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ANABOLIC RESPONSE TO AMINO ACID SUPPLEMENTATION IN CRITICAL ILLNESS

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Anabolic Response to Amino Acid Supplementation in Critical Illness

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough.

Richard P. Feynman

ABSTRACT

Background Critically ill patients suffer from protein catabolism with losses of skeletal muscle and whole-body proteins associated with morbidity and mortality. Catabolism is difficult to overcome, but nutritional supplementation with enteral protein or parenteral amino acids may limit protein losses. It is unknown which dose, mode, and timing of feeding is optimal for critically ill patients.

Aim The aim of this project was to evaluate a technique of quantifying the response of protein turnover to feeding, then apply it to study effects of protein or amino acid supplementation in critical illness. A main question was whether exogenous protein/amino acid is utilized for improved protein balance, or else consumed in catabolic pathways.

Methods In study 1, a previously characterized cohort of viscerally obese, insulin-resistant women was studied. Postprandial muscle and whole-body protein turnover were quantified by stable-isotope labeled phenylalanine tracers. In study 2, the whole-body technique was adapted to investigate the effects of early enteral feeding in critically ill patients. In study 3, the response of critically ill patients to a three-hour course of intravenous supplemental amino acids was studied. In study 4, the time course of uptake of stable-isotope-labeled phenylalanine from the intestine into arterial blood was studied in healthy subjects and critically ill patients during continuous enteral feeding.

Results In study 1, the technique was found workable to quantify postprandial protein metabolism, and it was found that viscerally obese women are resistant to postprandial stimulation of anabolism. In study 2, it was found in critically ill patients that enteral feeding of a small amount of protein yields a detectable gain in protein balance and no increase in amino acid oxidation. In study 3, an improvement in whole-body protein balance after three hours of intravenous amino acid supplementation was found in critically ill patients. In study 4, the uptake of dietary phenylalanine from continuous enteral feeding was found to reach a tentative steady state, but with high intra- and interindividual variability.

Conclusion Exogenous amino acids from enteral or intravenous nutrition improve protein balance in healthy subjects and critically ill patients, and are not predominantly consumed in catabolic pathways.

LIST OF SCIENTIFIC PAPERS

I. **Upper-body obese women are resistant to postprandial stimulation of protein synthesis**
Liebau F, Jensen M D, Nair K S, Rooyackers O
Clin Nutr, 2014. 33(5): p. 802-7

II. **Effect of initiating enteral protein feeding on whole-body protein turnover in critically ill patients**
Liebau F, Wernerman J, van Loon L J, Rooyackers, O
Am J Clin Nutr, 2015. 101(3): p. 549-57

III. **Short-term amino acid infusion improves protein balance in critically ill patients**
Liebau F, Sundström M, van Loon L J, Wernerman J, Rooyackers O
Crit Care, 2015. 19(1): p. 106

IV. **Uptake of dietary amino acids into arterial blood during continuous enteral feeding in critically ill patients and healthy subjects**
Liebau F, Király E, Olsson D, Wernerman J, Rooyackers O
Manuscript

CONTENTS

1 Introduction 1
1.1 Critical illness and protein catabolism 1
1.1.1 Critical illness 1
1.1.2 Body composition 1
1.1.3 ICU acquired weakness 2
1.1.4 Mechanisms of muscle wasting 2
1.1.5 Anabolic stimuli and anabolic resistance 3
1.1.6 Anabolic resistance in critical illness 3
1.2 Interventions targeting protein catabolism in critical illness 3
1.2.1 Non-nutritional 4
1.2.2 Nutritional support 4
1.2.3 Protein/amino acid dose 5
1.2.4 Protein/amino acid dose and ICU outcomes – observational and retrospective studies 5
1.2.5 Protein/amino acid dose and ICU outcomes – interventional studies 6
1.2.6 Protein/amino acid dose – experimental studies 7
1.2.7 Amino acid composition 7
1.2.8 Current recommendations on protein dose 7
1.2.9 Monitoring protein nutrition 8
1.2.10 Negative effects of nutritional support in critical illness 8
1.3 Summary 8
2 Aims 9
2.1 Research questions for studies 9
3 Studies overview 10
3.1 Study design and experimental protocols 10
3.2 Logistics and execution, recruitment, data acquisition 10
3.3 Ethical considerations 10
3.4 Registration 13
4 Methods 14
4.1 Physiology and in vivo quantification of protein turnover 14
4.1.1 Protein turnover 14
4.1.2 Regulation of protein turnover 16
4.1.3 Protein balance and metabolic pools 16
4.1.4 Isotope labeled amino acid tracers 17
4.1.5 Mass spectrometry 17
4.1.6 Gas chromatography-mass spectrometry for analysis of isotopic enrichment in AA tracer studies 18
4.1.7 Isotope dilution method 19
4.1.8 Steady-state whole-body protein turnover 20
4.1.9 Phenylalanine as a tracer 22

4.1.10 Phenylalanine hydroxylation 24
4.1.11 Splanchnic first-pass metabolism and quantifying the dietary contribution to amino acid availability 26
4.1.12 Summary of the phenylalanine-tyrosine whole-body protein turnover model 26
4.1.13 Muscle and leg amino acid kinetics 26
4.2 Diagnostic and analytical methods 28
4.2.1 Isotopic analysis 28
4.2.2 Calculation of leg protein turnover 28
4.2.3 Calculation of whole-body protein turnover 28
4.2.4 Contribution of dietary protein to WB protein turnover 31
4.2.5 Plasma aminograms 31
4.2.6 Indirect calorimetry 31
4.2.7 Insulin sensitivity 32
4.3 Statistical methods 32
5 Results 33
5.1 Study 1 33
5.1.1 Method evaluation 33
5.1.2 Body composition, insulin resistance and free fatty acid metabolism 33
5.1.3 Protein turnover 33
5.2 Study 2 33
5.2.1 Method evaluation 33
5.2.2 Patients and nutrition 34
5.2.3 Findings 35
5.3 Study 3 38
5.3.1 Method evaluation study 38
5.3.2 Whole-body protein turnover study 38
5.4 Study 4 41
5.4.1 Subjects and nutrition 41
5.4.2 Findings 41
6 Discussion 43
6.1 Methods 43
6.1.1 Whole-body protein turnover 43
6.1.2 Phenylalanine/tyrosine tracers 44
6.1.3 Phenylalanine/tyrosine tracers in critically ill patients 45
6.1.4 Splanchnic first-pass metabolism of dietary amino acids 46
6.2 Study subjects and (lack of) between-groups comparisons 48
6.3 Protein turnover 48
6.4 Continuous enteral feeding 51
6.4.1 Variability in plasma aminoacidemia 51
6.4.2 Anabolic stimulation 52

6.5 Outlook 52
7 Conclusion 53
8 Acknowledgements 54
9 References 56
10 Errata to published papers 68

LIST OF ABBREVIATIONS

AA	Amino acid(s)
A-V	Arterio-venous
BW	Body weight
EAA	Essential amino acid(s)
ERB	Ethics Review Board
FFA	Free fatty acid(s)
GCMS	Gas chromatography – mass spectrometry
ICU	Intensive care unit
ICUAW	ICU acquired weakness
MS	Mass spectrometry
Phe	Phenylalanine
R_a	Rate of appearance
R_d	Rate of disappearance
RCT	Randomized controlled trial
SOFA	Sequential Organ Failure Assessment score
TTR	Tracer/tracee ratio
Tyr	Tyrosine
UBO	Upper-body obese
WB	Whole-body
WHR	Waist-to-hip ratio

1 INTRODUCTION

Critically ill patients universally suffer from protein catabolism and associated morbidity and mortality. Protein catabolism is difficult to overcome, but nutritional supplementation has shown beneficial effects in observational studies. Nutritional strategies make use of enteral or parenteral supply of amino acids, though it is not well understood how the metabolism of critically ill patients reacts to such supply.

In the work here presented, the response to feeding in adult human subjects was investigated, with a focus on patients in the intensive care unit (ICU). Protein metabolism was assessed by quantifying whole-body and skeletal-muscle protein turnover, using intravenous and intragastric/enteral infusions of stable-isotope labeled phenylalanine and tyrosine tracers.

1.1 CRITICAL ILLNESS AND PROTEIN CATABOLISM

1.1.1 Critical illness

Critical illness emerged in the 20th century with technological advances such as mechanical ventilation, renal support, and advanced hemodynamic monitoring and pharmacotherapy. It is in some measure an artificial disease, as it occurs in conditions that are hardly survivable in their natural course. Challenges in critical care medicine arise from the necessity to handle physiological processes far outside their normal equilibria, where adaptive - or maladaptive - responses may react to intervention in unpredictable ways. For the same reason, extrapolation from the physiology of less severe disease or health may be inappropriate when studying the physiology of critical illness.

Although the causes of critical illness are diverse, many patients exhibit a phenomenologically rather uniform, multiphasic course of illness, characterized by multiorgan dysfunction and secondary morbidity. It is increasingly recognized that the pathophysiology and the spectrum of morbidities differ significantly between the early acute phase in the first few days of critical illness, the ensuing late acute phase, and a prolonged post-acute phase thereafter[1-4], and that ICU survivors may suffer chronic infirmity and late mortality[5,6].

1.1.2 Body composition

Changes in body composition are common in critical illness[7]. Along with changes of energy expenditure and body water content, loss of lean body mass is typical of critical illness[8], with excessive loss of skeletal muscle[9] and whole-body protein[10]. Skeletal muscle is quantitatively the dominant component of lean body mass. In the ICU, quantifying body composition, and especially skeletal muscle mass[11], may be difficult. Physical anthropometric measurements are unreliable due to varying degrees of edema. Techniques such as dual X-ray absorptiometry, neutron activation analysis, and magnetic resonance imaging offer theoretical advantages, but are problematic in practice because of logistic challenges and lack of

validation in relevant populations[12]. Computed tomography and ultrasound imaging are clinically applicable techniques[13] and have been used in observational studies to quantify the loss of muscle mass[14] and muscle quality[15]. By these techniques it was also demonstrated that macroscopic loss of muscle mass correlates with higher morbidity and poor outcome in critical illness[9,16]. Bioimpedance analysis is increasingly used to quantify lean body mass in ICU patients[17].

1.1.3 ICU acquired weakness

Critically ill patients typically lose both mass and function of skeletal muscle, but muscle wasting is not synonymous with ICU acquired weakness (ICUAW)[18,19], and the causal relationship between the two phenomena is not completely understood[20]. The etiology of ICUAW is multifactorial, and factors such as inflammation, sepsis, multiorgan failure, immobilization, age, medications, and hyperglycemia may all be involved[21]. Both critical illness polyneuropathy (CIP) and critical illness myopathy (CIM) may contribute to the development of ICUAW[19,22], and the precise etiology in individual cases may be difficult to determine. Because a stringent evaluation of ICUAW requires the patient's cooperation, diagnosis is in practice based on clinical tests that carry inherent limitations[21]. ICUAW may affect the respiratory muscles, and the subsequent need for prolonged ventilatory support poses a major clinical problem[23]. ICUAW contributes to both in-hospital and long-term mortality[24] and to long-term loss of function[21,25]. Although a majority of ICU survivors recover from the sequelae of ICUAW, others suffer from persistent infirmity up to several years after ICU admission[25].

1.1.4 Mechanisms of muscle wasting

Skeletal muscle undergoes a constant turnover of its protein components by catabolism, i.e. proteolysis and AA release, and anabolism, i.e. AA uptake and protein synthesis. To some degree, this turnover occurs within muscle tissue itself, constituting a local recycling of AA. However, muscle protein metabolism is also integrated into the organism's overall AA exchange. In that respect, skeletal muscle serves as a reservoir of AA, as mammalian physiology otherwise lacks organs or tissues for long-term protein storage.

Phenomenologically, muscle wasting in critical illness is characterized by fiber atrophy or necrosis[9,26,27]. The loss of lean body mass is driven by an increase of protein breakdown in skeletal muscle[9,28] that occurs largely in the early phases of illness and is mediated by both the proteasome and the lysosomal proteolytic enzyme systems[29]. Accelerated muscle protein breakdown coincides with an increased global protein turnover and a net flow of AA from skeletal muscle to the liver, lymphatic organs, and site of injury[28,30,31]. It is speculated that this mechanism serves to maintain protein synthesis in these sites, as well as hepatic gluconeogenesis[32].

1.1.5 Anabolic stimuli and anabolic resistance

Anabolic resistance is a term used to describe a reduced sensitivity of skeletal muscle to protein anabolic stimuli. It is seen in a variety of circumstances such as aging, inactivity, obesity, chronic kidney disease, chronic lung disease, and cancer. Mechanisms of anabolic resistance have been studied in various physiological and pathological conditions. Immobilization impairs the ability of skeletal muscle to react to AA provision[33]. In higher age[34], the response of skeletal muscle to the major anabolic stimuli - exercise, AA availability, and insulin - is altered through various mechanisms such as splanchnic sequestration of dietary AA[35], impaired insulin-mediated capillary recruitment[36], and altered AA transporter and signaling activity[37]. On the cellular level, the response to anabolic stimuli, specifically by the mammalian target of rapamycin (mTOR) pathways[38], is attenuated in aging subjects, but also in sepsis[39].

1.1.6 Anabolic resistance in critical illness

Several causes of anabolic resistance converge in critical illness, but their respective roles in the pathophysiology of muscle wasting are far from clear[32,38,40]. Immobilization removes a major stimulus of skeletal muscle anabolism. Non-pediatric ICUs typically have a large proportion of older patients who may be anabolically resistant by virtue of age. Although insulin promotes protein anabolism by several pathways, including recruitment of microvascular nutritive flow, the role of insulin resistance in muscle wasting of critical illness is not clear[32]. Availability of AA for muscle protein synthesis may be abnormal in septic patients, in that AA efflux from muscle cell to interstitium and venous blood is increased, while AA from arterial blood are shunted past the interstitium and muscle cell[41]. Glucagon acts as a catabolic signal regulating hepatic AA breakdown, and plasma AA stimulate islet α-cell growth in a feedback cycle independent of the glucose-insulin axis[42]. This mechanism is active in critically ill patients and in an experimental model of critical illness, where it interferes with feeding in the early phase of critical illness, in that exogenous AA supplementation accelerate AA breakdown, though apparently without effect on muscle wasting[43]. A derangement of the hypothalamic-pituitary-adrenocortical axis is seen in critical illness, characterized by elevated total and free plasma cortisol concentrations and low adrenocorticotropic hormone plasma concentrations[44]. Muscle wasting is characteristic of hypercortisolism and is partly mediated by direct and indirect inhibition of insulin action on skeletal muscle protein turnover[45]. Finally, systemic inflammation[32], hypoxia[46], and uremia[47] may further impair stimulation of anabolism. Thus, a variety of epidemiological, biophysical, hormonal and biochemical factors contribute to a milieu in the ICU that is hostile to gain or maintenance of body protein.

1.2 INTERVENTIONS TARGETING PROTEIN CATABOLISM IN CRITICAL ILLNESS

Pharmacological, physical, physiotherapeutic and nutritional strategies against protein catabolism and muscle wasting have been investigated.

1.2.1 Non-nutritional

Various pharmacological interventions have been investigated or are theoretically viable[48-50]. Human growth hormone is theoretically attractive as an anabolic agent and may be safe in specific patient groups[48,51], but was found to increase mortality of critically ill patients in large randomized trials[52]. The β-adrenergic receptor blocker propranolol[48,53] and the testosterone analog oxandrolone[54] have beneficial effects on lean body mass in children with severe burns, but have not gained ground in other ICU populations. Insulin, as a major physiological anticatabolic signal, is a plausible candidate drug. In a small trial in surgical patients, intensive insulin treatment during parenteral nutrition was associated with greater muscle protein synthesis and less negative nitrogen balance[55]. However, an independent role of exogenous insulin may be difficult to define because of the highly regulated homeostatic function of endogenous insulin secretion, as well as varying nutrient supply, varying degree of insulin resistance, and heterogeneity of ICU populations in general.

The biguanide metformin inhibits hepatic glucagon signaling[56,57] and appears to have an anticatabolic effect in severe burn injury[58,59]. Metformin improves insulin sensitivity when used for glycemic control in patients with severe burn injury[60]. Its use in general ICU populations remains to be explored. The leucine metabolite β-hydroxy β-methylbutyrate (HMB) acts as an anabolic signal on muscle protein metabolism[61], improves muscle mass in sarcopenia[62], and in conjunction with protein supplementation improves mortality in malnourished hospitalized patients[63]. In one RCT, hydroxymethylbutyrate had no effect on femoral muscle volume of critically ill patients[64].

Neuromuscular electrical stimulation was investigated in several studies and found in some[65,66], but not others[67], to improve muscle strength. Methodological questions remain[68], and the strength of currently available evidence is limited by the heterogeneity of methods and outcomes studied[65,66]. Functional challenge is an important stimulus of protein anabolism in skeletal muscle, and its absence in the immobilized ICU patient appears to play a major role in muscle wasting[28,69]. Interventions such as physiotherapy, reduced sedation, and early mobilization were found to improve muscle function[70-72] and ICU outcomes[53,71-74], but may be difficult to implement in practice[21,75]. Thus, increased physical activity is likely to improve ICU outcomes, but the heterogeneity of protocols and outcome measures limit the generalizability of such findings[11].

1.2.2 Nutritional support

Nutritional support is an essential therapeutic modality in critical care and has a role in modulating protein catabolism. Effects of nutritional support have been studied on all scales from physiological investigations in a few subjects to multicenter RCTs with several thousand patients. The following sections mostly focus on recent research, the rationale being that ICU treatment protocols in general - and feeding strategies specifically - are undergoing such a rapid change that older findings might not be applicable to patient populations currently treated at ICUs.

1.2.3 Protein/amino acid dose

It is well established that appropriate protein/AA provision is required to minimize catabolism, but there is much uncertainty over protein requirements in critical illness[76,77], and timing[78], route, and dose of protein/AA supplementation[79,80]. The existing uncertainty is partly due to methodological obstacles that affect ICU nutrition studies in general[81], such as:

- observational and/or retrospective study designs
- heterogeneity of ICU populations
- heterogeneity of study protocols
- heterogeneity of endpoints
- multiple confounding factors, due to the complexity of medical protocols and the physiology of critical illness itself.

One other problem more specific to studies of protein/AA dose is the fact that many studies do not consider protein/AA supplementation separately from total nutrient content. Taken together, these factors complicate any generalization of findings or generation of clinical recommendations[2,82]. Current recommendations stipulate a protein dose that is far lower than what is considered the highest safe intake in health[83]. On the whole, high level evidence supporting specific protein targets is scarce[76,79,84-86] and more research in this topic is considered a high priority[77].

1.2.4 Protein/amino acid dose and ICU outcomes – observational and retrospective studies

A relationship between protein/AA dose and ICU morbidity and mortality is reported in observational studies[2]. Weijs et al.[87] investigated 886 ICU patients in a prospective observational study design and found that patients where both energy and protein targets were reached had a lower mortality than those where only energy targets were reached. In a reanalysis of 843 patients from that cohort[88] it was found that energy overfeeding and sepsis were independent risk factors for mortality; in non-septic, non-overfed patients, a high protein intake was associated with better survival. Allingstrup et al.[89] found in 113 ICU patients that higher protein supply correlated with better survival, while measured resting energy expenditure, energy supply, or nitrogen balance had no effect. Nicolo et al.[90] retrospectively analysed 2828 patients and found higher survival when > 80 % of protein targets was reached, while higher energy intake did not affect survival. Elke et al.[91] analyzed 2270 patients with sepsis or pneumonia and found higher protein and caloric intake associated with higher survival. Compher et al.[92] found an association between protein intake and survival in several subgroups of a 2853 patient sample. Investigations by Song et al.[93] and Zusman et al.[94] came to similar conclusions. In a post hoc subgroup analysis of 729 patients in the PermiT RCT[95], patients were stratified into lower or higher enteral protein dose (with corresponding differences in caloric intake), and no difference in mortality was found, but higher protein intake was associated with higher urinary urea excretion. Finally, Koekkoek et al.[96] retrospectively analysed 445 patients and found that both overall low protein intake and early high protein intake was associated with higher mortality.

To summarize, evidence from observational studies generally supports an association between higher protein/AA intake and better outcomes. However, given the difficulties of feeding ICU patients, one reasonable interpretation might be that patients with a more stable physiology and better prognosis are more easily fed. Thus, these findings support, but do not prove, a causal role of higher dose protein supplementation for better ICU outcomes.

1.2.5 Protein/amino acid dose and ICU outcomes – interventional studies

Several interventional studies have targeted supplemental parenteral nutrition resulting in both higher caloric and higher AA supply[97-100], or different doses of enteral nutrition at identical daily protein/AA dose[101-103]. However, there are few published results from newer RCTs where the relationship of protein/AA dose and ICU outcomes was studied independent of total nutrition dose, although more RCTs are under way[104,105].

In the study by Ferrie et al.[106], 120 patients were randomized to receive either 1.2 or 0.8 g AA/kg BW per day, with doses actually delivered of 0.9 and 1.1 g/kg BW per day. Better handgrip strength and better nitrogen balance were seen in the higher-AA group at various timepoints. However, early energy intake was lower in the higher-AA group, and selection bias due to differences in mortality risk cannot be excluded[107].

In one RCT by Rugeles et al.[101], 115 patients were randomized to either high-protein or standard-protein hypocaloric enteral nutrition, with doses actually delivered of 12 kcal/kg BW and 1.4 g protein/kg BW per day vs. 14 kcal/kg BW and 0.76 g protein/kg BW per day. Patients in the high-protein group had slightly better Sequential Organ Failure Assessment (SOFA) scores. However, there was substantial risk of selection bias due to patients leaving the ICU early, and mortality was not reported in the per-protocol analysis.

In the Nephro-Protective Trial, a multicenter RCT designed to study acute kidney failure as the primary outcome[108], 474 patients were randomized to receive either standard nutrition or an additional supplement of intravenous AA, with doses delivered being 0.75 vs. 1.75 g AA/kg BW per day. There were between-group imbalances in baseline renal morbidity and severity of illness. Patients in the high-AA group had slightly shorter duration of respiratory failure and somewhat higher urine output and estimated glomerular filtration rates, but also higher fluid intake, higher serum urea concentration, and possibly greater use of renal replacement therapy. These findings show that AA dose had little effect on ICU morbidity, but the difference in serum urea values suggest that exogenous AA at the higher dose may not have been utilized for protein anabolism. A post hoc subgroup analysis[109] revealed that patients with kidney dysfunction or risk of progression of acute kidney failure at baseline did not benefit from the AA supplement, while patients without these risk factors who received the supplement had a lower mortality compared with those who did not.

In conclusion, evidence from prospective interventional trials where different AA doses were compared in isolation shows that higher-dose AA supplementation may confer some benefit in biochemical, biomechanical, and severity-of illness parameters, and possibly in mortality.

1.2.6 Protein/amino acid dose – experimental studies

Complementing ICU outcome studies, protein requirements in critical illness can be studied experimentally. This can be done by calculating nitrogen balance, i.e. measuring total intake of protein/AA and output of AA metabolic products over a period of time, or by measuring protein turnover using AA tracers. These techniques are discussed in more detail below in the context of the quantification of protein turnover.

In a prospective randomized experimental study in trauma and burn patients[110], a less negative cumulative 7-days nitrogen balance was seen with AA supply of up to 0.7 g/kg BW per day, but not above that level; an extrapolation beyond that specific patient cohort may not be warranted. In a nonrandomized study in trauma and sepsis patients[111], a protein dose of 1.2 g/kg preadmission BW was found to optimally support body protein mass. In an extensive review of the literature on protein requirements in the ICU[80], it was found that all available experimental studies showed methodological weaknesses, such as measurement during energy overfeeding, and inappropriate or unclear calculation of AA doses. Most available studies used nitrogen balance as outcome measure. Protein/AA doses varied from 0 to 2.8 g/kg BW per day. Due to the great heterogeneity of patient populations and study protocols, it is impossible to reconcile their results and define a single protein/AA dose that optimally maintains body protein[76]. However, it can be concluded that higher protein/AA supply generally appear to increase short-term nitrogen balance, that maintenance of whole-body protein may require a protein/AA dose in the region of 1.2 – 1.5 g/kg BW per day[76], and that an upper limit may be difficult to define[80].

1.2.7 Amino acid composition

Nutritional supplementation with individual AA - e.g. glutamine, leucine, arginine, or citrulline - can experimentally be shown to increase protein anabolism, but it remains controversial whether any such intervention improves ICU outcomes[61,107,112]. In the most recent European guidelines[2], AA composition in nutritional support is not specifically addressed, apart from recommendations to abstain from enteral glutamine supplementation except for burns or trauma, and to abstain from parenteral glutamine supplementation in complex cases and patients with unstable physiology.

1.2.8 Current recommendations on protein dose

Despite the limitations of available evidence, attempts have been made to synthesize current knowledge into clinical guidelines. This was most recently done in 2018 by the European Society for Clinical Nutrition and Metabolism after thorough review[2], in a revision to previous guidelines[113,114]. Based on strong consensus among the guideline's authors and reviewers, the current recommendation is that 1.3 g protein equivalent/kg BW per day can be delivered progressively to patients in the ICU[2]. In the corresponding American guidelines[115], a very low quality of evidence is noted. It is suggested that "sufficient (high-dose) protein should be provided", that "protein requirements are expected to be in the range of 1.2–2.0 g/kg actual body weight per day and may likely be even higher in burn or multitrauma

patients", and that "aggressive high-protein EN therapy" should be used in chronical critical illness.

1.2.9 Monitoring protein nutrition

It is recognized that risks, effects, and complications of nutrition in the ICU should be monitored, but the use of monitoring is not sufficiently studied[116]. For monitoring the effects of protein supply, few methods are practically available. Available measurements of body composition lack feasibility in the ICU setting and are therefore not widely used. Estimations of daily nitrogen balance can be made based on urinary urea losses, but are imprecise, and may not be useful in settings of rapid metabolic change. More exact measurements of nitrogen balance are usually not feasible in the ICU setting[117]. Measurements of blood urea and ammonia may detect overt protein overfeeding[116], but their interpretation is complicated by effects of organ dysfunction and comorbidities. Plasma aminograms are a useful research tool. ICU patients with sepsis show a wide variation in plasma aminoacidemia and several parameters derived from the aminogram correlate with severity of illness and survival[118-120]. However, measurements of plasma AA require specialized equipment and are not clinical routine in the ICU. The usefulness of other routine laboratory analyses for monitoring of protein nutrition is not established.

1.2.10 Negative effects of nutritional support in critical illness

Possible risks of early nutritional support in critical illness are increasingly recognized. Subgroup analyses of large RCTs in adult[121] and pediatric[122] critically ill patients have shown that early full-dose nutritional support is associated with higher complication rates. From these data, it appears that specifically AA, but not other macronutrients, are implicated[122]. An antagonism of autophagy is discussed as a physiological mechanism[123]. Autophagy is a cellular mechanism that is activated by inflammation, hypoxia and various other stressors[124] and modulated by availability of nutrients, especially AA[125]. Previously thought of as a pathway to cell death, autophagy is nowadays rather considered an essential housekeeping mechanism. It may have an adaptive role in critical illness and its inhibition by early nutrition may be detrimental[126], although inhibition of autophagy on a cellular level can be shown in only a fraction of critically ill patients[127]. Even if the pathophysiology remains unclear, current guidelines discourage early full-dose feeding, and note that intentional hypocaloric feeding may be safe in certain circumstances[2,78].

1.3 SUMMARY

Protein catabolism in critical illness is common, difficult to ameliorate, and insufficiently understood. While nutritional strategies appear promising, the physiology of protein/AA feeding in critical illness is not sufficiently characterized, and it remains unclear how nutrition should be composed, dosed, delivered, timed, and monitored to give the greatest possible benefit to specific individuals or patient groups.

2 AIMS

The overall aim of this project was to mechanistically study the effects of protein/AA supplementation on protein turnover in critical illness. We sought to establish a technique of measuring whole-body protein turnover by stable-isotope labeled phenylalanine/tyrosine tracers, then apply it to quantify the effects of parenteral and enteral protein/AA feeding on protein metabolism in critically ill patients. A central question was whether exogenous protein/AA is utilized for improved protein balance, or else consumed in catabolic pathways.

2.1 RESEARCH QUESTIONS FOR STUDIES

Study 1: Quantify the postprandial response of muscle and WB protein turnover by stable-isotope labeled phenylalanine tracers in a previously characterized population of viscerally obese, insulin-resistant subjects.

Study 2: Quantify the postprandial response of WB protein turnover to an initial dose of continuous enteral feeding by intravenous and enteral stable-isotope labeled phenylalanine/tyrosine tracers in healthy volunteers and patients in an early phase of critical illness.

Study 3: Evaluate the usability of a free phenylalanine tracer to quantify the availability of dietary protein from continuous enteral feeding in critically ill patients; and quantify the response of WB protein turnover to a three-hour course of supplemental parenteral AA in patients at two time points during the postacute phase of critical illness.

Study 4: Characterize the time course of uptake into arterial blood of dietary isotope labeled phenylalanine during continuous enteral feeding in healthy subjects and critically ill patients.

3 STUDIES OVERVIEW

3.1 STUDY DESIGN AND EXPERIMENTAL PROTOCOLS

An overview of study designs is shown in Table 1 and an overview of nutrition and tracers used in Table 2. The details can be found in the individual papers.

3.2 LOGISTICS AND EXECUTION, RECRUITMENT, DATA ACQUISITION

Study 1 is a retroactive analysis of data that had been obtained, but previously not analyzed or published, in conjunction with another set of experiments[128] at Endocrine Research Unit, Mayo Clinic, Rochester, Minnesota, USA. Studies 2-4 were executed at the experimental facility and the ICU of the Department of Perioperative Medicine and Intensive Care (formerly Department of Anesthesia and Intensive Care), Karolinska University Hospital Huddinge in Stockholm, Sweden.

For study 1, volunteers were recruited in accordance with the host institution's regulations and as stipulated by the relevant Ethics Review Board (ERB) decisions. For studies 2 and 4, healthy subjects were recruited from the department's volunteer database and informed consent was obtained in accordance with Swedish law and as stipulated in the ERB decisions. For studies 2-4, patients were recruited from the department's ICU and informed consent was obtained from them or from their next of kin, in accordance with Swedish law and as stipulated in the ERB decisions.

For study 1, anthropometric and clinical data were obtained in conjunction with the earlier investigation [128]. For patients in studies 2-4, anthropometric and clinical data were obtained from hospital records and from the ICU's patient data management system. For healthy subjects, relevant information was obtained by interview, and where appropriate from hospital records. For study 1, laboratory analyses were performed at the host institution. For studies 2-4, laboratory analyses were performed at the department's research laboratory.

3.3 ETHICAL CONSIDERATIONS

Study 1 was approved by Mayo Institutional Review Board at Mayo Clinic, Rochester, Minnesota, USA. Studies 2-4 were approved by the regional ERB in Stockholm. Assessments of risk and benefit to individual study subjects included in the Stockholm ERB applications concluded that participation in the experiments carried minimal risks, insofar as: medical procedures followed established standards, were performed by qualified staff and in the setting of the department's ICU and experimental facilities; additional procedures were limited to study nutrition, tracer infusion, and sampling and – for healthy subjects – placement of nasogastric feeding tubes, arterial, and venous catheters; risk associated with exposure to study nutrition and tracers was minimal; and blood loss by sampling was minimized to safe levels.

	Study population(s)	Study questions	Intervention, measurements	Primary outcome variables	Other outcome variables
Study 1	Upper-body obese (n=6) vs. lower-body obese (n=7) women	Effect of feeding on leg and WB protein kinetics in upper-body obesity vs. lower-body obesity	Leg and WB protein kinetics before/after 5 hrs enteral nutrition	Leg protein turnover, WB protein turnover	Plasma AA, FFA, glucose, insulin
Study 2	Healthy subjects (n=6), ICU patients (n=10)	Effect of low-dose enteral feeding on WB protein kinetics	WB protein kinetics before/after 6 hrs low-dose enteral nutrition using intrinsically labelled casein	WB protein turnover, Phe splanchnic extraction fraction	Plasma AA
Study 3 (method evaluation)	ICU patients (n=6)	R_aPhe from dietary protein-bound Phe identical to that from dietary free Phe?	Simultaneous enteral infusion of intrinsically labeled casein and free Phe tracer	R_a from protein-bound Phe, R_a from free Phe	
Study 3 (main study)	ICU patients (n=13)	1) Effect of extra iv. AA on WB protein kinetics? 2) Change of baseline WB protein kinetics during ICU stay? 3) Effect of extra iv. AA maintained during ICU stay?	1) WB protein kinetics before/after iv AA infusion 2, 3) Protocol repeated after 2 to 4 days	WB protein turnover, Phe hydroxylation, Phe splanchnic extraction fraction	Plasma AA, urea
Study 4	Healthy subjects (n=10), ICU patients (n=10)	Does continuous enteral feeding result in constant uptake of dietary Phe into plasma?	Continuous enteral feeding for 12 hrs run-in, then 12 hrs study period	Variability of plasma Phe isotopic enrichment	Plasma AA

Table 1 Study designs.
Abbreviations: AA, amino acid(s); FFA, free fatty acid(s); ICU, intensive care unit; Phe, phenylalanine; R_a, rate of appearance; REE, resting energy expenditure; Tyr, tyrosine; WB, whole-body

	Study nutrition: type, dose	Study nutrition: route, mode, duration	Other nutrition	Enteral AA tracers	I.v. AA tracers	Timing of measurements (hrs)
Study 1	Liquid feeding formula, total 40% of daily REE within 5 hrs	Oral, frequent bolus, 5 hrs total	none	$^{2}H_5$-Phe ^{15}N-Phe	$^{2}H_5$-Phe ^{15}N-Phe	Baseline (t=0), after run-in (t=3.0), after intervention (t=8.0)
Study 2	Maltodextrin 2.73 g/hr casein (intrinsically labeled) 0.73 g/hr	Intragastric, continuous, 6 hrs total	Complete parenteral nutrition solution, intravenous, continuous	^{13}C-Phe (protein-bound)	$^{2}H_5$-Phe $^{2}H_4$-Tyr $^{2}H_2$-Tyr	Baseline (t=0), after run-in (t=2.5), repeated every 15 min to t=8.5
Study 3 (method evaluation)	Maltodextrin 2.7 g/hr casein (intrinsically labeled) 1.5 g/hr	Intragastric, continuous, 6 hrs total		^{13}C-Phe (protein-bound), $^{2}H_5$-Phe (free AA)		Baseline (t=0) repeated every 15 min to t=6.0
Study 3 (main study)	Mixed AA, (equiv. 1 g/kgBW/d)	Intravenous, continuous, 3 hrs	Enteral nutrition by clinical routines	^{13}C-Phe	$^{2}H_5$-Phe $^{2}H_4$-Tyr $^{2}H_2$-Tyr	Baseline (t=0) after run-in (t=4.5) after intervention (t=7.5)
Study 4	Liquid feeding formula, 25 kcal/kgBW/d (healthy subjects), >80% of REE (patients)	Intragastric/ intrajejunal, continuous, 12 hrs + run-in	none	^{13}C-Phe	none	Baseline (t=0) Repeated every 30 min to t=12.0

Table 2: Nutrition and tracers.

Abbreviations: AA, amino acid(s); FFA, free fatty acid(s); ICU, intensive care unit; Phe, phenylalanine; R_a, rate of appearance; REE, resting energy expenditure; Tyr, tyrosine; WB, whole-body

3.4 REGISTRATION

Study 2 was registered at Australian New Zealand Clinical Trials Registry, with registration number ACTRN12614000333617, and study 4 with registration number ACTRN12616000593437.

4 METHODS

4.1 PHYSIOLOGY AND IN VIVO QUANTIFICATION OF PROTEIN TURNOVER

All models are wrong, but some are useful *

4.1.1 Protein turnover

Protein metabolism is unique in its functions and its regulation. AA serve a unique variety of physiological roles, from building block of protein to signaling molecule in metabolism, to reserve energy fuel, and to precursor for bioactive small molecules, such as hormones, transmitters and signaling molecules, with a vast number of biological functions[129].

The protein content in all tissues undergoes a constant turnover, i.e. cycling, of AA through protein synthesis and breakdown[130,131]. In the adult human, WB protein turnover is estimated to be 4 g/kg BW per day, i.e. three to four times the dietary intake[132,133]. Turnover varies widely between organs and tissues, with liver and intestine representing 50% of WB turnover at only 8% of lean body mass, while skeletal muscle accounts for 25% of WB turnover at 55% of lean body mass[130]. Protein turnover is not energetically cheap and in a normal adult is estimated to account for 20% of the basal metabolic rate[130]. As the daily total amount of AA cycling substantially exceeds dietary protein intake, a fine-tuned regulation both in individual tissues and the organism as a whole is necessary to maintain both homeostasis and adaptability[134].

Sources of AA are dietary intake, proteolysis, and - for non-essential AA - endogenous synthesis. While there appears to be a buffer, or short-term storage, of mainly dietary AA in the intestine[135], there is no long-term storage of AA outside functional tissues. Instead, skeletal muscle serves as the major reservoir of AA when dietary AA are not available. AA concentrations in individual cells, tissues, and the organism as a whole are homeostatically controlled to minimize loss through AA oxidation[134]. AA from protein breakdown are either recycled locally within organs or tissues, or released into the circulation as free AA. Free AA constitute only 2% of the body's total AA content[136] but serve as a vehicle for substrate traffic between organs and tissues. A constant exchange through the blood circulation distributes AA for interorgan traffic[130]. Interorgan traffic is an active and regulated process[137] that involves transportation of free AA, peptides and proteins by blood and lymph circulation. Different organs and tissues have widely varying content of individual AA and distinct metabolic patterns[129]. For example, skeletal muscle serves as a site of synthesis for glutamine

* Alternatively, 'Since all models are wrong the scientist cannot obtain a "correct" one by excessive elaboration. On the contrary following William of Occam he should seek an economical description of natural phenomena. Just as the ability to devise simple but evocative models is the signature of the great scientist so overelaboration and overparameterization is often the mark of mediocrity.' George Box, Science and Statistics. Journal of the American Statistical Association, 1976, Vol. 71, pp. 791-799

and alanine which it exports to other organs where these AA are used for specific metabolic functions (Fig. 1)[138].

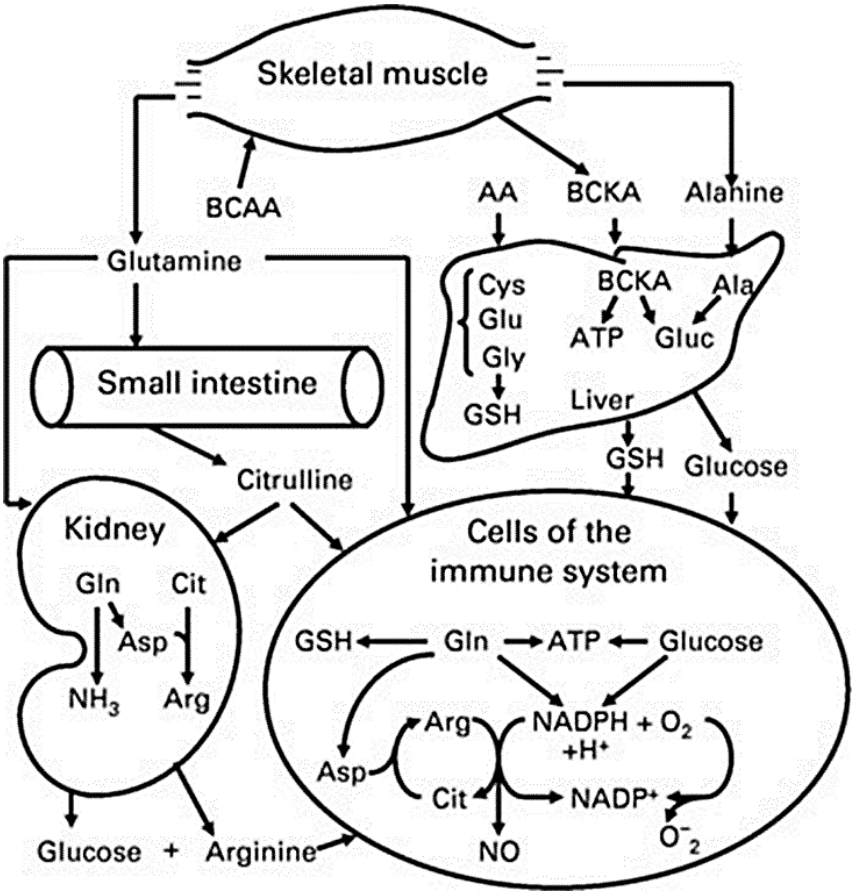


Figure 1 Inter-organ metabolism of branched-chain amino acids, glutamine and arginine, and their role in immune function. Skeletal muscle takes up BCAA from the arterial blood, synthesises both alanine and glutamine from BCAA and α-ketoglutarate, and releases these two amino acids into the circulation. The small intestine utilises glutamine to synthesise citrulline, which is converted into arginine in kidneys, cells of the immune system and other cell types. The liver is the primary organ for the synthesis of glutathione from glutamate, glycine and cysteine, and of glucose from alanine for use by extrahepatic cells (including leukocytes) and tissues. Abbreviations: Arg, arginine; Asp, aspartate; Cit, citrulline; BCAA, branched-chain amino acids; BCKA, branched-chain α-ketoacids; Gluc, glucose; GSH, glutathione.

Image and caption source: [138]. Reproduced by permission.

The splanchnic organs have a highly active AA metabolism, and dietary AA undergo significant splanchnic first-pass metabolism that is mediated by both the intestinal microbiome and the host[83]. Enterocytes actively regulate AA and di- and tripeptide absorption and release[139], and AA are cycled between mucosa and lumen[140,141]. Splanchnic retention of dietary AA varies between different AA[83,140,142] and systemic availability of a quantity fed enterally can be as low as 10% or less for glutamine, glutamate, or aspartate, but over 100% - i.e. net synthesis in the splanchnic organs - for alanine, arginine, or tyrosine[142].

4.1.2 Regulation of protein turnover

Regulatory mechanisms of protein turnover exist on the whole-body, organ/tissue, and cellular level and have growth-related, long-term homeostatic, cyclic, and adaptive components[130]. Protein turnover is controlled through synthetic and proteolytic pathways in a complex system that is modulated at transcriptional and translational levels[130]. Proteolysis and protein synthesis are intracellular processes that are specifically regulated depending on the substrates involved[130].

Global and regional protein metabolism varies cyclically between the fed vs. fasted state. Protein utilization and AA metabolism are modulated by substrate availability and hormonal response to food intake. Postprandial metabolism is characterized by a net flow of AA from the splanchnic organs to skeletal muscle, with plasma insulin and AA concentrations as the most important regulators and mTOR-dependent pathways the most important intracellular signal transduction systems[130,134]. In fasting, net AA flow is reversed, with AA – predominantly glutamine and alanine – being released from skeletal muscle to provide substrates for protein synthesis and hepatic gluconeogenesis.

4.1.3 Protein balance and metabolic pools

Protein metabolism, being in a state of constant AA turnover, can be quantified using the concept of protein balance. Protein balance is, by definition, the arithmetic difference between protein synthesis and breakdown, where a negative value (i.e. breakdown > synthesis) represents a catabolic balance and a positive value (synthesis > breakdown) an anabolic balance. It can be defined for specific organs and tissues or the whole-body and can be measured by nitrogen balance or AA tracer techniques[143,144]. Calculation of nitrogen balance is based on measuring total input of protein/AA and output of AA metabolic products over a period of time. While simple in principle, its use in practice is restricted by tedious and imprecise procedures of collecting complete or representative samples of excreta, and by the slow turnover of WB protein, which makes the method unattractive for the study of rapid metabolic changes. Furthermore, nitrogen balance does not allow insights into specific processes of AA turnover, such as measures of protein synthesis and proteolysis or organ-specific metabolism.

In physiological study, metabolic pools can be defined, which are volumes of distribution that may correspond to body compartments, organs, or tissues, but may also be more abstract,

operationally defined, entities[145]. Mass transport of AA is then conceptualized as a system of metabolic fluxes, here understood as the rate of AA turnover into, out of, or between pools.

4.1.4 Isotope labeled amino acid tracers

Since their development in the early 1930's, tracers have been an indispensable tool in metabolism research[146,147]. A tracer is a molecular analogue of some target molecule - the tracee - that chemically and biologically behaves nearly identically, but can be distinguished analytically[148]. AA tracers are a tool for the study of human protein metabolism *in vivo*. They can be used in several ways, such as by enriching a substrate pool for protein synthesis and measuring tracer incorporation in the product, or by using variations of the indicator dilution method for measurement of substrate fluxes between AA pools.

To be usable as a tracer, an AA is labeled, i.e. chemically modified to contain radioactive or stable isotopes in specific positions of the molecular structure. In studies of human metabolism, stable isotope labels are preferred to minimize radiation exposure. With stable isotope labels of e.g. hydrogen, carbon, nitrogen, or oxygen, the molecular mass of the labeled compound differs from that of the most common natural form and this difference can be measured by mass spectrometry. In the context of AA metabolism, the effects of stable isotope labeling on chemical and biological processes[149] of interest are mostly negligible, thus the tracer's biological behavior can be assumed to reflect that of the tracee.

4.1.5 Mass spectrometry

Mass spectrometry (MS) is used to measure the molecular mass of ions. Its fundamental principle is the acceleration of ions in a vacuum chamber, where said ions are separated – e.g. through deflection in an electromagnetic field - allowing their sorting by electric charge and molecular mass, and subsequently recorded in a detector[150]. Besides its more common use to detect, identify and quantify unique compounds in a sample, MS can be used to separate different isotopic species of a compound - or fragments of a compound - by molecular mass, and determine their relative abundance. Thus it is possible to analyze the proportion of tracee to tracer in a sample, which makes MS the essential analytical tool in stable isotope labeled tracer studies[151].

Several isotopic species of a molecule may exist. Within a given molecular structure, the position of any atom may be occupied by any isotope of the respective element. This results in differences of molecular mass depending on the number and atomic mass of isotopes in the individual molecule. The details of the isotopic configuration may be described by the terms isotopologue (syn. mass isotopomer), referring to species of a molecule that differ in molecular mass, and constitutional isotopomer, referring to species that share a summary isotopic composition but differ in the position of specific isotopes within the molecular structure.

A mass spectrum is the output of MS in which the abundance of molecule species (technically, abundance of ion species) within a sample is plotted against molecular mass

(technically, mass-to-charge ratio, m/z). Terminology of ion masses in MS is somewhat complex[152]. When studying known small metabolites, exact mass measurements are not required. Masses may instead be described in rounded, integer values as "M + n", where M represents the mass of the most abundant species of a molecule or fragment ion ("base"), and n the additional mass of heavier isotopes in atomic mass units. Ion masses are then denoted as M + 0 for the "base", and M + 1, M + 2, … for subsequent heavier isotopologues.

4.1.6 Gas chromatography-mass spectrometry for analysis of isotopic enrichment in AA tracer studies

One of many technical implementations of the physical principle of MS, gas chromatography – mass spectrometry (GCMS)[153] is mostly used for analyzing AA in biological samples. In GCMS, a gas chromatograph is linked to a mass spectrometer, enabling isolation of compounds of interest from a biological sample before MS analysis. Gas chromatography requires analytes that are chemically stable and sufficiently volatile at operating temperatures in the region of 250-300 °C, which many metabolites - including AA - are not. Therefore, preparation of AA samples may require chemical derivatization by e.g. silylation to enable analysis by GCMS[151]. Samples of derivatized AA are then fed from the gas chromatograph into the mass spectrometer, where they are ionized and fragmented in an ion source, separated by molecular mass in a radiofrequency quadrupole filter, and recorded in a detector[151].

The interpretation of mass spectra from GCMS is not straightforward and requires knowledge of possible molecular configurations after sample preparation, derivatization, and fragmentation in the ion source. To reconstruct the molecular configurations of the ionized fragments of the derivatized forms of tracer and tracee, one must consider the isotopic background in the natural, unlabeled biological sample and in the final analytes used for GCMS, which may include additional compounds used for derivatization[151,154]. In experimental setups using multiple or highly labeled tracers, there may be significant overlap of m/z values between tracers, their metabolic products, and naturally occurring species of the tracee, which may require dedicated consideration[155].

The isotopic background arises from the natural abundance of isotopes, i.e. the natural, relatively stable and approximately uniform isotopic composition of chemical elements in the planetary environment. Natural abundance is largely reflected in the biochemistry of living organisms. It is assumed that available isotopes may occupy positions in a biomolecule stochastically according to their abundance. However, isotopic background is not exactly uniform within the biosphere, both because of physical and chemical effects resulting in local isotopic fractionation, and because certain pathways of photosynthetic carbon fixation result in preferential enrichment of specific isotopologues in some plants[156]. These effects propagate through the food chain and result in subtle differences of isotopic background in biological samples. Furthermore, natural abundance implies that several isotopic species of a compound, including such that are used as tracers, may occur naturally in a biological

sample. These factors necessitate measurements of the baseline isotopic background in tracer studies.

The most abundant naturally occurring isotopes of hydrogen, carbon, nitrogen, oxygen, and several other biochemically relevant elements are also the lightest[157]. For any organic compound, a configuration exists that is entirely composed of the lightest respective isotopes. For small molecules, this species typically represents the largest fraction occurring biologically, which makes it the reference in measurements of isotopic enrichment, denoted M + 0 in mass spectra. For calculation of isotopic excess in a sample, MS is run in selected ion monitoring (SIM) mode, i.e. monitoring only the part of the mass spectrum that contains the ions of interest, and ratios between M + 0 and subsequent isotopologues are recorded. Taking into account the isotopic background in the natural, unlabeled sample, this output can then be recalculated to determine the isotopic excess[154].

4.1.7 Isotope dilution method

One major field of application of AA tracers is the measurement of substrate fluxes between AA pools by variations of the indicator dilution method. The principle of indicator dilution is that an unknown quantity, such as a volume or flux, can be determined by adding a known quantity of an indicator to the compartment of interest. The indicator then undergoes dilution by mixing with the compartment's content, and indicator concentration is measured in a sample. The quantity of interest can then be calculated from the dilution of the indicator. (In the simplest case, it is assumed that

$$\frac{S}{s} \approx \frac{N}{n}$$

where *N* is the unknown quantity of interest, *n* the amount of indicator given, *S* the size of the sample, and *s* the amount of indicator recovered in the sample. Then, N can be calculated by

$$N \approx \frac{S \times n}{s}$$

the so-called Petersen-Lincoln estimator.)

The applicability of indicator dilution methods relies on a number of assumptions, the most fundamental of which is that the indicator behaves similarly to the target entity. Further assumptions[158] include that:

- the system is in a steady state;
- pool size is stable;
- loss of indicator is zero;
- mixing is complete;
- perturbation of the system by the indicator is negligible.

Variations of indicator dilution methods have been used for a wide variety of purposes, from estimation of wildlife populations in the 19th century[159], to epidemiology of birth defects in

the 20th century[160], to measurements of body composition (D_2O dilution), regional blood volume and flow (dye dilution) and cardiac output (thermodilution, lithium chloride dilution)[158,161].

In metabolism research, dilution of an isotope labeled tracer can be used to determine the size of a metabolic pool or flux and is then imprecisely termed isotope dilution. In accordance with the aforementioned fundamental assumption, the tracer is assumed to undergo the same metabolic processes as the target metabolite in the organism. With respect to AA/protein metabolism, the concept is applied to dilution of AA tracers in the body's AA and protein pools. Specifically, an AA tracer is injected or infused into a metabolic pool, and samples are taken from the same pool, or another pool where the tracee and tracer and/or their metabolic products are found. Fluxes may then be calculated from the rate of tracer infusion and isotope dilution, as expressed by the ratio of isotopic enrichment in infusate and sample. They can be expressed as rate of appearance or disappearance of the AA corresponding to the tracer. Rate of appearance (R_a) or rate of disappearance (R_d) is generically defined as the concentration change of a compound over time[162], but is in this context understood to represent a flux into or out of a metabolic pool.

4.1.8 Steady-state whole-body protein turnover

Besides nitrogen balance techniques, WB protein turnover may be determined by isotope dilution of an AA tracer injected or infused into the blood[144]. WB protein turnover is then quantified using some model, which can generically be defined as "a simplified representation of a physiological system which has a structure (compartments) with routes of exchange of material between them"[163]. More specifically, AA turnover is conceptualized as a system of fluxes, i.e. mass transports, of AA between a body protein pool and AA pools, involving processes of AA conversion/synthesis, oxidation/excretion, and dietary intake. Under specific assumptions, measured AA fluxes may then be extrapolated to approximate processes of protein metabolism *.

A generic model of WB metabolism could look like the one shown in Fig. 2[144]. One fundamental property of such WB models is that in the steady state the total turnover of AA is assumed to equal the flux of AA into the central pool, R_a (defined as the sum of dietary intake and protein breakdown), which in turn is equal to the flux of AA out of the central pool, R_d (defined as the sum of protein synthesis and loss to metabolic end products)[133]. To be experimentally usable, any such model requires modification depending on the choice of tracers. For example, the generic model subsumes metabolic processes such as AA

* A technical note. Model pools are an abstraction and do not necessarily correspond to anatomical or physiological compartments. For example, parameters for some free AA pool may be calculated from plasma samples, suggesting that this pool might correspond to the body's plasma volume. However, distribution volumes for purposes of tracer studies may be incompletely defined, e.g. due to exchange with other compartments whose kinetics are unknown. Therefore, absent measurements or explicit assumptions about specific compartments, any extrapolations about quantities not included in the model should be made with caution, if at all.

conversion and synthesis into the central pool, and is as such only usable with tracers for AA that take part in these processes[133].

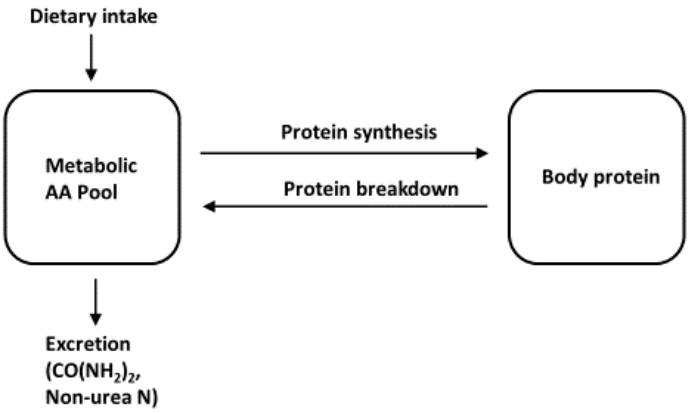


Figure 2 A simple generic model of whole-body protein metabolism, showing two metabolic pools and principal pathways of protein turnover

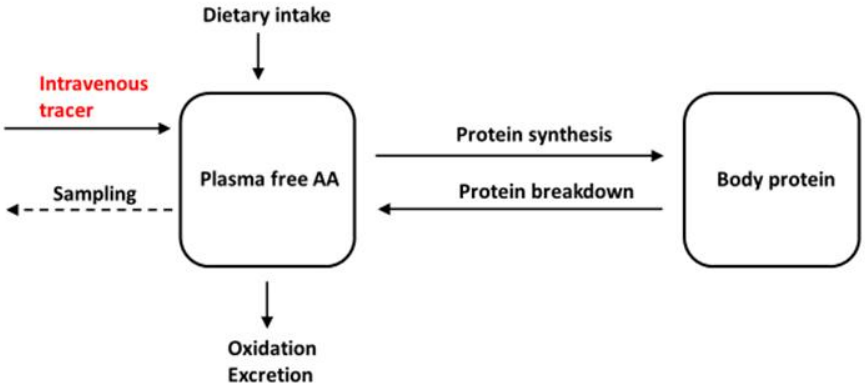


Figure 3 A highly simplified model of whole-body protein metabolism used for EAA tracer studies. De novo synthesis and conversion (other than oxidation) of AA are omitted from the model.

A direct measurement of protein synthesis is not possible without sampling from the protein pool. However, AA oxidation or some other step in the final catabolic pathway can be measured[132]. Dietary intake can be recorded, or measured using a tracer, or in the fasting state set as zero. As in the generic model, it is then assumed that in metabolic steady state, total flux = R_a = R_d, and R_a is taken to represent (WB protein breakdown + dietary intake), and R_d to represent (WB protein synthesis + oxidation). Using the three known quantities - R_a, dietary intake, and oxidation - protein synthesis can then be calculated as (R_d – oxidation).

Using an AA tracer, only pools of the respective AA and its metabolites are labeled, enabling measurement of turnover of that AA (Fig. 3). The method of interest here is based upon an EAA tracer infused into the blood at a constant rate until isotopic equilibrium is reached, followed by plasma sampling and measurement of isotopic enrichment. From the isotopic dilution of the AA tracer, R_a of that AA can be calculated. It is then postulated that fluxes of the representative AA can be extrapolated to total body protein[133,144].

The utility of such measurements relies on a number of underlying assumptions [132,133,144] beyond those inherent in all indicator dilution techniques, chiefly that:

- measurements are made in a metabolic steady state;
- and the metabolism of the representative AA reflects that of total body protein.

4.1.9 Phenylalanine as a tracer

In principle, any EAA can be used as a tracer to determine WB protein turnover, but the individual metabolic pathways require specific adaptations of the general method[133]. In the most widely accepted method, 1-^{13}C-leucine infusion is used with sampling of exhaled $^{13}CO_2$ and plasma leucine or metabolites[132]. Alternatively, phenylalanine tracers are usable for measurements of both WB and muscle protein turnover[164,165].

The sources of phenylalanine in humans are dietary intake, and release from protein by proteolysis (Fig. 4). The chief metabolic fates of phenylalanine are incorporation into protein, and hydroxylation to tyrosine. The sources of tyrosine are phenylalanine hydroxylation, dietary intake, and release from protein by proteolysis. The chief metabolic fates of tyrosine are incorporation into protein, and oxidation to fumaric acid and acetoacetate[136,165]; its role as precursor for bioactive amines such as the catecholamines and the trace amines[136,166] is quantitatively insignificant.

The method here described comprises several phenylalanine and tyrosine tracers. The first step is to use a phenylalanine tracer (such as L-ring-$^2H_5$-phenylalanine) to determine the total appearance of phenylalanine into plasma (Fig. 5, shown in blue).

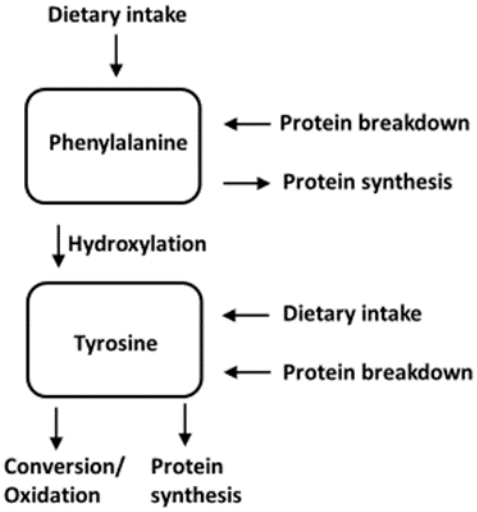


Figure 4 A schematic overview of phenylalanine and tyrosine metabolism. Modified from [165]

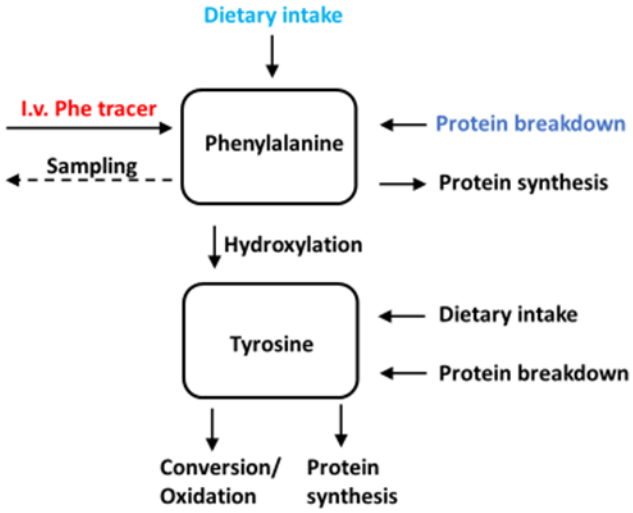


Figure 5 A phenylalanine tracer used to determine the appearance of phenylalanine into plasma. Modified from [165]

4.1.10 Phenylalanine hydroxylation

Hydroxylation to tyrosine is the first, irreversible, and rate-limiting step[167] of phenylalanine catabolism and is catalyzed by phenylalanine hydroxylase, which is primarily located in the liver[168]. Conversion to other metabolites is negligible, unless there is phenylalanine hydroxylase deficiency such as in phenylketonuria. Hydroxylation quantitatively approximates oxidation to CO_2 under certain experimental conditions[169], but does not in itself represent the final metabolic fate of phenylalanine and is therefore not a direct measure of oxidation. This must be accounted for when considering WB metabolism in a broader context, e.g. in comparison with studies using other AA tracers such as L-1-^{13}C-leucine, where AA oxidation can be measured from the end product CO_2.

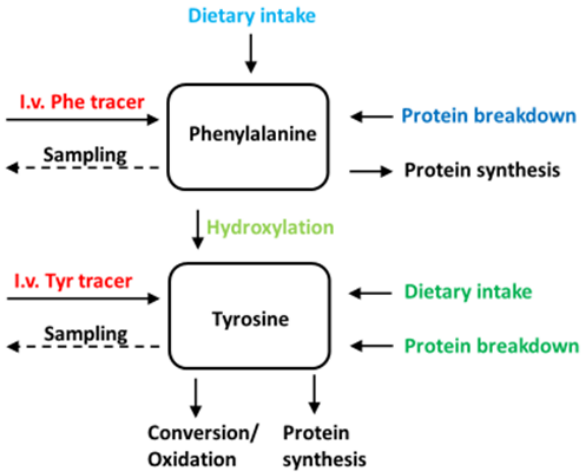


Figure 6 A phenylalanine tracer and a tyrosine tracer used to determine the appearance of phenylalanine and tyrosine into plasma and phenylalanine hydroxylation. Modified from [165]

For purposes of WB turnover, phenylalanine hydroxylation can be quantified by tyrosine tracers[132,164,169,170]. To obtain a complete picture, appearance of tyrosine from phenylalanine hydroxylation is quantified separately from total appearance of tyrosine. The appearance of tyrosine from phenylalanine hydroxylation (Fig. 6, shown in light green) is found from the ratio of fluxes of the corresponding compunds, i.e. L-ring-$^{2}H_4$-tyrosine and its parent compound L-ring-$^{2}H_5$-phenylalanine. Another tyrosine tracer (such as L-3,3-$^{2}H_2$-tyrosine) is used to measure the total appearance of tyrosine from all sources (Fig. 6, shown in light + dark green).

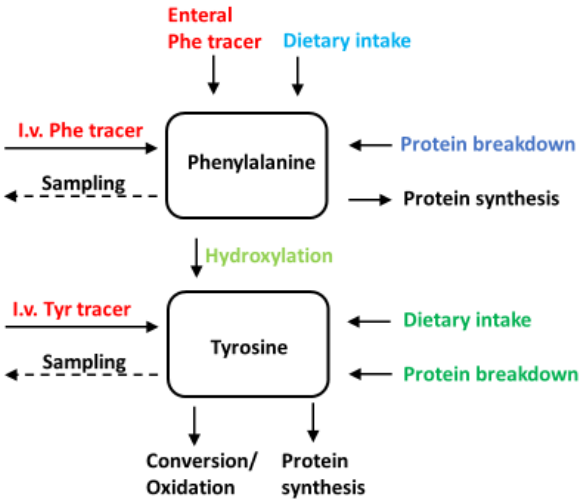


Figure 7 Two phenylalanine tracers and a tyrosine tracer used to determine the appearance of phenylalanine into plasma, the enteral contribution to phenylalanine appearance, the appearance of tyrosine into plasma, and phenylalanine hydroxylation. Modified from [165]

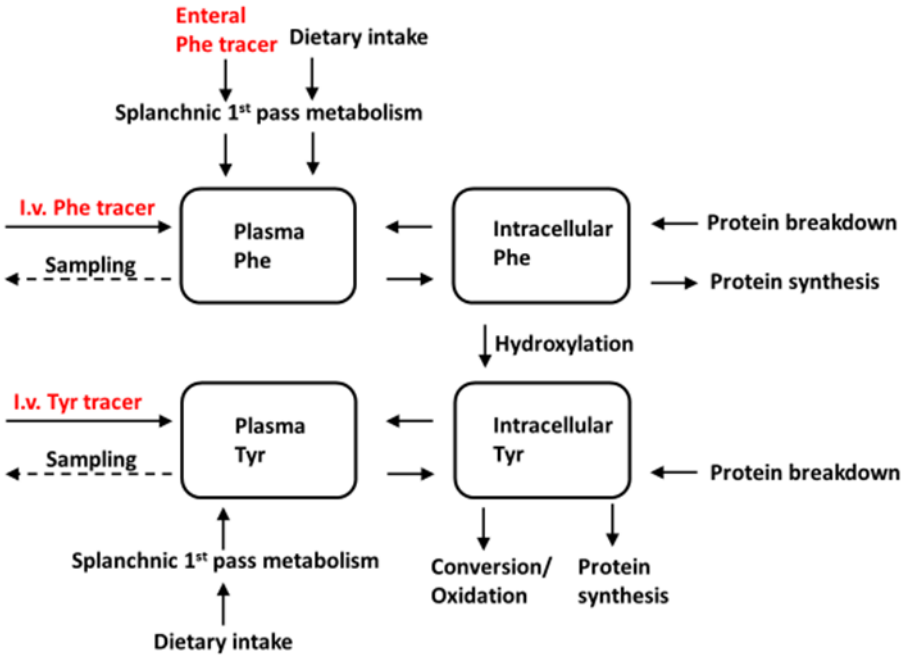


Figure 8 A model of whole-body protein metabolism as measured using phenylalanine and tyrosine tracers, showing processes explicitly measured, processes derived from measurements, and processes included as assumptions. Modified from [165]

4.1.11 Splanchnic first-pass metabolism and quantifying the dietary contribution to amino acid availability

In the fasting state, the only source of an EAA is from proteolysis. In the fed state however, EAA from dietary protein intake must be considered separately when a comprehensive description of protein turnover is sought[133]. For that purpose, it is not sufficient to record the amount of dietary intake, because like most AA[129,142], phenylalanine undergoes extensive first-pass metabolism in the splanchnic circulation[171]. To quantify first-pass metabolism, a second phenylalanine tracer (such as L-1-^{13}C-phenylalanine) is given by the enteral route, either as a free AA added to food, or incorporated into dietary protein as so-called intrinsically labeled protein[172,173]. From the appearance of the enteral tracer into plasma, the contribution of dietary phenylalanine (Fig. 7, shown in light blue) to the total systemic appearance into plasma can be calculated, and conversely the splanchnic extraction fraction of dietary phenylalanine[133].

4.1.12 Summary of the phenylalanine-tyrosine whole-body protein turnover model

To summarize, the WB model here outlined (Fig. 8) uses a multitude of phenylalanine and tyrosine tracers (Fig. 9) and includes processes that are either explicitly measured (viz., total appearance of phenylalanine into plasma, appearance of dietary phenylalanine into plasma, total appearance of tyrosine into plasma, and appearance of tyrosine from phenylalanine hydroxylation into plasma), included as assumptions (phenylalanine hydroxylation as approximation of true oxidation), or calculated from measured values (protein breakdown, protein synthesis, splanchnic extraction fraction).

4.1.13 Muscle and leg amino acid kinetics

AA kinetics in specific organs or tissues can be determined if the organ of interest is accessible for biopsies and/or catheterization of regional vessels. Such regional techniques are applied extensively in animal experiments, but their use in humans is limited by ethical considerations. In the simplest case, substrate net balance can be calculated from arterio-venous (A-V) mass balance. This requires only measurement of blood flow and substrate concentrations in arterial and regional venous samples, but does not allow determination of intraorgan breakdown and synthesis rates[132,174].

For the study of muscle AA metabolism, leg or forearm models using AA tracers are used extensively, and their physiological properties are well characterized. Properties of multiple-pool models, including arterial, venous, interstitial, and muscle tissue compartments, may be calculated from A-V mass balances in combination with AA tracers[132,174]. Such techniques usually require biopsies. However, a model is possible in which an approximation of muscle protein breakdown and synthesis can be quantified, without muscle biopsies, by intravenous phenylalanine tracer infusion[164]. This model makes use of the fact that phenylalanine is not metabolized in muscle, other than in protein breakdown and protein synthesis[174]. Therefore, appearance and disappearance of phenylalanine over the arm or leg can be calculated from

arterial and regional venous phenylalanine concentrations and isotopic enrichments, plus regional blood flow measurement.

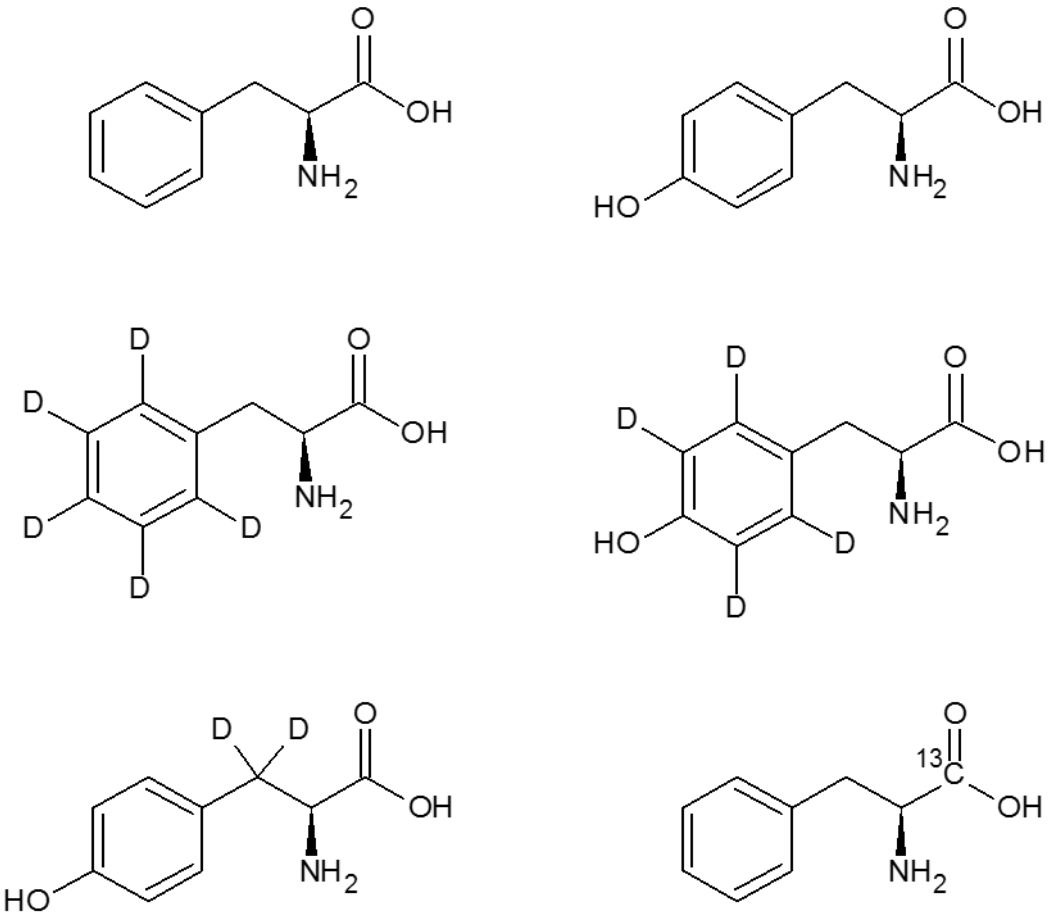


Figure 9 A molecule zoo.
- Upper left, unlabeled L-phenylalanine, $C_9H_{11}NO_2$, 165.19 g/mol
- Upper right, unlabeled L-tyrosine, $C_9H_{11}NO_3$, 181.19 g/mol, hydroxylation product of phenylalanine
- Middle left, L-ring-$^2H_5$-phenylalanine, 170.22 g/mol
- Middle right, L-ring-$^2H_4$-tyrosine, 185.21 g/mol, hydroxylation product of L-ring-$^2H_5$-phenylalanine
- Lower left, L-3,3-$^2H_2$-tyrosine, 183.20 g/mol
- Lower right, L-1-^{13}C-phenylalanine, 166.18 g/mol
Molecular masses indicated are those of the natural isotopic mix or of the tracer preparation, sources: PubChem Open Chemistry Database [175] and manufacturer's data. Graphics created using ACD/ChemSketch 2018.1.1

4.2 DIAGNOSTIC AND ANALYTICAL METHODS

An overview of diagnostic and analytical methods is shown in Table 3. The details are found in the respective papers.

4.2.1 Isotopic analysis

GCMS is here applied to measure relative abundances of isotopic species of phenylalanine and tyrosine, performed on fragments of the silylized AA from plasma samples. For that purpose, mass/charge ratios (m/z) are reported as rounded integer values. Ion m/z values here used are 336 for phenylalanine, 337 for ^{13}C-phenylalanine and ^{15}N-phenylalanine, 341 for $^{2}H_5$-phenylalanine, 466 for tyrosine, 467 for ^{15}N-tyrosine, 468 for $^{2}H_2$-tyrosine, and 470 for $^{2}H_4$-tyrosine, in atomic mass units.

The value required for further calculations is the isotopic enrichment of the tracer in a sample. To arrive at that value, a correction for isotopic background is applied. First, abundances of tracer and tracee in baseline samples are expressed as tracer/tracee ratio (TTR). Then, in subsequent samples from the same subject, abundances are also expressed as TTR and the background value is subtracted. From that result, the tracer's isotopic enrichment is expressed as atom percent excess (APE) by the formula[154]

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

and that value is used for further calculations.

4.2.2 Calculation of leg protein turnover

Leg protein turnover is reported in study 1. As noted above, the only pathways of phenylalanine metabolism in skeletal muscle are those of protein synthesis and breakdown. Thus, measurements of phenylalanine concentrations, tracer isotopic enrichments, and blood flow are sufficient to approximate R_a and R_d over the leg without a need for muscle biopsies[164,174]. Blood flow was here determined by dye dilution using indocyanine green[176].

4.2.3 Calculation of whole-body protein turnover

WB protein turnover is reported in studies 1-3. The principles are described in the physiology section above, and calculations are elaborated in study 1[177] and in reference[164]. Specific assumptions underlying the model are detailed in[164] and are chiefly that:

- free phenylalanine and tyrosine pools are homogenous and mixed;
- protein breakdown is the only source of free phenylalanine in the fasting state;
- hydroxylation to tyrosine and incorporation into protein are the only metabolic fates of phenylalanine;
- implicitly, phenylalanine fluxes are representative of WB protein.

	Isotope analysis	Plasma AA	Other biochemical analyses	Other
Study 1	GCMS for t-butyldimethylsilylether derivatized $^{2}H_5$-Phe, ^{15}N-Phe, $^{2}H_4$-Tyr, ^{15}N-Tyr	HPLC	Plasma FFA: HPLC Plasma insulin: Radioimmunoassay	Blood flow: Indocyanine green Body composition: Dual energy x-ray absorptiometry and single slice computed tomography Energy expenditure: Indirect calorimetry Insulin sensitivity: Quicki index
Study 2	GCMS for MTBSTFA derivatized Phe, ^{13}C-Phe, $^{2}H_5$-Phe, Tyr, $^{2}H_2$-Tyr, $^{2}H_4$-Tyr	HPLC using OPA/3-MPA on-column derivatization		Energy expenditure: Indirect calorimetry (DeltaTrac II)
Study 3 (method evaluation and main study)	GCMS for MTBSTFA derivatized Phe, ^{13}C-Phe, $^{2}H_5$-Phe, Tyr, $^{2}H_2$-Tyr, $^{2}H_4$-Tyr	HPLC using OPA/3-MPA on-column derivatization		Urea: photospectrometric analysis
Study 4	GCMS for MTBSTFA derivatized Phe, ^{13}C-Phe	HPLC using OPA/3-MPA on-column derivatization		

Table 3. Abbreviations used: AA, amino acids; FFA, free fatty acids; GCMS, Gas chromatography – mass spectrometry;HPLC, high pressure liquid chromatography; MTBSTFA, N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide; OPA/3-MPA, ortho-phtaldialdehyde/3-mercaptopropionic acid

Specifically, values calculated are:

- WB flux for phenylalanine, $Q_p = R_aPhe$, representing WB protein breakdown, calculated as

$$Q_P = i \times \left(\frac{E_i}{E_p} - 1\right)$$

where i is the tracer infusion rate, E_i is the isotopic enrichment of the infusate, and E_p is the isotopic enrichment in plasma;

- WB conversion (hydroxylation) of phenylalanine to tyrosine, Q_{PT} (without tyrosine tracer, as in study 1), calculated as

$$Q_{PT} = (0.73 \times Q_P) \times \frac{Q_P}{\left(\frac{E_P}{E_T} - 1\right) \times (Q_P + i_P)}$$

where E_p and E_T are isotopic enrichments of phenylalanine and tyrosine in plasma, respectively, i_P is the phenylalanine tracer infusion rate, and 0.73 is a factor representing the molar ratio of fluxes of tyrosine and phenylalanine from protein breakdown[164,178];

- WB conversion (hydroxylation) of phenylalanine to tyrosine, Q_{PT} (with tyrosine tracers, as in studies 2-3), calculated as

$$Q_{PT} = R_a\,(indep.Tyr\ tracer) \times \frac{E_T(hydrox.prod.of\ Phe\ tracer)}{E_P} \times \frac{R_aPhe}{R_aPhe + i_P}$$

using values for the hydroxylation product of the phenylalanine tracer (i.e. L-ring-$^2H_4$-tyrosine from hydroxylation of L-ring-$^2H_5$-phenylalanine) and the independent tyrosine tracer (i.e. L-3,3-$^2H_2$-tyrosine), where E_p and E_T are isotopic enrichments of phenylalanine and tyrosine in plasma, respectively, and i_P is the phenylalanine tracer infusion rate;

- WB incorporation of phenylalanine into protein, R_dPhe, representing WB protein synthesis, calculated as

$$R_d = Q_P - Q_{PT}$$

- WB protein net balance, calculated as

$$R_dPhe - EndoR_aPhe$$

where $EndoR_aPhe$ is the endogenous rate of appearance of phenylalanine;

- Endogenous rate of appearance of phenylalanine, representing WB protein breakdown, assumed – in the fasting state – to equal Q_p

$$EndoR_aPhe\ (fasting) = Q_p$$

and - in the fed state - calculated as

$$EndoR_aPhe\ (fed) = Q_p - dietary\ R_aPhe$$

4.2.4 Contribution of dietary protein to WB protein turnover

The contribution of dietary protein or AA to WB metabolism is reported in studies 1-3. As noted above in the physiology chapter, a second phenylalanine tracer is added to the enteral nutrition formula. Calculations are elaborated in study 1[177] and are modified from reference[171]. To arrive at the dietary R_aPhe used in the previous formula, splanchnic first-pass metabolism is accounted for by the following calculations:

- the rate of appearance of dietary phenylalanine tracer, calculated as

$$R_a(dietary\ Phe\ tracer) = \frac{i_{iv}}{\left(\frac{E_{iv}}{E_{dietary}}\right)}$$

where i_{iv} is the infusion rate of the intravenous phenylalanine tracer and E_{iv} and $E_{dietary}$ are isotopic enrichments of the intravenous and dietary tracers in plasma, respectively;

- which is then used to calculate the rate of appearance of dietary unlabeled phenylalanine by

$$dietary\ R_aPhe = \frac{R_a(dietary\ Phe\ tracer)}{E_{Phe\ (nutrition)}}$$

where $E_{Phe(nutrition)}$ is the enrichment of the tracer in nutrition;

Furthermore, splanchnic uptake, calculated as

$$Phe\ splanchnic\ uptake = Phe\ total\ dietary\ intake - dietary\ R_aPhe$$

which when expressed as a percentage of intake is termed splanchnic extraction fraction.

4.2.5 Plasma aminograms

Plasma amino acids were measured by high-performance liquid chromatography (HPLC), which can be considered the standard technique for this purpose[179,180]. The details of the technique employed in studies 2-4 are elaborated in reference[181].

4.2.6 Indirect calorimetry

Resting energy expenditure was determined by indirect calorimetry, using the Deltatrac II instrument[182], to determine doses of nutrition in healthy subjects (studies 1-2) and in some, but not all, ICU patients (studies 2-3). Measurement of energy expenditure by indirect calorimetry is the standard technique to guide nutrition in the ICU[183] and is recommended in current guidelines[2]. In particular, measurement by the Deltatrac instrument is considered a standard technique the ICU[184,185].

4.2.7 Insulin sensitivity

In study 1, insulin sensitivity was assessed by QUICKI ("quantitative insulin sensitivity check index")[186]. The Quicki index is easily calculated from glucose and insulin values in a single blood sample taken in the fasting state. It is extensively validated against direct tests such as glucose-insulin clamp studies, and yields a meaningful estimation of insulin sensitivity under most circumstances[187,188].

4.3 STATISTICAL METHODS

An overview of statistical methods is shown in Table 4. All studies in this project comprise small numbers of study subjects, necessitated by resource intensive experimental setups and laboratory procedures. It is acutely understood that results from such unique, small, mechanistic studies are to be interpreted with great caution and in the context of relevant physiology. It is furthermore understood that any method of statistical inference may be of limited usefulness in such a setting[189]. That notwithstanding, results are presented using conventional, and mostly very conservative, statistics for both descriptive and inferential purposes.

In study 4, a test was sought to define a possible periodicity in the time series for tracer enrichments and plasma AA. Simulations demonstrated that the Ljung-Box test[190] may be useful. The Ljung-Box test tests for serial autocorrelation and was here used on residuals from a model that removes linear trends, to highlight possible periodicity.

	Descriptive statistics	**Statistical tests**	**Other**
Study 1	Mean, standard deviation	Repeated measures ANOVA with post hoc analysis using Bonferroni correction Mann-Whitney U test t test for independent samples	
Study 2	Median, range Mean, standard error of the mean	Wilcoxon matched pairs test	
Study 3	Median, range Pearson's correlation coefficient	Mann-Whitney U test Wilcoxon matched pairs test Friedman test t test for paired samples	
Study 4	Median, range Coefficient of variation	Spearman's rank correlation coefficient	Ljung-Box test

Table 4

Abbreviations: ANOVA, analysis of variance

5 RESULTS

All descriptive statistics are reported as median (range) unless indicated otherwise.

5.1 STUDY 1

5.1.1 Method evaluation

This work was dedicated to both method evaluation and the investigation of the anabolic response to protein feeding in a population of viscerally obese, insulin resistant subjects. Specifically, muscle protein[164] and WB protein turnover was calculated as described above in detail. The main finding from the methodological perspective was that the tracer methods used were workable in this setting.

5.1.2 Body composition, insulin resistance and free fatty acid metabolism

Two cohorts of obese women were studied who differed in body fat distribution. The criterion for grouping into upper-body obese (UBO) vs. lower-body obese was a waist-to-hip ratio (WHR) of >0.85 vs. <0.80. Although the WHR criterion has its limitations for this purpose[191], it has been shown to sufficiently discriminate between metabolic phenotypes, specifically with respect to deficient insulin-mediated postprandial lipolysis suppression[192], but also to insulin sensitivity of carbohydrate metabolism[191].

In the cohorts here studied, UBO subjects had a greater amount of visceral fat as measured by single-slice computed tomography. Baseline insulin sensitivity was lower, baseline plasma free fatty acid (FFA) concentrations were similar and postprandial FFA suppression was less pronounced in the UBO group. These findings confirm that the UBO cohort is sufficiently representative of the viscerally obese, insulin resistant phenotype of obesity[191].

5.1.3 Protein turnover

Protein turnover was calculated before and after a mixed meal. The main findings for muscle protein turnover were that net protein balance shifted from negative, i.e. catabolic, in the fasting state to zero after feeding. There were no differences between groups. The main findings for WB protein turnover were that in response to feeding, phenylalanine hydroxylation increased somewhat and WB net protein balance switched from negative, i.e. catabolic, to positive, i.e. anabolic. Net protein balance after feeding was less positive in the UBO group.

5.2 STUDY 2

5.2.1 Method evaluation

Here the experimental protocol previously used in study 1 was modified in two ways. Firstly, enteral nutrition contained casein intrinsically labeled with L-1-^{13}C-phenylalanine[172] to determine the contribution of dietary protein to WB protein turnover. Secondly, tyrosine

tracers were used for a more accurate determination of WB phenylalanine hydroxylation. The protocol was first investigated in a proof-of-concept experiment in six healthy subjects to ensure workability and the attainment of sufficient isotopic enrichment in plasma samples. The response of WB protein turnover to feeding was then studied in ten critically ill patients.

5.2.2 Patients and nutrition

The cohort studied consisted of ten patients in an early phase of critical illness. They were recruited from the ICU at Karolinska University Hospital Huddinge and represent a population fairly typical of a mixed adult ICU at a tertiary referral hospital, in that they were elderly with an age of 73.5 (65-79) years and were rather ill with APACHE 2 scores of 25.5 (19-40) and SOFA scores of 7.5 (3-12). Main diagnoses at ICU admission were surgical in three and medical in seven. Six patients died within 30 days, one within one year after ICU admission, and three were alive after one year.

Experiments were performed at 55.5 (34-87) hours after ICU admission. Patients were not previously enterally fed but received parenteral nutrition. Study nutrition was composed of intrinsically labeled casein and maltodextrin at a hypocaloric-hyponitrogenous dose and was administered by continuous infusion over 6 hours. The dose of parenteral nutrition was not standardized for purposes of the study and patient charts were reviewed in two-hours intervals to document the amount of nutrients given parenterally. There was variable nutrient supply during the experiment in five patients, with variation exceeding 50% of the maximum value in three.

	Healthy (n=6)			Patients (n=10)		
	PN	PN+EN	p	PN	PN+EN	p
Essential AA	1248 (940-1566)	1382 (1070-1539)	0.116	924 (497-1533)	1009 (571-1730)	0.241
Nonessential AA	1839 (1478-2072)	1900 (1364-2281)	0.753	1524 (703-2702)	1607 (676-2760)	0.333
Phenylalanine	96 (83-130)	114 (80-132)	0.116	105 (64-299)	121 (76-309)	0.005
Leucine	145 (116-190)	154 (126-169)	0.753	105 (73-195)	118 (67-170)	0.646

Table 5 Plasma amino acid concentrations in healthy subjects and in critically ill patients on parenteral vs. parenteral plus hypocaloric-hyponitrogenous, continuous, enteral protein feeding. Data denote median (range). P values for within-group comparison by Wilcoxon matched pairs test.Values in µmol/L, averaged from measurements at timepoints 120-150 min for PN, at 480-510min for PN+EN. Essential AA represent His, Thr, Val, Met, Trp, Phe, Ile, Leu, Lys; nonessential AA represent Glu, Asn, Ser, Gln, Gly, Arg, Ala, Tyr. Abbreviations used: AA, amino acid(s), PN, parenteral nutrition, EN, enteral nutrition

5.2.3 Findings

Results are reported for both healthy subjects and critically ill patients. However, between-group comparisons by statistical tests were not done in consideration of the great differences in physiology between those groups.

Plasma AA concentrations, calculated as the sum of essential AA and the sum of nonessential AA, showed great variability, particularly in critically ill patients (Table 5). Changes between fasting and fed states could not be statistically ascertained. Except for phenylalanine and leucine, detailed analyses of individual AA were not performed.

Uptake of dietary phenylalanine into arterial plasma was documented as the time course of plasma ^{13}C-phenylalanine isotopic enrichment in 30 minutes intervals during the experiment. The main findings here were that:

- there is great inter-individual variability
- uptake is detectable promptly in healthy in healthy subjects but delayed in patients (Fig. 10)
- uptake remains near zero in several patients
- uptake reaches a tentative steady state after approximately 3 hours in healthy subjects, but later in patients (Fig. 11)

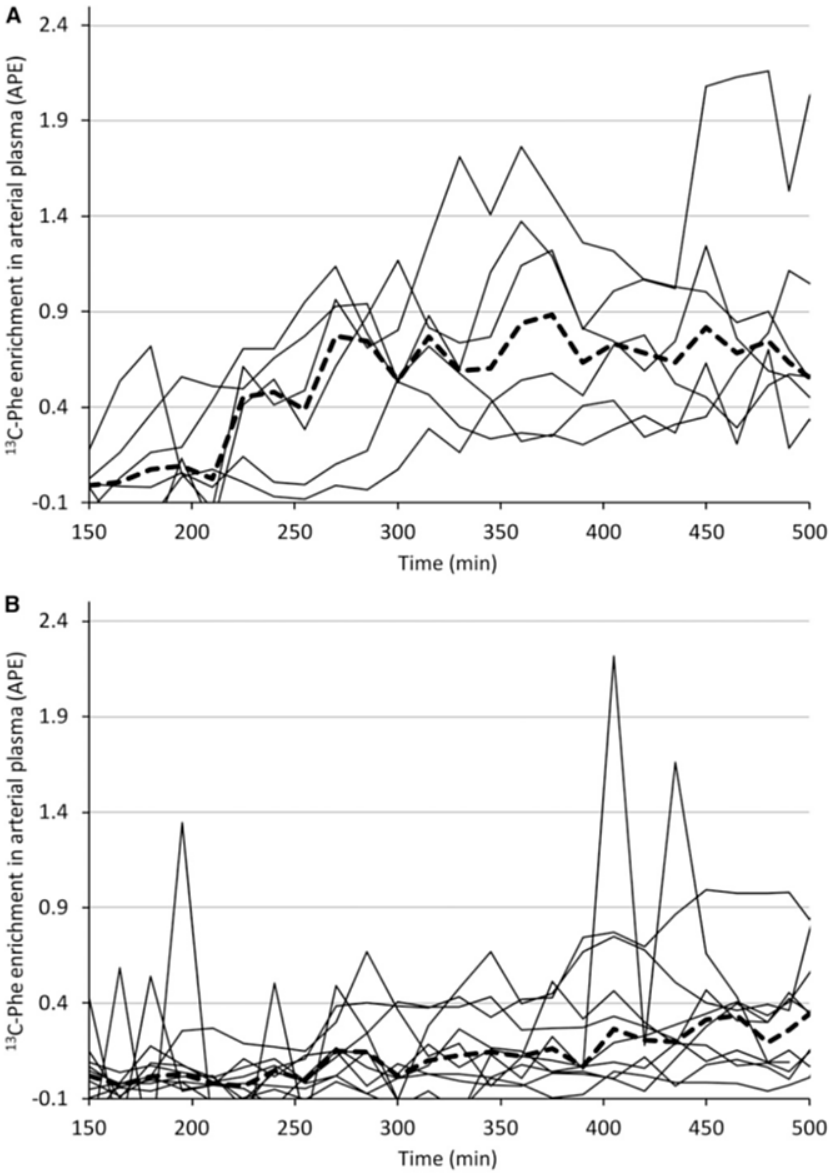


Figure 10 Isotopic enrichment of L-1-^{13}C-phenylalanine from dietary intrinsically labeled casein in arterial plasma of n=6 healthy volunteers (A) and n=10 critically ill patients (B) receiving early enteral feeding. Data are shown for individual subjects and medians (dashed line). APE, atom percent excess.

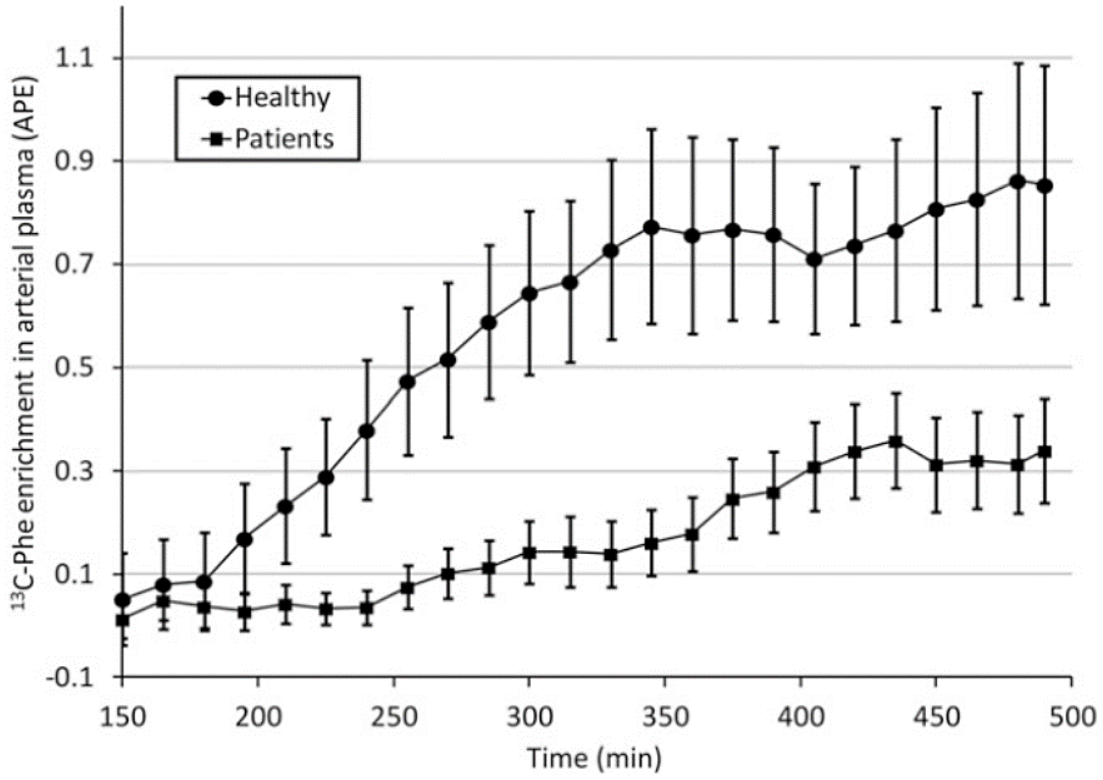


Figure 11 Mean (±SEM) isotopic enrichment of L-1-^{13}C-phenylalanine from dietary intrinsically labeled casein in arterial plasma of healthy volunteers (n=6, shown as circles) and critically ill patients (n=10, shown as squares) receiving early enteral feeding. Unweighted moving averages were calculated for individual subjects over n=5 consecutive measurements. APE, atom percent excess.

The related parameter of splanchnic extraction fraction of dietary phenylalanine was found to be high with values of 80 (60-91) % in healthy subjects and 92 (86-99) % in patients.

Protein turnover was characterized as WB protein breakdown, synthesis, and net balance, as well as phenylalanine hydroxylation (here termed Phe conversion). In healthy subjects, no changes of these parameters before vs. after feeding were seen ($p = 0.173$ to 0.917 by Wilcoxon's matched pairs test). In critically ill patients, there was great inter-individual variation of all parameters at both timepoints and changes between timepoints were small ($p = 0.047$ to 0.139).

In three patients, ongoing parenteral nutrition was found to be highly variable over time. Results for the remaining seven patients were analyzed separately, the rationale being that WB protein turnover is modulated by total AA availability, hence effects of nutritional intervention would be difficult to discern when confounded by varying AA supply from another source. In these seven patients, changes from fasting to fed states were small on average. A small gain in protein net balance was found, with patients becoming less catabolic with a net balance of -8.6 (-22.6 to +1.3) µmol phenylalanine x kg^{-1} x h^{-1} before feeding vs. -5.8 (-20.0 to +3.4) µmol phenylalanine x kg^{-1} x h^{-1} after feeding ($p = 0.018$).

5.3 STUDY 3

In study 3, results are reported on:

- a method evaluation study designed to investigate the equivalence of two phenylalanine tracers given enterally, namely the protein-bound L-1-^{13}C-phenylalanine used previously in study 2, and L-ring-$^{2}H_5$-phenylalanine given as a free AA together with study nutrition
- an investigation of WB turnover in critically ill patients before vs. after three hours of parenteral AA supplementation
- a repeated investigation of WB turnover, by the same experimental protocol and in the same patients, after several days in the ICU.

5.3.1 Method evaluation study

Here it was investigated whether the intrinsically labeled casein previously used in study 2 might be replaced with a free AA tracer. The relevant physiological differences between free vs. protein-bound dietary AA are related to digestion and enteral absorption, processes which may be affected by critical illness. Therefore, this study was conducted in critically ill patients and not in healthy subjects.

Six patients were recruited from the ICU at Karolinska University Hospital Huddinge. Their age was 70 (61-79), SAPS III score was 73 (41-76), SOFA score was 8 (1-12), and main diagnoses at ICU admission were surgical in four and medical in two. The study was performed on day 4 (3-48) after ICU admission. Enteral feeding was given in a protocol similar to that from study 2, combining maltodextrin, casein intrinsically labeled with L-1-^{13}C-phenylalanine, and the free L-ring-$^{2}H_5$-phenylalanine tracer. Isotopic enrichments of the two tracers were measured in plasma samples over 6 hours and their rates of appearance into plasma were calculated, with no correction for splanchnic extraction.

No uptake into plasma of either tracer was seen in one patient. In the remaining five, R_a of the protein-bound tracer was initially higher, but R_a values for both tracers equilibrated after no more than three hours of infusion. It was concluded that given sufficient infusion time, the phenylalanine tracer administered as a free AA in combination with enteral nutrition is suitable to represent phenylalanine from dietary protein in critically ill patients.

5.3.2 Whole-body protein turnover study

5.3.2.1 Patients and nutrition

For the WB turnover study, fourteen patients were initially recruited, but several were lost to subsequent experiments because of logistical or medical problems. Thirteen had WB protein turnover studied at the first timepoint before and after intervention, ten of these at the second timepoint before intervention, and seven of these ten at the second timepoint before and after intervention. Patients' age was 69 (46-77), 71 (46-77) and 71 (46-77), respectively, for the

above-named subgroups; SAPS III score was 70 (54-93), 70 (54-93), and 73 (54-93); main diagnoses at ICU admission were (surgical/medical) 6/7, 6/4, and 4/3.

Nutrition schedules were not standardized for purposes of this study. Total energy and protein intake varied between patients and timepoints and were generally in the hypocaloric/hyponitrogenous range (Table 6).

	Total energy intake (kcal/kg/day)	Total protein intake (g/kg/day)
Q1 effect AA (n =13)		
First basal	17 (0 to 26)	0.7 (0.0 to 1.0)
First AA	21 (4 to 30)	1.6 (1.0 to 2.0)
P-value	0.001	0.001
Q2 time effect basal (n =10)		
First basal	16 (0 to 21)	0.6 (0.0 to 1.0)
Second basal	18 (0 to 36)	0.7 (0.0 to 1.4)
P-value	0.09	0.11
Change from first to second	8 (−21 to 23)	0.3 (−1.0 to 0.9)
Q3 time effect AA (n =7)		
First basal	14 (0 to 20)	0.5 (0.0 to 0.8)
First AA	17 (4 to 24)	1.5 (1.0 to 1.7)
P-value	0.017	0.017
Second basal	20 (10 to 36)	0.8 (0.4 to 1.4)
Second AA	24 (14 to 40)	1.7 (1.4 to 2.3)
P-value	0.017	0.017

Table 6 Total energy and protein intake in ICU patients before/after intravenous amino acid supplementation. Q1: change between basal and post-supplementation values. Q2: change between first measurement and second measurement several days later. Q3: effect of amino acid supplementation maintained at second measurement?

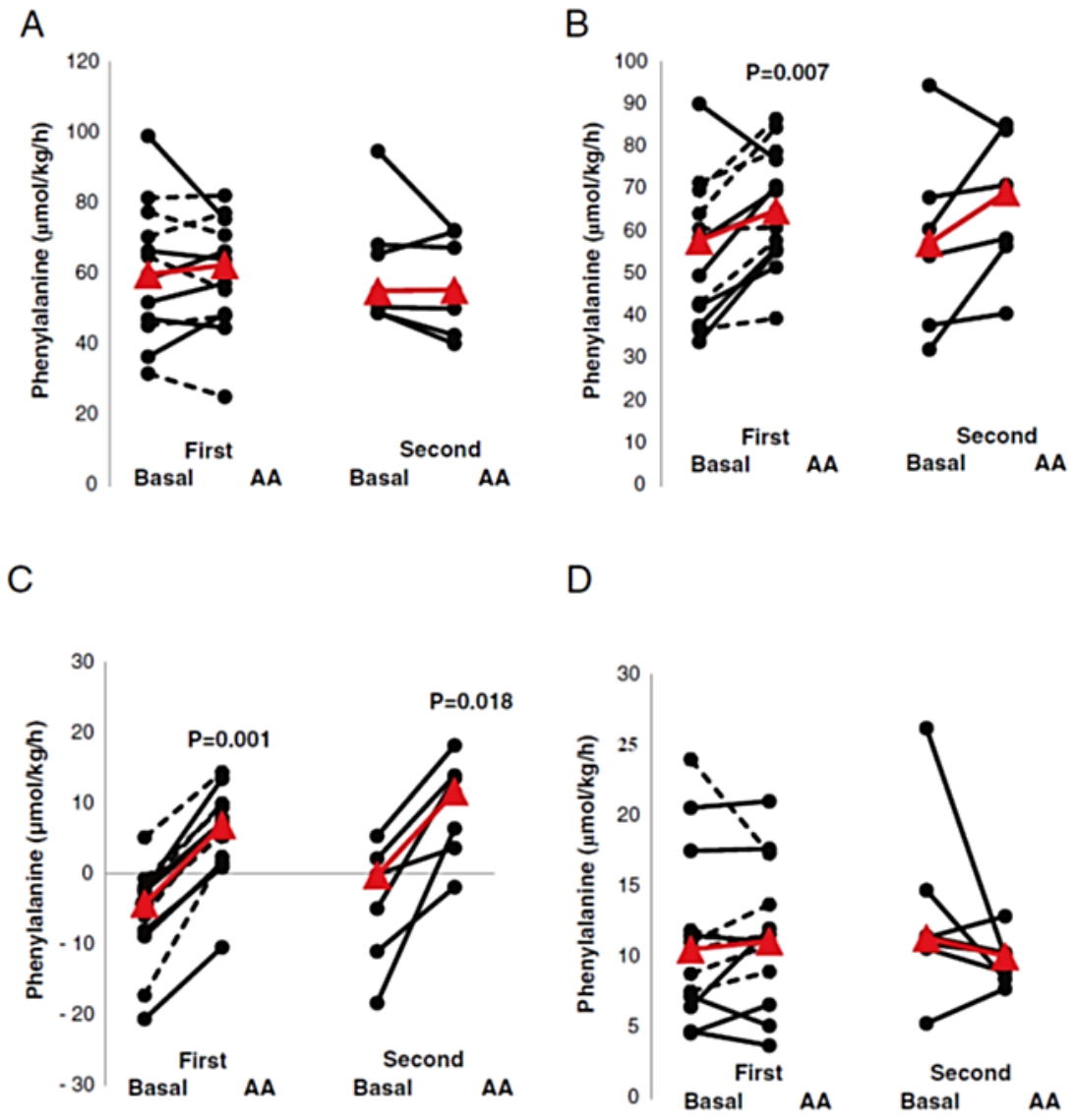


Figure 12 Whole-body protein breakdown (A), protein synthesis (B), protein balance (C) and phenylalanine oxidation (D) in critically ill patients during baseline and parenteral amino acid supplementation (AA) on 2 study days. On the first study day, 13 patients were studied, and on the second study day, 7 of these patients were still being treated in the intensive care unit and were studied again. The seven patients studied twice are indicated by continuous lines, and the other five studied on day 1 only are indicated by dashed lines. Individual values are shown, with the median in red.

5.3.2.2 Response to parenteral amino acid supplementation

In response to parenteral AA supplementation, plasma AA concentrations increased in all patients at both timepoints, and plasma urea concentrations remained unchanged.

All parameters of WB protein turnover showed a wide inter-individual variation at all timepoints. When WB protein turnover was first studied on day 7 (2-14) after ICU admission, it was found that WB protein breakdown and phenylalanine hydroxylation remained

unchanged after parenteral AA supplementation. WB protein synthesis increased from 58 (34 to 90) to 65 (40 to 87) µmol phenylalanine x kg^{-1} x h^{-1}, and WB net protein balance switched from catabolic at -4 (-21 to +5) to anabolic at +7 (-10 to +14) µmol phenylalanine x kg^{-1} x h^{-1} (Fig. 12). Splanchnic extraction fraction of dietary phenylalanine decreased from 0.4 (0.2 to 0.6) to 0.2 (-0.1 to 0.9).

Furthermore, parameters of WB protein turnover were studied at a second timepoint two to four days later, with an experimental protocol identical to the first experiment. No change over time was seen in basal (i.e. before intervention) WB protein breakdown, phenylalanine hydroxylation, WB protein synthesis, net balance, or splanchnic extraction.

Finally, the response to AA supplementation was compared between the first vs. second timepoint of intervention. No difference was seen in any parameter of WB protein turnover.

5.4 STUDY 4

In study 4, the time course of uptake of phenylalanine from continuous enteral nutrition was studied in healthy subjects and critically ill patients. After a run-in time of 12 hours, ongoing nutrition was supplemented with a with free L-1-^{13}C-phenylalanine tracer whose uptake into arterial blood was then measured over 12 hours.

5.4.1 Subjects and nutrition

Seven male and three female healthy subjects of 23 (20-46) years of age were studied. Furthermore, ten critically ill patients were recruited from the ICU at Karolinska University Hospital Huddinge. Patients' age was 64 (44-74) years, SAPS III score was 60.5 (57-85), SOFA score was 5.5 (1-10), and main diagnoses were surgical in three, medical in four and neurological in three. One patient died on day 37 after ICU admission, and nine were alive 90 days after ICU admission. Experiments were performed on day 20 (5-41) after ICU admission. Nutrition was provided by continuous infusion via nasogastric tube or jejunostomy, using a complete enteral formula providing 25 kcal/kg per day for healthy subjects, or >80% of calculated or measured energy requirements for patients.

5.4.2 Findings

Plasma ^{13}C-phenylalanine isotopic enrichments and concentrations of EAA and total AA showed large inter-individual variations at all timepoints, in both healthy subjects and critically ill patients. A tentative steady state of ^{13}C-phenylalanine isotopic enrichment - here defined as a rolling average value that does not change by more than 5% by including additional samples - was reached in all individuals after no more than 270 minutes.

Individual time series were dominated by timepoint-to-timepoint variation. Expressed as coefficient of variation for individual time series, variability of plasma ^{13}C-phenylalanine isotopic enrichment was 20.9 (12.3-46.3) % in patients and 25.8 (17.5-30.3) % in healthy subjects, variability of plasma EAA concentrations was 10.2 (7.8-13.2) % in patients and

10.5 (8.5-18.3) % in healthy subjects, and variability of plasma total AA concentrations was 8.1 (7.3-9.9) % in patients and 8.6 (6.4-13.3) in healthy subjects.

To mathematically characterize variability over time, individual time series were tested for possible intra-individual periodicity. No evidence of periodicity was found, though simulations showed that a shorter sampling interval would be required to give the test an appropriate sensitivity for the type of data at hand.

Finally, co-variation between timepoint-to-timepoint changes of ^{13}C-phenylalanine isotopic enrichment and plasma EAA concentrations was found to be significant in three of ten patients and in nine of ten healthy subjects.

6 DISCUSSION

6.1 METHODS

Tracer studies allow unique insights into metabolic processes but require caution in the interpretation of findings. In this section, it is outlined what may and may not be concluded from the findings previously described.

Tracer studies are subject to a trade-off between invasiveness and the information that can be gained. In human in vivo experiments, ethical and practical considerations restrict the range of anatomical sites accessible for tracer administration and sampling. The techniques discussed here require access to: arterial and venous blood, plus possibly enteral infusion for WB protein turnover; arterial and femoral venous blood for muscle protein turnover; and arterial blood and enteral infusion for enteral AA uptake. These are minimally invasive methods and consequently, the information obtainable is restricted to AA turnover in plasma and in skeletal muscle, and a limited description of dietary AA uptake into plasma.

Furthermore, calculations of substrate turnover rest upon assumptions about physiology and methodology that are not always explicitly stated but should be understood to appraise results. Underlying assumptions for tracer techniques are outlined in the respective sections above and are discussed further in the following sections.

6.1.1 Whole-body protein turnover

No method of measuring WBPT in human subjects can be regarded as "true" and (…) even if we had such a model, we would not be able to determine that it was "correct". John Waterlow[163]

A fundamental weakness of steady state "whole-body" protein turnover is that it is poorly defined what it measures, both on a technical and a conceptual level[133]. Furthermore, the validity of the concept rests on assumptions that may be difficult to verify[163]. A few areas of uncertainty are outlined below.

First, by using plasma labeling and sampling, only such processes can be studied whose precursors and products are found in plasma. However, protein breakdown and synthesis are intracellular processes and all AA turnover is affected by the exchange between the intracellular space and other compartments, as well as by intracellular AA cycling. Neither process can be quantified by plasma sampling. Therefore, it may remain uncertain to which degree true AA turnover is systematically underestimated in a WB model[144].

Second, tracer recycling, i.e. tracer being incorporated into protein and then released by proteolysis during the study period, will affect AA turnover calculations[144]. Recycling is probably not significant under many experimental conditions[133], but it cannot be quantified from plasma samples.

Third, the assumption that relevant compartments equilibrate in steady-state conditions may or may not hold true for specific experimental conditions. Slowly equilibrating metabolic pools may exist, whose metabolism is not quantitatively represented in plasma fluxes[133].

Fourth, positive changes in some organs or tissues may be offset by negative ones elsewhere, so that effects of any intervention may remain undetected, or underestimated, by WB methods.

Fifth, a direct measurement of protein synthesis is not possible without access to the target protein pool e.g. by biopsy. Therefore, protein synthesis and balance are calculated indirectly from values of total AA appearance into plasma, catabolism, and intake. Of these, catabolism ("oxidation") of the target AA can be measured from isotopic enrichment in metabolic end products such as CO_2 or urea, or approximated from measurement of the product of some irreversible step in the catabolic pathway. These measurements require further assumptions about kinetic parameters of metabolic pools in the catabolic pathway, introducing additional sources of error.

Sixth, the necessary underlying assumption of steady state conditions is inherently an abstraction, and even in a resting subject changes in metabolism may exist that go undetected unless specifically looked for.

These uncertainties do not invalidate the method, but emphasize that its limitations should be recognized. The predicament may be resolved by understanding WB protein turnover as an operationally defined concept, i.e. accepting WB turnover as whatever is measured by the specific tracer and experimental protocol used. Under that premise, WB turnover studies are a reasonable choice to investigate the response to disease or intervention, and to compare different cohorts of subjects.

6.1.2 Phenylalanine/tyrosine tracers

The method here used in studies 1-3 is minimalistic in that only plasma sampling is required. Equally minimalistic are the findings, as only plasma substrate fluxes (of total and dietary phenylalanine, plus phenylalanine hydroxylation) are directly measured, and AA intake recorded, and no compartment modeling takes place. Two peculiarities of the phenylalanine/tyrosine method should be considered, that is to say, the representativeness of phenylalanine hydroxylation as a measure of catabolism, and the representativeness of phenylalanine turnover as a measure of WB protein turnover.

Phenylalanine catabolism is assumed to be quantitatively represented by hydroxylation, i.e. total catabolism is represented by the first irreversible catabolic step. The assumption is valid if, firstly, hydroxylation leads to the only relevant catabolic pathway in phenylalanine metabolism. This seems to be the case, as of the other possible pathways of phenylalanine catabolism, such as transamination to phenylpyruvate[167], none are quantitatively relevant in healthy adults[165]. Secondly, the fraction of total tyrosine flux going into protein synthesis vs.

catabolism should be quantifiable[165]. This is done by using a tyrosine tracer as described above.

The representativeness of phenylalanine turnover as a measure of WB protein turnover is an inherent assumption of the method. Fundamentally, WB turnover can be measured as turnover of any representative EAA. Tracers for different EAA should then give similar results for WB turnover when the respective AA content in protein is taken into account. This premise is indeed correct for the endogenous R_a of various EAA when normalized to their content in muscle protein[133,144]. Also, when directly compared in fasting healthy subjects, WB turnover calculated from infusions of L-ring-$^{2}H_5$-phenylalanine and L-1-^{13}C-leucine was found to be similar[164]. Here the authors extensively discuss the significance of values derived from leucine oxidation to CO_2 vs. phenylalanine hydroxylation, suggesting that the different catabolic pathways and different tissues involved imply an independent validation of the phenylalanine/tyrosine method.

6.1.3 Phenylalanine/tyrosine tracers in critically ill patients

The theoretical advantage of the leucine method – direct measurement of AA oxidation from the metabolic end product CO_2 – makes it less suitable for ICU patients. To standardize values calculated from isotopic enrichment in exhaled CO_2, the fraction of CO_2 recovered from the body's HCO_3^- pool during the measurement period must be taken into account. Given ICU patients' alterations in body composition and acid-base balance, and possible losses through renal replacement therapy, such recovery would have to be measured individually by additional tracer studies, adding theoretical uncertainties and logistical challenges. In one study from our group[31], both leucine and phenylalanine/tyrosine tracers were found to be workable, and it was found that healthy subjects increase AA oxidation in response to parenteral feeding (Fig. 13), while ICU patients do not. In another study, WB turnover was measured to investigate the response to varying doses of parenteral nutrition in critically ill patients, using simultaneous infusions of L-ring-$^{2}H_5$-phenylalanine and L-1-^{13}C-leucine[193]. While WB protein balance was similar when calculated from either tracer, it was noted that the phenylalanine tracer showed a change in WB protein synthesis while the leucine tracer did not. This discrepancy is not readily explained from the experimental data, but it was speculated that the two tracers may reflect different weightings of AA metabolism, in that leucine may be more representative of skeletal muscle and phenylalanine more representative of liver metabolism.

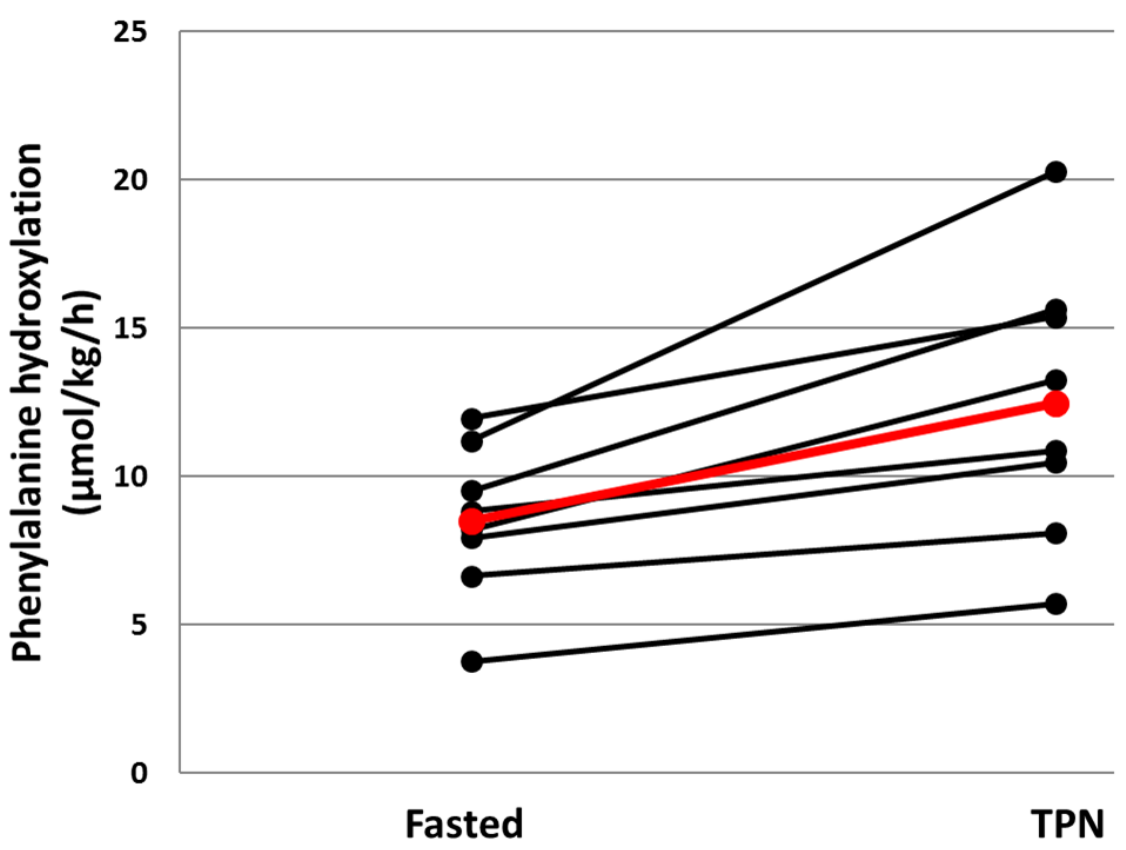


Figure 13 Phenylalanine hydroxylation in healthy subjects before and after parenteral nutrition corresponding to 20% of daily energy expenditure given over 3 hrs. p = 0.003 by paired t test (unpublished data from [31], reproduced by the authors' permission)

6.1.4 Splanchnic first-pass metabolism of dietary amino acids

Measuring WB turnover is conceptually simple when AA intake is either zero, i.e. in fasting subjects, or easily measured, i.e. during parenteral feeding. However, when there is an exogenous source of AA from dietary protein, that contribution must also be quantified[133,144]. This cannot be done from theory, because digestion, absorption, and splanchnic first-pass metabolism vary greatly between different AA[142], experimental protocols, and subject cohorts. Neither is it practical to experimentally quantify all the physiological processes affecting an AA from intake to release into hepatic venous blood and further on to sampling in arterial blood (Fig. 14). Rather, these processes in their entirety are treated as a black box when measuring the R_a of a dietary AA using a tracer in the nutrition.

The techniques here discussed rely on a tracer that is either incorporated in intact dietary protein (calculation of dietary R_a for WB turnover in study 2 and method evaluation in study 3) or added to nutrition as free AA (calculation of dietary R_a for WB turnover in study 3 and labeled phenylalanine uptake in study 4). As discussed in more detail in study 2, values for the splanchnic extraction fraction of phenylalanine have been reported from below 30 to nearly 60 percent[194,195], depending on experimental protocol and choice of tracer.

Intrinsically labeled protein is used extensively[172,173] to quantify the R_a of AA from dietary sources. However, the validity of the technique has been criticized on conceptual grounds, in that tracer dilution by unlabeled AA at various stages in the splanchnic organs may not properly be accounted for[196,197]. That controversy cannot be resolved here. Rather, its existence is taken to support the view that findings obtained by these techniques should be considered in the context of their respective experimental protocols.

These arguments support the notion that the phenylalanine/tyrosine method is valid within its operational definition, and is to some degree also independently corroborated. It may yield meaningful results for the global response of protein metabolism to intervention. However, findings from such studies will not directly reveal the location or mechanism of effects, and should ideally motivate investigations of the anatomical and biochemical details.

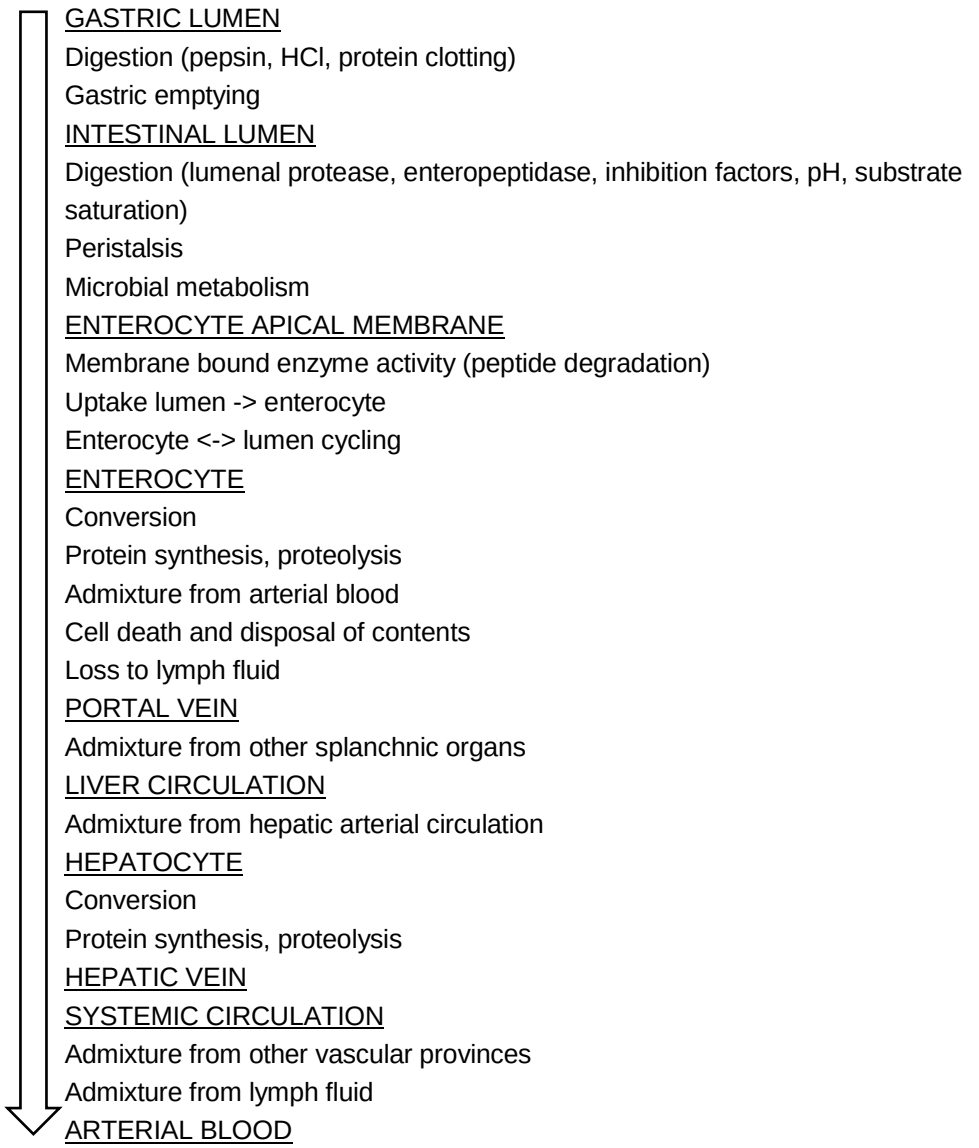


Figure 14 A simplified schematic of transport and metabolic steps for dietary amino acids

6.2 STUDY SUBJECTS AND (LACK OF) BETWEEN-GROUPS COMPARISONS

Even though many critically ill patients show a degree of similarities in their clinical course, populations of critically ill patients are notoriously heterogenous in their demographics, comorbidities, diagnoses, physiology, and outcomes[198-200]. The patient cohorts seen in studies 2-4 are fairly representative of an adult mixed ICU in a tertiary center, but are in themselves heterogenous, as exemplified by the diversity of diagnoses, severity of illness scores, length of stay, and length of survival. Therefore, any generalization of findings should be made with caution.

Extensive physiological differences between healthy subjects and critically ill patients are expected, related to both preexisting morbidity and manifestations of critical illness itself. Thus, the findings of between-groups differences in protein metabolism in studies 2 and 4 are unsurprising. In study 2, between-groups differences in AA concentrations and parameters of WB protein turnover were noted but not formally evaluated. In study 4, characterization of the physiological differences between healthy subjects and ICU patients was one aim. Variability over time of plasma AA concentrations and ^{13}C-phenylalanine enrichment was compared between groups and differences could not be statistically ascertained. Given the exploratory nature of the study and the insufficient sampling rate, this finding should not be considered definitive.

6.3 PROTEIN TURNOVER

The main findings in study 1 were that upper-body (viscerally) obese women have a diminished sensitivity of postprandial stimulation of WB protein anabolism, and that they are insulin resistant as demonstrated by lower Quicki index and lower postprandial lipolysis suppression, compared with lower-body-obese women. Visceral obesity is known to be associated with postprandial insulin resistance of fatty acid metabolism[192], and insulin resistance of glucose metabolism. The pathophysiology of insulin resistance in visceral obesity is complex and so is the role of FFA[191,201]. A "protein-sparing" effect of FFA is established[202,203], and protein metabolism in obesity has been described as characterized by anabolic resistance, although some such findings may be explained by experimental conditions with unphysiological plasma insulin or AA concentrations[204]. As discussed in the paper, defective insulin-mediated lipolysis suppression with subsequently elevated plasma FFA concentrations and an FFA-mediated suppression of protein anabolism might explain the finding of lower postprandial protein accretion. An alteration of myocellular or vascular insulin action might be an alternative explanation[201]. The data available do not allow to locate the defect, however.

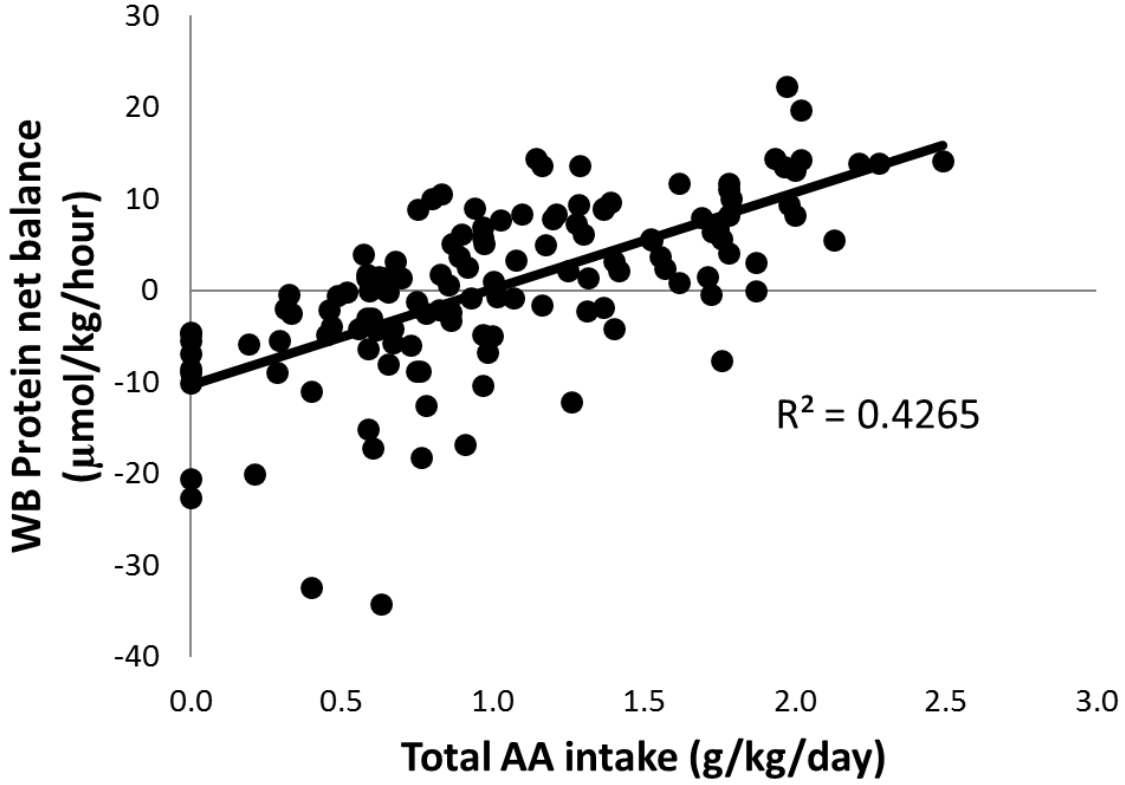
One central finding in studies 2 and 3 was that in critically ill patients, net protein balance typically increases in response to protein/AA feeding, while AA oxidation does not. Specifically, the small amount of enterally supplied protein in study 2 yielded small changes of protein turnover, but when analyzed in the subgroup with stable parenteral nutrition, a less negative net balance was seen. In the WB turnover investigation in study 3, a more

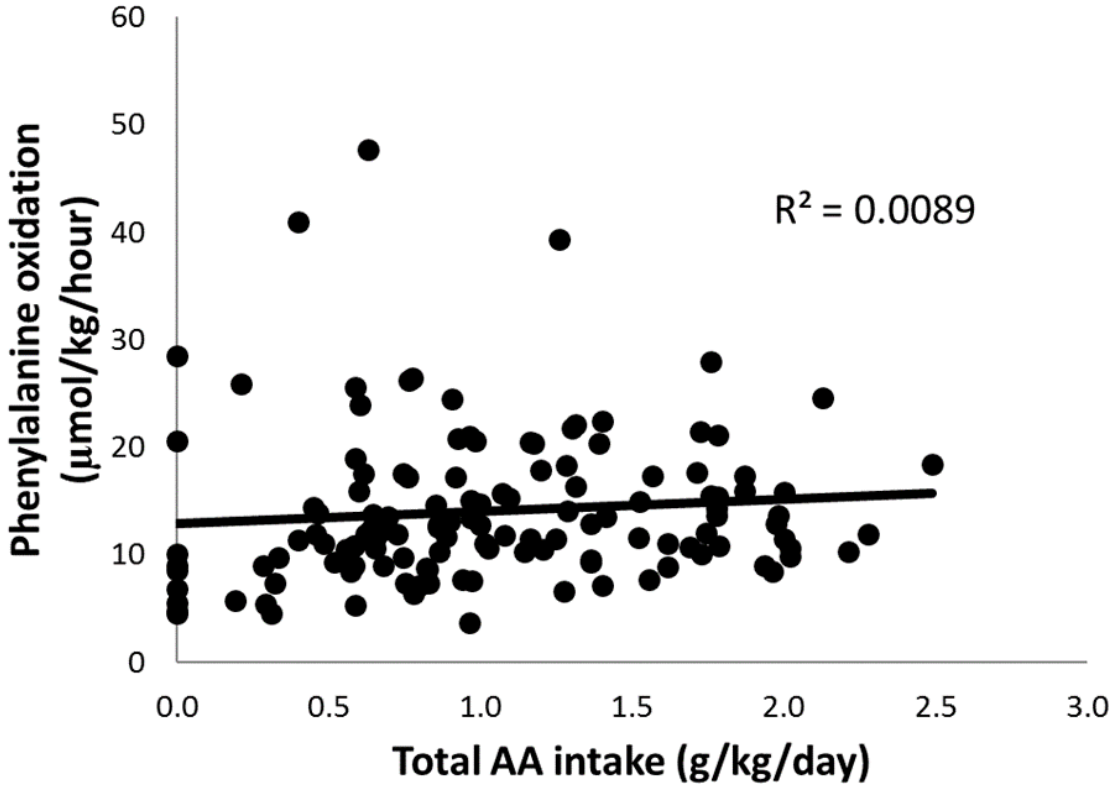
substantial amount of AA supplementation given over three hours was on average sufficient to shift net protein balance from the catabolic to the anabolic range. This response remained stable when the study was repeated at a later timepoint.

While these findings show that protein/AA supplementation affects protein turnover in diverse cohorts of ICU patients, they are strictly only valid for the specific populations and nutrition protocols studied. A broader picture emerges in aggregation with results from other studies from our group, where the short-term response to feeding was studied using identical, or very similar, methods (Fig. 15). These aggregated data represent diverse ICU patient populations, as well as diverse treatment and nutrition protocols, and must therefore be interpreted with caution. Nonetheless, several trends are apparent:

- The relationship between supplemental AA/protein dose and WB protein balance holds over a wide range of AA doses
- By linear regression from these values, it appears that a protein/AA dose in the region of 1.2 g/kg BW per day is sufficient for a neutral protein balance
- Lower and upper thresholds may exist, as no measurements show a positive net balance with a protein/AA dose below the region of 0.5 g/kg BW, and no measurements show a negative net balance with a dose above the region of 1.8 g/kg BW
- Phenylalanine hydroxylation ("oxidation") remains independent of protein/AA dose over a wide range of doses

Integrating these findings, it might be concluded that in short-term studies, protein/AA supplementation in critically ill patients appears to improve WB protein balance, in a dose-dependent manner, largely irrespective of patient specifics, and that increased oxidation of supplemental AA is not induced even at higher doses.





<u>Figure 15</u> Whole-body net protein balance and phenylalanine hydroxylation vs. total AA/protein intake. Aggregate values from critically ill patients representing diverse ICU patient cohorts and nutrition schemes, studied using phenylalanine/tyrosine tracers. Data from references [31], [195], [205], [193] and [206]. Regression line and R^2 values by linear regression.

6.4 CONTINUOUS ENTERAL FEEDING

Since all models are wrong the scientist must be alert to what is importantly wrong. It is inappropriate to be concerned about mice when there are tigers abroad. George Box[207]

6.4.1 Variability in plasma aminoacidemia

Few publications were found where nutrient uptake during continuous enteral feeding was reported with high resolution on the time axis. In a study of intragastric vs. jejunal continuous feeding in healthy subjects[208], aminoacidemia was followed through 12 hrs, though with infrequent sampling after the first 4 hrs. Inter-individual variability of total AA and EAA was apparently small and a steady state appeared to exist. In a study in enterally fed critically ill children[209], aminoacidemia and plasma enrichments of enterally infused leucine and phenylalanine tracers were documented over 24 hrs with a sampling frequency of one sample per hour. There was a high intra-individual variability, with a coefficient of variation of plasma enrichments over 24 hrs of up to 38 % for leucine and 35 % for phenylalanine, and temporal patterns of variation were described as highly individual and non-circadian. Despite the differences in study populations, these findings are similar to those in study 4, where variation over 12 hrs was in a comparable range, and similar individual patterns of variability were seen. These findings suggest that during continuous enteral feeding, a great temporal variability of AA uptake into blood is not unusual in either healthy or critically ill subjects.

The mechanisms underlying temporal variability in AA uptake may lie in any of the stages listed in Fig. 14 and cannot be anatomically localized from the available data. However, one candidate mechanism is gastric emptying. Gastric emptying is shared among liquid stomach contents and could therefore represent a common source of the variability of ^{13}C-phenylalanine enrichment and plasma concentrations of EAA derived from dietary protein. Significant co-variation in changes of ^{13}C-phenylalanine enrichment and plasma concentrations of EAA was seen in 9 of 10 healthy subjects in study 4, which appears to support a causal role of gastrointestinal transport. EAA from dietary protein share gastrointestinal transport mechanisms with free AA, but require digestion before absorption can occur, and individual AA differ in molecular transport mechanisms[139] and degree of splanchnic first-pass metabolism.

Furthermore, when measurements of gastric volume are not available, the role of gastric emptying might indirectly be inferred by comparing study subjects with intragastric vs. postpyloric feeding. In the study by de Betue et al.[209], the lowest temporal variation of plasma enrichments from dietary leucine and phenylalanine tracers was consistently seen in the 5 (of n=8) patients with postpyloric feeding; in our data, the temporal variation for plasma ^{13}C-phenylalanine enrichment of the two patients fed by jejunostomy was the 2nd and 5th lowest of n=10 patients and lower than that of all but one healthy subjects.

6.4.2 Anabolic stimulation

Enteral nutrition in the ICU is typically supplied by continuous infusion, under a rationale of minimizing complications such as gastric retention and pulmonary aspiration of gastric contents. However, continuous feeding is in many respects unphysiological[210,211] and its effects on metabolism in critical illness are not well characterized[212,213]. Research comparing continuous and intermittent feeding has mostly focused on pulmonary complications, gastrointestinal tolerance[214-219], nutrient delivery[212,215-217], and glycemic control[214]. Continuous feeding may yield suboptimal anabolic stimulation in critical illness, but evidence is scarce and is to some degree based on indirect measurements of protein metabolism[211,220,221]. Indirect evidence suggests that intermittent feeding may result in a better anabolic response. In healthy humans, muscle protein synthesis reacts to a rapid rise, rather than absolute values, of aminoacidemia[222]. In a neonatal piglet model, muscle protein accretion is greater with intermittent than with continuous feeding[223,224], which is ascribed to a greater effect of AA- and insulin-mediated proanabolic signaling[225]. However, intermittent feeding appears not superior in terms of muscle protein anabolism in healthy adults during prolonged bed rest[226] or otherwise[227], although hospitalized, malnourished, geriatric patients gain more muscle mass when dietary protein is fed in pulses rather than evenly distributed over the day[228]. It remains to be tested whether protein turnover in critically ill patients reacts to variations of daily nutrient distribution.

6.5 OUTLOOK

After decades of clinical and experimental research in nutrition in critical illness, fundamental questions remain insufficiently understood. Current areas of uncertainty related to protein/AA feeding are the pathomechanisms of harmful effects, optimal mode and timing of feeding, monitoring of nutrition, definition of protein requirements, and individualization of treatment[77,82]. For answers to these interrelated questions, experimental and interventional trials with clinical endpoints will be needed. The work here presented does not by itself address clinical outcomes. However, designing future studies will require better understanding of the unique physiological mechanisms of critical illness. By providing findings such as the utilization of exogenous protein/AA for improved WB protein balance and the lack of their oxidation, the high splanchnic first-pass metabolism of dietary AA, and the variability of AA uptake during continuous enteral feeding, this work should contribute to such improved understanding.

7 CONCLUSION

A popular narrative in support of AA/protein supplementation in critical illness is that:

- protein loss and muscle wasting are common in critical illness and are associated with poor ICU outcomes;
- greater muscle mass affords better muscle function, therefore muscle wasting should be minimized;
- AA feeding improves protein balance, therefore AA feeding should ameliorate muscle wasting, therefore AA feeding should improve ICU outcomes.

Apart from its first point, this chain of argument - while physiologically plausible - is unproven. Fundamental questions remain open, such as how to best define protein requirements in critical illness, how to monitor feeding, and how to allow for the heterogeneity of ICU patient groups, diagnoses, stages of critical illness, physiological measures such as body composition, and specifics of feeding protocols.

The studies here presented explore the region of the argument that feeding improves protein balance. The main findings are that:

- the phenylalanine/tyrosine method is usable to study the effects of nutrient supply on whole-body protein turnover in healthy subjects as well as in critically ill patients;
- in populations of critically ill adults, enteral and parenteral protein/AA supplementation increases whole-body protein balance;
- in these patients, protein/AA supplementation does not result in increased AA oxidation;
- splanchnic first-pass metabolism of dietary phenylalanine is high, and in critically ill patients very high;
- continuous enteral feeding yields highly variably uptake of dietary phenylalanine into blood.

Any extrapolation of these findings towards possible clinical benefits of specific feeding protocols remains speculative, and interventional studies with clinical endpoints will be required to address outcomes in ICU patients. Nonetheless, it is concluded that the findings support an essential argument in favor of protein/amino acid supplementation as an anticatabolic strategy, namely that amino acids from nutritional supplementation are utilizable to improve protein balance.

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[*] For this work's title, that is. Olav's original suggestion was *Something with protein kinetics*.
Mine was *The protein anabolic response to dietary protein, as supplied by continuous or frequent-sip enteral feeding, or to supplementary amino acids by continuous intravenous infusion, quantified as skeletal muscle or whole-body amino acid turnover in the steady state, calculated from measurements of isotopic enrichments in plasma amino acids by gas chromatography - mass spectrometry, through enteral and intravenous infusions of stable isotope labeled phenylalanine and tyrosine tracers, using experimental protocols established first in viscerally obese, insulin resistant subjects, subsequently refined in healthy subjects, then studied in various cohorts of critically ill patients, as well as supplementary analyses of plasma aminograms and of other metabolites, and including an investigation of the uptake of dietary amino acids into arterial blood during continuous enteral feeding, as well as a partial literature review of nutrition-related outcomes in critical illness, mechanisms of anabolic resistance, muscle wasting, gastric emptying, splanchnic first pass metabolism of dietary amino acids, and all that.*
Which did not fit on the title page, needless to say.

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10 ERRATA TO PUBLISHED PAPERS

Study 1

P. 804, in section 3.1, last sentence,
"The UBO women had […] a higher fraction of body fat …"
should read:
"The UBO women had […] a *lower* fraction of body fat …"

P. 804, in section 3.2, third sentence,
"… Q_{pt} was unchanged, R_d Phe increased …"
should read:
"… *Q_{pt} and R_d Phe* increased …"

Study 2

P. 552, in section "WB protein kinetics",
"… steady state WB Phe kinetics were calculated as described (21, 22) by using the equations defined in reference 23."
should read:
"… steady state WB Phe kinetics were calculated as described (21, 22) by using the equations defined in reference 23, *except WB conversion of Phe to Tyr (Qpt), which was calculated as*

$$Q_{PT} = R_a\,(2^{nd}Tyr\ tracer) \times \frac{E_T(1^{st}Tyr\ tracer)}{E_P} \times \frac{R_aPhe}{R_aPhe + i_P}$$

where the 1st tyrosine tracer is the one corresponding to the hydroxylation product of the phenylalanine tracer (i.e. L-ring-$^2H_4$-tyrosine from hydroxylation of L-ring-$^2H_5$-phenylalanine), the 2nd tyrosine tracer is the independent tyrosine tracer (i.e. L-3,3-$^2H_2$-tyrosine), E_p and E_T are isotopic enrichments of phenylalanine and tyrosine in plasma, respectively, and i_P is the phenylalanine tracer infusion rate."

Study 3

P. 4/10, 2nd paragraph, 4th sentence,
"… at a rate of 0.083 g/kg/hr total amino acids (equivalent to 1 g/kg/day)…"
should read:
"… at a rate of *0.042* g/kg/hr total amino acids (equivalent to 1 g/kg/day)…"