (Figure 3). This in contrast with plasma concentrations of several EAA that were less reduced or elevated in comparison to the Control group. In addition, plasma myofibrillar protein metabolite mHis concentration was increased in the septic group. Urea concentration in the sepsis group was higher than in the Control group.

Table 2: Arterial Plasma Amino Acid Concentrations

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>POST - Baseline (Δ)</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
</tr>
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<tbody>
<tr>
<td>Histidine</td>
<td>78 (2)</td>
<td>-19 (7)*</td>
<td>-11 (4)*</td>
<td>0.3219</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>67 (2)</td>
<td>1 (8)</td>
<td>23 (7)*</td>
<td>0.0526</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>131 (35)</td>
<td>-11 (10)</td>
<td>4 (9)</td>
<td>0.2747</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>81 (8)</td>
<td>49 (10)*</td>
<td>61 (12)*</td>
<td>0.4574</td>
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<td>-87 (6)*</td>
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<td>Glutamine</td>
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<td>-141 (20)*</td>
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<td>-33 (12)*</td>
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<td>Serine</td>
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<td>Tyrosine</td>
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<td>BCAA</td>
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<td>TAA</td>
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<td>3-Methyl-Histidine</td>
<td>3.0 (0.1)</td>
<td>-0.1 (0.1)</td>
<td>0.2 (0.04)*</td>
<td>0.0211</td>
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<tr>
<td>Urea</td>
<td>2992 (157)</td>
<td>-2063 (302)*</td>
<td>165 (248)</td>
<td>&lt;0.0001</td>
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</table>

Arterial plasma concentrations before (Baseline: T = -2 to 0h) and after (POST: T=16 to 18h) start of Pseudomonas aeruginosa infusion. Data are expressed as mean (SEM) in µmol/l. “Baseline” is concentration before start PM infusion N=22. “POST-Baseline” is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. BCAA: Sum of the branched-chain amino acids: Valine, Leucine and Isoleucine. EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine; TAA: EAA+ alanine, arginine, asparagine, citrulline, glutamate, glutamine, ornithine, proline, serine, tyrosine. Statistics POST: Different from zero tested with Wilcoxon Signed Rank test, *) p<0.05. Effect of sepsis was tested with Unpaired T-test. Significance different compared to Control, P<0.05 (bold). Tendency: P<0.1
Figure 3. Glutamate arterial concentrations and organ net balances (NB)
Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. HQ is hindquarter (muscle compartment), PDV is Portal drained viscera, SPL is splanchnic area. Control n=9, Sepsis n=13. Statistics: unpaired Student t-test, Significance p<0.05 (hook), Tendency p<0.1 (dashed hook).
Figure 4. Glutamine arterial concentrations and organ net balances (NB)
Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. HQ is hindquarter (muscle compartment), PDV is Portal drained viscera, SPL is splanchnic area. Control n=9, Sepsis n=13. Statistics: unpaired Student t-test, Significance p<0.05 (hook).
Figure 5. Alanine arterial concentrations and organ net balances (NB)
Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. HQ is hindquarter (muscle compartment), PDV is Portal drained viscera, SPL is splanchnic area. Control n=9, Sepsis n=13. Statistics: unpaired Student t-test, Significance p<0.05 (hook). Tendency p<0.1 (dashed hook)
Rate of appearance and disappearance of Phe/Leu/Tyr as proxy for WbPS, WbPB, netPS (Table 3)
WbPB, WbPS and net PB were higher in the Sepsis group in comparing to Control group, indicating more protein catabolism in sepsis. The increase in PB was observed both in the Ra of Phe and Leu, albeit more prominent in Phe. Sepsis also increased the fraction of WbPB derived from myofibrillar protein breakdown. In addition, total urea production and extra hepatic urea production from arginase was substantially increased.

Hindquarter
Transorgan NB of AAs (Table 4)
Except for Gln and Ala, most AA were taken up by the hindquarter in the BL period (Figure 4,5). Although most of the individual EAA were taken up less or even released in the sepsis group, this was only significant for Leu, Met and Thr. Lys was taken up substantially more in both groups, but was not different between the Sepsis and Control group. We found no significant difference between these groups for total EAA. This in contrast with TAA that showed a disappearance of the uptake (no significant net release/uptake) in the HQ in the sepsis group (Figure 2). Sepsis increased the release of mHis by the HQ.

Protein metabolism (Table 5)
The HQ showed net PS during BL period. In POST period net PS is decreased significant (with the Tyr tracer) in the sepsis group. However not significant in comparing to the Control group. Also PB in the sepsis group was not significant different from the Control group. Therefore, the diminished net PS (NB of Leu or Tyr) in the sepsis group is not the result of a clear enhanced PB and/or diminished PS in the HQ in present severe septic state.

Portal Drained Viscera
Transorgan NB of AAs (Table 6)
In the BL period, the PDV is taken up almost all AA and is releasing Ala and Cit (Figure 2, 5). In the POST period, the Control group showed a different profile of uptake. For instance, we found an increase in uptake of individual AA like Leu, Thr, Arg, Glu and Ser and a reduced uptake of Pro. In the sepsis group the uptake of AAs is decreased in comparison to control group. And this was significant for the individual non-EAA: Arg, Gln (Figure 4) and Ser. No significant uptake of Glu was found (Figure 3). Cit production was the same between the control and sepsis group.

Protein metabolism (Table 5)
In the BL period PDV protein metabolism is in balance: no significant net PS or PB is observed. After administration of PA, Both PDV PS as PB were reduced in sepsis in comparison to control, but this decreased protein turnover did not change net PDV protein balance.
Whole body protein metabolism related fluxes before (Baseline: $T = -2$ to $0$h) and after (POST: $T=16$ to $18$h) start of 
*Pseudomonas aeruginosa* infusion. Data are expressed as mean (SEM) in nmol/kg bw/min. "Baseline" is flux before start PM infusion $N=22$; positive NB value is uptake; negative NB value is release. "POST-Baseline" is difference between POST and baseline period; Control: $N=9$; Sepsis: $N=13$; negative value is decrease. "P" of sepsis was tested with Unpaired T-test. Significance different compared to Control, $P<0.05$ (bold). Tendancy: $P<0.1$.

<table>
<thead>
<tr>
<th>Flux</th>
<th>Baseline</th>
<th>POST</th>
<th>T-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WbRa_Phenylalanine</td>
<td>$0.0001$</td>
<td>$0.0001$</td>
<td>$0.0001$</td>
<td>$0.0001$</td>
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<tr>
<td>Mysoblastic protein breakdown</td>
<td>$25 (1)$</td>
<td>$28 (1)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Urea production</td>
<td>$2517 (6.17)$</td>
<td>$3523 (6.41)$</td>
<td>-185 (5.73)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein breakdown (PB)</td>
<td>$420 (3.9)$</td>
<td>$29 (1.2)$</td>
<td>$66.44 (30)$</td>
<td>$-37.8 (4.7)$</td>
</tr>
<tr>
<td>Protein hydroxylation $Q_{\text{phe}&gt;\text{tyr}}$</td>
<td>$154 (3.1)$</td>
<td>$1234 (3.2)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein synthesis (PS)</td>
<td>$112.7 (11.2)$</td>
<td>$25.6 (21)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Net Protein catabolism (netPB)</td>
<td>$17 (1)$</td>
<td>$136 (3.1)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein breakdown (PB)</td>
<td>$28 (1)$</td>
<td>$107 (1.7)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Extra hepatic arginase activity</td>
<td>$49 (3.7)$</td>
<td>$163 (2.7)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein turnover (Q_Arg&gt;Urea)</td>
<td>$1215 (3.1)$</td>
<td>$77 (1.1)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Myofibrillar protein breakdown</td>
<td>$28 (1)$</td>
<td>$107 (1.7)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>$3204 (7.4)$</td>
<td>$29 (1.2)$</td>
<td>$66.44 (30)$</td>
<td>$-37.8 (4.7)$</td>
</tr>
<tr>
<td>Protein breakdown (PB)</td>
<td>$420 (3.9)$</td>
<td>$29 (1.2)$</td>
<td>$66.44 (30)$</td>
<td>$-37.8 (4.7)$</td>
</tr>
<tr>
<td>Protein hydroxylation $Q_{\text{phe}&gt;\text{tyr}}$</td>
<td>$154 (3.1)$</td>
<td>$1234 (3.2)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein synthesis (PS)</td>
<td>$112.7 (11.2)$</td>
<td>$25.6 (21)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Net Protein catabolism (netPB)</td>
<td>$17 (1)$</td>
<td>$136 (3.1)$</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein breakdown (PB)</td>
<td>$28 (1)$</td>
<td>$107 (1.7)$</td>
<td>0.0001</td>
<td>0.0001</td>
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</tbody>
</table>

Table 3. Whole body protein metabolism related fluxes

**Transorgan protein kinetics in sepsis**

Chapter 11

239
## Table 4. Hindquarter amino acid net balances

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Baseline</th>
<th>POST - Baseline (Δ)</th>
<th>P&lt;Wilcoxon</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>215 (40)</td>
<td>-100 (71)</td>
<td>&lt;0.0001</td>
<td>-249 (77)*</td>
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<tr>
<td>Isoleucine</td>
<td>213 (30)</td>
<td>-60 (37)</td>
<td>&lt;0.0001</td>
<td>-163 (92)</td>
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<td>0.4064</td>
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<td>Leucine</td>
<td>237 (47)</td>
<td>54 (42)</td>
<td>&lt;0.0001</td>
<td>-257 (92)</td>
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</tr>
<tr>
<td>Lysine</td>
<td>235 (48)</td>
<td>1842 (293)*</td>
<td>&lt;0.0001</td>
<td>-2160 (404)*</td>
<td></td>
<td>0.5824</td>
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<tr>
<td>Methionine</td>
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<td>-133 (50)</td>
<td>&lt;0.0001</td>
<td>-266 (33)*</td>
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<td>0.0331</td>
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<tr>
<td>Phenylnalanine</td>
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<td>-188 (85)</td>
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<td>Threonine</td>
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<td>-484 (92)*</td>
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<td>0.0066</td>
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<tr>
<td>Tryptophan</td>
<td>47 (8)</td>
<td>-46 (19)</td>
<td>&lt;0.0001</td>
<td>-100 (17)*</td>
<td></td>
<td>0.0515</td>
</tr>
<tr>
<td>Valine</td>
<td>514 (67)</td>
<td>-206 (78)*</td>
<td>&lt;0.0001</td>
<td>-477 (132)*</td>
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<td>Alanine</td>
<td>-1156 (298)</td>
<td>1114 (340)*</td>
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<td>-1093 (669)</td>
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<td>Arginine</td>
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<td>&lt;0.0001</td>
<td>-520 (102)*</td>
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<td>Asparagine</td>
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<td>-119 (64)</td>
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<td>-220 (46)*</td>
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<td>Citrulline</td>
<td>92 (13)</td>
<td>-37 (33)</td>
<td>&lt;0.0001</td>
<td>-63 (18)*</td>
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<td>0.4626</td>
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<tr>
<td>Glutamate</td>
<td>1072 (45)</td>
<td>-114 (75)</td>
<td>&lt;0.0001</td>
<td>-682 (95)*</td>
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<tr>
<td>Glutamine</td>
<td>-270 (233)</td>
<td>151 (783)</td>
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<td>0.6297</td>
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<td>Ornithine</td>
<td>217 (33)</td>
<td>-129 (78)</td>
<td>&lt;0.0001</td>
<td>-208 (122)</td>
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<td>0.3878</td>
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<td>Proline</td>
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<td>-469 (127)</td>
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<td>-621 (122)</td>
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<td>0.3878</td>
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<td>Serine</td>
<td>453 (63)</td>
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<td>Tyrosine</td>
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<td>-37 (29)</td>
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<td>-214 (45)*</td>
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<td>BCAA</td>
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<td>&lt;0.0001</td>
<td>-898 (290)*</td>
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<tr>
<td>EAA</td>
<td>1864 (288)</td>
<td>1090 (542)</td>
<td>&lt;0.0001</td>
<td>-53 (648)</td>
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<td>0.2204</td>
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<td>TAA</td>
<td>2887 (593)</td>
<td>1675 (920)</td>
<td>0.0002</td>
<td>-5148 (1636)*</td>
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<td>3-Methyl-Histidine</td>
<td>0.3 (1.4)</td>
<td>-3.2 (3.3)</td>
<td>0.7854</td>
<td>4.4 (0.9)*</td>
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<td>0.0137</td>
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</table>

Hindquarter net balances (NB) before (Baseline: T = -2 to 0h) and after (POST: T=16 to 18h) start of *Pseudomonas aeruginosa* infusion. Data are expressed as mean (SEM) in nmol/kg bw/min. Baseline net balance before start PM infusion N=22; positive NB value is uptake; negative NB value is release. “POST-Baseline” is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. BCAA: Sum of the branched-chain amino acids: Valine, Leucine and Isoleucine. EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine; TAA: EAA+ alanine, arginine, asparagine, citrulline, glutamate, glutamine, ornithine, proline, serine, tyrosine. Statistics: Baseline: Different from zero tested with Wilcoxon Signed Rank test, POST: Different from zero tested with Wilcoxon Signed Rank test, *) p<0.05. Effect of sepsis was tested with Unpaired T-test. Significance different compared to Control, P<0.05 (bold). Tendency: P<0.1.
Table 6. Portal drained viscera net balances

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Baseline</th>
<th>POST - Baseline (Δ)</th>
<th>PWilcoxon</th>
<th>Healthy</th>
<th>Sepsis</th>
<th>PT-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>106 (39)</td>
<td>-21 (42)</td>
<td>0.009</td>
<td>-116 (84)</td>
<td>0.3832</td>
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<tr>
<td>Isoleucine</td>
<td>75 (24)</td>
<td>85 (80)</td>
<td>0.0063</td>
<td>-32 (64)</td>
<td>0.2651</td>
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<td>Leucine</td>
<td>-43 (44)</td>
<td>201 (56)*</td>
<td>0.157</td>
<td>139 (80)</td>
<td>0.5615</td>
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<td>Lysine</td>
<td>-12 (52)</td>
<td>63 (74)</td>
<td>&gt;0.9999</td>
<td>4 (153)</td>
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<td>Methionine</td>
<td>133 (29)</td>
<td>-135 (67)</td>
<td>0.0002</td>
<td>-106 (18)*</td>
<td>0.6571</td>
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<td>Phenylalanine</td>
<td>-20 (39)</td>
<td>21 (53)</td>
<td>0.7593</td>
<td>48 (62)</td>
<td>0.7627</td>
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<td>Threonine</td>
<td>198 (33)</td>
<td>58 (20)*</td>
<td>&lt;0.0001</td>
<td>-42 (59)</td>
<td>0.1692</td>
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<tr>
<td>Tryptophan</td>
<td>51 (10)</td>
<td>-17 (19)</td>
<td>0.0002</td>
<td>-34 (13)*</td>
<td>0.4619</td>
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<tr>
<td>Valine</td>
<td>344 (57)</td>
<td>-136 (93)</td>
<td>0.0001</td>
<td>-273 (115)*</td>
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<td>Alanine</td>
<td>-725 (254)</td>
<td>1828 (878)</td>
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<td>836 (505)</td>
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<tr>
<td>Arginine</td>
<td>162 (58)</td>
<td>418 (142)*</td>
<td>0.0127</td>
<td>-119 (97)</td>
<td>0.0041</td>
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<tr>
<td>Asparagine</td>
<td>-34 (43)</td>
<td>26 (60)</td>
<td>0.7502</td>
<td>57 (49)</td>
<td>0.6948</td>
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<tr>
<td>Citrulline</td>
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<td>-76 (46)</td>
<td>0.0329</td>
<td>-3 (52)</td>
<td>0.3278</td>
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<tr>
<td>Glutamate</td>
<td>280 (46)</td>
<td>154 (38)*</td>
<td>&lt;0.0001</td>
<td>-320 (90)*</td>
<td>0.0005</td>
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<tr>
<td>Glutamine</td>
<td>2209 (190)</td>
<td>107 (399)</td>
<td>&lt;0.0001</td>
<td>-1402 (343)*</td>
<td>0.0099</td>
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<td>Ornithine</td>
<td>122 (55)</td>
<td>-95 (90)</td>
<td>0.0425</td>
<td>-20 (96)</td>
<td>0.7805</td>
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<tr>
<td>Proline</td>
<td>465 (101)</td>
<td>-495 (117)*</td>
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<td>-360 (88)*</td>
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<td>Serine</td>
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<td>-119 (97)</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>BCAA</td>
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<td>-359 (308)</td>
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<td>EAA</td>
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<tr>
<td>TAA</td>
<td>3502 (717)</td>
<td>2286 (1437)</td>
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<td>-2445 (1161)</td>
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<tr>
<td>Methyl-Histidine</td>
<td>-2.7 (1.8)</td>
<td>4.0 (1.5)*</td>
<td>0.2099</td>
<td>5.5 (2.6)</td>
<td>0.6971</td>
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</table>

Portal Drained Viscera net balances (NB) before (Baseline: T = -2 to 0h) and after (POST: T=16 to 18h) start of *Pseudomonas aeruginosa* infusion. Data are expressed as mean (SEM) in nmol/kg bw/min. PRE is net balance before start PM infusion N=22; positive NB value is uptake; negative NB value is release. "POST-Baseline" is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. BCAA: Sum of the branched-chain amino acids: Valine, Leucine and Isoleucine. EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine; TAA: EAA+ alanine, arginine, asparagine, citrulline, glutamate, glutamine, ornithine, proline, serine, tyrosine. Statistics: Baseline: Different from zero tested with Wilcoxon Signed Rank test, POST: Different from zero tested with Wilcoxon Signed Rank test, *p<0.05. Effect of sepsis was tested with Unpaired T-test. Significance different compared to Control, *P<0.05 (bold). Tendency: *P<0.1.
Table 5. Organ protein breakdown and protein synthesis

<table>
<thead>
<tr>
<th>Organ</th>
<th>Parameter</th>
<th>Baseline</th>
<th>PWilcoxon</th>
<th>POST - Baseline (Δ)</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
</tr>
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<tbody>
<tr>
<td>Hindquarter</td>
<td>Phe disposal</td>
<td>463 (52)</td>
<td>&lt;0.0001</td>
<td>12 (80)</td>
<td>-126 (128)</td>
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<td>(PS)</td>
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<td></td>
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<tr>
<td></td>
<td>Phe production</td>
<td>350 (34)</td>
<td>&lt;0.0001</td>
<td>99 (96)</td>
<td>-71 (98)</td>
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<tr>
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<td>(PB)</td>
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<tr>
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<td>Phe NB</td>
<td>104 (22)</td>
<td>&lt;0.0001</td>
<td>-119 (31)</td>
<td>-188 (85)</td>
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<tr>
<td></td>
<td>Leu disposal</td>
<td>949 (91)</td>
<td>&lt;0.0001</td>
<td>39 (100)</td>
<td>-177 (151)</td>
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<td>Leu production</td>
<td>712 (65)</td>
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<td>-14 (92)</td>
<td>106 (148)</td>
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<tr>
<td></td>
<td>Leu NB</td>
<td>237 (47)</td>
<td>&lt;0.0001</td>
<td>54 (42)</td>
<td>-257 (92)</td>
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<tr>
<td></td>
<td>Tyr disposal</td>
<td>281 (42)</td>
<td>0.0001</td>
<td>-106 (59)</td>
<td>-208 (55)*</td>
<td>0.2283</td>
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<tr>
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<td>(PS)</td>
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<tr>
<td></td>
<td>Tyr production</td>
<td>242 (19)</td>
<td>&lt;0.0001</td>
<td>-84 (39)</td>
<td>21 (50)</td>
<td>0.1425</td>
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<tr>
<td></td>
<td>Tyr NB</td>
<td>82 (24)</td>
<td>0.0025</td>
<td>-37 (29)</td>
<td>-214 (45)*</td>
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<tr>
<td>Portal drained viscera</td>
<td>Phe disposal</td>
<td>483 (53)</td>
<td>&lt;0.0001</td>
<td>238 (168)</td>
<td>-105 (141)</td>
<td>0.1343</td>
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<tr>
<td></td>
<td>(PS)</td>
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<tr>
<td></td>
<td>Phe production</td>
<td>533 (52)</td>
<td>&lt;0.0001</td>
<td>101 (76)</td>
<td>-248 (101)*</td>
<td>0.0199</td>
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<tr>
<td></td>
<td>Phe NB</td>
<td>-20 (39)</td>
<td>0.7593</td>
<td>21 (53)</td>
<td>48 (62)</td>
<td>0.7627</td>
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<tr>
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<td>Leu disposal</td>
<td>931 (105)</td>
<td>&lt;0.0001</td>
<td>242 (125)</td>
<td>-381 (180)</td>
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<tr>
<td></td>
<td>Leu production</td>
<td>921 (96)</td>
<td>&lt;0.0001</td>
<td>41 (100)</td>
<td>-544 (115)*</td>
<td>0.0016</td>
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<tr>
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<tr>
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<td>Leu NB</td>
<td>-43 (44)</td>
<td>0.157</td>
<td>201 (56)*</td>
<td>139 (80)</td>
<td>0.5615</td>
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<tr>
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<td>Tyr disposal</td>
<td>416 (319)</td>
<td>&lt;0.0001</td>
<td>-15 (49)</td>
<td>-297 (80)*</td>
<td>0.0144</td>
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<td>Tyr production</td>
<td>356 (38)</td>
<td>&lt;0.0001</td>
<td>-28 (29)</td>
<td>-274 (39)*</td>
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<tr>
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<tr>
<td></td>
<td>Tyr NB</td>
<td>84 (31)</td>
<td>0.0145</td>
<td>-55 (51)</td>
<td>-70 (53)</td>
<td>0.8549</td>
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Organ protein breakdown (PB) and protein synthesis (PS) by measuring amino acid disposal, production and net balance (NB) before (Baseline: T = -2-0h) and after (POST: T=16-18h) start of *Pseudomonas aeruginosa* infusion. Data are expressed as mean (SEM) in nmol/kg bw/min. "Baseline" is disposal, production and NB before start PM infusion (N=22); positive NB value is uptake; negative NB value is release. "POST-Baseline" is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. Phe is phenylalanine. Leu is Leucine, Tyr is Tyrosine. Statistics: Baseline: Different from zero tested with Wilcoxon Signed Rank test, POST: Different from zero tested with Wilcoxon Signed Rank test, *) p<0.05. Effect of sepsis was tested with Unpaired T-test. Significance different compared to Control, P<0.05 (bold). Tendency: P<0.1.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Parameter</th>
<th>Baseline</th>
<th>PWilcoxon</th>
<th>POST - Baseline (Δ)</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
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<tbody>
<tr>
<td>Splanchnic area</td>
<td>Phe disposal</td>
<td>853 (315)</td>
<td>0.0103</td>
<td>168 (339)</td>
<td>-3 (31)</td>
<td>0.7168</td>
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<tr>
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<td>Phe production (PB)</td>
<td>557 (286)</td>
<td>0.0737</td>
<td>-4 (343)</td>
<td>-162 (315)</td>
<td>0.7429</td>
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<tr>
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<td>Phe NB</td>
<td>282 (86)</td>
<td>0.0047</td>
<td>172 (133)</td>
<td>362 (97)*</td>
<td>0.2522</td>
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<tr>
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<td>Leu disposal</td>
<td>1243 (505)</td>
<td>0.0141</td>
<td>324 (330)</td>
<td>156 (607)</td>
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<td>Leu production</td>
<td>1110 (485)</td>
<td>0.0103</td>
<td>-242 (307)</td>
<td>-247 (581)</td>
<td>0.9943</td>
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<tr>
<td></td>
<td>Leu NB</td>
<td>-1 (79)</td>
<td>0.721</td>
<td>565 (149)*</td>
<td>553 (178)*</td>
<td>0.9594</td>
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</table>
Splanchnic area

Transorgan NB of AAs (Table 7)

In the BL period the SPL area is taken up EAA except for Leu. In addition, Arg, Gin, Pro and Tyr are taken up. Glu is released from the splanchnic area. In the POST period, we observed an overall reduction of uptake of Trp, Cit, Pro and enhanced uptake of Lys in the Control group. In the sepsis group, the SPL area the uptake of AA was tripled in comparison to the control group.

Table 7. Splanchnic area net balances

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>POST - Baseline (Δ)</th>
<th>PWilcoxon</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>337 (60)</td>
<td>&lt;0.0001</td>
<td>69 (119)</td>
<td>108 (96)</td>
<td>0.7993</td>
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<tr>
<td>Isoleucine</td>
<td>284 (75)</td>
<td>0.0006</td>
<td>306 (162)</td>
<td>123 (117)</td>
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<tr>
<td>Leucine</td>
<td>-1 (79)</td>
<td>0.721</td>
<td>565 (149)*</td>
<td>553 (178)*</td>
<td>0.9594</td>
<td></td>
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<tr>
<td>Lysine</td>
<td>205 (86)</td>
<td>0.0390</td>
<td>347 (264)</td>
<td>661 (196)*</td>
<td>0.3417</td>
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<tr>
<td>Methionine</td>
<td>595 (79)</td>
<td>&lt;0.0001</td>
<td>-327 (137)*</td>
<td>-256 (67)*</td>
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<tr>
<td>Phenylalanine</td>
<td>282 (86)</td>
<td>0.0047</td>
<td>172 (133)</td>
<td>362 (97)*</td>
<td>0.2522</td>
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<tr>
<td>Threonine</td>
<td>479 (61)</td>
<td>&lt;0.0001</td>
<td>-18 (176)</td>
<td>802 (81)*</td>
<td>0.0003</td>
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<td>Tryptophan</td>
<td>141 (17)</td>
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<td>-94 (29)*</td>
<td>-15 (19)</td>
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<tr>
<td>Valine</td>
<td>872 (124)</td>
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<td>-310 (199)</td>
<td>748 (133)</td>
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<tr>
<td>Alanine</td>
<td>897 (469)</td>
<td>0.0586</td>
<td>1923 (567)*</td>
<td>4877 (1258)*</td>
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<tr>
<td>Arginine</td>
<td>372 (112)</td>
<td>0.0049</td>
<td>380 (225)</td>
<td>326 (126)*</td>
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<td>Asparagine</td>
<td>114 (63)</td>
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<td>415 (202)</td>
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<td>Citrulline</td>
<td>85 (65)</td>
<td>0.2755</td>
<td>-160 (95)</td>
<td>-323 (85)*</td>
<td>0.2203</td>
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<td>Glutamate</td>
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<td>574 (414)</td>
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<td>Glutamine</td>
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<td>&lt;0.0001</td>
<td>-1123 (718)</td>
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<td>Ornithine</td>
<td>100 (115)</td>
<td>0.7023</td>
<td>-12 (164)</td>
<td>-286 (145)</td>
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<td>Proline</td>
<td>1258 (134)</td>
<td>&lt;0.0001</td>
<td>-732 (292)*</td>
<td>89 (169)</td>
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<td>Serine</td>
<td>161 (171)</td>
<td>0.4244</td>
<td>366 (292)</td>
<td>64 (293)</td>
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<td>Tyrosine</td>
<td>267 (58)</td>
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<td>-169 (111)</td>
<td>204 (104)</td>
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<td>BCAA</td>
<td>1366 (202)</td>
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<td>561 (449)</td>
<td>887 (279)*</td>
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<tr>
<td>EAA</td>
<td>3072 (511)</td>
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<td>710 (979)</td>
<td>1994 (1023)*</td>
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<tr>
<td>TAA</td>
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<td>Methyl-Histidine</td>
<td>-2.8 (4.4)</td>
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<td>0.4 (8.1)</td>
<td>10.8 (6.3)*</td>
<td>0.3554</td>
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</table>

Splanchnic area net balances (NB) before (Baseline: T = -2 to 0h) and after (POST: T=16 to 18h) start of Pseudomonas aeruginosa infusion. Data are expressed as mean (SEM) in mmol/kg bw/min. Baseline is net balance before start PM infusion N=22; positive NB value is uptake; negative NB value is release. POST-Baseline is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. BCAA: Sum of the branched-chain amino acids: Valine, Leucine and Isoleucine. EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine; TAA: EAA+ alanine, arginine, asparagine, citrulline, glutamate, glutamine, ornithine, proline, serine, tyrosine. Statistics: Baseline: Different from zero tested with Wilcoxon Signed Rank test, POST: Different
Protein metabolism (Table 5)

In the SPL area we are only able to determine PB. PS and netPS measurement was not possible due to the disposal of the used tracers to other metabolic routes than PS. PA induced sepsis did not change PB in the SPL area.

Liver

Transorgan NB of AAs (Table 8)

Overall the liver is taken up AAs in the BL period and that is diminished in the POST period in the Control group (Figure 2). In contrast with the Control group, the sepsis group tripled the AA uptake in as well the EAA as the non-EAA AA fractions. Normal Glu release is decreased more than 50%. No differences were observed in the BCAA’s NB.

Table 8. Liver amino acid net balances

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Net balance</th>
<th>P_{Wilcoxon}</th>
<th>Control</th>
<th>Sepsis</th>
<th>PT-test</th>
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<tr>
<td>Histidine</td>
<td>217(59)</td>
<td>0.0006</td>
<td>89(110)</td>
<td>230(103)*</td>
<td>0.3692</td>
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<td>Isoleucine</td>
<td>191(62)</td>
<td>0.0053</td>
<td>221(152)</td>
<td>188(53)*</td>
<td>0.8229</td>
</tr>
<tr>
<td>Leucine</td>
<td>42(97)</td>
<td>0.5234</td>
<td>364(158)</td>
<td>524(169)*</td>
<td>0.5166</td>
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<tr>
<td>Lysine</td>
<td>108(75)</td>
<td>0.1464</td>
<td>497(176)*</td>
<td>792(206)*</td>
<td>0.3239</td>
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<td>Methionine</td>
<td>443(67)</td>
<td>&lt;0.0001</td>
<td>-192(90)</td>
<td>-28(84)</td>
<td>0.2061</td>
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<tr>
<td>Phenylalanine</td>
<td>331(87)</td>
<td>0.0006</td>
<td>34(68)</td>
<td>248(81)*</td>
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<tr>
<td>Threonine</td>
<td>307(87)</td>
<td>0.0004</td>
<td>-38(166)</td>
<td>672(145)*</td>
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<td>Tryptophan</td>
<td>106(21)</td>
<td>&lt;0.0001</td>
<td>-77(26)*</td>
<td>4(22)</td>
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<tr>
<td>Valine</td>
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<td>-174(167)</td>
<td>235(206)</td>
<td>0.1665</td>
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<td>Alanine</td>
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<td>1112(535)</td>
<td>3470(1374)*</td>
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<td>15(116)</td>
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<tr>
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<td>-249(124)</td>
<td>329(105)*</td>
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<td>Citrulline</td>
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<td>-152(47)*</td>
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<td>59(225)</td>
<td>1597(317)*</td>
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<td>0.0101</td>
<td>-1230(838)</td>
<td>1501(888)</td>
<td>0.0428</td>
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<td>0.7502</td>
<td>47(164)</td>
<td>-412(208)*</td>
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<td>-52(328)</td>
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<td>-114(80)</td>
<td>253(90)*</td>
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<td>411(453)</td>
<td>1004(202)*</td>
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<tr>
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<td>0.0003</td>
<td>415(1020)</td>
<td>2989(704)*</td>
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<tr>
<td>TAA</td>
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<td>-3.0(6.8)</td>
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Liver net balances (NB) before (Baseline: T = -2 to 0h) and after (POST: T=16 to 18h) start of *Pseudomonas aeruginosa* infusion. Data are expressed as mean(SEM) in nmol/kg bw/min. “Baseline” is net balance before start PM infusion N=22; positive NB value is uptake; negative NB value is release. “POST-Baseline” is difference between POST and baseline period; Control: N=9; Sepsis: N=13; negative value is decrease, positive value is increase. BCAA: Sum of the branched-chain amino acids: Valine, Leucine and Isoleucine. EAA: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine; TAA: EAA+ alanine, arginine, asparagine, citrulline, glutamate, glutamine, ornithine, proline, serine, tyrosine. Statistics: Baseline: Different from zero tested with Wilcoxon Signed Rank test, POST: Different from zero tested with Wilcoxon Signed Rank test, *) p<0.05. Effect of sepsis was tested with Unpaired T-test. Significance different compared to Control, P<0.05 (bold). Tendency: P<0.1

**Discussion**

**Summary data**

The presented acute severe sepsis model is characterized by increased whole body net catabolism and increased plasma EAA concentrations. We tried to find the responsible organs and tissues responsible for the increased whole body catabolism. Although the increased WbRa of mHIS indicates accelerated muscle myofibrillar protein breakdown, we could not detect this by an increased EAA net release and/or PB in the muscle compartment (HQ). This is likely because of increased reincorporation of EAAs from PB back into protein without entering plasma. Protein turnover was reduced in gut tissue, and there was no net release of AA substrates. The liver increased AA uptake substantially, in agreement with the increased urea rate of appearance. Taken together, these data indicate severe tissue protein wasting in this acute severe sepsis phase. We conclude that protein catabolism also occurred in tissues and organs we did not directly measure, as the increased net release of AA substrates from the HQ and PDV cannot explain the observed net catabolism on whole body level and substantial AA substrate wasting by the liver. In addition, disturbed interorgan Glu metabolism with a key role for the liver, suggests a potential role of this AA in the initial sepsis response due to the fact that decreased systemic Glu availability is associated with higher mortality in humans (13).

**Changes in whole body metabolism**

The use of primed continuous tracer infusion approach, the single pool model to describe substrate kinetics (24), makes detection of changes in protein metabolism related substrates in (patho)physiological conditions possible (2, 17, 25). Tracer steady state is an important condition to determine the whole body rate of appearance. However, it can be challenging to keep the enrichment stable during tracer steady state, due to dynamics of the sepsis condition, the rapid and unpredictable changes in the pool size that makes priming very difficult. A long period is needed to achieve a steady state in metabolites and the use of some tracers are more
difficult to achieve a steady state, particularly urea and 3-mHis, a slow turnover rate relative to the pool size or multiple pool compartments (21, 26). Therefore we chose to measure the WbRa in those time ranges in which the animals were metabolically relative stable (16). In addition, instead of taking one single sample at a theoretically time point of steady state, we took multiple samples over time, so we were able to create an enrichment curve and theoretically calculate the tracer enrichment plateau. We determined the tracer steady state plateau value by fitting each individual steady state enrichment curve using one-phase association fitting (see examples in Supplemental figure 1). We believe that this approach improved the interpretation of the data.

In the Control group, whole body metabolism is characterized by reduced systemic AA concentration, WbPB, WbPS and lower muscle PB, but no change in the net catabolic state in the POST period. This is probably caused by the change in nutritional state from early post-absorptive (the BL period was 6-8 hours after the last meal) to an early starvation condition (POST period is 18-28h after the last meal). This downregulation of protein metabolism can be considered as an process of natural adaptation to a new nutritional state wherein availability of energy and protein substrate is limited (27). Additionally we cannot exclude a potential effect of the experimental conditions, such as fluid resuscitation, on metabolism (28). This supports the fact that the Control group is crucial in the study design to be able to discriminate between sepsis and non-sepsis specific changes in metabolism.

The changes we observed in systemic AA changes in sepsis agree with findings in human studies. The elevated Phe and decreased Glu concentrations are characteristic of septic, traumatized or ICU patients (2, 3, 13, 29, 30). Therefore, we conclude that our systemic Phe and Glu observations can be considered as a general phenomenon in critical ill conditions. In addition, in sepsis in the present study, higher WbPB and WbPS resulting in more severe catabolic state and enhanced AA substrate wasting (higher ureagenesis), are in line what we and others observed in septic humans (2, 3, 7). Also the observed higher extrahepatic ureagenesis by arginase in sepsis could well affect the availability of Arg locally in organ/tissues, resulting in a different metabolic fate of Arg (31). Additionally, with the use of 3-mHis tracer we were able to quantify on a whole body level that myofibrillar muscle protein is accelerated in sepsis. Overall, we can conclude that our severe sepsis pig model mimics well the Wb protein metabolism in septic/critically ill humans.

Transorgan AA and protein metabolism in the Control group

In the Control group the HQ showed an increased lysine uptake and a diminished Ala release in the POST period. However, this observation had no relation with HQ PB/PS. Across the PDV, we did not found a difference between BL and POST in net uptake of EAA and TAA. However, the profile of the AA of this uptake changed.
The increase of Leu uptake could indicate an increase in PDV PS, but the PS and PB data obtained from Leu tracer were inconclusive and did not confirm the disposal/production data of the Phe and Tyr tracers. Therefore we conclude that other metabolic pathways in PDV were changed in Control group in the POST period, potentially related to the difference in nutritional state between POST and BL and/or the influence of experimental factors like fluid resuscitation (27, 28). In the SPL area, we observed an overall reduction of uptake of some AA's, but this was not related to overall PS and PB changes in SPL area. Therefore, we conclude that protein turnover didn’t change in the SPL area in the Control group. Overall we conclude that in the Control group, the whole body downregulation of protein metabolism in the post period, most likely caused by changes in nutritional status and fluid resuscitation, cannot be related to PS/PB changes in one single or more individual organs that are measured.

**Transorgan AA and protein during severe sepsis**

In the present study, we tried to study transorgan protein NB to unravel which organs play a key role in the enhanced WbPB and WbPS resulting in an enhanced net catabolism in acute severe sepsis. The systemic WbRa of Phe was used as proxy for WbPB. In the HQ, we did not observe a significant net release of Phe although there was a significant net release of Tyr and Leu and TAA. HQ PS and PB, using 3 different tracers, were inconclusive. This in contrast with the specific myofibrillar muscle WbRa mHIS data. We conclude that enhanced muscle PB is occurring in the HQ but is relative low in the present acute severe sepsis phase in the pig or the reincorporation of AA into protein in the cell is enhanced. Other type of tissues, variability in protein kinetics in different muscle groups in HQ (24) and/or relative small HQ A-V concentration differences in early starvation state could mask the PB in the muscle. Previously described unchanged FSR in muscle in the present cohort of animals (Chapter 10), confirmed the observed unchanged PS in the HQ. Therefore, we conclude that in acute PA induced severe sepsis muscle PB is enhanced, but not sufficiently to explain the observed WbPB (4). In addition, PS in muscle does not contribute to the enhanced WbPS.

The PDV showed a diminishing PB and PS resulting in diminished protein turnover, which is mainly caused by a compromised jejunal mucosa (16)(Chapter 10). This in contrast with earlier observations in models of endotoxin induced sepsis wherein the PDV was preserved (17), indicating that virulent factors coming from bacteria induce a different response on PDV protein metabolism than endotoxin alone. The PDV diminished protein turnover indicates that the PDV is not contributing at all to the enhanced Wb protein turnover.

During sepsis, the liver consumes three times the amount of AAs compared to the Control group accompanied with a three times higher urea rate of appearance. This
indicates severe AA wasting in the phase of acute severe sepsis. The unchanged SPL protein turnover and diminished PDV turnover indicates that liver PB and PS should be enhanced. An enhanced PS is indeed detected as increased fractional synthesis rate in liver tissue (Chapter 10) and is in line with protein acute phase response in sepsis (11). However, unchanged SPL protein turnover also indicates that this part of the body is not contributing to the enhanced WbPB and WbPS. Therefore in general, we conclude that it is still unclear which tissue other than muscle is contributing to the enhanced WbPB and which other tissue than the liver is contributing to enhanced WbPS.

Figure 6 shows an estimation of the contribution of the individual organ PB to WbPB in which we compared the organ Phe production with the WbRa of Phe. We published earlier that WbRa measured with a primed continuous infusion is an underestimation of the real WbRa due to the used non-compartmental assumptions for the data analysis (19). Therefore, we corrected the measured WbRa with a factor 1.6 for this comparison. Overall with this figure and with the presented interorgan metabolism, we conclude that enhanced WbPB and WbPS measured in the systemic plasma pool cannot be explained by significant protein metabolism changes in one single or multiple measured organs, despite prominent changes in muscle and the SPL area protein metabolism. What can potentially explain this discrepancy? Due to influx of bacteria in all tissues and subsequently influx of inflammatory cells in tissue, a local acute phase reaction is initiated (32) resulting in a small local increased PS and PB that can contribute to WbPB and WbPS, but is too small to detect with our organ net balance measurements in post-absorptive/early starvation state where

![Graph showing the contribution of organ Phe production to whole body rate of appearance of Phe (Hypothetical)](image-url)

**Figure 6.** Contribution of organ Phe production to whole body rate of appearance of Phe (Hypothetical)

Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. Height of the bar = whole body rate of appearance of PHE corrected with factor of 1.6 (19). HQ is hindquarter (muscle compartment), HQ*2 = whole body muscle compartment. PDV is Portal drained viscera. LIVER = Splanchnic area – PDV.
A-V differences are relative small. Other more advanced tracer techniques like triple pulse tracer approach (33) could be helpful in future studies to study not only fractional synthesis but also fractional breakdown rates in more different organ/tissues/proteins to unravel the net catabolism phenomenon in severe sepsis. Finally, also changes in plasma protein synthesis and breakdown are suggested to play a role in the septic net catabolism. A severe decrease in the plasma albumin concentration in sepsis related to increased mortality in sepsis (14), are not only caused by redistribution to interstitial fluids but also potentially by diminished albumin synthesis in the liver and enhanced proteolysis or clearance (34). Data of albumin breakdown are very limited, but by measurements of disappearance of isotopic labeled plasma proteins such as albumin could be helpful to study further the contribution of plasma proteins to net catabolism (12).

Glutamate/Glutamine/Alanine interorgan metabolism

Glu, Gln and Ala metabolism are connected and characterized by high compartmentalization in different organs. In healthy situation the organs that have a net production of these AA are in balance with the organs that have a net uptake these AA. Interorgan transport of these substrates are known to be out of balance in certain pathological conditions (35). We found that severe systemic low Glu blood values are related to increased mortality in critically ill humans (13). Glu plays an intermediary role in many metabolic pathways and is therefore potentially critical for an adequate response to defeat bacteremia induced sepsis. In the present study, we observed that the systemic 3 fold decrease of plasma Glu concentration was associated with severely diminished release of Glu from the liver. Complete disappearance of the uptake by PDV preserve Glu availability for the rest of the body. The decreased uptake from the HQ is potentially caused by the decreased systemic availability.

In humans, Gln production by the HQ is considered to be comparable (in balance) with Glu production in the liver(35). However in the present study this is not observed. We observed decreased systemic Gln availability, but in contrast with systemic Glu levels, this was not sepsis related but probably caused by the nutrition state and/or fluid resuscitation because the response was observed in both the controls and septic animals. The SPL area still used the same amount of Gln, however in sepsis more uptake by the liver and less by the PDV was observed. Gln is used as energy substrate in the PDV and as precursor for purine and pyrimidine synthesis, necessary for DNA synthesis in rapidly dividing cells like enterocytes (36). The decreased uptake of Gln in the PDV indicates that beside the down regulated protein turnover, also the energy producing aerobic citrate cycle is down regulated. These results indicate potential compromised intestinal function, although the production of Cit by the PDV, a general gut function parameter (37), was not affected in this septic phase. The enhanced portal availability of Gln to the liver didn’t lead to more Glu
release. It is unlikely, that glutaminase activity that is used for urea synthesis (38), is compromised considering the huge increase of ureagenesis in the present septic state. Also HQ and PDV production of Ala could influence the liver Glu release. However, the substantial enhanced uptake of Ala by the liver, indicating an enhanced Ala-Glu transamination cycle resulting in enhanced glucose production (17) and ureagenesis, didn’t result in normalising the release of Glu by the liver. This indicates that Glu in the liver is routed to other metabolic routes as part/intermediary of an acute phase response to PA and therefore results in less availability for the rest of the body.

Overall we conclude, that in acute severe sepsis, systemic Glu depletion is caused by diminished release of Glu from the liver and enhanced uptake of Glu and Ala by the liver could not prevent this phenomenon. The consequences of the severely reduced systemic availability of glutamate are unclear with regard to the further progression of the pathological septic condition.

Conclusion

We have attempted to unravel the disturbed metabolic pathways that are involved in net catabolism in severe sepsis, by measuring AA substrate fluxes on Wb level and net balances across the PDV, SPL area, liver and HQ in a clinically relevant pig model. With the use of tracers, we established that enhanced WbPB and WbPS measured in the systemic plasma pool could not be explained by significant protein metabolism changes in one single or multiple measured organs, despite prominent changes in muscle and the SPL area protein metabolism. That the PDV is not responsible for the enhanced Wb protein turnover and that beside muscle multiple other organ/tissue/proteins should be involved. Further tracer studies on tissue/protein level might be helpful in future studies to determine this. Finally with the present results, we hypothesize, that in the acute phase of severe sepsis, the overall metabolic downregulated PDV and the compromised liver reflects severe AA substrate wasting, and decreased release of Glu to the whole body, that could not be compensated by interacting Gln and Ala multiorgan pathways, are potentially key metabolic factors for further progression of multi-organ failure in sepsis.

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References


Supplemental Figure

Supplemental Figure 1. Tracer/tracee ratio (TTR) curves. Example of one-phase association fitting of Tracer/Tracee ratio (TTR) curves. Plateau values of curves will be used for steady state enrichment. Before (Baseline) and POST infusion of Pseudomonas aeruginosa. Dash line is Control, solid line is Sepsis group. Control n=9, Sepsis n=13.
SECTION 4

Summary and conclusions
A healthy human body needs nutrients to be kept in a metabolic balance. To keep balance, complex interactions between multiple specialized organs for transportation, digestion, absorption, redistribution, utilization, conversion, breakdown of nutritional substrates during post-prandial and/or post-absorptive stage must occur. These interactions are highly dynamic and are subject to disturbances in many pathophysiological conditions (e.g. sepsis or liver failure) (1-10). To unravel (disturbed) complex nutritional substrate metabolism, quantitative and dynamic substrate flux measurements are needed to support new nutritional strategies in health and disease. To measure complex fluxes/trafficking (amounts in time) of substrates transorgan/interorgan, is extremely difficult in humans due to the multitude of ethical issues and the limitations to perform invasive procedures, especially in pathophysiological conditions (e.g. critical-ill patients). Therefore, clinically relevant animal models are essential to study (patho) physiological interorgan metabolism in a controlled, repeatable way and to be able to use invasive techniques, providing multiple measurements and mechanistic data within the same animal in several nutritional/pathological stages. The pig, with its remarkable similarity to humans, is used often in biomedical research and more generally in the field of nutrition and associated metabolic disease states.

The present dissertation is a reflection of my longstanding experience in metabolic research using a multi-catheterized pig model. I describe interorgan/ transorgan measurement of metabolism of mainly amino acids/protein, their metabolites and interaction with other macronutrients using advanced stable isotope tracer methods in healthy and disease states. The main focus is on a conscious disease model with acute severe sepsis induced by *Pseudomonas aeruginosa* bacteremia. In addition, we present a study in an unconscious devascularized acute liver failure model.

**Section 1: Interorgan balance studies during feeding**

In this section a review is given of the current knowledge of absorption kinetics of protein, peptides and amino acids (Chapter 2) (11). We stated that the small intestine acts as interface and regulator between the gut lumen and the rest of the body and controls the degree and rate of transport of amino acids derived from dietary protein via the portal vein to the liver and the systemic circulation. In a balanced-protein meal, the gut dietary amino acid utilization (30–50%) for protein synthesis will fill a labile protein pool in the gut that can be beneficial during the postabsorptive state.
Despite the absence of different absorption kinetics between protein, peptides, and amino acids, specific attributes can still be beneficial. For instance, when the digestibility function in the gut is compromised (critical ill patients), or in a postexercise situation in which a hydrolysate works insulinotropically. In this review, we have discussed the factors that lead to enhanced, reduced, or modified absorption kinetics. We concluded that to study these factors, a sophisticated multi-catheterized pig model and the use of isotopes to calculate absorption and gut metabolism is necessary. Data obtained from those studies in health and disease condition will support new/improve clinical nutritional strategies.

In Chapter 3 a catheterized conscious pig model is described that is developed to enable measurement of substrate fluxes across organs (12). To ensure that the model was reproducible and stable with a relative short recovery time, meticulous attention was given to optimize recovery conditions and to enhance the long term patency rate of the sampling catheters. Patency rate was probably due to the prevention of catheter-related infections using a gentamicin (20 mg/ml), α-chymotrypsin (225 D/ml) solution as catheter filling. The model enables simultaneous substrate flux measurement across the portal drained viscera, intestine, spleen, liver, kidney and hindquarter in the conscious pig and is used throughout the thesis.

In Chapter 4 and 5, the catheterized pig model is extensively used to study dietary factors that could influence the absorption rates from the lumen to the gut, metabolism of dietary component in the gut, the release of amino acids to the portal circulation from digested protein, the effect on liver metabolism and the resulting systemic availability.

In Chapter 4 we stated that gut health relates to a diet with a high digestibility and quality, but it is unknown what the acute consequences are of low quality foods on gut and liver metabolism (13). Therefore, a meal with the low quality protein gelatin (GEL; tryptophan deficient and low amount of essential amino acids) was compared to a meal with the high quality protein whey (Whey) and a tryptophan supplemented gelatin meal (GEL + TRP) (Figure 1). In the GEL group, the average net portal appearance of amino acids was 99.8 ± 14.6% of the intake versus 61.4 ± 9.0% in the Whey group (Figure 2). Despite the absence of tryptophan in the GEL group, a net portal appearance of tryptophan (42% of the amount in the Whey group) was observed in the GEL group. Intestinal energy metabolism and citrulline production was not affected. Amino acid gut retention for the GEL+TRP group was comparable with the Whey group. We concluded that in a postprandial phase after intake of a low quality protein meal, net anabolism in the healthy intestine is absent. It is likely that the intestine responds with a net breakdown of endogenous (labile) proteins to secure amino acid availability for the body. The addition of the first limiting essential amino acid to this meal (TRP) improved protein anabolism in the intestine. We concluded
Figure 1. Arterial amino acid nitrogen
A: amino acids (AA_N)  B: essential amino acid nitrogen (EAA_N); C: and non-essential amino acid nitrogen (NonEAA_N); concentrations, in µM, in post-absorptive state (Control, n=9) and during 6 hours after administration of Whey protein (Whey, n = 8), gelatin (Gel, n = 9) or gelatin + tryptophan (Gel+TRP, n = 9) meals. Values are mean ± SEM. ANOVA tested, significant meal effect (p < 0.05): (A) protein meals vs. Control; (B) Whey vs. all other test meals; (C) proteins vs. control, Whey vs. Gel groups. Significant interaction (p<0.05): (A) Control vs. protein meals, Gel vs. Whey; (B) Control vs. all protein meals, Whey vs. all test meals; (C) Control vs. Gel groups, Gel vs. Whey.
that protein quality of a meal is related to the anabolic response of the intestine to the meal.

In the last chapter of this section (Chapter 5) we studied the bioavailability kinetics of some food-derived peptides that possess bioactive properties, and may affect health positively (14). We stated that the potential blood pressure lowering C-terminal lacto-tri-peptides Ile-Pro-Pro (IPP), Leu-Pro-Pro (LPP) and Val-Pro-Pro (VPP) (together named here XPP) depends on their availability at the site of action. We hypothesize that the composition of the meal will modify their systemic availability. We studied trans-organ XPP fluxes to determine systemic and portal availability, as well as renal and hepatic uptake of a water-based single dose of synthetic XPP and a XPP containing protein matrix (casein hydrolyte, CasH). In a second experiment, we compared the CasH-containing protein matrix with a CasH-containing meal matrix and the modifying effects of macronutrients (high carbohydrates, low quality protein, high fat, and fiber) in a meal on the availability. Portal availability of synthetic XPP was $0.08 \pm 0.01\%$ of intake and increased when a protein matrix was present. Subsequent renal and hepatic uptake is about 75% of the portal release. In a meal with all macronutrients present, XPP are more portally available, albeit at a low percentage (0.2–0.3%). We concluded that the digestion rate of the meal, the quality of protein, and fiber contents, mainly determine systemic XPP bioavailability after a meal.
Section 2: Use of isotopes in pig sepsis models

In this section, we described a new developed *Pseudomonas aeruginosa* (PA) induced porcine sepsis model as a clinically relevant model for acute severe sepsis (*Chapter 6*) (15). Muscle wasting in sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection in critically ill, has symptoms like gut atrophy and stimulation of liver acute phase response, indicating complex interactions between organs. To study the multi-organ metabolism, in this new sepsis model, bacteremia was induced by intravenous administration of *Pseudomonas aeruginosa* (10⁹ CFU/hour) in 13 catheterized pigs for 18 hours. Both the septic and control animals received fluid resuscitation and were continuously monitored. The systemic inflammatory response (SIRS) diagnostic scoring system

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**Figure 3. Hemodynamics and body temperature**

Hemodynamic response on the *Pseudomonas aeruginosa* (PA) induced bacteremia in the pig. A: MAP is mean arterial pressure. B: MPAP is mean pulmonary arterial pressure. C: Heart rate. D: Body temperature. Control N=9; PA N=13. Values are mean ± S.E.M. Statistics: Two-Way ANOVA comparison; when appropriate a post-hoc Bonferroni multiple comparison test was done; hook or *: p<0.05.

A. MAP: Interaction p<0.0001, Time effect p<0.0001, PA effect p<0.0001.
B. MPAP: Interaction p=0.0021, Time effect p=0.0053, PA effect p<0.0001.
C. HR: Interaction p<0.0001, Time effect p<0.0001, PA effect p<0.0001.
D. Body Temp: Interaction p<0.0001, Time effect p<0.0001, PA effect p<0.0001.
was used to determine the clinical septic state. Within 6 hours after start PA, a septic state developed, as reflected by hyperthermia and cardiovascular changes (Figure 3). The period of 12-18h was characterized by severe sepsis caused by disturbed cardiovascular function, decreased portal drained viscera plasma flow as well as moderate villous injury in the small intestines (Figure 4). No lung, kidney or liver failure occurred during the study period. Significant metabolic changes such as enhanced protein breakdown, hypocalcemia and hypocholesterolemia were found. We concluded that the new *Pseudomonas aeruginosa* induced porcine sepsis model is a clinically relevant model to study metabolic changes during acute severe sepsis and instrumentation enables study of complex multi-organ metabolism.

The use of stable isotopes *in vivo* is considered to be the “gold standard” to study metabolism of substrates throughout the body and tissues to obtain knowledge on the rate of use of these substrates in metabolic pathways (16). Implementation of more advanced models needs to be done carefully. Therefore in Chapter 7, we compared the use of two different stable tracer models to obtain knowledge of whole body protein breakdown in a disease state like severe sepsis (17). The primed-continuous (PC) phenylalanine (PHE) stable isotope infusion methodology is often used as a proxy for measuring whole body protein breakdown (WbPB) in sepsis. It is unclear, if WbPB data obtained by an easy-to-use single intra-venous PHE isotope pulse administration (PULSE) are comparable to those by PC. The non-compartmental analysis with the PC is greatly limited by the necessary assumption that production appears in the sampled compartment (i.e. plasma) into which the tracer is also administered (16) (Figure 5). This is not the case for amino acids. Therefore, compartmental modeling with the decay curve of the PULSE could provide us more insight in the real WbPB in sepsis. Seventeen hours after sepsis induction, we compared the Whole body rate of appearance (WbRa) of PHE obtained by PC (L-[ring-13C_6]-PHE) and PULSE(L-[15N]-PHE) in arterial plasma using LC-MS/MS and (non)-compartmental modeling. PULSE-WbRa was highly correlated with PC-WbRa and WbPB independent of the septic state (Figure 6). PULSE-WbRa was 1.6 times higher than PC-WbRa. Compartmental and non-compartmental PULSE modeling provided comparable WbRa values, although compartmental modeling was more sensitive. Both PULSE-WbRa and PC-WbRa are an underestimation of the real WbPB. WbPB was elevated in sepsis. With PULSE, sepsis was characterized by an increase of the metabolic shunting (amount of PHE flux between intra- and extra-cellular pool that is not irreversible lost). Membrane transport capacity was the same. We concluded that both PC and PULSE-methods are able to assess changes in WbRa of plasma PHE reflecting WbPB changes with high sensitivity, independent of the (patho-) physiological state. The WbRa obtained from the easy-to-use (non-) compartmental PULSE reflects better the real WbPB than PC-WbRa. With PULSE compartmental analysis, we conclude that the membrane transport capacity for amino acids is not compromised in severe sepsis.
Figure 4. Organ Plasma flow

Organ plasma flows changes before and after *Pseudomonas aeruginosa* (PA) induced bacte-remia in the pig. “Pre” is average plasma flow one hour before induction bacteremia. “Post” is average plasma flow between 14 and 18 hours after start induction bacteremia. Control N=9; Sepsis N=12. Values are mean ± S.E.M.. Statistics: Two-Way ANOVA repeated measures for time, when appropriate a post-hoc Tukey multiple comparison test; hook: p<0.05.

A. Portal drained viscera: Interaction p=0.0008, Time effect p=0.5682, PA effect p=0.1297
B. Splanchnic area: Interaction p=0.7225, Time effect p=0.2849, PA effect p=0.2992
C. Hindquarter: Interaction p=0.6024, Time effect p<0.0001, PA effect p=0.2992
Figure 5: Calculation models for whole body protein breakdown

Two calculation models for whole body protein breakdown (WbPB) by determination of whole body rate of appearance (WbRa) of phenylalanine (Phe). (A) Non-compartmental model using a primed-continuous infusion of L-[ring-13C6]-Phe (PC). WbRa is the tracer infusion rate divided by the fraction of tracer found in plasma/extra cellular pool (TTR=tracer/tracee ratio) in a tracer steady state (B) Compartmental model using bolus infusion, L-[15N]-Phe (PULSE) and SAAMII computer modeling. Computer parameters: Q= pool sizes of the compartments; $k_{21}$= rate parameter to pool 2, from pool 1; $k_{02}$ rate parameter of irreversible loss from pool 2; $U_2$: rate of appearance of Phe in pool 2. (C) Physiological assignment of SAAMII computer model: Flux ($F_{12}$) of Phe from the intra-cellular pool to the extra cellular pool represents the $k_{21}$ times $Q_1$ and is equal to $F_{21}$ in physiological steady state; $U_2$ ($=F_{20}$) represents WbPB; Flux of Phe from the intra-cellular pool to hydroxylation and protein synthesis represents the irreversible loss ($F_{02} = k_{02}Q_2$); WbRa of Phe is the fraction of Phe from protein breakdown that appears in $Q_1$ that is not irreversible lost; The amount of Phe flux between $Q_1$ and $Q_2$ that is not irreversible lost is shunt back to $Q_1$ ($F_{21}$-WbRa).
Section 3: Interorgan balance studies in disease models.

In this section we described the use of stable tracers to unravel different metabolic routes in disease states. Chapter 8 and 9 discusses arginine-nitric oxide metabolism in a model of acute liver failure in unconscious pigs. Chapter 8 provides a review of existing literature considering the different aspect of arginine de novo and nitric oxide (NO) production in disease states (18). Arginine is a conditionally essential amino acid in healthy adults, and therefore there is no specific...
nutritional requirement. However, dietary arginine is required in neonates, infants, and in certain conditions and diseases. L-arginine was identified as the precursor for NO. Although first discovered in endothelial cells, NO appeared to be a ubiquitous molecule present in a variety of cells including cells from the cardiovascular and nervous systems and also inflammatory cells. As such, NO has many physiological functions, and the relationship between arginine availability and NO production emphasizes the functional relevance of arginine.

In Chapter 9, we studied the arginine-NO metabolic route in acute liver failure (ALF) in the pig, because the hyperdynamic circulation is believed to be the result of overproduction of nitric oxide (NO) in the splanchnic circulation (19). However, it has been suggested that arginine concentrations (the substrate for NO) are believed to be decreased in ALF, limiting substrate availability for NO production. To characterize the metabolic fate of arginine in early-phase ALF, we systematically assessed its interorgan transport and metabolism and measured the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) in a porcine model of ALF. Female adult pigs (23–30 kg) were randomized to a sham or hepatic devascularization ALF procedure for 6 h. We measured plasma arginine, citrulline, ornithine levels; arginase activity, NO, and ADMA (Figure 7). Whole body metabolic rates and interorgan flux measurements were calculated using stable isotope-labeled amino acids. Plasma arginine decreased >85% of the basal level at \( t = 6 \) h, whereas citrulline and ornithine progressively increased in ALF. No difference was found between the groups in the whole body rate of appearance of arginine or NO. However, ALF induced a significant increase in \textit{de novo} arginine synthesis. Interorgan data showed that citrulline net intestinal production and renal consumption was related to net renal production of arginine and ornithine. Both plasma arginase activity and plasma ADMA levels significantly increased in ALF. In this model of early-phase ALF, arginine deficiency or higher ADMA levels do not limit whole body NO production.
Figure 7. Plasma arginine (A) concentration, de novo arginine (B), Plasma ornithine (C) and plasma arginase activity (D) in Sham-operated and acute liver failure (ALF) pigs throughout the experimental period.

There is a significant increase in the plasma ornithine levels with a corresponding significant increase in the plasma arginase activity at the end of the experimental period. *) p < 0.05; ***) p < 0.001; 2-way ANOVA. Data shown are means ± SE.
Therefore, we conclude that arginine deficiency is caused by arginase-related arginine clearance in which arginine production is stimulated de novo.

In Chapter 10 and 11 we studied multi-organ metabolic disturbances in the Pseudomonas aeruginosa (PA) induced severe sepsis model. Maintenance of gut integrity has long been recognized as crucial for survival in sepsis but alterations in gut protein metabolism have not previously been documented. Therefore in Chapter 10, we studied the effect of a PA induced sepsis, on fractional protein synthesis (FSR) and breakdown rates (FBR) in jejunal mucosa in a fasted conscious state. We determined FSR by the measurement of the incorporation of stable isotope labeled amino acid (primed continuous infusion, L-[ring-13C6]-phenylalanine) into tissue protein, and FBR using the relation between blood arterial enrichment and intracellular enrichment in consecutive mucosal biopsies after a pulse of L-[15N]-phenylalanine. Additionally, we determined the FSR of other splanchnic tissue (jejunum, ileum, liver) and compared them with muscle and lung tissue. We found in this sham controlled acute severe sepsis pig model that jejunal mucosal protein turnover is reduced with both decreased FSR and FBR (Figure 8). We also found that FSR was unchanged in ileum and muscle, while it was higher in the liver and tended to be higher in the lung (Figure 9. In conclusion, our data suggest that jejunal mucosal protein metabolism is diminished in acute severe sepsis. Comparison with other tissues indicates that the most serious acute changes in severe sepsis occur in the gut rather than muscle.

In Chapter 11 inter- and multi-organ amino acid (AA) kinetics in sepsis was studied. We studied in our septic pig model the metabolic pathways that are involved in net catabolism in severe sepsis, by measuring AA substrate fluxes with multiple stable amino acid tracers on whole body (Wb) level by measuring rate of appearances (Ra)
of AA, and AA net balances (NB), PB and protein synthesis (PS) across the portal drained viscera (PDV), splanchnic area (SPL), liver and hindquarter (HQ). We measured before (baseline; BL) and 12 to 18 hours (POST) after start of sepsis induction by PA. We established in this sham controlled acute severe sepsis pig model that enhanced WbPB, WbPS, Wb net catabolic state and myofibrillar muscle breakdown occurs in sepsis. Across PDV, both PB and PS are decreased. The liver showed substantial increased uptake (3 times higher than control) of amino acids and severe increase of ureagenesis. Overall the SPL and HQ NB could not explain the enhanced whole body PB (Figure 10). Interacting glutamate, glutamine and alanine multiorgan pathways showed that severe systemic glutamate decrease (50% of Control), in humans related to enhanced mortality, was caused by decreased glutamate release of the liver to the systemic plasma pool that could not be compensated by interacting glutamine and alanine multiorgan pathways (Figure 11). We conclude that whole body enhanced protein turnover and net catabolism cannot be explained by significant changes in protein metabolism in one single or multiple measured organs, despite prominent changes in muscle and the SPL area protein metabolism. The PDV is not responsible for the enhanced whole body protein turnover and thus other organ/tissue/proteins must be involved. Further tracer studies on tissue/protein level will be helpful to delineate this. On the basis of the present results we hypothesize that in the acute phase of severe sepsis, the overall metabolic downregulated PDV and the compromised liver are key metabolic factors in sepsis.
Figure 10. Contribution of organ Phe production to whole body rate of appearance of Phe (hypothetical)
Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. Height of the bar = whole body rate of appearance of PHE corrected with factor of 1.6 (17). HQ is hindquarter (muscle compartment), HQ*2 = whole body muscle compartment. PDV is Portal drained viscera.

Summary of conclusions

- To study factors that lead to enhanced, reduced or modified absorption kinetics, a sophisticated model in multi-catheterized pigs along with the use of isotopes that can calculate absorption and gut metabolism is necessary.

- Data coming from a catheterized model that enables simultaneously liver, portal drained viscera, spleen, liver, kidney, and hindquarter flux measurement of many metabolic substances in the pig are highly translational to humans due to the remarkable physiological similarity of the pig to humans.

- Protein quality of a meal is related to the postprandial anabolic response of the intestine.

- Systemic bio-availability of potential bioactive lacto-peptides are mainly determined by the digestion rate of the meal, the quality of protein, and fiber contents.

- The presented *Pseudomonas aeruginosa* induced porcine sepsis model is a new clinically relevant model for acute severe sepsis and instrumentation enables study complex multi-organ metabolism.

- The whole body rate of appearance of phenylalanine in plasma obtained by the easy-to-use (non-)compartmental stable tracer pulse infusion approach, reflects better the real whole body protein breakdown than the traditional primed-continuous infusion.
Figure 11. Glutamate arterial concentrations and organ net balances (NB)
Before (Baseline) and post infusion of *Pseudomonas aeruginosa*. HQ is hindquarter (muscle compartment), PDV is Portal drained viscera, SPL is splanchnic area. Control n=9, Sepsis n=13. Statistics: unpaired Student t-test, Significance p<0.05 (hook), Tendency p<0.1 (dashed hook).
• Membrane transport capacity for amino acids is not compromised in severe sepsis.

• Nitric Oxide (NO) has many physiological functions, and the relationship between arginine availability and NO production emphasizes the functional relevance of arginine.

• In early-phase of acute liver failure, arginine deficiency or higher ADMA levels do not limit whole body NO production. Therefore, we conclude that arginine deficiency is caused by arginase-related arginine clearance in which arginine production is stimulated de novo.

• Jejunal mucosal protein metabolism is diminished in acute severe sepsis. Comparison with other tissues indicates that the most serious acute metabolic changes in severe sepsis occur in the gut rather than the muscle.

• Enhanced whole body protein turnover and net catabolism in acute severe sepsis cannot be explained by significant protein metabolism changes in one single or multiple measured organs, despite prominent changes in muscle and the splanchnic area protein metabolism. We hypothesize that in the acute phase of severe sepsis, the overall metabolic downregulated portal drained viscera and the compromised liver are key metabolic factors.

Future perspectives

Multiorgan and transorgan research

In the present dissertation, I presented several examples of multiorgan and transorgan metabolic research. Presented data illustrates an process of ongoing insight in the complex dynamics of nutritional substrate fluxes in (patho)physiological conditions, development of new and improved approaches of measurement due to the availability of advanced and improved analytical and tracer modeling technologies. However depending on the scientific question, future improvements like measurement of other organs, tissues, proteins, macronutrients, disease states with a variety of tracer models other than described in this dissertation, could be considered to continue the process of unraveling quantitatively highly compartmentalized, dynamic (disturbed) substrate metabolism and potential testing of new nutritional interventions.

Organs

Data showed that transorgan and multi-organ substrate flux measurements play an important role to understand whole body metabolic balances. It is clear that both
in a health or disease state the gut and liver have a crucial role in the systemic bioavailability of nutrients which is highly dynamic and complex. Our knowledge about substrate metabolism and trafficking across the lung is however very limited and need to be considered in future studies, especially in disease states in which complex multiorgan substrate trafficking and lung and multiorgan failure is expected. We think that can be done by implantation of a Swan Ganz catheter to accommodate afferent blood sampling (pulmonary artery) for the lung.

**Tissues and proteins**

For protein metabolism, measuring absolute substrate balances across organs is the gold standard to understand protein breakdown and synthesis in certain areas, but it can be a challenge to obtain valid measurements in organs with a high level of metabolism and blood flow where arterial venous differences are small. Therefore, additional fractional synthesis and breakdown rate measurement on tissue/protein level could provide extra information with the use of for instance a triple pulse measurement (20).

**Macronutrients**

We showed the interacting role of other macronutrients like carbohydrates and fats during feeding on protein/peptide/amino acid absorption kinetics. However, in carbohydrate and fat metabolism, knowledge of interorgan dynamics are still limited (21-23). For instance, our knowledge of interorgan dynamics of carbohydrate and fat metabolism can be expanded by unraveling disturbed organ metabolism in diseases like obesity or metabolic syndrome and develop new/improved nutritional strategies.

**Nutritional intervention**

In disease states like acute severe sepsis, we showed major changes in the splanchnic area (low mucosal protein turnover, high amino acid wasting in the liver) indicating that early support of gut metabolism should be imminent to prevent or attenuate gut dysfunction. In the current clinical practice, early provision of enteral nutrition via the gastrointestinal tract to critically ill patients has been established as a standard marker of quality of care for critically ill patients (7, 24). However, due to complexity of the disease states (often the occurrence of multi-organ failure) in these patients, there is still a lot of challenges to overcome for adequate diagnostics (e.g. personalized requirements to determine dosing), adequate delivery (e.g. volume in time when gastro-intestinal intolerance occur), and optimal composition (e.g. the use of supplementation of specific micro and/or macro nutrients). Therefore, we conclude that multorgan and transorgan measurements will be a valuable necessity in future (clinical) metabolic/nutritional research to collect supporting information for new/improved clinical nutritional strategies.
Role of the pig as multi-organ and transorgan model in metabolic research

Although the use of an animal for human (clinical) research can be controversial, experiments in animal models are relevant and essential, due to the fact that it is extremely difficult to perform studies in human subjects to the multitude of ethical issues and the limitations of invasive procedures. However, the principles of the 3Rs (reduction, refinement, replacement) are ongoing considerations for future studies.

Reduction
As described in the present dissertation, the pig is a clinically relevant large animal model to study multiorgan metabolism, but the invasive multi-catheterization that is required for this, demands for intensive post-surgical care (chapter 3) and highly skilled personnel. However, the surgical implantation of the catheters, easy adaptation of the pig to a small cage when performing experiments and the size of the animal ensures that we can measure over time and in a physiological relevant conscious condition in the post-absorptive and/or postprandial state. With the implementation of the use of existing and new commercial available stable tracers and new combined multiple stable tracer measurements with diverse “high throughput” mass spectrometry applications, we have the opportunity to study more scientific questions simultaneously in the same animal and therefore reduce the amount of animals needed.

Refinement
Despite widespread discussions in literature, the perfect clinically relevant animal model does not exist. Most of the time, concessions need to be made for practical and ethical reasons. For instance, with the presented pig model (20-30kg, female), you could argue that the pig is still very young (and female only) and therefore is not representing the humans to whom we want to translate the data to. We made these concessions for practical reasons (handling, catheter protection with the used harness), but with the knowledge that the development stage of the gastrointestinal tract in these animals is already mature (25). Therefore, we can still translate the obtained data to a broader age range. Nevertheless, if older age is critical for the scientific question to solve, the presented catheter model could also be used in long-term studies in older minipigs. Future efforts to refine for instance disease animal models like sepsis as described in chapter 6 and liver failure in chapter 9, will increase the quality of the data deducted from these studies and therefore its translational validity.

Replacement
Ongoing development of new multi stable tracer model approaches and technologies that are able to measure on systemic level, different organ related metabolic
fluxes in for instance the splanchnic area (digestion, gut absorption, gut extraction, liver extraction of amino acids) simultaneously, can be translational to humans and could therefore potentially be replaced in humans. The pig could play a crucial role to validate these new models.

General conclusion

With the use of a unique highly clinical relevant catheterized pig model in health and certain disease states like severe sepsis and acute liver failure, we are able to unravel complex interaction between multiple specialized organs for transportation, digestion, absorption, redistribution, utilization, conversion, breakdown of nutritional substrates during post-prandial and/or post-absorptive stage(s). Presented results indicates the complex interactions of organs in the splanchnic area are highly dynamic. In feeding condition, food macronutrient composition and quality (e.g. amino acid profile of the used protein) are major factors in splanchnic utilization and systemic bioavailability. In pathophysiological conditions like acute severe sepsis the splanchnic area is highly vulnerable and therefore more quickly disturbed than muscle compartments. Together with ongoing new developments in the use of stable tracer models, the multi-organ metabolic research in the pig will continue to be of high value in the future for quantitative substrate flux measurements that are needed to support new nutritional strategies in health and disease to keep our body in nutritional balance.

References

SUMMARY AND FUTURE PERSPECTIVES

CHAPTER 12


Samenvatting


Dit proefschrift is een weergave van stofwisselingsonderzoek, met name aan varkens met acute bloedvergiftiging veroorzaakt door de Pseudomonas aeruginosa bacterie. Door middel van hoogwaardige stabiele tracer methoden wordt het stofwisselingstransport van voornamelijk aminozuren en eiwitten, hun metabolieten en de interactie met andere macronutriënten op (inter)orgaan niveau toegedicht. Daarnaast presenteert dit proefschrift een studie van een onder anesthesie gedevasculariseerd leverfal model.

Deel één brengt de huidige kennis van absorptie(snelheden) van eiwitten, peptiden, en aminozuren in kaart (hoofdstuk 2) (11). De dunne darm gedraagt zich als een intermediair tussen het darmlumen en de rest van het lichaam. Het transport van aminozuren uit een eiwitmaaltijd wordt gecontroleerd en gereguleerd door de dunne darm. Vervolgens gaan de aminozuren via de vena porta naar de lever en de grote circulatie. Na inname van een gebalanceerde eiwitmaaltijd gebruikt de darm 30-50% van de aminozuren uit de voeding voor opbouw van een tijdelijke eiwitopslagplaats die vervolgens gebruikt kan worden in de nuchtere toestand. Ook de afwezigheid van een verschil in absorptie van eiwitten, peptiden en aminozuren
komt aan bod; andere factoren leiden tot veranderingen in absorptie kinetiek van deze voedingsstoffen. Om het effect van deze factoren te bestuderen op de darmstofwisseling is een geavanceerd gekatheteriseerd varkensmodel nodig dat gebruik maakt van stabiele tracers. Hoofdstuk 3 beschrijft zo’n model dat gelijktijdig de substraattransporten over de lever, portaal gedraineerde organen (darm, milt), de nier en de achterbenen (welke voornamelijk bestaan uit spieren) meet (12). De stofwisselingsgegevens afkomstig van dit varkensmodel kunnen gemakkelijk vertaald worden naar die van de mens. Verder laten hoofdstuk 4 en 5 zien dat de kwaliteit van het eiwit in een maaltijd bepalend is voor hoeveel eiwitopbouw er uiteindelijk plaatsvindt in de darm (hoofdstuk 4) (13). Tenslotte toont dit proefschrift dat voornamelijk de verteringssnelheid en kwaliteit van het eiwit in de voeding, en de aanwezigheid van vezels de beschikbaarheid in de grote circulatie van potentieel bioactieve lacto-peptiden bepalen (hoofdstuk 5) (14).

Deel twee beschrijft een nieuw (door Pseudomonas aeruginosa bacterie veroorzaakt) varkens bloedvergiftigingsmodel dat bruikbaar is als een nieuw klinisch relevant model voor acute ernstige sepsis. Het inbrengen van de verschillende katheters in dit model geeft de mogelijkheid om complexe orgaanstofwisselingsroutes in dit ziektebeeld te bestuderen (hoofdstuk 6) (15). Hoofdstuk 7 betreft de vergelijking van twee stabiele tracermethoden die gebruikt kunnen worden om eiwitafbraak op heel lichaamsniveau te meten. De conclusie luidt dat de mate van verschijning van het aminozuur phenylalanine in het bloed met behulp van een eenmalige tracer-pulse injectie een betere waarde geeft van de echte eiwitafbraaksnelheid dan een meer traditioneel gebruikte methode waarbij de tracer met een constante snelheid wordt geïnfundeerd (16). Bovendien blijkt de membraan transportcapaciteit voor aminozuren niet verminderd bij ernstige sepsis.

Deel drie beschrijft het gebruik van stabiele tracers om verschillende stofwisselingsroutes in ziektebeelden te kunnen ontrafelen. Een review beschrijft de vele fysiologische functies van stikstofoxide (nitric oxide, NO) en de relaties tussen de NO productie en de beschikbaarheid en functionele relevantie van het aminozuur arginine (bouwstof van NO) (hoofdstuk 8) (17). Hoofdstuk 9 toont dat in een vroege fase van acuut leverfalen het aminozuur arginine deficiënt is en de waarde van asymmetrisch dimethylarginine (ADMA, endogene NO synthese remmer) verhoogd. Dit resulteert echter niet in een verminderde NO productie. Uit grondige bestudering van de stofwisseling tussen organen blijkt dat de arginine deficiëntie veroorzaakt wordt door een verhoogde arginase activiteit buiten de lever, terwijl de NO productie op peil blijft door een verhoogde arginine productie in de nier (18).

Hoofdstukken 10 en 11 laten zien dat de eiwitstofwisseling in de darmmucosa verminderd is bij acute ernstige bloedvergiftiging. Vergelijking van deze resultaten met die van andere weefsels toont dat de grootste veranderingen in de stofwisseling
tijdens bloedvergiftiging plaatsvinden in de darm en niet in de spier (hoofdstuk 10). Ook blijkt dat de verhoogde eiwitstofwisseling en afbraak op lichaamsniveau niet verklaard kunnen worden door de bestudeerde veranderingen in stofwisseling in de organen, ondanks duidelijke veranderingen gevonden in de spiereiwitstofwisseling en aminozuurstofwisseling in het darm-lever gebied (hoofdstuk 11). De conclusie op basis van de resultaten beschreven in het proefschrift luidt dat in de acute fase van ernstige bloedvergiftiging de stofwisselingsvertraging in de darm en de verstoorde leverstofwisseling belangrijke metabole factoren zijn in het verdere beloop van de aandoening.

**Conclusie**

Het beschreven klinisch relevant gekatheteriseerd varkensmodel stelt onderzoekers in staat om inzicht te krijgen in de stofwisseling in gezonde toestand en tijdens ziekte zoals ernstige bloedvergiftiging en acuut leverfalen. De beschreven resultaten laten zien dat er complexe relaties zijn tussen de organen in het darm-lever gebied en dat deze relaties zeer dynamisch zijn. In gevoede toestand zijn de samenstelling en kwaliteit van macronutriënten in voeding (zoals de aminozuursamenstelling van het eiwit) belangrijke factoren die de beschikbaarheid van voedingsnutriënten in de circulatie beïnvloeden. In pathofysiologische condities zoals tijdens ernstige bloedvergiftiging is het darm-lever gebied zeer kwetsbaar en de stofwisseling hier eerder verstoord dan in een spier compartiment. In combinatie met nieuwe stabiele tracermethoden zal het metabool onderzoek op (inter) orgaan niveau in het varken nieuwe belangrijke inzichten blijven genereren in de toekomst. Kwantitatie en dynamische transportmetingen van substraten zijn noodzakelijk om nieuwe voedingsstrategieën tijdens ziekte te ontwikkelen met als doel de stofwisseling in balans te houden.

**Referenties**


CHAPTER 14
Valorization

A healthy human body needs nutrients to sustain its metabolic balance. To this end, complex interactions between specialized organs for transportation, digestion, absorption, redistribution, utilization, conversion, breakdown of nutritional substrates during post-prandial and/or post-absorptive stages must occur. These interactions are highly dynamic and subject to disturbances in many pathophysiological conditions (e.g. sepsis or liver failure) (1-10). To unravel (disturbed) complex nutritional substrate metabolism, quantitative and dynamic substrate flux measurements are needed. This knowledge will support the development of new nutritional strategies in health and disease, which are needed in light of many major worldwide nutritional problems:

World Health Organization (WHO) malnutrition factsheet (May 2017) (11):
Malnutrition, in all its forms, includes undernutrition (wasting, stunting, underweight), inadequate vitamins or minerals, overweight, obesity, and resulting diet-related non-communicable diseases.

• 1.9 billion adults are overweight or obese, while 462 million are underweight.

• 52 million children under 5 years of age are wasted, 17 million are severely wasted and 155 million are stunted, while 41 million are overweight or obese.

• Around 45% of deaths among children under 5 years of age are linked to undernutrition. These mostly occur in low- and middle-income countries. At the same time, in these same countries, rates of childhood overweight and obesity are rising.

• The developmental, economic, social, and medical impacts of the global burden of malnutrition are serious and lasting, for individuals and their families, for communities and for countries.

Malnutrition occurs in all countries and is considered one of the greatest global health challenges. Optimizing nutrition needs to start early in life to ensure long-term benefits. Poverty is considered a major risk factor for malnutrition and subsequently increases health care cost, reduces productivity and slows economic growth. Led by WHO and the Food and Agriculture Organization of the United Nations (FAO), the UN Decade of Action on Nutrition calls for policy action across several key areas. One of the areas is creating sustainable, resilient food systems for healthy diets. A healthy diet is a very generic term, and usually defined in terms
of the needs of the organism, i.e. metabolic demands, and the dietary amount which will satisfy those needs, i.e. efficiency of utilization, thus: dietary requirement = metabolic demand/efficiency of utilization (12). Therefore, an appropriate diet in health is different from that in disease. For instance, we recently published that appropriate clinical nutrition is lowering mortality in hospitalized adults (7). In critically ill patients, muscle wasting is a life-threatening organ dysfunction that can be treated with appropriate nutrition (13, 14). To determine the “metabolic demand” and “efficiency of utilization”, understanding of nutritional substrate metabolism, quantitative and dynamic substrate flux measurements in health and disease are needed and is the general focus of this dissertation.

Measuring complex fluxes and trafficking (amounts in time) of substrates in and between organs, is extremely difficult in humans due to the multitude of ethical issues and the limitations to perform invasive procedures, especially in pathophysiological conditions like in critically ill patients. Therefore, clinically relevant animal models are essential to study (patho) physiological metabolism in a controlled, repeatable way and to be able to use invasive techniques, providing multiple measurements and mechanistic data within the same animal in several nutritional and pathological stages. The pig, with its remarkable metabolic similarity to humans, is used often in biomedical research and more generally in the field of nutrition and associated metabolic disease states. Therefore the presented highly translational pig experiments generated important information that can be used for multiple purposes in the nutrition field.

We are aware that translational preclinical studies in the present dissertation are the “baby steps” in the journey to develop products and activities to solve worldwide nutritional health problems. However, the fact that eight of the ten described research projects in the present dissertation are published and received 184 citations (status 19th of July 2017) indicates the value for the scientific community (15). Besides the scientific recognition, the societal relevance of this research was also recognized by the food industry, the ministry of economic affairs (The Netherlands) and the National Institutes of Health (USA). The findings of this dissertation can be used and translated in multiple ways for the food industry (development new products, improve consumers information), agriculture and environmental field (better understanding dietary protein quality in relation to meat production and emissions of nitrogen), clinical field (new food strategies, improvement design of clinical trials, better understanding of disturbed metabolism in critically ill patients, new clinical food products) and policy makers for general public (improved healthy food definitions/ claims/ information, importance of a healthy gut).
Here an example:

In section 1 we presented research that shows the fate of amino acids (building blocks of protein) that are coming from dietary protein. We found that a high quality protein meal is healthy for your gut. In contrast with a low quality protein meal that breaks down the gut. Although these scientific findings were published a while ago, the discussion of dietary protein is currently a hot topic in the food social media. Online and on packages in the supermarket consumers are overwhelmed with beneficial health claims ("super foods") of protein. This however is misinformation if the protein involved is of low quality. For instance a gelatin pudding with 10% of low quality protein has no health benefits, this in contrast with 10% of high quality protein in yogurt. The “nutrition facts” sheet of a food package contains information of different types of fat and carbohydrates but protein quality grades are still missing. Therefore, I strongly recommend policy makers (US Food & Drug Administration, European Food Safety Authority) to add the quality grade of the used food proteins to the “Nutrition Facts” sheet of a food package to ensure consumers are better informed and can make healthier choices.

In section 2 and 3 we presented with innovated techniques and preclinical models, new insights of how metabolic substrates are changed in critically ill pigs. The role of gut and liver metabolism on the disturbed whole body metabolism was studied in detail. This information gives physicians and (specialized) dieticians more knowledge how metabolic substrates behave in critically ill patients, which cannot be obtained with human research. These data are sound information for further design, planning and development of randomized controlled trials by physicians, specialized dieticians, scientists and the clinical nutrition industry, to improve nutritional interventions and strategies in critically ill humans.

References


Biography

Gabriëlla Adriana Maria ten Have was born on the second of March 1964 in Vleuten-De Meern, The Netherlands. After high school graduation (VWO) in 1982 at the St. Bonifatius College in Utrecht, she began her advanced leveled professional research laboratorium education (HLO/Life Science, BSc) at Dr. Ir. W.L. Ghijsen institute (Utrecht, The Netherlands) and graduated in 1986. Although she had a broad interest in multiple disciplines (i.e. chemistry, medicine, physics, computer science), she specialized in biology, which gave her a strong fundamental education in conducting scientific research in the biomedical field.

During her nine-month internship in the research group of Prof. dr. F.P. Nijkamp at the Dept. of Pharmacology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands, she had the opportunity to develop her professional skills by working on a pharmacological in vitro receptor detection method, which resulted in her first scientific publication (1). In 1986, she acquired her molecular biology skills as research assistant of Dr. A. Berns at the Dept. of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands. In 1987, she continued her research on pharmacological interactions of immune cells with endothelium in endotoxin disturbed pathophysiological conditions in animals using in vitro measurements. Together with Dr. A.J. van Oosterhout and Dr. D. van Heuven-Nolsen, she was involved in the validation, conduct, and design of new in vitro animal models, which resulted in four publications (2-5).

Her appointment in 1990 as research assistant at the Metabolic Research Centre (MRC) of Dr. N.E.P. Deutz and Prof. dr. P.B. Soeters at the Dept. of Surgery, Faculty of Medicine, Maastricht University, The Netherlands, gave her the opportunity to expand her research experience in the preclinical field. She worked with complex in vivo research models using large instrumented animals (pigs), and was heavily involved in the quantitative analysis of metabolic pathways, using isotopic tracers, and metabolic substrates by advanced analytical technology (HPLC and LC-MS/MS). This work enhanced her ambition to conduct multidisciplinary translational research. She improved a catheterized in vivo pig model that is used for transorgan measurements of macronutrient fluxes, and introduced stable tracer models in multiple (patho)physiological clinical relevant conditions in international collaborative research (6-15). Alongside the multi-organ pig model, she developed with Dr. Deutz a multi-organ metabolic flux model in mice. She was involved in the design and conduct of multiple (transgenic) in vivo mice/rat studies in (patho) physiological conditions with and without use of stable isotope metabolic tracer models in collaboration with (inter)national researchers (16-22). In the 25 years of research
with Dr. Deutz, she gained metabolic research expertise which involved writing of scientific reviews, developing specific LC-MS/MS lab analytical methods, validation of new stable isotope model approaches, and developing a new clinically relevant sepsis model in the pig. Furthermore, she collaborated in human studies that involved stable isotope methods (23-28).

Ten Have’s longstanding research experience gave her strong leadership, mentoring, and teaching skills which resulted in the present dissertation. In 2007, she moved with the metabolic research group of Dr. Deutz to the Center for Translational Research on Aging & Longevity (CTRAL), at the Donald W. Reynolds Institute on Aging, University of Arkansas for Medical Sciences, Little Rock, AR, United States. In 2012, she subsequently moved to her current CTRAL employment at the Dept. of Health and Kinesiology, Texas A&M University, College Station, TX, where she got the opportunity to gain further experience in managing and designing multidisciplinary laboratories. Furthermore, she was involved in the design and construction of the new Human Clinical Research Facility on Texas A&M University campus in 2016. She is currently residing with her family in Montgomery, TX, United States. She aspires to become an independent researcher in the preclinical critical care metabolic research field.
Publications


Position and Employment

1986 – 1987: Research assistant at the Department of Molecular Biology, NKI (Netherlands Cancer Institute) in Amsterdam. The Netherlands.

1987 – 1990: Research assistant at the Department of Pharmacology, faculty of Pharmacy, Utrecht University. The Netherlands.

1990 – 2003: Research assistant at the Metabolic Research Centre (MRC) of Dr. N.E.P. Deutz of the Department of Surgery, faculty of Medicine, Maastricht University, The Netherlands.
2003 – 2007: Senior Instructor, at the Metabolic Research Centre (MRC) of Dr. N.E.P. Deutz of the department of Surgery, faculty of Medicine, Maastricht University, The Netherlands.

2007 – 2012: Research associate, Center for Translational Research on Aging & Longevity. Donald W. Reynolds Institute on Aging. University of Arkansas for Medical Sciences, Little Rock, AR, USA

2012 – : Senior Research Associate, Center for Translational Research on Aging & Longevity. Dept. Health and Kinesiology, Texas A&M University, College Station, TX, USA

Honors

2012 ESPEN Research Fellowship Grant 2012

Oral en Poster presentaties:

1. Glutathione (GSH) Absolute Synthesis Rates (ASR) of Multiple Organs in a Pseudomonas aeruginosa (PM) induced Hyperdynamic Sepsis Pig Model, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 30 (1 Supplement), 742.17-742.17

2. Severely Compromised Anabolic Response to Nutrition in a Pseudomonas aeruginosa (PM) Induced Hyper-dynamic Sepsis-Recovery Pig Model, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 30 (1 Supplement), 682.16-682.16

3. Increased muscle myofibrillar protein breakdown rates using 3-methyl-histidine (tau-mHIS) stable isotopes in a Pseudomonas aeruginosa (PM) induced hyperdynamic sepsis pig model, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 30 (1 Supplement), lb761-lb761,

4. SUN-PP026: Reduced Protein Synthesis and Breakdown in Jejunal Mucosal Biopsies in a Live Bacteria Induced Hyperdynamic Sepsis Pig Model, GA Ten Have, MP Engelen, RR Wolfe, J Thaden, NE Deutz, Clinical Nutrition 34, S33

5. OR004: Protein Fractional Synthesis Rates (FSR) of Multiple Organs in a Pseudomonas aeruginosa (PM) Induced Hyperdynamic Sepsis Pig Model, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, Clinical Nutrition 34, S1

6. Reduced lung glutamate (GLU) production is the cause of decreased systemic glutamate availability in hyperdynamic sepsis, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 26 (1 Supplement), 715.3-715.3,

7. Muscle breakdown determines Arginine (ARG) availability during hyperdynamic sepsis in the pig, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 26 (1 Supplement), 43.7-43.7

8. Using the phenylalanine (PHE) stable isotope pulse method to measure intracellular protein breakdown and metabolic shunting in the context of sepsis in the pig, GA Ten Have, MP Engelen, RR Wolfe, NE Deutz, The FASEB Journal 26 (1 Supplement), 42.1-42.1
9. Decreased circulating arginine in ALF mice does not compromise whole body NO production, GAM Ten Have, R Jalan, NEP Deutz, EUR J GASTROEN HEPAT 19 (10), A16-A16

10. Decreased circulating arginine in ALF mice does not compromise whole body NO production, GAM Ten Have, R Jalan, NEP Deutz, The FASEB Journal 20 (5), A1041


12. Neuropsychological and behavioural characterisation of a fully-reversible acetaminophen-induced model of hepatic encephalopathy (HE) in mice, GA Ten Have, A Blokland, R Jalan, NE Deutz, JOHN WILEY & SONS INC 42 (4), 358A-359A

13. Evidence for marked disturbance in whole body and brain glutamate (GLU) metabolism with no alteration of glutamine II (GLN) metabolism in an acetaminophen induced fulminant hepatic encephalopathy (HE) mouse model. GA Ten Have, R Jalan, NE Deutz, JOHN WILEY & SONS INC 42 (4), 364A-365A
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