Nutritional interventions with protein or amino acid supplementation in metabolic stress: translational studies from piglets to surgical and critically ill patients

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ABSTRACT

Background: Metabolic responses to stress result in insulin resistance and net catabolism. Nutritional substrates, such as amino acids (AAs), are reprioritized from anabolism to support immune and stress responses, altering whole-body protein and glucose turnover, nitrogen balance, AA concentrations, hepatic protein synthesis, and glutathione (GSH) metabolism. Stress from inflammatory bowel disease, surgery, and critical illness share some metabolic characteristics. Therefore, a series of studies was designed to investigate: 1) protein and glucose metabolism under malnutrition superimposed with metabolic stress response; and 2) the nutritional and metabolic benefits of protein or AA supplementation.

Methods: (Study 1) Colitis piglets (n =16) received a protein-deficient enteral diet for 10 days. They were then randomized to either control or supplementation with adequate protein or N-acetyl-cysteine (NAC). Protein and glutathione syntheses were measured by L-[ring-$^{2}H_5$]phenylalanine and [^{15}N,1,2-$^{13}C_2$]glycine, respectively. (Study 2) Patients undergoing colorectal surgery with (n = 8) or without diabetes (n = 10) received parenteral AAs at 20% of their individually measured resting energy expenditure (REE) perioperatively for 72 hours. (Study 3) Patients undergoing cardiac surgery while on a hyperinsulinemic-normoglycemic clamp received parenteral AAs equivalent to either a high dose of 35% (n = 8) or a moderate dose of 20% (n = 8) of REE. (Study 4) Critically ill patients received either exclusive enteral nutrition (n=8) or enteral nutrition combined with parenteral AA supplementation to achieve a total protein intake of 1.75 (n = 9) or 2.5 g/(kg·d) (n = 9). Stable isotope tracers, such as D-[6,6-$^{2}H_2$]glucose, L-[1-^{13}C]leucine and L-[ring-$^{2}H_5$]phenylalanine, were infused to determine glucose and protein kinetics in all three patient studies.

Results: Protein repletion ameliorated plasma protein and tissue protein turnover and systemic GSH kinetics in piglets with colitis and pre-existing protein deficiency, whereas NAC supplementation alone increased cysteine concentrations, but it did not change GSH status. In patients with colorectal surgery, perioperative supplementation of AA alone improved protein balance in patients with diabetes similar to their non-diabetic counterparts. However, postoperative hyperglycemia was prevented only in patients with good glycemic control, but not in those with poor glycemic control before surgery. In patients receiving a hyperinsulinemic-normoglycemic clamp during cardiac surgery, high dose parenteral AAs prevented hypoaminoacidemia. In critically ill patients, high dose parenteral AA ameliorated plasma AA concentrations and improved protein balance compared to exclusive enteral nutrition without supplementation. The dietary protein response (net protein balance) was negatively correlated with clinical scores at ICU admission.

Conclusion: Protein or AA supplementation in metabolic stress states improved protein and glucose metabolism. These studies provided insights into the limitations of current nutrition support strategies and a paradigm to improve nutritional interventions to develop optimal regimens. These may be applied in the design and conduct of larger clinical trials.

RÉSUMÉ

Contexte: Les réponses métaboliques au stress entraînent une résistance à l'insuline et un catabolisme net. Les substrats nutritionnels, tels que les acides aminés (AAs), sont priorisés pour soutenir les réponses immunitaires et au stress au lieu de l'anabolisme. Par conséquent, le renouvellement des protéines et du glucose du corps entier, le bilan azote, les concentrations en acides aminés, la synthèse des protéines hépatiques et le métabolisme du glutathion (GSH) sont altérés. Le stress dû aux maladies inflammatoires de l'intestin, à la chirurgie et aux maladies graves partage certaines caractéristiques métaboliques. C'est pourquoi une série d'études translationnelles ont été conçues pour étudier 1) le métabolisme des protéines et du glucose lorsque la malnutrition est superposée au stress métabolique et 2) les avantages nutritionnels et métaboliques d'une supplémentation en protéines ou en AAs.

Méthodes: (Étude 1) Des porcelets atteints de colite (n=16) ont reçu un régime entéral déficient en protéines durant 10 jours. Ils ont ensuite été randomisés pour contrôler ou compléter leur alimentation avec une protéine adéquate ou de la N-acétyl-cystéine (NAC). Les synthèses des protéines et du glutathion ont été mesurées avec de la L-[anneau-$^{2}H_{5}$]phénylalanine et la [^{15}N,1,2-$^{13}C_{2}$]glycine, respectivement. (Étude 2) Des patients subissant une chirurgie colorectale avec (n=8) ou sans diabète (n=10) ont reçu par voie parentérale des AAs de 20% de leur dépense énergétique au repos (DER) mesurée individuellement en périopératoire pendant 72 heures. (Étude 3) Des patients subissant une chirurgie cardiaque alors qu'ils étaient sous clamp hyperinsulinémique-normoglycémique ont reçu par voie parentérale une dose élevée d'AAs ou une dose modérée (35% ou 20% de la DER; n=8). (Étude 4) Des patients gravement malades ont reçu une nutrition entérale exclusive (n=8), ou une nutrition entérale combinée à une supplémentation parentérale en AAs pour atteindre un apport total en protéines de 1,75 ou 2,5 g/(kg-j) (n=9). Des traceurs isotopiques

stables tels que le D-[6,6-$^{2}H_{2}$]glucose, la L-[1-^{13}C]leucine et la L-[anneau-$^{2}H_{5}$]phénylalanine ont été infusés pour déterminer la cinétique du glucose et des protéines dans les trois études sur les patients.

Résultats: La complétion protéique a amélioré le renouvellement des protéines plasmatiques et tissulaires et la cinétique systémique du GSH chez les porcelets atteints de colite et de carence protéique préexistante, tandis que la supplémentation en NAC seule a augmenté les concentrations de cystéine, sans toutefois contribuer au métabolisme du GSH. Chez les patients ayant subi une chirurgie colorectale, la supplémentation en AAs périopératoire seule a amélioré la balance protéique chez les patients diabétiques, de même que chez leurs homologues non diabétiques. Cependant, l'hyperglycémie postopératoire n'a été évitée que chez les patients dont le diabète était bien contrôlé, mais pas chez ceux dont la glycémie était mal contrôlée avant l'opération. Chez les patients recevant le clamp hyperinsulinémique-normoglycémique lors d'une chirurgie cardiaque, les AA parentéraux à forte dose ont empêché l'hypoaminoacidémie. Chez les patients gravement malades, l'AA parentérale à forte dose a amélioré les concentrations plasmatiques d'AAs et la balance protéique par rapport à une nutrition entérale exclusive sans supplémentation. La réponse protéique alimentaire (balance protéique nette) était négativement corrélée avec les scores cliniques à l'admission.

Conclusion: La supplémentation en protéines ou en AAs dans un état de stress métabolique a amélioré le métabolisme des protéines et du glucose. Ces études ont permis de mieux comprendre les limites des stratégies de soutien nutritionnel actuelles et d'établir un paradigme pour améliorer les interventions nutritionnelles afin de mettre au point des régimes optimaux. Celles-ci pourront être appliqués dans la conception et la conduite d’essais cliniques plus vastes.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis investigates the efficacy of nutritional interventions to improve nutrient metabolism under various states of metabolic stress. The application of stable isotope tracer infusions to quantify protein and glucose kinetic responses allows translational studies in a piglet model of colitis to clinical metabolic studies in patients undergoing colorectal surgery, cardiac surgery, or critical illness. The originality and the contribution to scientific knowledge are detailed below:

Study 1 intervention: Protein or N-acetylcysteine supplementation in piglets with colitis.

- This is the first study to investigate nutritional interventions in a piglet model with pre-existing protein deficiency and colitis. Protein repletion ameliorated systemic antioxidative status and fostered more rapid growth and protein synthesis, but it did not attenuate colitis disease activity. A longer than 5-day adaptation to nutritional interventions may be needed due to the compromised gastrointestinal functions and microbiome environment from protein deficiency and colitis.
- NAC supplementation increased cysteine concentration in several tissues, but this did not translate to an increase in GSH concentration. Increased GSH synthesis and concentration in RBCs with protein repletion, but not NAC, suggests that cysteine may not be the only rate-limiting AA in glutathione synthesis under combined challenges from protein deficiency and colitis. The use of combinatorial supplementation may ameliorate glutathione status.

Study 2 intervention: Parenteral AA infusion individualized to 20% of REE in patients with T2D undergoing colorectal surgery.

- Preoperative HbA1c > 6.5% was associated with increased endogenous glucose production

and hyperglycemia after colorectal surgery. This finding provides in-depth kinetic evidence of how maintaining good preoperative glycemic control, as an important aspect of multimodal pre-habilitation before surgery, prevents postoperative hyperglycemia.

- Parenteral AA infused at 20% of REE was sufficient to increase protein balance above fasting rates before surgery. However, this finding that protein balance remained slightly negative suggests that higher parenteral AA intakes may be more effective to achieve a net anabolic response characterized by positive protein balance.
- In patients with good glycemic control, glucose should be an important component of perioperative nutrition support in addition to AAs to decrease endogenous glucose production, spare protein, and achieve a positive protein balance.

Study 3 intervention: Parenteral AA infusion individualized to 35% of REE in patients receiving a hyperinsulinemic-normoglycemic clamp during cardiac surgery.

- The hyperinsulinemic-normoglycemic clamp decreases plasma AA concentrations to below fasting levels. Parenteral AA infusion at 35% of REE at approximately 1.8 g/(kg·d), but not 20% of REE (0.9 g/(kg·d)), is sufficient to prevent the hypoaminoacidemia induced by the clamp.
- Our finding that plasma tyrosine concentration remained below preoperative fasting levels during the 35% AA infusion suggests the need to investigate alternative strategies to supply this insoluble AA in parenteral AA formulations.

Study 4 intervention: Parenteral AA supplementation in enteral-fed critically ill patients.

- This is the first randomized controlled trial to assess the effect of AA intake on protein kinetics using stable isotope tracers in critically ill ventilated patients. Our study provides

a protein kinetic metabolic basis to support the application in phase 3 clinical trials, and provides a study paradigm for future studies investigating molecular regulatory and nutrient-sensing mechanisms in this vulnerable population.

- Parenteral AA supplementation of standard enteral nutrition to achieve a total nominal protein intake of 2.4 g/(kg·d), but not 1.6 g/(kg·d), resulted in a slightly positive protein balance. This finding suggests that current guidelines may underestimate protein requirements for critically ill patients.
- Low plasma tyrosine concentration may have limited the anabolic effect from high AA intake. This finding provides additional evidence to investigate strategies to improve AA profile in parenteral formulations to achieve better functional and anabolic outcomes in critically ill patients.
- Patients with lower disease severity and nutritional risk showed a greater increase in leucine balance. Our finding supports the use of individualized approaches in critical care research and practice.

CONTRIBUTION OF AUTHORS

Manuscript 1: M Hong was the primary author, performed the statistical analysis, interpreted the results and drafted the manuscript. M Hong and Wykes LJ designed the study protocol and applied for ethics approval. M Hong, Xiao JY and Wykes LJ conducted surgery and collected samples. M Hong and Xiao JY provided animal care and carried out all study measurements. M Hong and Nitschmann E conducted all sample analysis. Wykes LJ supervised all aspects of the study, provided guidance on the sample and statistical analyses, data interpretation and manuscript revisions.

Manuscript 2: M Hong was the primary author, performed the statistical analysis, interpreted the results and drafted the manuscript. Schricker T and Wykes L designed the study protocol and applied for ethics approval. Lattermann R and Schricker T recruited patients. Manjrekar A, Nitschmann E, Lattermann R and Schricker T conducted isotope tracer studies, supervised nutrition delivery and collected samples. Manjrekar A and Nitschmann E analyzed samples. Roque PS aided the statistical analyses and manuscript revisions. Wykes L supervised all aspects of the study and provided guidance on statistical analyses, data interpretation and manuscript revisions.

Manuscript 3: M Hong was the primary author, performed the statistical analysis, interpreted the results and drafted the manuscript. Wykes L, Schricker T and Hatzakorzian R designed the study protocol and applied for ethics approval. Nakazawa K, Codere-Maruyama T, Schricker T and Hatzakorzian R recruited patients. Shum-Tim D conducted surgery. Nitschmann E, Nakazawa K, Schricker T and Hatzakorzian R collected samples. M Hong and Nitschmann E analyzed all samples. Bui H aided the manuscript revisions. Wykes L and Hatzakorzian R provided guidance on statistical analyses, data interpretation and manuscript revisions.

Manuscript 4: M Hong was the primary author, performed the statistical analysis, interpreted the results and drafted the manuscript. All authors were involved in the design of the study protocol. M Hong recruited patients. Campisi J and Port M aided patient recruitment. M Hong and Port M designed the diet protocol for each patient. M Hong and Nitschmann E prepared the isotope tracers. M Hong, Hatzakorzian R and Nitschmann E conducted study measurements and collected samples. Kristof A and Schricker T aided the sample collection. M Hong and Nitschmann E analyzed all samples. Wykes L supervised sample analyses and statistical analyses. Hatzakorzian R and Kristof A supervised patient recruitment and sample collection. Wykes L, Kristof A and Schricker T provided guidance on data interpretation and manuscript revisions.

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LIST OF ABBREVIATIONS

AA	Amino acid
AAA	Aromatic AA
ALB	Albumin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APACHE II	Acute Physiology and Chronic Health Evaluation II
APP	Acute-phase protein
ASR	Absolute synthesis rate
BCAA	Branched-chain AA
BMI	Body mass index
CABG	Coronary artery bypass surgery
CAD	Coronary artery disease
CPB	Cardiopulmonary bypass
CRP	C-reactive protein
DSS	Dextran sulfate sodium
EAA	Essential AA
EEN	Exclusive enteral feeding
eIF2α	Eukaryotic translation initiator factor 2α
EN	Enteral nutrition
ERAS	Enhanced recovery after surgery
ESPEN	European Society for Clinical Nutrition and Metabolism
FIB	Fibrinogen
FMOC	Fluorenylmethyl chloroformate
FRAP	Plasma ferric reducing ability
FSR	Fractional synthesis rate
GC/MS	Gas chromatography-mass spectrometry
GCN2	General control nonderepressible 2

GCS	Glasgow coma scale
GCS	γ-glutamylcysteine synthetase
γ-GC	γ-glutamylcysteine
GGT	γ-glutamyl transferase
GH	Growth hormone
GI	Gastrointestinal
GL	Glutamyl-leucine
GPx	Glutathione peroxidase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
h	hours
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostatic model assessment-insulin resistance
IBD	Inflammatory bowel disease
IC	Indirect calorimetry
ICU	Intensive care unit
IGF-1	Insulin-like growth factor
IRMS	Isotope ratio-mass spectrometry
KIC	Ketoisocaproic acid
LC-MS	Liquid chromatography/mass spectrometry
LOS	Length of stay
MT	Metallothionein
mTORC1	Mammalian target of rapamycin complex 1
MUHC	Mcgill University Health Centre
NAC	N-acetyl-cysteine
NB	Nitrogen balance
NEAA	Non-essential AA

NEM	N-ethylmaleimide
NRC	National research council
NUTRIC	Nutrition Risk in Critically ill
OPA	O-phthalaldehyde
PN	Parenteral nutrition
QQQ	Triple quadrupole
Ra	Rate of appearance
RBC	Red blood cell
RCT	Randomized controlled trial
Rd	Rate of disappearance
REE	Resting energy expenditure
RM	Repeated measure
ROS	Reactive oxygen species
RQ	Respiratory quotient
SAA	Sulfur amino acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOFA	Sequential organ failure assessment
T2D	Type 2 diabetes
TCA	Trichloroacetic acid
TPP	Total plasma protein
UCAA	Urea cycle AA
UHPLC	Ultra high-performance liquid chromatography
UPS	Ubiquitin-proteasome system

CHAPTER 1 – INTRODUCTION

1.1 Background

Metabolic stress response during surgery or disease encompasses hypermetabolic and catabolic responses and results in many physiological consequences, including insulin resistance, hyperglycemia, muscle wasting, inflammation, and oxidative stress **(Figure 1.1.1)** [1,2]. These stress-induced changes have been associated with increased morbidity and mortality, contributing to significant health and socio-economic burden. The rate of morbidity after major surgery ranged from 5% to 15% [3], resulting in an extra cost of at least $ 8,084 USD per patient in the United States [4]. The costs of adverse events in acute care ranged from $4,028 to $12,648 CAD in Canada [5]. Research on metabolic stress response provides insight into the underlying physiological mechanisms and guides the investigation of potential therapeutic approaches. Interventions that focused on increasing physiological reserves and reducing the stress response have been shown to reduce complications, shorten the length of hospital stay, improve long-term survival, and enhance the quality of life [6].

The study of metabolic responses to stress started almost a century ago when Cuthbertson observed an increased systemic loss of nitrogen, phosphorus and sulphur after a leg fracture; the rate of loss gradually decreased but could last up to two months [7]. Subsequent research has discovered that the neuroendocrine and immune systems mediate the response to stress ("fight or flight") and that the metabolic alterations are similar after injury, surgery, infection, and even psychological trauma [8]. The stress response over time has been categorized into three phases:
1) In the ebb phase (< 48 hours after injury), the patient is hemodynamically unstable with a decline in overall metabolism, including reduced cardiac output, systemic arterial blood pressure, body temperature, and oxygen consumption [9].

2) In the flow phase (> 48 hours to up to two weeks after injury), hypermetabolism results in a rise in thermogenesis, oxygen consumption, and energy expenditure [9].

3) In the chronic phase, energy expenditure is further increased to support the metabolic needs and to resynthesize substrates for post-injury recovery [10,11].

The hypercatabolic state in the flow phase dramatically alters the metabolism and interactions among whole-body amino acid (AA), protein, and glucose pools: the accelerated catabolism of skeletal muscle lead to a release of AAs for protein synthesis to support inflammatory responses [12], and for gluconeogenesis in response to stress-induced insulin resistance [13-15] **(Figure 1.1.2)**. The gain in the protein pool from either biosynthesis or diet is not sufficient to replenish the net loss of skeletal muscle, leading to a negative nitrogen balance [12]. Since nutrient substrates are reprioritized away from anabolism towards supporting the acute-phase responses, potential nutritional strategies should be investigated to attenuate catabolism and improve anabolism.

Protein or AA supplementation provides exogenous substrates to 1) increase nutrient reserves before surgical stress [16], 2) compensate the elevated demand and loss of protein and nitrogen during the hypercatabolic state [17], and 3) help to restore and recover muscle mass and function in the chronic phase [18,19]. However, the effect of protein or AA supplementation on protein and glucose kinetics under metabolic stress has rarely been studied. This thesis aims to fill this gap and provide kinetic evidence to support the application of protein or AA supplementation in future research. Specific objectives and hypotheses of studies 1 to 4 were stated in Section 1.3.

Figure 1.1.1 Metabolic stress consequences and the relationship to nutrient metabolism. Adapted from [1,2].

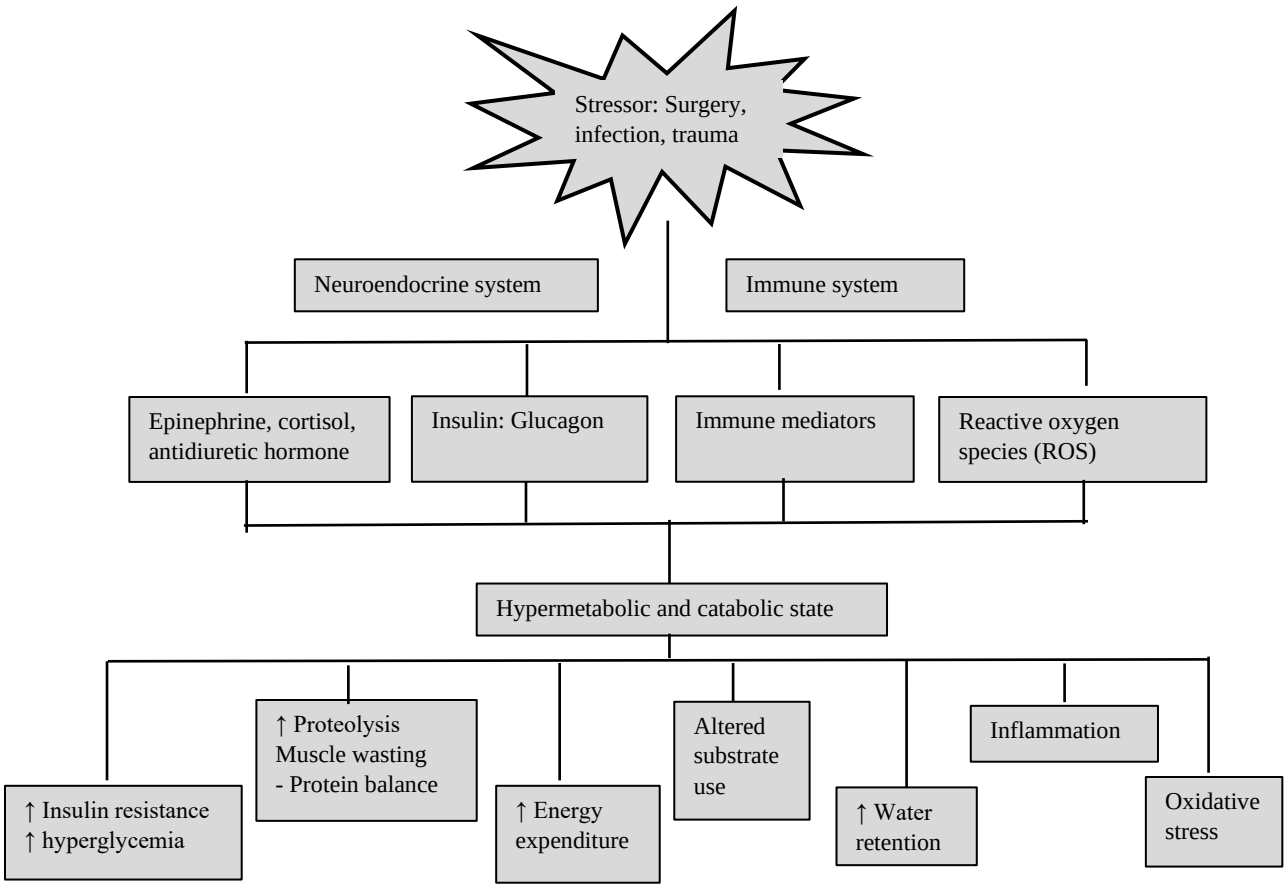
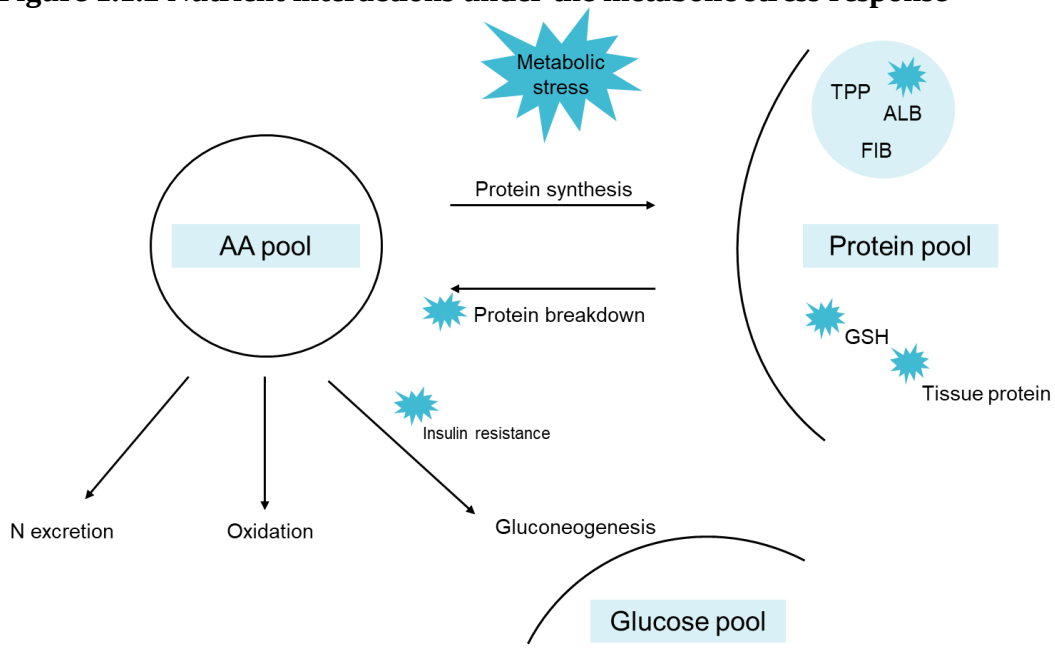


Figure 1.1.2 Nutrient interactions under the metabolic stress response



Metabolic stress response affects protein, amino acid, and glucose metabolism. Abbreviations: AA, amino acids; TPP, total plasma protein; ALB, albumin; FIB, fibrinogen; GSH, glutathione.

1.2 Rationale

Metabolic stress response results in a series of physiological consequences and alters the interactions among whole-body AA, protein, and glucose pools, especially when the exogenous supply is limited. Many animal and clinical studies have demonstrated that protein and AA supplementation may attenuate the stress response, improve mucosal healing, and increase nitrogen balance [17,18,20]. However, few studies to date have investigated the kinetic changes of the AA, protein and glucose pools using stable isotope tracers, especially when malnutrition superimposes illness. Therefore, this work aims to investigate protein and glucose kinetic responses under metabolic stress before and after protein or AA supplementation.

1.3 Objectives and hypotheses

The global objective is to determine how protein and AA supplementation can ameliorate nutrient metabolism under metabolic stress in an animal model using stable isotope tracer techniques and to apply the knowledge to surgical and critically ill patients.

Study 1: Colitis piglets

Objective: To determine the anabolic responses and GSH metabolism of nutritional intervention through protein repletion and NAC supplementation in a protein-deficient piglet model with DSS-induced colitis.

Hypothesis: Protein repletion elicits anabolism in both plasma and tissue proteins and increases GSH concentration and synthesis, whereas NAC has a moderate effect on GSH metabolism.

Study 2: T2D-AA

Objective: To examine whether parenteral AA can promote leucine balance without causing hyperglycemia in patients with T2D undergoing colorectal surgery.

Hypothesis: Parenteral AA infusion at 20% REE perioperatively avoids hyperglycemia in patients with T2D undergoing colorectal surgery and promotes leucine balance to a similar extent as in patients without diabetes.

Study 3: CABG-AA

Objective: To investigate whether parenteral AA can alleviate hypoaminoacidemia in patients undergoing cardiac surgery receiving hyperinsulinemic-normoglycemic clamp.

Hypothesis: Parenteral infusion with high dose AA at 35% REE prevents hypoaminoacidemia, whereas moderate dose AA at 20% REE does not achieve the same results.

Study 4: ICU-AA

Objective: To investigate whether parenteral AA supplement in addition to standard enteral nutrition exerts an anabolic response to increase the leucine balance in critically ill patients.

Hypothesis: High AA supplementation to achieve a total protein intake of 2.5 g/(kg·d) results in a higher leucine balance in patients, compared to moderate AA supplementation or exclusive enteral nutrition.

CHAPTER 2 – LITERATURE REVIEW

2.1 Metabolic stress response

The metabolic stress response is an adaptive mechanism to maintain physiological homeostasis and increase survival [21]. Stressors, such as tissue injury, surgery, infections, activate the central and sympathetic nervous systems and the hypothalamic-pituitary-adrenal axis and trigger the release of numerous counterregulatory hormones (e.g., catecholamines, glucocorticoid, and cortisol) [22]. The immune system is also activated in response to these stressors and releases cytokines (e.g., TNF-α, IL-1β, and IL-6) and other immune mediators [1,23,24]. These hormonal and immune mediators lead to both short-term (e.g., hyperglycemia, insulin resistance, fluid imbalance) and long-term (e.g., muscle wasting) metabolic consequences [1,2]. As part of the adaptive mechanism, nutrient substrates are diverted away from anabolic pathways to support the immune responses and the increased energy demand from vital tissues [3]. This leads to peripheral resistance to anabolic hormones, such as growth hormone and insulin [25], resulting in increased amino acid (AA) release from the muscle reservoir to support gluconeogenesis [22]. In the context of a compromised nutritional status and resultant catabolism due to stressors severe enough to induce metabolic stress responses, the goal of nutritional interventions is to restore the nutritional status and potentially improve clinical outcomes [1,23,26]. In the following sections, specific disease conditions related to metabolic stress response **(Section 2.1)**, nutrient metabolic changes **(Section 2.2)**, and current nutritional interventions **(Sections 2.3-2.5)** are discussed.

2.1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a relapsing and remitting gastrointestinal inflammatory condition [27]. The two major types of IBD, ulcerative colitis and Crohn's disease, differ in the extent of inflammation, affected areas in the gastrointestinal tract, and clinical

manifestations [28-31]. The etiology of IBD remains unknown; however, immunological, microbial, genetic, and environmental factors (diet, smoking and drugs) are believed to contribute to the development of IBD [32,33]. For example, infection or the use of nonsteroidal anti-inflammatory drugs (NSAID) may compromise the integrity of the mucosal barrier, leading to increased intestinal permeability and disrupted tight junctions [34]. Moreover, IBD only develops in the presence of gut flora but not in a germ-free environment in murine models [35]; and clinical studies have demonstrated that IBD patients have an altered intestinal microbiota composition [31]. Intestinal microbial may also play an important role in the pathogenesis of IBD.

Since approximately 25% of newly diagnosed IBD are found in patients younger than 20 years [36], investigation in pediatric IBD may also provide insight into the etiology of this life-long disease [37]. Pediatric IBD may result in growth failure, delayed puberty, and reduced bone mineralization [38-40]. Higher levels of inflammatory cytokines produced in response to IBD, such as IL-6, IL-1β, and TNF-α, dysregulated the growth hormone (GH)-insulin-like growth factor (IGF-1) axis [41] and decreased growth plate chondrocyte proliferation [42], leading to growth failure [43,44]. Since the impaired growth and development is possibly irreversible [45], research is needed to devise optimal strategies to improve growth and induce IBD remission for the younger population.

2.1.2 Colorectal surgery

Colorectal cancer is the third most diagnosed cancer and ranks second in the leading cause of death from cancer [46]. Although up to 55% of colorectal cancer cases occur in developed countries, a strikingly increased incidence has been observed in developing countries, presumably as a consequence of an increased prevalence of Western diets [47]. Dietary factors (e.g., high intake of red meat, saturated fat, and refined carbohydrates), obesity, and a sedentary lifestyle may

activate hyperinsulinemia and inflammatory pathways, resulting in increased cell proliferation and decreased apoptosis, which may ultimately increase the risk of carcinogenesis [48]. Cancer cells dysregulate the immune system and alter nutrient substrate use to support tumor growth [49].

Colorectal surgery is the most common treatment for colorectal cancer. Muscle wasting prior to surgery is associated with a greater risk of postoperative infections, longer hospital stay, and a higher rate of rehabilitation care [50,51]. Skeletal muscle catabolism is further exacerbated by surgical induced-insulin resistance and hyperglycemia [52]. Perioperative interventions to replenish metabolic and nutrient reserves and manage surgical stress may improve postoperative outcomes and potentially reduce muscle wasting.

2.1.3 Coronary artery bypass surgery

Coronary artery disease (CAD) is the primary cause of death globally and is caused by the buildup of plaques (atherosclerosis) in the arteries that limits myocardial blood flow and oxygen supply [53]. Intravascular plaque formation can be attributed to multiple factors, including genetic, sedentary lifestyle, smoking, and diet [54,55]. Severely malnourished patients had a 2.9-fold higher risk of poor clinical outcomes after cardiac surgery than patients with normal nutritional status [56]. Nutritional screening before surgery and perioperative interventions may improve surgical outcomes.

Coronary artery bypass surgery (CABG)—a standard treatment for CAD—creates a bypass around the area blocked by plaques to regain access for blood flow [57]. This surgery mitigates angina and the risk of cardiac arrest and reduces the 5-year mortality rate, especially in the presence of complex vascular lesions [58,59]. However, hyperglycemia is common in patients undergoing CABG, and the use of cardiopulmonary bypass (CPB) further increases the risk of hyperglycemia during and after surgery [60]. CPB elevated catecholamines and cortisol and exacerbated insulin

secretion; the use of hypothermic CPB and exogenous glucocorticoids also contributed to postoperative hyperglycemia [60,61]. Since hyperglycemia and insulin resistance are associated with poor surgical outcomes, recent research has focused on methods to provide tight glycemic control in cardiac patients [60].

2.1.4 Critical illness

Patients admitted to the intensive care unit (ICU) require specialized care to closely monitor their respiratory, hemodynamic, and metabolic status [62]. The average ICU length of stay is approximately three days, and the mortality rate ranges from 9% to 29% in the United States, Canada, and Europe [63-65]. The clinical assessment tools used frequently in the ICU include the following:

1) Acute Physiology and Chronic Health Evaluation II (APACHE II) score—to predict the risk of mortality [66];

2) Sequential Organ Failure Assessment (SOFA)—to evaluate the rate of organ failure to predict morbidity and mortality, especially during sepsis [67];

3) Glasgow Coma Scale (GCS)—to assess the impairment of conscious level;

4) Nutrition Risk In Critically ill (NUTRIC)—to assess nutritional risk in ICU patients [68].

Critically ill patients may have different baseline physiological status, disease progression, and treatment responses; therefore, a “one size fits all” approach may not be ideal. Personalized nutritional support, based on clinical assessment tools such as the APACHE II and the NUTRIC, may be necessary for this heterogeneous population.

2.2 Nutrient metabolism in metabolic stress response

2.2.1 Protein metabolism

Metabolic stress response alters protein metabolism—it increases the concentration of positive acute-phase proteins (APPs) (e.g., C-reactive protein (CRP) and fibrinogen), whereas decreases the concentration of negative APPs (e.g., albumin and prealbumin). Albumin concentration decreases further when exogenous protein source is limited, despite an increase in its synthesis rate, which is mostly due to increased albumin catabolism and loss [69-72]. Moreover, skeletal muscle, the body's largest protein reservoir, releases AAs into the circulation to support APP synthesis, gluconeogenesis, and immunological response rather than anabolism [72-74], leading to muscle wasting after prolonged metabolic stress [23,71,75,76].

Metabolic stress response leads to an imbalance between protein breakdown and synthesis. Whole-body protein loss of 13% was observed in patients with severe sepsis [77] and that of 16% in trauma patients within three weeks [78]; the muscle loss occurred mainly in the skeletal muscle and then shifted to visceral organs in the later stage [77]. Although new proteins are synthesized both systemically and in the affected tissues to support immune responses, the rate of whole-body protein breakdown usually exceeds synthesis, resulting in negative protein balance [79,80]. The goal of nutritional interventions under metabolic stress is to compensate for the protein loss and improve protein balance.

2.2.1.1 Amino acids

AAs are categorized into nutritionally essential, non-essential, and conditionally essential, depending on whether the AA can be synthesized de novo or provided exclusively from diet. Traditionally, nine AAs were considered essential. However, cysteine and tyrosine may be considered conditionally essential since their carbon skeletons cannot be synthesized de novo [81].

Moreover, AAs such as glutamine and arginine may metabolize differently under certain physiological conditions (e.g., pregnancy, growth and development, and critical illness) and, therefore, may be conditionally essential, although more evidence is required [82,83].

AAs not only serve as building blocks of proteins but also regulate protein metabolism through nutrient-sensing mechanisms. AA deprivation leads to an accumulation of uncharged tRNAs and activates general control nonderepressible 2 (GCN2), resulting in an inhibition in eukaryotic translation initiator factor 2α (eIF2α) that restricts translation and protein synthesis [84]. GCN2 detects the lack of any AA, although all the other AAs may be sufficient [84]. Moreover, AA abundance activates RAS-related GTP-binding protein GTPases and induces the localization of mammalian target of rapamycin complex 1 (mTORC1) to the lysosomal surface [85,86]. The activation of mTORC1 triggers a cascade of signalling transduction, such as through p70S6K and S6, and eventually upregulates protein synthesis [85,86]. Leucine is a particularly important AA in mTOR activation, especially during metabolic stress response when the substrates are limited [87-89]. Protein synthesis is a high-energy dependent metabolic process and is therefore tightly regulated through GCN2 and mTORC1 according to AA levels to maintain homeostasis [90].

AA depletion also triggers protein degradation to recycle AA substrates [91]. Two mechanisms are involved in the regulation of protein catabolism, the ubiquitin-proteasome system (UPS) and autophagy. The protein targeted for degradation is first marked by the ubiquitin chain, and it then binds to the proteasome through the loosely folded region and degrades into small peptides in the proteolytic chamber [92]. When UPS is impaired or AA is depleted, the autophagy process engulfs protein aggregates in the autophagosome and releases AA substrates [84]. Under metabolic stress, mTORC1 inhibition increases protein catabolism through UPS and autophagy to recycle AAs for energy or for the synthesis of APPs and other proteins related to the immune

response [92]. Avoid AA scarcity by providing exogenous AAs may help to prevent protein catabolism under metabolic stress.

2.2.1.2 Glutathione

Glutathione (GSH) plays a major role in antioxidative activities through the GSH/GSSG redox cycle [93]. Reactive oxidative species (ROS) oxidize the reduced GSH to glutathione disulfide (GSSG) via GSH peroxidase (GPx); GSSG is then reduced back to GSH by NADPH-dependent GSSG reductase [94,95]. GSH synthesis involves two steps: the first step is considered rate-limiting and is catalyzed by γ-glutamylcysteine synthetase (GCS) to form γ-glutamylcysteine (γ-GC) from glutamate and cysteine; glycine is then added to γ-GC via glutathione synthetase (GS) to form GSH [94,95].

Metabolic stress response leads to an imbalance in ROS and antioxidants, which is the characteristic of oxidative stress [40,41]. ROS compromises GSH redox cycle [96-98] and results in lower concentrations of GSH, cysteine, and cystine, and higher levels of homocysteine and GSSG in the inflamed tissues [96,99-101]; the activities of the rate-limiting enzyme GCS is also lower [102]. Supplementation with AAs such as cysteine may promote GSH synthesis and reverse the compromised GSH redox cycle.

In addition, glutathione reduces cysteine residues through protein S-glutathionylation to form disulfide protein-glutathione. This reaction is a reversible antioxidant defence mechanism that protects the free protein thiols from irreversible oxidation [103] and responds to the physiological redox state (e.g., GSH/GSSG ratio) [104,105]. Protein S-glutathionylation has become a potential biomarker for various oxidative stress-related conditions such as diabetes (glutathionylated hemoglobin) and cardiovascular disease [104].

Compared to GSH concentration as an intracellular marker of antioxidative status, ferric reducing antioxidant power (FRAP) assay measures the plasma antioxidant activity by reducing ferric ions to ferrous ions [106]. However, FRAP reacts slowly to protein and amino acids with a thiol group, including GSH [106]. This limitation may be negligible in samples such as plasma with a very low concentration of GSH.

2.2.2 Glucose metabolism

Glucose is the major source of energy and is the preferred source for the brain and red blood cells. At the beginning of fasting, glucose is released from hepatic glycogen for peripheral uptake to provide energy [107]. When all the stored glycogen is depleted (~ 12 to 18 hours), gluconeogenesis is initiated to produce glucose through the following glucogenic substrates [108]:

1) Glucogenic AAs (such as alanine and glutamine) via muscle protein breakdown;

2) Lactate from glycolysis in the muscle that returns to the liver through the Cori cycle;

3) Glycerol from lipolysis [108].

Metabolic stress response further stimulates gluconeogenesis through elevated catecholamines, cortisol, glucagon, and proinflammatory cytokines (e.g. TNF-α, IL-6). This scenario leads to insulin resistance, decreased glucose uptake, and, consequently, “stress hyperglycemia” [109]. Since cortisol and catecholamine concentrations are correlated with the intensity of stress, a high-stress level (such as in sepsis shock) leads to more severe hyperglycemia [109]. Stress-induced hyperglycemia is associated with postoperative adverse events, such as surgical site infection, sepsis, longer hospital stay, morbidities, and mortality [110].

Comorbidities such as diabetes may aggravate postoperative hyperglycemia and increase the rate of adverse events [111,112]. However, a postoperative glucose level higher than 10 mmol/L was associated with surgical site infection, regardless of whether patients had diabetes or

not [110]; the rate of adverse events was associated with the magnitude of hyperglycemia, but this relationship was only observed in patients without diabetes [112,113]. Perioperative glycemic control plays a vital role in improving postoperative clinical outcomes.

2.3 Nutrition and inflammatory bowel disease

2.3.1 Nutritional challenges

IBD decreases food intake and causes nutrient malabsorption; it also increases nutrient losses, resulting in malnutrition and weight loss—this scenario further aggravates the immune response and IBD remission [43,114]. The decreased food intake is mainly due to early satiety, pain associated with eating, impaired taste sensation, and food avoidance through fear of diarrhea and anorexia [43,115]. The malabsorption of both macro- and micronutrients, particularly vitamin B_{12} and fat-soluble vitamins, is caused by the inflamed mucosa epithelial surface of the GI tract [43]. Drugs, such as glucocorticoids, also reduce nutrient absorption [116]. The increased nutrient loss is mainly due to blood loss (loss of iron), diarrhea (loss of minerals such as Zn^{2+}, K^{+} and Mg^{2+}), exudative enteropathy (protein loss), and steatorrhea (loss of fat and fat-soluble vitamins) [43]. Long-term nutritional impairment in IBD results in various complications, such as osteoporosis and anemia [115].

Compromised nutrient metabolism also influences the immune response [117]. Protein deficiency in IBD triggers changes in APPs, which may alter cytokine production and can eventually modulate inflammatory pathways [117]. A lack of vitamin B_6, riboflavin and sulfur AAs in the glutathione metabolic cycle may further decrease glutathione and aggravate oxidative stress and inflammation in patients with IBD [117].

The goal of IBD treatment is to heal the intestinal lining, induce and maintain remission, and restore and maintain nutrition [118-120]. Compared to pharmacological agents, most dietary

supplements are safer, cheaper and more accessible [29]. Minerals (calcium, zinc, and iron), whey protein, omega-3 fatty acids, and carbohydrates (fructo-oligosaccharides and plant-derived polysaccharides) are all associated with clinical and cellular improvements in IBD [29]. AA supplements such as cysteine, glutamine, arginine, tryptophan, threonine, and glycine, whether solely or combined with other AAs, have demonstrated to reduce intestinal inflammation or induce IBD remission [20,121-125]. Probiotics have also been shown to maintain remission, reduce inflammation, and decrease the occurrence of pouchitis [118,126]. However, probiotics did not reverse the impairment from a macronutrient-restricted diet in colitis piglets [127]. Maintaining adequate nutrition is, therefore, an essential component of IBD management.

2.3.2 Nutritional interventions

2.3.2.1 Protein

Protein repletion after a period of under-nutrition normalizes metabolism and body function. Nutritional repletion improved whole-body protein turnover and synthesis [128] and gastrointestinal mucosal protein synthesis, indicating that repletion exerts a trophic effect and provides rapid restoration in the digestive tract mucosa [129]. Refeeding with adequate protein resulted in rapid catch-up growth [130], liver weight gain, higher nitrogen retention, and increased plasma IGF-1, leucine and albumin; moreover, gastrointestinal morphology was also restored [131,132].

2.3.2.2 Cysteine

IBD impairs GSH synthesis and compromises the GSH redox state [133], especially with inadequate sulfur AA (SAA) intake [134]. Supplementation with cysteine, N-acetyl-cysteine (NAC), SAA, or proteins rich in cysteine may exert anti-inflammatory and antioxidative effects [135]. Cysteine supplementation via gastric infusion decreased pro-inflammatory cytokine expression, improved intestinal permeability, and increased apoptosis of immune cells in colitis

piglets [20]. Combined cysteine and glycine infusion restored glutathione synthesis and attenuated oxidative stress in patients with diabetes [136]. Oral supplementation with cysteine-rich whey protein isolates elevated plasma GSH and antioxidant capacity in non-alcoholic steatohepatitis patients [137]. Dietary SAA supplementation reduced the thiol/disulfide redox state, increased ileal adaptation and mucosal growth in rats with massive small-bowel resection [138]. NAC, as a cysteine derivative, showed a protective effect on redox states in colitis mice [139] and was also associated with higher remission rates and lower disease severity index in patients with colitis [140].

2.4 Perioperative nutrition

2.4.1 Perioperative care

Perioperative practice guidelines require complete gastric emptying to decrease the risk of aspiration after anesthesia induction. Preoperative fasting typically starts 6 to 8 hours before surgery; however, in clinical practice, fasting periods sometimes last more than 12 hours [141-143]. Oral feeding is only resumed when the patient is hemodynamically stable, and the bowel function has returned, which usually occurs on the second or third postoperative day [142]. In total, patients may be fasted for at least 48 to 72 hours. The length of fasting is further prolonged if the patient has to return to the operating room (e.g. delayed sternal closure after cardiac surgery). Moreover, poor preoperative nutritional status—such as unintentional weight loss of more than 10% within six months before surgery or low albumin concentration at admission—increases the risk of postoperative complications [144].

The Enhanced Recovery After Surgery (ERAS) protocols have been developed to promote recovery after surgery [6,145]. This approach applies evidence-based advancements in surgery, anesthesia, and nutrition to attenuate patients' stress responses and induce earlier recovery, leading

to better clinical outcomes and lower cost [6]. ERAS has been shown to reduce surgical complications and hospital stays by up to 50% [145].

Nutrition support plays a vital role in ERAS, including preoperative nutritional screening, assessment and support, preoperative oral carbohydrate load, post-operative early intake on the day of surgery, and high protein supplementation [146]. Preoperative nutrition started 7 to 10 days before surgery reduced morbidity among malnourished patients [142,147]. Early oral feeding on postoperative day 1–2 decreased length of hospital stay and the first flatus time without influencing morbidities and mortality [148,149].

2.4.2 Nutritional interventions

2.4.2.1 Glucose

Perioperative glucose has been shown to improve postoperative nutrient metabolism and clinical outcomes. Preoperative oral carbohydrate load up to 2 hours before surgery decreased insulin resistance and hospital length of stay compared to overnight fasting [143,150]. Parenteral glucose administered before surgery and continued for two days after surgery for a total of 72 hours decreased endogenous glucose production relative to the baseline [151]. Similarly, 3-hour postoperative glucose infusion also markedly reduced endogenous glucose production compared to fasting [152]. Moreover, intraoperative glucose may attenuate AA oxidation and spare protein from catabolism, although postoperative hyperglycemia was more pronounced [153].

Glucose therapy should be used with caution in surgical patients with diabetes [154]. Although gastric emptying time after preoperative oral carbohydrate did not differ [155], patients with diabetes had greater peak glucose (13.4 vs. 7.6 mmol/L) and took longer to restore to baseline glucose compared to healthy subjects [155]. Postoperative parenteral glucose infusion in patients with diabetes also resulted in higher glucose concentration and a lower rate of glucose uptake

compared to patients without diabetes [152]. However, little is known in patients with poorly controlled diabetes since the patients had an average hemoglobin A1c (HbA1c) of 6.2% [155], which indicates good glycemic control.

2.4.2.2 Insulin clamp

Hyperinsulinemic-normoglycemic clamp technique involves an administration of a supraphysiological dose of insulin, followed by glucose titration [156] to maintain glucose concentration between 4 to 6 mmol/L [157,158]. Patients who are highly insulin resistant have poor peripheral glucose uptake and require relatively less glucose infusion to maintain normoglycemia than patients who are insulin sensitive.

The clamp provides tighter glycemic control than a traditional sliding scale approach using intermittent boluses of insulin [61]. It also decreases the risk of infectious complications after cardiac surgery [159]. High dose insulin optimizes whole-body glucose and suppresses endogenous glucose production, and, therefore, decreases protein breakdown and subsequent release of gluconeogenic AAs [158,160].

2.4.2.3 Amino acids

AAs administered either alone or in combination with glucose have been investigated as a preoperative metabolic preparation regimen to prevent prolonged fasting and insulin resistance. Oral intake of carbohydrate (50 g maltodextrin) together with glutamine (40 g) 8 and 2 hours before cholecystectomy decreased insulin resistance compared to the fasting controls [161]. Supplemental glutamine also increased nitrogen balance and glutathione concentration compared to oral carbohydrate fluid only [161]. Whey protein combined with oral carbohydrate fluid fed in the evening and again 3 hours before surgery lowered insulin resistance measured by homeostatic model assessment-insulin resistance (HOMA-IR) and the acute phase response assessed by CRP-

to-albumin ratio [162]. Moreover, AA supplementation via a parenteral route, compared to oral or enteral routes, bypasses the gastrointestinal tract and allows a longer administration time, thereby increasing substrate availability throughout the surgery. Parenteral glucose (50% REE) and AAs (20% REE), started 24 hours before and extended until two days after surgery, reduced protein breakdown and oxidation, increased albumin synthesis, and achieved a slightly positive protein balance compared to patients who had undergone surgery fasted [163].

As opposed to glucose, AAs may provide a better solution for patients with concomitant diabetes in preventing surgical-induced hyperglycemia and promoting protein anabolism. Postoperative AA infusion maintained glucose concentration at the preoperative level (7.9 mmol/L), whereas glucose infusion led to hyperglycemia (14.3 mmol/L) [164]. AA infusion also attenuated protein breakdown and markedly increased protein balance (leucine) to 30 μmol/(kg·h) compared to – 29 μmol/(kg·h) in patients receiving glucose [164]. It remains to be investigated whether AA infusion initiated before surgery for a more extended period has further metabolic benefits.

Parenteral AA administration rapidly replenishes plasma AA and corrects the hypoaminoacidemia induced by the hyperinsulinemic-normoglycemic clamp [160,165]. AA infusion at 20% of individually determined resting energy expenditure (REE) (1.0 g/(kg·d)) resulted in higher plasma concentrations of the majority of AAs and a positive protein balance (leucine) in patients treated with the clamp [165]. However, most AAs did not exceed their preoperative baseline levels, and no change was observed in protein synthesis [165]. In fact, parenteral AAs at 2.9 g/(kg·d) in septic adolescents restored plasma AA concentrations to baseline and led to higher protein balance than the ones receiving AAs at 1.5 g/(kg·d) [166]. Whether a

similar effect can be found in adult surgical patients receiving a high dose of AAs remains to be investigated.

2.5 Critical care nutrition

2.5.1 Nutritional practice in critical illness

Based on Cuthbertson's "ebb and flow" theory about the metabolic stress response, the 2019 ESPEN guidelines categorize ICU progression into three phases: 1) early acute phase (< day 3)—patients are usually hemodynamically and metabolically unstable, and early feeding is recommended to be initiated; 2) late acute phase (day 3-7)—patients are hypercatabolic to re-allocate substrates to support stress responses, and a full feeding is recommended to be achieved within this phase; 3) chronic phase (> day 7)—recovery begins with increased anabolism accompanied by moderate catabolism [167-169].

A wide range of recommendations on protein requirements for critically ill patients exists. The Society for Critical Care Medicine (SCCM) and the American Society of Parenteral and Enteral Nutrition (ASPEN) suggest a target of 1.2 to 2.0 g/(kg·d) of protein for critically ill patients whose BMI is less than 30 [170,171]. A protein intake of ≥ 2.0 g/(kg·d) and ≥ 2.5 g/(kg·d) (based on ideal body weight) is recommended for critically ill patients with class I and II or class III obese, respectively [170,171]. The ESPEN guidelines recommend 1.3 g/(kg·d) of protein for both regular critically ill patients and those with obesity (based on adjusted body weight) [167].

These guidelines were based on nitrogen balance studies and some observational studies (without any systemic review or meta-analysis) and the notion that patients may require higher protein intake than healthy adults to compensate for the increased protein breakdown induced by critical illness [167,170,171]. However, the recommended protein intakes from ESPEN and the lower bound of that from SCCM/ASPEN are, in fact, similar to the population-safe protein

requirement for healthy adults—1.2 g/(kg·d) using the indicator amino acid oxidation (IAAO) method [172,173]. By contrast, a high protein intake of 2.0–2.5 g/(kg·d) is considered safe for critically ill patients [12,174,175]. This is based on a systemic review by Hoffer *et al.* on prospective studies with various levels of protein intakes and also studies reported risks of hyperammonemia and hyperaminoacidemia to determine the potential upper limit of protein [12]. The wide range of recommended protein intakes from guidelines and expert opinions is mostly due to the lack of well-designed RCTs and the controversial view on high protein intake in critical illness (discussed below in **section 2.5.2**).

2.5.2 Controversies related to protein intake

2.5.2.1 Research evidence

High protein intake corrects or attenuates catabolism in response to prolonged malnutrition and metabolic stress response; it also provides additional substrates for protein synthesis to support the increased demands during injury and recovery [12,175,176]. This notion is supported by numerous critical care studies comparing the impact of different levels of protein intakes on nitrogen balance and protein balance.

Nitrogen balance

Higher protein intake has been shown to improve nitrogen balance (NB) in critical care studies with small sample sizes ($n \leq 50$) [12]. A large-scale NB study in trauma patients ($n = 249$) confirmed this benefit and demonstrated that a protein intake of at least 1.7 g/(kg·d) was associated with a significantly higher NB, and more than 54% of these patients reached NB equilibrium with protein intakes ≥ 2 g/(kg·d) [177]. Since the urine samples of this study were obtained within 5 to 14 days following trauma, the result may only apply to the chronic phase of critical illness. Evidence from the early acute phase also showed that higher protein intake at 1.8 g/(kg·d)

improved NB on both day 1 and 3 after ICU admission (n = 40, mixed medical and surgical ICU) [178]. However, these two recent NB studies excluded patients with acute or chronic renal disease, liver dysfunction, or obesity; thus, the finding might not be universally applicable.

Acute renal or liver failure decreased protein synthesis and accelerated protein breakdown and loss in critically ill patients [179]. NB remained negative (- 2 g/d) with a protein intake of 2.5 g/(kg·d) among patients with acute renal failure receiving continuous dialysis [180]. Open abdomen procedures also increased nitrogen losses to 3.5 g per day via abdominal fluid, which should be considered when calculating NB in critically ill patients [181]. Moreover, ICU patients older than 60 years may have a negative NB due to the decreased anabolism and increased muscle loss that occurs with age [17].

Current evidence suggests that a protein intake of at least 2.0 g/(kg·d) may be required to equilibrate NB; however, the optimal level of protein intake may vary depending on the types of ICU patients studied, measurement time points, disease severity, and concurrent medical treatments. Large scale RCTs are required with a protein intake ≥ 2 g/(kg·d) to determine the cut-off dose for nitrogen equilibrium.

Protein balance

Nitrogen balance provides information on whole-body net nitrogen based on the differences between nitrogen intake and excretion. By contrast, protein balance—assessed using stable isotope tracers—provides additional information on the rates of protein synthesis and breakdown. Protein balance in critically ill patients is negative at -9 μmol/(kg·h) compared to an average of +4 μmol/(kg·h) in healthy adults receiving the same nutrition regimen [182], indicating that protein breakdown exceeds synthesis under critical illness.

Protein supplementation may increase protein balance in critically ill patients. A 3-hour parenteral AA supplementation increased total protein intake to 1.6 g/(kg·d) and resulted in a significantly higher protein balance of 7 μmol/(kg·h) compared to - 4 μmol/(kg·h) at baseline (n = 10)[183]. A second 3-hour supplementation during the 2 to 4 days showed similar improvements in protein balance relative to baseline [183]. A follow-up study by the same research group extended the supplementation time to 24 h and demonstrated that the protein balance increased from 2 to 7 μmol/kg/h when a total protein of 2.0 g/(kg·d) was administered (n = 9)[184]. These two studies were both conducted during the late acute phase (day 3 to 7) and showed that AA supplementation effectively improved protein anabolism. However, an RCT may be needed to reduce bias, increase the quality of evidence, and identify whether the increase in protein balance is due to the high AA supplementation or time.

Clinical outcomes

Clinical RCTs performed to date have only provided limited evidence on the optimal protein dose in critical illness. A meta-analysis of 14 RCTs in ICU patients receiving protein for at least 48 hours showed no difference in mortality (odds ratio 0.93, 95% CI 0.72-1.22; heterogeneity I^2 = 48%) [185]. However, protein intake averaged at only 1.02 ± 0.42 g/(kg·d) in the high protein intake groups, which is below the minimum recommended intake per critical care guidelines. In addition, the average dose difference between low and high protein groups was only 0.35 g/(kg·d). This difference might be too small to produce a statistically significant difference in clinical outcomes, such as mortality. It is noteworthy that most of these RCTs were not designed to randomize patients to different levels of protein intake, but they had rather focused on other types of interventions related to feeding routes, timing, or total energy intake [185]. Another meta-

analysis of 5 RCTs that randomized patients based on protein intake also showed no difference in mortality (risk ratio 0.94, 95% CI 0.74-1.21; heterogeneity $I^2 = 0\%$) [186].

Two RCTs included in both meta-analyses had randomized patients solely based on protein intakes, without any concurrent independent variables such as energy intake. Supplemental AAs achieving a total protein intake of approximately 1.75 g/(kg·d) increased the glomerular filtration rate and urine output of the ICU patients, but the duration of renal dysfunction did not differ (Nephro-Protect study, n = 474) [187]. Comparing ICU patients who received either 0.9 or 1.1 g/(kg·d) parenteral AAs, the higher AA intake group had greater handgrip strength, increased muscle thickness (measured by ultrasound at the forearm and thigh area on day 7), and lower fatigue scores [18]. Despite the beneficial effects on renal and muscle function, protein intake in these studies remained relatively low. Further studies designed with an intervention at the high end of the recommended protein intake (≥ 2.0 g/(kg·d)) may be more appropriate to examine its impact on clinical outcomes [188].

2.5.2.2 Is protein harmful?

Concerns related to the possible harmful effects of high protein intake in critically ill patients have been raised due to the autophagy suppression effect by AAs. Previous mechanistic studies have demonstrated that AAs suppress autophagy through two major mechanisms—mTORC1 and Beclin1-PIK3C3 complexes [189]. Autophagy repairs cellular damage, adapts to starvation and stress, and promotes survival [190]. Insufficiency of this process may lead to mitochondrial dysfunction, resulting in organ failure [191]. However, evidence from clinical studies for this relationship remains scarce.

Clinical studies associated protein or AAs with poor clinical outcomes were based on post-hoc analysis, and none of them had randomized patients based on protein intake levels. In the

EPaNIC trial [192,193], protein per 10% increments of target intake (14.2 to 47.2 g/d, equivalent to 0.19 – 0.63 g/(kg·d)), compared to cumulative glucose, was associated with delayed ICU discharge [192,193]. Similarly, in the Intensive Insulin Therapy trial, protein intake (40 to 53 g/d, equivalent to 0.57–0.76 g/(kg·d)) was negatively correlated with autophagy markers in the skeletal muscle [194,195]. An observational study had similarly found a significant positive linear relationship between protein intake (2.3 to 7.3 g/kg over ten days, equivalent to 0.23–0.73 g/(kg·d)) and muscle wasting [196,197]. However, the protein intakes from these three studies were lower than the protein requirement for healthy subjects from DRI (0.8 g/(kg·d)) and far below that determined by the IAAO method (1.2 g/(kg·d)) [172]; these intake levels were insufficient even for maintaining normal physiology [198]. It remains to be determined whether these associations will vary with protein intake within or above the recommended intake range from current guidelines. Moreover, mere associations need to be interpreted with caution as they do not explain causation.

2.5.3 Research gap

It remains controversial whether high protein intake is beneficial for patients with critical illness. Existing evidence of potential harm by the administration of large amounts of protein is weak, and the association with autophagy suppression in critically ill patients requires further investigation. In fact, current evidence from nitrogen and protein balance and clinical outcomes appears to favour higher protein intake. However, these studies are often difficult to compare to each other because their study designs differ in many aspects:

1) nutritional interventions with a wide range of protein intake levels, timing of feeding (early or late), routes (EN or PN), duration, types of protein (e.g., intact protein, peptides, or AAs), and AA profiles (e.g., added or lack of glutamine, cysteine, tyrosine);

2) ICU population (e.g., medical, surgical, burn, trauma);

3) study endpoints (e.g., nitrogen balance, muscle function, length of stay, mortality).

It is, therefore, impossible to draw a general conclusion. The severity of the disease and the small sample size may also mask any potential clinical impact from nutritional interventions [168,199].

Compared to clinical parameters, an investigation of high protein intake on outcomes that are more quantifiable and directly linked to protein intake should be attempted, such as protein kinetics (measured by stable isotope tracers), muscle thickness (measured by ultrasound), muscle function (measured by handgrip test), insulin resistance and glucose kinetics, and antioxidative status [200]. There is a lack of kinetic data on protein metabolism in the presence of various protein intake levels. Moreover, it is essential to understand whether and how protein metabolism depends on the disease severity and nutritional risk of the individual patient.

Achieving a high protein intake is challenging in critical care. Average protein intake in critically ill patients was approximately 0.6 g/(kg·d) (n = 2388) [201] or half of the prescribed protein intake (n = 1199) [68]. This poor adherence to clinical recommendations was mainly due to failure to initiate early nutrition support, rate-based feeding strategy with frequently interrupted schedule, and intolerance with high gastric residual volume [202]. Volume-based feeding, use of motility agents, and small bowel feeding have been recommended to improve tolerance and compliance [203].

Prescribing an adequate protein to meet target requirements may also be challenging. Considering that one water molecule is lost when a peptide bond is formed from the AA mixtures, an AA-protein conversion factor of approximately 83% should be applied when calculating the parenteral AA intake based on protein requirement [174]; ignoring this factor may result in protein underfeeding. Moreover, when a large dose of propofol (a lipid-based sedative drug) is required,

the energy from propofol (1.1 kcal/ml) may contribute a significant amount to the total energy intake. The proportion of energy from EN or PN has to be decreased to avoid overfeeding. Consequently, protein intake is decreased as a result of reduced amounts of nutrition support provided [204]. In addition, the readily available parenteral AAs are often part of the fixed ratio AA/glucose mixture rather than as in separate solutions, which decrease the flexibility of prescribing high protein intake without energy or glucose overfeeding. Together, these patients may, therefore, particularly benefit from protein supplementation or high protein-to-energy ratio enteral formula to achieve target protein intake.

2.6 Methodology in metabolic research

2.6.1 Colitis piglet model

The pig model is widely used in research due to its morphological, anatomical and physiological similarities to humans. Pigs have been identified as the closest animal to humans, apart from primates [205]. Piglets and human infants have similar postnatal nutrient metabolism, intestinal development, digestive enzymes, and microbiome composition. Piglets have rapid growth rates and higher metabolic rates than human infants and can be considered as an accelerated model for pediatric research [206-208]. In addition, the size of the piglet (compared to rodents) allows for extensive surgical manipulation, which makes piglets ideal for nutritional studies using enteral or parenteral feedings or isotope infusions [206].

The dextran sulfate sodium (DSS)-induced colitis model is a well-established and widely used method to investigate IBD. DSS damages the integrity of the colonic epithelial barrier and increases the colonic mucosal permeability. By adjusting the amount, length, and frequency of DSS, researchers can induce colitis with different levels of disease severity [209] and disease

characteristics such as acute, chronic, or dysplastic lesions [210] in experimental animals; these models provide a foundation to investigate different therapeutic strategies.

2.6.2 Stable isotope tracer method

Whole-body protein turnover can be assessed by the precursor method in which a labelled AA is infused at a constant rate until a steady-state is achieved. At this state, the amount of AA entering the pool (Ra, rate of appearance) equals to that leaving the pool (Rd, rate of disappearance). This model is simplified when the tracer is an essential AA with only one metabolic pathway (*e.g.* leucine) and without *de novo* synthesis or AA contributing to other metabolic fates [211]. Therefore, AA from dietary AA intake and AA released from protein breakdown are entering the pool, whereas AA from AA disposal for protein synthesis and AA oxidation are leaving the pool. The AA balance can be calculated from the difference between AA disappearance for protein synthesis and AA appearance from protein breakdown. Total protein balance can then be extrapolated from the AA balance; for instance, leucine is approximately 8% of total AAs at all ages [212]. Several assumptions underlying this model are as follows [212]:

1) The tracer AA is well distributed in the whole body;

2) The metabolism of tracer AA represents the metabolism of all AAs at the whole-body level;

3) The amount of tracer AA is minimal compared to dietary AA and should not influence protein turnover;

4) The tracer AA metabolizes the same as unlabeled AA;

5) There is no recycling of tracers from protein breakdown.

Both leucine and phenylalanine/tyrosine tracer models are commonly used to determine whole-body protein turnover in clinical studies. Leucine metabolizes into ketoisocaproic acid (KIC) or contributes to protein synthesis. The enrichment of plasma labeled KIC, thus, can be used as a

surrogate to measure intracellular enrichment of leucine tracer. In contrast, phenylalanine either converts to tyrosine or contributes to protein synthesis in the liver. When tyrosine is limited in the diet or tyrosine requirement is increased in certain disease conditions (e.g., liver and renal failure) [213], excessive phenylalanine converts into tyrosine instead of contributing to protein synthesis, resulting in an underestimation of protein balance.

Protein balance measured by leucine and phenylalanine/tyrosine tracers were similar in premature infants receiving parenteral nutrition; however, while leucine oxidation increased vastly, phenylalanine hydroxylation did not change [214]. The author speculated that the limited supply of phenylalanine and tyrosine in the parenteral nutrition led to increased oxidation of excess AAs such as leucine. In addition, when tyrosine is limited in the parenteral nutrition, the amount of tyrosine tracer may have contributed significantly to total tyrosine intake, which might have contradicted the model's assumption that the tracer amount should be "unnoticeable". In this case, leucine tracer may be a preferred choice to determine whole-body protein balance.

Protein synthesis under metabolic stress at a tissue level may be different compared to that measured at a whole-body level. Therefore, a constant infusion method is used to investigate the synthesis rate of tissue-specific protein or peptides. The enrichment of tracer AA in the precursor free pool typically takes 3 to 8 hours to reach a steady-state. By sampling the tissue protein (e.g., liver protein) or isolating a specific type of protein (e.g., albumin), the enrichment of tracer AA incorporated into the target protein can be determined. Fractional protein synthesis can then be calculated from the enrichment of bound protein against the free pool enrichment. This method is ideal for measuring proteins with a slow turnover rate [215]. However, the infusion time should not be too long to cause recycling—tracer incorporated into the protein is broken down and re-

enter the free pool. It occurs mainly in proteins with rapid turnover rates, such as proteins in the liver and gut, resulting in an overestimation of AA enrichment in the free pool [215].

2.7 Conclusion

Nutritional status is compromised from the metabolic response to stress. The stable isotope tracer technique quantifies the changes in nutrient metabolism in the stress state. Such research would also foster the exploration of possible nutritional intervention strategies that could be applied in future clinical research and practice.

CHAPTER 3 – MANUSCRIPT 1

Protein repletion, but not N-acetylcysteine supplementation, ameliorates systemic protein and glutathione kinetics in a malnourished colitis model in the piglets

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3.1 Abstract

Background Inflammatory bowel disease (IBD) is often associated with compromised nutritional status. The combined inflammation and malnutrition aggravate the metabolism of protein and antioxidants such as glutathione (GSH) and eventually lead to growth impairment in pediatric patients. Our objective was to determine if supplemental protein or N-acetylcysteine (NAC, a GSH precursor) would improve protein and GSH kinetics using a colitis piglet model with pre-existing protein deficiency.

Methods All piglets (n=16) received a protein-deficient diet enterally and were administered with dextran sulfate sodium (DSS) to induce colitis. After the discontinuation of DSS, piglets were randomized to continue with the protein-deficient diet (PD, n = 5), or to receive either adequate protein (AP, n = 6) or NAC supplement (NAC, n = 5). Stable isotope tracers were administered at the end of the study to determine protein and GSH kinetics.

Results Protein repletion led to higher catch-up growth and nitrogen balance. Both albumin fractional (FSR) and absolute synthesis rate (ASR) were 4-fold higher in AP than PD piglets. Similar patterns were found in the liver and plasma total proteins. Moreover, protein repletion resulted in higher GSH concentrations and synthesis ($p < 0.02$) in red blood cells (RBCs) with the correspondingly higher ferric-reducing ability of plasma (FRAP) ($p < 0.002$) than the other two groups. In contrast, although NAC supplementation led to a 10-fold higher cysteine in the jejunum (NAC vs. PD $p = 0.003$) and moderately higher in spiral colon and liver, it did not translate into GSH concentration nor synthesis when piglets remained in protein-deficient state.

Conclusion Our data suggest that protein repletion is an effective strategy to improve systemic protein and GSH kinetics in piglets with colitis. However, when protein deficiency is coupled with

colitis, NAC alone is not effective in improving GSH status despite the increased cysteine concentration.

3.2 Introduction

Inflammatory bowel disease (IBD) is a multifactorial gastrointestinal (GI) inflammatory condition that can be diagnosed at any age. Due to its complexity in adulthood and the fact that 25% of its cases occur in childhood, investigating the pediatric disease pathogenesis may provide more insight on potential treatment [38]. The inflammatory process and oxidative stress compromise nutritional and antioxidative status in patients with IBD [216-218]. The high risk of malnutrition in IBD is mainly caused by decreased food intake, increased nutrient demand, and losses or poor absorption from the gastrointestinal tract [43,74]. Recent clinical nutrition guidelines recommend an increased protein intake (1.2 – 1.5 g/(kg·d)) in patients with active IBD [219]. Protein deficiency impairs growth and dramatically reduces plasma and visceral protein synthesis [69,220,221]. Inflammation superimposed with protein deficiency further aggravates growth, protein anabolism [69,72,74,222]. Previous piglet study has also demonstrated that GSH concentration and synthesis both decreased while under combined challenges of colitis and macronutrient deficiency [222].

Animal models are widely used to investigate these therapeutic approaches and potentially apply them to humans [72]. Dextran sodium sulfate (DSS)-induced colitis can mimic the typical characteristics of ulcerative colitis and is one of the most commonly used models [223]. Compared to rodents, piglets have a more similar physiological structure, digestive systems, nutrient requirements and metabolism to children [206]. Besides, due to the more rapid growth and metabolic rate in piglets, it can be considered as an accelerated model for pediatric research [206].

Moreover, the piglet model allows for surgical manipulation for exclusive enteral feeding and stable isotope infusion to study nutrient kinetics [224,225].

Nutrient repletion has been shown to normalize the protein synthesis and resting energy expenditure in patients with coexistent diseases [128]. Likewise, in animal studies, protein repletion fosters rapid catch-up growth, increases albumin concentration, nitrogen retention, liver growth, and gastrointestinal morphology restoration [130-132]. Moreover, nutritional interventions are also essential in ameliorating antioxidative status in malnutrition coupled with inflammation [222,226]. GSH and its related sulfhydryl metabolites constitute the major thiol/disulfide redox system and participate in antioxidant defence, regulation of protein synthesis, and immune response [138,227]. Since cysteine is a rate-limiting substrate in GSH synthesis, supplementation of cysteine alone ameliorates GSH status [226]. N-acetyl-cysteine (NAC), a cysteine derivative, shows protective effects on redox states and clinical improvement [139,140]. Compared to other cysteine derivatives such as L-cysteine hydrochloride, NAC is more soluble and stable in aqueous solution and allows for potential use in enteral and parenteral feedings [228].

A previous study observed that colitis piglets fed with a well-nourished diet were able to decrease disease severity and synthesize proteins faster in the colon, compared to piglets with a macronutrient-deficient diet [229]. Since malnutrition is prevalent in IBD, however, it is unknown whether the benefit from a well-nourished diet persists with pre-existing compromised nutrition status. Therefore, a piglet model of combined colitis and protein deficiency is developed in this study to mimic the concomitant malnutrition in IBD. Our objective was to determine the impact of protein repletion on growth, protein anabolism, and GSH metabolism. In addition, we would also like to investigate whether NAC supplementation alone, as a quick and simple approach, could foster moderate improvement in GSH status while remaining malnourished.

3.3 Methods

Experimental protocol

This study was approved by the McGill University Animal Care Committee in accordance with the Canadian Council on Animal Care Guidelines. Sixteen female piglets (7-10 days old) underwent surgery upon arrival to implant catheters into the stomach for enteral feeding and DSS administration, into the femoral vein for tracer infusion and jugular vein for blood sampling [72,127,229] **(Figure 1)**. All piglets initiated a protein-deficient diet enterally 2 hours post-surgery and acclimated to full feeding rates on study day 3. DSS was administered to induce colitis from day 3 to the end of day 9. On day 10, piglets proceeded to the intervention phase and were randomized into one of three groups to continue on the protein-deficient diet (PD, n=5), or an adequate protein diet (AP, n=6), or continued protein-deficient diet and NAC supplementation (NAC, n=5). On day 15, piglets received a 6-hour stable isotope infusion to determine protein and glutathione kinetics. At the end of the infusion, piglets and were euthanized with an injection of sodium pentobarbital at 400 mg/kg (Euthansol, Schering Plough Canada Inc. Pointe-Claire, QC, CA).

Diet

Isoenergetic diets were formulated based on the National Research Council (NRC) nutrient requirement for growing piglets [230,231] and were given continuously through the gastric catheter and adjusted daily based on weights **(Table 1)**. Protein consisted of 4% and 27% of energy requirements in the PD and AP diet, respectively. N-acetylcysteine (NAC) solution (3.936 g/L) was infused with the liquid diet to achieve a dose of 6.03 mmol/(kg·d). This dose was extrapolated from cysteine supplementation studies in malnourished children [232,233]. There were 0.39 and 2.57 mmol/(kg·d) cysteine in the PD and AP diet, respectively.

Disease activity

DSS solution (200 g/L, 40,000 MW, ICN Biomedicals Inc., OH, USA) was administered twice daily (0.5 g/(kg.d)) via the gastric catheter to induce colitis. Feces were tested twice daily for occult blood with Hemoccult Sensa Strips (Beckman Coulter Inc., Fullerton, CA, USA), and stool consistency was recorded daily to calculate Disease Activity Index without Weight (DAINWT) [234,235].

Growth, Nitrogen balance

Body weight was obtained daily. Nitrogen balance was determined over 48 hours at the end of the DSS phase and the intervention phase. Urine, fecal and diet samples were analyzed through the Tru-Spec N system (LECO, St Joseph, MI, USA) to determine the nitrogen content. Nitrogen balance was calculated by subtracting nitrogen excretion from nitrogen intake. Nitrogen retention % was calculated by the percentage of nitrogen balance from intake.

Stable isotope infusion and sampling

Stable isotope tracers L-[ring-$^{2}H_5$]phenylalanine and [^{15}N, 1,2-$^{13}C_2$]glycine (Cambridge Isotope Laboratories, Cambridge, MA) were prepared in sterile saline and sterilized through a 0.22 μm filter. Primed continuous infusion of L-[ring-$^{2}H_5$]phenylalanine was given for 6 hours (priming: 52.5 μmol/kg, continuous: 35 μmol/(kg·h)). After the first hour, primed continuous infusion of [^{15}N, 1,2-$^{13}C_2$]glycine was administered for 5 hours (priming: 150 μmol/kg, continuous 75 μmol/(kg·h)). Blood was sampled from the jugular vein at baseline and hourly thereafter until the end of the 6-h infusion. All blood samples were transferred into EDTA tubes and centrifuged immediately after each draw. Hematocrit was measured at the beginning and 5 hours during the isotope infusion. Tissue sections, including liver, jejunum mucosa, spiral and distal colon, were

collected after euthanasia. Plasma, RBC, and tissue were immediately frozen in liquid nitrogen and stored at -80 ºC for later analysis.

Protein concentration and synthesis

Plasma total protein and albumin concentration were measured by clinical biochemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, CA). Plasma fibrinogen concentration was determined by ELISA (Kamiya Biomedical Company, Seattle, USA). Tissue total protein concentrations were quantified by Bradford protein assay (Thermo Scientific, Rockford, IL).

Plasma and tissue proteins were isolated to determine their synthesis rates. Protein from plasma and tissue homogenates were precipitated in ice-cold 10% trichloroacetic acid (TCA). Fibrinogen and albumin were isolated from plasma protein using their selective solubility/insolubility in ethanol and further purified with gel electrophoresis. All proteins were hydrolyzed overnight in 6 mM HCl. Both free and bound amino acids were further purified by cation exchange chromatography (Dowex, Bio-Rad, CA) and esterified with 1-propanol. Samples were analyzed by Triple Quadrupole Liquid Chromatography/Mass Spectrometry (LC-MS: UHPLC 1290, QQQ 6460, Agilent Technologies, Palo Alto, CA).

Fractional synthesis rate (FSR) of the plasma total protein, albumin and fibrinogen were calculated using FSR (%/d) = $[(E_{t2}-E_{t1}) \times 24 \times 100]/[E_{free} \times (t_2-t_1)]$ where $E_{t2} - E_{t1}$ is the increase of enrichment in the relevant plasma protein at the steady state between t_1 and t_2, and E_{free} is the enrichment of the plasma protein precursor pool (liver free amino acids). Tissue protein FSR was calculated using FSR (%/d) = $(E_{bound} \times 24 \times 100)/(E_{free} \times t)$, where E_{bound} is the enrichment in the relevant tissue protein from baseline to tissue harvest time (t); E_{free} is the enrichment of tissue free amino acids.

Absolute synthesis rate (ASR) of the plasma proteins was calculated using ASR (mg/(kg·d)) = FSR × plasma protein concentration × PV, where PV is the plasma volume calculated by hematocrit and an average of 80 ml/kg blood volume. Tissue protein ASR was calculated by ASR (g/(g tissue·d)) = FSR × tissue protein concentration × tissue weight.

GSH concentration, synthesis and plasma ferric reducing ability (FRAP)

Plasma and RBC GSH samples were derivatized immediately with N-Ethylmaleimide (NEM) to preserve the active thiols. Glutamyl-leucine (GL) was also added as internal standards before further sample processing. After centrifugation, RBCs were lysed by $ZnSO_4$, and the protein was precipitated with ice-cold methanol. The liver, jejunum mucosa, and spiral and distal colon tissues were homogenized in NEM and GL, purified with methanol and centrifuged to obtain the supernatant. Concentrations of GSH and its sulfhydryl metabolites were measured by LC-MS as previously described [222]. GSH FSR from RBC and tissues were calculated using FSR (%/d) = $[(E_{t2}-E_{t1}) \times 24 \times 100]/[E_{free} \times (t_2-t_1)]$, where $E_{t2} - E_{t1}$ is the increase of GSH enrichment at the steady state between t_1 and t_2, and E_{free} is the glycine enrichment in the precursor pool. FRAP was measured by an assay with colorimetric method to determine the whole-body total antioxidant activity [229,236].

Plasma amino acid concentration

Plasma samples, along with internal standards norvaline and sarcosine, were filtered through a Captiva ND lipid extraction plate (Crawford Scientific Ltd., Scotland, UK) with methanol. Standard amino acids with internal standards were prepared in various concentrations (9, 22.5, 90, 225, and 900 pmol/μl) and run along with sample amino acids on Ultra High-Performance Liquid Chromatography (UHPLC 1290 with fluorescent detector, Agilent

Technologies, Santa Clara, USA). Reagents o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) were mixed in for pre-column derivatization.

Statistical analysis

Statistical analysis was conducted using SAS 9.4. Homogeneity of variances was tested using Bayesian Criteria. Normality was tested using PROC UNIVARIATE function and Shapiro-Wilk's test. Differences between groups in protein and GSH synthesis were analyzed with one-way analysis of variance (ANOVA) adjusted with Tukey's for multiple comparisons. Changes in variables over time were assessed using two-way repeated-measures ANOVA adjusted with Tukey's for multiple comparisons. A p value of < 0.05 was considered statistically significant.

3.4 Results

All piglets continued to gain weight even while receiving DSS and a protein-deficient diet. During the intervention phase, piglets that were switched to the adequate protein diet gained more than 3-fold faster compared to piglets in the other two groups, which continued on the same trajectory as in the DSS phase **(Figure 2)**. Piglets started to develop occult blood and loose feces on study day 8-9, approximately 5-6 days after the initiation of DSS. Disease activity score, which was not different among groups, kept increasing during the intervention phase, even after discontinuing DSS on study day 10 **(Figure 3)**.

Nitrogen retention was positive and similar amongst groups in the DSS phase in which piglets retained about 75% of dietary nitrogen (**Table 2**). In the intervention phase, the higher nitrogen intake in the AP piglets translated to a dramatically higher nitrogen balance. PD piglets continued to retain similar nitrogen as in the DSS phase and showed a modest positive nitrogen balance. NAC supplementation of 0.984 g/(kg·d) contributed 84 mg N/(kg·d) to the intake. Urinary nitrogen was on average 84 mg higher than that in the DSS phase, suggesting that most of the NAC

was excreted in the urine. Nitrogen balance in these piglets was unchanged compared to the DSS phase.

The effect of protein repletion on protein synthesis was focused on the liver. Hepatic protein synthesis and concentration were the highest in the AP piglets but were unaffected in the intestine **(Table 3)**. Plasma albumin synthesis was 4-fold higher in the AP piglets; however, that did not translate to an increase in concentration. Fibrinogen synthesis was modestly higher in the AP group. The total plasma protein pool, heavily weighed by albumin, doubled in FSR and ASR without a change in concentration.

The effect of protein repletion on GSH status was most apparent in RBCs where synthesis and concentration were higher even than with NAC supplementation **(Table 4)**. Systemic antioxidant power (FRAP) (AP 0.15 ± 0.05 vs. PD 0.10 ± 0.02, $p = 0.002$; vs. NAC 0.11 ± 0.03, $p = 0.009$) showed the same pattern. GSH FSR was the highest in AP piglets in the spiral colon ($p < 0.01$) and tended to be higher in the jejunum (AP vs. PD, $p = 0.07$); however, concentrations of GSH or cysteine were not increased. Conversely, NAC supplementation resulted in higher cysteine concentration without an effect on GSH concentration or synthesis. NAC concentration was the highest in the jejunum (4.99 ± 2.00 μmol/g tissue) and was more moderate in other tissues (liver 0.36 ± 0.06, spiral colon 0.28 ± 0.04, distal colon 0.39 ± 0.08 μmol/g tissue, plasma 0.23 ± 0.03 μmol/ml).

Protein repletion increased plasma amino acid concentrations including GLX (glutamate and glutamine) **(Figure 4),** whereas NAC increased cysteine. Glycine was not different among groups in the intervention phase.

3.5 Discussion

The growing piglet model with pre-existing protein deficiency mimics the clinical reality of concomitant malnutrition in children with IBD. Our findings suggest that protein repletion should be the primary nutritional strategy in colitis piglets with prolonged protein deficiency in order to foster growth, anabolism and ameliorate systemic antioxidative status. NAC supplementation was a demonstrated precursor of cysteine; however, it was not effective in increasing GSH status or achieving antioxidative benefits when protein deficiency remains. Moreover, our previous studies reported that a well-nourished diet attenuated histological damage and disease severity in colitis piglets compared to a malnourished diet [229]. Although neither protein repletion nor NAC demonstrated clinical improvement, this study provides a foundation to investigate nutritional strategies with pre-established protein deficiency during colitis.

In this study, disease severity continued to increase even five days after discontinuing DSS in all groups. In contrast, Kim *et al.* provided adequate nutrition to colitis piglets from the start of the study and resulted in an attenuation of disease activities, even though the DSS dose was more than double during the induction phase compared to that in our study [20]. Similarly, with standard diet supply, a study in mice also observed a decrease in disease activity starting three days after DSS was discontinued [237]. Our data suggest that pre-existing protein deficiency dramatically compromised colitis recovery.

Despite the active colitis during the 5-day intervention phase, our study demonstrated that protein repletion was effective in ameliorating hepatic-derived plasma protein synthesis, resulting in synthesis rates that were 4-fold higher in albumin and 2-fold higher in plasma total protein than PD piglets. In fact, the synthesis rates reached levels similar to colitis piglets in a previous study fed a well-nourished diet without pre-existing protein deficiency [229]. However, protein concentration from plasma did not differ among groups. This is possibly due to the loss of protein

into the interstitial fluid caused by increased vascular permeability from acute-phase response [72,229,238]. Moreover, protein repletion resulted in higher liver protein synthesis and reached a similar level to that of the well-nourished piglets [229]; as a result, liver protein concentrations were higher. Therefore, a 5-day intervention with protein repletion was sufficient to increase hepatic protein metabolism even with a pre-established protein-deficiency in piglets with active inflammation. However, this nutritional intervention was not as effective in spiral and distal colons in improving protein metabolism [229]. We speculate that although protein was replenished to match the requirement for growing piglets, the demand might be increased with pre-existing protein deficiency at the inflamed sites. In addition, protein might be lost in the intestinal tract due to increased intestinal permeability, a common phenomenon in colitis [239,240].

Protein repletion was demonstrated to be effective in improving systemic antioxidative status. The total antioxidative power in the plasma measured by FRAP was higher in AP compared to PD piglets. In addition, protein repletion resulted in 2-3 fold higher GSH FSR and ASR, and 50% higher GSH concentration in the RBCs compared to piglets with continued protein-deficient diet. Our finding is in agreement with studies in children with severe edematous malnutrition, which demonstrated increased RBC GSH kinetics with protein repletion of 1.2 g/(kg·d) [233,241]. However, GSH synthesis did not differ with protein repletion in intestinal tissues except in spiral colon, which might be due to high variability among the AP piglets and small sample size. Moreover, GSH concentrations were not improved from protein repletion in all intestinal and liver tissues. In contrast, a previous study observed that when colitis piglets were fed with a well-nourished diet from the beginning of the study, GSH concentration in distal colon was higher compared to piglets with a malnourished diet [222]. It is possible that protein repletion might not

be long enough in our study to increase GSH-related amino acids in these tissues from the malnourished state and observe a change in GSH concentration.

Enteral NAC supplementation resulted in higher cysteine in all tissues and RBC. NAC deacetylated to cysteine via aminoacylase I to provide intercellular substrates for GSH synthesis [242]. Our study observed that the concentrations of NAC and cysteine were considerably higher in the jejunum compared to other tissues, highlighting the role of jejunum in metabolizing dietary NAC. In this study, NAC was supplemented at 0.984 g/(kg·d) through the enteral route in our study, whereas Shoveller *et al.* administered 0.27 g/(kg·d) NAC parenterally [243]. Plasma NAC concentration was similar in both studies. Together these studies suggest extensive first-pass metabolism of enteral NAC [244]. Urinary NAC but not fecal NAC was very high, suggesting that plasma NAC was modulated by urinary excretion [245] and that NAC supplementation was greater than the capacity for its conversion to cysteine.

Higher cysteine generated from NAC did not translate into higher γ-glutamylcysteine (γGC) nor GSH concentrations. It is possible that other substrates, such as glutamate and glycine, might be needed to foster GSH synthesis, especially in a protein-deficient state. Similarly, protein repletion resulted in higher liver γGC, yet GSH concentration did not differ, suggesting that glycine might be a limiting factor during the second step of glutathione synthesis. Several studies from Sekhar *et al.* reported the combination of cysteine and glycine supplementation to improve glutathione status and decrease oxidative stress [136,246,247]. Furthermore, glutamate-cysteine ligase (GCL) is feedback inhibited by GSH and is recognized as the rate-limiting enzyme in GSH synthesis [248,249]. GCL has been found to be down-regulated in IBD patients [133]. Subsequently, a study supplemented γGC directly and reported an increase in GSH concentrations in lymphocytes in human subjects [250].

The fact that neither protein repletion nor NAC exerts clinical improvement in this study may be presumably due to 1) the prolonged protein deficiency, 2) insufficient adaptation and recovery time, and 3) the modest colitis. Firstly, previous studies with NAC or cysteine supplementation demonstrated protective effects on intestinal tissues; however, the results were based on a well-nourished diet [251,252]. Pre-existing protein deficiency in our study limited the availability of the substrates for anti-inflammatory and antioxidative activities in active colitis. Secondly, although intestinal epithelial cells regenerate every 4-5 days, a 5-day intervention phase may not be sufficient for these cells to recover and renew when piglets had colitis coupled with protein deficiency [253]. Moreover, the intestinal microbiome has a significant impact on colitis progression and responds dramatically to diet change [254]. Since the protein intake changed from 4 to 27% of total calories in the AP group during the intervention, a more extended acclimation period may be needed to adapt to this significant diet change and stabilize the microbiome environment in the intestinal tract. Lastly, our study administered DSS at 0.5 g/(kg.d) to induce mild colitis, as compared to other piglet studies with a much higher DSS of 1-2 g/(kg.d) [20,72,127]. It might be, therefore, difficult to observe changes in disease severity in modest colitis.

In summary, our study demonstrated that protein repletion improved protein, GSH kinetics and antioxidative status systemically, however, did not change disease severity. NAC alone increased cysteine concentration but had no effect on GSH kinetics or systemic antioxidative status during protein deficiency. Adequate protein combined with a protocol of glutathione related amino acids and longer nutritional intervention time may be a more effective nutritional intervention to improve protein anabolism and antioxidative status.

Figure 3.1 Study protocol.

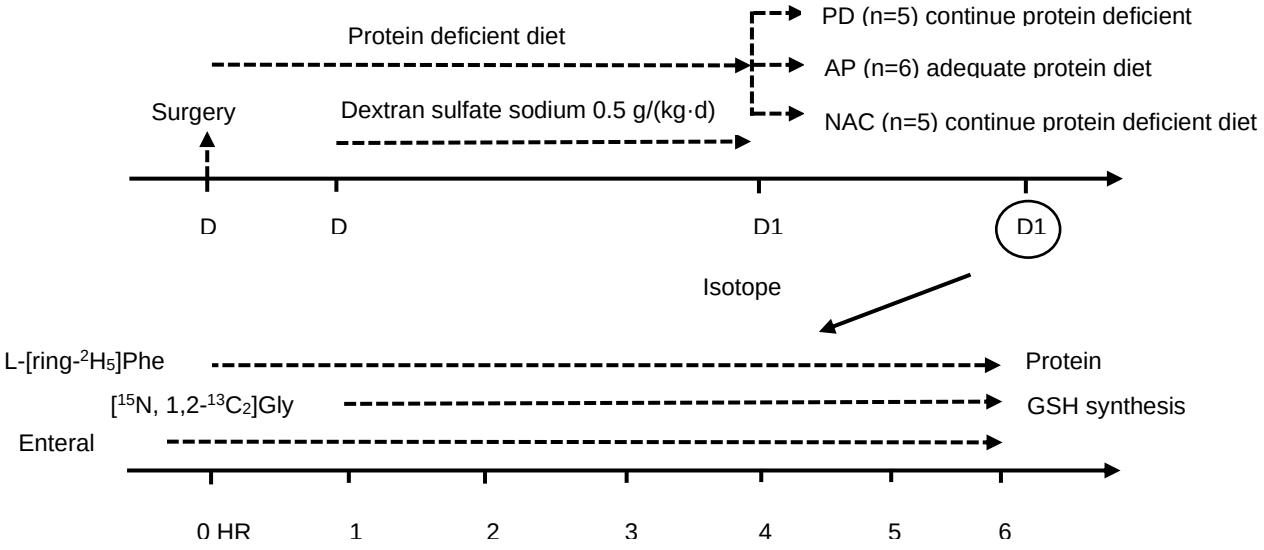


Table 3.1 Enteral feeding diet composition.

a) Diet ingredients.

	Protein deficient diet	Adequate protein diet
Egg white g/L	5.34	34.95
Whey protein g/L	5.50	36.00
Lactose g/L	86.63	56.73
Maltodextrin g/L	22.15	22.15
Corn oil g/L	24.52	18.49
Canola oil g/L	21.64	16.31
Mineral mix g/L	13.70	10.80
Vitamin mix g/L	0.38	0.38

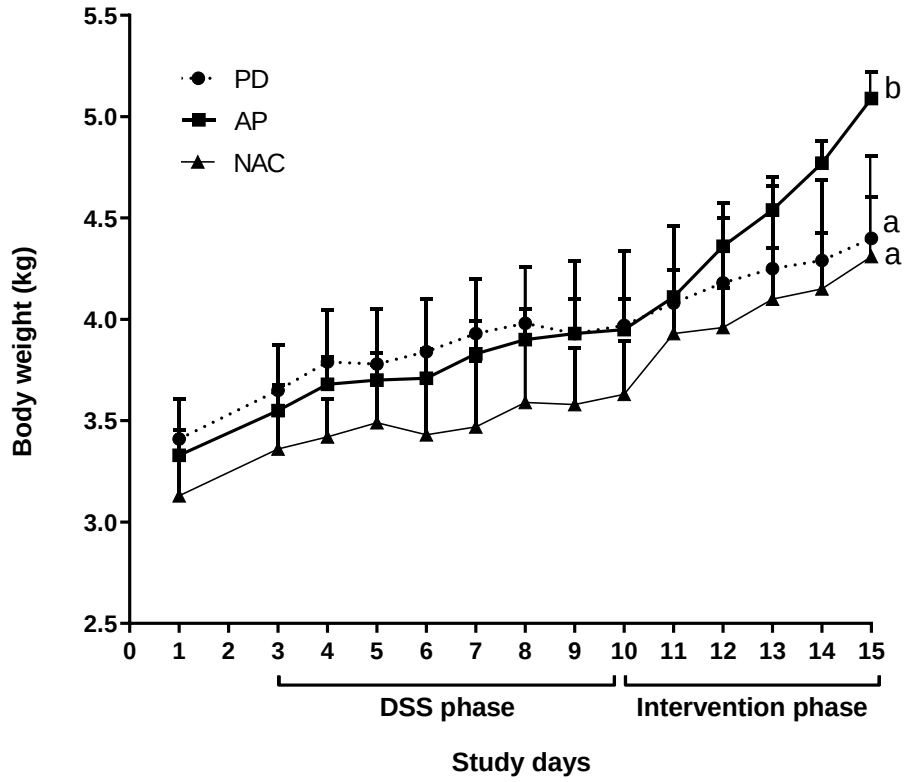
b) Nutrient and energy intakes.

	Protein deficient diet			Adequate protein diet		
	g/(kg·d)	kcal/(kg·d)	kcal %	g/(kg·d)	kcal/(kg·d)	kcal %
Protein	2.2	8.8	4.1	14.4	57.6	26.9
Carbohydrate	26.0	103.9	48.0	19.6	78.3	36.6
Lipids	11.5	103.9	48.0	8.7	78.3	36.6
Minerals	3.4	0	0	2.7	0	0
Vitamins	0.1	0	0	0.1	0	0
Total energy		216.5	100		214.2	100

c) *Diet amino acid composition.*

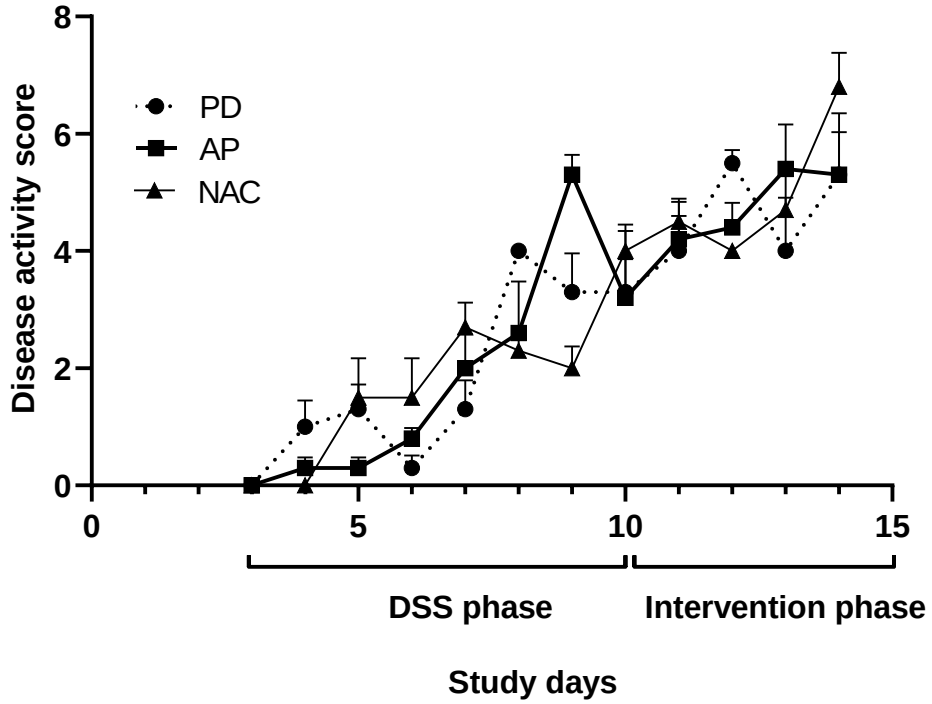
Amino acids	**NRC req't**	**Protein deficient diet**		**Adequate protein diet**	
	g/(kg·d)	Intake g/(kg·d)	% Req't	Intake g/(kg·d)	% Req't
Arginine	0.34	0.093	27	0.621	183
Histidine	0.26	0.046	18	0.309	119
Isoleucine	0.44	0.121	27	0.805	183
Leucine	0.83	0.201	24	1.337	161
Lysine	0.82	0.163	20	1.089	133
Cysteine/Cystine	0.25	0.047	19	0.311	124
Methionine	0.22	0.062	28	0.416	189
Methionine + cysteine	0.46	0.109	24	0.727	158
Tyrosine	0.28	0.071	26	0.476	170
Phenylalanine	0.48	0.104	22	0.691	144
Phenylalanine + tyrosine	0.77	0.175	23	1.167	152
Threonine	0.51	0.104	20	0.696	137
Tryptophan	0.15	0.040	26	0.265	177
Valine	0.55	0.135	25	0.901	164
Alanine	n/a	0.117	n/a	0.781	n/a
Aspartic acid	n/a	0.205	n/a	1.369	n/a
Glutamic acid	n/a	0.352	n/a	2.345	n/a
Glycine	n/a	0.060	n/a	0.399	n/a
Proline	n/a	0.108	n/a	0.720	n/a
Serine	n/a	0.130	n/a	0.868	n/a

Figure 3.2 Daily body weight.



Mean (SEM).

Figure 3.3 Disease activity.



Mean (SEM).

Table 3.2 Nitrogen balance.

	PD	AP	NAC
DSS phase			
Nitrogen intake, mg/(kg·d)	305 (4)	311 (7)	314 (5)
Fecal nitrogen, mg/(kg·d)	9 (3)	10 (3)	6 (1)
Urinary nitrogen, mg/(kg·d)	70 (6)	95 (30)	55 (4)
Nitrogen balance, mg/(kg·d)	228 (4)	206 (38)	253 (1)
Nitrogen retention, %	75 (2)	65 (12)	81 (1)
Intervention phase			
Nitrogen intake, mg/(kg·d)	308 (15) [a]	1964 (90) [c]	402 (6) [b]
Fecal nitrogen, mg/(kg·d)	23 (13) [a]	67 (23) [b]	6 (1) [a]
Urinary nitrogen, mg/(kg·d)	60 (13) [a]	560 (106) [c]	139 (16) [b]
Nitrogen balance, mg/(kg·d)	230 (34) [a]	1338 (171) [b]	257 (14) [a]
Nitrogen retention, %	72 (9)	67 (6)	64 (4)

Values are mean (SEM). Nitrogen balance, intake and excretion of piglets with DSS-induced colitis consuming a protein deficient diet (PD) in the DSS phase from study day 3 to day 10, and then either continue PD diet (PD), or consuming adequate protein diet (AP), or continue PD diet but supplemented with N-acetylcysteine (NAC) in the intervention phase from day 10 to day 15. Means without a common superscript differ, $p < 0.05$.

Table 3.3 Tissue and plasma protein concentration and synthesis rate.

	PD	AP	NAC
Spiral colon total protein			
FSR, %/d	22 (4)	31 (2)	22 (2)
ASR, mg/(g tissue·d)	18 (8)	20 (4)	14 (3)
Concentration, mg/g tissue	65 (18)	58 (14)	53 (12)
Distal colon total protein			
FSR, %/d	22 (4) [ab]	24 (1) [b]	18 (2) [a]
ASR, mg/(g tissue·d)	9 (3)	11 (1)	8 (2)
Concentration, mg/g tissue	37 (5)	48 (5)	47 (7)
Liver total protein			
FSR, %/d	30 (2) [a]	42 (2) [b]	31 (3) [a]
ASR, mg/(g tissue·d)	30 (5) [a]	61 (4) [b]	32 (4) [a]
Concentration, mg/g tissue	98 (13) [a]	145 (9) [b]	106 (8) [a]
Plasma albumin			
FSR, %/d	5 (2) [a]	21 (1) [b]	7 (1) [a]
ASR, mg/(kg·d)	57 (19) [a]	285 (20) [b]	77 (10) [a]
Concentration, g/L	20 (1)	22 (1)	18 (1)
Plasma fibrinogen			
FSR, %/d	42 (6)	52 (4)	41 (4)
ASR, mg/(kg·d)	79 (15) [a]	128 (16) [b]	84 (26) [ab]
Concentration, g/L	3 (0.3)	4 (0.7)	3 (0.9)
Plasma total protein			
FSR, %/d	21 (3) [a]	42 (3) [b]	26 (2) [a]
ASR, mg/(kg·d)	450 (76) [a]	1011 (44) [b]	535 (68) [a]
Concentration, g/L	39 (2)	39 (2)	35 (2)

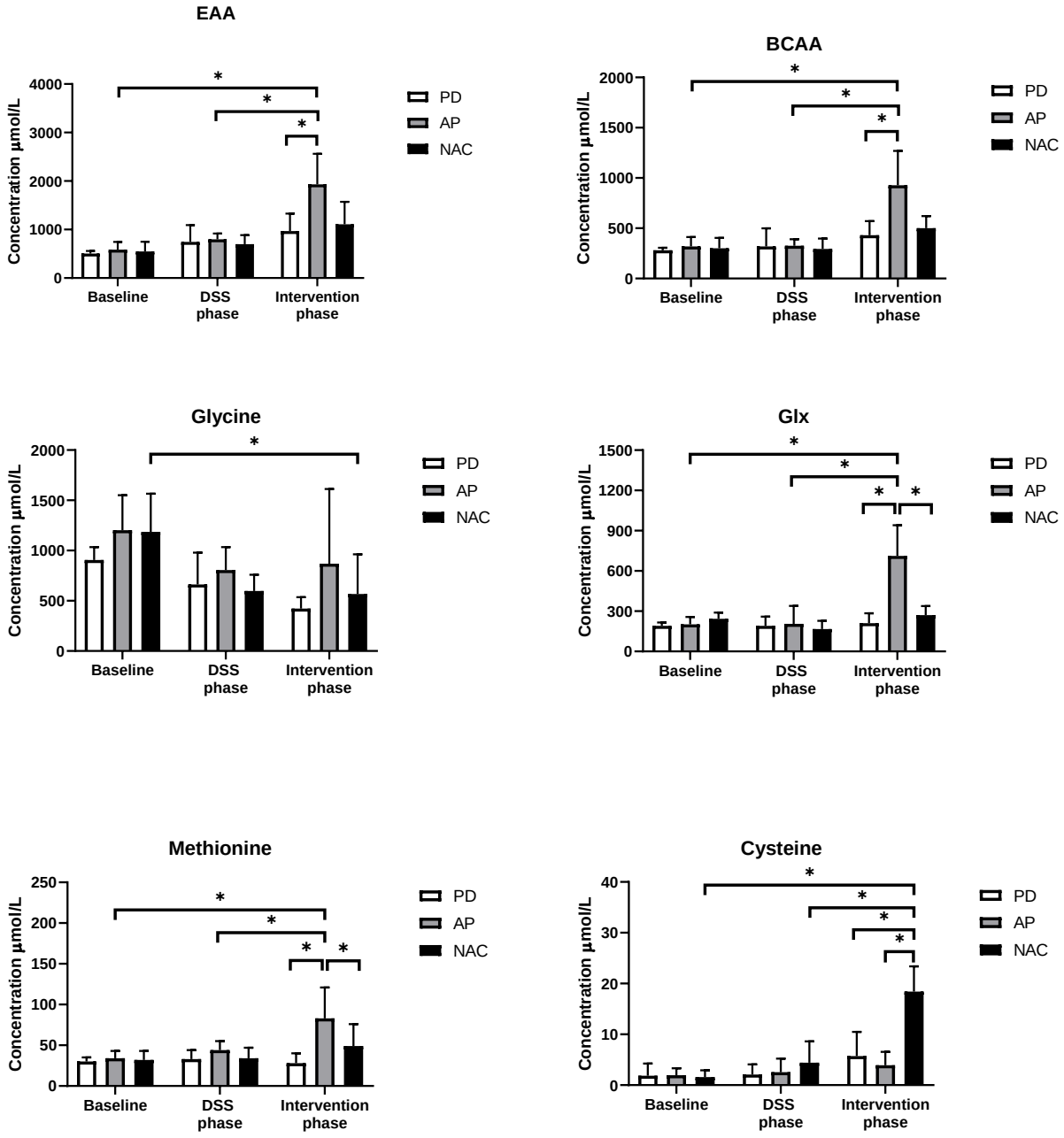
Mean (SEM). Means without a common superscript differ, $p < 0.05$.

Table 3.4 Synthesis and concentrations of glutathione and related sulfhydryls.

	PD	AP	NAC
RBCs			
GSH FSR, %/d	28 (2) [a]	53 (2) [b]	24 (2) [a]
GSH ASR, nmol/(g RBC·d)	327 (23) [a]	924 (82) [b]	329 (54) [a]
GSH concentration, nmol/g RBC	1163 (61) [a]	1757 (180) [b]	1488 (162) [a]
γGC concentration, nmol/g RBC	17.5 (3.7) [a]	31.0 (4.4) [b]	23.0 (4.0) [ab]
Cysteine concentration, nmol/g RBC	28.0 (4.2) [a]	64.0 (7.6) [b]	77.6 (8.3) [b]
Jejunum mucosa			
GSH FSR, %/d	112 (5)	207 (33)	140 (10)
GSH ASR, µmol/(g protein·d)	23 (6)	42 (10)	28 (1)
GSH concentration, µmol/g protein	21 (5)	20 (8)	20 (1)
γGC concentration, µmol/g protein	0.16 (0.03)	0.24 (0.04)	0.45 (0.21)
Cysteine concentration, µmol/g protein	0.7 (0.2) [a]	0.8 (0.1) [a]	6.9 (1.6) [b]
Spiral colon			
GSH FSR, %/d	104 (4) [a]	160 (11) [b]	106 (4) [a]
GSH ASR, µmol/(g protein·d)	13 (2)	25 (7)	21 (8)
GSH concentration, µmol/g protein	15 (4)	15 (3)	23 (7)
γGC concentration, µmol/g protein	0.08 (0.01)	0.11 (0.02)	0.16 (0.05)
Cysteine concentration, µmol/g protein	0.4 (0.1) [a]	0.4 (0.1) [a]	1.4 (0.5) [b]
Distal colon			
GSH FSR, %/d	100 (10)	152 (23)	115 (16)
GSH ASR, µmol/(g protein·d)	20 (3)	22 (6)	17 (3)
GSH concentration, µmol/g protein	20 (3)	14 (2)	12 (3)
γGC concentration, µmol/g protein	0.14 (0.03)	0.08 (0.01)	0.12 (0.02)
Cysteine concentration, µmol/g protein	0.5 (0.1)	0.4 (0.1)	0.8 (0.4)
Liver			
GSH FSR, %/d	404 (19)	349 (11)	393 (15)
Total liver GSH ASR, µmol/d	1382(173)	1534 (93)	1897 (180)
Total liver GSH, µmol	343 (44)	469 (37)	483 (43)
Total liver γGC, µmol	3.2 (0.5) [a]	13.8 (1.2) [b]	5.4 (1.4) [a]
Total liver cysteine, µmol	11.4 (2.9) [a]	12.2 (2.7) [ab]	18.2 (5.5) [b]

Mean (SEM). Means without a common superscript differ, $p < 0.05$.

Figure 3.4. Plasma amino acid concentrations.



Mean (SD). BCAA, branched-chain amino acid; Glx, sum of glutamate and glutamine.

BRIDGE STATEMENT 1

The previous manuscript demonstrated that protein repletion in piglets with colitis and pre-existing protein deficiency ameliorated growth, protein synthesis, and systemic antioxidative status, whereas NAC did not. Stable isotope tracer infusions quantified a dynamic change of protein and antioxidant metabolism that otherwise cannot be characterized using traditional concentration measurements. Since colitis and malnutrition form a vicious circle [218], nutritional interventions examined in this unique animal model with combined challenges from colitis and protein deficiency may be applicable in future clinical research.

Malnutrition is also highly prevalent in patients undergoing colorectal surgery [255]. Patients reported decreased food intake (from appetite loss and taste change) and significant weight loss of more than 10 % in the past six months before the surgery [256]. This suboptimal nutritional status is associated with postoperative outcomes [51]. In particular, hypoalbuminemia is common in patients with colon cancer requiring surgery, and it is also associated with adverse clinical outcomes after colorectal surgery [257]. In the past, low albumin concentration was often used as an indicator of protein deficiency in clinical practice; however, now it is recognized that hypoalbuminemia is predominantly triggered more by inflammation than malnutrition. Albumin concentration remains low despite a dramatic increase in its synthesis rate, and this is mostly due to the increased intestinal and vascular permeability induced by the metabolic stress response, leading to accelerated albumin loss into the interstitial fluid [258].

Manuscript 1 showed that colitis piglets receiving protein repletion resulted in a higher albumin synthesis rate, but albumin concentration remained low, similar to the control piglets. Similarly, a previous clinical study showed that patients receiving perioperative nutrition had almost 2-fold higher albumin synthesis than preoperative fasting patients, although albumin

concentrations were similar [163]. Albumin synthesis rate measured by stable isotope tracers may be a better indicator for the effectiveness of protein supplementation than albumin concentration.

In manuscript 1, enteral supplementation of high-quality intact proteins (sourced from egg albumin and whey) was digested to small peptides and AAs in the intestine. In contrast, providing AAs through the parenteral route delivers nutrients directly into the systemic circulation bypassing the first-pass splanchnic uptake. Parenteral nutrition and decreases the risk of aspiration during surgery and anesthesia [259].

Patients receiving a perioperative infusion of AA and glucose to minimize perioperative fasting achieved slight positive leucine balance, increased albumin synthesis, while maintaining normoglycemia by minimizing endogenous glucose production [163]. Since surgical patients with pre-existing insulin resistance and T2D are at higher risk of hyperglycemia during and after surgery [260], we hypothesized that parenteral infusion of AAs alone without glucose would be an effective strategy to achieve positive protein balance while avoiding hyperglycemia in patients with and without T2D.

Therefore, we designed the following study in which patients with and without T2D received infusions of stable isotope tracers to investigate changes in whole body glucose kinetics and protein balance, including albumin synthesis. Tracer infusions were conducted during fasting before surgery and again on the second postoperative day while receiving a parenteral nutrition regimen consisting of AA alone for a total of 72 hours. The regimen was individualized to deliver AAs at 20% of each patient's measured REE and started immediately after the first tracer infusions to minimize perioperative fasting.

CHAPTER 4 – MANUSCRIPT 2

Perioperative nutrition with amino acids alone prevents hyperglycemia in surgical patients with good glycemic control, but not with poorly controlled diabetes

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4.1 Abstract

Background Surgical stress-induced insulin resistance promotes a catabolic response. Avoiding perioperative fasting with parenteral amino acids (AAs) and glucose reduces protein catabolism. However, glucose infusion increases the risk of postoperative hyperglycemia in patients with type 2 diabetes (T2D). We hypothesized that perioperative AAs alone might induce protein anabolism in patients with T2D while avoiding hyperglycemia.

Methods Patients undergoing colorectal surgery with (n=8) or without diabetes (n=10) received parenteral AAs perioperatively. Patients with T2D were further stratified into well-controlled and poorly controlled groups with a cutoff of HbA1c at 6.5%. Whole body leucine balance, glucose kinetics and plasma protein synthesis were measured by stable isotope tracers.

Results Perioperative nutritional intervention with parenteral AAs alone prevents hyperglycemia in patients with well-controlled T2D similarly to patients without diabetes. However, hyperglycemia was still observed in patients with poorly controlled T2D (11.2 ± 2.8 mmol/L, $p < 0.001$), with elevated endogenous glucose production (18.8 ± 5.1 vs. without diabetes 11.3 ± 2.3 μmol/(kg·min), $p = 0.0207$). Moreover, regardless of preoperative glycemic control, parenteral AAs increased postoperative leucine balance from baseline fasting rates in patients with T2D at levels comparable to their counterparts without diabetes (T2D pre -18.2 ± 6.8 to post -9.3 ± 5.0, without T2D pre -12.7 ± 4.0 to post -7.7± 7.6 μmol/(kg·h)). Plasma protein synthesis showed a similar pattern.

Conclusions Individualized perioperative nutrition support with AAs alone prevents postoperative hyperglycemia in patients with good glucose control, but not poorly controlled T2D. AA supplementation is effective in increasing leucine balance and plasma protein synthesis in patients with T2D. However, the significant postoperative endogenous glucose production,

hypoalbuminemia and improved but persistently negative leucine balance suggest potential benefits of including glucose in perioperative nutrition support regimens for patients with good glycemic control.

4.2 Introduction

The stress of surgery imparts immunological and neuroendocrine responses, which trigger the release of cytokines, as well as counter-regulatory hormones (e.g. growth hormone, cortisol, catecholamines) via the hypothalamic-pituitary-adrenal axis [261]. The resulting insulin resistance (IR) induces hyperglycemia, proteolysis, muscle wasting, and delayed convalescence [262].

Various strategies were developed to attenuate the adverse metabolic outcomes induced by surgical stress. These include intensive insulin therapy, enhanced anesthetic and analgesic techniques (e.g. epidural blockade), and nutritional interventions. Hyperinsulinemic-normoglycemic clamp allows tight glycemic control and promotes anabolic protein response [158]. Epidural blockade blunts the sympathetic adrenergic response to reduce protein catabolism and insulin resistance; moreover, combining epidural blockade with parenteral amino acids (AAs) after surgery promotes a more efficient substrate utilization to achieve positive protein balance while avoiding hyperglycemia [263,264]. Furthermore, avoiding preoperative fasting is an intuitive and relatively simple approach to promoting protein anabolism [163]. Our finding that omitting glucose from parenteral nutrition in the postoperative period prevents hyperglycemia [265] led to the question of whether glucose is necessary for perioperative nutritional interventions.

Type 2 diabetes (T2D) in surgical patients aggravates the stress response due to pre-existing insulin resistance. Compared to patients without diabetes, T2D exacerbates stress-induced hyperglycemia, insulin resistance and protein catabolism [152]. Patients with T2D who received high dose parenteral AAs alone without glucose achieved positive protein balance while

maintaining normoglycemia after colorectal surgery [164]. It is noteworthy that this study was designed to deliver AAs over 3 hours at 2.9 g/(kg·d) to simulate a single meal. Achieving a net positive anabolic response while maintaining normoglycemia over the longer perioperative term is particularly challenging in patients with T2D.

Considering the exaggerated catabolic response of patients with T2D and their predisposition to hyperglycemia, we aimed to develop a perioperative nutrition strategy to (1) mitigate hyperglycemia by eliminating parenteral glucose, 2) enhance anabolism by providing individualized parenteral nutrition support with a moderate dose of AAs, and (3) avoid preoperative fasting by starting parenteral AAs before surgery. We hypothesized that our nutrition intervention would promote protein balance without hyperglycemia to a similar extent in both patients with and without T2D undergoing surgery for colorectal cancer resection. Pre- and postoperative stable isotope infusions were performed to determine protein and glucose kinetics.

4.3 Methods

Participants and study design

The study was approved by the Research Ethics Board of the McGill University Health Centre (MUHC) and performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from patients who were scheduled for colorectal cancer surgery. Patients were then allocated to two groups according to pre-established T2D diagnosis (**Figure 1**). Exclusion criteria included evidence of metastatic disease, body mass index (BMI) >30 kg/m^2, severe anemia (hemoglobin < 100 g/L), type 1 diabetes mellitus, pregnancy, advanced cardiac, respiratory, hepatic, or renal dysfunction, or use of corticosteroids.

Stable isotope infusion studies were performed in both patient groups one day prior to surgery after an overnight fast, and again on the second postoperative day. Immediately after the

first isotope infusion, patients received nutrition support with parenteral AAs. AA intake was based on 20% of measured resting energy expenditure (REE) of each patient and maintained until the second postoperative day, for a total of 72 hours **(Figure 2)**.

Experimental protocol

One day before surgery, after overnight fasting, REE was determined by indirect calorimetry (TrueOne 2400, Parvo Medics, USA) while each patient was lying in a semi-recumbent position and breathing in a hood for 30 minutes. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured simultaneously to calculate the respiratory quotient (RQ). Parenteral AA solution (Travasol®10%, Baxter, Canada) was administered from the end of preoperative isotope infusion to the end of the postoperative isotope infusion. AA infusion rate was individualized based on 20% of each patient's measured REE. This is approximately 1 g/(kg·d) AA or 0.83 g/(kg·d) protein after correcting for AAs loss of 18 mass units as it formed a peptide bond [266]. The composition of Travasol® (g per 100g of total AAs) is as follows: isoleucine 6.0, leucine 7.3, valine 5.8, lysine 5.8, methionine 4.0, phenylalanine 5.6, tyrosine 0.4, threonine 4.2, tryptophan 1.8, histidine 4.8, arginine 11.5, glycine 10.3, alanine 20.7, proline 6.8, and serine 5.0. HbA1c was measured preoperatively to assess long-term glycemic control status. Oral antihyperglycemic medications were discontinued at the beginning of the study.

Upon arrival to the anesthetic room, an epidural catheter was inserted in vertebral levels between T10-T12. Bupivacaine was administered to achieve and maintain sensory block throughout the surgery. General anesthesia was induced with propofol and maintained by nitrous oxide and isoflurane. Postoperative analgesia was adjusted to keep pain scores at rest below 4 [265].

Stable isotope tracer (Cambridge Isotope Laboratories, Tewksbury, MA, USA) solutions were tested to be sterile and free of pyrogens. Glucose kinetics was determined by a 3-hr primed continuous infusion of D-[6,6-$^{2}H_2$]glucose (priming: 19 μmol/kg, continuous: 0.22 μmol/(kg·min), *i.v.*). Whole body protein kinetics were determined by L-[1-^{13}C]leucine (priming: 4 μmol/kg, continuous: 3.6 μmol/(kg·h), *i.v.*). Na$H^{13}CO_3$ (1 μmol/kg, *p.o.*) was administered to prime the bicarbonate pool. Fractional and absolute synthesis rates of the hepatic-derived proteins (total protein, albumin and fibrinogen) were measured by a 6-hr primed continuous infusion of L-[ring-$^{2}H_5$]phenylalanine (priming: 12 μmol/kg, continuous: 9 μmol/(kg·h), *i.v.*) **(Figure 2)**.

Blood and breath samples were collected at baseline, 150, 160, 170 and 180 minutes of the isotope infusion to measure whole body leucine and glucose kinetics. In addition, hourly blood samples were taken from hour 3 to the end of the isotope infusion to measure the incorporation of phenylalanine into plasma proteins. Blood samples were also collected to measure the concentrations of plasma proteins and other metabolites at 240 minutes of each isotope infusion **(Figure 2)**. All blood samples were transferred immediately into sodium EDTA vacutainers and centrifuged at 4°C. Plasma samples were stored at -80°C for later analysis.

Sample analysis

Plasma glucose was derivatized by acetylation with acetic anhydride and dissolved in ethyl acetate for analysis of D-[6,6-$^{2}H_2$]glucose enrichment in the gas chromatography-mass spectrometry (GC/MS 5988A, Agilent Technologies, Palo Alto, USA) with electron impact ionization using selected ion monitoring at *m/z* 200 to 202 [163].

Plasma α-ketoisocaproic acid (KIC), a marker of intracellular leucine enrichment, was derivatized to its pentafluorobenzyl ester and analyzed by GC/MS using methane negative chemical ionization (NCI) and selected-ion monitoring at *m/z* 129 and 130 [165]. $^{13}CO_2$

enrichment in breath samples was analyzed by isotope ratio-mass spectrometry (IRMS, Analytical Precision AP2003, Manchester, UK).

In general, proteins were processed by isolation from plasma, purification, and then hydrolysis [163]. Total plasma proteins were precipitated with trichloroacetic acid, vortexed and centrifuged. The pellet was washed with TCA and hydrolyzed overnight with 6N HCl at 110°C. Albumin and fibrinogen were isolated by differential solubility in ethanol for further purification on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel bands were excised and hydrolyzed overnight. All hydrolyzed amino acids were isolated using cation exchange chromatography (Dowex, Bio-Rad, CA, USA). VLDL ApoB100, representing the hepatic precursor pool enrichment, was isolated by ultracentrifugation 104,447×g at 12 ºC for 20 hours and precipitated with isopropanol [267]. Amino acids released from each protein were derivatized to their heptafluorobutyrate derivatives and analyzed by GC/MS monitoring ions at *m/z* 383 to 388 [163].

Plasma total protein, albumin and fibrinogen concentrations were measured in the hospital clinical laboratory. Plasma glucose was measured by glucose oxidase method with glucose analyzer 2 (Beckman Instruments, Fullerton, CA). Serum insulin levels were measured by chemiluminescent immunoassay (CMIA, Access Immunoassay system, Beckman Instruments, Fullerton, CA). Plasma amino acids were measured by Ultra High-Performance Liquid Chromatography (HPLC1290 with UV detection, Agilent Technologies, Palo Alto, USA) after pre-column derivatization with o-phthalaldehyde (OPA).

Calculations

Whole body glucose and leucine kinetics were calculated from the dilution of the tracers into their free pools at steady state. The rate of appearance (Ra) can be calculated by $Ra = i \cdot (E_i/E_p$

-1); where i is the infusion rate of the labeled tracer, E_i is the tracer enrichment in the infusate, and E_p is the mean enrichment at steady state.

At steady state, the rate of leucine appearance equals the rate of disappearance, such that Ra = S + O = B + I; where S is the rate of leucine incorporation into protein synthesis, O is the rate of leucine oxidation, B is the rate of leucine disposed from protein breakdown. Whole body leucine balance was calculated as the difference between S and B, serving as an indicator of protein balance. In the calculation of leucine oxidation, correction factors were applied to account for a fraction of $^{13}CO_2$ released from leucine but retained within slowly turning over bicarbonate pools [268].

Fractional synthesis rates (FSR) of plasma total protein, albumin, and fibrinogen were calculated by FSR (%/d) = $[(E_{t2}-E_{t1}) \times 24 \times 100] / [E_{free} \times (t_2-t_1)]$, where Et_2-E_{t1} is the enrichment of labeled phenylalanine incorporated in plasma proteins during the t_2-t_1 hours of the infusion; and E_{free} is the enrichment of precursor pool at steady state. Absolute synthesis rate (ASR mg/(kg·d)) = FSR × conc. × PV, where conc. is the plasma concentration of the protein, and PV is the estimated plasma volume [163].

Statistical analysis

The sample size was determined by prospective power analysis with a power of 80% and the ability to detect a difference in protein balance with a type I error of 5% [151,152]. Patient characteristics were compared using independent *t*-test or chi-square test. Differences in other outcomes were determined by repeated-measures analysis of variance (ANOVA) with Bonferroni correction as a post-hoc test. Homogeneity and normality were tested using Bayesian Criteria and Shapiro-Wilk's test, respectively. A $p < 0.05$ was considered statistically significant. All data are

presented as mean ± SD unless otherwise specified. All statistical analyses were performed using SAS 9.4.

4.4 Results

Eighteen patients were included in the study **(Figure 1)**. Patient characteristics were similar between the two groups, except that HbA1c, as expected, was higher in the T2D group **(Table 1)**. Surgical parameters were also similar, and the perioperative course was uneventful in all patients.

REE and VCO2 were higher in patients with T2D vs. without diabetes **(Table 2)**. In contrast, RQ was not different between patient groups but was lower post-surgery. Neither insulin concentration nor HOMA-IR index was different between groups, either with or without patient stratification based on their glycemic control levels. Cortisol increased postoperatively in both groups.

Fasting plasma glucose was higher in patients with T2D than without ($p = 0.0054$) **(Table 3)**. Similarly, glucose Ra was higher in patients with T2D ($p = 0.0387$). Neither parameter was affected by surgery.

Due to the large variability in postoperative glucose concentration and Ra within the T2D group, patients were further stratified according to the level of glycemic control: well-controlled with HbA1c < 6.5% and poorly-controlled with HbA1c > 6.5% **(Table 4)**. Using Bonferroni's post-hoc comparisons, all patients had similar preoperative glucose concentrations. However, patients with poorly controlled T2D (HbA1c > 6.5%) had higher postoperative glucose concentration compared to patients without diabetes ($p < 0.0001$) and patients with well-controlled T2D ($p = 0.0003$). Patients with poorly-controlled T2D trended to have higher postoperative glucose concentrations compared to baseline ($p = 0.06$), which might be attributed to a higher postoperative glucose Ra ($p = 0.0207$). In contrast, patients without diabetes and those with good

control showed no difference between pre and postoperative glucose concentrations. HbA1c was positively correlated with postoperative glucose concentration, endogenous glucose production and preoperative branched-chain amino acid concentrations (BCAA) **(Figure 3)**.

At baseline fasting before surgery, whole body leucine kinetics were not different between groups, and leucine balance was negative (T2D -18.2 vs. without diabetes -12.7 μmol/(kg·h)) **(Table 5)**. Leucine balance increased in both groups ($p = 0.0027$) post-surgery while receiving amino acids; however, values remained negative (T2D - 9.3 vs. without diabetes - 7.7 μmol/(kg·h)). Leucine Ra and oxidation increased significantly after surgery in both groups with $p = 0.0121$ and $p < 0.0001$, respectively. Stratification based on glucose control did not reveal any differences between groups.

No difference in plasma protein kinetics while fasting before surgery was observed **(Table 6)**. Albumin concentrations decreased in both groups postoperatively ($p < 0.0001$). The trend for an increase in albumin FSR ($p < 0.07$) was insufficient to maintain concentration. In contrast, the increase in fibrinogen concentration was achieved by an increase in FSR, which resulted in a dramatic increase in ASR. FSR of the total plasma protein pool doubled after surgery; however, the decrease in concentration was comparable to the decrease in plasma albumin. Total essential ($p < 0.001$) and branched-chain ($p < 0.0001$) amino acid concentrations increased postoperatively, whereas total AA and NEAA concentrations were not different by time **(Figure 4)**. There was no difference between groups. Stratification of plasma protein kinetics and plasma AAs based on glucose control did not show any difference between groups.

4.5 Discussion

Pre-existing insulin resistance in patients with T2D amplifies surgical stress-induced hyperglycemia and catabolism [262,269,270]. Although exogenous glucose has been used as a

strategy either before or after surgery to reduce adverse postoperative clinical outcomes, it leads to hyperglycemia in patients with T2D [152,155]. In the present study, an individualized nutrition support regimen providing AAs alone and avoiding preoperative fasting was effective in preventing hyperglycemia in colorectal cancer patients with well-controlled T2D (HbA1c < 6.5%). Furthermore, AA infusion improved leucine balance and total plasma protein synthesis in patients with T2D to a level similar to their counterparts without diabetes.

Stratifying patients based on preoperative HbA1c revealed that replacing glucose with AAs in nutrition support was not effective in preventing hyperglycemia in patients with poorly controlled T2D. Indeed, endogenous glucose production in these patients was substantially higher than in patients with well-controlled T2D. This suggests that the level of preoperative glycemic control directly influences the patients' postoperative glucose kinetics and plasma glucose concentration, which highlights the importance of establishing good preoperative glycemic control. Moreover, patients with undiagnosed T2D should be identified to allow preoperative interventions, which may alleviate hyperglycemia and improve postoperative clinical outcomes [271].

HbA1c, a marker of long term hyperglycemia, has been shown to predict insulin sensitivity as assessed by the clamp during cardiac surgery [272]. Our study agrees with the previous study and found that preoperative HbA1c was positively correlated with postoperative glucose concentration and its endogenous production. Moreover, HbA1c greater than 6-6.5% has been associated with increased postoperative adverse events such as infections and longer hospital stays after various surgical procedures [272-277]; however, systemic reviews have failed to show a definite association due to the heterogeneity of studies and small sample sizes [278,279]. In contrast, HOMA-IR did not show a difference amongst groups, which is not surprising since it was based on a single blood sample that reflects momentary measure and may be complicated by

the pulsatile nature of insulin release [280]. Moreover, HbA1c was also positively correlated with preoperative BCAA concentration, which is mainly due to the impairment of the BCAA catabolic pathway caused by insulin resistance [281,282]. Collectively, while this study was not powered for clinical outcomes and more large scale studies are needed, it does provide evidence to support the importance of long term glycemic control in mediating postoperative glucose kinetics and glycemic responses.

Regardless of the level of glycemic control, nutritional intervention with perioperative AAs alone ameliorated protein metabolism in patients with T2D, to the extent that was comparable to patients without diabetes. This benefit was demonstrated by elevated whole body leucine balance, plasma protein synthesis rates and plasma essential and branched-chain amino acid concentrations in this study. In addition, leucine balance was considerably higher than in a previous study in which patients with T2D were fasted [152]. In fact, the 20 μmol/(kg·h) difference in leucine balance could correspond to a sparing of 55g protein per day from lean body mass breakdown in a 70 kg person, assuming an average of 8% leucine in whole body protein [283]. This is particularly important in a surgical patient with metabolic stress due to the potential functional significance related to wound healing and muscle strength recovery [284-286].

Nutrition guidelines for surgical patients recommend protein intakes in the range of 1.5-2.5 g/(kg·d) [285,287]. In this study, AAs infused without glucose at a moderate dose of 20% REE (approximately 1g/(kg·d) AAs) did not result in a positive leucine balance even when perioperative fasting was avoided. A previous study with an infusion of parenteral AAs at 2.9 g/(kg·d) without glucose postoperatively for a short term of 3 hours resulted in a positive leucine balance of 30 ± 8 μmol/(kg·h) [164]. This higher leucine balance was achieved primarily by a reduction in whole body protein breakdown. Provision of AAs at a higher dose than in the current

study would likely spare more protein from catabolism and provide more substrates for synthesis; besides, the short-term bolus effect in our previous study may induce insulin secretion, thus increase protein anabolism via initiating mRNA translation through mTOR signaling pathway [288-290].

The protein kinetic response was similar in patients with and without diabetes in this study. Although leucine balance was higher than during the preoperative fasting study, it remained negative. Leucine oxidation was substantially increased, and endogenous glucose production was unchanged compared to preoperative fasting. This contrasts with our previous study of a similar design in which AAs were infused at 20% REE with glucose [163]. Neutral leucine balance was achieved with a more moderate increase in leucine oxidation and a decrease in endogenous glucose production compared to the current study. Thus, AAs were not used as efficiently as expected to contribute to protein anabolism. The same AA solution used in both studies (Travasol®10%, Baxter, Canada) is high in glycine, alanine and arginine (43% of total AAs). These glucogenic AAs are important substrates for gluconeogenesis and major contributors to endogenous glucose production, especially since the length of fasting before surgery allows one to assume depletion of glycogen. Perioperative glucose via either intravenous glucose or oral carbohydrate had been shown to attenuate postoperative endogenous glucose production [152,153,291]. Therefore, a comparison with our previous study [163] reveals that the inclusion of glucose with AAs in perioperative nutrition support is an important strategy to achieve net protein anabolism.

AA infusion at the current rate increased total protein and fibrinogen syntheses compared to before surgery, although albumin synthesis did not change, and albumin concentration significantly decreased. Interestingly, our previous study with combined AA and glucose facilitated a doubling of albumin fractional synthesis (to 38 ± 11 %/d) after surgery, which

maintained albumin concentration [163]. As mentioned above, glucose infusion might have decreased hepatic gluconeogenesis to spare AAs for albumin synthesis. Hypoalbuminemia is associated with increased postoperative adverse events such as respiratory failure, surgical site infection, and mortality [255,292-294]. The cause might be multifactorial, presumably through increased capillary permeability and shortened half-life from accelerated degradation from surgical trauma and underlying inflammation [295]. Therefore, we speculate that, despite the infusion of parenteral AAs, albumin synthesis rate was lower than its catabolic and redistribution rate (e.g. moving to the interstitial space) in patients under surgical stress.

4.6 Conclusion

The most novel part of the study was to reveal the distinct metabolic responses to perioperative AA infusion in patients with different levels of glycemic control before surgery. An individualized nutrition support regimen of parenteral amino acids alone at approximately 1 g/(kg·d) in patients with good glycemic control, but not in patients with poorly controlled T2D, prevents hyperglycemia while improving whole body leucine balance. However, leucine balance remained negative, suggesting that the inclusion of glucose for patients with good glycemic control may be important to stimulate a net anabolic response and to prevent postoperative hypoalbuminemia. In patients with poor preoperative glycemic control, omitting glucose from nutrition support regimen is effective in avoiding postoperative hyperglycemia. Taken together, this illustrates the importance of improving long term glycemic control in patients with T2D before surgery.

Figure 4.1 CONSORT diagram.

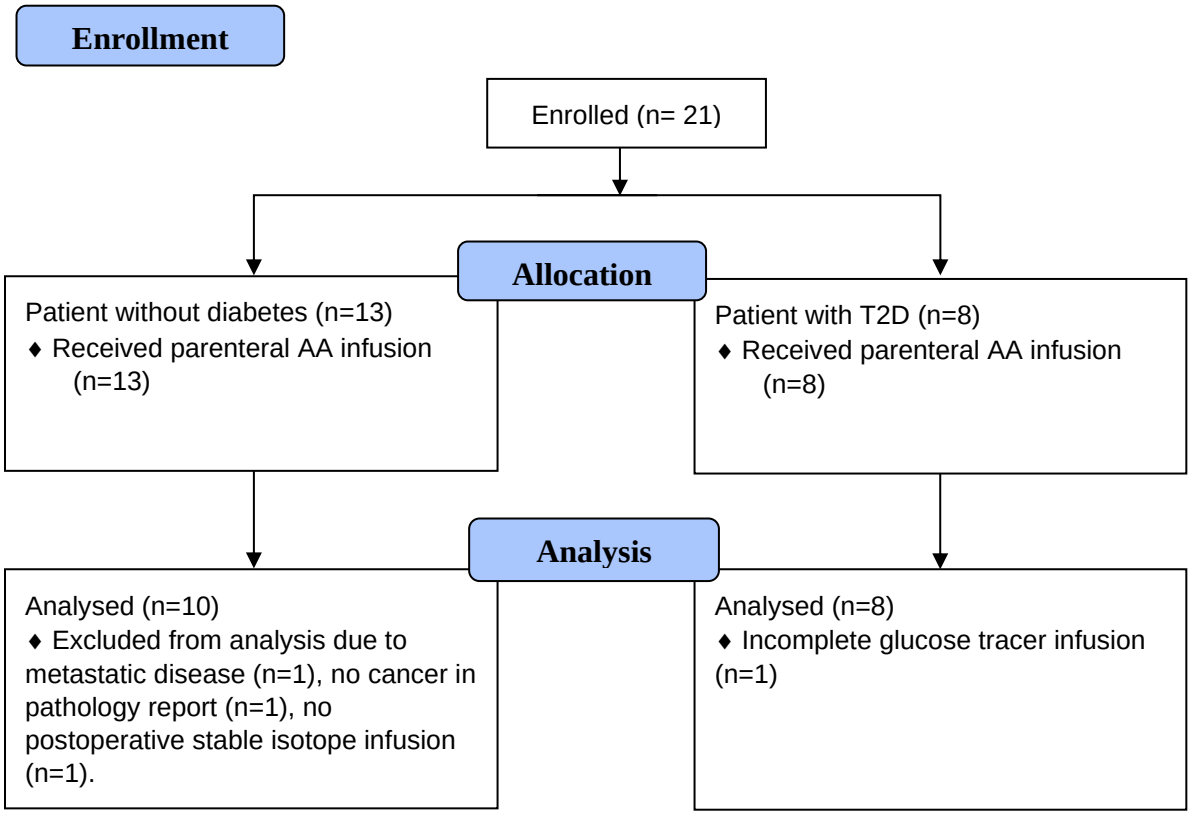


Figure 4.2 Study protocol.

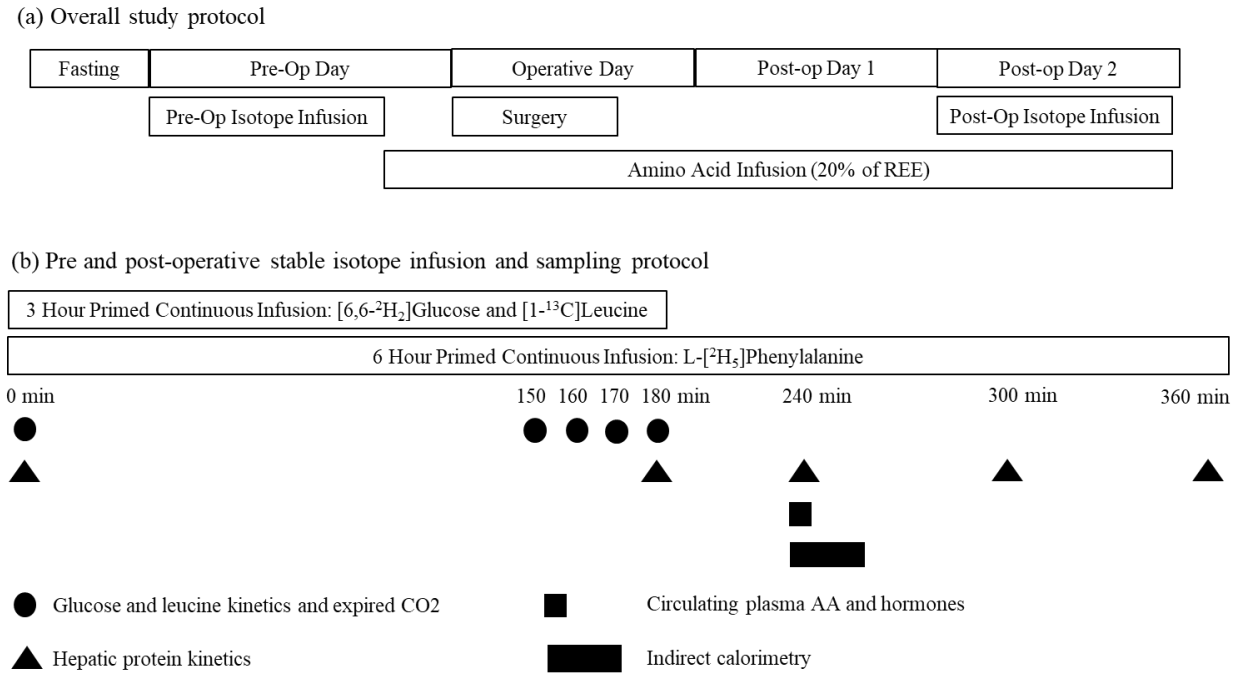


Table 4.1 Patient characteristics.

	Without diabetes n = 10	T2D n = 8
Age, yr	65 (16)	70 (11)
Sex, M/F	6/4	5/3
Weight, kg	74 (10)	75 (11)
BMI, kg/m^2	25 (2)	26 (4)
HbA1c, %	5.5 (0.3)	6.8 (1.2) *

Data are expressed as mean (SD). M, male; F, Female; BMI, body mass index; HbA1c, hemoglobin A1c.

* $p < 0.05$ by independent t-test or chi-square test.

Table 4.2 Resting energy expenditure and metabolic hormones.

	Without diabetes n = 10		T2D n = 8		*P* value		
	Pre	Post	Pre	Post	Group	Time	Interaction
VCO_2, mL/min	167 (24)	151 (27)	187 (40)	177 (23)	***0.0398***	0.2419	0.7565
VO_2, mL/min	205 (20)	200 (42)	225 (51)	239 (42)	0.0575	0.7853	0.5410
RQ	0.81 (0.07)	0.76 (0.06)	0.83 (0.06)	0.76 (0.09)	0.8437	***0.0268***	0.6261
REE, kcal/d	1327 (217)	1360 (273)	1558 (350)	1695 (439)	***0.0191***	0.4653	0.6548
kcal/(kg·d)	18 (3)	19 (4)	21 (3)	23 (4)	***0.0152***	0.3647	0.6566
Insulin, pmol/L	41 (30)	29 (19)	30 (13)	26 (13)	0.3129	0.2675	0.5664
HOMA-IR	1.52 (1.40)	1.02 (0.81)	1.28 (0.67)	1.14 (0.66)	0.8613	0.3511	0.5936
Cortisol, nmol/L	224 (59)	339 (233)	243 (68)	379 (157)	0.5806	***0.0255***	0.8459

Data are expressed as mean (SD). REE, resting energy expenditure; RQ, respiratory quotient. HOMA-IR, homeostatic model assessment-insulin resistance. Statistical analysis: repeated measures ANOVA.

Table 4.3 Perioperative whole body glucose kinetics.

	Without diabetes n = 10		T2D n = 7		*P* value		
	Pre	Post	Pre	Post	Group	Time	Interaction
Glucose concentration, mmol/L	5.4 (0.9)	5.2 (0.9)	6.8 (1.1)	7.8 (3.4)	***0.0054***	0.4551	0.3383
Endogenous glucose Ra, µmol/(kg·min)	11.7 (1.9)	11.3 (2.3)	13.4 (3.1)	14.8 (5.4)	***0.0387***	0.7131	0.5161
Glucose clearance ml/(kg·min)	2.2 (0.4)	2.2 (0.6)	2.0 (0.3)	2.0 (0.3)	0.1269	0.9584	0.8959

Data are expressed as mean (SD). *p* value shows the main effect of time (pre vs. post), group and time × group interaction by repeated measures ANOVA.

Table 4.4 Perioperative glucose kinetics in patients with T2D stratified according to glucose control.

	Without diabetes n = 10		Well controlled T2D n = 4		Poorly controlled T2D n = 3		*P* value		
	Pre	Post	Pre	Post	Pre	Post	Group	Time	Interaction
Glucose concentration, mmol/L	5.4 (0.9)	5.2 (0.9) ^{a}	6.2 (1.2)	5.5 (1.8) ^{a}	7.5 (0.4)	11.2 (2.8) ^{b*}	***<0.0001***	0.1022	***0.0157***
Endogenous glucose Ra, µmol/(kg·min)	11.7 (1.9)	11.3 (2.3) ^{a}	13.2 (4.2)	11.8 (3.2) ^{ab}	13.9 (1.5)	18.8 (5.1) ^{b}	***0.0108***	0.4178	0.1637
Glucose clearance ml/(kg·min)	2.2 (0.4)	2.2 (0.6)	2.1 (0.3)	2.2 (0.3)	1.9 (0.1)	1.7 (0.1)	0.1061	0.8741	0.8528

Data are expressed as mean (SD). *p* value shows the main effect of time (pre vs. post), group and time × group interaction by repeated measures ANOVA; means with different letter superscripts show difference between groups ($p < 0.05$) and with * show difference within group ($p < 0.05$) after adjusted with Bonferroni post-hoc analysis.

Figure 4.3 Correlations of HbA1c with glucose and amino acids
(A) postoperative endogenous glucose Ra, (B) postoperative glucose concentrations, and (C) preoperative BCAA concentrations.

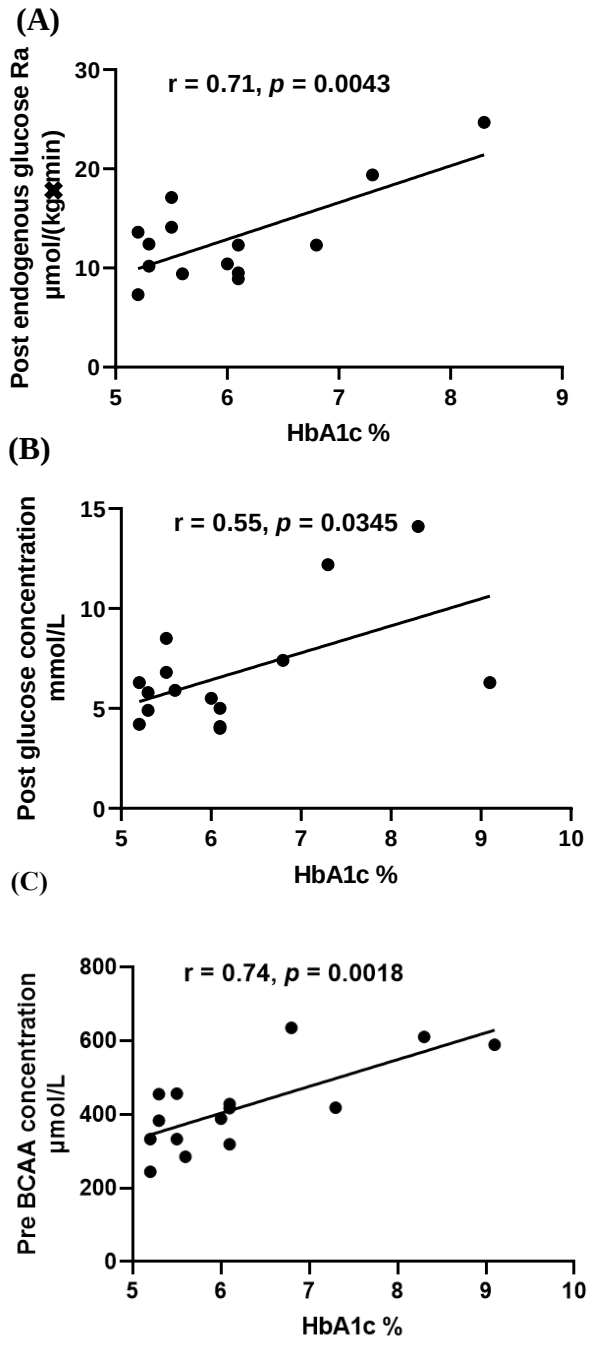


Table 4.5 Perioperative whole body leucine kinetics.

	Without diabetes n = 10		T2D n = 8		*P* value		
	Pre	Post	Pre	Post	Group	Time	Interaction
Leucine Ra, µmol/(kg·h)	99.3 (17)	123.3 (13.8)	110.5 (20.6)	122.7 (24.9)	0.4419	***0.0121***	0.3920
Leucine oxidation, µmol/(kg·h)	12.7 (4.0)	25.1 (8.4)	18.2 (6.8)	28.3 (4.4)	0.0580	***<0.0001***	0.5884
Protein synthesis, µmol/(kg·h)	86.6 (18.0)	98.2 (12.7)	92.3 (14.7)	94.4 (22.6)	0.8631	0.2735	0.4489
Protein breakdown, µmol/(kg·h)	99.3 (17.0)	105.9 (13.0)	110.5 (20.6)	103.7 (23.0)	0.4979	0.9860	0.3143
Leucine balance, µmol/(kg·h)	-12.7 (4.0)	-7.7 (7.6)	-18.2 (6.8)	-9.3 (5.0)	0.1080	***0.0027***	0.3563

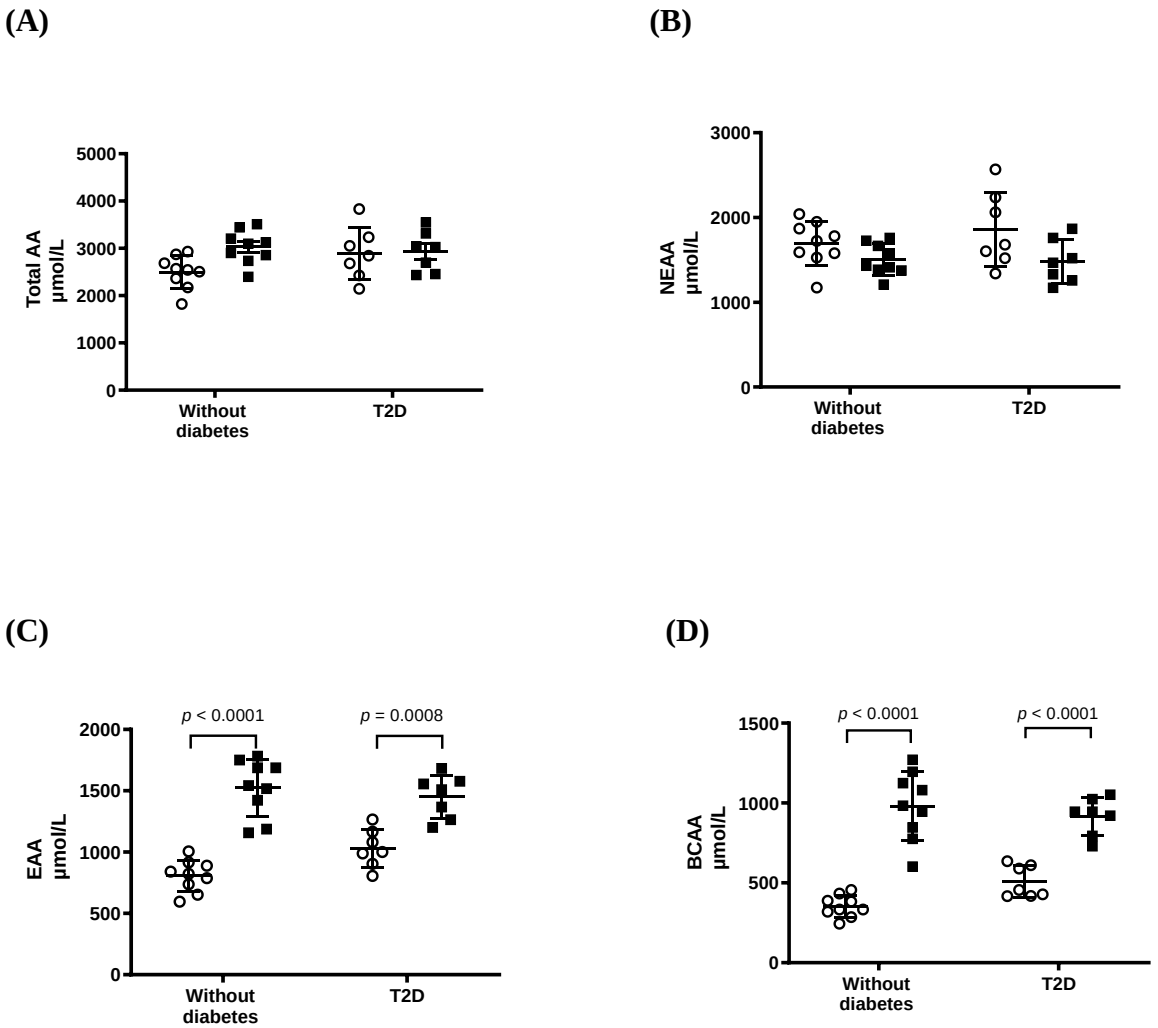
Data are expressed as mean (SD). *p* value shows the main effect of time (pre vs. post), group, and time × group interaction by repeated measure ANOVA.

Table 4.6 Perioperative plasma protein kinetics.

	Without diabetes n = 9		T2D n = 7		*P* value		
	Pre	Post	Pre	Post	Group	Time	Interaction
Albumin							
FSR, %/d	16 (4)	19 (10)	11 (4)	26 (23)	0.7981	0.0693	0.2300
ASR, mg/(kg·d)	188 (51)	171 (95)	141 (47)	247 (242)	0.7709	0.3722	0.2210
Concentration, g/L	36 (4)	26 (5)	38 (2)	27 (5)	0.3828	***<0.0001***	0.9670
Fibrinogen							
FSR, %/d	37 (10)	52 (14)	36 (16)	56 (18)	0.8516	***0.0043***	0.6968
ASR, mg/(kg·d)	38 (12)	95 (33)	36 (17)	92 (43)	0.8132	***<0.0001***	0.9357
Concentration, g/L	3 (0)	5 (1)	3 (0)	5 (1)	0.8346	***<0.0001***	0.8052
Total Protein							
FSR, %/d	22 (4)	39 (9)	18 (6)	36 (8)	0.1887	***<0.0001***	0.8521
ASR, mg/(kg·d)	417 (78)	625 (137)	354 (113)	581 (113)	0.2233	***<0.0001***	0.8267
Concentration, g/L	60 (6)	47 (7)	62 (4)	48 (5)	0.4595	***<0.0001***	0.8620

Data are expressed as mean (SD). *p* value shows the main effect of time (pre vs. post), group and time × group interaction by repeated measure ANOVA.

Figure 4.4 Perioperative plasma amino acid concentrations.



(A) Total AA, (B) NEAA, (C) EAA, and (D) BCAA. Data are expressed as mean (SD). EAA, essential amino acid. NEAA, non-essential amino acid. BCAA, branched-chain amino acid.

BRIDGE STATEMENT 2

The previous manuscript demonstrated that parenteral AA infusion individualized to 20% of REE prevented postoperative hyperglycemia in patients with good glycemic control, but not in patients with poorly controlled T2D as assessed by HbA1c > 6.5%. Protein balance was higher compared to preoperative fasting rates, but it failed to achieve a net anabolic response. Our previous study with a combined infusion of glucose and AAs showed a much higher protein balance [163] than in manuscript 2 with AA infusion alone. Therefore, we conclude that surgical patients with good glycemic control may benefit from concurrent glucose infusion to spare AAs from gluconeogenesis.

Various strategies have been shown to be effective in preventing surgical-induced hyperglycemia and improving clinical outcomes [271]. Manuscript 2 showed that preoperative HbA1c > 6.5% was associated with increased endogenous glucose production and hyperglycemia after surgery. Preoperative glycemic control strategies, including hypoglycemic agents, insulin and lifestyle interventions, reduce preoperative glucose and HbA1c [296] and decrease the risk of postoperative complications [297]. Intra- and postoperative strategies, including epidural blockade and intensive insulin therapy, have also been investigated in the past two decades. Epidural anesthesia and analgesia blunt catabolic hormone secretion and reduce insulin resistance induced by surgical stress [264]. Tight glucose control by intensive insulin therapy decreases adverse surgical outcomes [194].

Hyperinsulinemic-normoglycemic clamp administers high dose insulin while infusing glucose to maintain normoglycemia during and after surgery. High insulin limits endogenous glucose production, mainly from gluconeogenesis; it also reduces protein breakdown. This results in a reduction of plasma AA availability, especially when exogenous AA supply is limited [158].

Parenteral AA infusion is a strategy to prevent hypoalbuminemia induced by the clamp and to provide AAs for protein synthesis. AA infused at 20% REE in cardiac patients receiving the clamp resulted in higher plasma AAs (13 out of 20 AA) compared to patients without AA infusion; however, only 7 AAs reached their preoperative baseline levels [165]. Parenteral AA infused at the same level in study 2 showed increased essential and branched-chain AAs but not total or non-essential AAs. Protein balance in both studies remained negative after AA infusion at 20% REE. Therefore, we hypothesize that a parenteral AA intake higher than 20% REE may be needed to improve protein anabolism.

The following study aims to determine whether parenteral AA at 35% REE infused perioperatively, compared to AA at 20% REE, can prevent postoperative hypoaminoacidemia in the patient receiving hyperinsulinemic-normoglycemic clamp during and after coronary artery bypass surgery. Parenteral AA infusion was based on each patient's individualized REE and begun at the beginning of surgery until 6 hours after surgery while receiving hyperinsulinemic-normoglycemic clamp. Plasma AA concentrations were measured before and after the AA supplementation to compare the effect of these two AA infusion levels.

CHAPTER 5 – MANUSCRIPT 3

Parenteral amino acid supplementation with high dose insulin prevents hypoaminoacidemia during cardiac surgery

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5.1 Abstract

Objective Surgery triggers a stress response that produces insulin resistance and hyperglycemia. During cardiac surgery, administration of high dose insulin along with dextrose titration maintains normoglycemia, but dramatically decreases plasma amino acids (AAs) compared to pre-operative fasting levels. Hypoaminoacidemia limits protein synthesis and prevents anabolic responses after surgery. We investigated whether a parenteral infusion of AA during and immediately after cardiac surgery would prevent hypoaminoacidemia in patients receiving high dose insulin therapy.

Methods Sixteen patients undergoing coronary artery bypass grafting (CABG) surgery were randomly allocated to receive AAs with % kcal equivalent to either 20% (n=8) or 35% (n=8) of their measured resting energy expenditure (REE). Insulin was infused at a constant rate of 5 mU/(kg·min), while dextrose was titrated to maintain normoglycemia during and until 5 hours after surgery. Plasma AA concentrations were measured at baseline before and after surgery.

Results Compared to the 20% AA group after surgery, AA concentrations were significantly higher in the 35% AA group for 12 out of 20 AAs ($p < 0.032$), including all branched-chain AAs (BCAAs). In the 20% AA group, total essential AA (EAA) decreased by 21%, and non-essential AA (NEAA) decreased by 14% after surgery compared to pre-operative fasting levels. In contrast, giving 35% AA prevented this unfavorable decrease in AAs, and in fact "allowed" a 23% and 12% increase in EAA and NEAA, respectively.

Conclusion AA supplementation at 35% REE, but not 20% REE, can effectively prevent hypoaminoacidemia caused by high dose insulin therapy during cardiac surgery.

5.2 Introduction

Surgical stress is characterized by a hypermetabolic and catabolic response that results in increased energy expenditure, hyperglycemia, altered nutrition substrate use and acute-phase protein synthesis [24,200,298]. The mechanism of the stress response is mediated through the neuroendocrine and immune systems [1]. Activation of the central nervous system and the hypothalamic-pituitary-adrenal axis triggers the release of counterregulatory hormones such as epinephrine, glucocorticoids, growth hormone, and catecholamines [1,299,300]. Stress-induced insulin resistance increases glycogenolysis and gluconeogenesis, and decreases glucose and AA uptake, leading to hyperglycemia and muscle protein breakdown [13,300]. Maintenance of normoglycemia perioperatively is associated with a lower risk of infection, morbidity, mortality, and hospital length of stay [13,300,301].

Previous studies have shown that high dose insulin of 5 mU/(kg·min) along with dextrose titration can tightly control blood glucose in both diabetic and non-diabetic patients during cardiac surgery [301,302]. High dose insulin therapy inhibits glycogenolysis and gluconeogenesis [158] and decreases whole-body protein breakdown; however, it causes hypoaminoacidemia [158,160]. Plasma AA concentrations were 40-50% lower than preoperative fasting, and BCAAs were 60-70% lower than in patients who did not receive high dose insulin therapy [158,160]. Furthermore, parenteral AA supplementation in an amount equivalent to 20% of REE attenuated the decrease in circulating EAA and NEAA but did not achieve isoaminoacidemia.

The purpose of this study was to examine whether a parenteral infusion of AA during and immediately after cardiac surgery would prevent hypoaminoacidemia caused by high dose insulin therapy. We hypothesize that the delivery of AA in an amount equivalent to 35% of REE will achieve isoaminoacidemia compared to 20% of REE in cardiac surgical patients receiving high dose insulin therapy.

5.3 Materials and Methods

Study subjects

This study was approved by the Research Ethics Board of McGill University Health Centre and conducted at the Royal Victoria Hospital, Montreal, Canada. Adult patients scheduled for elective coronary artery bypass grafting (CABG) surgery requiring cardiopulmonary bypass (CPB) were approached, and informed consent was obtained prior to surgery. Patients who were severely malnourished (weight loss >20% in the preceding three months, body mass index (BMI) < 20 kg/m^2), severely obese (BMI >35 kg/ m^2), on dialysis, had chronic liver disease, severe left ventricular dysfunction, or active cancer were excluded. Study subjects and data analysis personnel were blinded to the dose of parenteral AAs.

Study protocol

Sixteen eligible patients were randomly allocated to receive parenteral AAs (Travasol® 10%, Baxter, Canada) infused at either 20% of measured REE (n=8) or 35% (n=8) (Figure 1). Both groups received AA infusion during and until 5 hours after surgery. High dose insulin with dextrose was administered to maintain euglycemia. Standard surgical and anesthetic care was provided per hospital guidelines. Heparin (400 U/kg) was administered to obtain an activated clotting time of >500 s before CPB. The ascending aorta and the right atrium were cannulated, the aorta was cross-clamped, and cardioplegia was administered. Once coronary anastomoses were sutured, and the aortic cross-clamp was removed, CPB was stopped. The patient was transferred to the Intensive Care Unit (ICU), and high dose insulin therapy and AA were continued for 5 more hours until the end of the study.

Indirect calorimetry

Compared to predictive equations to calculate energy requirements, indirect calorimetry is the gold standard to measure energy expenditure and is recommended for critically ill patients to

give an accurate and individualized measurement of REE and protein needs [303]. Indirect calorimetry (Quark RMR ICU, Cosmed, Italy) was conducted for a 20-min period the day before surgery and after surgery to determine REE, oxygen consumption (VO2), carbon dioxide production (VCO2) and respiratory quotient (RQ).

Individualized amino acid dose

Based on pre-operative REE measured from indirect calorimetry, an AA dose of 20% or 35% REE was calculated and administered at the beginning of surgery until 5hr after surgery. The composition of the AA solution from Travasol® 10% (g per 100g of total AAs) was as follows: isoleucine 6.0, leucine 7.3, valine 5.8, lysine 5.8, methionine 4.0, phenylalanine 5.6, tyrosine 0.4, threonine 4.2, tryptophan 1.8, histidine 4.8, arginine 11.5, glycine 10.3, alanine 20.7, proline 6.8, and serine 5.0.

High dose insulin therapy

In this study, both study groups received high dose insulin therapy. A bolus of 2U insulin (Humulin® R, Eli Lilly, IN) was infused, followed by a 5 mU/(kg·min) insulin infusion until 5 hours after surgery. Additional insulin boluses were given if the blood glucose remained > 6.0 mmol/L with incremental 2U of insulin for each 2 mmol/L increase in blood glucose. Blood glucose was tightly maintained between 4-6 mmol/L. Glucose (dextrose 20%) supplemented with potassium (40 mEq/L) and phosphate (30 mmol/L) was administered when the blood glucose was < 6.0 mmol/L starting 10 min after commencing the insulin infusion. Blood glucose was measured prior to the induction of anesthesia for baseline measurement and every 15-20 min during and until 5 hours after surgery using a point of care glucose monitor (Accu-chek®, Roche Diagnostics, Switzerland).

Plasma amino acids

Blood samples collected in EDTA tubes before and 4 hours after surgery were centrifuged at 4 ºC, and plasma was then isolated and stored at -80 ºC for later analysis. Each 100 µL plasma sample contained 100 pmol norvaline and sarcosine as internal standards and was deproteinated and then measured on Ultra High-Performance Liquid Chromatography (1290 UHPLC, Agilent Technologies, Santa Clara, USA) with automated pre-column derivatization by o-phthalaldehyde 3-mercatoproprionic acid (OPA-3MPA) and 9-fluorenylmethyl chloroformate (FMOC) for primary and secondary amino acids, respectively. One microliter of the derivatized sample was then injected onto a reverse-phase column (Agilent Poroshell 120 EC-C18 4.6 x 150 mm 2.7 µm) with a constant flow rate of 1.5 mL/min from two mobile phases (A: 10mM Na2HPO4, 10mM Na2B4O7, 5mM NaN3, pH 8.2; B: acetonitrile: methanol: water = 45:45:10). The initial gradient was 5% B for 0.5 min, then 5% to 57% B from time 0.5 to 20 min and 57% to 95% at 20.1 min. At 23 min the column was reconditioned to starting conditions for 2 minutes before the next injection. AAs were detected by fluorescence with emission and excitation wavelengths of 230 nm and 450 nm, respectively.

Statistical Analysis

Independent t-test was used to compare group differences (20% AA vs. 35% AA) in patient characteristics, and AA % change. Repeated measures ANOVA was used to assess within group (baseline vs. after surgery) and between group (20% AA vs. 35% AA) differences in all other measurements. Homogeneity and normality were tested using Levene's and Anderson-Darling tests, respectively. Post hoc pairwise comparisons were performed with Bonferroni correction. We expected a difference of 40% in BCAAs between 2 groups. A group size of 8 was chosen to detect

that expected difference with α of 0.05 and power of 80%. Analyses were performed with SAS 9.4.

5.4 Results

Patients

All 16 patients completed the study (Table 1). Patient and surgical characteristics were not different except BMI (p=0.01). High dose insulin given at 5 mU/(kg·min) maintained the blood glucose within 4-6 mmol/L range in both groups during the study period without any incidence of hypoglycemia (blood glucose < 3.5 mmol/L).

Respiratory exchange, REE and parenteral AA dose

There was no difference in pre-operative baseline REE between groups (Table 2). Whole body CO_2 and O_2 consumption, REE, and RQ increased in both groups post-operatively ($p < 0.003$). Parenteral AA doses of 20% and 35% REE were equivalent to 0.90 g/(kg·d) and 1.83 g/(kg·d) of AA, respectively. Based on the elimination of water with each peptide bond, these AA intakes were equivalent to protein intakes of 0.75 g/(kg·d) and 1.52 g/(kg·d).

Plasma AA concentrations

Plasma AA concentrations at pre-operative fasting were similar between the groups, except for citrulline and methionine. Compared to baseline, 6 out of 20 AAs (histidine, phenylalanine, glycine, alanine, arginine, and tyrosine) were significantly increased after surgery in the 35% AA group ($p < 0.03$), however, in the 20% AA group, AA concentrations were either decreased or remained the same after surgery. AA concentrations were significantly higher after surgery in the 35% AA group compared to 20% AA group ($p < 0.032$) for 12 out of 20 AAs including all BCAAs. Tyrosine was the only amino acid that decreased significantly after surgery in both groups (20% AA: 56 ± 14 μmol/L and 25 ± 4 μmol/L baseline vs. after surgery, $p = 0.003$; 35% AA: 72 ± 19 μmol/L and 37 ± 12 μmol/L baseline vs. after surgery, $p = 0.001$) (Table 3).

Plasma AA group % of change

The percent change (from baseline fasting to post surgery) in the concentration of each AA group was calculated (Figure 1). In the 20% AA group, EAA decreased by 21% and NEAA decreased by 14%; whereas in the 35% AA group, AA increased by 23% and 12% for EAA and NEAA, respectively. BCAAs decreased by 19% in the 20% AA group but increased by 33% in the 35% AA group. Similarly, but to a lesser extent, aromatic AAs (AAAs) and urea cycle AAs (UCAAs) decreased in the 20% AA group and increased in the 35% AA group from baseline to after surgery. For the individual AAs (Supplementary Figure 1), 13 out of 20 AAs had a positive % change in the 35% AA group; whereas only 6 out of 20 AAs changed positively in the 20% AA group. Lysine and citrulline decreased after surgery in both 20% and 35% group (lysine -23% and -15%, citrulline -24% and -31%). Notably the AA with the greatest decrease in both groups was tyrosine (-56% and -49%).

5.5 Discussion

High dose insulin along with dextrose titration maintains target blood glucose perioperatively, however it limits available amino acids and leads to decreased protein synthesis [158,160,302]. Our study demonstrated that high dose parenteral AA at 35% REE significantly increased plasma AA availability in cardiac surgery patients receiving high dose insulin therapy. AA given at a low dose of 20% REE failed to restore plasma AA back to baseline pre-operative fasting state levels.

Patients fasted overnight before surgery used more fatty acids as an energy source than later when AAs and glucose were given during and after surgery, as shown by the increase in RQ and VCO_2 after surgery, especially in the 35% AA group. REE increased after surgery in both groups by 34-39% due to the hypermetabolic state from surgical stress [304].

Supplying AAs at 20% and 35% of each patient's measured REE was equivalent to AA intakes of 0.9 g/(kg·d) and 1.8 g/(kg·d), respectively. Each AA loses 18 mass units when forming a peptide bond. Therefore, converting intake of free amino acids to an equivalent intake of protein requires consideration of both the AA profile and the 'hydrated' nature of free AAs in parenteral amino acid solutions [174]. Travasol 10% used in this study provided effective protein intake of 0.75 g/(kg·d) and 1.52 g/(kg·d) for the 20% and 35% groups, respectively. The AA intake of the 20% group is similar to the recommended protein intake of 0.8 g/(kg·d) for healthy adults. The AA intake of the 35% group is toward the lower end of the range of the intake recommended for critically ill patients, 1.2-2.0 g/(kg·d) or 2.0-2.5 g/(kg·d) [147,167,176,285,305,306]. However, the higher intake is rarely achieved in critically ill patients in the immediately perioperative period.

Insulin action is dose-responsive to not only glucose disposal, but to AA availability as well [307]. Previous studies showed that supraphysiological levels of insulin were associated with a decrease in plasma AAs by 30-60% and BCAAs by 70% [158,160]. However, with a 20% AA supplementation, compared to the control with no AA, high dose insulin decreased the availability of plasma AA to a much lesser extent, but was still not sufficient to restore AAs back to baseline fasting levels [165]. In this study, a high dose parenteral AA at 35% REE attenuated hypoaminoacidemia and dramatically increased plasma concentrations of 12 out of 20 AAs with an average increase of 14%, and 5 out of 20 AAs were significantly higher than their pre-operative levels. BCAAs are the most sensitive amino acids to high dose insulin [281,304], and since leucine has a major role in triggering the mTOR signalling pathway and increasing protein synthesis, it is essential to ensure that BCAA levels are well maintained [281,308]. The 20% AA group showed a negative change after surgery in all three BCAAs; whereas 35% AA dose was associated with significantly increased concentrations for all BCAAs from their pre-operative state by 33%.

To our knowledge this is the first time an AA dose of 35% REE was given to cardiac patients under high dose insulin therapy. Similar studies have been done with healthy individuals. Chevalier *et al*. conducted a study in 10 healthy young men, and an isoaminoacidemic clamp was used in additional to hyperinsulinemic-normoglycemic clamp (HNC) [308]. An AA mixture of TrophAmine 10% (B. Braun Medical, Bethlehem, PA) was used with an average infusion dose of 1.02 g AA/(kg·d) required to maintain plasma BCAAs at their baseline levels. This dose is very close to our 20% AA group with 0.9 g/(kg·d) AA infused. Their results showed that by maintaining constant BCAA levels under HNC, whole body protein breakdown was attenuated and protein synthesis was increased, resulting in improved protein balance [308]. They showed an average decrease of AA by 3.8% and increase of BCAA by 5.87%, which were much higher than in our 20% AA group [308]. This is likely due to the following reasons: first, patients in our study underwent cardiac surgery with more metabolic stress, insulin resistance and higher demand on acute phase amino acids [1]. Second, our patients were given a much higher dose of insulin, which likely decreased protein breakdown and thereby AA availability of AAs to a greater extent. Third, the type of AA solution (TrophAmine 10%) contains much more EAAs and BCAAs proportionally than the Travasol 10% used in our study. In another study reported by Luzi *et al.*, healthy young and middle-aged subjects were infused an equivalent of 1.58 g/(kg·d) AA from Travasol 10% along with an insulin clamp. They also found a significant increase in leucine disposal and oxidation compared to baseline fasting state [309]. Studies using stable isotopes to measure protein synthesis and breakdown will be beneficial to determine the optimal AA dose for better protein balance in surgical and critical care patients.

Total aromatic AA concentrations increased postoperatively only in the 35% AA group after surgery, mainly due to increases in phenylalanine and tryptophan. Tyrosine concentration,

however, dramatically decreased after surgery in both groups. Tyrosine can be synthesized endogenously by hepatic hydroxylation of phenylalanine. In this study, even though phenylalanine significantly increased in the 35% AA group (from 77 ± 16 to 126 ± 31 μmol/L after surgery), systemic tyrosine availability was still 50% lower than before surgery. This might be due to three possible reasons. First, Travasol, like most parenteral AA formulations, contains an extremely low amount of tyrosine because of its low solubility [310]. Second, high dose insulin administration suppresses the availability of tyrosine by decreasing protein breakdown, which removes the endogenous supply. Third, phenylalanine converted to tyrosine in hepatocytes might be shunted to make other proteins within the liver and thus not have an impact on plasma tyrosine levels during this stressed state.

Urea cycle AAs showed a similar pattern with a slightly positive change in the 35% AA group compared to negative change in the 20% AA group. Arginine is considered a conditionally essential amino acid in a surgical stress state because it is involved in immune response, nitric oxide (NO) production, collagen formation and wound healing [311-314]. Synthesis of NO from arginine has been shown to support collagen synthesis in fibroblasts [315]. Arginine can also be metabolized via ornithine into proline, which is a major component of collagen structure [316]. On the other hand, glutamine can also be converted to proline via pyrroline-5-carboxylate [317], which may contribute to collagen synthesis as well. Studies have shown effects of large dose arginine and/or glutamine supplementation on collagen synthesis and wound healing [318-321]. The AA solution used in this study is high in arginine (11.5% by mass, 100-210 mg/(kg·d)), which resulted in significant increase in arginine concentration in 35% group but not in 20% group (46% increase in 35% group vs. 6% in 20% group). Citrulline level slightly decreased after surgery in both groups. As a precursor for arginine synthesis, decreased citrulline might have a delayed

impact on arginine concentration post-operatively, which may eventually decrease NO production and potentially increase the risk of post-operative pulmonary hypertension [322-324].

Glutamine is the most abundant amino acid in plasma and a conditionally essential amino acids, especially in catabolic stress state. It's a major fuel for immune and oxidative function and cell proliferation in the intestine, muscle, lymphocytes and fibroblasts [325]. When supplemented in large doses, it has been shown to facilitate glutathione and antioxidant activity, reduce infections, improve arginine synthesis and wound repair, etc. in certain type of surgical and critically ill patients [167,325-329]; however, a large RCT (REDOXS study) in critically ill patients with multiorgan failure did not find any improvement in ICU length of stay or mortality [330]. Glutamine is not stable and is easily degraded into glutamic acid in aqueous solution [331]. In this case, glutamine is not included in parenteral nutrition solutions, or is included as its dipeptide format such as alanyl-glutamine or glycyl-glutamine [327]. The AA solution used in this study (Travasol® 10%) does not contain any glutamine/glutamate, therefore glutamine/glutamate had to be sourced from protein breakdown or derived from other amino acids. This explains why glutamine/glutamate concentrations were not significantly different between groups nor after surgery. Future studies should consider using AA solutions with a source of glutamine to ensure sufficient supply in metabolic stress.

Lysine is an indispensable amino acid in a strict metabolic with no involvement in transamination reactions [332]. In this study, lysine intakes of 52 mg/(kg·d) in the 20% REE group, and 104 mg/(kg·d) in the 35% group, were higher than the recommended intake for healthy adults (12-45 mg/(kg·d)) [332]. However, of all the essential AAs, plasma lysine decreased from pre-operative fasting levels in both groups. This suggests that under the conditions of surgical stress

and high dose insulin infusion, the requirement for lysine may be higher than the intake delivered in the 35% group.

We acknowledge some limitations in this study. A control group of patients was not included because our previous study with no AA supplementation established the dramatically lower AA concentrations induced by the high dose insulin, and clinical characteristics of patients were similar [160]. Due to the small sample size, clinical outcomes were not investigated. It would be important for a future trial with a large sample size to examine the impact of perioperative AA infusion on relevant clinical outcomes such as ICU and hospital length of stay, morbidity and mortality. We also recognize the need to study nitrogen and protein balance in the perioperative period in patients receiving AA supplements with or without high dose insulin therapy.

5.6 Conclusion

This study demonstrated that parenteral amino acids given at 35% REE significantly increased plasma AA availability in patients undergoing cardiac surgery with high dose insulin therapy. 20% AA was not sufficient to restore AA levels back to their preoperative state. We identified several amino acids that did not show an increase with the 35% AA dose, such as tyrosine, lysine, and citrulline. These amino acids may be the limiting factor for improving protein synthesis. Further studies on surgical and critically ill patients are needed to determine the best AA profile and dose to improve protein balance and, ultimately, improve clinical outcomes.

Table 5.1 Patient and surgical characteristics

	20% AA	35% AA
n	8	8
Age, yr	59 ± 9	61 ± 11
Sex (M/F)	8/0	8/0
Weight, kg	88 ± 12	79 ± 6
BMI, kg/m^2	28 ± 2	26 ± 1*
Diabetes, *n*	3	1
HbA1c, %	6.4 ± 1.1	5.9 ± 0.5
β-blockers, *n*	5	6
LVEF, %	52 ± 13	53 ± 9
Anesthesia time, min	373 ± 48	356 ± 27
Surgical time, min	281 ± 36	273 ± 23
CPB time, min	137 ± 22	132 ± 24
X-clamp time, min	117 ± 21	115 ± 19
Grafts, *n*	5.1 ± 0.9	4.9 ± 0.9
PRBC transfusions, units	2.1 ± 1.6	2.2 ± 1.6
Inotropic and vasopressor therapy		
Dobutamine (2.5-5.0 μg/(kg·min), *n*	1	2
Norepinephrine (1-10 μg/min), *n*	7	6

Mean ± SD or number of patients (n). M, male; F, Female; BMI, body mass index; HbA1c, hemoglobin A1c; LVEF, left ventricular ejection fraction; CPB, cardiopulmonary bypass; PRBC, packed red blood cells. * $p < 0.05$ 20% AA vs. 35% AA by independent *t*-test or Chi-square test.

Table 5.2 Respiratory exchange, REE and parenteral AA dose

	20% AA		35% AA	
	Baseline	**After Surgery**	**Baseline**	**After Surgery**
VCO_2 mL/min[a]	188 ± 41	293 ± 82	181 ± 30	272 ± 62
VO_2 mL/min[a]	224 ± 62	312 ± 88	243 ± 50	316 ± 69
RQ[a, b]	0.86 ± 0.12	0.94 ± 0.06	0.75 ± 0.07	0.86 ± 0.09
REE kcal/d[a]	1554 ± 407	2165 ± 624	1648 ± 321	2205 ± 479
kcal/(kg·d)[a]	18 ± 5	24 ± 6	21 ± 4	28 ± 7
AA ml/h	32 ± 8		60 ± 12	
AA equivalent g/(kg·d)	0.90 ± 0.26		1.83 ± 0.35	
Protein equivalent g/(kg·d)	0.75 ± 0.21		1.52 ± 0.29	

Mean ± SD. REE, resting energy expenditure; RQ, respiratory quotient.

[a.] Main effect of time (before surgery vs. after surgery), $p < 0.003$

[b.] Main effect of treatment (20% AA vs. 35% AA), $p < 0.004$

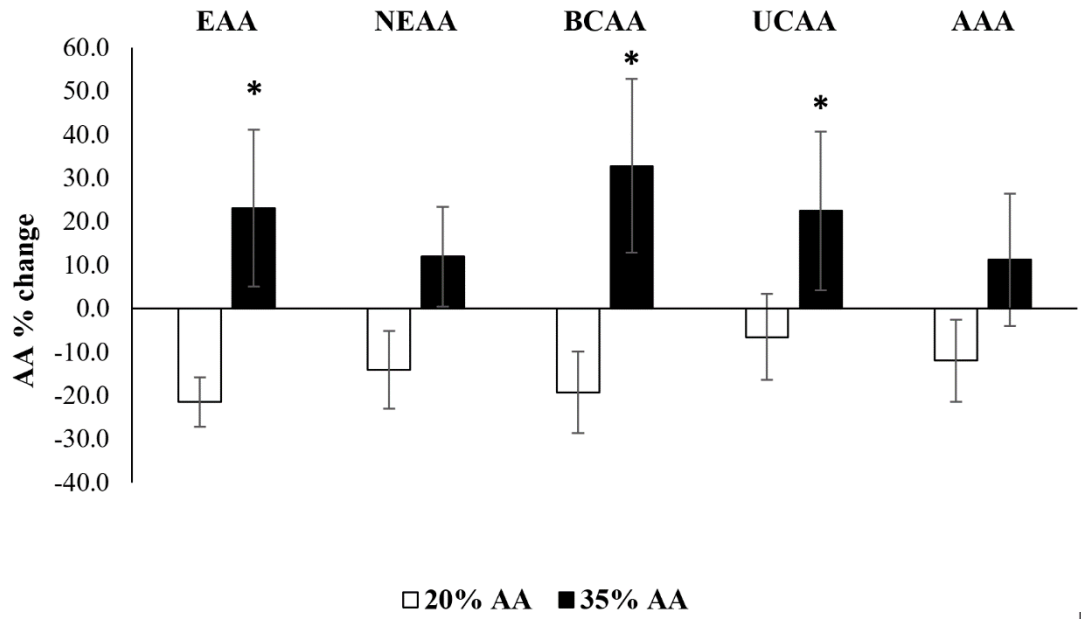
[c.] Treatment x time interaction, $p < 0.05$

Table 5.3 Plasma AA concentration

	20% AA		35% AA		P Value			
µmol/L	**Baseline**	**After Surgery**	**Baseline**	**After Surgery**	**Between groups at baseline**	**Between groups after surgery**	**20% AA baseline vs. after surgery**	**35% AA baseline vs. after surgery**
	Essential AA concentration µmol/L							
Histidine	74 ± 12	85 ± 21	67 ± 18	97 ± 24	-	-	-	0.026
Threonine	128 ± 41	95 ± 20	168 ± 45	164 ± 54	-	0.031	-	-
Methionine	18 ± 7	18 ± 8	36 ± 20	55 ± 24	0.016	<0.001	-	-
Tryptophan	47 ± 8	41 ± 6	66 ± 19	65 ± 16	-	0.005	-	-
Phenylalanine	68 ± 16	77 ± 21	77 ± 16	126 ± 31	-	0.001	-	0.001
Isoleucine	87 ± 17	60 ± 22	87 ± 20	133 ± 51	-	<0.001		
Leucine	164 ± 29	106 ± 24	174 ± 29	189 ± 67	-	0.004	-	-
Valine	278 ± 48	238 ± 47	262 ± 40	351 ± 106	-	0.016	-	-
Lysine	179 ± 29	137 ± 17	222 ± 48	188 ± 42	-	-	-	-
	Non-essential AA concentration µmol/L							
Glutamate	78 ± 29	73 ± 15	71 ± 23	85 ± 36	-	-	-	-
Glutamine	616 ± 85	435 ± 95	780 ± 116	609 ± 179	-	-	-	-
Proline	264 ± 82	206 ± 44	243 ± 53	279 ± 61	-	-	-	-
Asparagine	40 ± 7	12 ± 3	57 ± 21	14 ± 10	-	-	-	-
Serine	98 ± 16	76 ± 18	137 ± 23	144 ± 56	-	<0.001	-	-
Glycine	193 ± 51	240 ± 41	259 ± 63	412 ± 160	-	0.002	-	0.012
Alanine	375 ± 95	380 ± 103	376 ± 100	622 ± 163	-	0.002	-	0.001
Citrulline	24 ± 13	18 ± 5	47 ± 20	32 ± 13	0.017	-	-	-
Arginine	73 ± 20	77 ± 22	110 ± 19	161 ± 42	-	<0.001	-	0.006
Ornithine	75 ± 18	57 ± 10	91 ± 23	91 ± 20	-	0.011	-	-
Tyrosine	56 ± 14	25 ± 4	72 ± 19	37 ± 12	-	-	0.003	0.001

Mean ± SD.

Figure 5.1 Plasma AA groups % of change from baseline to after surgery



Mean ± SEM. EAA, essential amino acid; NEAA, non-essential amino acid; BCAA, branched-chain amino acid; UCAA, urea cycle amino acid; AAA, aromatic amino acid.

* $p < 0.05$ 20% AA vs. 35% AA by independent t-test.

BRIDGE STATEMENT 3

Manuscript 3 demonstrated that parenteral AA infused at 35% REE prevented postoperative hypoaminoacidemia induced by the hyperinsulinemic-normoglycemic clamp in patients undergoing CABG. This AA intake, approximating 1.8 g/(kg·d), increased plasma concentrations of 12 out of 20 AAs, including all branched-chain AAs and most of the essential AAs, to above preoperative fasting levels. By contrast, AA infusion at 20% REE, approximating 0.9 g/(kg·d), failed to restore most of the AAs to their preoperative levels.

High AA supplementation at 1.8 g/(kg·d) may be more effective for increasing plasma AA concentrations and protein balance (assessed from leucine kinetics). Manuscript 2 showed that an infusion of AA at 1.0 g/(kg·d) increased protein balance from preoperative fasting levels but remained negative. Parenteral AA infused at 2.8 g/(kg·d), however, resulted in a positive protein balance in critically ill adolescents receiving hyperinsulinemic-normoglycemic clamp [166]. Since the current nutritional guidelines and field experts recommend a protein intake of 1.2 to 2.5 g/(kg·d) for critically ill patients [167,176], protein intake at the higher end of the recommendation should be attempted to elevate protein balance.

When AAs are the sole exogenous nutritional substrates, as in the previous manuscripts, they may partially contribute to endogenous glucose production. Moreover, as shown in manuscript 3, plasma glutamate and tyrosine concentration remained low after AA infusion. This was due to the limited amount and low solubility in the parenteral AA solution. Concomitant enteral nutrition supplies all macronutrients while avoiding limiting AAs from parenteral AA solution, which may be more effective to foster protein anabolism.

The following chapter aims to determine whether parenteral AA supplementation could improve protein balance in critically ill patients. As mentioned in the literature review (section

2.5.1), full enteral feeding should be achieved during the “flow” phase (> day 3 after ICU admission) when patients are hemodynamically stable [167]. AA intake is calculated in addition to the protein in standard enteral nutrition to achieve a total protein intake of 1.75 or 2.5 g/(kg·d); these protein targets are designed to match the middle and upper range of recommended protein intake from clinical guidelines [167,171].

CHAPTER 6 – MANUSCRIPT 4

Parenteral amino acid supplementation increases leucine balance in critically ill patients

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*This clinical trial was approved by MUHC Centre for Applied Ethics Research Ethics Board (Study #15-155-MUHC) and Health Canada (NOA #HC6-24-c 193639), and the trial was registered at ClinicalTrials.gov (NCT02865408).

6.1 Abstract

Background. The stress response and nutritional restriction in critically ill patients lead to net protein catabolism. The objective of this study is to determine whether supplementing enteral nutrition support with parenteral amino acids (AAs) induces a net anabolic response assessed by leucine balance.

Method Twenty-six ICU patients receiving standard enteral nutrition (EN) support were randomized into one of three groups: the group with exclusive enteral nutrition (EEN) without parenteral AAs, two groups combined standard EN with parenteral AA supplementation to achieve target total protein intakes of 1.75 g/(kg·d) (moderate AA) and 2.5 g/(kg·d) (high AA). Protein and glucose kinetics were measured with stable isotope tracers before and 48 hours after beginning supplementation.

Results High AA supplementation led to higher leucine balance compared to moderate AA (p = 0.047) or EEN group (p = 0.036); moderate AA supplementation failed to increase leucine balance significantly. Consistent with increased whole body protein (leucine) balance, high dose AA administration significantly increased nitrogen balance, plasma albumin and total protein syntheses. The pre- to post-intervention change in leucine balance was negatively correlated with baseline clinical severity scores (*i.e.*, APACHE, NUTRIC).

Conclusion High-dose AA supplementation to achieve an actual total protein intake of 2.4 g/(kg·d) induces an anabolic response in critically ill patients, as indicated by increased leucine balance, nitrogen balance, and plasma protein synthesis.

6.2 Introduction

The stress response of critical illness—via the hypothalamus-pituitary axis and inflammatory pathways—stimulates the release of series hormones and immune mediators, such as catecholamines, cortisol and proinflammatory cytokines (e.g. TNF-α, IL-6) [1]. As part of the survival mechanism, these metabolic changes decrease anabolism and increase catabolism to prioritize substrates to support the stress response and the energy demand of vital tissues [1]. Stress-induced insulin resistance triggers peripheral protein breakdown to support the increased glucose demand via gluconeogenesis [109]. Metabolic stress also increases protein degradation through the ubiquitin-proteasome system and autophagy to recycle amino acids (AAs) for the use as energy sources or to synthesize protein required for the stress response [92]. By contrast, protein synthesis is downregulated to preserve energy and AA substrates through several nutrient-sensing pathways, including the general control nonderepressible 2 (GCN2) and mammalian target of rapamycin complex 1 (mTORC1) [333]. This increased protein degradation and decreased synthesis lead to negative net protein balance, resulting in muscle atrophy and functional impairment in prolonged critical illness [334].

Current practice guidelines recommend a protein intake range of 1.2-2.0 g/(kg·d) for critically ill patients [167,171]. Some experts recommend even higher protein intake of 2.0-2.5 g/(kg·d) [12,198]. However, in part due to significant heterogeneity in the metabolic response to critical illness, it has been challenging to investigate the impact of nutritional interventions in prospective randomized controlled trials (RCTs) in this patient population [204]. Some studies suggested an association between higher protein and decreased mortality and shorter time to discharge alive [335,336]; others found no change in the duration of renal dysfunction, mortality and length of stay [187]. A meta-analysis on 14 RCTs, with average high protein intakes at 1.02 g/(kg·d) vs. low intake at 0.67 g/(kg·d), showed that protein intake did not affect mortality [185].

Nonetheless, current clinical studies rarely randomized patients solely based on protein doses, and the protein doses assigned to the higher intake group were frequently at the lower bound of the current recommended intake range. The impact of high protein intake on clinical outcomes remains to be established in future large-scale RCTs [188].

As a first step, it is essential to understand the impact of high protein on surrogate markers of response to protein, including nitrogen balance (NB) and the kinetics of protein synthesis and breakdown. NB was higher with protein given at 1.2 compared to 0.8 g/(kg·d) [18], and NB equilibrium was achieved with a protein intake higher than 2.0 g/(kg·d) [177]. Parenteral AA supplementation to achieve a total protein intake of 2.0 g/(kg·d) was recently shown to increase protein balance by comparing to baseline measures of the same group of patients [184]. However, to reduce bias and increase the quality of evidence, a randomized controlled dosing trial on protein balance is especially pertinent; it may also determine how total protein intake higher than 2.0 g/(kg·d) affects protein metabolism compared to lower intake levels.

Achieving a high protein intake is challenging, which may contribute to the net protein catabolic state in critically ill patients [334]. The average actual protein intake levels—approximately 60% of the prescribed protein dose [337]—are far below the current recommended dose in critically ill patients. Common barriers include intolerance, frequently interrupted feeding, low protein to energy ratio in the commercial enteral nutrition product [167,175,337]. Post-pyloric feeding, prokinetic agents and supplemental protein powder are often used to improve intolerance and increase protein intake. Compared to protein powder given enterally as a bolus, parenteral AA infusion is less likely to be interrupted or skipped, and it can be infused at a constant rate to facilitate protein kinetics measurements via stable isotope tracers.

Therefore, an RCT was designed to investigate the anabolic effect of protein targeting the middle or upper range of the recommended intake via parenteral AAs supplementation. We hypothesized that supplementing EN with parenteral AAs to achieve a total protein intake of 2.5 g/(kg·d) would ameliorate protein anabolism measured by the primary outcome leucine balance, as well as plasma AA availability, nitrogen balance, and plasma protein synthesis.

6.3 Methods

Participants and study design

This study (ClinicalTrials.gov NCT02865408) was approved by the Research Ethics Board of the McGill University Health Centre and Health Canada (PI: A. Kristof) and performed in accordance with the Declaration of Helsinki. The study was performed in a single center at the MUHC-Glen Intensive Care Unit under the supervision of the ICU clinical research coordinator. Informed written consent was obtained from patients or their substitute decision-maker. Eligibility was assessed in mechanically ventilated adults with an expected ICU dependency of at least three days **(Figure 1)**. Exclusion criteria included: moribund state, already on PN, unable to tolerate EN, no central venous access, on extracorporeal membrane oxygenation or carbon dioxide removal, required renal replacement therapy, acute fulminant hepatitis, organ transplantation, or bronchopleural fistula.

Patients were randomized to one of three groups with one group receiving exclusive enteral nutrition (EEN), and two intervention groups with standard EN supplemented with parenteral AAs to reach a total protein intake of 1.75 (moderate AA) or 2.5 g/(kg·d) (high AA). The primary outcome was leucine balance. Secondary outcomes included nitrogen balance, glucose kinetics, plasma protein synthesis and plasma AA concentrations. Parenteral AA supplementation was initiated after conducting a baseline multiple stable isotope infusion studies to assess AA, protein

and glucose kinetics, as well as other metabolic substrates **(Figure 2.a)**. Post-intervention measurements of kinetics and metabolic substrates were completed after 48 hours of AA supplementation. Stable isotope tracers $NaH^{13}CO_3$ and L-[1-^{13}C]leucine were infused to measure leucine kinetics, [ring-$^{2}H_5$]phenylalanine for hepatic derived plasma protein kinetics, and D-[6,6-$^{2}H_2$]glucose for glucose kinetics.

Study protocol

Patient clinical scores were calculated at ICU admission, including Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) II to assess the severity of illness [66], Sequential Organ Failure Assessment (SOFA) to evaluate the rate of organ failure [67], and Nutrition Risk in Critically ill (NUTRIC) to quantify nutrition risk in ICU patients [68]. All patients initiated enteral feeding within 72 hours of admission, and the study was started after patients reached a full rate of enteral feeding, which was after the 72 hours of admission as per current ICU nutrition guidelines. After randomization, the ICU dietitian calculated each patient's enteral nutrition from estimated resting energy expenditure (REE) based on the current practice used in this ICU (Penn State Equation 2003b and 2011 [338,339]) with actual body weight or last known historical body weight. Calculated REE was then validated by indirect calorimetry (Quark RMR ICU, Cosmed, Rome, Italy) when clinically feasible. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured simultaneously to calculate respiratory quotient (RQ). Parenteral AA doses in the two intervention groups were calculated in addition to the protein from their standard enteral feeding to achieve total protein intake levels of 1.75 or 2.5 g/(kg·d). These regimens were not adjusted to be isoenergetic. AA composition of the parenteral AA solution (Prosol®20%, Baxter, Illinois) and enteral nutrition (Peptamen® 1.5 [340], Nestle, Switzerland) are

in **Suppl. Table 1**. If Propofol (a lipid-based sedative) was administered, adjustments to enteral feeding rates were made to account for the lipid-derived energy from propofol.

Stable isotope tracer (Cambridge Isotope Laboratories, MA) solutions were tested to be sterile (USP <71>) and free of endotoxins (USP <85>) and pyrogen (USP<151>). Primed continuous tracer infusions were conducted before the start of the AA supplementation and before the discontinuation of AAs at 48 hours **(Figure 2.b)**. During each 6-h infusion period, enteral and parenteral nutrition infusion rates were held constant. Primed continuous infusion of $NaH^{13}CO_3$ (priming: 1 μmol/kg, continuous 3 hrs: 1 μmol/(kg·h), *i.v.*) and L-[1-^{13}C]leucine (priming: 3.8 μmol/kg, continuous 3 hrs: 3.8 μmol/(kg·h), *i.v.*) were given to determine whole-body leucine kinetics; and L-[ring-$^{2}H_5$]phenylalanine (priming: 15 μmol/kg, continuous 6 hrs: 9 μmol/(kg·h), *i.v.*) to determine fractional and absolute synthesis rates of the hepatic-derived plasma proteins (total protein, albumin, and fibrinogen); and D-[6,6-$^{2}H_2$]glucose (priming: 18.7 μmol/kg, continuous 3 hrs: 13.2 μmol/(kg·h), *i.v.*) to determine glucose turnover.

Samples were taken at each time point according to the protocol **(Figure 2.a and 2.b)**. Expired air samples were taken at baseline and every 10 mins from 150-180 mins and 330-360 mins to determine $^{13}CO_2$ isotopic enrichment. Blood was sampled from the arterial line for both glucose and leucine kinetics at baseline and 330, 340, 350 and 360 mins, and for plasma protein synthesis from phenylalanine enrichment were taken at 0, 180, 240, 300 and 360 mins. Blood samples for metabolic substrates such as plasma AAs were taken at 360 mins. All blood samples were immediately transferred into Na_2EDTA vacutainers, except in lithium heparin vacutainer for total protein and albumin concentration, or sodium citrate vacutainer for fibrinogen concentration. All blood samples were centrifuged, and the plasma samples were stored at -80 ºC for later analysis.

Urine was collected for approximately 6 hours pre- or post-intervention with urine volume recorded, and samples were stored at -80 ºC to assess nitrogen balance.

Whole-body leucine kinetics

Dynamic changes in whole-body protein metabolism pre- and post-intervention were assessed using the leucine / α-ketoisocaproic acid (KIC) reciprocal pool model in which plasma [1-^{13}C]α-KIC enrichment is used to represent intracellular leucine enrichment [341]. Plasma KIC was derivatized to its pentafluorobenzyl ester derivative and extracted in hexane [163,342]. KIC enrichment was measured by Gas Chromatography Mass Spectrometry (GC-MS) (Agilent Technologies, Palo Alto, CA) by methane negative chemical ionization (NCI) and selected-ion monitoring at *m/z* 129 and 130. Whole-body leucine flux was calculated as (Q) = i [E_i/E_p -1], where i is the infusion rate of leucine tracer, E_i is leucine enrichment of the infusate, and E_p is the mean enrichment of plasma KIC at steady state.

Enrichment of breath $^{13}CO_2$ was used to measure leucine oxidation after assessment of each patient's individualized bicarbonate retention fraction using the model of Kien *et al.* [343]. CO_2 enrichment was analyzed by isotope ratio mass spectrometry (IRMS) (Analytical Precision, UK). Leucine oxidation was calculated as (Ox) = ($E_{leu\ CO2} \times i$)/($E_{bicarb\ CO2} \times E_p$), where $E_{leu\ CO2}$ is the CO_2 enrichment during L-[1-^{13}C]leucine infusion, i is the infusion rate of $NaH^{13}CO_3$ tracer, $E_{bicarb\ CO2}$ is the CO_2 enrichment during the 3-h $NaH^{13}CO_3$ infusion, and E_p is the mean enrichment of plasma KIC at steady state [343].

At isotopic steady state, the rate of inflow to the pool (through intake (I) and appearance from protein breakdown (B)) is balanced by the rate of outflow (through oxidation (Ox) plus non-oxidative leucine disappearance or incorporation into protein (S)), creating the equation: Q = I +

B = Ox + S. After measuring Q and Ox, and by knowing intake I, S and B were calculated. Whole-body leucine balance was calculated by subtracting breakdown (B) from synthesis (S).

Nitrogen balance

Timed 6-h urine collections were used to estimate 24-hour nitrogen balance as previously validated by Graves *et al.* when patients were fed with continuous parenteral or enteral nutrition without hepatic or renal failure [344]. Nitrogen excretion was calculated from nitrogen loss in the urine (from measured urea and creatinine, and estimated uric acid and ammonia) [345], and estimated nitrogen loss in fecal and miscellaneous source [346]. Nitrogen balance was calculated by subtracting nitrogen excretion from intake (enteral and parenteral). Patients with oliguria (urine output of < 0.5 ml/(kg·h), n = 4) were excluded.

Whole-body glucose kinetics

Plasma glucose was derivatized to its penta-acetate compound, and D-[6,6-$^{2}H_{2}$]glucose enrichment was determined by GC/MS using electron impact ionization and selected ion monitoring at *m/z* 200 to 202 [163]. Glucose rate of appearance was calculated as Ra = i [E_i/E_p -1] where i is the infusion rate of the labeled glucose, E_i is glucose enrichment in the infusate, and E_p is the mean enrichment of glucose at steady state. Endogenous glucose Ra was then calculated by subtracting intake from Ra. Since at steady state, the rate of appearance and disappearance (Rd) are equal, glucose clearance (glucose utilization corrected by glucose concentration) was calculated from Rd /glucose concentration.

Hepatic-derived plasma protein synthesis

All hepatic-derived plasma proteins were isolated to measure their individual synthesis rates [163]. Total plasma proteins were precipitated with trichloroacetic acid (TCA), vortexed, and centrifuged. Fibrinogen and albumin were isolated by using their selective solubility/insolubility

in ethanol, followed by further purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Plasma ApoB-100—the apolipoprotein of very-low-density lipoprotein that represents the phenylalanine precursor pool enrichment [163]—was isolated by ultracentrifugation at 100,000×g at 12 ºC for 20-h, followed by isopropanol precipitation. ApoB-100 had to be separated from apoB-48 from chylomicrons due to enteral feeding by SDS-PAGE.

Gel bands from fibrinogen, albumin and ApoB-100 were excised. Total protein pellet and the excised gel bands were hydrolyzed overnight in 6N HCl at 110 ºC. All released AAs were isolated using cation exchange chromatography (Dowex, Bio-Rad, CA) and esterified with 1-propanol. The enrichment of phenylalanine was then analyzed by triple quadrupole mass spectrometry (LC-MS/MS: UHPLC 1290, QQQ 6460, Agilent Technologies, Palo Alto, CA) by MRM monitoring ions at *m/z* 208 to 213. Plasma total protein, albumin and fibrinogen concentrations were measured by Synchron Clinical System (Beckman Coulter, CA) as per MUHC clinical lab protocol.

Fractional synthesis rate (FSR) of hepatic-derived plasma proteins (total protein, fibrinogen and albumin) were determined from the rate of increase in enrichment of protein-bound phenylalanine over the steady-state enrichment of phenylalanine in the precursor pool represented by ApoB-100 in VLDL. FSR (%/d) = $[(E_{t2}-E_{t1}) \times 24 \times 100]/[E_{free} \times (t_2-t_1)]$, where $E_{t2}-E_{t1}$ is the phenylalanine enrichment difference during the two steady-state time points t_2-t_1, E_{free} is the enrichment of the precursor pool. Absolute synthesis rate (ASR mg/(kg·d)) was calculated as FSR × conc. × PV, where conc. is the plasma concentration of the protein, and PV is the estimated plasma volume [347].

Plasma AA concentration

Plasma samples, along with internal standards norvaline and sarcosine, were filtered through a Captiva ND lipid extraction plate (Crawford Scientific Ltd., Scotland, UK) with methanol. Samples were measured on Ultra-High-Performance Liquid Chromatography (UHPLC 1290 with fluorescence detector, Agilent Technologies, Palo Alto, CA) after pre-column derivatization with ortho-phthalaldehyde (OPA) and fluorenylmethoxy chloroformate (FMOC) and calculated against standard curves.

Statistical analysis

The sample size was determined based on leucine balance from a previous study in surgical patients [163]. Ten patients per group were required with a power of 80% and the ability to detect a difference with a type I error of 5%. Patient characteristics were compared using independent *t*-test or chi-square test. Diet intake data were analyzed by one-way analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was conducted to determine the treatment effect on the kinetics of leucine, nitrogen, and glucose, and plasma AA concentrations, while controlling baseline measures of each outcome as well as other covariates (e.g. APACHE, corticosteroids, insulin) to eliminate bias. Tukey's post hoc test was used to adjust for multiple comparison errors. Homogeneity and normality were tested using Bayesian Criteria & Levene's test, and Shapiro-Wilk's test, respectively. Pearson correlation was used for continuous variables. $P < 0.05$ was considered statistically significant. All data were presented as mean ± SD. All statistical analyses were performed using SAS 9.4.

6.4 Results

In total, 26 patients completed the primary outcome measurements, with 8 patients from EEN group and 9 patients in each of the two AA intervention groups **(Figure 1)**. Patient characteristics such as age, sex, BMI, LOS and mortality did not differ amongst groups **(Table 1)**. Energy intakes from EN and Propofol averaged 21.50 kcal/(kg·d) and did not differ amongst groups **(Table 2)**. Similarly, actual protein intakes from EN averaged 0.83 g/(kg·d) and were not different amongst groups. Total actual protein intakes were 0.82 ± 0.21, 1.62 ± 0.07 and 2.41 ± 0.10 g/(kg·d) for the EEN, moderate AA, and high AA group, respectively, which were 93% and 96% of the targeted protein intake of the moderate and high AA groups, respectively. AA intake levels were converted to protein equivalent by correcting for the elimination of a water molecule when AAs form each peptide bond [174]; therefore, the corrected protein equivalent intakes were 0.82 ± 0.21, 1.48 ± 0.06, and 2.16 ± 0.09 g/(kg·d) for the EEN, moderate AA and high AA group, respectively. Eighteen patients completed the indirect calorimetry measurements, and no difference was found in VCO_2, VO_2, RQ, and REE amongst three groups **(Table 3)**.

The primary outcome, leucine balance, was higher in patients receiving high AA supplementation, compared to moderate AA ($p = 0.047$) and EEN groups ($p = 0.036$), whereas AA supplemented at the moderate dose did not impact leucine balance ($p = 0.893$) **(Table 4)**. One extreme outlier (> -3 IQR) was found in the high AA group and was therefore excluded, presumably due to the extremely high dose of corticosteroids, which may alter protein kinetics through increase protein loss and muscle protein breakdown [198]. Despite the additional AAs, protein synthesis rates were not higher in the two intervention groups (high AA $p = 0.143$ and moderate AA $p = 0.117$); however, higher parenteral AA did contribute to higher leucine oxidation (high AA $p < 0.001$ and moderate AA $p = 0.002$). The same pattern was evident with higher endogenous glucose production at high AA dose compared to moderate AA ($p = 0.036$) and EEN

group (p = 0.042) **(Table 5)**. Glucose concentration was not different amongst groups. Regardless of AA dose, net leucine balance was negatively correlated with disease severity as assessed by APACHE score (r = - 0.45, p = 0.031) and nutritional risk as assessed by NUTRIC score (r = - 0.51, p = 0.014) **(Figure 3)**.

Plasma protein concentrations were not different. However, AA supplementation increased albumin absolute synthesis rates with both high (p = 0.004) and moderate AA doses (p = 0.010); fractional synthesis rates were also higher with high AA (p = 0.013) and tended to be higher with moderate AA (p = 0.089) **(Table 6)**. Nitrogen intake was higher in the two intervention groups as planned, which led to higher nitrogen balance with high AA (p = 0.005) and tended to be higher in moderate AA supplementation (p = 0.088) **(Table 7)**. Nitrogen excretion tended to be higher as well in the high AA group (p = 0.083). Furthermore, regardless of intervention group, results from nitrogen and leucine kinetics were similar. Nitrogen excretion was highly correlated with leucine oxidation (pre: r = 0.81, p < 0.0001; post: r = 0.90, p < 0.0001; net: r = 0.73, p < 0.0002), which led to a strong correlation between nitrogen and leucine balance (pre: r = 0.77, p < 0.0001; post: r = 0.90, p < 0.0001; net: r = 0.60, p < 0.0038) **(Figure 4)**.

Total essential AA concentrations were higher with high dose AAs, which was mainly due to tryptophan, histidine, isoleucine and valine **(Table 8)**. Similarly, total non-essential AA concentrations were also higher. However, despite the supplemental AAs, tyrosine concentration remained low and did not differ amongst groups, notably the net increase in tyrosine was < 10% in all groups **(Figure 5)**.

6.5 Discussion

Our study was designed to test the objective of whether increasing protein intake through parenteral AA supplementation increases protein balance. To our knowledge, this is the first RCT to systematically compare different AA doses on protein kinetics in critically ill patients. In this study, high dose parenteral AA supplementation resulted in higher whole-body leucine balance, nitrogen balance, hepatic-derived plasma protein synthesis, and plasma AA concentrations. We also demonstrated that the net change of leucine balance is negatively correlated to APACHE and NUTRIC scores, regardless of AA doses.

Patients receiving high AAs achieved an actual total protein intake of 2.4 g/(kg·d); moreover, leucine balance, the primary outcome, reached a positive level, representing a net anabolic response. However, leucine balance remained negative in the moderate AA group, suggesting that an actual total protein intake of 1.6 g/(kg·d) was not sufficient to achieve a net anabolic response. Similar to our findings, Rehal *et al.* conducted a non-randomized study and found that parenteral AA supplementation increased total protein intake to 2.0 g/(kg·d) and protein balance to 6.9 ± 5.1 μmol/(kg·h) from a negative baseline level after 24 hours [184]. Therefore, parenteral AA supplementation is an effective strategy to increase protein intake and improve protein balance in critically ill patients, and that a protein intake of at least 2.0 g/(kg·d)—the upper end of current recommended protein intake—is required to achieve positive protein balance.

Nitrogen balance (NB) showed a similar pattern to leucine balance, becoming positive with high AA supplementation, whereas remaining negative in patients with moderate AA or EEN. Our data is in agreement with studies that reported improved NB with protein intake in between 1.0 to 2.0 g/(kg·d), and reached equilibrium or positive NB with protein intake higher than 2.0 g/(kg·d) [18,177]. However, to our knowledge, the correlation between NB and protein kinetics has not yet been demonstrated in critically ill patients with various doses of supplemental AAs. These two

methods assess protein anabolism using different assumptions. NB was calculated by following the nitrogen from all AAs from intake to excretion into urine, feces, and other miscellaneous sources; however, it is not possible to quantify the nitrogen used for protein synthesis and released from protein breakdown. On the other band, leucine balance was calculated by following the labelled carbon used for protein synthesis and released from protein breakdown. An estimate of protein balance can be extrapolated from leucine balance based on the 8% contribution of leucine to total body protein [348]. Although these two approaches have different assumptions and limitations, we demonstrated strong correlations between nitrogen and leucine balance in critically ill patients. NB, therefore, confirmed the results from leucine balance on the anabolic benefit of AA supplementation.

High AA supplementation increases the synthesis of plasma albumin to 27 ± 9 %/d in critically ill patients. Similarly, in a previous study, perioperative AA and glucose supplementation increased albumin synthesis to 38 ± 11 %/d [163]. However, the increased albumin synthesis failed to translate into albumin concentration significantly in both studies. This low albumin concentration is mostly due to albumin loss into the interstitial fluid through the inflammatory response associated with surgery and critical illness [349].

Whole-body protein synthesis did not change significantly after high AA supplementation, which is in agreement with Rehal *et al*. [184]. It could be that the sample sizes were too small in both studies to detect the difference. Moreover, protein synthesis might be high in some tissues and low in others under the stress response. Hence, a significant change in protein synthesis might not be observed at the whole-body level. Alternatively, some of the supplemental AAs might take alternative pathways such as gluconeogenesis and leucine oxidation, rather than protein synthesis in certain conditions.

The increased gluconeogenesis in our study was found in the high AA group, as evidenced by a higher endogenous glucose Ra, although glucose intake levels were similar among groups. Our data is in agreement with a study in critically ill adolescents that high AA at 3 g/(kg·d) led to higher endogenous glucose Ra compared to standard AA infusion [166]. AAs induce insulin secretion and foster protein anabolism in a healthy condition; however, when insulin sensitivity is impaired by metabolic stress from critical illness, AAs may rather convert to glucose and increase glycemia [350], suggesting a concurrent tighter glycemic control may be beneficial.

The imbalanced AA profile in the parenteral AA solution might have also contributed to the increased gluconeogenesis and leucine oxidation after high AA supplementation. The high proportion of glucogenic AAs (*e.g.,* glycine and alanine consist of 24% of total AAs) in the parenteral AA solution may contribute to increased gluconeogenesis rather than protein synthesis, creating an extra burden to glucose metabolism in critical illness. Moreover, limited endogenous and exogenous tyrosine might contribute to high leucine oxidation. Plasma tyrosine concentration remained low after AA supplementation, which is similar to a previous study with 0.8 or 1.5 g/(kg·d) AA supplementation [351]. Endogenous tyrosine converted from phenylalanine might be rapidly used up in the hepatocytes before releasing into the plasma under metabolic stress, whereas exogenous tyrosine in the parenteral AA solutions was extremely low due to its low solubility. Therefore, the AAs that require tyrosine to produce peptides might be considered excessive and eventually be oxidized or excreted. In this study, high AAs contained the most "excessive" leucine with respect to tyrosine had the highest leucine oxidation among groups. A more balanced AA profile in the nutrition support regimen may improve protein anabolism.

The AA solution contains only 0.25% tyrosine/total AAs in this study, which is much less than in other AA solutions such as Glavamin® (1.7% tyrosine/total AAs, Fresenius Kabi) and

TrophAmin® (2.4% tyrosine/total AAs, B. Braun). It is unknown whether and how much the tyrosine difference would contribute to protein kinetics among studies using different parenteral AA solutions [184,308]. Nonetheless, the profile and quality of parenteral AA solution should be carefully investigated, especially in critical illness in which AA requirement might be altered [352].

The net increase of leucine balance was negatively correlated with APACHE or NUTRIC score regardless of AA doses, suggesting that patients with higher disease severity or higher nutritional risk might be less likely to respond to nutrition in general. Moreover, it revealed that the heterogeneity of patients' baseline status led to different metabolic responses to nutrition, which in part explains why a "one size fits all" approach in nutrition support fails to demonstrate clinical improvement [11]. Indeed, the baseline values of the leucine balance in this study highly varied with a pooled average of -18 ± 14 μmol/(kg·h). The variability of leucine balance was much less in a more homogenous study population. For instance, the baseline leucine balance was -17 ± 7 μmol/(kg·h) in patients with colorectal surgery [163] and -20 ± 8 μmol/(kg·h) in cardiac surgery [165]. Future studies should stratify patients based on APACHE and NUTRIC scores to reduce the heterogeneity and to quantify more accurately the impact of high AA supplementation on nutrient metabolism.

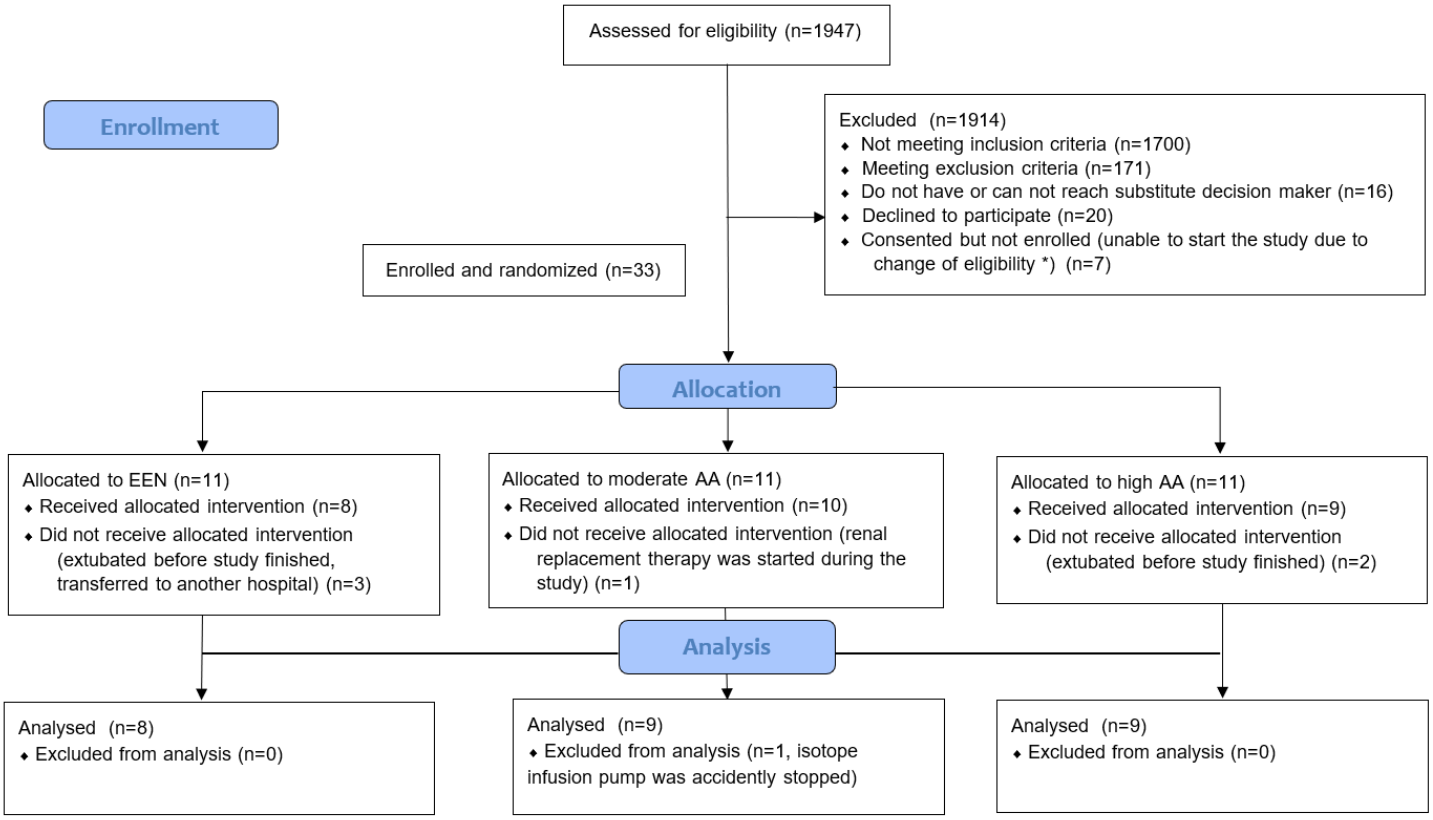
Together, our study demonstrated that high AA supplementation improved leucine balance and other kinetics outcomes. Most importantly, the understanding of nutrients kinetics and metabolic responses to AA supplementation provides a foundation to study further the clinical outcomes [188] or to investigate more in-depth into the molecular responses of high dose AA in critically ill patients. For instance, these high AAs may trigger nutrient-sensing mechanisms through mTOR/GCN2 pathways [353]. Conversely, this high dose may suppress autophagy and delay recovery from organ failure [193,195].

We acknowledge some limitations in this study. We observed heterogeneity of the baseline nutritional status and disease severity, although the randomization procedure was followed. These baseline characteristics were considered as covariates in the statistical model; however, future studies should stratify patients according to their clinical scores to reduce potential bias. Furthermore, the supplemental AAs contribute partially to oxidation/excretion and gluconeogenesis rather than protein synthesis. This is most likely due to the insulin resistance from metabolic stress and the imbalanced AA profile in the parenteral solution, revealing the importance of glycemic control and the quality of nutrition support regimens in critical care.

6.6 Conclusion

High AA supplementation to target protein intake at 2.5 g/(kg·d), but not moderate AA for a protein intake of 1.75 g/(kg·d)), elicits a net anabolic response in the critically ill patients demonstrated by higher leucine balance, nitrogen balance, plasma albumin synthesis, and AAs availability. Furthermore, the net change of leucine balance was found to be correlated to the APACHE and NUTRIC scores, and net nitrogen balance. This metabolic study of protein kinetics provides a framework to fully assess the value of high protein intakes through future large-scale trials investigating clinical outcomes and molecular mechanisms.

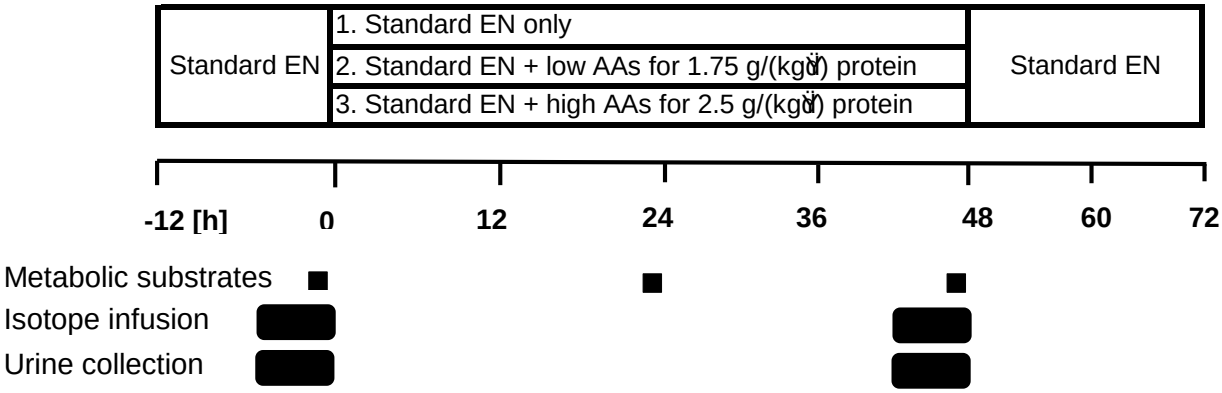
Figure 6.1. CONSORT Flow Diagram for patient recruitment.



* Patients were extubated, or transfer to another unit or hospital, or change of level of care after consent before enrollment.

Figure 6.2 Study protocol.

a) Overall study protocol.



b) Stable isotope infusion protocol.

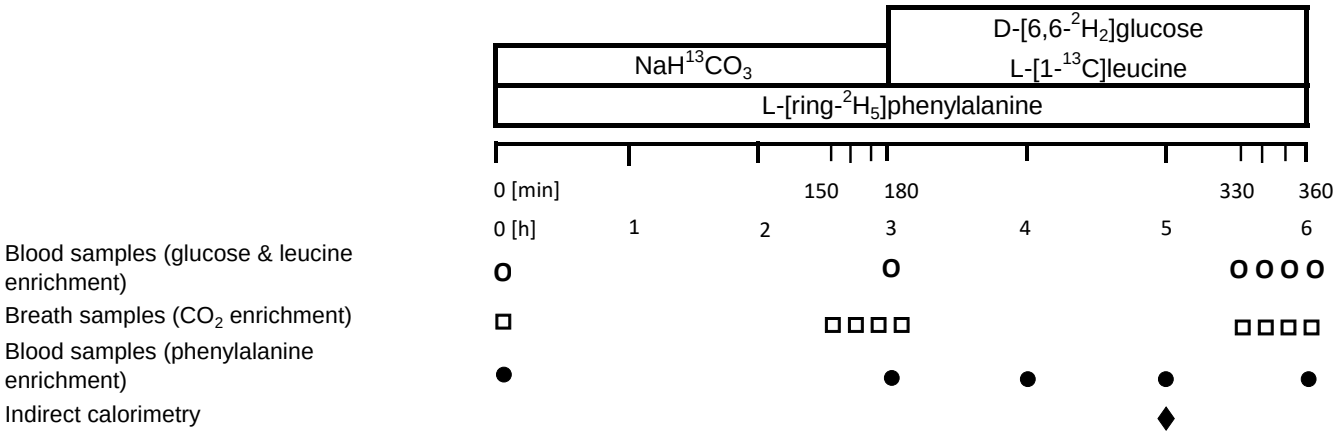


Table 6.1 Patient characteristics.

	EEN	Moderate AA	High AA
n	8	9	9
Age, y	67 (14)	63 (7)	64 (13)
Sex, M/F	4/4	4/5	4/5
Weight, kg	84 (19)	82 (16)	70 (10)
BMI, kg/m^2	28 (6)	30 (5)	26 (4)
Scoring at ICU admission			
APACHE II	24 (8)	21 (7)	22 (8)
SOFA	9 (2)	9 (4)	9 (5)
NUTRIC	6 (2)	6 (2)	5 (3)
LOS			
Days in the ICU when study started	4 (1)	4 (2)	3 (1)
ICU LOS, days	18 (9)	23 (13)	15 (10)
Hospital LOS, days	32 (16)	41 (20)	41 (42)
Mortality			
ICU, *n* (%)	2 (25%)	1 (11%)	3 (38%)
Hospital, *n* (%)	2 (25%)	1 (11%)	4 (44%)
Mechanical ventilation, days	13 (7)	20 (14)	17 (10)
Corticosteroids, *n*	3	2	4
Insulin, *n*	3	3	3
Propofol dose before the study, µg/(kg·min)	26 (13)	21 (18)	9 (11)
Propofol average dose during the study, µg/(kg·min)	19 (17)	21 (17)	18 (21)

Mean (SD). BMI, body mass index; LOS, length of stay; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; NUTRIC, nutrition risk in critically ill.

Table 6.2 Energy and protein intakes from enteral nutrition supplemented with parenteral AAs

	EEN	Moderate AA	High AA	Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
				P values		
Prescribed energy, kcal/(kg·d)	23.7 (4.5)	22.7 (2.2)	23.4 (2.2)	0.826	0.253	0.530
Achieved energy without AA, kcal/(kg·d)	21.1 (4.8)	19.7 (3.1)	23.7 (4.9)	0.827	0.486	0.197
Achieved energy with AA, kcal/(kg·d)	21.1 (4.8)	22.6 (2.8)	28.0 (4.7)	0.744	**0.007**	**0.028**
Achieved protein intake from EN, g/(kg·d)	0.82 (0.21)	0.75 (0.14)	0.93 (0.15)	0.681	0.455	0.114
Achieved AA intake, g/(kg·d)	0 (0)	0.88 (0.16)	1.48 (0.17)	**< 0.001**	**< 0.001**	**< 0.001**
Achieved total protein intake, g/(kg·d)	0.82 (0.21)	1.62 (0.07)	2.41 (0.10)	**< 0.001**	**< 0.001**	**< 0.001**
Achieved total protein equivalent, g/(kg·d)	0.82 (0.21)	1.48 (0.06)	2.16 (0.09)	**< 0.001**	**< 0.001**	**< 0.001**

Mean (SD). REE, resting energy expenditure; EN, enteral nutrition; Achieved total protein intake was calculated from protein intake from EN + AA g/(kg·d); Achieved total protein equivalent was calculated from protein intake from EN + adjusted AA to protein equivalent g/(kg·d).

Table 6.3 Indirect calorimetry.

	EEN		Moderate AA		High AA		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post		*P* values	
VCO_2 mL/min	285 (78)	270 (90)	263 (82)	274 (114)	242 (26)	270 (24)	0.581	0.290	0.700
VO_2 mL/min	329 (87)	309 (97)	300 (93)	334 (152)	264 (37)	297 (31)	0.318	0.349	0.973
RQ	0.87 (0.07)	0.87 (0.04)	0.88 (0.07)	0.84 (0.08)	0.92 (0.05)	0.91 (0.05)	0.947	0.422	0.233
REE, kcal/d	2285 (623)	2166 (685)	2101 (650)	2305 (1026)	1872 (250)	2095 (204)	0.403	0.386	0.953
REE, kcal/(kg·d)	26.9 (7.3)	25.4 (7.9)	25.9 (4.8)	27.9 (9.3)	30.4 (10.8)	33.6 (10.1)	0.630	0.502	0.922

Mean (SD). RQ, respiratory quotient; REE, resting energy expenditure.

Table 6.4 Whole body leucine kinetics.

	EEN		Moderate AA		High AA		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post		*P* values	
Leucine Ra, µmol/(kg·h)	145 (35)	137 (26)	131 (20)	153 (19)	129 (44)	155 (24)	**0.004**	**0.002**	0.916
Leucine oxidation, µmol/(kg·h)	53 (18)	47 (12)	45 (11)	55 (11)	40 (13)	57 (9)	**0.002**	**< 0.001**	0.633
Protein synthesis, µmol/(kg·h)	92 (21)	90 (20)	86 (16)	98 (18)	89 (38)	99 (19)	0.117	0.143	0.988
Protein breakdown, µmol/(kg·h)	116 (29)	109 (21)	104 (22)	109 (19)	96 (42)	97 (23)	0.252	0.942	0.364
Leucine balance, µmol/(kg·h)	-25 (13)	-19 (9)	-18 (12)	-12 (12)	-7 (12)	2 (9)	0.893	**0.036**	**0.047**

Mean (SD). Ra, rate of appearance.

Table 6.5 Glucose turnover.

	EEN		Moderate AA		High AA		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post		*P* values	
Glucose, mmol/L	8.9 (1.1)	8.8 (1.1)	9.3 (2.6)	9.1 (2.2)	9.9 (2.9)	12.1 (5.0)	0.783	0.149	0.383
Glucose intake, µmol/(kg·min)	9 (2)	9 (2)	8 (2)	9 (2)	11 (2)	11 (2)	0.634	0.978	0.832
Glucose Ra, µmol/(kg·min)	19 (6)	20 (5)	21 (4)	20 (4)	21 (5)	29 (11)	0.985	**0.030**	**0.036**
Endogenous glucose Ra, µmol/(kg·min)	10 (6)	11 (5)	13 (5)	11 (3)	11 (5)	19 (11)	0.997	**0.042**	**0.044**
Glucose clearance ml/(kg·min)	2.1 (0.5)	2.2 (0.4)	2.3 (0.2)	2.2 (0.7)	2.4 (1.0)	2.9 (1.7)	0.929	0.292	0.151

Mean (SD).

Figure 6.3 Correlations between leucine kinetics and clinical scores.

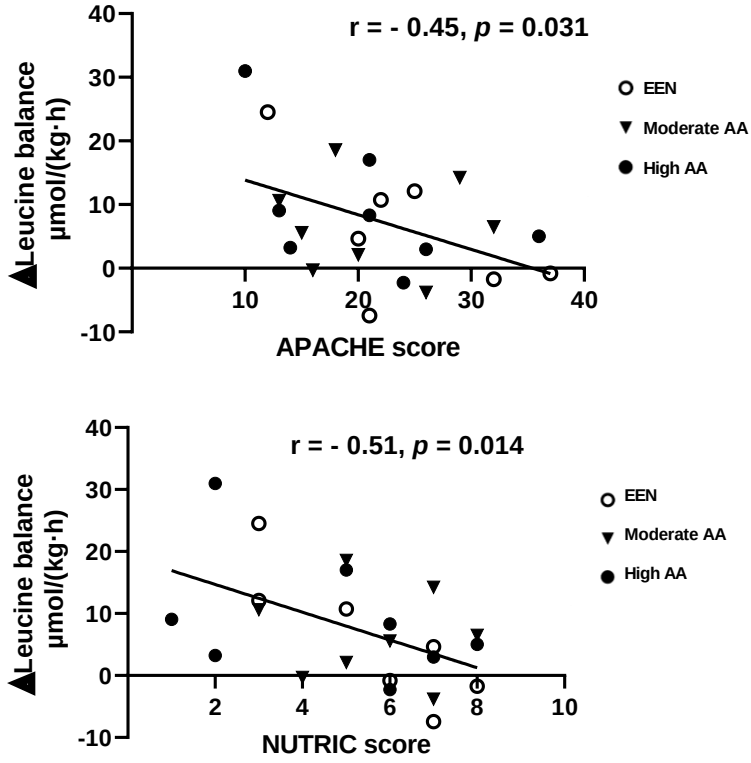


Table 6.6 Hepatic derived plasma protein synthesis.

	EEN		Moderate AA		High AA		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post	*P* values		
Total Protein									
FSR, %/d	39 (9)	29 (6)	33 (9)	33 (5)	42 (13)	36 (18)	0.105	0.141	0.988
ASR, mg/(kg·d)	1050 (272)	797 (219)	916 (344)	963 (217)	1068 (335)	999 (406)	**0.028**	0.090	0.845
Concentration, g/L	57 (5)	57 (4)	55 (6)	59 (7)	59 (9)	60 (6)	0.448	0.667	0.939
Albumin									
FSR, %/d	22 (8)	17 (6)	17 (6)	21 (4)	19 (8)	27 (9)	0.089	**0.013**	0.525
ASR, mg/(kg·d)	280 (107)	222 (72)	233 (108)	290 (60)	218 (102)	324 (114)	**0.010**	**0.004**	0.815
Concentration, g/L	28 (3)	27 (4)	27 (3)	27 (4)	26 (2)	26 (3)	0.370	0.773	0.778
Fibrinogen									
FSR, %/d	41 (33)	32 (14)	39 (14)	40 (15)	55 (17)	52 (26)	0.628	0.233	0.697
ASR, mg/(kg·d)	105 (78)	76 (41)	83 (43)	88 (29)	124 (66)	124 (75)	0.753	0.324	0.699
Concentration, g/L	5 (2)	5 (1)	4 (1)	5 (1)	5 (2)	5 (2)	0.959	0.975	0.863

Mean (SD).

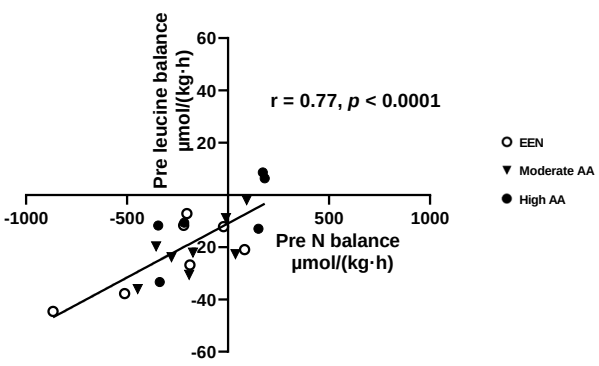
Table 6.7 Nitrogen balance.

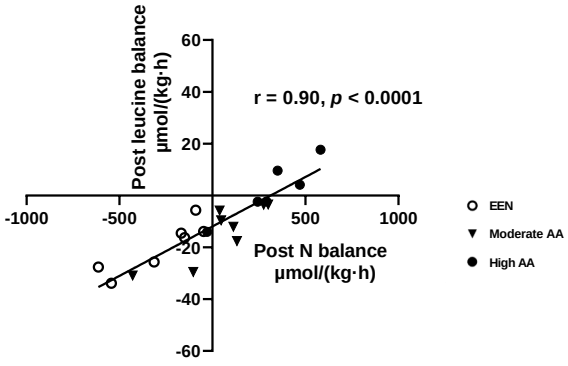
	EEN		Moderate AA		High AA		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post	*P* values		
Nitrogen intake									
µmol/(kg·h)	383 (83)	383 (83)	382 (67)	823 (9)	451 (76)	1184 (10)	**< 0.001**	**< 0.001**	**< 0.001**
Urine urea nitrogen									
µmol/(kg·h)	575 (340)	576 (269)	468 (165)	699 (214)	431 (210)	784 (192)	0.193	0.078	0.635
Nitrogen excretion									
µmol/(kg·h)	659 (349)	658 (277)	548 (166)	777 (214)	517 (209)	866 (189)	0.210	0.083	0.624
Nitrogen balance									
µmol/(kg·h)	-276 (296)	-276 (207)	-166 (181)	47 (217)	-66 (238)	316 (192)	0.088	**0.005**	0.101

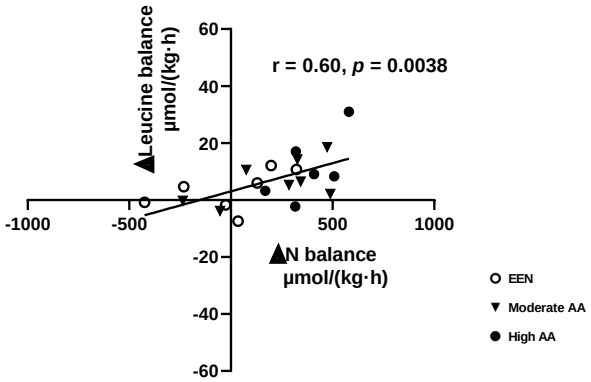
Mean (SD).

Figure 6.4 Correlations between nitrogen and leucine kinetics.

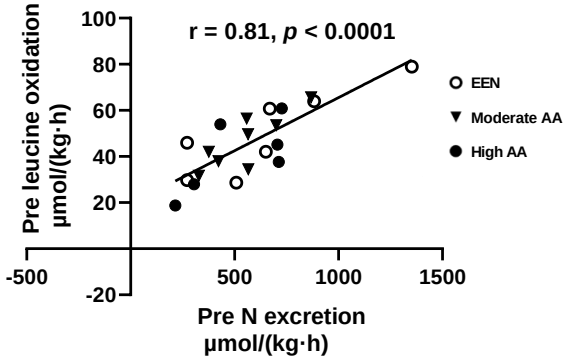
a) Correlation between nitrogen balance and leucine balance

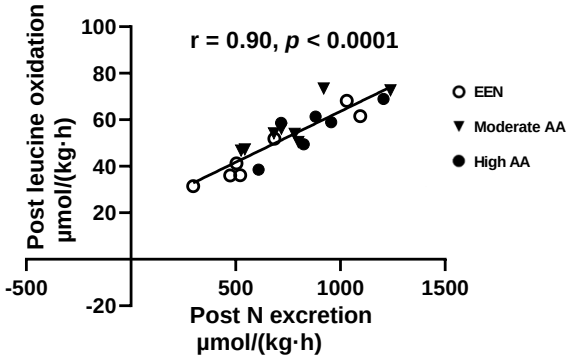






b) Correlation between nitrogen excretion and leucine oxidation





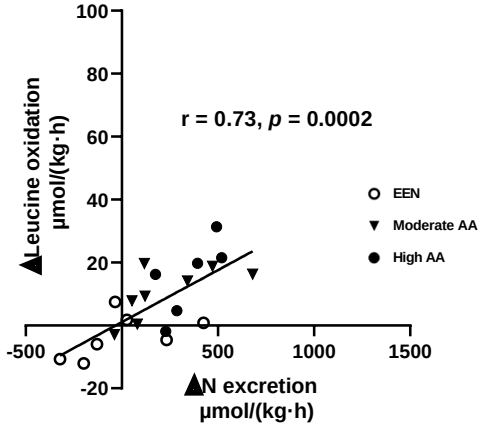
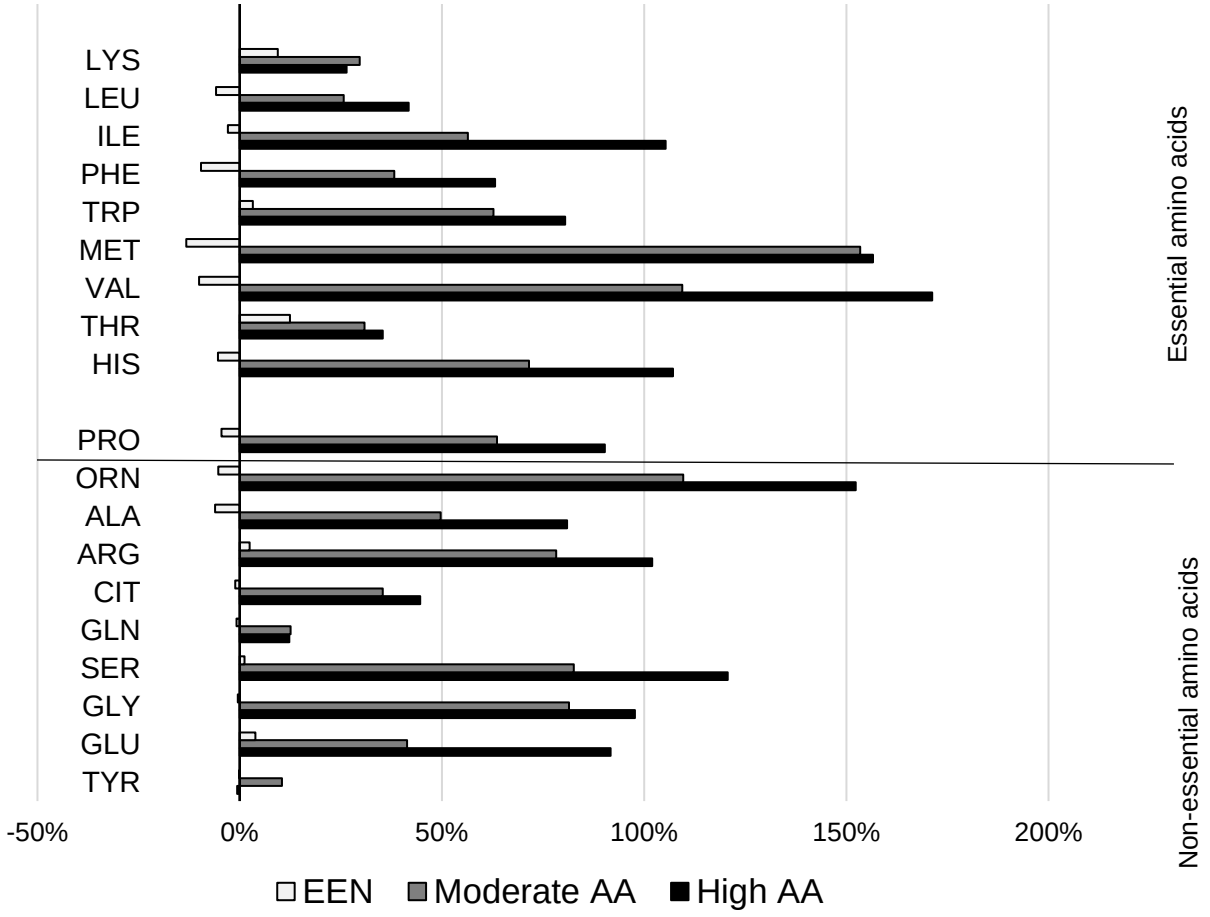


Table 6.8 Plasma amino acid concentration.

µmol/L	EEN (n=7)		Moderate AA (n=7)		High AA (n=6)		Moderate AA vs. EEN	High AA vs. EEN	High vs. Moderate AA
	Pre	Post	Pre	Post	Pre	Post		*P* values	
Essential AA concentration									
Lysine	201 (66)	220 (53)	215 (76)	279 (48)	284 (126)	359 (110)	0.415	0.105	0.569
Leucine	182 (66)	172 (44)	177 (85)	223 (90)	177 (54)	251 (83)	0.318	0.105	0.745
Isoleucine	90 (34)	87 (24)	101 (57)	158 (71)	88 (31)	181 (44)	0.126	**0.013**	0.460
Phenylalanine	167 (51)	151 (31)	116 (34)	160 (47)	155 (50)	253 (163)	0.341	0.052	0.580
Tryptophan	41 (15)	42 (18)	34 (13)	55 (14)	32 (13)	58 (22)	**0.018**	**0.005**	0.687
Methionine	33 (16)	28 (12)	32 (15)	80 (75)	28 (9)	73 (33)	0.322	0.317	0.999
Valine	302 (119)	271 (68)	258 (101)	540 (221)	260(104)	708 (230)	**0.046**	**0.005**	0.388
Threonine	155 (64)	175 (61)	139 (63)	181 (29)	199 (133)	269 (132)	0.950	0.384	0.544
Histidine	66 (21)	62 (14)	53 (10)	92 (19)	65 (19)	135 (59)	0.110	**0.005**	0.236
Branched-chain AAs	574 (212)	531 (126)	536 (242)	921 (378)	526 (179)	1139 (349)	0.075	**0.009**	0.450
Total essential AAs	1238 (363)	1210 (267)	1124 (405)	1768 (450)	1290 (429)	2287 (615)	0.075	**0.006**	0.326
Non-essential AA concentration									
Proline	207 (67)	198 (54)	197 (86)	322 (148)	196 (85)	373 (92)	0.057	**0.016**	0.728
Ornithine	106 (31)	100 (21)	115 (81)	242 (180)	85 (32)	215 (46)	**0.045**	**0.020**	0.910
Alanine	354 (197)	332 (149)	298 (150)	446 (132)	340 (121)	615 (246)	0.095	**0.003**	0.148
Arginine	81 (45)	83 (40)	70 (35)	126 (23)	83 (37)	168 (64)	0.103	**0.009**	0.345
Citrulline	33 (18)	33 (16)	22 (17)	30 (19)	26 (20)	38 (19)	0.497	0.257	0.862
Glutamine	707 (228)	701 (193)	542 (164)	611 (86)	712 (354)	799 (166)	0.950	0.260	0.398
Serine	78 (32)	79 (31)	72 (28)	131 (20)	81 (34)	179 (69)	0.149	**0.009**	0.266
Glycine	223 (85)	222 (76)	156 (55)	284 (57)	212 (87)	420 (154)	0.280	**0.014**	0.247
Asparagine	62 (34)	61 (31)	42 (19)	43 (9)	55 (23)	47 (19)	0.783	0.582	0.938
Glutamate	104 (64)	108 (45)	74 (63)	105 (74)	50 (30)	97 (51)	0.589	0.495	0.968
Tyrosine	85 (24)	85 (22)	83 (32)	92 (30)	86 (36)	85 (36)	0.757	0.984	0.848
Total non-essential AAs	2046 (709)	2008 (579)	1677 (622)	2435 (625)	1932 (756)	3041 (729)	0.098	**0.010**	0.400

Mean (SD).

Figure 6.5 Plasma amino acids % net change from pre- to post-intervention.



Supplemental Table 6.1 Diet product amino acid composition

Product **Feeding route**	**Prosol 20%** **Parenteral**	**Peptamen 1.5** **Enteral**
	%	
Isoleucine	5.40	5.60
Leucine	5.40	10.30
Valine	7.20	5.20
Lysine	6.75	9.50
Methionine	3.80	2.20
Cysteine	0.00	2.50
Phenylalanine	5.00	3.10
Tyrosine	0.25	3.00
Threonine	4.90	7.10
Tryptophan	1.60	2.10
Histidine	5.90	1.70
Arginine	9.80	2.70
Glycine	10.30	1.90
Alanine	13.80	4.90
Aspartate	3.00	10.50
Glutamate	5.10	10.70
Proline	6.70	5.30
Serine	5.10	5.50
Glutamine	0.00	6.20
Total	**100**	**100**

CHAPTER 7 – GENERAL DISCUSSION

The overall objective of this thesis is to determine whether and how supplementation of protein and AA contributes to protein anabolism under metabolic stress. Study 1 built a foundation for the use of stable isotope tracers to examine the kinetic response of nutritional interventions in piglets; this technique was then applied in subsequent patient studies. Study 2 to 4 investigated the effect of nutritional interventions on protein metabolism with parenteral AA infusion either as a sole nutrient source in surgical patients or as a supplementation to standard enteral nutrition in critically ill patients. In summary, this thesis:

(1) contributes to the understanding of nutrient kinetic responses under the interactions between metabolic stress and malnutrition,

(2) improves the current nutrition support regimens with the application of protein or AA supplementation in patients under metabolic stress,

(3) provides a study paradigm for large clinical trials to associate nutrition with patients' clinical outcomes and for mechanistic studies to determine the effect of nutritional interventions on cell signaling pathways and nutrient-sensing mechanisms.

This chapter provides an overview of the main outcomes as well as specific considerations and constraints involved in the studies to guide future research.

7.1 Main outcomes

Metabolic stress response activates neuro-hormonal and immunological systems, resulting in hypermetabolism with increased energy expenditure and stress-induced insulin resistance [1]. As glucose demand increases, skeletal muscle protein is broken down to provide AAs for gluconeogenesis [19]. The studies in this thesis quantified the metabolic changes before and after nutritional interventions using stable isotope tracers. The results demonstrate that protein or AA

supplementation fosters protein anabolism through increasing protein balance (leucine), nitrogen balance and plasma protein synthesis. However, some AAs undergo oxidation and gluconeogenesis rather than protein synthesis; this may limit the anabolic potential of protein and AA supplementation in patients under metabolic stress **(Figure 7.1).**

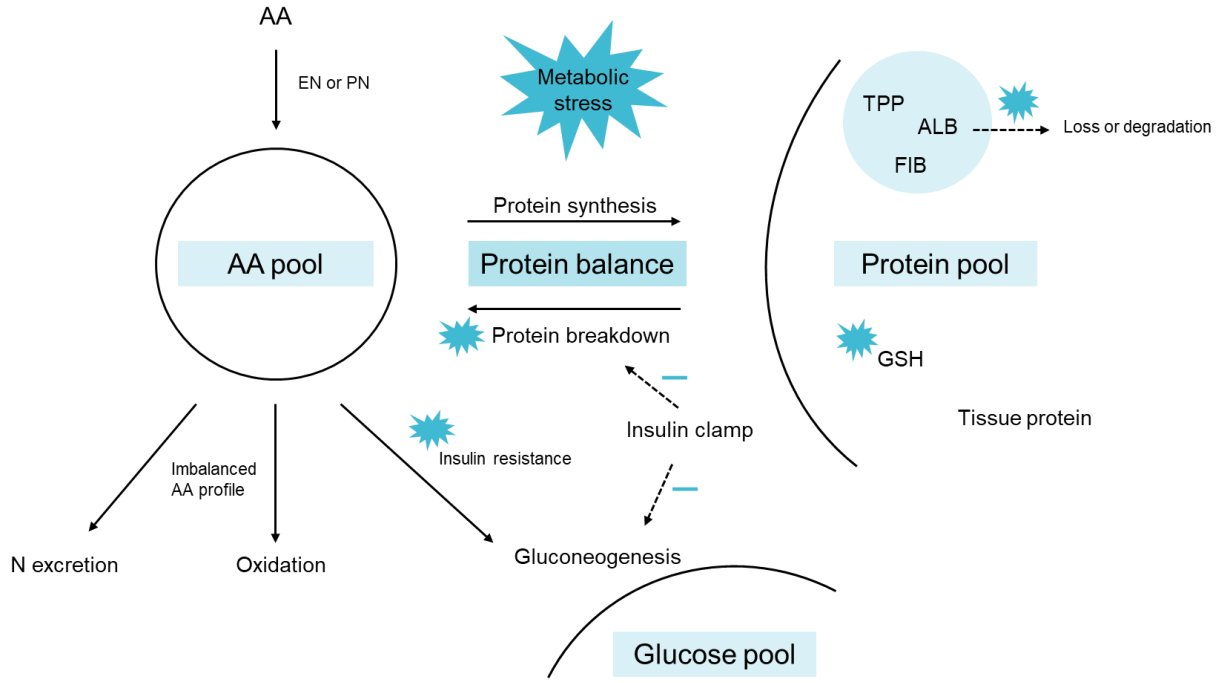
Depending on the composition of the protein and AA supplementation, the magnitude of change in AA concentrations may vary. Both essential (EAAs) and branched-chain AAs (BCAAs) were increased in all studies after the infusion of either parenteral AA solution or intact protein via the enteral route. Parenteral AAs from Travasol® (study 2–3) or Prosol® (study 4) contain a limited amount of tyrosine and cysteine due to their low solubility in aqueous solutions. As a result, plasma concentrations of these limiting AAs remained low after AA infusion, although these AAs can be synthesized *de novo*. In contrast, study 1 sourced protein from whey and egg white and showed elevated tyrosine after protein repletion. A more detailed discussion on the quality of nutrition support regimens can be found in **Section 7.2**.

Protein or AA supplementation elevated the synthesis of tissue and plasma proteins, such as albumin and fibrinogen, and the tripeptide glutathione. Albumin constitutes a major portion of total plasma proteins and is a negative acute-phase protein. However, albumin concentration remained low despite an increased synthesis rate during protein or AA supplementation. The main underlying reasons for this have been suggested to involve: 1) increased vascular permeability from inflammatory response causing albumin to accumulate in the interstitial fluid [292], 2) intestinal epithelial leakage resulting in albumin loss into the intestinal tract [237], 3) increased rate of albumin degradation into AAs to serve as substrates for protein synthesis [351], and 4) increased the blood volume due to fluid resuscitation with crystalloid (without albumin) in patients with hypovolemia [354].

Protein balance is a calculated difference between protein synthesis and breakdown, but not a direct measurement. Our studies showed that parenteral AA supplementation tended to increase protein synthesis and decrease protein breakdown; however, none of the changes was significant, resulting in a relatively minor increase in protein balance. A significant attenuation of protein breakdown was only found in patients receiving a supraphysiological dose of insulin as part of the hyperinsulinemic-normoglycemic clamp in previous studies [158,165]. Several potential reasons may be related to the limited increase in protein balance after AA supplementation.

Our data suggest that both insulin resistance and an unbalanced parenteral AA profile may limit the anabolic effect of AA supplementation. Insulin resistance, whether induced by surgery, critical illness, or T2D with poor glycemic control, exposes the gluconeogenic potential of AAs [350]; higher AA intake provides more exogenous substrates to enter gluconeogenesis rather than for protein synthesis. Insulin sensitivity, therefore, plays an important role in modulating the anabolic effects of AA supplementation. Moreover, imbalances in the AA profile of parenteral AA solutions—characterized by a limited amount of tyrosine and excessive glucogenic AAs—results in AA degradation into CO_2 and H_2O via oxidation (measured by labelled CO_2 in breath samples from the oxidation of leucine tracers) and nitrogen excretion (in urine, feces, and other miscellaneous losses). Follow-up mechanistic studies and larger clinical trials are necessary to understand the limitations of AA supplementation and to determine the optimal protein intake in patients under metabolic stress (further discussed in **Sections 7.4 and 7.5**).

Figure 7.1 Overall outcomes from protein or amino acid supplementation under metabolic stress.



Dietary AA, either via EN or PN, increased AA availability in the AA pool. AA substrates contribute to whole-body protein synthesis in the protein pool, such as the synthesis of hepatic derived plasma protein (TPP, FIB and ALB), GSH and tissue proteins. In addition, insulin clamp shuts down gluconeogenesis and attenuates protein breakdown. As a result, protein balance, the difference between protein synthesis and breakdown, may increase. However, ALB loss increases in the interstitial fluid or intestinal tract under metabolic stress. Moreover, AA substrates may choose to take the degradation pathway to 1) contribute to the glucose pool via gluconeogenesis in an insulin-resistant condition, 2) increase oxidation and N excretion when the profile of the additional AAs is imbalanced. Abbreviations: EN, enteral nutrition; PN, parenteral nutrition; AA, amino acids; insulin clamp, hyperinsulinemic-normoglycemic clamp; TPP, total plasma protein; ALB, albumin; FIB, fibrinogen; GSH, glutathione; N, nitrogen.

7.2 Evaluation of nutrition support regimens

7.2.1 AA to protein conversion

In studies in which AAs rather than intact proteins are administered, protein intake may be overestimated, considering that AA only converts to approximately 83% of protein (varies slightly depending on the AA composition in the nutrition support solutions) [174]. Consequently, the Atwater physiological fuel value of 3.3 kcal/g, rather than 4 kcal/g, should be applied to calculate the rate of AA infusion based on energy expenditure [174]. However, these factors have rarely been taken into consideration in clinical research. A 1.2 g/(kg·d) AA infusion to match the lower bound of the recommended protein intake for critically ill patients, however, was equivalent to a protein intake of only 1.0 g/(kg·d) [18]. Moreover, in study 2, a parenteral AA infusion of 1.2 g/(kg·d) should have been calculated (using 3.3 kcal/d) to match the designed dose at 20% REE; however, the actual AA infusion rate was at 1.0 g/(kg·d) (using 4 kcal/d), which is equivalent to a protein intake of 0.8 g/(kg·d). A similar calculation was found in study 3, resulting in a lower AA intake than the designed level. Future studies with AA infusion should consider these factors in the study design stage to avoid discrepancies between intended protein intake levels and those actually delivered to patients.

7.2.2 AA composition

AA composition varies among parenteral AA products **(Table 7.2.2)** and may affect metabolic and kinetic responses. We postulated that the increased leucine oxidation and insignificant changes in protein synthesis in studies 2 and 4 **(chapters 4 and 6)** were mainly due to the imbalance of the AA profile in the parenteral AA solutions used (Travasol® and Prosol®). Both solutions have low concentrations of tyrosine due to the low solubility of this AA in aqueous solutions. The tyrosine in these parenteral AA solutions accounts for 0.3–0.4% of the total AAs

and is approximately 10-fold lower than the tyrosine content in the reference egg protein. In contrast, glucogenic AAs, such as alanine and glycine, account for 24.6–31.4% of the total AAs in these two parenteral AA solutions compared to only 8.6% in the reference egg protein. The potential impact of low tyrosine and high glucogenic AAs on protein and glucose metabolism is discussed in the following sections.

7.2.2.1 Tyrosine

Tyrosine may be considered a conditionally essential AA. Under the normal condition, tyrosine is synthesized from phenylalanine in the liver and kidney via phenylalanine hydroxylase; in other tissues without phenylalanine hydroxylase, tyrosine is mainly supplied from the liver [355]. However, tyrosine synthesis is reduced in patients that suffer from hepatic or renal failure [356,357]. The peripheral supply of tyrosine may be further limited when its sole exogenous source is from parenteral AA infusion that skips the first-pass splanchnic uptake.

The tyrosine requirement, determined by the indicator amino acid oxidation method, is 21 mg/(kg·d) for healthy adults with the presence of a moderate level of phenylalanine [358]. However, parenteral AA solutions in our studies can not meet this requirement. For instance, Travasol® contains 0.4% tyrosine, and an AA infusion of 1.5 g/(kg·d) provides only 6 mg/(kg·d) of tyrosine. When tyrosine becomes the limiting AA, the body may consider other AAs, such as leucine, to be in excess and thus shift from protein synthesis to oxidation.

Some manufacturers of parenteral AA solutions replace tyrosine with N-acetyl-tyrosine or dipeptide glycyl-tyrosine as an attempt to improve its issue with solubility. Previous studies have indicated that parenteral AA solutions with these tyrosine substitutes have a high proportion of tyrosine, such as 2.3% in Aminosyn®, 1.6% in Glavamin®, and 2.4% in Trophamine®, resulting in either an increase in protein synthesis or no change in AA oxidation [166,184,308]. Future studies

may explore the effect of tyrosine substitutes in parenteral AA solutions to achieve a balanced AA profile and avoid excessive oxidation of other AAs.

7.2.2.2 Glucogenic amino acids

Under insulin resistance, glucogenic AAs, such as alanine, glycine, serine and cysteine, convert to pyruvate to enter the gluconeogenic pathway in the liver. The level of alanine aminotransferase—the enzyme involved in the conversion of alanine to pyruvate—increases with the existence of hepatic insulin resistance [359].

High AA intake of 3 g/(kg·d), compared to 1.5 g/(kg·d), resulted in higher endogenous glucose production in critically ill adolescents [166]. Although the author speculated that the high AA intake might elevate gluconeogenesis under insulin resistance, the high proportion of glucogenic AAs (10% alanine) in the parenteral AA solution (Aminosyn®) may also play a role. Similarly, in study 4, Prosol® contains 14% alanine and might have contributed to the increase in gluconeogenesis in the high AA group. More evidence is needed to determine whether elevated gluconeogenesis occurs primarily due to high AA intake or due to a higher proportion of glucogenic AAs relative to other AAs.

7.2.2.3 EN to PN ratio

Commercially available EN formulas to date can rarely reach a high protein intake of 2.5 g/(kg·d) without energy overfeeding. This risk of overfeeding is even greater if a large dose of lipid-based sedative Propofol (1.1 kcal/ml) is administered. Supplementation with protein alone is a flexible strategy to increase protein intake without modifying other macronutrients. Parenteral AA supplementation is superior to enteral protein powder bolus for preventing dose skipping, reducing high gastric residuals, and increasing dietary tolerance.

The AA composition of EN formulas differs from that of parenteral solutions. EN protein sourced from milk or soy contains more AAs that are not soluble in aqueous solutions (e.g., tyrosine) and fewer exclusively glucogenic AAs. This AA profile, compared to that of parenteral AA solutions, is more balanced and may contribute to protein synthesis rather than oxidation and gluconeogenesis.

The higher the protein content in the EN formulas, the easier it is to achieve target protein intake. Enteral nutrition formula Peptamen 1.5 (Nestle) was chosen in study 4 for a few reasons: 1) the peptides hydrolyzed from whey protein increases absorption; 2) the calorie-dense formula provides 1.5 kcal/ml, which meets the volume restriction requirement for patients in the ICU; 3) the protein content of 6.8 g/100 ml is relatively higher compared to other EN formulas available at the time in the ICU where we recruited patients for study 4, which helps to meet the protein goal. However, this formula can only provide 0.91-1.13 g protein/kg with an EER at 20-25 kcal/kg without energy overfeeding. Alternatively, enteral formulas with higher protein-to-energy ratio have become commercially available in recent years, such as the Peptamen intense ((Nestle, 1.0 kcal/ml) with a protein content of 9.2 g/100 ml. This formula can achieve a protein intake of 1.84-2.3 g/kg with an EER at 20-25 kcal/kg. Future studies in critical care should consider using EN with a high protein-to-energy ratio to meet high protein goals without energy overfeeding, and it may also facilitate greater manipulation of the ratio between EN and supplemental AAs to generate a more balanced AA profile to ameliorate protein anabolism.

Table 7.2.2 Amino acid composition in enteral and parenteral nutrition.

% total AA	Egg (USDA)	Whey & egg white (Study 1)	Peptamen 1.5, Nestle [340] (Study 4)	Travasol, Baxter (Study 2 & 3)	Prosol, Baxter (Study 4)	Aminosyn, Hospira	Glavamin, Fresenius Kabi	Trophamine, B.Braun
Alanine	5.3%	5.4%	4.9%	20.9%	14.1%	10.2%	11.9%	5.4%
Arginine	6.3%	4.3%	2.7%	11.6%	10.0%	10.5%	8.4%	12.0%
Aspartic acid	10.2%	9.5%	10.5%	-	3.1%	7.2%	2.5%	3.2%
Cysteine	3.1%	2.2%	2.5%	-	-	-	-	0.2%
Glutamine	-	-	6.2%	-	-	-	13.7%	-
Glutamic acid	13.0%	16.3%	10.7%	-	5.2%	7.6%	4.2%	5.0%
Glycine	3.3%	2.8%	1.9%	10.4%	10.5%	5.2%	7.7%	3.6%
Histidine	2.3%	2.1%	1.7%	4.9%	6.0%	3.1%	5.1%	4.8%
Isoleucine	4.9%	5.6%	5.6%	6.1%	5.5%	6.8%	4.2%	8.2%
Leucine	8.4%	9.3%	10.3%	7.4%	5.5%	10.3%	5.9%	14.0%
Lysine	6.7%	7.6%	9.5%	4.7%	4.9%	7.7%	6.7%	8.2%
Methionine	3.3%	2.9%	2.2%	4.0%	3.9%	1.8%	4.2%	3.4%
Phenylalanine	5.3%	4.8%	3.1%	5.7%	5.1%	3.1%	4.4%	4.8%
Proline	4.5%	5.0%	5.3%	6.9%	6.8%	7.4%	5.1%	6.8%
Serine	7.4%	6.0%	5.5%	5.1%	5.2%	5.5%	3.4%	3.8%
Threonine	4.8%	4.8%	7.1%	4.2%	5.0%	4.1%	4.2%	4.2%
Tryptophan	1.3%	1.8%	2.1%	1.8%	1.6%	2.1%	1.4%	2.0%
Tyrosine	4.1%	3.3%	3.0%	0.4%	0.3%	2.3%	1.6%	2.4%
Valine	5.9%	6.3%	5.2%	5.9%	7.3%	5.2%	5.4%	7.8%
Taurine	-	-	-	-	-	-	-	0.2%

7.3 Heterogeneity and statistical interpretation

Heterogeneity in study participants may reduce the statistical power of a study and lead to under- or overestimation of the impact of nutritional interventions.

In study 1, we minimized heterogeneity by controlling the study block as a random effect in the statistical analysis and calculating tissue kinetics by tissue protein instead of tissue weight to limit variation during tissue washing and processing. However, the results indicated that protein repletion failed to increase GSH synthesis in several tissues due to large standard errors, although the mean was 50% higher than that of the control group. This variation might be due to the uncontrolled genetic and environmental factors in piglets obtained from a local farm.

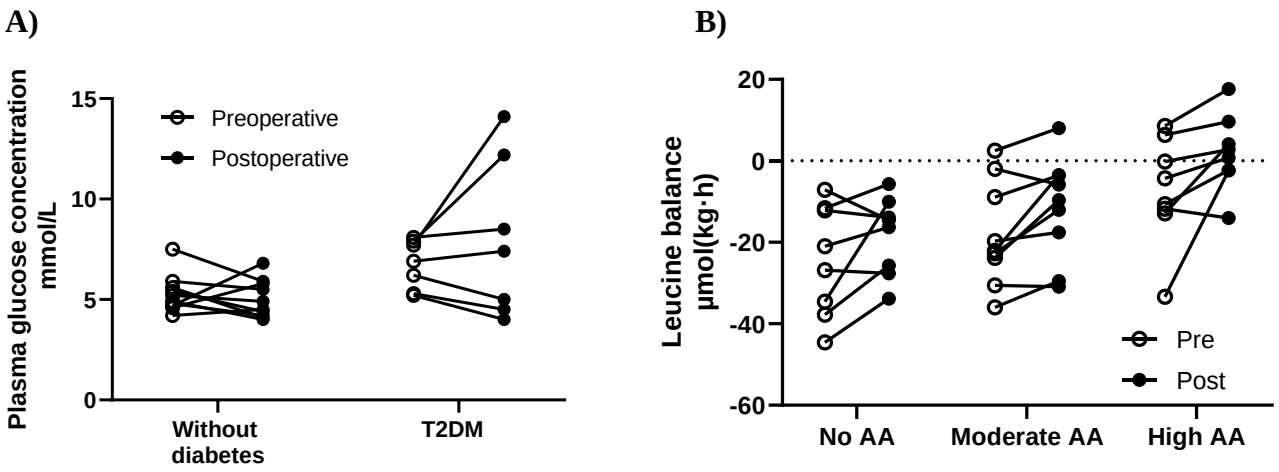
In study 2 **(Figure 7.3 A)**, the T2D group had more heterogeneous glucose parameters than the group without T2D. Variations in glucose concentration were small at baseline but increased after surgery and AA supplementation in patients with T2D, which revealed the impact of pre-existing insulin resistance on glucose responses to surgical challenge and nutritional intervention. We, therefore, stratified patients with T2D based on their preoperative glycemic control to reduce the heterogeneity. In study 4 **(Figure 7.3 B)**, the leucine balance in critically ill patients was heterogeneous both at baseline and after nutritional intervention in all three groups. Statistical analysis controlling the baseline variability was therefore applied.

Clinical studies with pre-post design commonly use the following statistical models: ANOVA-post (compares post results only), repeated measure (RM) ANOVA (compares time and intervention interactions), ANOVA-gain (compares pre-post gain scores), ANCOVA-post (compares post results, with pre as a covariate), and ANCOVA-gain (compares pre-post gain scores, with pre as a covariate). ANOVA-post is less appropriate than others because it ignores baseline information and assumes baseline homogeneity. Moreover, RM ANOVA with time ×

intervention comparisons has been statistically proven to be equivalent to ANOVA-gain analysis in a pre-post design; similarly, ANCOVA-post is equivalent to ANCOVA-gain [360].

RM ANOVA was used in study 1 to 3, whereas ANCOVA-post was used in study 4 with the baseline measures as covariates. Only ANCOVA-post can answer the research question: Are participants of the same baseline measure different after different interventions? Although the randomization procedure was carefully followed, it is unrealistic to evenly distribute all baseline characteristics across the groups, especially in a study with a small sample size [361]. ANCOVA-post was particularly important in study 4, which had substantial baseline heterogeneity due to the complexity of critical illness. O'Connell *et al.* also demonstrated that ANCOVA-gain and ANCOVA-post are more effective than RM or ANOVA-gain [362]; however, it remains inconclusive whether the former methods are superior in studies in which the baseline measures are heterogeneous not only in pooled participants but also among groups with different interventions [362,363].

Figure 7.3 Heterogeneous responses of the sample population.



The heterogeneous responses from pre- to post-intervention in study 2 **(panel A)** and study 4 **(panel B)**.

7.4 Strengths and limitations

This thesis evaluated the impact of protein or AA supplementation on nutrient kinetic responses under metabolic stress. One strength of this research is the use of stable isotope tracers to investigate protein and glucose metabolism. This technique measures the dynamic movement of nutrients (e.g. albumin synthesis rate) as opposed to their momentary status (e.g., albumin concentration). Moreover, unlike nitrogen balance that calculates the difference between nitrogen intake and excretion, protein kinetic studies with leucine tracers identify how protein is metabolized, specifically through the rate of protein synthesis and breakdown. Similarly, glucose tracers, as opposed to measuring only glucose concentration, can provide information on how much glucose was synthesized endogenously and taken up by tissues.

Our study designs are clinically relevant and can fill the gap in nutrient kinetic research on protein supplementation in specific patient populations. In study 1, we designed a piglet model that combined challenges from protein deficiency and colitis, reflecting a realistic metabolic stress state in colitis. Previous studies have rarely considered the prevalence of protein malnutrition in IBD. While NAC or cysteine has been consistently shown to ameliorate intestinal GSH status in colitis, this benefit was masked by pre-existing protein deficiency as observed in our study, leading to the speculation that other AAs might be limiting in a protein deficient state. Similarly, the benefits of 5-day protein repletion on disease recovery might also be masked by pre-existing protein-deficiency, raising the question on an optimal adaptation length of time. In study 2, we investigated patients undergoing colorectal surgery with concomitant T2D. The effect of perioperative nutritional interventions in this patient population has rarely been studied due to the high risk of postoperative hyperglycemia, although T2D is highly prevalent in patients with colorectal cancer. In addition, studies rarely have stratified patients with T2D into groups with

good and poor glycemic control, even though they may show differential responses to nutritional interventions as demonstrated in our study. In study 4, we investigated parenteral AA supplementation in critically ill patients. Previous studies have examined the effect of high protein by measuring nitrogen balance, muscle functions or clinical outcomes, but little is known about protein metabolism under critical illness. To our knowledge, our study was the first RCT to determine the effects of moderate and high AA supplementations on protein kinetics. In addition, although guidelines and experts recommended protein intake of 1.2 to 2.5 g/(kg·d), previous studies have rarely targeted protein intake as high as 2.5 g/(kg·d). Our study demonstrates the efficacy of a high target dose on protein anabolism in critically ill patients.

In addition to elucidating the effect of protein and AA supplementation on protein kinetics, other metabolic responses such as glucose kinetics, nitrogen balance, and the correlation between protein balance and clinical scores were also examined. Glucose kinetics was studied to explain the fate of AAs and to propose potential nutritional strategies for improving both protein anabolism and glucose metabolism. Moreover, measurements of nitrogen balance and protein balance rely on different assumptions but are rarely examined together in critically ill patients. We compared the results from both methods and found a strong correlation between them. Nitrogen balance confirmed the benefit of AA supplementation assessed by protein balance, and we propose this as an easy alternative for future studies when certain conditions (e.g., without severe hepatic and renal impairment) are met. In addition, clinical scores such as NUTRIC and APACHE were also found to be negatively correlated with the change in protein balance; to our knowledge, this is the first study to report such a relationship. These observations revealed that critically ill patients responded heterogeneously to protein and emphasized that personalized nutrition support is necessary for this patient population.

As mentioned in previous sections, the quality of parenteral AA solutions used in studies 2 to 4 was suboptimal due to low levels of tyrosine and high levels of glucogenic AAs. The imbalance in the AA profile may have led to increased leucine oxidation and endogenous glucose production. A parenteral AA solution using acetyl-tyrosine or tyrosine dipeptide with a lower proportion of alanine and glycine, such as Trophamine®, may be beneficial for patients undergoing surgery or with critical illness. Moreover, if a concurrent EN is administered, EN formulas with a higher protein-to-energy ratio may allow for a higher EN to PN ratio to reach the protein goal without energy overfeeding, as well as improving the AA profile. The duration of AA supplementation was short (48–72 hours) in these studies, and the long-term effect of AA supplementation on protein and glucose kinetics remains unknown.

The sample sizes were relatively small in all studies, limiting the statistical power to detect significant differences, especially when study participants were heterogeneous. In study 2, a post hoc analysis in patients with good and poor glycemic controls was conducted and revealed a significant difference in postoperative glucose concentration and endogenous glucose production after AA infusion. However, no differences were detected in leucine oxidation and glucogenic AA concentrations; we speculate that the small sample size might have masked the potential difference. Moreover, the sample size in our studies was not sufficient to determine changes in clinical outcomes such as length of stay and mortality. Nonetheless, protein kinetics studies are necessary for understanding the metabolic fate of protein and glucose, and they provide the basis for future large-scale studies to examine the optimal dose, route, formula and duration on clinical outcomes.

The sex difference was not examined in any of the studies. In study 1, male piglets were excluded due to immunological complications after routine castration at 3-7 days of age—a potential confounding factor to colitis challenge [364]. In study 3, patients were all males, and

this might be just due to chance because there are generally 3- to 4-times more males than females undergoing CABG [365]. Nonetheless, sex differences should be considered in future animal and clinical research.

Limited anthropometric measurements were conducted in studies 2-4 because most patients were sedated throughout or after the intervention. However, skin-folds and waist circumference, or surrogate muscle mass determined by ultrasound [196] can be attempted in future studies to examine the nutritional status and the effectiveness of interventions in surgical and critically ill patients.

It is essential to determine REE accurately to prescribe proper nutrition support regimens. Indirect calorimetry (IC) and predictive equations were both used to determine REE in this thesis. IC was used in surgical patients in studies 2 and 3, whereas a combination of both was used in ICU patients in study 4. IC is the gold standard for REE measurement and is calculated based on the momentary measurement of VCO_2 and VO_2. However, obtaining an accurate REE through IC is challenging in the condition of critical illness. For instance, it is recommended that IC should only be used when $FiO_2 < 0.8$, because VO_2 is calculated from FiO_2 based on the Haldane equation and would be largely overestimated with higher FiO_2 levels [366]. In fact, we frequently observed a high fluctuation of VO_2 in IC even when $FiO_2 \geq 0.5$ on day 2-3 after ICU admission. Moreover, other factors in critical illness such as hyper- or hypo-ventilation, metabolic acidosis/alkalosis, over- or under-feeding, sepsis, hyper- or hypothermia, and frequent endotracheal tube suction may also affect VCO_2 and VO_2 and result in an inaccurate REE from IC [366]. Despite these limitations, IC has been shown to be more accurate than predictive equations in critical illness [367]. Future studies should try to acknowledge these limitations while using IC rather than predictive equations to determine REE. Alternatively, a capnometer connected to the ventilator to obtain VCO_2 and

calculate REE via $VCO_2 \times 8.1$ can be used as a secondary method, as recommended by the 2019 ESPEN guideline [367].

Since ICU patients in our study were fed a mixed enteral and parenteral diet, the leucine kinetics determined by IV tracer neglected the leucine flux in the splanchnic beds. Consequently, leucine flux, protein synthesis and, to a lesser extent, leucine oxidation may have been underestimated in study 4 [283]. An intragastric (IG) leucine tracer may provide a more accurate measurement and calculation of leucine kinetics.

Despite the limitations, this thesis focused on nutrient kinetic changes via stable isotope tracers and demonstrated that protein kinetic response to protein supplementation was higher in patients with lower disease severity and risk of malnutrition. These results indicate the importance of an individualized approach in future clinical research. Finally, our work facilitates the potential application of protein or AA supplementation in patients under metabolic stress.

7.5 Future directions and clinical implications

The metabolic stress response in patients with IBD, T2D, surgery or critical illness compromises nutrient metabolism. This thesis extends our knowledge of protein and glucose kinetic responses under stress, provides potential nutritional intervention strategies for clinical practice, and lays a foundation for future mechanistic studies and large clinical trials. Each study opens specific possibilities for the future direction of related research.

Study 1 used a piglet model to examine the effect of protein repletion and NAC supplementation on colitis with prolonged protein deficiency. We demonstrated that protein repletion, but not NAC alone, improved protein anabolism and systemic GSH kinetics; however, 5-day protein repletion failed to attenuate disease activities. Future studies could extend the

acclimation time to allow piglets to adapt to the diet change and possibly surpass the active colitis state to observe any potential changes in microbiome profile and histology. Moreover, cysteine may not be the only rate-limiting AA for GSH synthesis in protein-deficient piglets. Supplementation of a protocol of GSH-related AAs, such as glutamate and glycine, should be compared with cysteine or NAC supplementation alone to see whether other AAs could be rate-limiting. In addition, the consequence of high cysteine resulted from NAC supplementation in these piglets remains unknown and should be investigated further [368].

Study 2 illustrated that AA supplementation increased protein balance while preventing hyperglycemia in patients with good glycemic control undergoing colorectal surgery. T2D is highly prevalent in patients undergoing colorectal surgery, and some cases may be undiagnosed or poorly controlled. Future studies should stratify patients based on their glycemic control using an HbA1c cut-off of 6.5%. HbA1c is a better predictor of insulin sensitivity during and after surgery than a one-time glucose measure before surgery because the concentration of glucose could be affected by many factors such as diet and medications right before sampling. Moreover, although protein balance assessed by leucine kinetics increased from baseline in this study, the values remained negative in all patients. In order to achieve a higher protein balance, patients with good glycemic control should be infused with glucose concomitantly with AAs to prevent AAs from entering gluconeogenesis. In addition, oral antihyperglycemic agents and insulin that are taken before surgery could be a confounding factor of the metabolic responses in patients with T2D and should be examined in future studies.

Study 3 assessed patients undergoing cardiac surgery while receiving insulin clamp and demonstrated that AA infusion at a higher dose of 35% REE significantly increased plasma AA availability compared to a moderate dose of 20% REE. However, some AA (e.g., tyrosine)

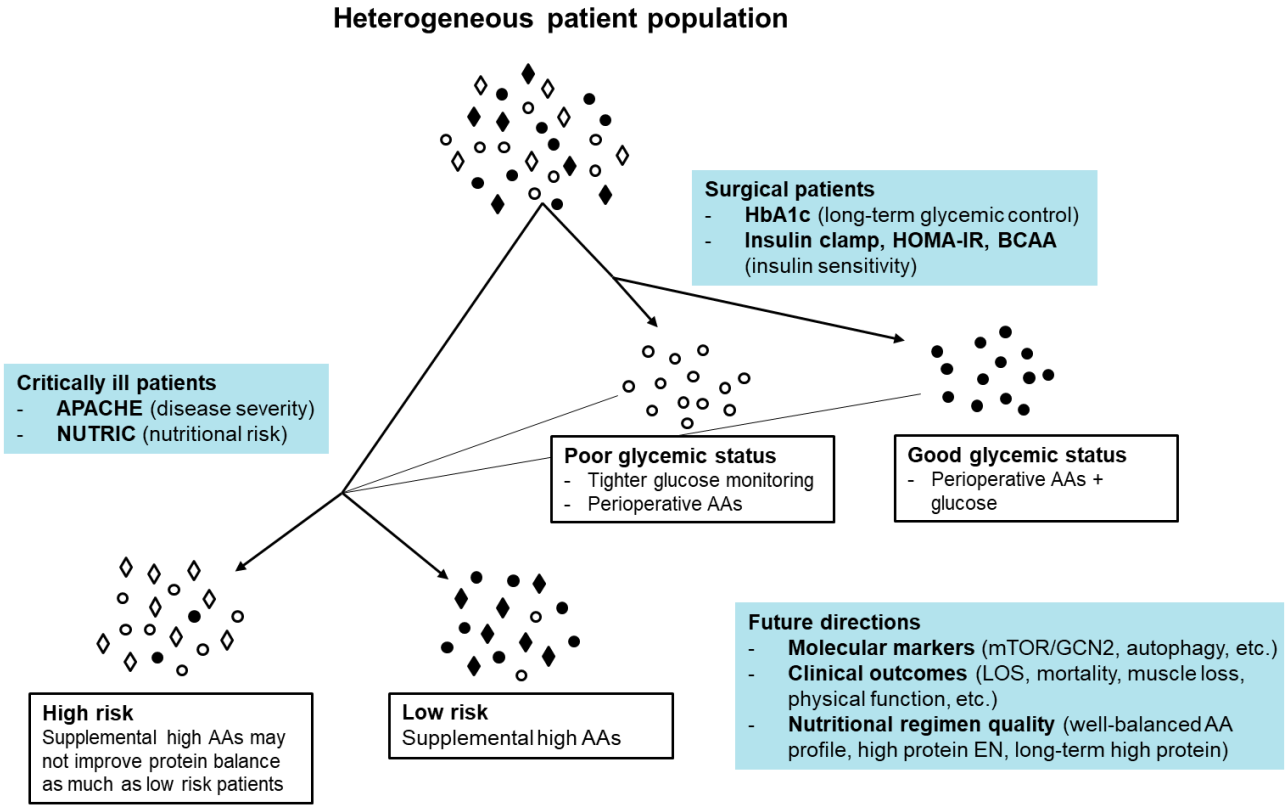
concentrations did not differ from baseline measures under either treatment, revealing the importance of a balanced parenteral AA profile. Future studies should assess the quality of parenteral AA solution and, if necessary, add additional supplemental AA such as tyrosine in the form of acetyl- or tyrosine dipeptide to balance the AA profile and prevent oxidation of other AAs.

Study 4 reported that high AA supplementation at 1.5 g/(kg·d) in addition to standard EN increased total nominal protein intake to 2.4 g/(kg·d), leading to increased protein balance (assessed by leucine kinetics) and nitrogen balance in critically ill patients; however, this was not observed in AA supplementation at 0.9 g/(kg·d) with total nominal protein intake at 1.6 g/(kg·d). Large-scale RCTs comparing the effect of low and high protein doses on clinical outcomes should be conducted to provide guidance in clinical practice. Since high AA resulted in higher leucine oxidation and endogenous glucose production rates, future studies should avoid limiting AAs at the study design stage to prevent other AAs from becoming excessive and contributing to oxidation and gluconeogenesis.

A more personalized nutrition support approach is proposed for future clinical research and practice in a heterogeneous patient population to better adapt to individual needs under metabolic stress **(Figure 7.5)**. Preoperative glycemic control status in surgical patients should be assessed using an HbA1c cut-off of 6.5% to identify undiagnosed or poorly controlled T2D and plan for nutrition support before, during and after surgery. Insulin clamp, HOMA-IR, and BCAA concentrations can be used to provide complementary information on insulin sensitivity. Patients with poor glycemic control require tighter glucose management before surgery and intensive monitoring during and after surgery to achieve optimal protein and glucose metabolism. These patients will also benefit from receiving perioperative AAs without glucose to increase protein balance. Alternatively, patients with good glycemic status may benefit from a combined infusion

of AAs and glucose perioperatively to maximize protein anabolism. Moreover, critically ill patients, either medical or surgical, should be screened with NUTRIC and APACHE scores at admission to divide them into high- and low-risk groups. Patients with high risk may not experience improved protein balance after high protein intake as much as low-risk patients; therefore, their nutrition support regimens should be closely monitored and adjusted accordingly.

Figure 7.5 Proposed nutrition support strategies to achieve better protein and glucose metabolism in a heterogeneous patient population.



Heterogenous patients undergoing surgery may be stratified based on glycemic status assessed from insulin clamp, HbA1c and BCAA concentration to predict postoperative glucose kinetics. Heterogenous critically ill patients may be stratified based on APACHE and NUTRIC scores to identify high or low-risk patients. Note that the strategies mentioned above were proposed for optimal glucose and protein kinetics responses. Future studies can explore better nutrition support strategies based on molecular markers, clinical outcomes or investigation into the quality of nutritional regimens. Adapted from [11,168]. Abbreviations: AA, amino acids; insulin clamp, hyperinsulinemic-normoglycemic clamp; HbA1c, hemoglobin; BCAA, branched-chain amino acids; APACHE, acute physiology and chronic health evaluation; NUTRIC, nutrition risk in critically ill; mTOR, mammalian target of rapamycin; GCN2, general control nonderepressible 2; LOS, length of stay; EN, enteral nutrition.

7.6 Conclusion

In conclusion, protein or AA supplementation improved protein anabolism under metabolic stress response induced by colitis, surgery, or critical illness. The use of stable isotope tracers provided quantitative evidence on the metabolic responses of protein and glucose during stress and after nutritional interventions. The efficacy of protein or AA supplementation depended on the baseline disease severity, nutritional status, and the supplementation duration, amount and quality. The results from this thesis may help to determine the optimal nutrition regimens in future clinical research in patients under metabolic stress. The results also provide a protein kinetic basis for nutritional intervention in the future investigation of nutrient-sensing mechanisms and clinical outcomes.

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