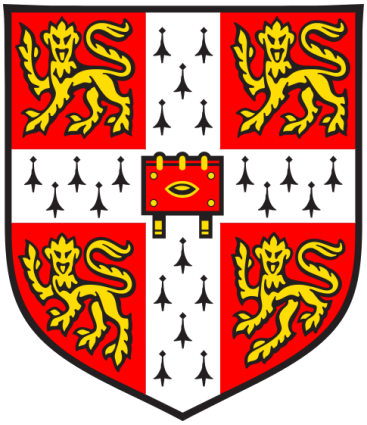
The relationship between enteral nutrition, energy metabolism and gut homeostasis during the course of critical illness.



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DECLARATION OF ORIGINALITY

I declare that this report is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text and bibliography.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my report has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text

ABSTRACT

Nutrition has an indirect effect on the gastrointestinal function of the host and thereby on health, mainly by influencing the composition and activity of the human gut microbiota. The aim of this PhD project was to investigate the effect of nutrition as a factor affecting the intestinal microbiome-host relationship in critically ill children and whether it has an impact on clinical disease severity.

The balance between requirement and delivery of energy and macronutrients was assessed in a cohort of 124 critically ill children. Then an integrated approach of metataxonomics and metabolomics analysis was undertaken to examine how feeding during critical illness affects the gut-host relationship. Collection of faecal samples was required for the assessment of faecal calprotectin, gut microbiota and their metabolites while serum samples were used for the analysis of inflammatory cytokines, and intestinal injury biomarkers.

Overall this project has recorded a cross-link between feed, gut homeostasis with systemic inflammation and host metabolism. Within the feed delivered, fat delivery was often above requirements compared to protein and carbohydrate. In addition, both protein deficit and higher delivery of fat were associated with elevation in the levels of pro-inflammatory cytokines. The results also showed that abnormalities in gut health biomarkers were associated with elevation in inflammatory cytokines. Finally this study also recorded a profound loss of diversity in the faecal microbiome of critically ill children. This was associated with the loss of key commensal species and increased levels of opportunistic pathogens. Consequently resulted in reduced functionality of the gut microbiome manifested by reduced production of SCFAs and abnormalities in BAs metabolism. The current study showed for the first time that energy underfeeding appeared to influence the microbial composition of critically ill children.

In conclusion this work provided an insight about the potential contribution of nutrition as a factor to improve the disease state in critically ill children, if targeted to modulate gut microbiome and host response to critical illness.

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Of course I would like to thank my family, who although far away, has supported me throughout my PhD. Words cannot express how grateful I am to my dad for being my rock and my mum for her constant love and support, my grandma your prayers are what sustained me this far. Of course I cannot forget my two beloved sisters who always encouraged and supported me during the rough times.

I would like to dedicate this work to my grandfather who I lost last year; if it wasn't for him I would not have the courage to pursuit a research degree. You were my number one supporter and my personal cheerleader. I miss you every day and I wish if you could see me finish my PhD.

TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS

ASPEN	American Society for Parenteral and Enteral Nutrition
BAs	Bile acids
CA	Cholic acid
CDCA	Chenodeoxycholic Acid
CHO	Carbohydrate
CRP	C reactive protein
EN	Enteral nutrition
ESPGHAN	European Society for Paediatrics Gastroenterology, Hepatology and Nutrition
FC	Faecal calprotectin
FiO_2	Fraction of inspired oxygen
FSMP	European Commission of Food for Special Medical Purposes
GCDCA	Glycochenodeoxycholic Acid
GHICI	Gut Homeostasis in Critical Illness
GI	Gastrointestinal
GLP-1	Glucagon Like Peptide-1
GPR	G protein-coupled receptors
IC	Indirect calorimeter
IL-10	Interleukin-10
IL-6	Interleukin-6
IL1-β	Interleukin-1 beta
ILCA	Isolithocholic Acid
LCA	Lithocholic acid
LPL	Lipoprotein Lipase
MEE	Measured energy expenditure
MOF	Multi Organ Failure
NHS	National Health Service
PCA	Principal component analyses
PERMANOVA	Permutational multivariate analysis of variance
PICU	Paediatric Intensive Care Units

PIM2	paediatric index mortality 2
PLSDA	Partial Least Square Discriminant Analysis
PMODS	Paediatric multiple organ dysfunction score
PN	parenteral nutrition
PREE	Predicted Resting Energy Expenditure
PYY	Peptide YY
REE	Resting energy expenditure
RQ	Respiratory quotient
SACN	Scientific Advisory Committee on Nutrition
SCFAs	Short chain fatty acids
SDD	Selective decontamination of the digestive tract
SIRS	Systemic Inflammatory Response Syndrome
TCA	Taurocholic Acid
THCA	Taurohyocholic Acid
TNF-α	Tumour necrosis factor alpha
UDCA	Ursodeoxycholic acid
VCO_2	Volume of Carbone dioxide
VO_2	Volume of oxygen
WHO	World health organization
12 DHCA	12 Dehydrocholic Acid
23 nor 5ß- CA-3α, 12a-diol	23-nor-5b-Cholanic Acid-3a, 12a-diol
3,12-DKCA	3,12-Diketocholanic Acid
3a-H-12 KLCA	3a-Hydroxy-12 Ketolithocholic Acid
3aH-6,7-DCA	3a-Hydroxy-6,7-DiketoCholanic Acid
3DHCA	3 Dehydrocholic Acid
3KCA	3-Ketocholanic Acid
5ß- CA-3ß, 12a-diol	5beta-Cholanic Acid-3beta, 12a-diol

Papers arising from this thesis:

- **Association between enteral macronutrient delivery and inflammatory response in critically ill children**

  Sara Zaher, Deborah White, Jenna Ridout, Frederic Valla, Ricardo Branco, Rosan Meyer, Nazima Pathan.

  (Accepted in Clinical Nutrition Journal).

- **Relationship between inflammation and metabolic regulation of energy expenditure in critically ill children**

  Sara Zaher, Deborah White, Jenna Ridout, Frederic Valla, Ricardo Branco, Rosan Meyer, Nazima Pathan.

  (Submitted to Clinical Nutrition Journal, under review).

1 Chapter one: Introduction

Critical illness represents the most complex derangement of cellular and organ function, and consumes a large portion of the National Health Service (NHS) budget. There are over 20,000 admissions to Paediatric Intensive Care Units (PICUs) in the UK annually (Parslow & Draper 2017). The care given in PICUs is costly and resources are often limited (Turner et al. 2016). The majority of children admitted to PICU have a brief stay and recover well, but a significant proportion may develop severe multi-organ failure (MOF) and require prolonged hospitalisation (Villeneuve et al. 2016; Hamshary et al. 2017). MOF in critical illness imposes significant clinical challenges, often persisting beyond the period of acute illness (Taylor et al. 2003; Typpo et al. 2009).

The human intestine is host to a large and complex population of resident microbes (the gut microbiota). These bacteria (microbiota), and their metabolic products (together comprising the microbiome) have a complex synergistic relationship with the host (Clark & Coopersmith 2007). The intestinal microbiome contributes significantly to the nutritional, metabolic, endocrine and inflammatory activity of the host (Ramakrishna 2013; Krajmalnik-Brown et al. 2012; Vinolo et al. 2011; Byrne et al. 2015). Because of its array of beneficial functions, the microbiome is considered an organ that is important for maintaining the health and wellbeing of the host.

Dysbiosis of the intestinal microbiome may have systemic consequences, due to extensive vascular, nerve and lymphatic connections between the intestinal tract and other distal organs (Petrova & Koh 2018; Furness 2008). Emerging evidence suggests that the intestinal microbiome may interact with host factors during critical illness to drive MOF and other adverse outcomes (Clark & Coopersmith 2007; Mittal & Coopersmith 2014; Dickson 2016; Wolff et al. 2018). There are many examples of how the composition and diversity of the microbiome is associated with a range of diseases in both children and adults, including obesity, heart disease, kidney injury and asthma

(Kasai et al. 2015; Parekh et al. 2014; Kelly et al. 2016; Zhang et al. 2018; Noval Rivas et al. 2016).

A better understanding of the relationship between gut failure and multi-organ dysfunction in critical illness will allow us to define criteria for the therapeutic modulation of the intestinal environment. Whilst many of the triggers of intestinal dyshomeostasis may be unchangeable in critical illness, it seems a common-sense approach to support the intestinal environment in patients with organ failure. Nutrition is an important factor determining intestinal microbiota composition. It plays a critical role in the colonisation, maturation and stability of the gut residing microbes (Zhang & Yang 2016). Enteral feeding is the mainstay of nutrition in most critically ill children, with 73% receiving enteral nutrition (EN), commenced and progressed where possible (Kerklaan et al. 2016). Therapeutic modulation of the intestinal environment by EN could be a potential effective therapy to restore homeostatic state in affected children.

EN modulation of the body's response has been tried with immune nutrition in critically ill children and it has been associated with lower infection rates and improvements in nutritional indices (Briassoulis et al. 2005; Jacobs et al. 2013). Nonetheless, Carcillo et al. (2012) showed that a targeted EN intervention was not associated with clinical benefits and the assigned EN intervention made no difference in the overall population with respect to the onset of nosocomial infection or sepsis (Carcillo et al. 2012). Targeted manipulation of the intestinal microbiome by EN has not been explored fully in this population. Given the extensive vascular and lymphatic links between the intestines and other organs (Petrova & Koh 2018), it is possible that enhancing the growth of commensal bacteria and their metabolites by EN could be of systemic clinical benefit.

1.1 Critical illness

1.1.1 Pathophysiology of critical illness

The acute insult of critical illness is characterised by disturbances in oxygen supply and consumption, resulting in reduced tissue oxygenation (Top et al. 2011; Hayes et al. 1994). The body adopts different mechanisms to maintain tissue oxygenation and

normal organ function (Mansjoer & George 2008). This is characterised by dynamic changes in host physiology, including the induction of neuro-endocrine, immunologic and metabolic responses (Joosten et al. 2016).

The stress response varies according to the phase of illness. The recent European Society for Paediatrics Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines on paediatric parenteral nutrition (PN) proposed the following three phases of stress response in critically ill children (Mesotten et al. 2018):

- **The acute phase:** the resuscitation phase when the child requires vital organ support.

- **The stable phase:** characterised by stabilisation and weaning from vital support.

- **The recovery phase:** the mobilisation phase where the neuro-endocrine, immunologic and metabolic responses are resolved (Mesotten et al. 2018; Joosten et al. 2016).

The acute phase of stress response may last from hours up to days, depending on the severity of insult (Joosten et al. 2000). It is initiated by activation of the inflammatory cytokine and the central nervous system to stimulate secretion of counter-regulatory hormones, aimed at maintaining tissue perfusion and delivering energy substrate to the injured organ (Chwals 2014; Joosten et al. 2016). It is associated with hypovolemia and hypoxia. In this phase, there is a decrease in body temperature, cardiac output, oxygen consumption and energy expenditure (Stahel et al. 2010; Chwals 2014; Şimşek et al. 2014; Sobotka et al. 2009). Abnormalities of the endocrine and metabolic responses in critically ill children were recorded in non-survivors compared to survivors (Verhoeven et al. 2011; Joosten et al. 2000). This was manifested by variation in glycaemic control and lipid profiles, as well as cortisol and adrenocorticotropic hormone responses (Verhoeven et al. 2011; Joosten et al. 2000).

The stable phase of critical illness is characterised by a reduction in serum inflammatory

cytokines and suppression of catabolic counter-regulatory hormone stimulation (Preiser et al. 2014). The duration of the stable phase could be from days to weeks (Marcin et al. 2001). Lastly, the recovery phase is the mobilisation and anabolic phase, where signs of the stress response are resolved. It is also associated with an increased rate of protein synthesis, tissue repair and restoration of mitochondrial function (Joosten et al. 2016; Singer 2014).

1.1.2 Inflammatory response in critically ill children

The acute phase stress response is characterised by the aggressive release of inflammatory mediators. These cytokines are the main regulators of the immune response to the acute insult; they can either have a protective or damaging effect (Brix-Christensen 2001). The acute pro-inflammatory response is typically followed by an induction of anti-inflammatory mediators (Oberholzer et al. 2000). The interaction between the pro-inflammatory and anti-inflammatory mediators appears to determine the severity of outcome in critically ill patients (Oberholzer et al. 2000; Wang & Ye 2015).

The term Systemic Inflammatory Response Syndrome (SIRS) refers to the widespread of inflammation in areas remote form the primary injured site (Ye-Ting & Dao-Ming 2018). The dissemination of inflammation may result in the impaired function of other distal organs, or what is known as MOF syndrome (Figure 1.1). The clinical outcome of the inflammatory response depends mainly on the intensity and concentration of released cytokines and the type of cell they are acting on, as well as the balance between pro- and anti-inflammatory responses (Brix-Christensen 2001). Uncontrolled inflammation is not only associated with adverse outcomes for tissue and organ function but it also affects host metabolism (Wang & Ye 2015; Chaudhry et al. 2013).

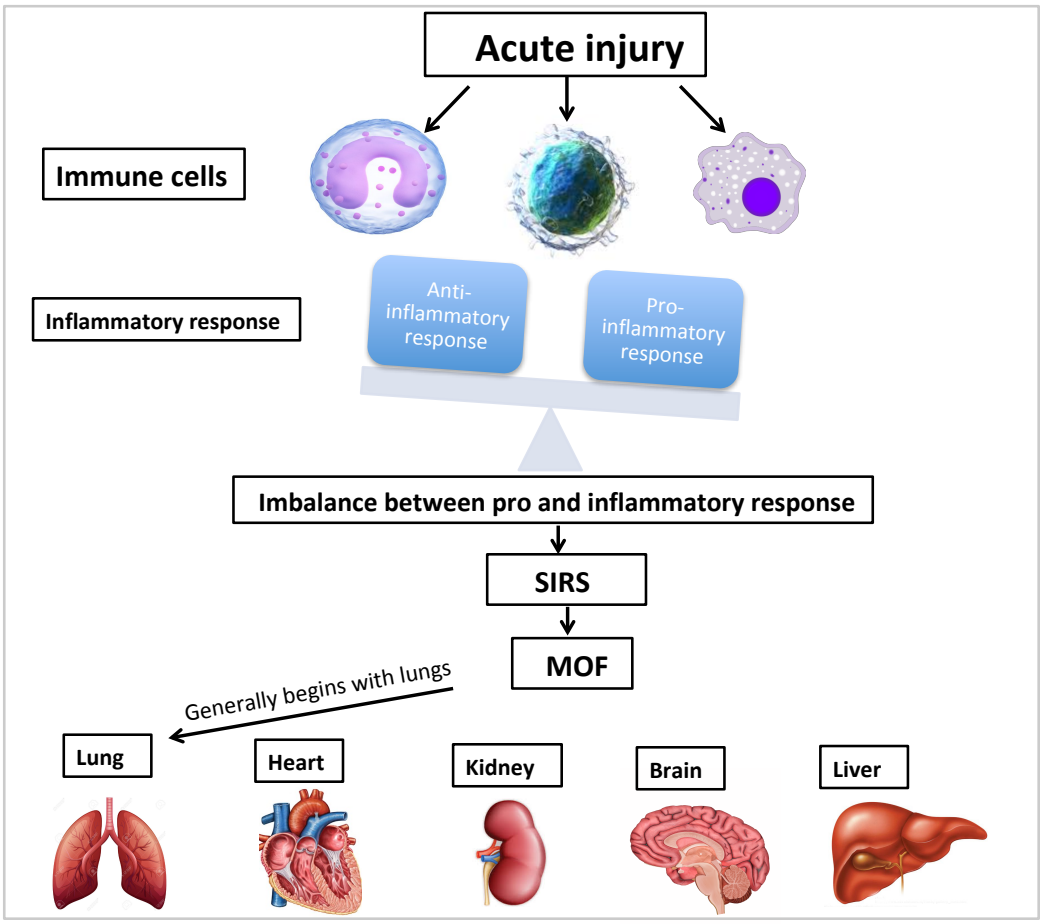

Figure 1.1 Development of SIRS and MOF in critical illness

Figure 1.1 shows the process of SIRS and MODS development in critically ill patients. Following the acute insult the immune cells, including monocytes, macrophages and lymphocytes, produce pro- and anti-inflammatory mediators. The uncontrolled dissemination of inflammatory mediators to distal organs affects their function and results in MOF. This generally begins with lung failure followed by failure of other organs.

* The pictures used in this figure have been obtained from MedScape.org

1.1.3 Metabolic derangement in paediatric critical illness

Following acute injury, the release of inflammatory cytokines triggers the secretion of counter-regulatory hormones. As a result, the body adopts a cascade of metabolic events. This includes increased glycogenolysis, gluconeogenesis, fatty acid oxidation and ketogenesis as well as reduced energy expenditure (Srinivasan 2012; Caresta et al. 2007; Briassoulis et al. 2000; Chwals 2014; De Cosmi et al. 2017) (see Figure 1.2).

1.1.3.1 Alteration in the pattern of energy expenditure in critically ill children

The typical response to acute injury in adults is a reduction in energy expenditure during the ebb phase followed by an increase in energy expenditure after the restoration of oxygen transport, where the body enters the flow phase. However, this adaptive pattern of energy expenditure is different in critically ill children; the dominant hyper-metabolic response seen in critically ill adults is not evident in children's studies (Briassoulis et al. 2000; Taylor et al. 2003; Hardy Framson et al. 2007). In fact, these studies have found that critically ill children have reduced energy expenditure during the acute phase of illness. However, energy expenditure may be increased transiently (4 to 6 hours) after acute injury (Pierro 2002). Reduced energy expenditure in critically ill children could be due to lack of physical movement, decreased work of breathing, and maintaining a thermo-neutral environment in PICU settings (Briassoulis et al. 2000). However, the most important distinctive physiological function in children in comparison to adults is somatic growth. During critical illness, growth, which is an anabolic process, is inhibited and restored again only after the acute metabolic stress has been resolved (Chwals 2014). Growth accounts for 30-35% of total energy expenditure in infants (Verhoeven et al. 1998). Unlike adults, critically ill children have the ability to reduce their energy expenditure through re-channelling the spare energy used for growth (Briassoulis et al. 2000; Vernon & Witte 2000). However, this may not be the case for older children as energy expenditure used for growth is very small and accounts for only 5% of the total energy expenditure (Butte et al. 2000; Hardy Framson et al. 2007).

The pattern of energy expenditure is variable during the clinical course of critical illness. Energy expenditure is increased by 1.3 times in the stable phase and by 2 times in the recovery phase as the body shifts from catabolic to anabolic state (Joosten et al. 2016; Joosten et al. 2018).

1.1.3.2 Shift in fuel utilisation

Critically ill children utilise fat preferentially as a substrate for their energy metabolism, thus the acute metabolic response is characterised by increased lipolysis and fatty acid

oxidation relative to glucose oxidation (Coss-Bu et al. 2001; Caresta et al. 2007). Lipolysis is accelerated in the early phase of illness under the hormonal control of adrenocorticotropic hormone, cortisol, catecholamine, glucagon, growth hormone and insulin resistance (Şimşek et al. 2014). In addition, the release of pro-inflammatory cytokines affects fatty acid uptake by adipose tissues and muscles by suppressing the activity of lipoprotein lipase in these sites (Jan & Lowry 2010). Muramoto et al. (2016) reported an elevation in serum triglycerides levels in critically ill children in response to an increase in inflammatory markers, which could be related to the effect of inflammatory cytokines on suppressing lipoprotein lipase activity.

Loss of endogenous protein stores is another feature of the acute metabolic response in children. Amino acids are mobilised to the liver where they provide substrate for the synthesis of acute phase proteins and glucose (Şimşek et al. 2014; Sobotka et al. 2009; De Cosmi et al. 2017). The risk of muscle atrophy is substantially increased during the stable phase of critical illness where the anabolic process is initiated (De Cosmi et al. 2017; Joosten et al. 2016; Jotterand Chaparro et al. 2016).

Glucose is a particularly important substrate for glycolytic tissues in which mitochondrial respiration is not possible, such as in leukocytes, erythrocytes, macrophages and the renal medulla. Glycolysis in these tissues leads to the release of lactate, which is returned to the liver and reconverted into glucose by the lactic acid cycle (Şimşek et al. 2014; Sobotka et al. 2009). In hypoxic states, mitochondrial oxidative phosphorylation is limited, limiting pyruvate metabolism, which as a result is preferentially reduced to lactate, resulting in increased concentrations of arterial lactate (Fuller & Dellinger 2012). Lactate accumulation reflects the state of mitochondrial oxidative phosphorylation in cells. It serves as a biomarker of mitochondrial function and a predictor of disease severity in the critically ill population (Pang et al. 2014).

1.1.3.3 Loss of glycaemic control in critically ill children

Loss of glycaemic control is frequently recorded in critically ill children due to the initiation of the inflammatory response, the dysregulation of endocrine stimulation and

substrate metabolism, as well as peripheral insulin resistance (Faustino & Apkon 2005; Patki & Chougule 2014; Srinivasan 2012). Hyperglycaemia is a common feature of the stress response in critically ill children. It is usually induced by the massive induction of counter-regulatory hormones. Increased blood glucose levels also might be secondary to the state of insulin resistance (Patki & Chougule 2014). Blood glucose levels appear to spontaneously normalise within 48 hours in septic children (Verhoeven et al. 2011). Tight glycaemic control has been shown to significantly reduce the rate of infection and length of PICU stay in children (Vlasselaers et al. 2009; Day et al. 2008; Jeschke et al. 2010).

1.1.3.4 Changes in gut endocrine function during critical illness

Glucagon-Like Peptide-1 (GLP-1) and Peptide YY (PYY) are gut hormones secreted from intestinal L-cells in response to nutrient stimulation. They play an important role in the regulation of the metabolism by controlling the appetite, gastric motility and to a lesser extend energy expenditure. Generally GLP-1 and PYY induce a state of negative energy balance by suppressing appetite, inhibiting gastric emptying and increasing energy expenditure (Vrieze et al. 2010; Barrera et al. 2011; Parekh et al. 2014).

New emerging studies have recorded an alteration in gut hormone levels in both critically ill children and adults (Lebherz et al. 2017; Nematy et al. 2006; Kahles et al. 2014). The inflammatory mediators are thought to contribute to the induction of GLP-1 and PYY secretion through stimulation of intestinal L-cells (Ellingsgaard et al. 2011; Nguyen et al. 2006). Studies also suggested that SCFAs produced from the bacterial fermentation of dietary carbohydrates may stimulate the secretion of GLP-1 and PYY through the activation of GPR41 and GPR43 receptors in the intestines (Kim et al. 2014, Tolhurst et al. 2012, Vrieze et al. 2010), leading to up regulation of GLP1 and PYY synthesis by intestinal L cells (Kim et al. 2014, Vrieze et al. 2010).

Kahles et al. (2014) indicated that septic adult patients had statistically higher levels of GLP-1 compared to non-septic. GLP-1 secretion also appeared to be increased in

response to endotoxin stimulation (Nguyen et al. 2014). In critically ill adults, the levels of GLP1 were positively correlated with the severity of disease (Kahles et al. 2014; Lebherz et al. 2017). These aforementioned studies suggest a potential association between gut hormones and immune system activation.

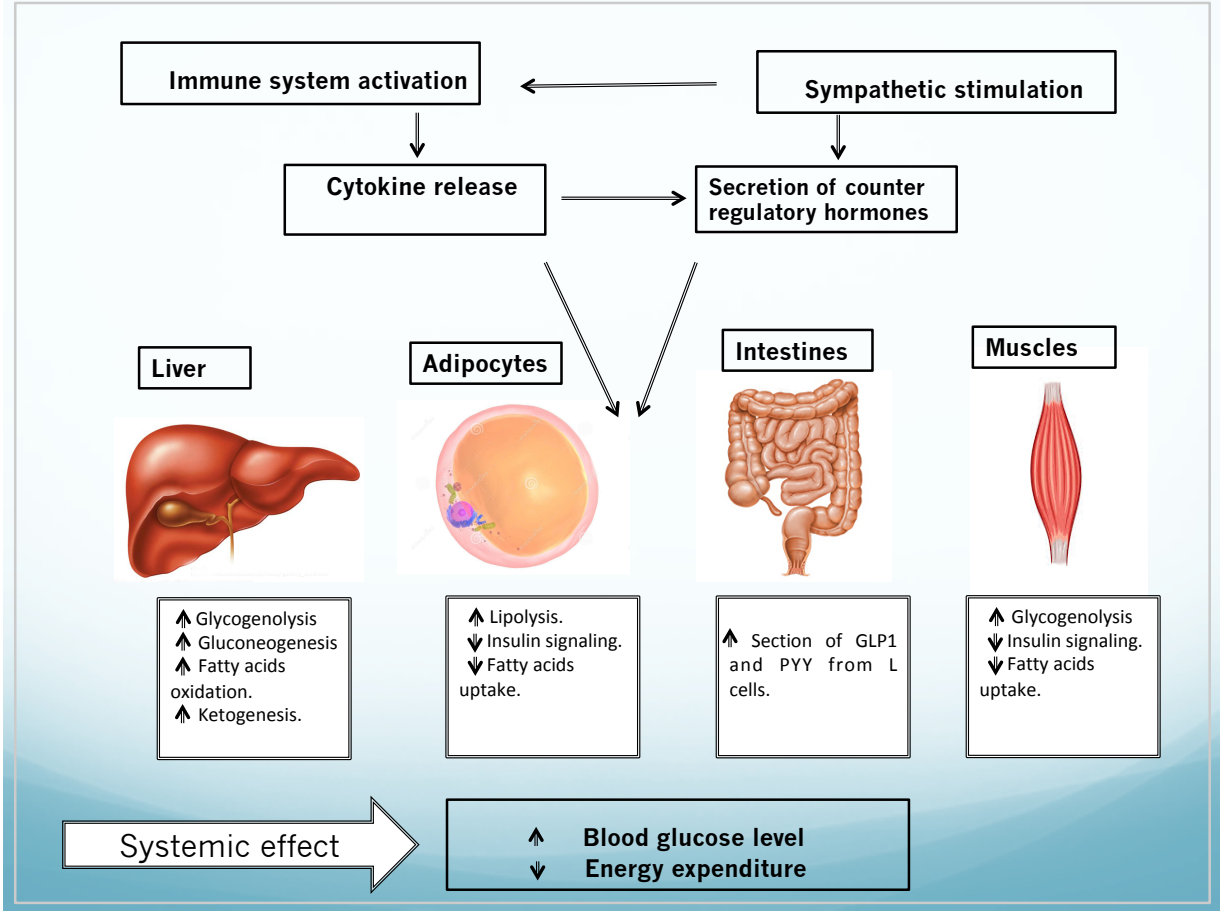

Figure 1.2 Summary of the metabolic response during the acute phase of illness

Figure 1.2 shows the adaptive metabolic cascade in response to acute illness. The metabolic response is initiated by the neurological stimulation of inflammatory cytokines and counter-regulatory hormone release, resulting in metabolic alteration of a local and systemic effect.

* The pictures used in this figure have been obtained from MedScape.org

1.1.4 Key difference in the stress response between children and adults

The acute stress response is distinctive between adults and children in many aspects. Therefore, results from adult studies may not be applicable in critically ill children. The initial stress response in critically ill children appears to be different from that of adults

(Top et al. 2011). Septic shock in critically ill children is characterised by a hypo-dynamic state, while in adults hyper-dynamic is the predominant pattern of hemodynamic instability (Mercier et al. 1988; Top et al. 2011). To compensate for hemodynamic instability, adult patients increase their cardiac output through ventricular dilatation and increased heart rate, while children lack this adaptive mechanism of ventricular dilatation (Top et al. 2011). Unlike adults, the release pattern and plasma concentrations of the pro-inflammatory response in children are characterised by a large variation (Brix-Christensen 2001; Brix-Christensen et al. 1998; Ozawa et al. 2000). Critically ill children utilise fat preferentially as a substrate for their energy metabolism, thus the acute metabolic response is characterised by increased lipolysis and fatty acid oxidation relative to glucose oxidation (Coss-Bu et al. 2001; Caresta et al. 2007). In critically ill adults, on the contrary, the rate of carbohydrates oxidation is increased more than lipid oxidation during the early phase of illness (Preiser et al. 2014; Tappy et al. 1998). Furthermore, the dominant hyper-metabolic response in critically ill adults is not evident in children (Briassoulis et al. 2000; Taylor et al. 2003; Hardy Framson et al. 2007). Due to variations in many parameters of the stress response between adults and children, the results derived from adult studies may not be relevant to the child population. Therefore, there is an urgent need for studies particularly performed with critically ill children.

1.2 Disruption of human microbiome in critical illness

Changes to the microbiome in response to environmental influences can affect overall health (Parekh et al. 2014; Kelly et al. 2016; Noval Rivas et al. 2016). Critical illness is associated with pathophysiological effects that are damaging to the intestinal microbiome. These include ischemia, hypoxia and hypotension along with the iatrogenic effects of therapeutic agents and the lack of enteral feeds (Krezalek et al. 2016; Ferrer et al. 2016; Maier et al. 2018). As a result, of alteration to intestinal microbial composition, the integrity of the intestinal epithelial barrier and intestinal mucosa absorptive function are compromised (Lupp et al. 2007; Kohler et al. 2005; Morowitz et al. 2011; Lara & Jacobs 2016; Btaiche et al. 2010).

Generally, the intestinal microbiome of critically ill children and adults is characterised by low diversity (Jacobs et al. 2017; Rogers et al. 2016). Critically ill children and adults appear to rapidly colonised with opportunistic pathogens (Ojima et al. 2016; Rogers et al. 2016). In addition, a loss of intestinal anaerobic bacteria has been frequently detected during the course of critical illness, and accordingly the colonic degradation capacity of undigested carbohydrates into lactic acid and short chain fatty acids (SCFAs) may be affected (Shimizu et al. 2006; Iapichino et al. 2008; Hayakawa et al. 2011; Yamada et al. 2015). The interaction between the host and intestinal microbiome has been shown to be highly relevant to pathophysiology and outcomes in severe and critical illness. Damage to the intestinal mucosal lining in severe disease may lead to the translocation of bacteria or their fragments into the bloodstream and this may contribute to inflammation, sepsis, MOF and death (Clark & Coopersmith 2007; Pathan et al. 2011; Deitch 2012). Depletion of the normal gut microbial population and metabolites, and alterations to the intraluminal gut environment have been shown to be associated with adverse outcomes in critically ill adults (Reintam et al. 2009). Indeed the gut is seen as an important driver of organ failure in critical illness (Mittal & Coopersmith 2014), (see Figure 1.3).

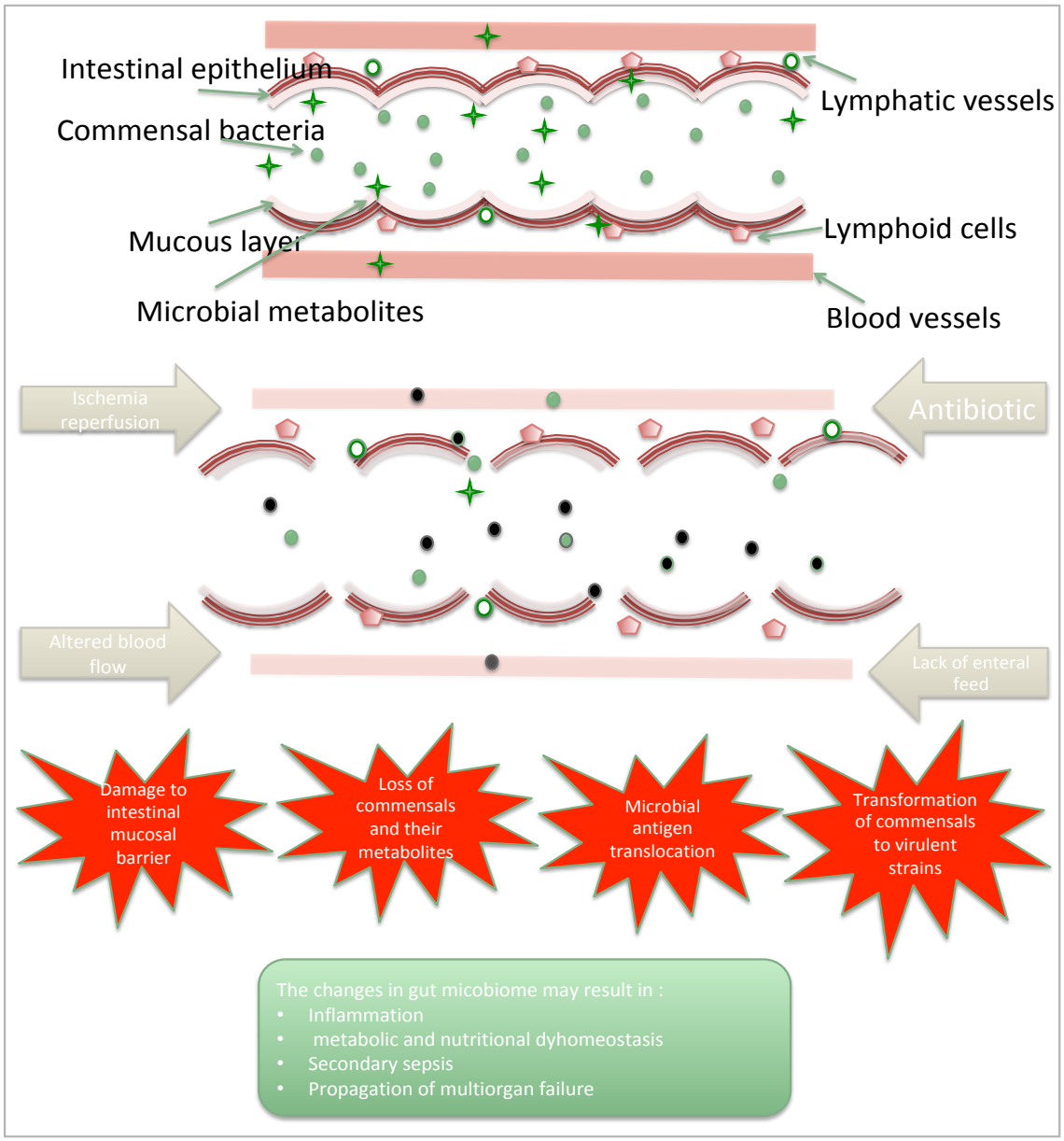

Figure 1.3 How critical illness affects the gut microbiome

Figure 1.3 summarises the changes that occur in the gut microbiome during critical illness. In response to acute insult, factors such as reperfusion, antibiotic therapy and the lack of enteral feed result in damage to the intestinal barrier and changes in microbial composition. Alterations to the gut microbiome are strongly related to the exacerbation of the inflammatory response, metabolic dysregulation sepsis and the propagation of MOF.

1.2.1 Factors affecting intestinal microbiome

1.2.1.1 Disease state

The intestinal microbiome is altered in response to the pathophysiological effects of critical illness. The pro-inflammatory stimulation appears to influence the tight junctions between enterocytes and results in increased epithelial permeability, the infiltration of luminal antigens and the induction of intestinal inflammation (Clark & Coopersmith 2007). Intestinal inflammation and hypoxia have significant impact on modulating the gut microbiome and microbiota composition during critical illness (Lupp et al. 2007; Kohler et al. 2005). Both promote a shift from commensal anaerobes to opportunistic pathogens (Lupp et al. 2007; Kohler et al. 2005). Furthermore, intestinal inflammation alters the responses of gut hormones and neurotransmitters and accordingly impacts gut motility (Btaiche et al. 2010). It was speculated that inflammation supresses the expression of certain hormones such as PYY by mature endocrine cells, resulting in impaired gut motility (El-Salhy et al. 2017). The activation of inflammatory cytokines and increased levels of luminal catecholamine have also been shown to promote the growth of pathogenic species (Dickson 2016; Alverdy et al. 2000). Impaired mucosal immunity and decreased IgA production are other features of acute illness, and are associated with decreased ability to eliminate intestinal pathogens (Dickson 2016).

1.2.1.2 Medication

The pharmaceutical agents commonly used in ICU such as antibiotics, inotropic drugs and vasopressors are known to impact the human microbiota (Lupp et al. 2007; Kohler et al. 2005; Sandrini et al. 2015; Freestone et al. 2008; Maier et al. 2018).

Critically ill patients are frequently treated with antibiotics. It has been estimated that the total antibiotic consumption in PICUs is nearly ten times greater than in general hospital wards (Malacarne et al. 2004). Several studies have recorded profound changes in gut microbial communities following the administration of antibiotic treatment, which can subsequently result in important functional alterations of the gut microbiome and

increase susceptibility to gastrointestinal infections (Palmer et al. 2007; Preidis & Versalovic 2009). The effect of antibiotic exposure on gut microbiota has been widely investigated. The main phyla influenced by antibiotic treatment appear to be *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria* (Theriot et al. 2014; Panda et al. 2014; Theriot et al. 2016). In a study conducted on antibiotic-treated infants, a massive reduction in total bacterial densities was observed after antibiotic treatment, accompanied by delayed colonisation by beneficial species such as *Bifidobacteria* and *Lactobacilli*, and induced colonisation by antibiotic-resistant strains (Schumann et al. 2005). The findings from these studies could explain the high prevalence of nosocomial infections with increased antibiotic consumption in critically ill patients (Weber et al. 1999).

The alteration in the balance of gut microbiota following antibiotic treatment could be short term or may last for a longer period. An example of the long-lasting effect of antibiotic use is reduced colonisation resistance against some pathogens such as *Clostridium difficile* (Theriot et al. 2014). As this population often receives extensive antibiotic treatment, it may, in addition to the functional loss of commensal species, affect the prevalence of antimicrobial-resistant genes within the intestinal microbiome.

Other therapeutic agents used in PICU are also known to impact the intestinal microbiome, including inotropic drugs that promote the growth of pathogens (Sandrini et al. 2015; Freestone et al. 2008). Vasopressors like calcium-channel blockers have recently been shown to inhibit the growth of commensal bacteria, however the exact mechanism has not been established yet (Maier et al. 2018).

1.2.1.3 Nutrition

Nutrition has an indirect effect on the gastrointestinal function of the host and thereby on health, mainly by influencing the composition and activity of the human gut microbiota. It is considered one of the major determinants of the intestinal microbial composition. The route of feeding greatly affects gut microbial population (Hodin et al. 2012; Shiga et al. 2012). EN helps to maintain the immunological function of the gastrointestinal tract; it decreases bacterial translocation and accordingly blunts the

systemic inflammatory response (Heyland et al. 1993; Yang et al. 2009). In addition, early compared to delayed enteral feeding is associated with less bacterial translocation and better mucosal integrity (Feng et al. 2017; Kotani et al. 1999). In contrast, bowel rest, as associated with total PN or delayed EN, results in gastrointestinal mucosal atrophy, which compromises the integrity of the mucosal barrier and enhances exposure to bacteria and endotoxins (Deplancke et al. 2002; Bjornvad et al. 2008; Heyland et al. 1993). The composition of EN is also known to greatly influence the colonisation, maturation and stability of the intestinal microbiota (Zhang & Yang 2016). The effect of the dietary component on intestinal microbiota varies; it may promote the growth of opportunistic microbes while other dietary factors could endorse beneficial microbes (Brown et al. 2012).

Dietary fibre is the primary energy source for most commensal species and, therefore, can directly impact their growth (D. Graf et al. 2015). It is also the main substrate for the microbial production of important bacterial metabolites known to influence the host's health (den Besten et al. 2013a). The effect of fibre supplementation on gut microbiota has been widely investigated in healthy subjects, and data showed that the main species influenced *were Bifidobacteria*, *Lactobacilli, Faecalibacterium prausnitzii* and *Roseburia* (Bouhnik et al. 1996; Vulevic et al. 2008; Ramirez-Farias et al. 2009; Benus et al. 2010; Sawicki et al. 2017). The nutrition status is also an important factor determining the diversity of intestinal microbiome (Krajmalnik-Brown et al. 2012). Therefore, the state of energy deficit, particularly during the acute phase of illness, might have a profound effect on the gut microbiota and environment.

1.2.2 Contribution of intestinal microbiota to the host health

The gut microbiota use ingested dietary components (carbohydrates – mainly resistance starch, proteins, and lipids) to generate energy for cellular processes and for growth (Ramakrishna 2013). During the process of utilising these substrates, the microbiota produce several metabolites that influence host health and metabolism (Krajmalnik-Brown et al. 2012; Ramakrishna 2013).

1.2.2.1 Macronutrient metabolism

Preclinical data and animal studies have suggested possible mechanisms of the contribution of intestinal microbiota to the regulation of macronutrient metabolism.

a. Carbohydrates

The fermentation of polysaccharides (fibre) by anaerobic bacteria leads to the production of SCFAs, which are utilised by the host. Colonic epithelial cells derive up to 70% of their energy from the oxidation of butyrate (Blaut & Clavel 2007). The microbial gluconeogenesis from propionate reduces hepatic gluconeogenesis and promotes energy homeostasis (De Vadder et al. 2014; Rowland et al. 2018). Gut microbiota are also known to regulate glucose metabolism through the stimulation of GLP-1 and PYY hormone secretions from intestinal L-cells (Psichas et al. 2015; Tolhurst et al. 2012).

b. Protein

Gut microbiota contribute to host nitrogen balance through de novo synthesis of amino acids and intestinal urea recycling. Studies with radiolabelled tracers suggest that gut microbes synthesise nearly 20% of circulating threonine and lysine in healthy adult humans (Metges 2000). The intestinal microbiota also contribute to host nitrogen balance by participating in urea nitrogen salvaging (Morowitz et al. 2011). Elevated urease expression in gut microbes results in the metabolism of urea in the GI tract into ammonia and carbon dioxide. Some of the ammonia can be utilised for the microbial synthesis of amino acids. More importantly, the nitrogen generated during this process can re-enter the host circulation and serve as a substrate for synthetic processes (Morowitz et al. 2011). Reduced urea recycling has been reported in germ-free animals and in humans receiving antibiotic therapy (Levenson & Crowley 1959; Walser & Bodenlos 1959). Therefore, the state of negative nitrogen balance frequently observed during critical illness could be partially attributed to disturbances in the gut microbiome, which impacts the intestinal urea recycling process.

c. Lipids

The impact of dietary lipids on the microbiota in critically ill children has not yet been established. However, preclinical studies in animal models indicate that the intestinal microbiota regulate fat metabolism by suppressing the activity of a circulating inhibitor of lipoprotein lipase (LPL) (Bäckhed et al. 2004). This results in increased levels of circulating LPL, which stimulate hepatic triglyceride production and promote the storage of triglycerides in adipocyte (Janssen & Kersten 2017; Goldberg et al. 2009; Bäckhed et al. 2004). Suppression of LPL inhibitors is promoted by intestinal microbiota through transcriptional suppression of the intestinal epithelial gene encoding for LPL inhibitors (Bäckhed et al. 2004). Abnormalities in lipid metabolism commonly observed during the course of critical illness could be also related to the disturbance in the functional capacity of the gut microbiota.

1.2.2.2 Short chain fatty acids (SCFAs)

SCFAs arising from the anaerobic bacterial metabolism of indigestible dietary fibre in the colon are beneficial to the host health. SCFAs support the integrity of the gut barrier by regulating the release of mucus by colonic cells and acting as a fuel source to colon cells. They also have immune modulating activity and are involved in the release of gut hormones (Vinolo et al. 2011; Byrne et al. 2015). The principal SCFAs seen in the colon are acetate, propionate and butyrate. SCFAs have been shown to be depleted in adults with critical illness, often due to the effect of the acute insult and other environmental factors on SCFAs-forming species (Shimizu et al. 2006; Osuka et al. 2012; Hayakawa et al. 2011; Yamada et al. 2015; Vermeiren et al. 2012).

a. SCFAs as a source of energy

The bacterial formation of SCFAs enables the host to salvage some of the energy contained in dietary fibre that would otherwise be lost, while various tissues in the body are able to oxidise SCFAs for energy generation (Blaut & Clavel 2007). SCFAs are absorbed by passive diffusion across the colonic epithelium, and utilised by different organs. Colonic epithelial cells derive up to 70% of their energy from the oxidation of

butyrate (den Besten et al. 2013). Propionate serves as a substrate for microbial gluconeogenesis (De Vadder et al. 2014; Rowland et al. 2018). Acetate is used by skeletal and cardiac muscle and also can be utilised by adipocytes for lipogenesis (Hooper et al. 2002). Butyrate is metabolised primarily in the gut epithelium to yield ketone bodies or CO_2 and propionate is transported to the liver for gluconeogenesis (Morowitz et al. 2011).

b. Physiological functions of SCFAs

Besides being used as fuel by different organs, SCFAs have other physiological functions (Topping & Clifton 2001; Kasubuchi et al. 2015). For instance, butyrate appears to affect cell differentiation and protects cells from carcinogens, either by slowing growth and activating apoptosis in colon cancer cells (Scharlau et al. 2009), or by upregulating the detoxifying enzymes, such as glutathione-S-transferases (Pool-Zobel et al. 2005). SCFAs also impact water absorption, local blood flow, and epithelial proliferation in the large intestines (Morowitz et al. 2011). SCFAs have a direct protective effect on strengthening the gut barrier in the normal colon by increasing the production of mucus by colonocytes (Lewis et al. 2010). Most importantly, SCFAs are involved in the regulation of inflammation. Generally, SCFAs exert their physiological functions by acting as signal molecules that activate target receptors in various cells and organs.

c. SCFAs as signalling molecules

The physiological effects of SCFAs depend on the activation of G protein-coupled receptors (GPRs) (Vinolo et al. 2011; Kim et al. 2014). In adipocytes, the activation of GPR43 by SCFAs results in the suppression of insulin signalling and accordingly inhibits lipogenesis and enhances glucose and lipid utilisation by the muscles. On the other hand, activated G-protein-coupled membrane receptor GPR41 enhances the production of leptin, and activated GPR109A suppresses lipolysis (Kim et al. 2014; Kimura et al. 2014). In addition, it has been suggested that the SCFAs activation of GPR41 in adipocytes increases fatty acid oxidation and energy expenditure (den Besten et al. 2013b). The expression of GPR41 and GPR43 by SCFA in the intestines promotes gut

hormone secretion, which regulates energy homeostasis (Psichas et al. 2015; Tolhurst et al. 2012) (see Figure 1.4).

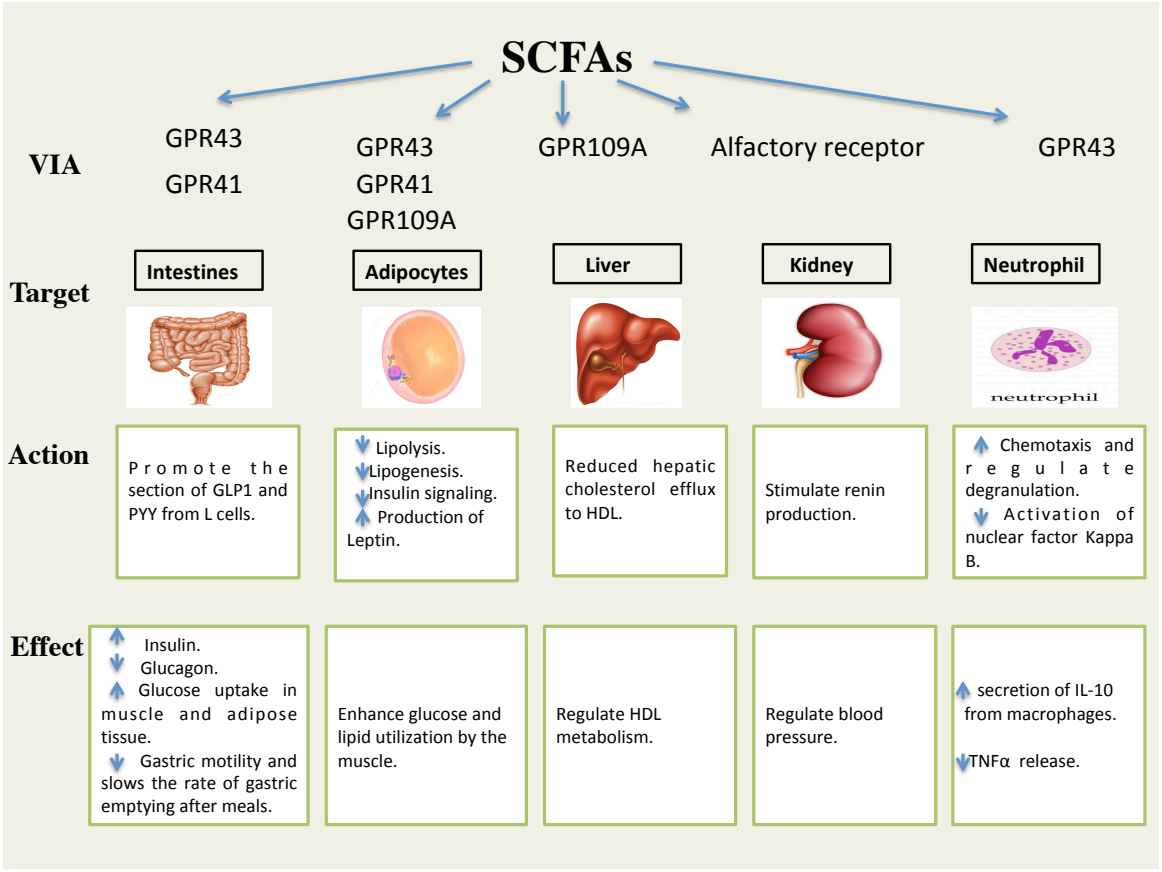

Figure 1.4 SCFAs as signalling molecules activating G-protein-coupled receptors

Figure 1.4 summaries the physiological function that SCFAs exert by activating G-protein-coupled receptors in different target organs and cells.

* The pictures used in this figure have been obtained from MedScape.org

d. SCFAs and inflammation

An important driver of MOF in critical illness is the dysregulation of innate immune pathways and the loss of balance between pro-inflammatory and anti-inflammatory mechanisms (Cox 2012). SCFAs appear to play a role in modulating inflammatory and immune responses, since they modify the migration of leukocytes to the site of inflammation, as well as modifying the release and production of chemokine (Vinolo et al. 2011). The activation of GPR43 by SCFAs induces the chemotaxis and regulates degranulation of neutrophils (Kim et al. 2014). The anti-inflammatory properties of

SCFAs have been investigated in patients with inflammatory bowel diseases (IBD). In a previous study in patients with IBD, impaired butyrate metabolism was reported (Canani et al. 2011). SCFAs, in particular butyrate, reduce inflammatory cytokine production and inflammation in the intestine through mechanisms including nuclear factor kappa B signalling (Ramakrishna 2013; Tedelind et al. 2007). SCFAs exert an inhibitory effect on both Tumour Necrosis Factor-alpha (TNF-α) mediated activation of the nuclear factor kappa B pathway and lipopolysaccharide-induced TNF-α release (Tedelind et al. 2007). On the other hand they increase the secretion of anti-inflammatory Interleukin-10 (IL-10) from macrophages (Kim et al. 2014).

1.2.2.3 Regulation of bile acid metabolism

Bile Acids (BAs) are synthesised in the liver from cholesterol and stored in the gall bladder. They are released into the small intestines following food ingestion to aid the digestion process by facilitating the emulsification of dietary fats. The physiological functions of BAs in the human body are not only restricted to facilitating lipid digestion and absorption; they appear to be also involved in the overall regulation of host metabolism (Ramírez-Pérez et al. 2017). Recently, preclinical studies in murine and in vitro models indicated that BAs contribute to the activation of farnesoid X receptor (FXR) and the GPR5 receptor. These receptors activate the expression of genes involved in BAs, lipids, and carbohydrates metabolism as well as energy expenditure regulation (Cariou et al. 2006; Abdelkarim et al. 2010; Watanabe et al. 2006).

The human BA pool is made up of primary, secondary and tertiary BAs. The primary BAs [Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA)] are synthesised in the liver, while secondary BAs are produced in the gut via the modification of primary BAs through dehydroxylation, epimerisation and oxidation. The tertiary BAs are formed in both the liver and gut microbiota via modification of secondary BAs through sulfation, glucuronidation, glucosidation and N-acetylglucosaminidation (Marschall et al. 1992; Ridlon et al. 2006; Wahlströ et al. 2016).

Primary BAs synthesised by the liver are conjugated with taurine or glycine and during enterohepatic circulation, they are then deconjugated through gut microbial action

(Ridlon et al. 2006). The resulting free amino acids are then metabolised by intestinal microbiota (e.g. Bifidobacterium) for energy supply (Tanaka et al. 2000).

The intestinal microbiota possess enzymes involved in the regulation of the BA pool such as bacterial bile salt hydrolases, bacterial hydroxysteroid dehydrogenases and 7α/β-dehydroxylase (Ridlon et al. 2006). The majority of intestinal microbial species facilitate deconjugation and dehydrogenation of bile salts through expression of bacterial bile salt hydrolases and bacterial hydroxysteroid dehydrogenases. Limited intestinal species (e.g. *Clostridium* clusters XI and XVIa, such as *C. sordellii*, *C. sordelliifell*, and *C. scindens)* express 7α/β-dehydroxylase enzymes that catalyse the dehydrogenation reaction of primary BAs (Ridlon et al. 2006; Nie et al. 2015) (see Figure 1.5)

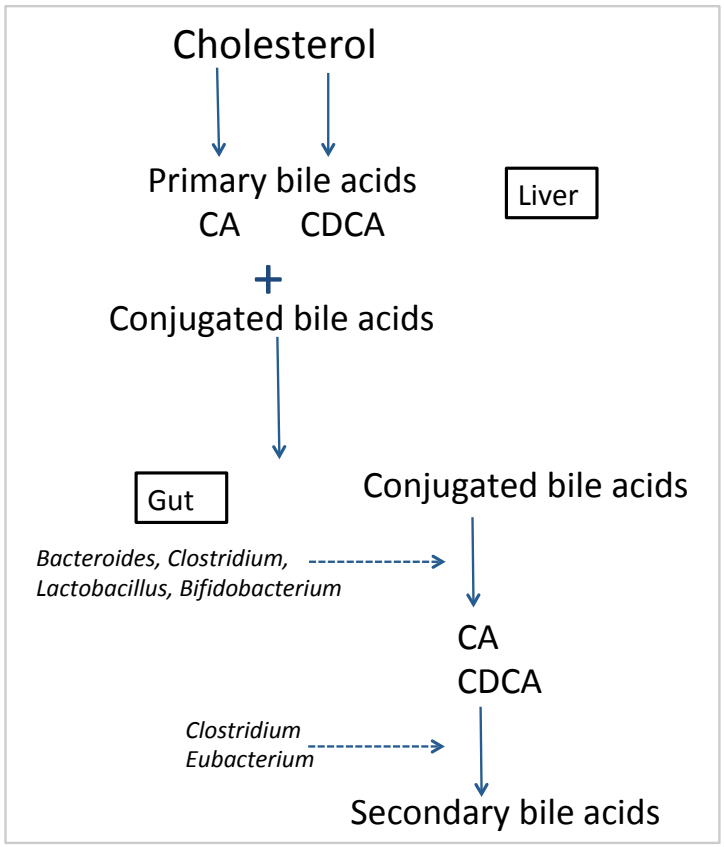

Figure 1.5 Regulation of bile acid metabolism by intestinal microbiota

Figure 1.5 illustrates the contribution of *Bacteriodes Clostridium, Lactobacillus Bifidobacteria* and *Eubacterium* to the regulation of bile acid metabolism.

1.3 Nutrition support in critically ill children

Nutrition is an important component in the management of critically ill children. The goals of nutrition support in paediatric critical care is to minimise catabolism, support basal physiological functions and aid anabolism during the recovery phase (Gurgueira et al. 2005; Meyer and Elwig 2007; Joosten et al. 2016). Under- and overfeeding has been shown to compromise this goal and to substantially complicate the stay in PICU (Mehta et al. 2012). The role of nutrition in shaping gut microbiome has been recently highlighted (Hodin et al. 2012; Shiga et al. 2012; Morowitz et al. 2011; Krajmalnik-Brown et al. 2012), therefore the restoration of gut homeostasis should be one of the goals of nutrition support in critically ill children.

Achieving optimal nutrition prescription is challenging in critically ill children, as they undergo different phases of illness and accordingly the body response to injury vary over time (Wischmeyer 2013; Joosten et al. 2016). Understanding energy needs during the phases of illness is necessary to avoid imbalances between energy intake and requirements (Joosten et al. 2016). To maintain energy balance, energy intake should ideally be equivalent to energy expenditure. As energy expenditure is changeable during the phases of illness, the nutritional requirements should be adjusted accordingly (Joosten et al. 2016). Macronutrients, carbohydrates, fat and protein are the main fuel used for the generation of energy to maintain normal body function and they are the main substrate of intestinal microbiota. Therefore, substrate metabolism (i.e. carbohydrate, protein, fat sources) should also be considered when choosing feeds for these children.

1.3.1 Assessment of energy and macronutrient requirements in critically ill children

To ensure adequate nutrition delivery, a proper assessment of energy and macronutrient requirements should be undertaken. Energy requirements for critically ill children ideally should be measured by indirect calorimetry (IC). However, generally in

clinical practice, energy needs are often estimated by a range of prediction equations due to the limitation of using IC in PICU settings.

1.3.1.1 Measurement of REE by IC

IC measures energy expenditure by determining the oxygen consumption and carbon dioxide production of the body over a given period of time. The values of VO_2 and VCO_2 are converted to energy expenditure via a metabolic chart using the Weir equation (Weir 1949). The principle of IC is based on the assumption that the inspired oxygen is completely utilised to oxidise degradable fuels, and during this process carbon dioxide is generated (Gupta et al. 2017). IC is considered the gold standard to determine energy requirements in critically ill children (Mehta et al. 2017). It ensures that the specific conditions of the critically ill patient are taken into account, thereby preventing potential complications from over- and underfeeding (Mehta et al. 2017). IC also allows for identification of energy substrates that are being predominantly metabolised by the body during the course of admission (Coss-Bu et al. 2001a). A number of factors may restrict the regular use of IC in PICU settings. These are mainly related to the high cost of machines and a lack of trained clinical staff to perform IC testing (Mehta et al., 2017). In addition, clinical conditions such as air leak, fraction of inspired oxygen (FiO_2) >60%, and failure to achieve a steady state are known to compromise the accuracy of energy expenditure measurements by IC (Kyle et al., 2012). Measurements of energy expenditure may also be limited in unstable children, where medical care cannot be interrupted for such measurements (Framson et al., 2007). As a result, the feasibility of IC testing in intensive care settings is constrained, in a worldwide survey of 156 PICUs, IC was used in only 14% of the units (Kerklaan et al. 2016).

1.3.1.2 Prediction equations used in PICU settings to estimate energy requirements

In clinical practice, energy requirements are often estimated by prediction equations. The most commonly used prediction equations in PICU settings are: the Schofield equations, the World Health Organization (WHO) equations and the Harris-Benedict equations (Table 1-1), (Schofield 1985; Joint FAO/WHO/UNU Expert Consultation on Energy and Protein Requirements 1981: Rome 1985; Harris & Benedict 1918). The

Recommended Daily Allowance (RDA) for healthy children was used in some units in the past to guide energy prescription. However, it is no longer recommended to use RDAs in PICU settings as they integrate an activity factor resulting in the over-estimation of energy requirements (Mehta et al. 2017). The Schofield and WHO equations are recommended by the recent American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines to estimate energy requirement in critically ill children in the absence of IC.

The use of predictive equations to develop nutrition regimens can be problematic in the critical care setting, as the effects that disease, injury, and stress have on energy expenditure are often varied and unpredictable (Joosten et al. 2016; Coss-Bu et al. 2001; Briassoulis et al. 2000). Multiple studies have demonstrated that most published predictive equations are inaccurate and lead to over- or underestimation of energy requirements (Table 1) (Christine M. Hardy Framson et al. 2007; Jotterand Chaparro et al. 2017; Briassoulis et al. 2000). As the feasibility of IC measurements in PICU is limited there is still an urgent need for formulas that closely estimate the energy needs of critically ill children (Kyle, Arriaza, et al. 2012). Efforts have been made to create predictive equations adapted to clinical changes during critical illness (White et al. 2000; Meyer et al. 2012). However, still the degree of agreement between measured energy expenditure and estimated energy requirements by the available equations is not satisfactory.

Table 1-1: Prediction equations commonly used in PICU settings

Name of the equation	Equation	Accuracy
Schofield Equation (weight and height)	**Male** < 3 years: (0.167 x W) + (1517.4 x H) - 616.6 3-10 years: (19.59 x W) + (130.3 x H) + 414.9 10-18 years: (16.25 x W) + (137.2 x H) + 515.5 **Female** < 3 years: (16.252 x W) + (1023.3 x H) - 413.5 3-10 years: (16.969 x W) + (161.8 x H) + 371.2 10-18 years: (8.365 x W) + (465 x H) + 200.0	Recommended by ASPEN guidelines to estimate energy needs in critically children in the absence of IC. Potential over-estimation of energy needs in critically ill children.
WHO Equation **(only weight)**	**Male** 0-3 years: (60.9 x W) - 54 3-10 years: (22.7 x W) - 495 10-18 years: (17.5 x W) + 651 **Female** 0-3 years: (61 x W) -51 3-10 years: (22.5 x W) + 485.9 10-18 years: (17.686 x W) + 658.2	Recommended by ASPEN guidelines to estimate energy needs in critically children in the absence of IC. Potential over-estimation of energy needs in critically ill children. Does not incorporate the height of the child in the calculations of energy requirements.
Harris-Benedict Equation **(weight and height)**	**Male** 66.4730+ (5.0033 x H) + (13.7516 x W) – (6.7550 x age) **Female** 655.0955 + (18496 x H) + (9.5634 x W) – (4.6756 x age)	The limits of agreement between measured energy expenditure and Harris-Benedict equation is poor. Not recommended to estimate energy requirements in PICU settings.

1.3.1.3 Macronutrients requirements of critically ill children

Current recommendations for macronutrient requirements in this population are based on the understanding of their metabolism and their handling during the course of their illness (Mehta et al. 2009; Mehta et al. 2017). The ASPEN guidelines recommend protein intakes of at least 1.5 g/kg (Mehta et al. 2017). The requirements may vary by age; the recommendation for children aged 0–2 years is 2–3 g/kg/day; for 2–13 years it is 1.5–2 g/kg/day and for >13 years it is 1.5 g/kg/day) (Mehta et al. 2009). The available guidelines regarding carbohydrate and fat requirements are based on maximal doses of parenteral glucose and fatty acids permitted (Mesotten et al. 2018; Lapillonne et al. 2018). There is a lack of published guidelines regarding enteral fat and carbohydrate

requirements for critically ill children. The new ESPGHAN guidelines for PN suggested that energy and macronutrient requirements of PN are close to those of EN (Joosten et al. 2018; Mesotten et al. 2018; Lapillonne et al. 2018). Their argument is based on findings from 20th-century experimental studies by Atwater where the metabolisable energy of IV macronutrients was similar to that provided by EN. However, their findings remain controversial as there are key differences in the metabolisms of enteral versus parenteral carbohydrates, lipids and protein related to intestinal absorption of nutritional substrates, insulin and inflammatory stimulation and visceral protein synthesis (Suchner et al. 1996).

1.3.2 Nutrition delivery in the acute phase of illness

The recent ASPEN guidelines target the delivery of at least two-thirds of the prescribed daily energy requirements by the end of the first week in the PICU, to avoid undesired clinical outcomes associated with cumulative nutrition deprivation (Mehta et al. 2017). Provision of protein in the acute phase is also recommended to achieve protein goals and promote positive nitrogen balance (Mehta et al. 2017).

Early initiation of EN is recommended for critically ill children for several reasons, in particular to avoid the deleterious effect of fasting. In addition, EN helps to maintain the gut function. Lastly, it promotes energy metabolism and supports the functions of body organs and systems (Meyer and Elwig 2007). In a study of critically ill children, the early initiation of EN within 48 hours of admission was associated with a lower mortality rate (Mikhailov et al. 2014). In addition, the implementation of an aggressive early EN protocol was significantly effective in improving nitrogen balance in critically ill children (Briassoulis et al. 2002; Botrán et al. 2011; Briassoulis et al. 2005).

Although energy requirements are reduced during the acute phase of critical illness, still the nutritional needs are often not fulfilled via the enteral route due to poor estimation of energy and macronutrient requirements, lack of routine nutritional assessments, fluid restrictions, feeding intolerance, and frequent interruption of enteral feeding (Hulst et al. 2006; Mehta et al. 2012b). Mehta et al. (2012) indicated that around 60% of the

prescribed calories are not being delivered via the enteral route due to the aforementioned reasons. The development of PN substantially improved nutrient delivery in a critically ill population, however the early use of PN has been shown to be associated with numerous problems such as increased rate of infections, metabolic disturbances, and higher hospital costs (Cotogni 2017; Netto et al. 2017; Fivez et al. 2016). Early initiation of PN was also associated with prolonged period of mechanical ventilation and PICU stay (Fivez et al. 2016; van Puffelen et al. 2018). The recent ESPGHAN guidelines for PN provision suggest that critically ill children may benefit from withholding PN while providing micronutrients during the first week of hospital admission (Van Goudoever et al. 2018). There is evidence that withholding PN during the acute phase of illness is useful for enhancing the process of autophagy, a cellular survival mechanism that involves the self-breakdown of damaged components, aimed at recycling intracellular nutrients and generating energy during starvation (Levine et al. 2011; Vanhorebeek et al. 2017; Joosten et al. 2016). The inhibition of autophagy by forced overfeeding was linked to an increased risk of organ failure and cell death, and is associated with adverse clinical outcomes (Joosten et al. 2016).

Although the majority of critically ill children are fed via the enteral route, there are no clear guidelines on the optimal amounts for enteral carbohydrate and fat. There is often a clinical focus on delivering the energy and protein requirements of a patient, but the balance of macronutrients is often neglected. The only guidelines available for enteral nutritional support in this population are for energy and protein.

1.3.3 Nutrition delivery in the stable and recovery phase

The stable and recovery phases are characterised by early normalisation of counter-regulatory hormones, accompanied by an increase in energy expenditure. Generally during this phase the nutritional requirements are increased. The goal of nutrition support at this point is to support the anabolic growth. Therefore, energy and macronutrient intake should gradually be increased to enable recovery, growth and catch-up growth (Joosten et al. 2018; Mesotten et al. 2018; Mehta et al. 2017). The recent ESPGHAN guidelines suggested an increase by 1.3 times of energy requirements

during the stable phase of critical illness and further increases in the recovery phase were recommended (Joosten et al. 2018).

1.3.4 The role of nutrition in contributing to clinical outcomes of critically ill children

The role of nutrition in the progression of adverse clinical outcome has been highlighted in the recent ASPEN guidelines for nutrition support (Mehta et al. 2017). Optimal nutrition support helps dramatically to improve the care and clinical outcomes of critically ill infants and children. Inadequate nutrition intake is associated with poor clinical outcomes such as decreased muscle function, poor wound healing, impaired gut function and an increased risk of developing infections due to poor immune defence (Hulst et al. 2006; Maslowski & Mackay 2011). Under-nutrition, particularly protein energy malnutrition, also has been strongly linked with mortality, prolonged ventilation and hospital stay (Mehta et al. 2012b; Correia 2003; Hulst et al. 2006; Maslowski & Mackay 2011).

Overfeeding critically ill children may lead to undesirable outcomes such as steatosis and cholestasis, resulting in impaired liver function (Mehta et al. 2009). In addition, hyperglycaemia associated with overfeeding in parenterally fed children increases the risk of infection and has been linked with prolonged ventilation and PICU stay (Alaedeen et al. 2006; Fivez et al. 2016). Overfeeding is also associated with an increase in carbon dioxide production, which may be implicated in delayed weaning from mechanical ventilator support (Chwals 1994; Meyer and Elwig 2007). In addition, overfeeding during the acute phase was linked to inhibition of autophagy and increased risk of organ failure and cell death (Joosten et al. 2016).

There is a growing body of evidence suggesting that a pattern of alteration in the gut microbiome would be observed during a period of nutrient deprivation (Krajmalnik-Brown et al. 2012). Critically ill children are often underfed, and the state of energy deficit might have a profound effect on the gut microbiota and environment. Studies are needed to define the role of starvation or underfeeding during critical illness on the gut microbes.

1.4 Targeted modulation of intestinal microbiome to improve clinical outcomes in critically ill children

Given the substantial clinical and experimental evidence supporting the gut-lymph hypothesis (Deitch et al. 2006; Deitch 2012), the targeted modulation of intestinal microbiome has been proposed as a therapy to support gut health in critically ill patients.

The suppression of pathogenic gut bacteria was the first therapeutic line of microbiome modulation tried on critically ill patients. Selective decontamination of the digestive tract (SDD) was first introduced in the 1980s (Unertl et al. 1987), aimed at keeping the overgrowth of potential pathogens in the gut to the minimum through the administration of tailored antibiotic treatment (Unertl et al. 1987; Dickson 2016). Ever since, SDD intervention has been tested in many clinical trials in critically ill patients (de Jonge et al. 2003; de Smet et al. 2009; Daneman et al. 2013). Although proven effective and shown to significantly reduce the rate of infections, the number of patients with MOF and mortality (Silvestri et al. 2012; de Jonge et al. 2003; de Smet et al. 2009), there still remains concern about the potential risk of antimicrobial resistance (Francis et al. 2014; Cavalcanti et al. 2017). All these data are derived from studies on critically ill adults, and there is an absence of paediatric-specific clinical trials. In a recent survey by Murthy et al. (2017) to determine the baseline knowledge of health care providers about SDD, they indicated that there is still uncertainty about implementing SDD protocols in PICU, mainly due to concerns about antimicrobial resistance (Murthy et al. 2017).

Given the extensive vascular and lymphatic links between the intestines and other organs, it is possible that enhancing the growth of commensal bacteria and their metabolites by EN could be of systemic clinical benefit. Certain nutrients have been verified for their capacity to modulate the gut bacterial profile, such as glutamine and prebiotics (dietary fibre that promotes the growth of beneficial bacteria). In a previous

study to evaluate the effect of glutamine supplementation on the gut microbial content, they reported significant changes in the composition of the gut microbiota in obese subjects (de Souza et al. 2015). As regards to prebiotics, lactulose added to infant formulas was used for long enough to increase the numbers of lactobacilli in infant intestines (Collins & Gibson 1999). However, this practice has evolved with extensive research on the role of prebiotics in enteral feeds. The most common prebiotics are fructooligosaccharides and galactooligosaccharides; they have been added to a number of infant formulas and they demonstrated modulation of the gut bacterial population (Vandenplas et al. 2014; Fanaro et al. 2005). Using probiotics containing *Lactobacillus* and *Bifidobacteria* species has a beneficial effect on modulating the balance of the intestinal microbiota (Collins & Gibson 1999). Furthermore, using probiotics in combination with antibiotic therapy has proven to have a beneficial effect, as probiotics not only suppress gastrointestinal pathogens but may potentiate the efficacy of antibiotics by producing antibacterial factors (Preidis & Versalovic 2009). Generally, using probiotics in clinical settings has been proven to be safe, however they must be used with caution in certain patient groups (Srinivasan et al. 2006). A theoretical concern with the safety of using probiotics is that some of them may adhere to the intestinal mucosa and facilitate bacterial translocation and virulence (Boyle et al. 2006). However, Srinivasan et al. (2006) showed that the supplementation of probiotics in enterally fed critically ill children is safe and is not associated with any clinical complications (Srinivasan et al. 2006). In a recent study of critically ill adult patients, the administration of synbiotics, a combination of probiotics and prebiotics, was associated with a reduction in the rate of infections and an increase in the count of commensal bacteria, as well as SCFAs (Shimizu et al. 2018). This suggests that nutritional intervention targeting the gut microbiota in critically ill patients has the potential to improve clinical outcomes

1.5 Key identified gaps in the literature:

Having reviewed the literature, gaps and unaddressed challenges in the field are identified as follows:

- Studies on the effect of EN on clinical outcomes of critical illness have focused mainly on energy and protein delivery. There is a critical lack of guidelines on carbohydrate and fat requirements for critically ill children. It is not surprising that there have been no trials of enteral macronutrient targets in this population.

- There is a lack of studies investigating the effect of macronutrient delivery on disease features such as inflammation (systemic and intestinal).

- All microbiome studies in critical care settings have arrived at a similar conclusion that commensal species are replaced by pathogens. None of them investigated the effect of environmental influences (e.g. nutrition, antibiotic treatment) on the microbiome of critically ill patients.

- No studies have investigated the specific influences of intestinal dysbiosis on host metabolism and clinical outcomes in this setting.

2 Chapter Two: Methodology

2.1 Main aims and objectives

The main aim of this PhD project is to investigate the effect of nutrition on the intestinal microbiome-host relationship in critically ill children and whether it has an impact on clinical disease severity.

Objectives

- Describe feeding and energy metabolism during PICU admission.
- Identify and evaluate relationships between nutritional intake and systemic and intestinal inflammation.
- Describe changes in the gut microbiome and how it is influenced by current feeding practice in the PICU.
- Establish whether changes in gut homeostasis and the relationship between gut and host have clinical significance for disease progression and clinical outcomes.

2.2 Flow of the study

The research conducted for this thesis was undertaken using samples and clinical measurements collected from the Gut Homeostasis in Critical Illness (GHICI) study taking place at Addenbrooke's Hospital in Cambridge, UK and Hospices Civils de Lyon in France. The Evelyn Trust funded the study, and ethical approval was granted by the City and Hampstead LREC (Reference: 13/LO/0974). Children were transferred to hospital PICUs either through the accident and emergency (A&E) department or from regional hospitals by the Children's Acute Transport Service (CATS).

2.3 Ethical considerations

Parents were approached for consenting for their children to participate by the PICU research nursing team. For those children transported to Addenbrooke's Hospital PICU from regional hospitals by the CATS, a deferred consent process was implemented and approved by the local research ethical committee, and samples were taken on retrieval. Where consent was obtained the samples were processed and stored. If parents refused consent the samples were destroyed and no clinical data was collected.

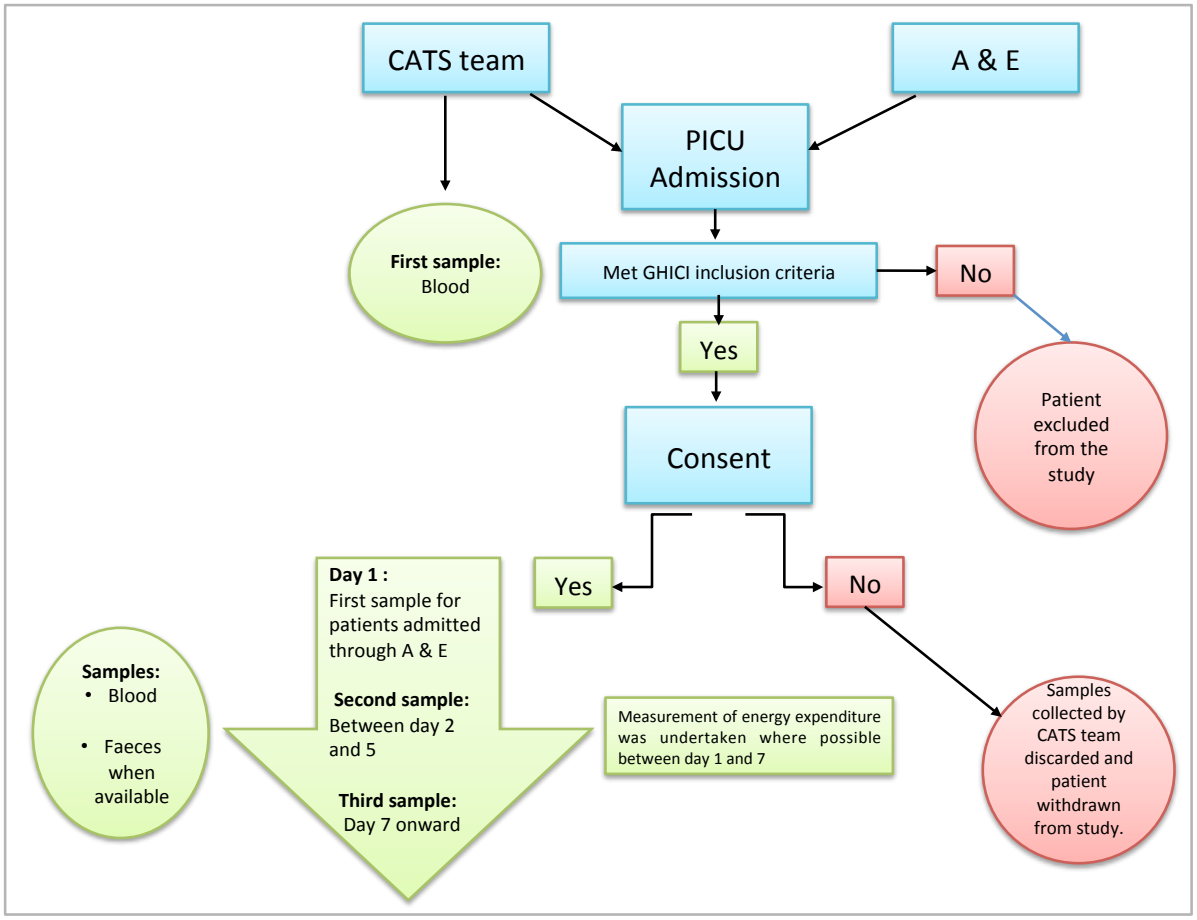

Figure 2.1 The flow of this study

The above figure shows the stages of the recruitment process and the time frame of samples collection and energy expenditure measurements.

2.4 Study participants

PICU research nurses at Addenbrooke's Hospital performed the consent process for participation. I was responsible for identifying the subjects eligible to participate in the study. Two groups of children were recruited; critically ill children admitted to the PICU alongside a cohort of healthy children. A subgroup of critically ill and control children were recruited from Hospices Civils de Lyon in France. Written informed consent was obtained from the parents and ethical approval was granted by the hospital. Where comparison is made between the two groups in the subsequent chapters, the analysis is restricted to the age-matched population.

2.4.1 Critically ill children

All children admitted to PICU and likely to remain ventilated for more than 24 hours were eligible to participate in the study.

Inclusion criteria

- One or more organ failures requiring intensive care.
- Mechanically ventilated.
- Age 0 month to 16 years.

Exclusion criteria

- Preterm gestation (birth < 37 weeks).
- Treatment with antibiotics in the last 2 weeks prior to admission.
- Known pre-existing immune paresis, oncological diagnosis and HIV.

Further specific exclusion criteria were applied, and will be mentioned in the relevant chapters.

2.4.2 Healthy children

Parents of children undergoing elective surgeries or from the community consented for their children's participation and were asked to provide samples of blood, urine and/or

faeces where possible. Those who had received antibiotic treatment in the last 2 weeks prior to the recruitment process were excluded.

2.4.3 Age matching patients and controls

The study participants were categorised into three age groups: 0-6 months, 6-12 months and >12 months based on the stages of diet progression. Critically ill children were age-matched with healthy children to allow for a valid comparison between the groups.

2.5 Biological samples

Samples were collected from both critically ill patients and from healthy children. Serum and faecal samples were collected by PICU research nurses at Addenbrooke's Hospital. I was responsible for the processing and the storage of the biological samples.

2.5.1 Critically ill children

Serial samples of serum were collected at 3 time-points (TP), (see Figure 2.1). Faecal samples were obtained whenever the child passed a stool.

Blood: 2.5ml of blood was collected if there was an indwelling line or venepuncture was taking place already for clinical reasons. A volume of between 0.5-1 ml of serum was obtained. The samples were centrifuged for 10 minutes at 4000× *g, 4*°C then the supernatant was removed and carefully transferred into micro-tubes and stored at -80°C.

Faeces: faecal samples of 300 mg were collected from the children's nappies into a sterile container. Samples were refrigerated immediately after collection and then stored at -80°C within 24 hours of collection until the time of the assay.

2.5.2 Healthy children

A single sample of each of the following was collected from each child:

Blood: 2.5ml of blood was obtained if venepuncture was taking place already for clinical reasons.

Faeces: the children were asked to defecate into a clinically clean bedpan, and then the specimen was collected using the stool pot.

2.5.3 Samples from Hospices Civils de Lyon in France

Samples were processed immediately after collection then stored at -80°C until they were shipped for analysis at Addenbrooke's Hospital laboratories. Serum and faecal samples were used for the assessment of Faecal Calprotectin (FC) and Cytokines in Chapter 7.

2.6 Demographic and clinical data

Patients' data were obtained from the hospital electronic system (EPIC, Madison, Wisconsin, United States). The system went live on 26 October 2014, therefore, the clinical information for the patients recruited before the initiation of the hospital's electronic system were not obtainable retrospectively.

Demographic and anthropometric data including gender, age, weight, and height were collected for each child. Patients' primary diagnosis necessitating PICU admission was recorded and then categorised into multi-organ system failure (MOF) or single organ failure categories as described in Table 2-1 (Goldstein et al. 2005). Information related to the presence of infection or signs of infections was also recorded from the patients' records. Sepsis was defined as ≥ 2 abnormalities of body temperature, heart rate, respiratory rate or abnormal white blood cells count plus suspected or proven infection (Goldstein et al. 2005).

The severity of disease and morbidity were assessed by the paediatric multiple organ dysfunction score (PMODS) (Graciano et al. 2005), the paediatrics index mortality 2 (PIM2) score (Slater et al. 2003) and the inotrope score (Gaies et al. 2010). Data on the highest blood lactate and highest C reactive protein (CRP) level were also recorded as indicators of disease severity. Hours free of mechanical ventilation (ventilator-free

hours, VFH) at 30 days, days free of intensive care at 30 days, hours free of inotrope and survival status were recorded and used as clinical outcome measures.

Table 2-1: Primary admission diagnosis categories:

Diagnosis	Diagnostic Category
• Cardiovascular and respiratory dysfunction. • Neurological and haematological abnormalities. • Renal and hepatic dysfunction **Examples of diagnoses included in this category:** Meningococcal Septicaemia Staphylococcus Sepsis Cardiorespiratory Arrest Diabetic Ketoacidosis leading to MOF Drug Overdose leading to MOF Any other condition leading to MOF	Multi-organ failure
Bronchiolitis Pneumonia Aspiration Pneumonia Asthma Croup Acute Respiratory Distress Syndrome Pleural effusion	Respiratory Failure
Supraventricular Tachycardia (SVT)	Cardiac Disorders
Tracheal repair Scoliosis repair Appendectomy Laparotomy Splenectomy	Surgical Disorders
Mallory Weiss tear	Gastrointestinal Disorders
Meningoencephalitis Status Epileptlicus Traumatic Head Injury Brain Tumour Encephalitis Arteriovenous Malformation (AVM) Idiopathic Intracranial Hypertension Near drowning	Central Nervous System (CNS) Disorders

2.7 Assessment of nutritional status and daily dietary intake

The nutritional status was assessed for both controls and critically ill children using z-scores (weight for age, height for age and weight for height), which incorporate anthropometric measurements of height/length and weight, according to the World Health Organization (WHO) child growth standards (Pichler et al. 2014). The z-scores were calculated using the WHO Anthro and AnthroPlus software (version 3.2.2, January 2011, Geneva) (Anon 2017b). The definitions of the WHO were used to define under-nutrition: moderate under-nutrition was considered if z-scores were between -2 and -3 standard deviation, and severe under-nutrition if below -3 z-scores (Anon 2010).

The actual nutritional intake was prospectively recorded for critically ill children using the patient clinical daily record. The type of feed (EN/PN/IV glucose) and total volume delivered was documented and from this the energy and macronutrient delivery was calculated for each patient. Calculation of breast milk composition was based on data from a previously published study on breast milk composition (Ballard & Morrow 2013).

To evaluate relationships between nutritional intake and the measured biomarkers, the amount of energy and macronutrients delivered were calculated for the 24-hour period prior to each sample collection. The cumulative intake of energy and macronutrient from admission was also calculated to assess the long-term effect of feeding on disease state. Energy and macronutrient intake were expressed in percentages of calculated requirements.

2.8 Standard measurement protocol of Indirect Calorimetry (IC)

Resting energy expenditure (REE) was measured using Ultima CCM™® IC. This metabolic cart employs a breath-by-breath gas analysis method. Gas sensors (*pneumotach* flow meter) measure both volume of oxygen consumed and carbon dioxide produced, which was then used to calculate REE automatically using the Weir equation:

REE = [3.9 (VO_2) + 1.1 (VCO_2)] 1.44 (WEIR 1949).

This indirect calorimeter has the capacity to measure REE in children over 10 Kg accurately.

The measurements were undertaken when the child was in a resting state. No nursing care or physical therapy had been provided for at least 1 hour before the test, to ensure the volume of oxygen (VO_2) and volume of carbon dioxide (VCO_2) was as stable as possible. IC was calibrated to reference gases prior to each participant according to the Manufacturer's Manual. The IC was then attached to the mechanical ventilator for at least 10 minutes before starting the test to ensure patient and ventilator stability prior to measuring REE. The test lasted for 40 minutes in a resting state, where intra variation between VO_2 and VCO_2 was less than 10% (Frankenfield et al. 2004). During the test, if any medical intervention had to be provided, the test was stopped and the measurement was repeated according to the above criteria, to ensure the continuity of a resting state. Information including respiratory quotient (RQ) and body utilisation of carbohydrate and fat was also obtained from IC and will be described in Chapter 4.

2.9 Faecal Calprotectin (FC) assay:

To assess the intestinal inflammation the levels of Calprotectin were measured in stool samples using *IDK®* Calprotectin ELISA (Bensheim, Germany), the lower limit of detection was 1.023 ng/ml. I performed the FC assays at the Cambridge University Department of Paediatrics laboratories.

Prior to performing the assay, the raw stool samples were allowed to thaw and were then mechanically homogenised. A volume of 15 mg of faeces was required. FC was extracted from the stool samples using *IDK®* Calprotectin ELISA kit (Bensheim, Germany), then the ELISA was performed according to the manufacturer's guidelines. The plate was read at an absorbance of 450nm against 620 nm. The obtained results were then multiplied by the dilution factor to obtain the actual FC concentrations in the sample.

2.10 Collaboration aspects:

2.10.1 Cytokine analysis

This assay was performed at the Core Biological Assay Laboratory (CBAL) in collaboration with Dr Keith Burling, Director of CBAL. I was responsible for processing and selecting the samples for the assay, analysing and processing the data and securing funds for the study.

Cytokines were measured using an electrochemical luminescence immunoassay from MSD, Rockville, Maryland, USA. A customised version of MSD 10-plex V-PlexProinflammatory Cytokine panel was used for the assay of pro- and anti-inflammatory cytokines. The lowest levels of detection are shown in Table 2-2.

Table 2-2: The lowest level of detection of measured inflammatory cytokines:

Cytokine	Lowest level of detection
Interleukin-6 (IL-6)	0.01-0.11 pg/ml
Tumour necrosis factor alpha (TNF-α)	0.01-0.13 pg/ml
Interleukin-10 (IL-10)	0.01-0.15 pg/ml
Interleukin-1 beta (IL1-β)	0.01-0.27 pg/ml

2.10.2 Metabolomics work

This work was done in collaboration with Dr. Anisha Wijeyasekara, Research Manager in the Division of Computational and Systems Medicine at Imperial College and Professor Elaine Holmes, the Head of the Division of Computational and Systems Medicine at Imperial College.

I was responsible for processing and selecting the samples for the assay, performing faecal water extraction and sample preparation procedure and I conducted the statistical analysis.

2.10.2.1 Faecal water extraction sample preparation

Faecal samples were subjected to 2 x freeze-thaw cycles. When the samples were defrosted they were mechanically homogenised using an inoculation loop. Roughly 100 mg of defrosted faecal sample was weighed and placed in a microtube with added 1.0mm Zirconia beads and 250µl of acetonitrile: water (ACN:H_2O) (1:3) (solvent). The tubes were placed in the Biospec bead beater and beaten for 1 x 10 seconds. Other tubes were centrifuged for 20 minutes at 16,000 × *g* and the filter was washed and spun through 3 times with (ACN):H_2O. Following this step, 250µl of supernatant from the sample was removed and placed into spin filter tubes and centrifuged for 30 minutes at 16,000 × *g.* The samples were stored at -80°C until the day of the run.

2.10.2.2 ^{1}H-NMR spectroscopy of faecal water

Faecal water samples were prepared for ^{1}H-NMR spectroscopy, for the assessment of SCFAs, according to published protocols (Gratton et al. 2016). The mixture of the sample and the buffer solution was vortexed and centrifuged for 10 sec, and 600 µL of the mixture was pipetted into NMR tubes with an outer diameter of 5 mm. Samples were analysed in a randomised order, and one-dimensional spectroscopic data were acquired according to established metabolic profiling protocols (Dona et al. 2014), using standard one-dimensional NMR pulse sequence with water pre-saturation.

2.10.2.3 ^{1}H-NMR spectral pre-processing and faecal water metabolic profile data analysis

Data was processed using the TopSpin 3.1 software package (Bruker Biospin, Rheinstetten, Germany) and SIMCA 13.0 software package (Umetrics AB, Umeå, Sweden) using unit variance (UV) scaling. Spectral peaks corresponding to the target compounds were integrated using the TopSpin 3.1 software package (Bruker Biospin, Rheinstetten, Germany), and the spectral integrals exported into Excel for further statistical analysis. This will be discussed thoroughly in the relevant chapter.

2.10.2.4 Liquid chromatography–mass spectrometry (LC/MS) :

Bile acid data were acquired by LC-MS in profiling mode (using a quadrupole time-of-flight (QToF) instrument. Data were acquired using protocols described by Sarafian et al. (2015). The serum citrulline data was also acquired using an LC-MS instrument but in targeted mode using a triple quadrupole (TQ) instrument, using the Absolute IDQ®P 180 kit (Biocrates Life Sciences, Innsbruck, Austria) which includes reference standards for targeted detection and quantification. Samples were prepared for analysis according to existing protocols (Sarafian et al. 2015). The identity of the detected compounds in the samples was confirmed by comparison of their retention times and their mass spectra with those of authentic standards and with reference spectra database.

2.10.3 Metataxonomic work

The principle of this work relies on the identification bacterial 16S ribosomal RNA (rRNA) gene in the faecal samples. The bacterial 16S rRNA gene has conserved regions which provide good PCR targets for broad range amplification and nine highly variable regions which provide good resolution of bacteria down to genus level and in many cases down to species level (Crawford et al. 2009). In this study we sequenced the bacterial 16S rRNA gene variable region one and two (V1-V2). The Illumina MiSeq 600 cycle kit enables the nearly full-length sequencing of the approximately 350 bp long V1-V2 region from both ends. A full-length sequencing from both ends is important to keep the sequencing error as low as possible.

This work was done in collaboration with Mrs Sarah Thurston, research scientist, department of paediatrics at University of Cambridge, and Dr Josef Wagner, senior postdoctoral scientist at the Welcome Trust Sanger institute. I was responsible for processing the samples after collection, and perform the DNA extraction; I also performed the statistical analysis.

Faecal samples were thawed before DNA extraction. Larger samples, especially those that came from healthy controls, were split for other analysis as well as for DNA

extraction. The DNA was extracted using the PowerFecal® DNA Isolation kit, MOBIO, CA, USA, following manufacturer guidelines. PCR's were then prepared to amplify the bacterial 16s rRNA gene (Figure 2.3), and produce libraries, as described by (Kozich et al. 2013).

PCR product was then cleaned up using magnetic bead methodology. Once cleaned the PCR production was quantified using a high sensitivity Qubit kit and fluorometer. The quantification permits the construction of a library equimolar mix to be prepared, which is then visualized using a 1% agarose gel and purified by using the Wizard® SV Gel and PCR Clean up system, Promega, WI, USA, following the manufacturers guidelines.

The equimolar library was then sequenced on the MiSeq sequencing platform following validation and quality control procedures at the Welcome Sanger Institute. The sequencing data was then quality filtered and analysed by Mothur, software package according to the MOTHUR MiSeq SOP (MOTHUR wiki at http://www.mothur.org/wiki/MiSeq_SOP). Sequencing reads were binned into operational taxonomic units (OTUs). The generated OTU tables were then used in the statistical analysis of the gut microbial profiles of critically ill and healthy children.

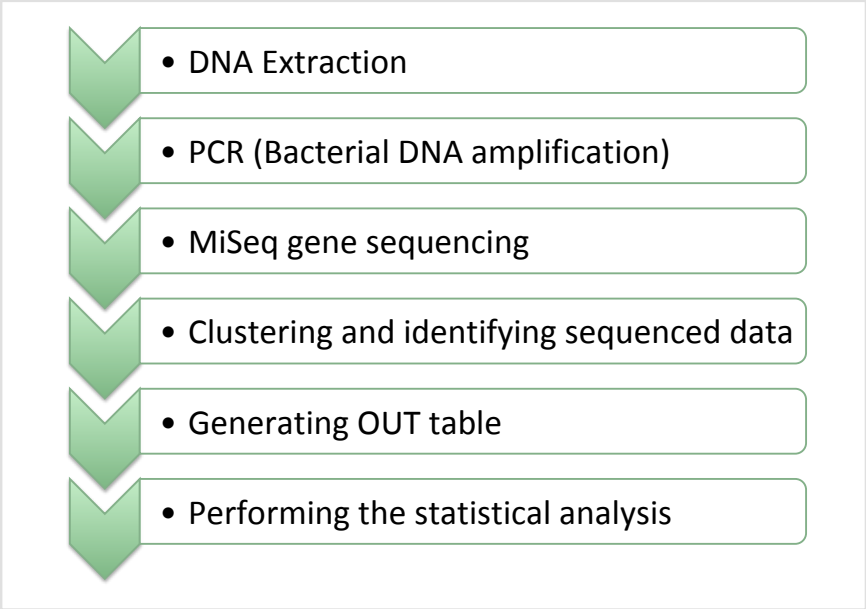

Figure 2.2 The process of sample handling and data analysis of the metagenomic work

The above figure summarises the steps of gut microbial analysis, from the DNA extraction to the generation of OUT tables and performing the statistical plan

Components	25 ul Reaction		MM x 26	Final Concentration
Nuclease free water	14.25	µl	370.5	Make up to 25 µl
5x Q5 PCR Buffer	5	µl	130	1X
10 mM dNTPs	0.5	µl	13	200 µM
10 µM forward primers (500 number primers)	Add individual 1.25	µl	32.5	0.5 µM
10 µM reverse primers (700 number primers)	1.25	µl	32.5	0.5 µM
Q5 Taq PCR enzyme	0.25	µl	6.5	0.02 U/µl
Template DNA	2.5	µl	65	< 1000ng
Total volume	**25**	**µl**	**650**	
Master mix volume	21.25	**µl**		

PCR conditions:		
98°C	2 mins	
98°C	30 secs	**20 cycles***
50°C	30 secs	
72°C	90 secs	
72°C	5 mins	
4°C	∞	

Figure 2.3 PCR mastermix set-up and amplification conditions for MiSeq library preparation

2.10.3.1 Contamination detection

By using various guidelines outlined in published work by Salter et al. (2014), the effects of exogenous kit-associated DNA contamination was mitigated. The methods used to reduce the effects of contamination included using and sequencing appropriate controls including biological replicates, recording the kit from which samples were extracted, reducing contamination as much as possible during collection and extraction by using sterile materials and applying general aseptic techniques, and randomising sample handling as much as logistically possible. Once the sequencing was performed, contaminant OTPs were identified using the negative controls, or association with extraction kit, batch or sequencing run. Many of these taxa were similar to those previously identified as contaminants (Salter et al. 2014).

2.11 Statistical analysis

2.11.1 Univariate statistics

The statistical package used for this analysis were IBM SPSS v25 and Microsoft® Excel® 2011. The statistical tests used were simple descriptive statistics for the majority of the study variables to show the counts and percentages as well as median and interquartile range (IQR). The Shapiro-Wilk Test was used to assess the normality of the data distribution as the sample size was not large.

For the normally distributed variables T-test was performed to compare two variables and one-way ANOVA was used to compare repeated measurement. For variables with skewed distribution, Mann- Witney-U for two group comparison and the Kruskal-Wallis tests for three or more group comparison were applied. Spearman correlations were performed to detect the statistical association between variables.

2.11.2 Multivariate statistics

Linear and logistic regression analysis was performed to assess the association between multiple variables. Additional multivariate analysis was undertaken for the metataxonomics and metabolomics work (this will be explained in more detail in the relevant chapters). Multivariate analysis was necessary for these datasets as the generated OTU tables contained hundreds of bacterial species. In addition, the digitised spectroscopic data also contained large number of variables from the same peak, or multiple peaks from the same metabolite. So multivariate analysis enables the assessment of these altogether, factoring in that there will be correlations between variables from the same class (or group). Principal component analysis (PCA) was used to observe the overall pattern/trends and to detect outliers. A permutational multivariate analysis of variance (PERMANOVA) test was used to assess if the observed differences (in multivariate space) were statistically significant (Anderson & Walsh 2013). Partial Least Square Discriminant Analysis (PLS-DA) was then performed to reduce the dimension, maximise the separation between groups and view important microbial

characteristics of the groups. The models were assessed based on goodness-of-fit (R^2 Y) and goodness-of-prediction (Q^2 Y) metrics.

2.11.3 Sample size calculation

Sample size calculation was performed using the statistical power analysis program G*Power 3 (Faul et al. 2007). Whilst much of the data were multiparametric, the level of faecal butyrate was selected as the primary outcome for this project. The sample size was chosen to detect a clinically statistical difference in total faecal butyrate between critically ill children and healthy controls. There are no data in the paediatric population. Data from studies in critically ill adults demonstrates a rapid, sustained decrease in faecal butyrate compared to healthy adults. This assumption was based on work by Yamada et al. (2015) in critically ill adults where a difference of 12 micromol/g faeces of butyrate was recorded between critically ill and healthy controls, it was estimated that 25 children per group would be needed to detect the same difference between critically ill and healthy children, assuming a 5% significant level (2-sided) and 90% power. Assuming a drop-out rate of 20%, it was estimated that 30 children would be needed in each group.

Many other studies have shown a rapid and dramatic loss total SCFAs in the critically ill adults compared to healthy subjects. In a study by Shimizu et al. (2006) mean faecal butyrate in critically ill patients was 0.9 micromol/g faeces compared to 16 micromol/g faeces in healthy controls with recorded difference of 15 micromol/g faeces. While Hayakawa et al. (2011) recorded a difference of 10 micromol/g faeces of butyrate between critically ill adults and healthy controls.

For the primary endpoint 30 children were required, but as the secondary endpoints included: intestinal and systemic inflammation and stool microbial composition I performed sample calculation for each sub-analysis. Sample size calculation was performed considering the specification of the research question in each chapter to ensure that sufficient data was collected to show statistical significance.

3 Chapter Three: An Overview of the Population Characteristics, Biological samples and Assays Undertaken

3.1 Introduction

Critical illness affects more than 20,000 children per year in the UK. It has been estimated that nearly 2% of children admitted to PICUs are discharged and then readmitted as an emergency within 48 hours (Parslow & Draper 2017). The recent Paediatric Intensive Care Audit Network (PICANet) report indicated that the majority of admitted children were under one year of age (Parslow & Draper 2017). There are many reasons for PICU admission; a national cohort study by Fraser & Parslow (2018) indicated that respiratory and cardiac diagnoses were the most common reasons for PICU admission (Fraser & Parslow 2018). The NHS national review showed that the mean length of PICU stay for children who survived was 6 days compared to 17 days for those who died (Anon 2017a). The PICANet report indicated that the crude mortality rates in PICUs is 4% (Parslow & Draper 2017). The effect of acute insult may extend until after discharge from PICU and compromise the functional outcome and quality of life in surviving children (Taylor et al. 2003; Typpo et al. 2009). Approximately 12% of children who were admitted to a PICU during the period 2002 – 2015 died either in the PICU or following discharge (Parslow & Draper 2017).

Growing evidence points to the role of gut barrier dysfunction during critical illness in the pathophysiology of inflammation and organ dysfunction (Pathan et al. 2011; Mittal & Coopersmith 2014). The main study of gut homeostasis (GHICI) was designed to examine the changes in gut-host relationship over a time-course from admission to discharge in critically ill children. A better understanding of the relationship between gut failure and multi-organ dysfunction in critical illness will allow us to define criteria for the therapeutic modulation of the intestinal environment using prebiotics and/or micronutrients. This PhD focuses particularly on examining the effect of nutrition on the

intestinal microbiome-host relationship in critically ill children and whether it has an impact on clinical disease severity.

The purposes of this chapter are to describe the subjects who were enrolled in the study, to present the number of samples collected from each study group and to highlight the assays undertaken in this PhD project.

3.2 Results

3.2.1 Population

A total of 186 critically ill children and 70 healthy children were recruited for this PhD project. Critically ill children were recruited from paediatric intensive care at Addenbrooke's Hospital in the UK (N=166) and Hospices Civils de Lyon in France (N=20) between November 2014 and May 2017. Healthy children were recruited from outpatient clinics and from the community in the Cambridge area. Due to the limitation of obtaining blood samples from these children, another control group of children undergoing elective surgeries was recruited.

The demographic characteristics of patients and controls are presented in Table 3-1. The results showed that the control group were significantly older and therefore also had higher growth parameters, than the patient group. There was no difference in the gender distribution of critically ill and healthy children. Eighty-five females and 101 males were recruited in the study group and 38 female and 32 males in the control group. All patients received at least one broad-spectrum antibiotic therapy and 61.8% (N=120) of them received more than one type of antibiotics. The median (IQR) number of antibiotic classes received per patients was 2 (1-3).

Among the study group the majority of the children were admitted to PICU due to MOF, respiratory and CNS disorders diagnosis. The distribution of the categories of primary diagnosis among the patient group is shown in Table 3-2. All patients received invasive mechanical ventilation. The PICU mortality among the study group was 5% (N=10).

The majority of the children recruited in this study were fed via enteral route (N=145), while 8 children received parenteral nutrition (PN) and 3 children who were enterally fed had also supplementary PN (Table 3-3). EN was commenced at a median (IQR) time of 8 (5 - 12) hours following PICU admission. Only 7 children started their enteral feeding >24 hours after PICU admission. The most common reasons for delaying enteral feed

were failed attempts of extubation (N=4), diagnostic tests (N=1), haemodynamic instability (N=1) and planning for PN (N=1).

Table 3-1: Anthropometric and clinical characteristics of children enrolled in the study

Population characteristics	All patients and controls		
	Patients (N=186)	Control (N=70)	P value
Age (years)	2.4 (0.65 – 6)	4.8 (2.9 – 6.6)	0.00**
Weight (Kg)	13 (7.8 – 20.1)	19.8 (14 – 28)	0.00**
Height (cm)	90 (73 – 112)	110 (92 – 123)	0.00**
Weight age Z score	-0.33 (-1.56 – 0.54)	0.36 (-0.07 – 0.72)	0.00**
Height for age Z score	-0.2 (-1.9 – 0.97)	0.64 (-0.27 – 1.1)	0.006**
Weight for height Z score	0.01 (-1.19 – 0.72)	0.14 (-0.44 – 0.81)	0.056
Disease severity variables			
PIM2 score	3.7 (1.2 – 7)		
PMODS	6 (5 – 7)		
Highest Lactate	1.9 (1.4 – 3.3)		
Highest CRP	74 (35 – 198)		
Inotrope score	0.0 (0.0 – 17)		
Duration of mechanical ventilation	93 (61 – 168)		
VFH at 30 days	625 (545 – 657)		
Hours of Inotrope	0 (0 – 28)		
Hours free of Inotrope at 30 days	720 (683 – 720)		
Length of PICU stay	7 (5 – 11)		
PICU free days at 30 days	23 (18 – 25)		

Data are presented as median (IQR)

** P value is significant at 0.01 level

* P value is significant at 0.05 level

VFH at 30 days: ventilation-free hours at 30 days

PICU free days at 30 days: days free of paediatric intensive care at 30 days

Table 3-2: Patient's primary admission diagnostic category

Primary admission diagnosis	N (%)
MOF	56 (30%)
Respiratory Failure	84 (45%)
CNS Disorders	35 (19%)
Surgical Disorders	8 (4.3%)
Cardiac Disorders	1 (0.5%)
Gastrointestinal Disorders	1 (0.5%)

Table 3-3: Number of enterally and parenterally fed critically ill children

Route of feeding	N (%)
Oral	3 (1.6%)
Nasogastric (NG)	129 (69.5%)
Gastrostomy	10 (5.4%)
Jejunostomy	2 (1 %)
Orogastric tube (OGT)	1 (0.5%)
PN	8 (4.4%)
NG with supplementary PN	3 (1.6%)
Unknown	30 (16%)

3.2.2 Samples

Serum

A total of 204 serum samples were collected from 106 patients. Of those 194 were serial samples collected from 86 patients (see Figure 3.1). Seventeen serum samples were collected from the control group.

Faecal samples

Eighty-nine critically ill children provided 119 faecal samples. Generally, a single sample was collected from each child. However, in a number of critically ill children more than one faecal sample was available within the study period. A single faecal sample was collected from 70 healthy children.

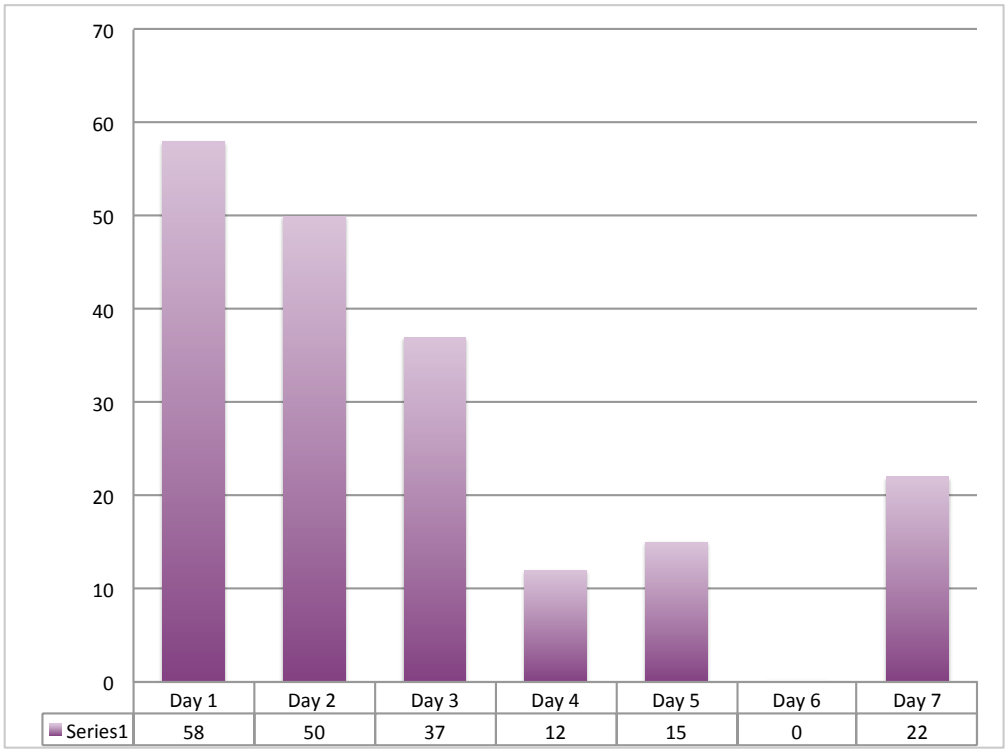

Figure 3.1 The number of longitudinal serum samples collected from critically ill children

3.2.3 Assays and measurements undertaken

Several measurements and assays were undertaken; these included:

- Collection of demographic, anthropometry, clinical and nutritional data.
- Measurements of energy expenditure by IC.
- Measurement of inflammatory cytokines and citrulline in serum.
- Measurement of faecal calprotectin.
- Faecal metagenomic and metabolomic analysis.

The numbers of patients included in each assay are presented in Figure 3.2.

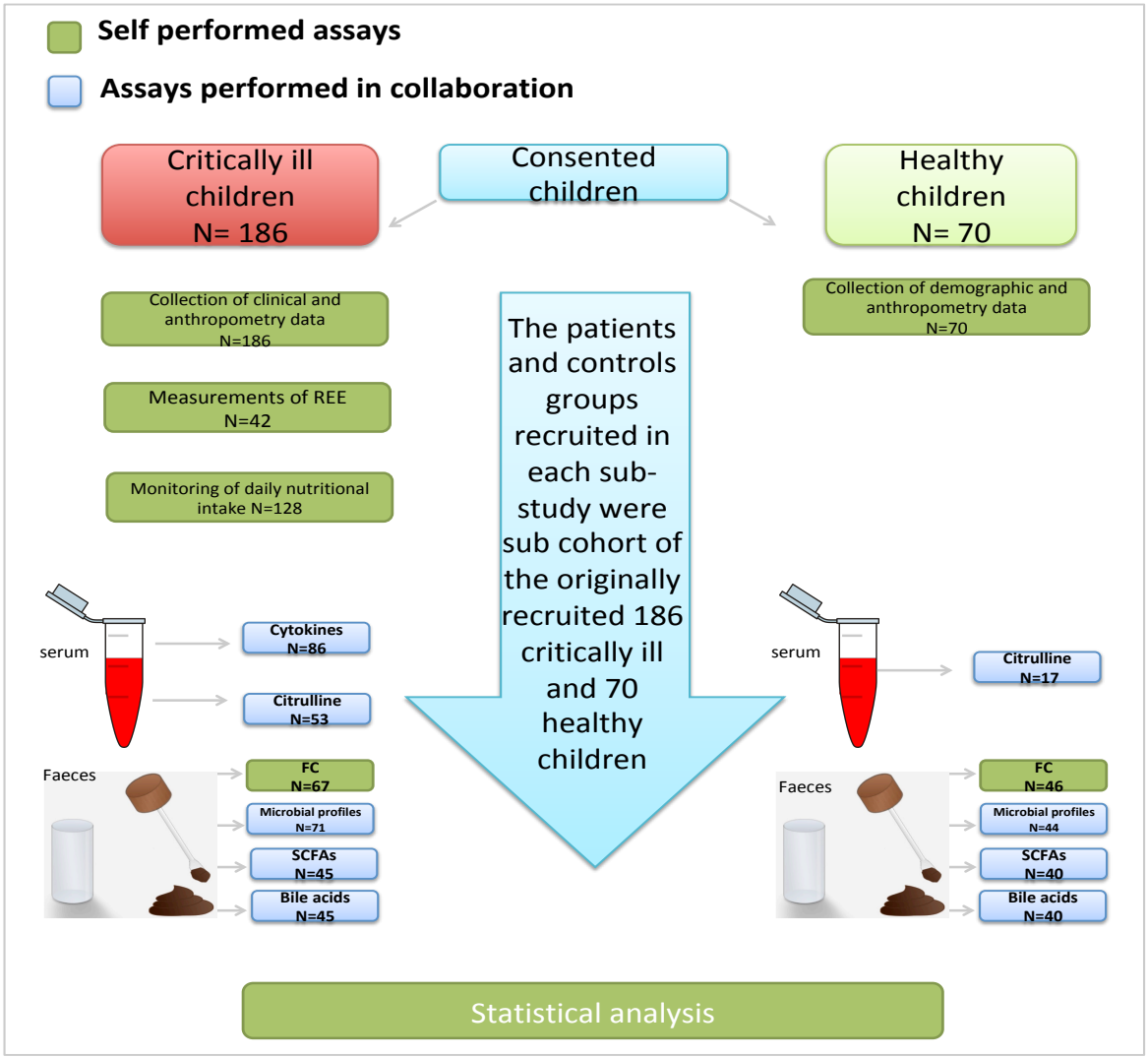

Figure 3.2 Patient and control enrolment algorithm showing the number of children included in each study of this PhD project

The patients and controls groups recruited in each sub-study were sub cohort of the originally recruited critically ill (N=186) and healthy children (N=70) groups, ie. For some patients multiple assays were preformed, the numbers will be specified in the relevant chapters.

3.3 Discussion

Studies investigating the changes in intestinal microbiome in critically ill children are limited. The majority of studies have been performed with adult populations (Howard et al. 2017; Mcdonald et al. 2016; Ojima et al. 2016; Shimizu et al. 2011; Hayakawa et al. 2011; Shimizu et al. 2006). Only one study by Rogers et al. (2016) investigated the microbiome of different body sites including the gut in critically ill children. They analysed gut microbial data in 37 critically ill children aged between 1 and 9 years old. This PhD project aims to investigate the effect of nutrition on the intestinal microbiome-host relationship in a larger cohort of critically children. This chapter describes the critically ill and control children groups recruited in the study.

The median age of the patients group was 2.4 (0.65-6) years old, which is similar to data from population-based studies in PICU settings where the majority of the children are aged between 1 and 4 years (Ibiebele et al. 2018; Fraser & Parslow 2018). The results of this chapter, however, showed that the healthy control group was statistically older than the study group with a median age of 4.8 (2.9-6.6) years. This is mainly because the majority of children admitted to PICU were under 4 years old, as was shown in the PICANet report and other national studies (Parslow & Draper 2017; Fraser & Parslow 2018; Ibiebele et al. 2018). In addition, the control group included children undergoing elective surgeries and those are usually older than 4 years. Selvadurai et al. (2018) indicated that the mean age of patients undergoing elective surgery was 4.8 ±2.0 years. Therefore, where comparison is made between patients and healthy children in the subsequent chapters, the analysis is restricted to the age-matched cohort.

The most common diagnostic categories dictating PICU admission among the patient group were respiratory failure, MOF and CNS disorders. Other studies utilising data collected from the PICANet clinical audit database have also shown a similar distribution of patients' diagnosis (Ibiebele et al. 2018; Green et al. 2016; Fraser & Parslow 2018; Morris et al. 2017). Around 45% of critically ill children included in this study were admitted due to respiratory conditions. PICU admissions for infants with bronchiolitis

have increased in recent years in the UK (Green et al. 2016). This is mainly due to the increasing prevalence of maternal and perinatal factors that predispose to severe disease (Green et al. 2016). The overall admission rates due to lower and upper respiratory tract infections are also increasing (Gill et al. 2013). Many children admitted to PICU have a brief stay and recover well, but a significant proportion may develop MOF (Hamshary et al. 2017). Nearly 30% of the patient group developed MOF. Watson et al. (2017) indicated that MOF is common among this population, occurring in up to 57% of critically ill children. CNS disorders were also frequent among the current cohort, occurring in 19% of the children. Generally, CNS disorders are a common reason for PICU admission and they are usually associated with higher mortality, morbidity and prolonged PICU stay (Aurangzeb et al. 2012).

The median (IQR) length of PICU stay among the study group was 7 (5-11) days. This matched the national data where a median of 6 days stay in PICU was recorded (Ibiebele et al. 2018). All critically ill children received invasive mechanical ventilation during the first day of PICU admission and the median duration of invasive mechanical ventilation was 93 hours. Based on PICANet data, 67.5% of children admitted to UK PICUs receive invasive mechanical ventilation compared to 31.7% who receive non-invasive mechanical ventilation (Morris et al. 2017). The duration of ventilation and length of PICU stay were also presented as hours free of mechanical ventilation at 30 days and days free of PICU stay at 30 days to correct for those who died.

The majority of the children included in the current study were fed via the enteral route. EN was started within a few hours of admission (median of 8 hours) according to the PICU's enteral feeding protocol. There is a strong consensus that the enteral route of providing nutrition is preferred over the parenteral route (Seres et al. 2013). Enteral feeding is a mainstay of nutrition in most critically ill children, with 73% receiving EN (Kerklaan et al. 2016). The current practice in many centres is the early initiation of EN within 24-72 hours of PICU admission; enteral feeding would even be started within 12 hours in some PICUs (Kerklaan et al. 2016; Mehta et al. 2009; Mara et al. 2014). Delayed initiation of enteral nutrition may often be due to extubation, intubation, feeding tube

displacement, diagnostic procedures, gastrointestinal bleeding, decreased bowel motility, abdominal distention, large aspirates and hemodynamic instability (Keehn et al. 2015; Lee et al. 2013). In the current cohort, the most common reasons for delaying enteral feed were failed attempts of extubation, diagnostic tests, haemodynamic instability and planning for PN. Early initiation of EN is associated with improvement in the nutritional status and clinical outcomes in critically ill children (Briassoulis et al. 2001; Kaplan & Frangos 2005; Mehta et al. 2012). PN, on the other hand, is being used to supplement or replace EN in some cases where the nutrition goal is not met by EN alone (Mehta et al. 2009). The results also suggested that our cohort is relatively normally nourished and representative of the general UK population (NatCen Social Research 2017).

With regard to the primary objective of this study, an integrated approach of metataxonomics and metabolomics analysis was used to examine how feeding during critical illness affects gut-host relationship. This required not only the collection of faecal samples; serum samples were also essential for the analysis of inflammatory cytokines and intestinal injury biomarkers. The results showed inconsistency in the number of serum serial samples collected at each time-point. Ideally all samples should be collected on the same day at the same time. This was unlikely to happen due to the nature of PICU settings, so the samples were taken when permissible. It was challenging to collect the whole set of serum samples for a number of reasons. Firstly, for some children it was not possible to consent the parents as early as required and accordingly samples were not taken on the early days of admission. The majority of critically children did not have an indwelling line until day 7, which limited the extent to which time-course analysis is possible. Furthermore, a considerable percentage of the children were extubated and the indwelling line for blood sampling was removed. All of these factors accounted for the inconsistency in the number of samples collected at each time-point. Collecting the faecal sample was also challenging; it is not surprising that not all the enrolled children provided a faecal sample within the course of their PICU stay. The sample volume was also problematic; the small volumes of blood and faecal samples

obtained from children required the prioritisation of assays. In some cases the volume was only enough for one assay and not the other.

A number of assays and measures were undertaken to achieve the objectives of this thesis. As not all the sub-studies of this PhD project included the same cohort of critically ill and healthy children, I will present the population characteristics of the cohort included in each study in the relevant chapter.

3.4 Conclusion

The demographic and clinical characteristics of the critically ill children included in this study match the national reports describing PICU population in the UK. The age variation between patients and controls might affect the validity of the comparison between the groups. Therefore, this should be taken into account when performing the statistical analysis in the subsequent chapters. A number of reason accounted for inconsistency in the number of serial biological samples, mostly related to limitations in the sample volumes and challenges in the sample collection process in this particular population and setting.

4 Chapter Four: Measuring Energy Expenditure in Critically Ill Children

4.1 Introduction

The nutritional needs for critically ill children are frequently not fulfilled in the acute phase of critical illness due to several reasons: lack of routine nutritional assessments, fluid restriction, feed intolerance, and frequent interruption of enteral feeding (Hulst et al., 2006; Mehta et al., 2012). Importantly, estimating energy and macronutrient requirements in this group of patients remains a clinical challenge (Hulst et al., 2006; Mehta et al., 2012).

As critically ill children undergo different phases of illness, the metabolic response to injury varies over time (Wischmeyer, 2013). Studies have found that critically ill children have reduced energy expenditure during the acute phase of illness (Briassoulis et al., 2000; Taylor et al., 2003; Framson et al., 2007; Mehta et al., 2012). The increase in energy expenditure only occurs in the later stable and recovery phases (Joosten et al., 2016). The variation in energy expenditure might be due to the different and often opposing effects of the neuro-endocrine, immunological and metabolic components (Joosten et al., 2016). As it is extremely challenging to estimate requirements during the course of illness, IC has been recommended as the gold standard to determine energy requirements in critically ill children (Mehta et al., 2017). During the oxidation of carbohydrates, proteins and fat, oxygen consumption and carbon dioxide production occur, which is measured by an IC to enable the automated calculation of REE (Frayn, 2003). The principle of IC is based on the assumption that the inspired oxygen is completely utilised to oxidise degradable fuels, and during this process carbon dioxide is generated (Gupta et al., 2017). Nearly 80% of REE is due to oxygen consumption, and the remaining 20% of energy expenditure is due to carbon dioxide production. Studies that performed serial measurements of REE recorded a minimal intrapersonal variation in REE of <10% during the first week of PICU stays (De Klerk et al., 2001; Framson et al.,

2007). These studies suggested that single measurement over the course of PICU admission of REE would provide an insight regarding the daily energy requirements of critically ill children. The ASPEN guidelines also recommend at least one REE measurement for children at high risk of metabolic alteration to guide energy administration (Mehta et al., 2017).

A number of factors may restrict the regular use of IC in PICU settings, therefore, there is still an urgent need for formulas that closely estimate the energy needs of critically ill children (Kyle et al., 2012). The aim of this study was to assess the energy metabolic patterns of critically ill children and identify an equation predicting energy requirements closest to measured resting energy expenditure. The study also aimed to explore factors that might affect energy expenditure, such as disease severity, drugs, body temperature and diagnosis.

4.2 Materials and Methods

One 40-minute measurement of REE was performed for critically ill children admitted to PICU between days 1 and 6 using Ultima CCM™® IC. The standard measurement protocol of IC has been described in the Method Chapter (Chapter Two), pages 52-53.

Inclusion criteria:

- Mechanically ventilated children for more than 24 hours.
- Weight >10 kg (as the IC is not accurate in children with a weight < 10 kg).

Exclusion criteria:

- Fraction of inspired oxygen (FiO_2) >60%.
- Endotracheal tube leak >10%.
- Chest drain.
- Peritoneal dialysis.
- Hemodialysis/hemofiltration.

4.2.1 Respiratory quotients and fuel utilisation

Information including respiratory quotient (RQ) and body utilisation of carbohydrate and fat was also obtained from IC.

RQ was automatically calculated by the IC based on the following equation:

RQ = VCO2 / VO2

Table 4-1: Interpretation of RQ and adequacy of intake

RQ	Feeding status
>1	Overfeeding with lipogenesis
0.85-0.95	Mixed substrate utilisation (adequate intake)
<0.82	Underfeeding

Values are adopted from The Clinical Dietitian's Essential Pocket Guide (Width & Reinhard, 2009).

The percentage of carbohydrate and fat utilised was obtained from IC automatically based on the assumption of the following equations:

Carbohydrate grams per day = 4.21*VO_2 - 2.91*VCO_2 (Ruppel, 1991).

Percentage of carbohydrate utilised = Carbohydrate (kcal per day) *100/REE

Fat grams per day =1.69*VO_2 - 1.69*VCO_2 (Ruppel, 1991).

Percentage of fat utilised = Fat (kcal per day) *100/REE

4.2.2 Estimation of energy requirements by predictive equations

The current protocol at Addenbrooke's Hospital for estimating energy requirements in critically children adopts the recommendations of the Scientific Advisory Committee on Nutrition (SACN) (Jackson, 2011). The SACN established energy reference values predicted using healthy reference body weights, corresponding to the 50th centile of UK–WHO growth standards for infants and children (Jackson, 2011). For the purposes of this study, energy requirements were also estimated by the Schofield equation and WHO equation, as suggested by ASPEN guidelines and other studies, in the absence of IC (Schofield, 1985; Joint FAO/WHO/UNU Expert Consultation on Energy and Protein Requirements, 1981; Rome, 1985; Meyer et al., 2012; Jotter et al., 2017; Mehta et al., 2017).

4.2.3 Assessment of hypo- and hyper-metabolic status

In order to categorise the children's metabolic patterns into normal, hypo- or hyper-metabolic states, measured energy expenditure (MEE) was compared with predicted norms that come from a set of predictive equations to determine predicted resting energy expenditure (PREE). In this cohort, PREE calculations were based on the Schofield and WHO equations, as these are currently recommended by ASPEN and ESGPHAN in the absence of IC. The patient's metabolic and energy expenditure pattern was determined using the previously published metabolic index (Briassoulis et al., 2010) of MEE/PREE previously used in studies: the indices of >1.1, 0.9–1.1 and <0.9 were classified as hyper-metabolic, normo-metabolic and hypo-metabolic patterns,

respectively. This is a known method that has been used in many studies to assess the energy expenditure patterns of critically ill children (Briassoulis et al., 2010; Coss-Bu et al., 2001; Mehta et al., 2017).

4.2.4 Dietary intake

The actual nutritional intake over 24 hours prior to IC measurement was recorded retrospectively using patient clinical electronic records. The types of feed (enteral or parenteral, IV glucose) and total volume delivered were recorded for each patient for the calculations of energy and macronutrient intake. Energy intake was expressed as percentages of the measured requirements.

4.2.5 Body temperature

Axillary temperature was taken hourly by the nursing team and logged into the patient's records. The value obtained for the analysis was taken within an hour of IC measurement.

4.2.6 Medications

Medications received within 24 hours of IC measurements were recorded and categorised into three groups: sedatives, inotropic agents and neuromuscular blocking agents.

Table 4-2: Medication categories

Category	Drugs
Sedatives	Midazolam, Propofol, Chloral hydrate, Fentanyl, clonidine
Inotropic agents	Adrenaline, Noradrenaline, Dopamine, Milrinone
Neuromuscular blocking agents	Vecuronium, Atracurium, Suxamethonium

4.2.7 Sample size calculation

As this study was observational in describing the energy metabolic pattern, and aimed at just documenting differences between predictive equations and MEE, no power calculation was used. All children enrolled in the GHICI study and met the inclusion criteria of the current study were included.

4.2.8 Statistical analysis

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as a median with interquartile ranges (IQR). The Wilcoxon signed rank test was used to compare REE measured by IC and estimated by prediction equations. Bland-Altman plots were used to evaluate the agreement between measured and estimated REE, and allow for the identification of difference between the measurements. These plots were also used to quantify bias and the range of agreement, within 95% confidence limits. The Mann-Witney U test was applied to assess differences in fuel utilisation between groups. A chi-squared test was performed to assess the relationship between the hyper-metabolic response and the diagnostic categories.

The Spearman correlation coefficient was used to establish the correlation between variables, which was followed by stepwise linear regression analysis to assess factors that impact REE. A p-value of ≤ 0.05 was considered statistically significant, and log transformation was applied on variables with skewed distribution prior to regression analysis.

4.3 Results

IC measurements were performed on 46 critically ill children; four children were excluded due to the interruption of measurements by routine medical care affecting VCO2/VO2, rendering measurements inaccurate. All measurements were performed between days 1–6 post admission, with a median (IQR) 3 (2–4) days. Forty-two children [21 (50%) males] with a median (IQR) age of 4 (2–10) years were included in this study. The anthropometric and clinical characteristics of the children are shown in Table 4-3. Amongst the cohort, hospital mortality was 5% (n=2).

Table 4-3: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometrics N (42)	**Median**
Age (years)	4 (2 – 10)
Weight (kg)	17.8 (13.8 – 25.8)
Height (cm)	101 (87 – 117)
Weight age Z- score	0.43 (-0.34 – 1.2)
Height age Z- score	-0.14 (-1.2 – 1.12)
Disease severity variables	**Median**
PMODS	6 (5 – 7)
PIM2 score	3.0 (0.87 – 6.0)
Highest lactate (on the day of REE measurement)	1.1 (0.7 – 1.7)
VFH of mechanical ventilation at 30 days	628 (544 – 649)
PICU free days at 30 days	22 (19 – 24)
Primary admission diagnosis	**N (%)**
MOF	9 (22%)
Respiratory Failure	16 (38%)
CNS Disorders	14 (33%)
Surgical Disorders	3 (7%)

Data are presented as median (IQR).

VFH at 30 days: ventilation free hours at 30 days

PICU free days at 30 days: days free of paediatric intensive care at 30 days

4.3.1 Comparison between measured energy expenditure (MEE) by IC and the estimated energy requirements with predictive equations

The first step of the analysis was to compare MEE to estimated energy requirements with different predictive equations. The median (IQR) of MEE was 922 (679-1348) kcal/day [49 (33-60) kcal/kg]. No statistical difference between energy expenditure measured by IC and estimated by the Schofield equations [median (IQR) 899 (753-1001) kcal/day, 46 (38-52) kcal/kg], p = 0.181 and WHO equation [median (IQR) 937 (795-1068) kcal/day, 50 (41-56) kcal/kg], p = 0.431 was recorded. Energy requirements estimated by SACN recommendation were statistically higher than MEE [median (IQR) 1378 (1092-1768) kcal/day, 49 (33-60) kcal/kg] (p=0.431, p=0.00) (Figure 4.1).

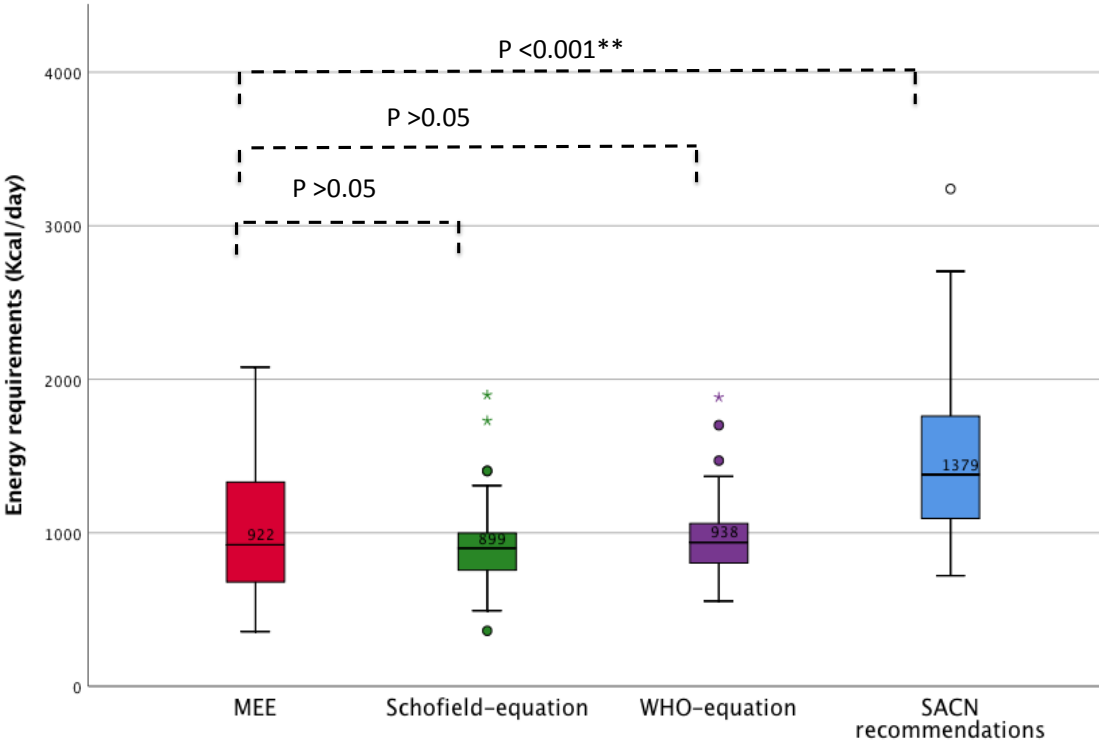

Figure 4.1 Comparison between measured energy expenditure and estimated energy with different predictive equations

Figure 4.1 shows that estimated energy requirements, according to SACN recommendations for healthy children, were statistically higher than the measured requirements. On the other hand, there was no statistical difference between measured and estimated energy with the Schofield and WHO equations.

The Bland-Altman method was also used to assess the limits of agreement between MEE and estimated energy needs by Schofield equation, WHO equation and SACN recommendations (Figures 4.2–4.4).

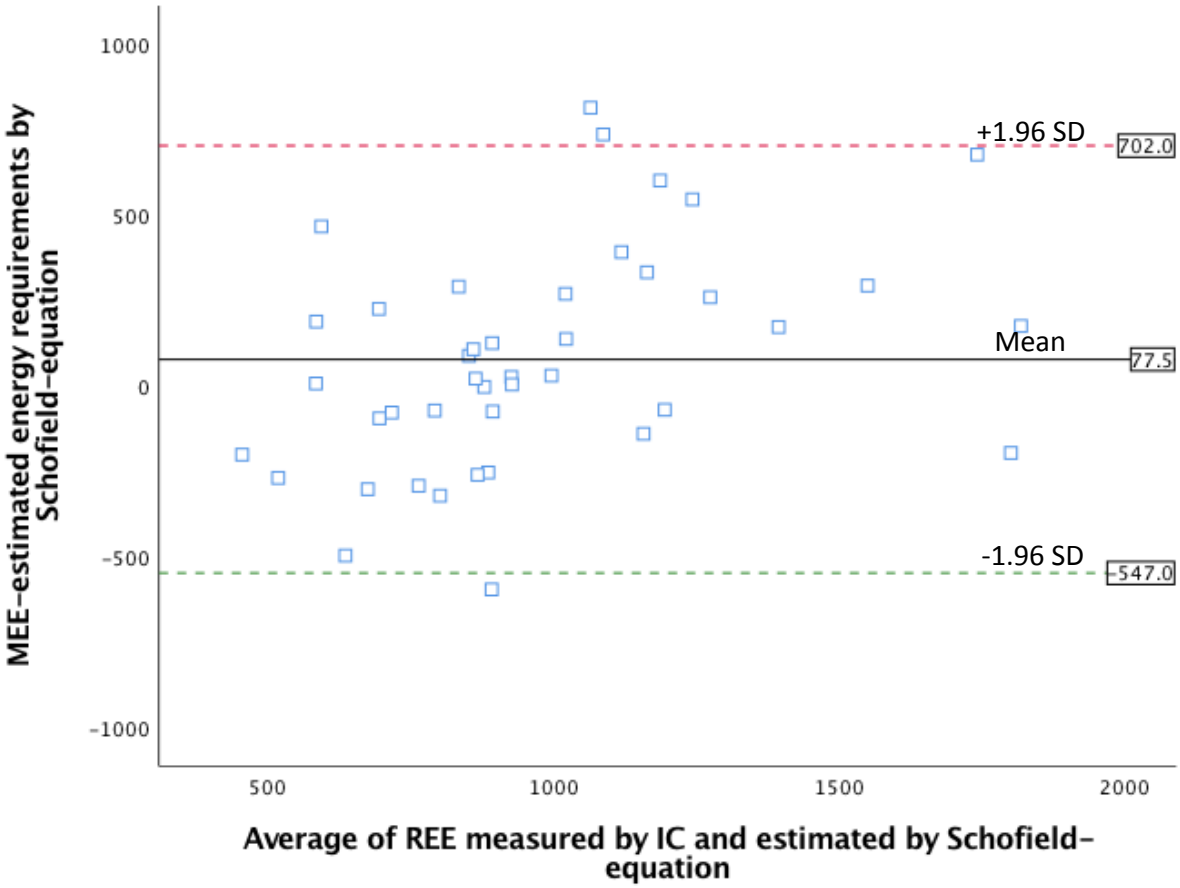

Figure 4.2 Bland-Altman plot comparing means of energy requirements measured by IC and estimated by Schofield equation

Figure 4.2 shows that the mean difference between the REE measured by IC and estimated by the Schofield equation was 77 kcal/day, and the SD of the differences was 318 kcal/day. The lower limit of agreement for REE was -547 kcal/day and the upper limit 702 kcal/day, with a wide scatter around the mean and three outliers outside upper and lower 95% CI.

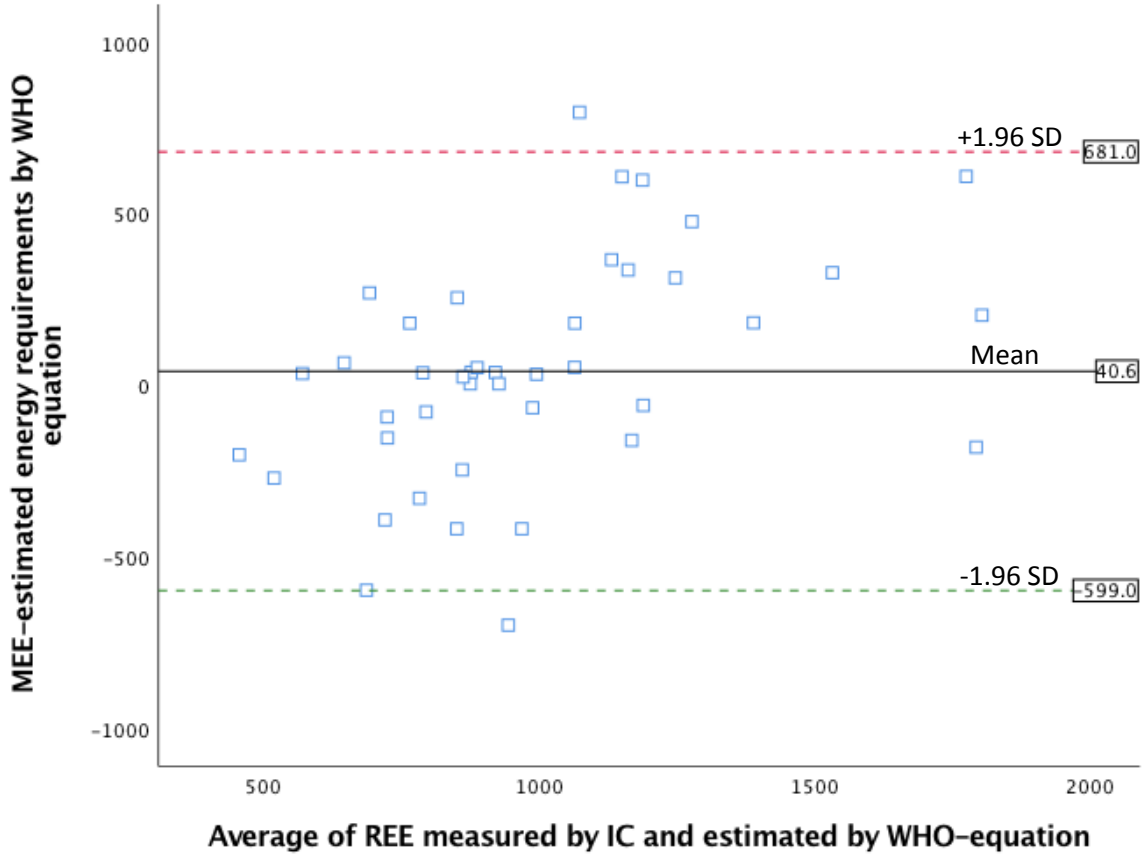

Figure 4.3 Bland-Altman plot comparing means of energy requirements measured by IC and estimated by the WHO equation

Figure 4.3 indicates that the mean difference between the REE measured by IC and estimated by the WHO equation was 40 kcal/day, and the SD of the differences was 326 kcal/day. The lower limit of agreement for REE was -599 kcal/day and the upper limit 681 kcal/day, with a wide scatter around the mean and two outliers outside upper and lower 95% CI.

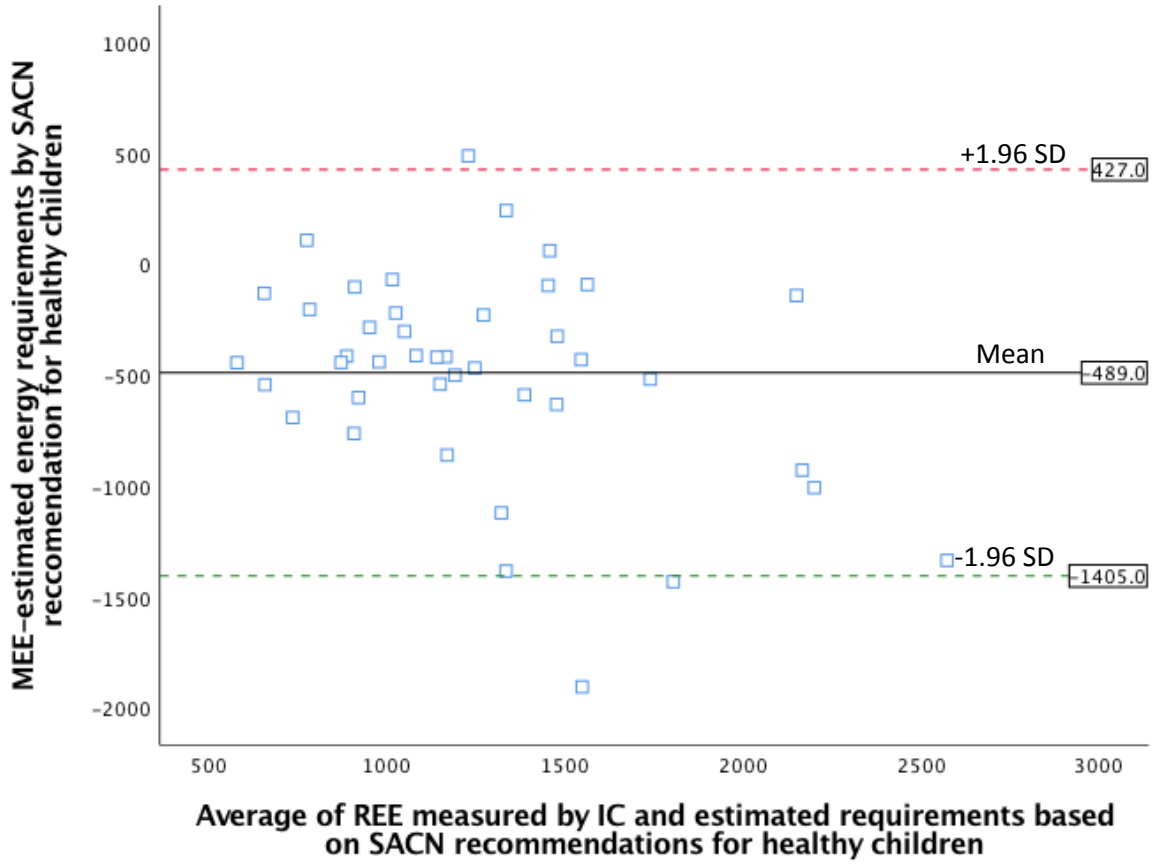

Figure 4.4 Bland-Altman plot comparing means of energy requirements measured by IC and estimated by SACN recommendation

Figure 4.4 shows a mean difference of -488 kcal/day (SD 467 kcal/day) between the REE measured by IC and estimated according to SACN for healthy children. The lower limit of agreement for REE was -1405 kcal/day and the upper limit 427 kcal/day. The figure also shows cluster scores where the mean is low and the difference is high.

4.3.2 Energy metabolic pattern in critically ill children

By using the Schofield equation to predict the normal metabolic response, data showed that 19 children exhibited a hyper-metabolic pattern, 13 were hypo-metabolic and 10 showed normal metabolic response. The WHO equation predicted slightly higher energy requirements, and therefore only 16 showed a hyper-metabolic pattern, 13 were hypo-metabolic and 13 showed normal metabolic response (Figure 4.5). The age and the weight of children did not vary statistically between the groups (p value ≥ 0.05). However, a slight variation in gender distribution was recorded (Table 4-4). The sex therefore was included in the regression model as it was thought be a confounding factor affecting the metabolic pattern.

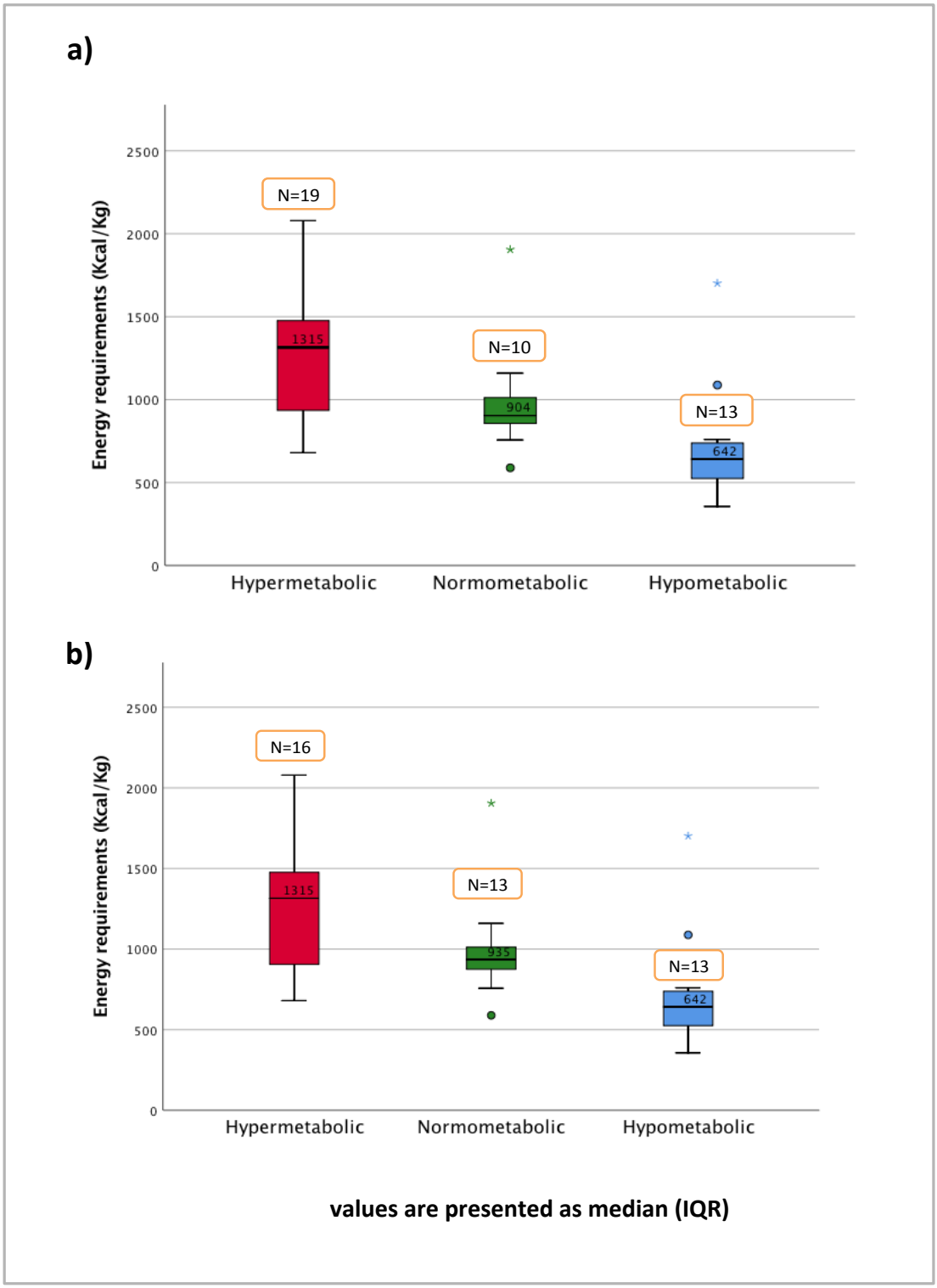

Figure 4.5 Metabolic pattern in critically ill children

Figure 4.5 shows the distribution of children based on their metabolic response. In Figure (a) the Schofield equation was used to predict the normal energy requirements, and the WHO equation was used in Figure (b). The median MEE for children who exhibited hyper-metabolic and normal metabolic pattern was lower in (a) compared to (b).

Table 4-4: anthropometric characteristics of patients based on their metabolic pattern

Metabolic pattern estimated by Schofield equation			
	Hyper-metabolic	Normo-metabolic	Hypo-metabolic
Age (Years)	3.9	5	4
Weight (kg)	17.4	20	18
Ratio of Males to females	9:10	8:2	2:11
Metabolic pattern estimated by WHO equation			
	Hyper-metabolic	Normo-metabolic	Hypo-metabolic
Age (Years)	3.9	4.5	4.9
Weight (kg)	16.9	17.8	21
Ratio of Males to females	11:5	8:5	2:11

4.3.3 Factors affecting MEE in critically ill children

Stepwise linear regression analysis was performed using the metabolic index as the primary outcome variable, to determine clinical factors contributing independently to variation in the metabolic index of critically ill children. The Schofield equation was used to predict REE to determine the clinical factors affecting the metabolic index. Results indicated that the severity of organ failure assessed by PMOD score and body temperature statistically impacted the metabolic index in critically ill children. This regression model accounted for 21% of the variation in REE (Table 4-5).

Table 4-5: Regression model to determine the factors affecting the metabolic index

Model	R	R^2	Adjusted R^2	R	P-value
Metabolic index [a]	**0.461**	**0.213**	**0.175**		
PMOD score [b]				-0.362	0.007*
Body temperature [b]				0.330	0.013*
Age [c]				-0.091	0.277
Sex [c]				-0.233	0.11
Weight for age Z-score [c]				-0.009	0.476
Heart rate [c]				0.136	0.187
PIM2 score [c]				-0.110	0.497
Highest lactate on the day of measurement [c]				-0.119	0.218

a. **Dependent variable**

b. **Predictors: (constant)**

c. **Excluded variables**

Twenty-six children with infections were admitted, and 16 presented with no signs of infections. A chi-squared test was performed and no relationship was found between the hyper-metabolic response and the presence of infection on admission, 0.278 (N = 42), p = 0.598. The chi-squared test was also performed to assess the relationship between the hyper-metabolic response observed in some children and the diagnostic categories. No statistical association was recorded between the hyper-metabolic state and MOF diagnosis [*1.6* (N = 42), p = 0.19], respiratory diagnosis [*2.9* (N = 42), p = 0.09] or CNS disorder diagnosis [*0.76* (N = 42), p = 0.300].

The effect of inotropic drugs and muscle relaxant on REE status was also evaluated. A chi-squared test indicated no statistical relationship between the hyper-metabolic response and treatment with inotropic drugs, 0.642 (N = 42), p = 0.423. However, a statistical relationship was recorded between the provision of muscle relaxant drugs and the hyper-metabolic response 4.12 (N = 42), p = 0.042. The Mann-Whitney test was performed to compare the metabolic index between children who received muscle relaxant within 24 hours of IC measurement and those who did not. The results showed that children who received muscle relaxant medication had a statistically higher metabolic index (p=0.008). To further investigate the effect of drugs on REE, regression analysis was performed. The regression model indicated that muscle relaxant agents were the only therapeutic agents that statistically impacted REE. Those who received muscle relaxants had a higher metabolic index (Table 4-6).

Table 4-6: Regression model to determine the factors affecting metabolic index

Model	R	R^2	Adjusted R^2	R	P-value
Metabolic index [a]	**0.556**	**0.309**	**0.277**		
PMOD score [b]				-0.348	0.009*
Muscle relaxants [b]				0.446	0.001*
Age [c]				-0.058	0.350
Morphine [c]				-0.022	0.441
Midazolam [c]				0.0.40	0.396
Inotropic drugs [c]				0.032	0.416

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

4.3.4 Fuel utilisation in critically ill children

Correlation analysis was performed to establish the relationship between energy intake and substrate utilisation. The results indicated that energy intake was positively correlated with RQ (r=0.550, p=0.00), the percentage of carbohydrate utilised (r=0.583, p=0.00), and negatively correlated with the percentage fat utilised (r=-0.583, p=0.00) (Figure 4.6). In addition, children who received less than 50% of their energy requirements utilised more fat and less carbohydrate compared to those who received more than 50% of their energy needs (p<0.001) (Figure 4.7). The results also showed that hyper-metabolic children utilised more fat (64%) compared to children showing normal (45%) and hypo-metabolic patterns (52%). However the variation in fat utilisation between the two groups was not statistically significant (p>0.05). On the other hand, the percentage of carbohydrate utilisation was lower in hyper-metabolic children (36%), but there was no evidence of statistical difference between the groups (p>0.05) (Figure 4.8).

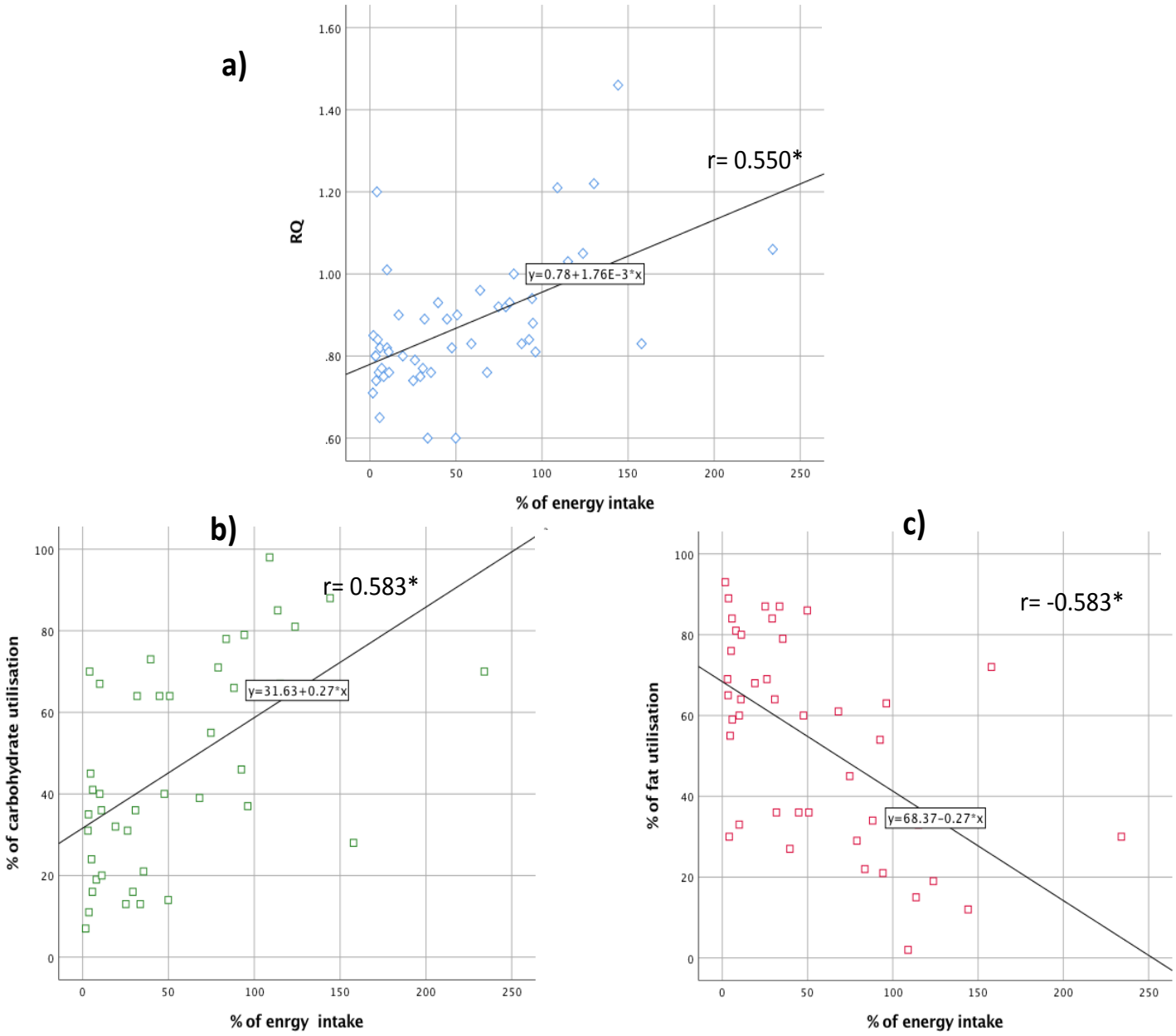

Figure 4.6 Relationship between energy intake and substrate utilisation

Figure 4.6 shows a positive correlation between energy intake with RQ (a) and the percentage of carbohydrate utilised (b), while a negative correlation is observed with the percentage of fat utilised (c). Indicating that carbohydrate is the primarily fuel utilised with sufficient energy intake while fat oxidation is usually stimulated in response to under-delivery of energy. The percentage of carbohydrate and fat utilisation can be used as a proxy to indicate the adequacy of the energy provided.

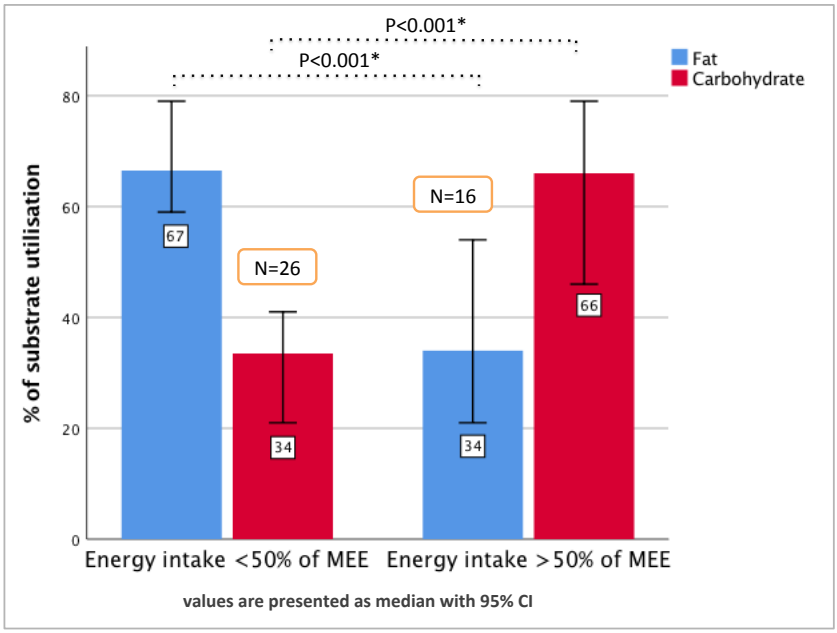

Figure 4.7 Comparison between the percentage of fat and carbohydrate utilisation based on the amount of energy received

Figure 4.7 indicates that children who received less than 50% of their energy requirements utilised more fat and less carbohydrate compared to those who received more than 50% of their energy needs.

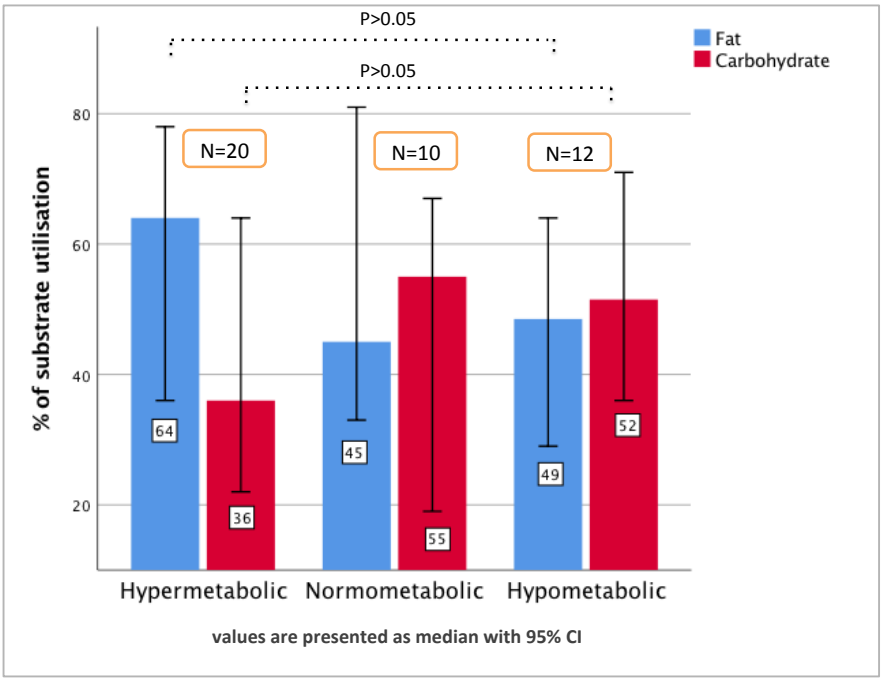

Figure 4.8 Comparison between the percentage of fat and carbohydrate utilisation based on the metabolic patter

Figure 4.8 shows that hyper-metabolic children utilised more fat (64%) and less carbohydrate (36%), compared to children who exhibited normal and hypo-metabolic patterns; however, there was no evidence of statistical difference between the groups (p>0.05).

4.3.5 The relationship between the metabolic index and clinical outcomes in critically ill children

The metabolic index and its association with clinical outcomes in critically ill children was also assessed. The data of 42 patients were included in this analysis. Two stepwise regression models were performed; in these regression models, PICU free days and VFH were the primary outcome variables. There was no evidence of statistical association between the metabolic index and clinical outcomes in this cohort. The severity of organ dysfunction assessed by PMOD score was the only variable statistically related to clinical outcomes in critically ill children (Table 4-7).

Table 4-7: Regression model to determine the factors affecting clinical outcomes in critically ill children

Model	R	R^2	Adjusted R^2	R	P-value
VFH	0.560	0.314	0.295		
PMOD score [b]				-0.560	0.00*
PIM2 score [c]				0.210	0.213
Age [c]				0.122	0.473
Weight for age Z-score [c]				-0.059	0.731
Metabolic index [c]				-0.191	0.256
PICU free days	0.662	0.439	0.424		
PMOD score [b]				0.662	0.00*
PIM2 score [c]				0.180	0.273
Age [c]				0.057	0.732
Weight for age Z-score [c]				0.244	0.134
Metabolic index [c]				-0.004	0.864

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

4.4 Discussion

In this study it was found that the median (IQR) of MEE was 922 (679-1348) kcal/day [49 (33-60) kcal/kg]. It was also shown that the Schofield equation and WHO equation were associated with the smallest level of bias when compared to MEE, confirming the recommendation to use these formulas, in the absence of IC. The hyper-metabolic pattern was recorded in the majority of children, which is thought to be related to patient factors such as the severity of disease, body temperature and medications received.

The mean difference between MEE and energy expenditure predicted by the Schofield and WHO equations was 84 kcal and 52 kcal, respectively. These results are in agreement with other authors' findings, where they evaluated the reproducibility of the Schofield equation in estimating energy requirements for critically ill children, and found it to be associated with the smallest level of bias compared to other available equations (Meyer et al., 2012; Jotter and Chaparro et al., 2017). On the other hand, energy requirements estimated by SACN recommendations, according to hospital protocol, tend to over-estimate energy needs. This is because SACN recommendations are intended to meet the needs of healthy children, and not critically ill children, as the recommendations take into account the activity factor. The motivation for using SACN to estimate energy requirements in Addenbook's Hospital PICU is to avoid underfeeding, as malnutrition is documented in 20–30% of critically ill children in developing countries (Skillman & Mehta, 2012). EN is frequently interrupted in PICU, resulting in under-delivery of energy; furthermore, young children are usually sensitive to fluid overload, and the amount of fluid permitted to feed is usually limited (Rogers et al., 2003; Kim et al., 2012). Due to the aforementioned factors, less than 65% of the prescribed energy goal (using various prediction equations) is usually delivered to patients (Bockenkamp et al., 2009). Therefore, although the energy goal estimated by SACN recommendations is higher than the measured requirements, the actual intake still may not exceed the measured energy requirements by IC or estimated by the Schofield equation. This speculation will be explored in more detail in Chapter Five.

In the current study a significant proportion of the children showed a hyper-metabolic response. Emerging evidence in the field of paediatric critical care nutrition suggests that during the acute phase critically ill children reduce their energy expenditure (Briassoulis et al., 2000; Taylor et al., 2003; Framson et al., 2007). Reduced REE in critically ill children could be due to lack of physical movement, decreased work of breathing, and maintaining a thermo-neutral environment in PICU settings (Briassoulis et al., 2000). Importantly, critically ill children may have reduced energy expenditure during the critical phase of illness, because IGF-1 and T3 remain low, implying that growth does not occur, thereby "sparing" this energy (Briassoulis et al., 2000; Vernon & Witte, 2000). Although the expected metabolic response in children involves a reduction of REE, a hyper-metabolic pattern was also reported in other studies, which is thought to be due to the heterogeneity of the cohort in terms of disease type/severity, interventions, comorbid conditions and nutritional status (Coss-Bu et al., 2001b; Mehta et al., 2017).

In this study the severity of organ failure assessed by PMOD score and body temperature both had a statistically significant impact on REE. An increase in PMOD score was associated with a reduction in the metabolic index. These findings indicate that children with multiple organ failure have reduced REE. Similar findings have been documented by other authors using other markers of disease severity; Mehta et al. (2012) recorded higher intraoperative peak serum lactate in hypo-metabolic children undergoing Fontan surgery. Lactate levels were used to indicate the degree of severity of postoperative illness, and it was correlated with morbidity after cardiac surgery. In addition, Forsberg et al. (1991) indicated that hypo-metabolic patterns become evident in adult patients with fatal outcomes during intensive care stays; their findings might explain why sicker children had lower REE. However, these findings are contradictory to previous work (Briassoulis et al., 2000; Taylor et al., 2003), where disease severity did not statistically influence REE. The discrepancies could be explained by the fact that those studies used paediatric risk of mortality (PRISM) score, which is mortality risk as a marker of severity. The association between REE and body temperature has been established by many previous studies. DuBois found that for each degree Celsius (ºC)

rise in temperature, metabolism increases by 13% (DuBois, 1921). The recent ASPEN guidelines referred to body temperature as a potential factor in increasing energy expenditure, since increase in body temperature may potentially lead to elevation in VO2 and VCo2, and accordingly result in increased REE (Mehta et al., 2017). Furthermore, an increase in energy expenditure has been recorded with an increase in body temperature beyond 38°C (Havalad et al., 2006; Meyer and Elwig, 2007). Fever in children with burn injuries was also associated with a degree of hyper-metabolism (Gore et al., 2003). In a study of critically ill adults, Frankenfield et al. (1997) suggested that fever, rather than severity of disease or diagnosis, was associated with an increase in energy expenditure. Faisy et al. (2003) affirmed that body temperature impacts REE in mechanically ventilated adults. In critically ill adults, targeted body temperature management at 33°C after cardiac arrest reduced REE by 20% compared to 36°C (Holzinger et al., 2015). With increasing evidence to support the implications of body temperature in changes in REE, a predictive equation utilised by White et al. (2000), developed for estimating REE in critically ill children, included body temperature as an independent variable. However, it is important to point out that White's formula has been shown to be inaccurate and impractical, as the calculations of energy requirements need to constantly change according to changes in body temperature. Mehta et al. (2011) failed to detect a statistical association between REE and body temperature in critically ill children; additionally, in a study by Briassoulis et al. (2014), REE did not differ among children based on their body temperature. This could be due to the implementation of a thermo-neutral environment in PICU to maintain body temperature within normal ranges – accordingly, it is difficult to detect a statistical association within the minimum rise in body temperature.

This study also evaluated the effect of inotropic drugs and muscle relaxant on REE. Surprisingly, children who received muscle relaxant medication within 24 hours of IC measurement had a statistically higher metabolic index. The regression analysis also showed that the use of muscle relaxant agents was statistically related to the variation recorded in REE. These findings are contradictory to previously published studies, where reduction in REE was recorded following the administration of neuromuscular blocking

agents (McCall et al., 2003; Vernon & Witte, 2000). However, these studies investigated the immediate action of those drugs. In studies of oxygen consumption in mechanically ventilated adults, an immediate reduction in oxygen consumption was recorded after administration of neuromuscular blocking agents, whereas improvements in oxygenation were observed 24 hours following the administration of the drugs (Price et al., 2012; Gainnier et al., 2004; Forel et al., 2006). These researchers' findings could justify or provide a suitable clinical explanation of the results from the current study. The findings of the current study may also be explained by the fact that usually those who are sicker (with higher PMOD score) are more likely to have higher doses of muscle relaxants.

Studies have shown that critically ill children utilise fat preferentially as a substrate for their energy metabolism, thus acute metabolic response is characterised by increased lipolysis and fatty acid oxidation relative to glucose oxidation (Coss-Bu et al., 2001; Caresta et al., 2007). In the current study, energy intake was positively correlated with RQ ($r=0.550$, $p=0.00$). RQ is known to be affected by body substrate utilisation, and therefore, it has been used before to identify the adequacy of nutritional intake in critically ill children (Liusuwan Manotok et al., 2008; Hulst et al., 2005). Hulst et al. (2005) similarly recorded a strong correlation between energy intake and RQ. Underfeeding appears to stimulate the use of fat, and subsequently causes a decrease in the RQ, whereas overfeeding causes an increase in the RQ (Liusuwan Manotok et al., 2008).

In the current study the amount of energy intake was positively correlated with the percentage of carbohydrate utilised ($r=0.583$, $p=0.00$), and negatively correlated with the percentage of fat utilised ($r=-0.571$, $p=0.00$). This is because fat oxidation is usually stimulated in response to under-delivery of energy, while with adequate delivery of energy carbohydrate is primarily utilised (Liusuwan Manotok et al., 2008; Frayn, 2003). Studies have shown that critically ill children utilise fat preferentially, which may be related to their feeding status (Coss-Bu et al., 2001; Caresta et al., 2007). The results of the current study also showed that hyper-metabolic children utilised more fat (64%)

compared to children exhibiting normal (45%) and hypo-metabolic patterns (52%), although the difference was not statistically significant ($p>0.05$). These findings are similar to previously published data, where Coss-Bu et al. (2001) reported an increased rate of fat oxidation in hyper-metabolic critically ill children. The exact mechanism has not been fully explored in the critically ill population. Increasing the rate of lipid oxidation could be an adoptive mechanism to provide energy substrate and compensate for increased requirements in hyper-metabolic children.

In this study, no evidence of statistical association was recorded between MEE and duration of ventilation and length of PICU stay. The association between the pattern of MEE and clinical outcomes has not been previously investigated in children. The findings from adult studies are inconsistent; Forsberg et al. (1991) suggested that hyper-metabolic patterns in adult patients were reduced with fatal outcomes during intensive care stays, while Rigaud et al. (2000) reported an increase in REE in dying patients.

4.5 Limitations

The study was limited by the inability to measure REE in infants, as the IC used in this study did not accurately measure REE in children under 10 kg.

The majority of the previously published studies used Deltatrac II® in REE assessment. Some other calorimeters such as MGA 1100®, Vmax Encore® and E-COVX® were also introduced (Coss-Bu et al., 1998; 2001; Mehta et al., 2011; 2012; Briassoulis et al., 2014; Spanaki et al., 2018). The IC machine used in this study was different to the Deltatrac II®, as it utilises a breath-by-breath method, whereas the latter uses a mixed-method sampling technique. In addition, this metabolic cart has not been validated against the Deltatrac II®. This may account for some of the discrepancies in the findings with previously published studies.

As the Deltatrac is no longer available on the market, new IC machines should be validated in order to be used in clinical settings. A recent study by Graf et al. (2015) compared the efficacy of the CCM express® and Quark RMR® to the Deltatrac II®. Their

findings suggested that both machines suffered from inaccuracy in estimating REE compared to the Deltatrac II®. In another study, Cooper et al. (2009) assessed the validity of five gas analysis systems against the Deltatrac II®. The five validated IC systems were MedGraphics CPX Ultima®, MedGem®, Vmax Encore 29 System®, TrueOne 2400® and Korr ReeVue®. The results were not satisfactory, as none of the instruments were within person reliability of the Deltatrac II®. The TrueOne and Vmax were the most valid instruments for REE assessment in comparison with the Deltatrac II®.

Future studies concerning measuring REE in intensive care settings should focus on the validation of the currently available IC machines against doubly labelled water or Douglas bags techniques, to further improve IC machines before recommending their clinical use in intensive care settings (Cooper et al., 2009). Measuring energy expenditure in healthy children and compare it against the RDA for energy could be a convenient validation tool for the available machine in the market. However, due the technical difficulties performing energy expenditure measurement and achieving steady state in healthy children, no control healthy children group were included in the current study.

4.6 Conclusion

Energy requirements have always been a matter of debate in PICU settings, often due to the unpredictability of the metabolic response among this population. The variability of metabolic state in critically ill children has been previously documented, and factors such as fever or type of illness have shown to influence REE. Understanding energy needs during the course of illness is necessary to avoid imbalances in energy intake and requirements. Inaccurate assessment of energy requirements results in both underfeeding and overfeeding in critically ill children. IC remains the gold standard in optimising nutritional support in PICU. Although several studies have concluded that predictive equations lack precision, the Schofield and WHO equations have shown to be the best available equations in predicting energy needs in this population.

5 Chapter Five: Prescribed versus delivered energy and macronutrients and their impact on clinical outcomes

5.1 Introduction

The imbalance between nutritional requirements and intake may predispose critically ill children to under- or overfeeding. Previous studies of critically ill children showed that only 58–65% of the prescribed energy goal is actually delivered to patients (Taylor et al., 2003; Bockenkamp et al., 2009). Kyle et al. (2012) recorded average energy and protein intake of 75% and 40%, respectively, of the estimated requirements (Kyle et al., 2012). Enteral feeding, the mainstay of nutrition in most critically ill children, is frequently interrupted in PICU, resulting in the under-delivery of energy and macronutrients (Rogers et al., 2003; Kerklaan et al., 2016). Furthermore, critically ill children are usually sensitive to fluid overload, and consequently the amount of fluid permitted is usually limited (Rogers et al., 2003; Kim et al., 2012). Patient factors such as age and nutritional status may also influence the delivery of energy in PICU settings. Younger children appear to accumulate the highest energy deficits, while the delivery of energy is usually better in the malnourished population (Hulst et al., 2004; Briassoulis et al., 2001).

Adequate nutritional delivery is critical in affecting the clinical outcomes of critically ill children. The effects of under- and overfeeding have been previously investigated, and both have been shown to be detrimental to critically ill children, in particular by extending the length of stay on the intensive care unit (Mehta et al., 2009; Hulst et al., 2006; Maslowski & Mackay, 2011; Hulst et al., 2006; Maslowski & Mackay, 2011). Therefore, optimal nutritional support, including accurate prescription and delivery of energy and macronutrients, is a fundamental element in improving the clinical outcomes of critically ill children (Briassoulis et al., 2001; 2002; Gurgueira et al., 2005; Mehta et al., 2012). The main focus of nutritional balance in critically ill children has been the delivery of energy and protein, but the balance of other macronutrients is often neglected. This is related to knowledge and guidelines focusing on energy and protein requirements

(Mehta et al., 2017), and the ideal balance of macronutrients, carbohydrates, protein and fats has not yet been established.

This study, therefore, sets out to assess the delivery of energy and macronutrients compared to the estimated requirements. The secondary aim of the study is to identify factors impacting cumulative energy intake in this population.

5.2 Material and Methods

The delivery of energy and macronutrients (protein, carbohydrates and lipids) was prospectively evaluated in critically ill children from November 2014 to May 2017, as part of a study assessing gut microbiome in critically ill children.

Inclusion criteria:

- Aged 1 week to 16 years.
- Enterally or parenterally fed for at least 3 days.

Exclusion criteria:

- Pre-term gestation (birth < 37 weeks).
- Known pre-existing immune paresis, oncological diagnosis and HIV.
- Children who require feed compositions outside of the norm, such as ketogenic feeds.

5.2.1 Nutrition prescription

5.2.1.1 EN prescription

For the purposes of this study, energy requirements were estimated using the Schofield equation (Schofield, 1985), in line with findings from Chapter Four, where the Schofield equation showed the closest agreement with MEE, and was suggested for use by the current ASPEN guidelines in the absence of indirect calorimetry (Mehta et al., 2017). Protein requirements were also calculated according to ASPEN guidelines for critically ill children (0–2 years, 2–3 g/kg/day; 2–13 years, 1.5–2 g/kg/day; and >13 years, 1.5 g/kg/day) (Mehta et al., 2009; 2017). Due to the lack of published guidelines regarding enteral CHO and fat requirements for critically ill children, the age-appropriate UK reference nutrient intake (RNI) for healthy children was used as a guide for establishing CHO requirements as 50% of the total energy requirements (Ashwell, 1991). Fat requirements of 40% and 35% of the total energy requirement were set for children <1 year of age and > 1 years, respectively, corresponding to ESPGHAN recommendations for healthy children (Koletzko et al., 2005; Uauy & Dangour, 2009).

5.2.1.2 PN prescription:

The unit used individualised PN; therefore, energy, carbohydrates (glucose), lipids and amino acids were delivered according to the patient's requirements based on weight. The calculations of energy requirements were based on the Schofield equation (Schofield, 1985; Joosten et al., 2018). Macronutrient requirements were calculated according to the recent ESPGHAN guidelines on paediatric parenteral nutrition (Mesotten et al., 2018; Van Goudoever et al., 2018; Lapillonne et al., 2018) (Table 5-1).

Table 5-1: PN substrate requirements based on ESPGHAN/ESPEN practical recommendations

Weight (kg)	Carbohydrate (glucose) (g/kg/day)
0-10	2 – 4
11-30	1.5 – 2.5
31-45	1 – 1.5
> 45	0.5 – 1
Age group	**Lipid (g/kg/day)**
Infants	3 – 4
Children	2 – 3
Age group	**Protein (amino acids) (g/kg/day)**
Pre-term neonate	1.5 – 3.5
Term neonates	1.5 – 3
2 months to 3 years	2.5
>3 years	2

5.2.2 Nutrition delivery

Actual intake was monitored for all recruited children for at least 3 days (1 day = 24 completed hours). The total volume, energy and macronutrient composition of enteral feed for each child was collected using the patient's charts and nutritional data card for each formula (Table 5-2). The calculation of breast milk composition was based on previously published data on breast milk composition (Ballard & Morrow, 2013).

Enteral formulas were classified into 3 types: the energy-dense formula (1 kcal/ml for infants < 1 year and 1.5 kcal/ml feed for older children), standard formulas (0.67 kcal/ml for infants < 1 year and 1 kcal/ml feed for older children) and breast milk. Energy and macronutrient intake were expressed in percentages of calculated requirements.

Feed selection:

The hospital protocol adopts feed choice that is nutrient dense option of those prescribable for a given age group. For infants under 1 year of age Infatrini is usually the formula of choice. Nutrini Energy was routinely prescribed for children over 1 year and Tenterini Energy for children aged 7-12 years or 21-45 kg in body weight. If an infant or a child was admitted on a specialist formula for clinical reasons (e.g Peptijunior, Nutramigen, Neocate LCP, Nutriprem 2 or fiber supplemented feeds) then this formula was continued and did not changed to an alternative one.

Table 5-2: Energy and macronutrient composition of breast milk and enteral formulas given during the course of study

Formula	Energy Kcal/ ml	Carbohydrate (g/ml)	Carbohydrate (% of total energy)	Protein (g/ml)	Protein (% of total energy)	Fat (g/ml)	Fat (% of total energy)
Breast milk	0.68	0.07	41.18%	0.01	5.88%	0.040	53.00%
Infatrini Nutricia®	1	0.103	41.20%	0.026	10.40%	0.054	48.60%
Neocate (LCP 0-12) Nutricia®	0.67	0.072	42.99%	0.018	10.75%	0.034	45.67%
Neocate advance Nutricia®	1	0.146	58.40%	0.025	10.00%	0.03	27.00%
SMA althera Nestle®	0.67	0.073	43.58%	0.017	10.15%	0.034	45.67%
Cow&Gate®	0.66	0.073	44.24%	0.013	7.88%	0.034	46.36%
Similac alimentum Abbott®	0.68	0.069	40.59%	0.0186	10.94%	0.037	48.97%
Nutriprem2 Cow&Gate®	0.75	0.075	40.00%	0.02	10.67%	0.04	48.00%
Nutrini Nutricia®	1	0.123	49.20%	0.027	10.80%	0.044	39.60%
Nutrini energy Nutricia®	1.5	0.185	49.33%	0.04	10.67%	0.067	40.20%
Nutrini energy multi-fiber Nutricia®	1.5	0.185	49.33%	0.041	10.93%	0.067	40.20%
Nutrini peptisorb Nutricia®	1	0.137	54.80%	0.028	11.20%	0.039	35.10%
Tentrini Nutricia®	1	0.123	49.20%	0.033	13.20%	0.042	37.80%
Tentrini energy Nutricia®	1.5	0.185	49.33%	0.048	12.80%	0.063	37.80%
Peptamen junior advance Abbott®	1.5	0.18	48.00%	0.045	12.00%	0.066	39.60%
Nutrison soya Nutricia®	1	0.123	49.20%	0.04	16.00%	0.039	35.10%
Nutrison energy Nutricia®	1.5	0.183	48.80%	0.06	16.00%	0.058	34.80%
Frebini energy Fresenius Kabi®	1.5	0.187	49.87%	0.038	10.13%	0.067	40.20%
Elemental 028 Extra Nutricia®	0.86	0.11	51.16%	0.025	11.62%	0.035	36.62%

5.2.3 Sample size calculation

This was an observational study to monitor nutritional intake in critically ill children. No specific sample size calculation was performed for this study, as all children enrolled in the GHICI who met the inclusion and exclusion criteria of the current study were included.

5.2.4 Statistical analysis

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as a median with interquartile ranges (IQR). The Wilcoxon signed rank test was used to compare the difference in energy and macronutrient delivery between day 1 and day 3 of admission, whilst the Mann-Witney U was applied to assess differences in disease severity variables between children. The Spearman correlation coefficient was used to establish the correlation between variables, which was followed by stepwise linear regression analysis to assess factors that impact on cumulative energy intake and clinical outcomes. A p-value of ≤ 0.05 was considered statistically significant.

5.3 Results

A total of 124 critically ill children [70 (57%) males], with a median (IQR) age of 1.9 (0.4 – 4.9) years, were enrolled in this study. The anthropometric and clinical characteristics of the children are shown in Table 5-3. Upon admission to the PICU, 14% of the children had moderate or severe malnutrition as classified by WHO criteria mentioned in the Method Chapter, page 52. Inpatient mortality was 4/124 patients (3%). A summary of the recruitment and consenting procedure is shown in Figure 5.1.

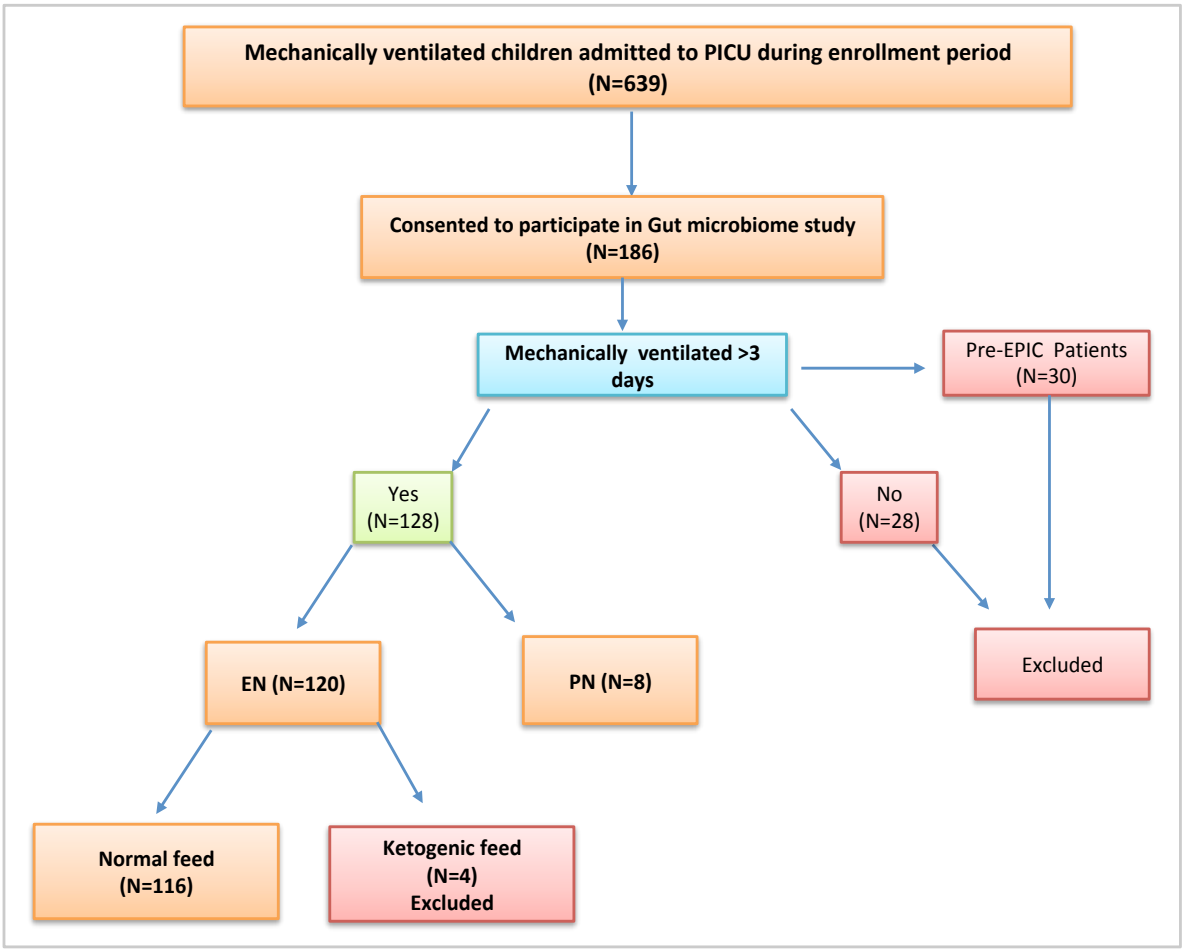

Figure 5.1 Summary of recruitment procedure

The above figure shows the stages of patient recruitment and consent.

Table 5-3: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometrics	**All children (N=124)**	**0-10 kg (N= 52)**	**10-20 kg (N=41)**	**>20kg (N=31)**
Age (years)	1.9 (0.4 – 4.9)	0.4 (0.1 – 0.8)	3.0 (2.0 – 4.1)	11 (7.9 – 13.4)
Weight (kg)	12.1 (6.9 – 18)	5.3 (3.5 – 8.4)	14.7 (13.1 – 16.0)	35 (25 – 43.8)
Height (cm)	89 (69 – 111)	60.5 (52 – 73)	96 (87 – 101)	132 (115 – 147)
Weight for age Z-score	-0.3 (-1.4 – 0.58)	-1.0 (-2.2 – -0.2)	0.24 (-0.377 – 0.88)	0.35 (-0.89 – 1.12)
% of children below <-2 Z-scoresz scores	14%	21%	7%	9%
Height for age Z-score	-0.15 (-1.9 – 1.09)	-0.7 (-3.9 – 0.8)	0.15 (-0.43 – 1.3)	-0.19 (-2.1 – 0.9)
% of children below <-2 Z-scoresz scores	15%	15%	12%	19%
Weight height Z-score	0.07 (-1.15 – 0.81)	-0.9 (-2.0 – -0.08)	0.26 (-0.93 – 0.94)	0.65 (-0.22 – 2.03)
% of children below <-2 Z-scoresz scores	6%	8%	9%	0%
Disease severity				
PMODS	6 (5 –7)	6 (5 –7)	5 (4 –6)	6 (5 –7)
PIM2 score	3 (0.93 – 6.1)	3.4 (0.89 – 6.8)	2.6 (0.97 – 5.6)	3.1 (0.92 – 5.0)
Inotrope score	0 (0 – 15.25)	0.0 (0.0 – 15)	0.0 (0.0 – 20.25)	0.0 (0.0 – 27)
Highest lactate	1.9 (1.3 – 3.22)	1.9 (1.4 – 3.1)	1.2 (1.7 – 2.8)	2.5 (1.2 – 3.3)
Highest CRP	97 (36 – 213)	52 (22.7 – 117.5)	105 (25 – 221)	139 (44 – 269)
VFH at 30 days	627 (549 – 653)	616 (573 – 656)	635 (562 – 652)	629 (495 – 651)
PICU -free days at 30 days	22 (18 – 24)	23 (19 – 24)	22 (20.0 – 24.0)	22 (18 – 25)
Primary admission diagnosis	N (%)			
MOF	40 (32%)			
Respiratory Failure	57 (46%)			
CNS Disorders	21 (17%)			
Surgical Disorder	4 (3%)			
Gastrointestinal Disorders	1 (1 %)			
Cardiac Disorders	1 (1%)			

Data presented as median (IQR)

- VFH at 30 days: ventilation -free hours at 30 days
- PICU -free days at 30 days: days free of paediatric intensive care at 30 days

5.3.1 Feeding in PICU

The majority of the children were enterally fed n=116 (93%), and only 8 (7%) children received PN (see Figure 5.1). Nutritional intake was analysed in both enterally and parenterally fed children.

PN fed children:

Eight children received PN, and 6 of them were admitted due to gastrointestinal (GI) diagnoses including GI infections and surgery. PN was started within 3 days of PICU admission for 4 children, and the other 4 children started PN after day 3. The daily intake of energy, fat, carbohydrate and protein during the first 3 days of PICU admission is presented in Figure 5.2; data are expressed as individual data points, as the numbers were small to show median (IQR).

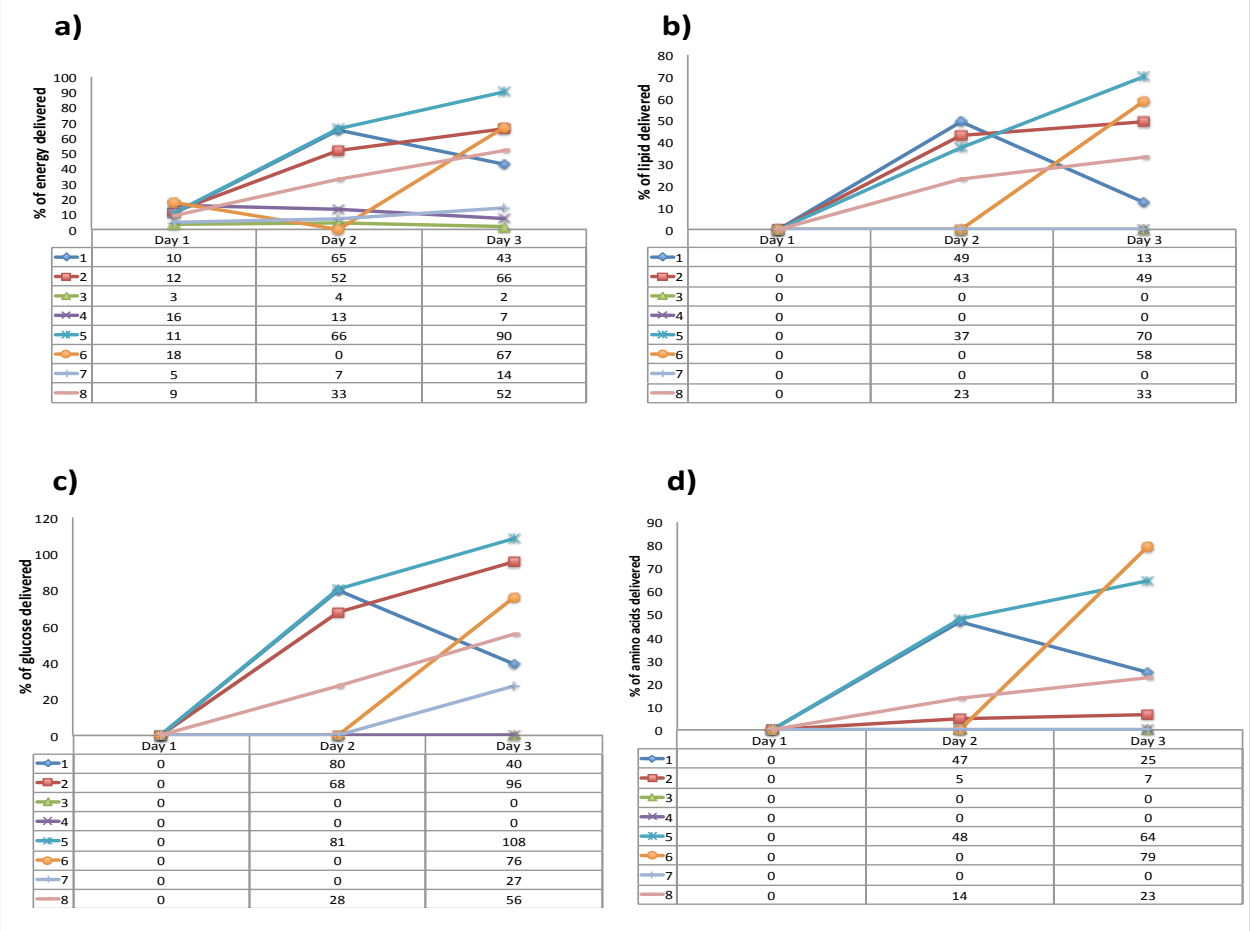

Figure 5.2 Percentage of energy and macronutrients delivered, expressed as a percentage of the calculated requirement

Figure 5.2 shows the daily intake of energy, fat, carbohydrate and protein expressed as individual data points. Each line represents one patient.

EN fed children:

Data regarding the time of commencing EN was recorded in 104 children: these were started at a median (IQR) time of 8 (5–13.75) hours following PICU admission, and 77 (66%) started enteral feeding within the first 12 hours of admission. EN was suspended for a median (IQR) duration of 19 (11–28) hours within the first 3 days of admission. The most common reason for the cessation of EN among this cohort was the presence of perceived large gastric aspirates. Failed attempts for extubation and diagnostic tests also accounted for withholding EN. Children commenced EN within 12 hours of admission, had statistically lower PIM2 (p=0.04) and inotrope scores (p=0.02) compared to those who started EN ≥12 hours of admission. Eighty-seven patients (75%) of the cohort received energy-dense formulas, 9 (8%) expressed breast milk, and 20 (17%) were on standard formulas (Table 5-4).

Table 5-4: Number of patients receiving standard and ED formulas

	All children (N=116)	0-10 kg (N= 51)	10-20 kg (N=40)	>20kg (N=25)
Breast milk	9 (8%)	9 (18%)	0 (0%)	0 (0%)
Standard infant formula	7 (6%)	7 (14%)	0 (0%)	0 (0%)
Standard paediatric feed	13 (11%)	0 (0%)	7 (17.5%)	6 (24%)
Energy -dense infant formula	31 (27%)	28 (55%)	3 (7.5%)	0 (0%)
Energy -dense paediatric feed	56 (48%)	7 (13%)	30 (75%)	19 (76%)

Table 5-4 shows the number of children receiving standard and ED formulas among each weight group.

The daily energy and macronutrient intake was calculated for the first 3 days of PICU admission (Figure 5.3). The enteral intake of energy and each macronutrient on day 1 and day 2 were lower than the calculated requirements. By day 3, the median (IQR) intake of energy, fat, carbohydrate and protein was 82% (53-108), 90% (52-125), 70% (47-101) and 49% (28-66), respectively. Fat intake on day 3 exceeded 100% of the requirements in 53 (45.7%) children. The delivery of energy and macronutrients was statistically improved by day 3 compared to day 1. I went on to examine how the daily

intake varied among different weight groups. The results showed that the children with lower weight (0-10 kg) received higher doses of energy and macronutrients. The average intake of energy and macronutrients from admission up to day 3 are presented in Table 5-5.

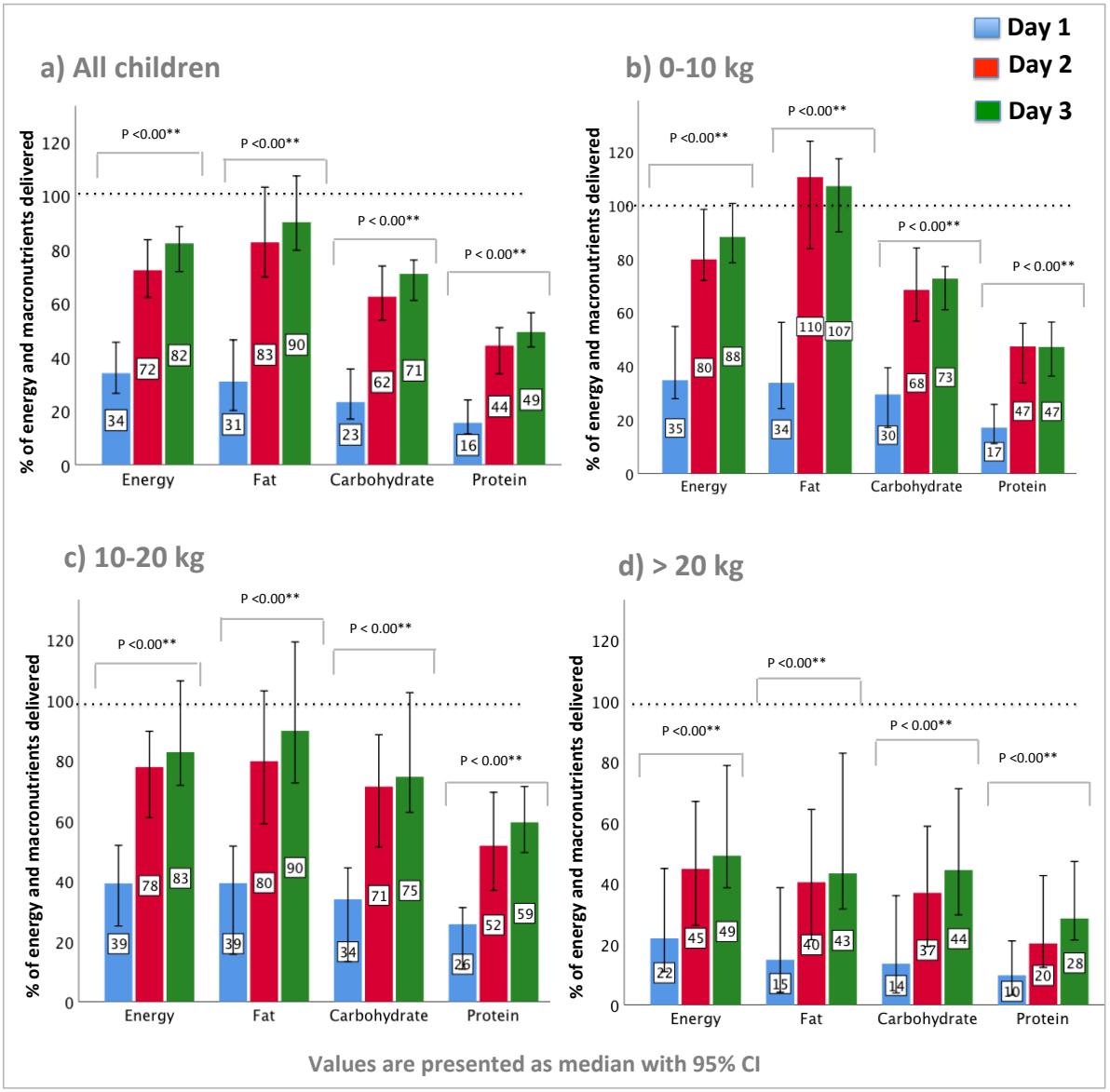

Figure 5.3 Percentage of enteral energy and macronutrients delivered expressed as a percentage of the calculated requirements

Figure 5.3. a) Shows the daily enteral intake (days 1-3) of energy and macronutrients in 116 critically ill children. Figure 5.3. b), c) and d) show the daily enteral intake (days 1-3) of energy and macronutrients in different weight groups. The above figure clearly indicates that smaller children (0-10 kg) received higher doses of energy and macronutrients. The enteral intake of both energy and macronutrients has statistically improved on day 3 compared to day 1 (p<0.001**).

Table 5-5: Average enteral intake of energy and macronutrients from admission up to day 3

Average intake up to day 3	0-10 kg (N= 51)		10-20 kg (N=40)		>20kg (N=25)	
	Crude value kcal or g/kg/day	%of reference intake	Crude value kcal or g/kg/day	%of reference intake	Crude value kcal or g/kg/day	%of reference intake
Energy	37 (31.0-49.0)	70 (55-88)	38 (26-44)	75 (48-83)	13 (6.3-25.9)	4 (23-65)
Fat	2 (1.4 -2.6)	86 (62-103)	1.6 (0.9-1.9)	76 (43 -93)	0.48 (0.18-1.07)	37 (18-67)
Carbohydrate	4.6 (3.8-4.6)	54 (44-76)	4.9 (3.5-5.6)	66 (40-80)	1.8 (1.2-3.3)	34 (17-60)
Protein	0.9 (0.6-1.2)	40 (24-51)	0.99 (0.56-1.1)	45 (32-63)	0.3 (0.17-0.69)	21 (9-42.7)

Data presented as median (IQR)

The above table shows the average doses of enteral energy and macronutrients presented as crude values and as a percentage of reference requirements.

5.3.2 Factors affecting delivery of enteral nutrition:

Linear regression analysis was performed with three days cumulative enteral energy intake as an outcome to determine the factors affecting the delivery of energy. The regression model suggested that age, weight for age z-score and PIM2 score statistically impacted the cumulative amount of energy delivered. This indicated that younger children and those who had lower weight for age z-score and PIM2 score had more cumulative energy intake. This regression model accounted for 30% of the variation in cumulative energy intake (Table 5-6).

Table 5-6: Regression model to determine the factors affecting cumulative energy intake

Model	R	R^2	Adjusted R^2	R	P-value
Cumulative Energy intake [a]	**0.549**	**0.302**	**0.283**		
Age [b]				-0.396	0.00*
Weight for age z –score [b]				-0.337	0.00*
PIM2 score [b]				-0.234	0.013*

a. **Dependent variable**
b. **Predictors: (constant)**

5.3.3 Effect of enteral feeding on clinical outcomes

In this section the association between clinical outcomes and the average intake of energy and macronutrients was investigated. Two stepwise regression models were performed; in these regression models PICU-free days and VFH were the outcome variables. There was no evidence of statistical association between the intake and clinical outcomes in this cohort (Table 5-7).

Table 5-7: Regression model to determine the effect of feeding on clinical outcomes:

Model	R	R^2	Adjusted R^2	R	P value
VFH [a]					
PIM2 score [c]				0.039	0.341
Age [c]				-0.092	0.165
Average energy received (3 days) [c]				-0.107	0.13
Average carbohydrate received (3 days) [c]				-0.032	0.367
Average protein received (3 days) [c]				-0.078	0.206
Average fat received (3 days) [c]				-0.088	0.177
PICU free hours at 30 days					
PIM2 score [c]				0.113	0.115
Age [c]				-0.115	0.111
Average energy received (3 days) [c]				-0.024	0.4
Average carbohydrate received (3 days) [c]				0.019	0.419
Average protein received (3 days) [c]				0.007	0.472
Average fat received (3 days) [c]				-0.011	0.456

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

5.4 Discussion

This study set out to assess the difference between prescribed and delivered energy and macronutrients. Previous data has indicated that there is a strong association between under-delivery of energy and protein with growth parameters and adverse clinical outcomes, including prolonged ventilation and hospital stays, multiple organ dysfunction and mortality (Hulst et al., 2004; Mehta et al., 2012; Bhutia et al., 2013; Correia, 2003). Conversely, over-nutrition, particularly excessive glucose delivery, has also been linked to negative PICU outcomes (Mehta et al., 2012; Bhutia et al., 2013). Therefore, it is crucial to take into account the proportion of macronutrients required and not only aim to achieve the energy goal.

In the current study the delivery of energy increased over the 3 days of the study period. By day 3, energy and carbohydrate intake reached 82% and 71%, respectively, of the estimated requirements. The recent ASPEN guidelines target the delivery of at least two-thirds of the prescribed daily energy requirement by the end of the first week in the PICU to avoid undesired clinical outcomes associated with cumulative nutrition deprivation (Mehta et al., 2017). The majority of the children achieved this energy goal by day 3, which was likely to occur, as EN generally commenced within a few hours of admission (median of 8 hours), in accordance with our unit enteral feeding protocol. However, over the first 3 days, protein delivery was 40% of the calculated requirements based on ASPEN guidelines, and reached only 49% of these requirements by day 3. In addition, our results showed that children > 20 kg were at the greatest risk of not fulfilling their protein requirements, only receiving an average of 21% of the requirements over 3 days. Insufficient delivery of protein is common, particularly in the early stages of admission. These findings are similar to previously published data where under-delivery of protein was also reported (De Neef et al., 2008; Mehta et al., 2012; Brasil de Oliveira Iglesias et al., n.d.; Kyle et al., 2012). On the contrary, on day 3, the median fat intake was 90% and exceeded the prescribed requirements in almost 46% of children.

To the knowledge of the author, this is the first study to assess the delivery of enteral fat and carbohydrate in PICU settings. This study recorded discrepancies in energy, carbohydrate, protein and fat requirements and delivery, with the under-delivery of energy and protein and over-delivery of fat. This is mainly related to two aspects: the use of standardised, ready-to-feed energy-dense formulas in PICU, and the aim requirements for macronutrients in critically ill children. The only guidelines available for enteral nutritional support in this population are for energy and protein (Mehta et al., 2009; 2017). However, none have been established for fat and carbohydrate. The age specific ESPGHAN guidelines for healthy children were used as a guide to estimate fat requirements in a critically ill population, as younger children, particularly breast-fed infants, might have higher fat requirements. RNI for healthy children was used as a guide for establishing carbohydrate requirements. In this study almost 80% of patients were fed energy-dense feeds to help meet their energy needs, and to compensate for restrictions in fluid intake and the often frequent interruptions to EN (Rogers et al., 2003; Kim et al., 2012). These feeds have a higher energy density and provide, on average, 50% of energy from carbohydrate, 10% from protein and 40% from fat. The macronutrient composition of all medical formulas in Europe is guided by the European Commission of Food for Special Medical Purposes (FSMP) guidelines. Many factors need to be taken into account with the development of these enteral feeds, including macronutrient composition, osmolality, volume of feed for nutritional adequacy, and safety ranges for macro and micro nutrients for children, with a wide range of diagnoses. As energy requirements are lower and protein requirements are higher in critically ill children (Meyer et al., 2012; Jotter & Chaparro et al., 2017; Mehta et al., 2009; 2017), the distribution of macronutrients in standardised feeds may not match the specific requirements of critically ill children.

The results showed that children who started EN within 12 hours of their PICU admission had lower inotrope and PIM2 scores. This indicates that less sick children start EN earlier than the sicker ones, which confirms that clinical and haemodynamic instability account for delaying EN (Lee et al., 2013). However, other studies showed that the implementation of aggressive early EN protocol was safe and significantly effective in

improving nitrogen balance in critically ill children (Briassoulis et al., 2002; 2005; Botrán et al., 2011).

Factors that impacted the cumulative intake of energy have also been investigated. It was found that age and weight for age z-score were the strongest predictors of cumulative energy intake. Younger children and those who had lower weight for age Z-scores and PIM2 scores had more cumulative energy intake. The findings regarding age are similar to those of Mehta et al. (2011), but contradictory to the work of Hulst et al. (2004), who found that younger children accumulated the highest energy deficits. This controversy could be explained by the fact that younger children had the highest percentage of malnutrition (21%), as assessed by weight for age Z-score. Due to the well-documented impact of early malnutrition (De Souza Menezes, et al. 2012; Aurangzeb, et al. 2012)(39, 40), it is likely that the nutritional support team focused more on providing full nutritional requirements, particularly for smaller (lower weight for age Z-scores) children, to avoid further deterioration of their nutritional status during PICU stays. Briassoulis et al. (2001) have similarly shown that malnourished critically ill children have better energy intake.

In the absence of guidelines regarding enteral macronutrient requirements, it is not surprising that there have been no trials of enteral macronutrient targets outside of protein in critically ill children. In a landmark study of parenteral nutrition in this patient group (the Paediatric Early versus Late Parenteral Nutrition In Critical Illness (PEPaNIC) trial by Vanhorebeek et al., 2017), the dose of amino acids delivered was associated with an increased rate of infection and a longer ventilation time. The authors postulated this to be due to the repression of autophagy by amino acids in critical illness. Another potential mechanism is the early induction of Resistin (a hormone derived from macrophages in humans), leading to repression innate immunity and changes in amino acids kinetics, including increased levels of phenylalanine and serine, and reduced glutamine concentrations (Spanaki et al., 2018; Tavladaki et al., 2017). The PEPaNIC trial indicated that parenteral delivery of glucose and lipids was associated with fewer infections and earlier PICU discharge, respectively. We did not observe such an

association between enteral nutritional intake and the duration of ventilation or the length of PICU stay. It is important to note that the route of delivering nutrition was different from our study; also, a distinctive statistical plan was followed. Unlike the PEPaNIC study, we monitored the intake for 3 days, which may be insufficient to detect the statistical effect of nutritional intake on clinical outcomes. Importantly, our cohort had significantly lower protein intake, compared to requirements, than patients enrolled in the PEPaNIC trial (40% vs 80%) (Vanhorebeek et al., 2017). However, we only measured intake for 3 days, compared to 7 in the PEPaNIC trial, making a comparison between data from the two studies challenging.

5.5 Limitations

There is a lack of published guidelines regarding enteral fat and carbohydrate requirements for critically ill children. Although the PEPaNIC study contributes significantly to our knowledge of PN nutrient delivery in critically ill children, the requirements used for PN are not applicable for enteral nutrition. The new ESPGHAN guidelines for PN suggested that energy and macronutrient requirements of PN are close to those of EN (Joosten et al., 2018; Mesotten et al., 2018; Lapillonne et al., 2018). This argument is based on findings from 20th-century experimental studies by Atwater, where the metabolisable energy of IV macronutrients was similar to that provided by EN. However, there are key differences in the metabolisms of enteral versus parenteral; carbohydrates, lipids and protein are related to intestinal absorption of nutritional substrates, insulin and inflammatory stimulation and visceral protein synthesis (Suchner et al., 1996). Therefore, guidelines from ESPGHAN for enteral fat and RNI for carbohydrate for healthy children were used, whilst utilising published guidance for recommended protein and energy intake in paediatric critical illness (Ashwell, 1991; Koletzko et al., 2005; Uauy & Dangour, 2009; Mehta et al. 2009; 2017).

5.6 Conclusion

The current data suggests that within the feed delivered, fat intake is often above requirements compared to protein and carbohydrate delivery. This imbalance of

nutrient provision may result from using the standard paediatric feeds that may not necessarily match the macronutrient requirements of critically ill children. To avoid imbalances of macronutrients delivery within the feed provided we have to take into account the proportion of macronutrients required for a child, and not only aim to achieve the energy goal. These findings are based on generalised guidelines for macronutrient requirements in healthy children, and future work should examine whether critically ill children have specific macronutrient needs.

6 Chapter Six: Association Between Enteral Macronutrient Delivery and Inflammatory Response In Critically Ill Children

6.1 Introduction

Critical illness is characterised by a state of systemic inflammation accompanied by the release of inflammatory cytokines (Joosten et al. 2016). These cytokines are the main regulators of the immune response to the acute insult (Jaffer et al. 2010). The acute pro-inflammatory response is typically followed by an induction of anti-inflammatory mediators (Oberholzer et al. 2000). The interaction between the pro-inflammatory and anti-inflammatory mediators appears to determine the severity of outcome in critically ill patients (Oberholzer et al. 2000; Wang & Ye 2015). The balance between pro- and anti-inflammatory mediators needs to be achieved in order to restore a homeostatic state. Excessive production of inflammatory cytokines can cause adverse outcomes on the host metabolism, tissue and organ function (Wang & Ye 2015; Chaudhry et al. 2013).

Cytokines act systemically to regulate energy metabolism. In the brain, inflammatory mediators inhibit energy intake through suppression of appetite. They also induce lipolysis in fat tissue and protein breakdown in muscles (Wang & Ye 2015). It is thought that pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α play a role in increasing energy expenditure in critically ill children (Puigserver et al. 2001). These inflammatory cytokines act through cell membrane receptors in the brain or peripheral tissues to induce energy expenditure (Wang & Ye 2015).

There is a growing body of evidence suggesting that there is interplay between nutrition and inflammation. Increased levels of inflammatory cytokines have been reported before in undernourished children with cirrhosis (Wilasco et al. 2017). Studies also suggested that a diet high in fat might induce systemic low-level inflammation in adult populations (Pendyala et al. 2012; Parekh et al. 2014; Sharman & Volek 2004).

The aim of this study was to map out the changes occurring in TNFα, IL-6, IL-10 and IL-1β during the course of PICU stay, and to investigate the effect of energy and macronutrients intake on the pattern of cytokine release.

6.2 Materials and Methods

This study included only enterally fed children, where the daily recording of intake was accessible. Children who were on full/supplemental PN or a ketogenic diet were excluded.

Measurements of circulating levels of key inflammatory mediators, including pro-inflammatory (TNF-α, IL-6, IL-1β) and anti-inflammatory response pathways (IL-10), were undertaken between day 2 and 7. The detailed methodology of the cytokines assay was described in Chapter Two, page 54.

To investigate the association between enterally delivered energy and macronutrients and cytokine levels, values for the amount delivered were calculated from the 24-hour period prior to each sample collection (1 day =24 hours). Cytokine samples were then categorised into early samples, measured during the early phase of illness when nutrient delivery may be lower (days 2-3) and a later sample taken 4-7 days after admission. As fat requirements varied between infants and older children, study participants were stratified into two age groups, >1 year of age and <1 year of age. The association between energy and macronutrient delivery with the inflammatory response was assessed amongst all children and in the stratified groups by age.

6.2.1 Sample size calculation

Sample size calculation was performed using the statistical power analysis program G*Power 3 (Faul et al. 2007). The sample size was chosen to test the hypothesis that higher dietary fat is associated with the stimulation of inflammatory cytokines. Since no studies have investigated the impact of feeding on inflammation in critical illness, this assumption was based on findings from studies linking inflammation to dietary factors in other populations, including obesity, heart disease and inflammatory bowel disease. Lennie et al. (2005) recorded a difference of 3 pg/ml of TNFα between patients having diets higher versus lower in saturated fat. The anti-inflammatory effect of caloric

restriction on plasma levels of TNFα was also examined in an experimental rat model; the mean TNFα in the calorie-restricted group was 14 pg/ml compared to 38 pg/ml (Ugochukwu & Figgers 2007) . For an effect size of 0.5 and 90% power it was estimated that 31 children would be needed to detect a statistical association between dietary fat and inflammatory cytokines. Since those children were part of the main GHICI study, rather than reduce the sample size available, all children who met the inclusion criteria were included in the study.

6.2.2 Statistical analysis

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as a median with interquartile ranges (IQR). The Mann-Witney U test was applied to assess differences in cytokine levels between day 1 and day 7. The Spearman correlation coefficient was used to establish the correlation between variables, which was followed by stepwise linear regression analysis to assess factors that impact inflammatory cytokines (TNF-α, IL-10, IL-6 and IL-1β). A p-value of ≤ 0.05 was considered to be statistically significant and log transformation was performed on cytokines prior to regression analysis.

6.3 Results

Cytokine levels were measured in 87 critically ill children (51 males) who were enterally fed. Fifty-eight samples were collected on day 1, 50 samples on day 2, 37 samples on day 3 and 39 samples between days 4 and 7. The population characteristics of the children recruited in this study are shown in Table 6-1.

Table 6-1: Anthropometric and clinical characteristics of children enrolled to the study

Anthropometrics	All children (N=87)	<1 year of age (N= 27)	> 1 year of age (N=60)
Age (years)	2 (0.6 – 4.9)	0.25 (0.1 – 52)	4 (2 – 10)
Weight (kg)	14 (8 – 21)	4.8 (3.4 – 7.6)	16 (13 – 28)
Height (cm)	95 (74 – 112)	58 (51 – 70)	102 (88 – 122)
Weight for age Z-score	-0.1 (-1.4 – 0.72)	-1.2 (-2.5 – -0.2)	0.2 (-0.7 – 0.8)
Height for age Z-score	-0.15 (-1.9 – 1.2)	-0.2 (-1.9 – 1.7)	-0.03 (-0.1 – 1.1)
Weight height Z-score	0.05 (-1.1 – 0.81)	-0.5 (-0.9 – 0.7)	0.20 (-1.1 – 1.09)
Disease severity			
PIM2 score	3.2 (0.9 – 6.2)	3.4 (0.8 – 6.2)	3.1 (1.1 – 6.8)
PMODS	6 (5 to 7)	6 (5 to 7)	6 (5 to 7)
Inotrope score	0 (0 – 22)	2 (0.0 – 17)	0.0 (0.0 – 29)
Maximum lactate	1.9 (1.3 – 3.3)	1.7 (1.3 – 3.9)	1.9 (1.3 – 3.1)
Maximum CRP	85 (29 – 193)	60 (32 – 165)	114 (33 – 223)
VFH at 30 days	622 (542 – 651)	609 (547 – 648)	623 (527 – 651)
PICU- free days at 30 days	23 (19 – 24)	22 (17– 25)	22 (18 – 24)
Primary admission diagnosis	**N (%)**		
MOF	32 (37%)		
Respiratory Failure	33 (37%)		
CNS Disorders	19 (22 %)		
Cardiac Disorders	1 (1 %)		
Gastrointestinal Disorders	1 (1 %)		
Surgical Disorders	1 (1 %)		

Data presented as median (IQR)

- VFH at 30 days: ventilation-free hours at 30 days
- PICU-free days at 30 days: days free of paediatric intensive care at 30 days

6.3.1 The inflammatory response in critically ill children

The levels of IL-6, IL-10 and IL-1β were higher in the early days of admission and then they started to decline gradually. There was a statistical difference in their levels between days 1 and 7, $p<0.05$. Whilst TNFα showed a fluctuating pattern, on day 2 the level of TNF-α was the highest and it was lowest on day 7, but remained relatively elevated and had not statistically altered between admission and day 7 (see Figure 6. 1).

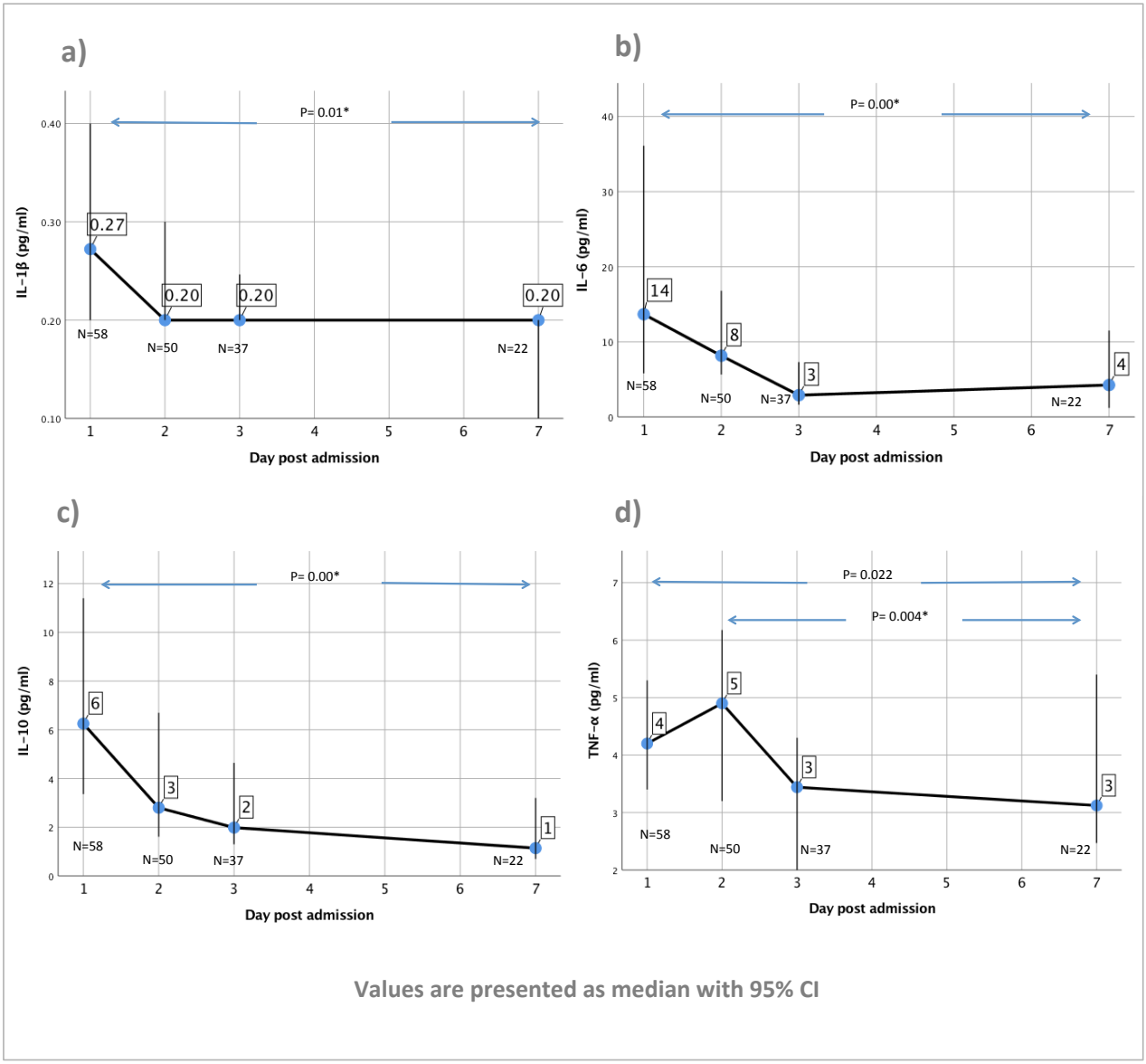

Figure 6.1 Changes in the levels of inflammatory cytokines following PICU admission

The above figures show that cytokines levels declined gradually over the course of PICU stay, except TNFα, which showed a fluctuating pattern.

6.3.2 Feeding and inflammatory response

Serum inflammatory markers were measured in a total of 125 samples collected from 87 children. The number of samples collected from each age group is presented in Table 6-2. The median amount of energy and macronutrient received during the early and late phase are presented in Figure 6.2. Prior to performing regression analysis, correlation analysis was used to explore the association between macronutrient intake and the inflammatory mediators.

Table 6-2: The number of samples collected from each age group

All children				
Early samples (Days 2-3)	**Number of samples**	**Median number of samples per patient**		
	87	1 (1-1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.8 (2.3 – 7.5)	6.5 (1.8 – 19)	2.5 (0.9 – 7.8)	0.2 (0.2 – 0.3)
Late samples (Days 4-7)	**Number of samples**	**Median number of samples per patient**		
	39	1 (1-2)		
	TNFα (pg/ml)	**1L-6(pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.7 (2.5 – 6.8)	3.5 (1.5 – 8.6)	1.6 (0.9 – 2.4)	0.2 (0.1 – 0.2)
<1 year				
Early samples (Days 2-3)	**Number of samples**	**Median number of samples per patient**		
	20	1 (1-1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	5.8 (4.5 – 9.6)	8.8 (2.8 – 14)	4.3 (1.1 – 28)	0.25 (0.2 –0.5)
Late samples (Days 4-7)	**Number of samples**	**Median number of samples per patient**		
	15	1 (1-2)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	6.8 (4 – 7)	3 (1.5 – 5.9)	1.8 (0.9 – 3.1)	0.2 (0.1 – 0.4)
>1year				
Early samples (Days 2-3)	**Number of samples**	**Median number of samples per patient**		
	67	1 (1-1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.2 (1.8 – 6.1)	5.9 (1.7 – 20.2)	2.2 (0.9 – 7)	0.2 (0.2 –0.21)
Late samples (Days 4-7)	**Number of samples**	**Median number of samples per patient**		
	24	1 (1-2)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.1 (2.2 – 4.3)	3.6 (0.8 – 10.9)	1.1 (0.7 – 2.3)	0.2 (0.1 – 0.2)

Data presented as median (IQR)

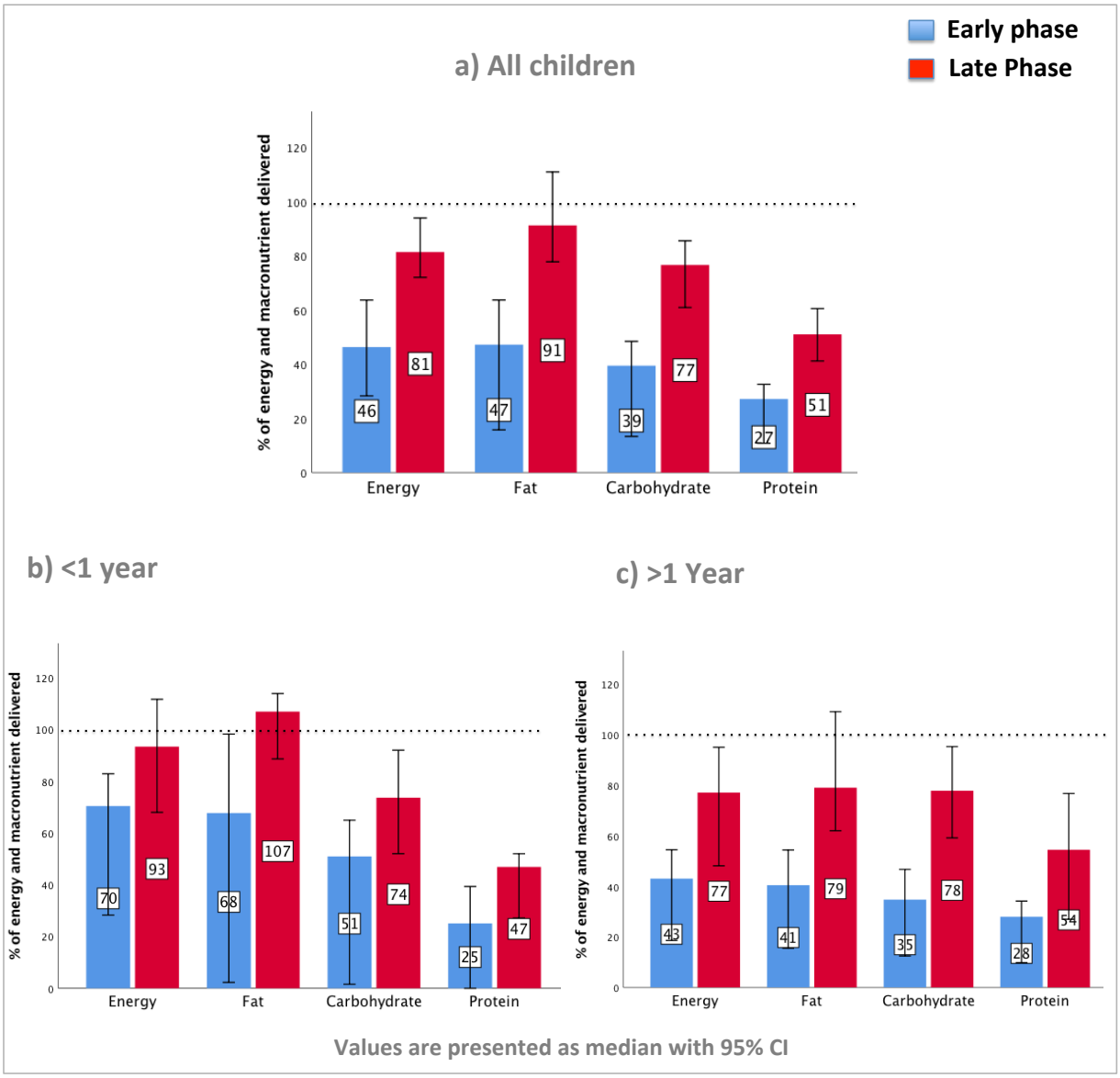

Figure 6.2 Average enteral energy and macronutrients delivered during early and late phase of illness

Figure 6.2 a) shows median intake of energy and macronutrients during the early and later phase of illness in 87 critically ill children. The early phase covers day 2-3, the later phase included days 4-7 post admission. Day 1 was excluded from this analysis because it was not feasible to record the feeds prior admission. Figures 6.2 b), c) and d) show the intake in different age groups. The above figure clearly indicates that younger children <1 year of received higher doses of enteral fat.

a. Early cytokines (samples obtained on day 2-3):

There was no correlation between macronutrients and early cytokines except for IL-6, which showed a weak negative correlation with energy (r= -0.22, p=0.04) and protein (r= -0.26, p=0.01).

Stepwise linear regression analysis was performed, with IL-6 as an outcome variable, to determine whether feeding contributed to a change in IL-6 independently from the severity of disease and age. The results indicated that both PIM2 score and % of delivered protein were statistically related to IL6 ($p < 0.01$). This regression model accounted for 20% of the variation in IL-6 levels (Table 6-3).

In children <1 year of age, a lower protein intake was associated with an increase in IL-6 levels, r= -0.554, p= 0.024, whilst in older children, no evidence of statistical association was recorded between the percentage of protein received and IL-6, r= -0.221, p= 0.077 (Table 6-3).

Table 6-3: Factors affecting IL-6 during the period of nutrition deprivation

Model	R	R^2	Adjusted R^2	R	P-value
IL-6 [a] (all children)	**0.457**	**0.209**	**0.189**		
PIM2 score [b]				0.346	0.001*
%of enteral protein received [b]				-0.295	0.007*
% of enteral energy received [c]				-0.053	0.638
% of enteral carbohydrate received [c]				0.104	0.351
% of enteral fat received [c]				0.077	0.490
Age [c]				0.213	0.055
IL-6 [a] (children < 1 year)	**0.544**	**0.296**	**0.249**		
%of enteral protein received [b]				-0.544	0.024*
% of enteral energy received [c]				-0.203	0.450
% of enteral carbohydrate received [c]				0.459	0.074
% of enteral fat received [c]				0.229	0.394
PIM2 score [c]				-0.074	0.0784
Age [c]				0.034	0.90
IL-6 [a] (children > 1 year)	**0.505**	**0.255**	**0.232**		
PIM2 score [b]				0.429	0.00*
Age [b]				0.300	0.014*
%of enteral protein received [c]				-0.221	0.077
% of enteral energy received [c]				-0.203	0.105
% of enteral carbohydrate received [c]				-0.156	0.216
% of enteral fat received [c]				-0.124	0.326

d. **Dependent variable**
e. **Predictors: (constant)**
f. **Excluded variables**

b. Late cytokines (samples obtained between day 4-8):

With the later samples, both TNF-α and IL-10 were positively correlated with the delivery of energy, fat and carbohydrate, whilst no evidence of statistically significant correlation was observed with protein intake.

Stepwise linear regression analysis was performed, with TNF-α as the outcome to determine whether energy and macronutrient intake contributed to an increase in TNF-α independently from the age and the severity of disease. The results indicated that

higher delivery of fat and lower delivery of protein statistically contributed to increases in TNF-α, $p < 0.01$, and this regression model accounted for 31% of the variation in TNF-α levels (Table 6-4).

I further investigated the effect of feeding on TNF-α, between different age groups. The regression models suggested that the percentage of enteral fat delivered was the only variable statistically related to the increase in TNF-α (r=0.440, p=0.032) in children >1 year of age. This was not replicated in the younger age group (r=0.387, p=0.077) (Table 6-4).

Table 6-4: Factors affecting TNF-α

Model	R	R^2	Adjusted R^2	R	P-value
TNF-α [a] (all children)	**0.561**	**0.315**	**0.277**		
% of enteral fat received [b]				0.534	0.001*
% of enteral protein received [b]				-0.347	0.033*
% of enteral energy received [c]				-0.004	0.983
% of enteral carbohydrate received [c]				0.008	0.963
PIM2 score [c]				0.187	0.269
Age [c]				-0.191	0.257
TNF-α [a] (children < 1 year)					
% of enteral fat received [c]				0.387	0.077
% of enteral protein received [c]				0.0.119	0.377
% of enteral energy received [c]				0.212	0.224
% of enteral carbohydrate received [c]				0.229	0.206
PIM2 score [c]				0.037	0.448
Age [c]				-0.202	0.337
TNF-α [a] (children > 1 year)	**0.440**	**0.193**	**0.157**		
% of enteral fat received [b]				0.440	0.032*
% of enteral protein received [c]				0.094	0.669
% of enteral energy received [c]				-0.094	0.669
% of enteral carbohydrate received [c]				-0.009	0.969
PIM2 score [c]				0.322	0.134
Age [c]				-0.010	0.963

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

This study also investigated the effect of energy and macronutrient intake in a stepwise linear regression analysis where IL-10 was the outcome variable. This model suggested that both energy intake and PIM2 score had a statistically significant effect on IL-10. This regression model accounted for 46% of the variation in IL-10 (Table 6-5).

In children <1 year both the percentage of energy intake and PIM2 score were statistically related to IL-10, whilst in older children PIM2 score was the only variable statistically related to an increase in IL-10 (Table 6-5).

Table 6-5: Factors affecting IL-10

Model	R	R^2	Adjusted R^2	R	P-value
IL-10 [a] (all children)	**0.679**	**0.461**	**0.415**		
% of enteral energy received [b]				0.575	0.00*
PIM2 score [b]				0.437	0.004*
%of enteral protein received [b]				0.163	0.018*
% of enteral carbohydrate received [c]				0.082	0.636
% of enteral fat received [c]				-0.082	0.636
Age [c]				-0.188	0.271
IL-10[a] (children < 1 year)	**0.758**	**0.575**	**0.504**		
% of enteral energy received [b]				0.698	0.006*
PIM2 score [b]				0.540	0.046*
%of enteral protein received [c]				-0.357	0.231
% of enteral carbohydrate received [c]				0.257	0.397
% of enteral fat received [c]				-0.185	0.546
Age [c]				-0.143	0.642
IL-10[a] (children > 1 year)	**0.510**	**0.260**	**0.227**		
PIM2 score [b]				0.510	0.011*
% of enteral fat received [c]				0.183	0.403
% of enteral energy received [c]				0.207	0.344
%of enteral protein received [c]				0.146	0.507
% of enteral carbohydrate received [c]				0.177	0.420
Age [c]				-198	0.365

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

6.4 Discussion

The initial insult in critical illness is usually characterised by its pro-inflammatory nature (Hall et al. 2017). In the current study, in response to stress, the inflammatory cytokines were higher in the early course of critical illness and then declined progressively, except for TNFα, which showed a fluctuating pattern. These data are consistent with the expected course of the systemic inflammatory response in critical illness as shown in previously published studies (Oberholzer et al. 2000; Briassoulis et al. 2010; Jaffer et al. 2010; Jawa et al. 2011). It is known that the degree of inflammation is associated with adverse outcomes (Hall et al. 2017); this study investigated the effect of macronutrient delivery on the cytokines' response in critically ill children.

It was found that both protein deficiency and a higher delivery of fat were associated with elevated levels of inflammatory cytokines; in particular, the under-delivery of protein was associated with an increase in serum IL-6 and TNF-α. The association between dietary protein and inflammatory markers has not been previously investigated in a critically ill population. However, increased levels of inflammatory markers were recorded in malnutrition studies where presumably energy and protein intake were compromised. An increased IL-6 level was reported in malnourished critically ill children and gastric cancer patients (Delgado et al. 2008; Correia et al. 2007). Protein intake was not assessed in neither of the aforementioned studies; however, low protein intake has been shown to be associated with increased serum IL-6 in a murine model of malnutrition (Ling et al. 2004). In addition, IL-6 and TNF-α have been proposed as possible biomarkers of nutritional deficits (Wilasco et al. 2017; Correia et al. 2007).

Despite the current knowledge about the contribution of dietary fatty acids to modulating the production of lipid mediators and signalling molecules in cells that are involved in immune regulation and inflammation (Galli & Calder 2009), to the knowledge of the author, no previous data have been published linking fat intake with elevated levels of circulatory cytokines in critical illness. The current study recorded an association between enteral fat delivery and elevation in TNF-α, particularly in children

>1 year of age. Although the younger group received higher doses of enteral fat and their median TNF-α was higher, there was no statistical association. This could be attributed to variations in fat requirements, as in young children, a higher fat intake is required for their physiological functions (Koletzko et al. 2005; Uauy & Dangour 2009). In breast milk nearly 50% of energy is provided from fat (Ballard & Morrow 2013). There are limited data describing the changes in lipid metabolism in relation to inflammation during critical illness. In septic patients the changes in plasma fatty acid profiles appear to be related to the intensity of the inflammatory response, and in addition, interleukins have been shown to be inversely related to low-LDL/low-HDL (Fitrolaki et al. 2016; Novak et al. 2017). In septic children, increased expression of Neutrophil CD64 was associated with low HDL and LDL levels (Fitrolaki et al. 2013). None of the above-mentioned studies recorded the EN intake or investigated the dietary factors. Studies linking inflammation to dietary factors do exist in other populations including patients with obesity, heart disease and inflammatory bowel disease. In patients with heart failure, TNF-α levels were elevated with a higher intake of saturated fat (Lennie et al. 2005). In an obese rat model, the use of a high-fat diet induced the expression of TNF-α, IL1β and IL-6 in skeletal muscle, visceral fat and blood (Borst & Conover 2005; Wu et al. 2016; Shoelson et al. 2006; Lee et al. 2010). Studies also suggest that the high-fat western diet might induce systemic low-level inflammation as a result of changes in gut microbiota (Pendyala et al. 2012; Parekh et al. 2014). In a study conducted on 15 overweight men, a low-fat diet resulted in a statistically significant reduction in TNF-α (Sharman & Volek 2004). However, it is important to point out that the type of fat delivered might also impact the inflammatory response. In an experimental induced colitis rat model, a medium-chain triglycerides (MCT) rich diet reduced IL-6 and IL-8 levels, indicating that MCT rich formulas exert an anti-inflammatory effect on colitis (Papada et al. 2014).

Although the exact mechanism that links dietary fat to inflammation is not fully understood, several mechanisms have been proposed. It is thought that pro-inflammatory fatty acids may act directly and activate receptors that signal an inflammatory response (Innes & Calder 2018). It has also been suggested that the

infiltration of macrophages associated with adiposity accounts for the increased adipose expression of TNF-α (Borst & Conover 2005). However, one of the recently proposed mechanisms that links dietary fat to inflammation is related to its effect on promoting the translocation of microbial products from the gut into the bloodstream (Fritsche 2015). The well-documented evidence of intestinal dysbiosis during critical illness makes this explanation plausible and clinically justifiable.

In the current study, the association between energy intake and IL-10 could be related to the stimulation of insulin secretion. IL-10 signalling has been proposed as a potential mechanism to increase energy expenditure and improve insulin sensitivity (Straczkowski et al. 2005; Ropelle et al. 2010).

This study suggests that the energy and macronutrient delivery in the later phase are associated with alterations in the inflammatory response when data was analysed in a single cohort. However, we recorded inconsistency in the findings with further stratification to different age groups, which could be related to the reduction in the sample size. This could also be attributed to variations in macronutrient requirements between infants and older children. It is worth mentioning that the changes in clinical condition could be contributory or indeed driving this association to a large extent and this needs to be explored in further studies.

6.5 Limitations

This study has several limitations including the small sample size and heterogeneity of the patient cohort. It would have been ideal to divide the children into more than two age groups and investigate the relationship between macronutrient and cytokines within each age group. As this study suggests that fat intake may drive a pro-inflammatory response, it seems prudent to investigate the relationship between n3:n6 in this study. In this smaller patient cohort, where formula delivery was protocolised and most children received a similar type of formula, this type of analysis was not feasible.

Within the linear regression model, there were some variables with a low r- value that limit how well the changes in inflammatory cytokines can be attributed to them. However, the results of the analysis appear to be in agreement with reported associations between macronutrient intake and inflammation in other disease states (Borst & Conover 2005; Wu et al. 2016; Shoelson et al. 2006; Lee et al. 2010; Sharman & Volek 2004; Delgado et al. 2008; Correia et al. 2007; Ling et al. 2004). It is clear that further work is needed to establish appropriate enteral fat intake in critically ill children, and also to examine the effect of specific fat sources such MCT and omega-3 on inflammatory response and other clinical outcomes.

6.6 Conclusion

The inflammatory response in critically ill children is complex and mediated by many factors. The current study suggests that the amount of enteral fat and protein delivery may influence the inflammatory response in children with prolonged PICU stay. In other words, macronutrients are rather a contributing factor that might worsen or improve the inflammatory condition. Finally, the findings of this chapter emphasize the importance of providing balanced macronutrients in the enteral feeds to avoid deleterious effect over or underfeeding on the inflammatory response.

7 Chapter Seven: Association between Intestinal Injury and Systemic Inflammation in Critical Illness

7.1 Introduction

The intestinal environment is very disrupted in critically ill children due to a combination of physiological stresses, pharmacological effects and a lack of EN (Krezalek et al., 2016; Ferrer et al., 2016; Maier et al., 2018). As a result, the synergy between the intestinal microbiome and the host is adversely affected (Krezalek et al., 2016; Ferrer et al., 2016). As an organ with extensive nutritional, metabolic, endocrine and inflammatory activities, intestinal dyshomeostasis may have systemic consequences (Petrova & Koh, 2018; Furness, 2008). Previous studies have speculated that the gastrointestinal tract might play a driving role in the development of sepsis, SIRS and MODS, due to impaired intestinal barrier function caused by the inflammation (MacFie et al., 1999; Earley et al., 2015; Clark & Coopersmith, 2007).

Assessment of intestinal integrity in clinical practice is challenging, due to difficulties in evaluating the condition of the gut with invasive tests, such as the oral administration of polyethylene glycols or dual sugar (Van Wijck et al., 2012). However, a number of biomarkers have been recently suggested as alternative diagnostic tools to assess intestinal permeability and inflammation (Derikx et al., 2010). Circulating or urinary fatty acid binding proteins (FABP) have been proposed as markers of mucosal tissue injury in neonates with suspected necrotising enterocolitis (Kanda et al., 1996; Derikx et al., 2007; Grootjans et al., 2010). Serum zonulin and urinary claudin-3 have been suggested as indicators of tight junction injury in septic and surgical patients (Klaus et al., 2013; Derikx et al., 2008). Serum citrulline appears to be a predictive marker of enterocyte loss and villous atrophy (Crenn et al., 2009). Faecal calprotectin (FC) has been widely utilised in clinical practice as an assessment tool of mucosal inflammation (Costa et al., 2003; Komraus et al., 2012; Moein et al., 2017).

Citrulline is an amino acid produced mainly by enterocytes of the small intestines, and has been well correlated with the functional enterocyte mass, and also with prognosis in ICU patients (Crenn et al., 2008; 2009; Hull et al., 2011; Piton et al., 2010). Evidence suggests that citrulline can be used as a predictive marker of enterocyte loss and villous atrophy in patients with HIV (Crenn et al., 2009). Reduced citrulline levels were also recorded in children with intestinal failure who developed catheter-related bloodstream infections, suggesting a potential role of citrulline as a marker of impaired intestinal barrier integrity (Hull et al., 2011). In addition, citrulline concentration was inversely correlated with highest endotoxin level in post-cardiac arrest patients (Grimaldi et al., 2013). Low plasma citrulline has shown to be an independent factor of mortality, and a marker of acute intestinal failure in critically ill patients (Piton et al., 2010). The findings of these studies underpin the idea that citrulline production is linked to intestinal functional mass, and therefore influenced by diseases affecting the gut (Sertaridou et al., 2015). Historically, gut permeability was assessed using chromatographic urinary analysis after oral administration of polyethylene glycols or dual sugars (Van Wijck et al., 2012). These pose several challenges in the clinical context of critical illness. Firstly, the need for administering polyethylene glycols or dual sugars, and secondly, the measurement requires the collection of urine over a prolonged period (4-6 hours). Spot measurement of serum citrulline is, therefore, a potentially attractive tool for the assessment of intestinal mucosal disease (Hull et al., 2011). The value of serum citrulline was utilised as a marker of reduced enterocyte mass, as it has been well correlated with the functional enterocyte mass, and also with prognosis in the critically ill adult population (Poole et al., 2015; Piton et al., 2010).

Calprotectin is a calcium and zinc-binding protein belonging to the S100 family; it is derived mainly from activated neutrophils present in the intestinal mucosa and lumen (Ho et al., 2015). It is largely stored in neutrophil granulocytes, and to a lesser extent in monocytes and activated macrophages (Benítez & García-Sánchez, 2015). Calprotectin is also known to have antibacterial activity and may play a role in the induction of apoptosis (Josefsson et al., 2007). Furthermore, it is probably involved in the regulation of inflammatory reactions (Costa et al., 2003). A faecal Calprotectin (FC) concentration

of <50 µg/g is considered the upper normal limit (Benítez & García-Sánchez, 2015). During bowel inflammation, raised levels of calprotectin are detected in faeces. Elevated levels of FC have been recorded in a number of gastrointestinal diseases, such as inflammatory bowel disease, colorectal cancer and Necrotizing Enterocolitis (NEC) (Costa et al., 2003; Komraus et al., 2012; Moein et al., 2017). FC has been previously used in the diagnosis and monitoring of inflammatory bowel diseases (Benítez & García-Sánchez, 2015; Ho et al., 2015; Moein et al., 2017). The levels of FC have shown a good correlation with the degree of inflammatory activity in inflammatory bowel diseases (Benítez & García-Sánchez, 2015). FC has also been investigated as a biomarker of mucosal inflammation in NEC and intestinal ischemia reperfusion (Ho et al., 2015). It has been shown that FC level is a more specific marker of mucosal inflammation than circulating CRP (Benítez & García-Sánchez, 2015). FC provides an objective evaluation of the gastrointestinal tract that is easy to use, particularly in children, for many reasons: it is easy to collect stool samples from children, FC is stable at room temperature for up to one week, and it can be measured easily using commercially available immunoassay (Ho et al., 2015; Andréasson et al., 2011).

The previous chapter established that enteral macronutrients are rather a contributing factor that might worsen or improve the inflammatory condition. The main aim of this study is to explore if critical illness is associated with abnormalities in gut health biomarkers and whether this is associated with increase in inflammatory cytokines. The secondary end point is to assess the effect of feeding on mucosal integrity during critical illness.

7.2 Materials and Methods

Both healthy and critically ill children were included in this study. Where comparisons were made between healthy and critically ill states, the analysis was restricted to samples obtained from an age-matched cohort of critically ill and healthy children.

Samples collected:

Faeces

The first available faecal sample was collected from the child's nappy (around 300 mg) in a sterile container. A volume of 15 mg was required for the analysis of FC.

Serum

Serial blood samples were collected from indwelling vascular catheters, for the analysis of serum citrulline and inflammatory cytokines levels. Samples were collected at day 1 (TP1), days 2-5 (TP2) and day 7 plus (TP3) following PICU admission. Another sample was collected for cytokine analysis, to match the time of the faecal sample in children who had indwelling vascular access at the time of faecal sampling.

The detailed methodology of FC, citrulline and inflammatory cytokine assays is described in Chapter Two, pages 53-56.

Nutritional information:

To investigate relationships between FC and citrulline with nutritional intake, the amount of energy and macronutrients delivered were calculated to the 24-hour period prior to each sample collection (1 day =24 hours). The cumulative intake of energy and macronutrients from admission was also calculated to assess the long-term effect of feeding.

7.2.1 Sample size calculation

Sample size calculation was performed using the statistical power analysis program G*Power 3 (Faul et al., 2007). The sample size was chosen to test the hypothesis that citrulline and FC levels are statistically increased during critical illness in response to intestinal injury and inflammation, respectively.

Since no studies in critically ill children have investigated citrulline as a potential marker of intestinal injury, this assumption was based on work by Poole et al. (2015) in critically ill adult population. The mean plasma citrulline level was 15.2 µmol/L in the critically ill group, compared to 26.5 µmol/L in healthy controls, with a recorded difference of 11 µmol/L. It was estimated that 16 children per group would be needed to detect the same difference assuming a 5% significance level (2-sided) and 90% power.

FC has also not been investigated before as a potential marker of intestinal inflammation in critically ill adults or children. The sample size calculation was based on studies of inflammatory bowel diseases. Many studies have shown a marked increase in FC levels among patients with inflammatory bowel diseases compared to healthy subjects (Komraus et al., 2012; Chen et al., 2012; Moein et al., 2017). These studies recorded a difference of (100-300) µg/g in FC levels between children with gastro-inflammatory disorders and healthy children. Based on this assumption, it was estimated that 34 children per group would be needed to detect the same difference assuming a 5% significance level (2-sided) and 90% power.

The second end point was the association between citrulline/FC and inflammatory cytokines. For an effect size of 0.5 and 90% power, it was estimated that 31 children would be needed to detect a statistical association between citrulline or FC and inflammatory cytokines. As these investigations were part of a larger study assessing gut homeostasis in critically ill children, rather than reducing the sample size, all children who met the inclusion criteria of the current study were included.

7.2.2 Statistical analysis

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as median with (25-75) interquartile ranges. Mann-Witney U was used to compare independent groups with non-normal distribution, such as differences in FC and citrulline between the patient and control groups. The Wilcoxon signed rank test was used to compare related samples with non-normal distribution, such as the difference in FC/citrulline levels in serial samples. Stepwise linear regression analysis was applied to assess the relationship between FC/citrulline with inflammatory cytokines and clinical outcomes. A logistic regression was also performed to assess the effects of citrulline/FC on the likelihood that critically ill children have sepsis. Log transformation was performed on FC, citrulline, cytokines and other non-normally distributed variables prior to regression analysis.

7.3 Results

The data used in this chapter was derived from two studies. The flow of each study and the number of patients and healthy controls included are presented in Figure 7.1.

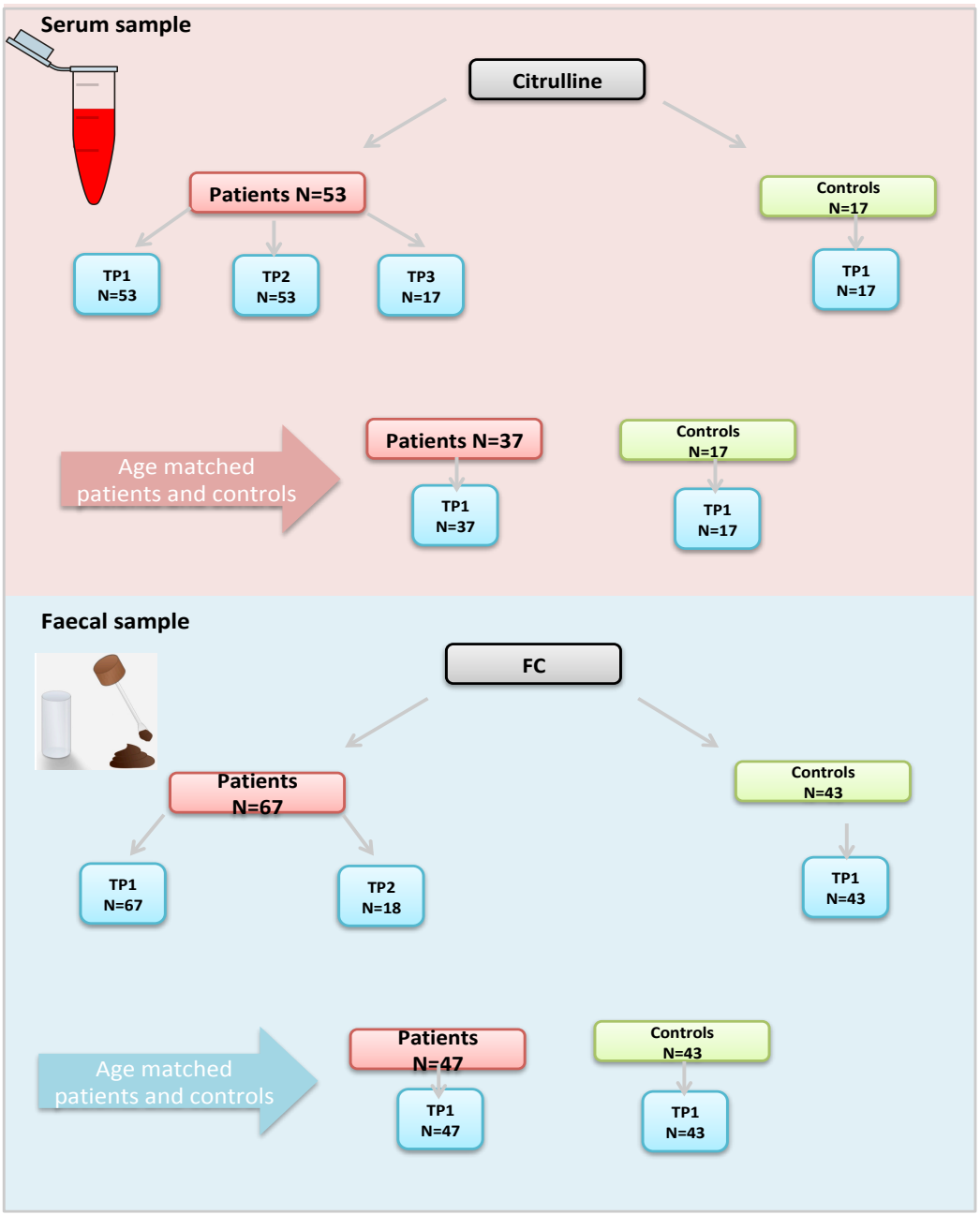

Figure 7.1 Study participant flow diagram

Figure 7.1 shows the number of study participants recruited in each study, as well as the number of age-matched healthy children.

7.3.1 Assessment of intestinal permeability using serum citrulline study

Serum samples were available from 53 critically ill children (30 males) and 17 healthy children (8 males). Amongst the patient cohort, the hospital mortality rate was 5.6% (3 out of 53 children). The demographics of patients and controls are shown in Table 7-1. The results showed that control group was older than the patients group even after the age matching, however, the difference in median age distribution between the patients and control was not statistically significant (p-value= 0.188). Since the serum citrulline levels are known to be affected by age, the citrulline was plotted against age for both control and critically ill to see if there were differences in citrulline at each age (Figure 7.2). The graph showed that the citrulline varied between the critically ill and healthy children in each age, indicating a good age matching.

All patients received at least one broad-spectrum antibiotic therapy and 75% (N=40) of them received more than one type of antibiotics. The median (IQR) number of antibiotic classes received per patients was 2 (1-3).

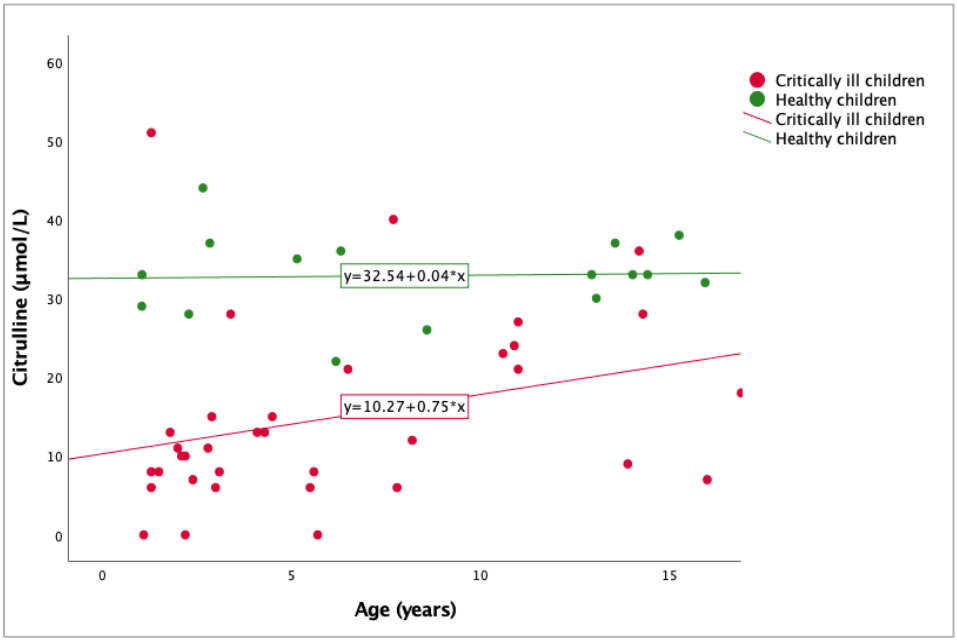

Figure 7.2 Age related changes of citrulline levels in critically ill children and their age matched controls

The graph showed that the citrulline varied between the critically ill and healthy children in each age, indicating a good age matching. In critically ill children the citrulline levels increased with age, the citrulline levels remained stable in controls.

Table 7-1: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometry	All patients and controls			Age-matched patients and controls		
	Patients (N=53)	**Control (N=17)**	**P- value**	**Patients (N=37)**	**Control (N=17)**	**P- value**
Age (Years)	2.2 (0.9 – 4.4)	6.3 (2.3 – 7.2)	0.016**	4.3 (2.1 –9.4)	7.4 (2.7 – 13.7)	0.188
Weight (Kg)	12 (8.5 – 17)	18 (3 – 25)	0.003**	14.8 (11.9 to 24)	18.9 (13.5 – 7.3)	0.032*
Height (cm)	88 (69 – 109)	112 (90 – 130)	0.00**	100 (85 – 121)	115 (93 – 133)	0.010*
Weight age Z-score	-0.3 (-1 – 0.46)	0.45 (-0.08 – 0.73)	0.423*	-0.33 (-1.41 – 0.54)	0.3 (-0.1 – 0.67)	0.678*
Height for age Z-score	-0.3 (-1 – 1)	-0.12 (- 0.65 – 0.6)	0.181	0.07 (-1.1 – 1.3)	0.14 (-0.5 – 0.7)	0.41
Disease severity variables						
PMODS	5 (4 – 6)			6 (4 – 6)		
PIM2 score	3.6 (1.1 – 6.4)			3.9 (0.96– 8.4)		
Highest Lactate	1.8 (0.97 – 3.3)			2.1 (0.90 – 3.2)		
Highest CRP	92 (39 – 257)			137 (34 – 262)		
Inotrope score	0.0 (0 – 15)			0.0 (0 – 15)		
VFH at 30 days	616 (541 – 648)			630 (501 – 653)		
PICU-free days at 30 days	22 (17 – 24)			22 (16 – 24)		
Inotrope-free hours at 30 days	710 (667 – 720)			715 (669– 720)		
Primary admission diagnosis	**N (%)**					
MOF	19 (36%)					
Respiratory failure	20 (38%)					
CNS Disorders	11 (20%)					
Surgical Disorders	2 (4%)					
Cardiac Disorders	1 (2%)					

Data are presented as median (IQR)

** P- value is significant at 0.01 level * P- value is significant at 0.05 level

- PICU-free days at 30 days: days free of Paediatric intensive care at 30 days
- VFH at 30 days: ventilation-free hours at 30 days

7.3.1.1 Citrulline in critically ill and healthy states

From all of the 53 critically ill children, TP1 and TP2 samples were collected. The TP3 sample was obtainable only from 17 critically children.

The results showed that critically ill children had statistically lower serum citrulline (measured in admission sample) compared to age-matched healthy children (P = 0.00) (Figure 7.3).

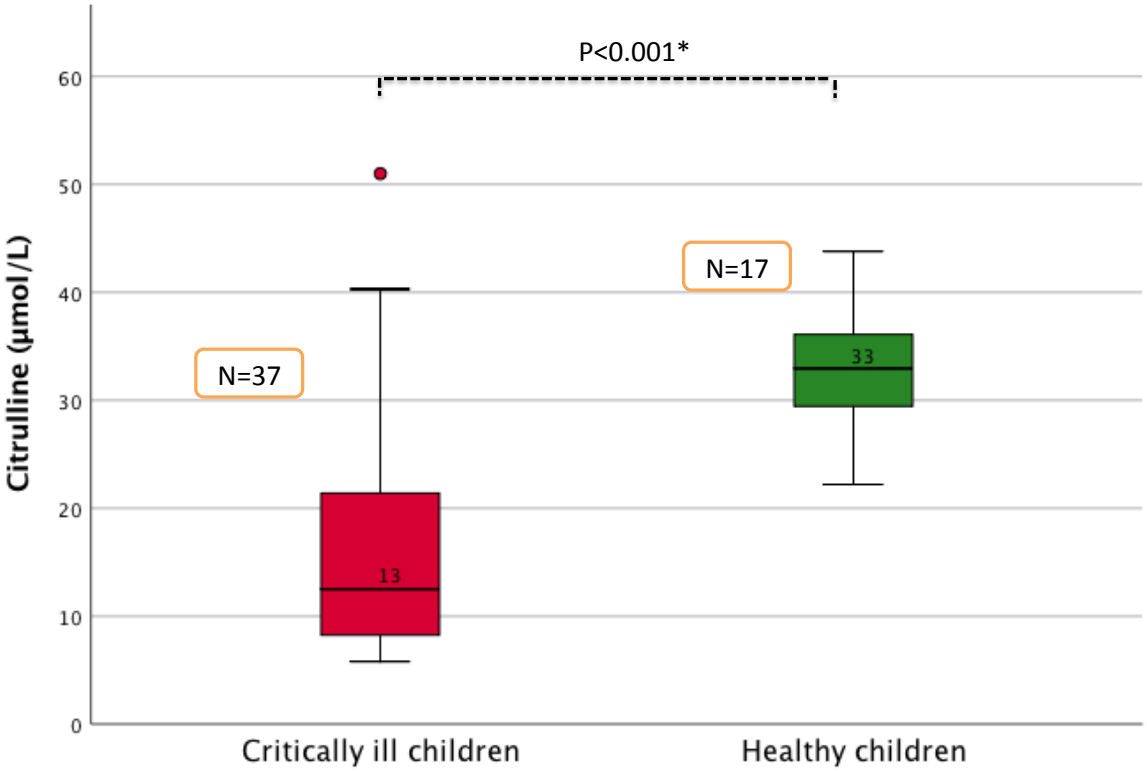

Figure 7.3 Differences in citrulline levels between patients and their age-matched healthy controls

Figure 7.3 shows that the level of citrulline measured in the first sample obtained from the patient group was statistically lower than that of the control group p-value = 0.00**.

A time course analysis of serum citrulline was undertaken and is presented in Figure 7.4. The results showed that citrulline levels increased gradually over the course of PICU stays. However, the citrulline levels were low in 8 patient samples obtained between days 8 and 10. These children were very sick upon admission to PICU; their median (IQR) PIM2 score was 5 (1.7–7.2) and 6 of them had MOF. Their median (IQR) admission citrulline level was 9.6 (2.1–18.7) and remained low in the later sample obtained [median (IQR) 9.7 (7.6 – 20.6)].

The variation in citrulline levels was then assessed in the paired samples, where the analysis was restricted to children who had samples from each of the time points being compared. No statistical difference in citrulline concentration was recorded between TP1 and TP2 samples (p=0.17). In a subset of 17 patients who had a PICU stay of at least 7 days, there was no evidence of statistical difference in citrulline concentration between TP1 and TP3 samples (p=0.30) (Figure 7.5).

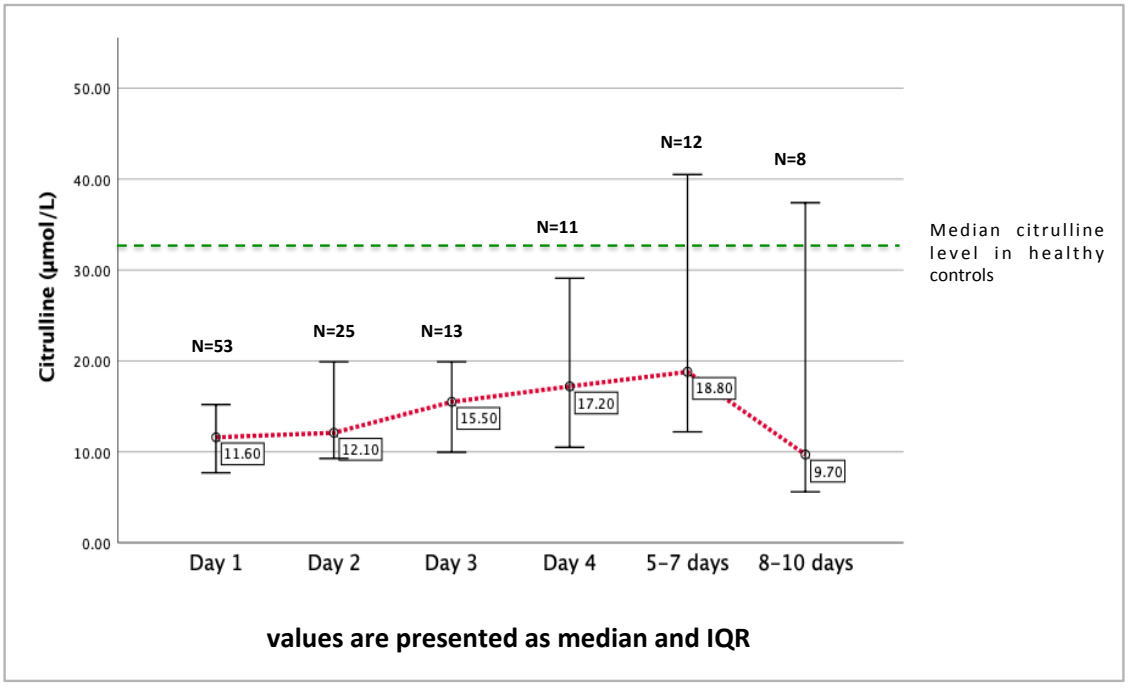

Figure 7.4 Changes in the levels of serum citrulline in different stages following PICU admission

The above figure shows that citrulline levels increased gradually over the course of PICU stays. However, the citrulline levels were low in the samples obtained between days 8 and 10.

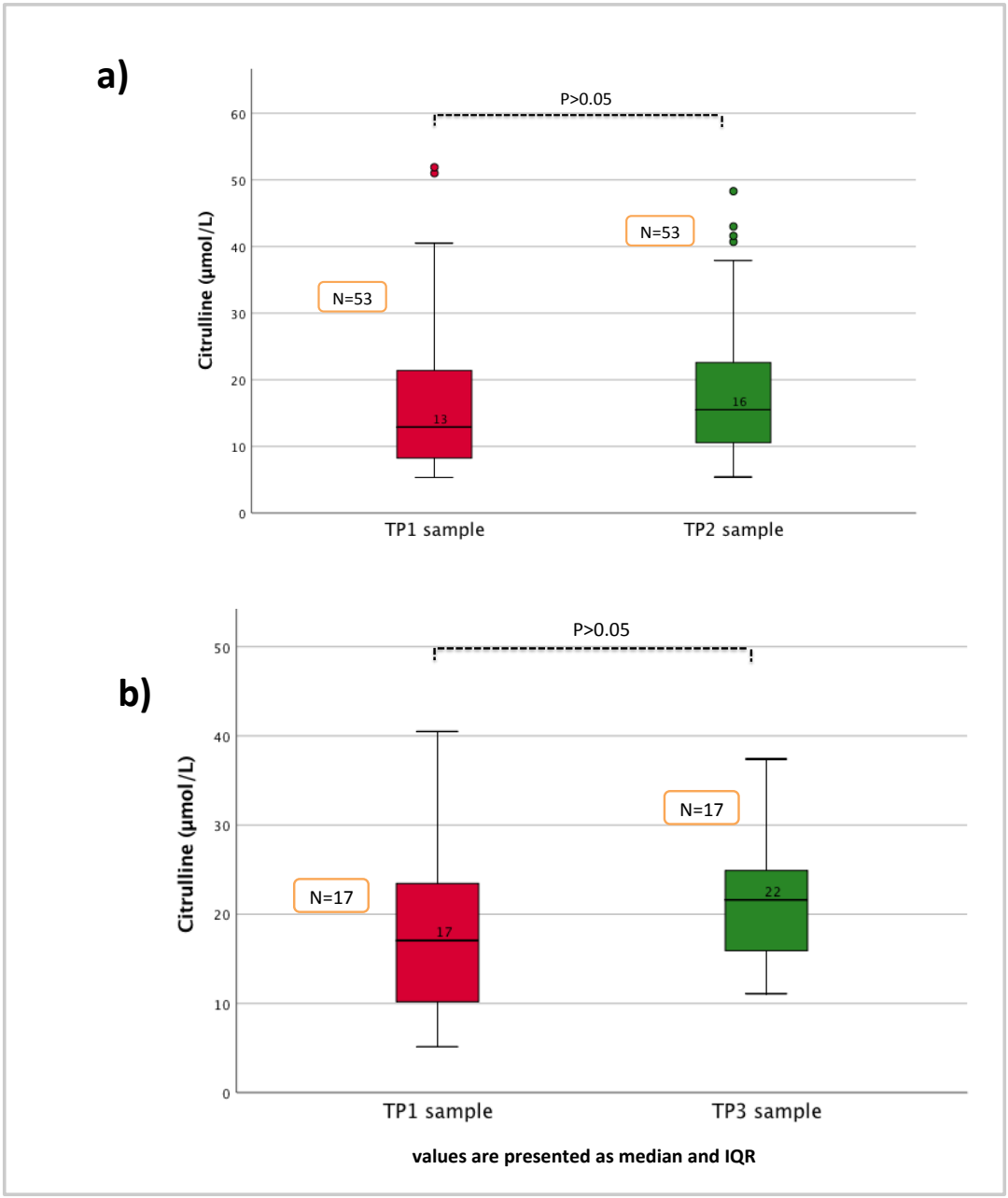

Figure 7.5 Comparing the levels of serum citrulline in the paired samples

Figure 7.5 a) shows the difference in citrulline levels between TP1 and TP2 samples in all recruited children. Figure 7.4 b) shows the difference in citrulline levels between TP1 and TP3 samples in a subset of children who stayed for a longer period. The above figure shows that the level of citrulline measured on admission was lower than in the other consecutive samples, though no evidence of statistical difference was recorded, p-value >0.05.

7.3.1.2 The effect of disease severity and medications on citrulline levels

This section of the analysis aims to explore whether disease severity, inotropic agents and duration of antibiotic therapy influenced intestinal permeability. Since TP1 samples were collected on day 1, it was not possible to investigate the effect of medications on citrulline concentration in those samples. Therefore, only TP2 and TP3 were included in this analysis. The regression analysis indicated that inotrope score was the only variable statistically related to citrulline (r= -0.279, p-value=0.013) (Table 7-2).

Table 7-2: Association between citrulline, medications and clinical variables

Model	R^2	Adjusted R^2	R	P-value
Citrulline[a]	**0.279**	**0.078**	**0.066**	
Inotrope score [b]			-0.279	0.013*
Age [c]			-0.043	0.710
PIM2 score [c]			-0.018	0.897
Days of antibiotic exposure [c]			0.188	0.099

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.1.3 The effect of feeding on citrulline concentrations

This section of the analysis aims to explore whether feeding in PICU influenced intestinal permeability during PICU stays, independently from age disease severity. Data from 17 children were included in the regression models. To investigate the effect of the cumulative nutritional intake on citrulline concentration, only citrulline measured in the later stages of illness (TP3 sample obtained from day 7 onward) was used for this analysis (N=17). The results showed that there was no statistical association between citrulline and the amount energy and macronutrients received during the 24hours prior the collection of serum samples. However, the regression analysis indicated that the cumulative intake of enteral fat was statistically related to citrulline, (r=0.540, p=0.017), (Table 7-3).

Table 7-3: Association between citrulline and the amount of nutrition received in PICU

Model	R^2	Adjusted R^2	R	P-value
Citrulline [a]	**0.290**	**0.243**		
Age [b]			-0.539	0.009*
PIM2 score [c]			0.174	0.238
% of energy received [c]			0.185	0. 225
% of carbohydrate received [c]			0.344	0.075
% of protein received [c]			0.183	0.225
% of fat received [c]			0.401	0.044
Citrulline [a]	**0.291**	**0.250**		
Cumulative fat received [b]			0.540	0.009*
Age [c]			-0.539	0.009
PIM2 score [c]			0.174	0.238
Cumulative energy received [c]			0.357	0.067
Cumulative carbohydrate received [c]			0.502	0.014
Cumulative protein received [c]			0.419	0.037

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.1.4 Association between intestinal injury and systemic inflammation

The relationship between intestinal injury and systemic inflammation was explored in 53 children where serum samples were collected on admission for the analysis of citrulline and the inflammatory cytokines. Stepwise linear regression analysis was performed to determine whether intestinal injury/permeability assessed by Serum citrulline independently influenced systemic inflammation. The regression models that included inflammatory cytokines as the outcome variables suggested that citrulline statistically related to IL-6, TNF-α, IL-1β and IL-10 (Table 7-4).

Table 7-4: Association between citrulline and inflammatory cytokines

Model	R^2	Adjusted R^2	R	P-value
IL-6 [a]	**0.366**	**0.336**		
Citrulline [b]			-0.407	0.002*
PIM2 score [b]			0.436	0.001*
Age [c]			-0.093	0.269
TNFα [a]	**0.501**	**0.466**		
Citrulline [b]			-0.315	0.037*
PIM2 score [b]			-0.521	0.002*
Age [b]			-0.469	0.007*
IL-1β [a]	**0.202**	**0.165**		
Citrulline [b]			-0.342	0.010*
PIM2 score [b]			0.284	0.028*
Age [c]			-0.111	0.224
IL-10 [a]	**0.261**	**0.226**		
Citrulline [b]			-0.344	0.010*
PIM2 score [b]			0.369	0.006*
Age [c]			-0.152	0.157

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.1.5 Association between serum citrulline and sepsis in critically ill children

Sepsis was diagnosed according to the criteria described in the method chapter (Chapter Two). Sepsis diagnosis was documented in 32 out of 53 children. In 15 children the primary site of infection was the respiratory system, 8 children in CNS, and 8 children in blood (see Table 7-5).

Table 7-5: Citrulline levels in three groups of patients with sepsis

	Primary site of infection		
	Respiratory N=16	CNS N=8	Blood N=8
Citrulline	12.2 (6.7–20.9)	20.9 (6.7–23)	8 (6.7– 17)
Day of sample collection	1	1	1
Days of antibiotic exposure	1	1	1

Data are presented as median (IQR)

The first step of the analysis was comparing the levels of admission citrulline in children who developed sepsis and those who did not. No evidence of statistical difference in serum citrulline was recorded between septic children and non-septic children (p = 0.729)**.** A logistic regression was also performed to assess the relationship between serum citrulline measured in the admission sample and sepsis (Table 7-5). The logistic regression model was not statistically significant, $\chi 2$ (6) = 9.3, p = 0.155. None of the included variables were statistically related to the incidence of sepsis (Table 7-6).

Table 7-6: Regression model to assess the relationship between serum citrulline and sepsis

Model	**Model statistics** $\chi 2$	**B**	**Exp (B)**	**Significance**
Sepsis [a]	$\chi 2$ (6) = 9.6, p-value= 0.155			
Citrulline [c]		0.239	1.27	0.485
Age [c]		0.37	1.447	0.566
PIM2 score [c]		0.432	1.54	0.598
Highest TNFα [c]		1.926	6.861	0.253
Highest IL-6 [c]		1.026	2.789	0.164
Highest IL-1 β [c]		-1.797	0.166	0.105

a. **Dependent variable**
b. **Predictors**
c. **Insignificant variables**

7.3.1.6 Association between citrulline and clinical outcomes in critically ill children

I also investigated the effect of intestinal injury assessed by serum citrulline measured in the admission sample on clinical outcomes. No statistical association was recorded between citrulline and length of ventilation, duration of PICU stay, or the duration of inotrope treatment (Table 7-7). In addition, no evidence of statistical difference was recorded between children treated with inotrope and those who did not receive inotrope treatment (p=0.231).

Table 7-7: Association between citrulline and clinical outcomes

Model	R^2	Adjusted R^2	R	P-value
VFH at 30 days [a] (N=53)				
Citrulline [c]			-0.193	0.096
Age [c]			-0.257	0.040
PIM2 score [c]			-0.159	0.143
PICU-free days at 30 days [a] (N=53)				
Citrulline [c]			-0.245	0.048
Age [c]			-0.203	0.085
PIM2 score [c]			-0.158	0.144
Hours free of Inotrope at 30 days [a] (N=20)				
Citrulline [c]			-0.153	0.260
Age [c]			0.244	0.150
PIM2 score [c]			0.009	0.486

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.2 Assessment of intestinal inflammation using FC study

FC was measured in 67 critically ill children (33 males) and 43 healthy children (21 males). Amongst the patient cohort, the hospital mortality rate was 5.9% (4 out of 67 children). The demographics of patients and controls are shown in (Table 7-8).

All patients received at least one broad-spectrum antibiotic therapy and 63% (N=42) of them received more than one type of antibiotics. The median (IQR) number of antibiotic classes received per patients was 2 (1-3).

Table 7-8: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometry	All patients and controls			Age-matched patients and controls		
	Patients (N=67)	**Control (N=43)**	**P- value**	**Patients (N=47)**	**Control (N=43)**	**P-value value**
Age	2.1 (0.9 – 4.4)	4.5 (2.3 – 7.2)	0.001**	3.25 (2 –6.1)	4.5 (2.3 – 7.2)	0.13
Weight (Kg)	12 (8.5 – 17)	18 (3 – 25)	0.00**	14.8 (11.9 – 24)	18 (3 – 25)	0.04*
Height (cm)	88 (69 – 109)	114 (91.0 – 130.0)	0.00**	100 (85 – 121)	114 (91.0 – 130.0)	0.010*
Weight age Z - score	-0.3 (-1 – 0.46)	0.45 (-0.08 – 0.73)	0.01*	-0.33 (-1.41 – 0.54)	0.45 (-0.08 – 0.73)	0.04*
Height for age Z - score	-0.3 (-1 – 1)	-0.12 (- 0.65 – 0.6)	0.18	0.07 (-1.1 – 1.3)	-0.12 (- 0.65 – 0.6)	0.50
Disease severity variables						
PMODS	5 (4 – 6)			6 (4 – 6)		
PIM2 score	3.8 (1.2 – 9.8)			3.9 (1.8 – 9.7)		
Highest Lactate	1.9 (1.9 – 3.3)			1.9 (1.2 – 3.3)		
Highest CRP	105 (42 – 210)			136 (35 – 260)		
Inotrope score	0.0 (0 – 20)			0.0 (0 – 26)		
VFH at 30 days	627 (549 to 669)			628 (524 – 669)		
PICU-free days at 30 days	22 (18 – 25)			22 (17 – 25)		
Inotrope- free hours at 30 days	720 (682 to 720)			712 (669– 720)		
Primary admission diagnosis	**N (%)**					
MOF	26 (39%)					
Respiratory failure	24 (34%)					
CNS Disorders	14 (21%)					
Surgical Disorders	4 (6%)					

Data are presented as median (IQR)

** P- value is significant at 0.01 level * P- value is significant at 0.05 level

- VFH at 30 days: ventilation-free hours at 30 days
- PICU-free days at 30 days: days free of Paediatric intensive care at 30 days

7.3.2.1 FC in critically ill and healthy states

Eighty-five samples were obtained from the patient group and 43 from the healthy control group. In most children, a single faecal sample was collected. Eighteen children had a second sample collected during their PICU stay. The age-matched population was represented by 47 critically ill children and 43 controls. Critically ill children had statistically higher levels of FC measured in the first sample obtained compared to healthy children (P = 0.02) (Figure 7.6). Among healthy children, FC was negatively correlated with age (p=0.01) and weight (p= 0.02), indicating that younger and smaller children had higher FC levels. However, no such correlation was recorded in critically ill children (Figure 7.7).

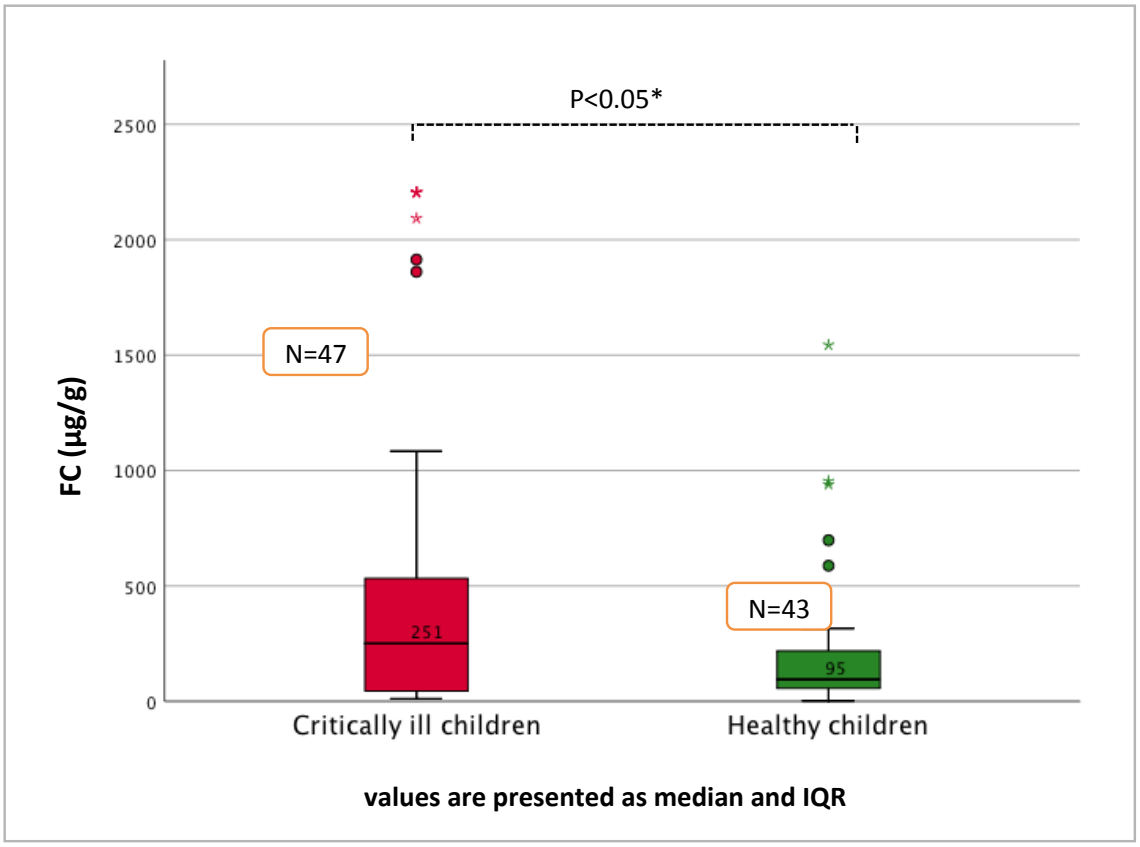

Figure 7.6 Differences in FC levels between patients and age-matched healthy controls

Figure 7.6 shows that the levels of FC measured in the samples obtained from the patient group were statistically higher than in those of the control group p-value = 0.031*.

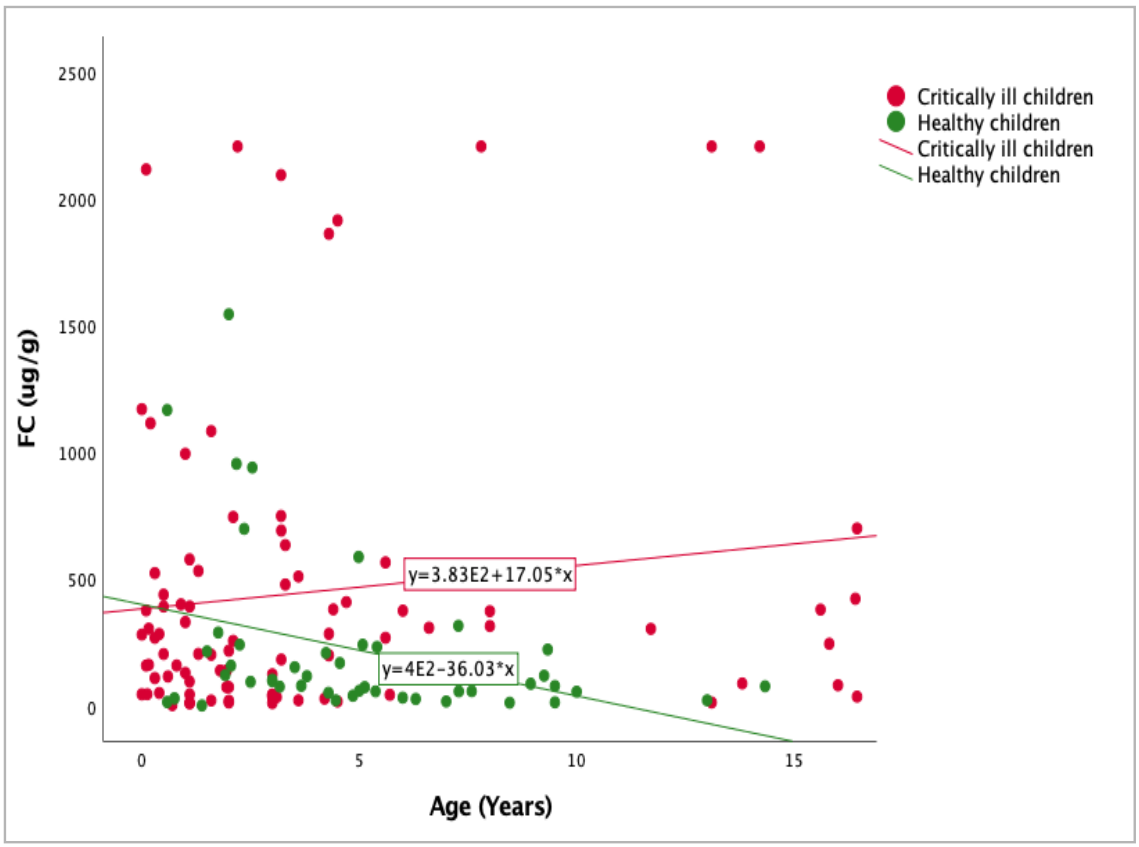

Figure 7.7 Age related changes of FC levels in critically ill children and their age matched controls

Figure 7.7 shows that FC in the healthy children is negatively correlated with age (p=0.01). No such correlation was recorded in critically ill children no particular pattern of FC was recorded with age.

The differences in FC levels between the first sample and second sample were compared in 18 critically ill children, where two faecal samples were available. The levels of FC appear to be reduced in the later stages of disease compared to the early stages (P = 0.02) (Figure 7.8).

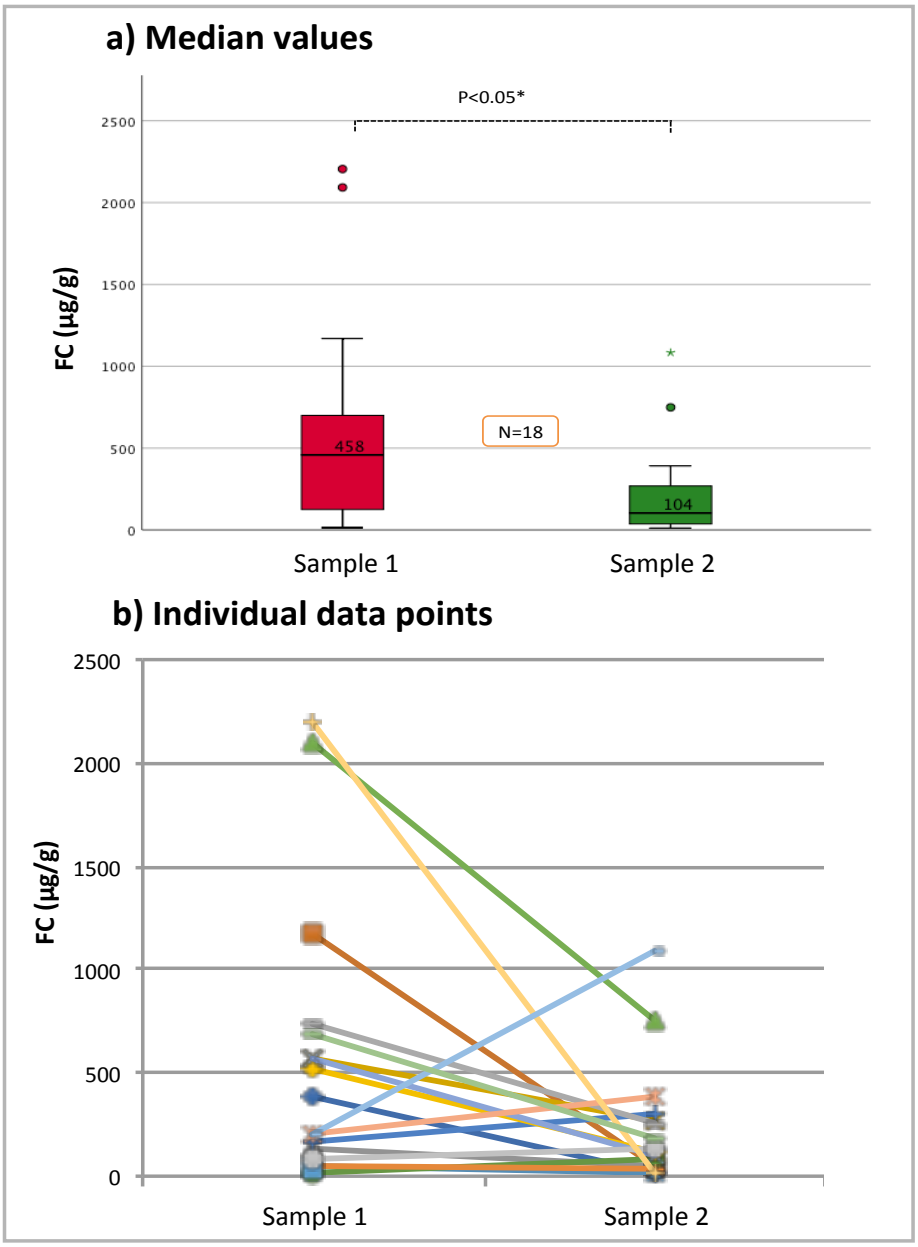

Figure 7.8 Changes in FC level during the course of illness

Figure 7.8 shows the differences in FC levels between sample 1 and sample 2 obtained from the same patients. Clearly FC levels were higher in the first sample, then gradually decrease over the PICU stay p= 0.021*.

In graph **a)** data are presented as median values, and graph **b)** is a graphic illustration of the data expressed as individual data points.

7.3.2.2 The effect of disease severity and medications on FC levels

This section of the analysis aims to explore whether disease severity, inotropic agents and the duration of antibiotic therapy influenced intestinal inflammation. The FC value measured in the first sample obtained from each child (between days 3-5) was included in the analysis. The regression analysis indicated that inotrope score was the only variable statistically related to FC (r= 0.293, p-value=0.018), (Table 7-9).

Table 7-9: Association between FC, medications and clinical variables

Model	R^2	Adjusted R^2	R	P value
FC [a]	**0.293**	**0.086**	**0.071**	
Inotrope score [b]			0.293	0.0.018*
Age [c]			-0.031	0.679
PIM2 score [c]			-0.061	0.634
Days of antibiotic exposure [c]			-0.128	0.314

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.2.3 The effect of feeding on FC levels

This section of the analysis aims to explore whether feeding in PICU influenced intestinal inflammation during PICU stays independently from age and disease severity. The FC value measured in the first sample obtained from each child (between days 3-5) was included in the analysis.

No evidence of statistical association was recorded between FC and the amount of energy and macronutrients received during the 24-hour period prior to the collection of faecal samples. In addition, the cumulative amount of nutrition received from admission to the time of the sample collection was not statistically associated with any variation in FC concentration (Table 7-10).

Table 7-10: Association between FC and the amount of nutrition received in PICU

Model	R^2	Adjusted R^2	R	P-value
FC [a]				
Age [c]			0.035	0.399
PIM2 score [c]			-0.031	0.412
% of energy received [c]			0.091	0.256
% of carbohydrate received [c]			-0.062	0.328
% of protein received [c]			-0.037	0.391
% of fat received [c]			-0.104	0.227
FC [a]				
Age [c]			0.035	0.399
PIM2 score [c]			-0.021	0.440
Cumulative energy received [c]			-0.101	0.232
Cumulative carbohydrate received [c]			-0.185	0.088
Cumulative protein received [c]			-0.184	0.089
Cumulative fat received [c]			-0.199	0.073

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.2.4 Association between intestinal and systemic inflammation

The relationship between intestinal and systemic inflammation was explored in 32 children, where serum samples were collected at the time of obtaining the faecal sample. Stepwise lineal regression analysis was performed to determine whether intestinal inflammation assessed by FC influenced systemic inflammation independently from age and disease severity. The regression models that included IL6 and TNFα as the outcome variables suggested that FC is statistically related to both IL6 and TNFα, but no evidence of statistical association was observed between FC and IL1β and IL-10 (Table 7-11).

Table 7-11: Association between FC and inflammatory cytokines

Model	R^2	Adjusted R^2	R	P-value
IL-6 [a]	**0.366**	**0.345**		
FC [b]			0.605	0.00*
Age [c]			-0.008	0.483
PIM2 score [c]			-0.132	0.235
TNFα [a]	**0.143**	**0.115**		
FC [b]			0.379	0.016*
Age [c]			-0.315	0.040
PIM2 score [c]			-0.001	0.997
IL-1β [a]				
FC [c]			0.324	0.035
Age [c]			0.156	0.197
PIM2 score [c]			-0.048	0.397
IL-10 [a]				
FC [c]			0.210	0.125
Age [c]			-0.014	0.947
PIM2 score [c]			-0.104	0.286

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.2.5 Association between FC and the incidence of sepsis in critically ill children

Sepsis was diagnosed according to the criteria described in the method chapter (Chapter Two). Sepsis diagnosis was documented in 25 out of 67 children. In 15 children the primary site of infection was the respiratory system, for 5 children in CNS, and 5 children in blood (see Table 7-12).

Table 7-12: FC levels in three groups of patients with sepsis

	Primary site of infection		
	Respiratory N=15	CNS N=5	Blood N=5
FC	374 (181 – 633)	361 (176 – 843)	1860 (320 – 2148)
Day of sample collection	5 (3 – 7)	3 (1 – 7)	4 (1 – 5)
Days of antibiotic exposure	5 (3 – 7)	3 (1 – 7)	4 (1 – 5)

Data are presented as median (IQR)

The first step of the analysis was comparing FC levels in children who had sepsis and those who did not. The results indicated that septic children had statistically higher FC levels compared to the non-septic group (p=0.03) (Figure 7.9). A logistic regression was then performed to assess the relationship between FC and sepsis (Table 7-10). The logistic regression model was statistically significant, χ2 (6) = 19.6, p-value= 0.003. The model explained 41.3% (Nagelkerke R2) of the variance in sepsis and correctly classified 81% of the cases. In this regression model, FC was the only variable statistically related to the incidence of sepsis. Increasing FC was associated with an increased likelihood of having sepsis. (Table 7-13).

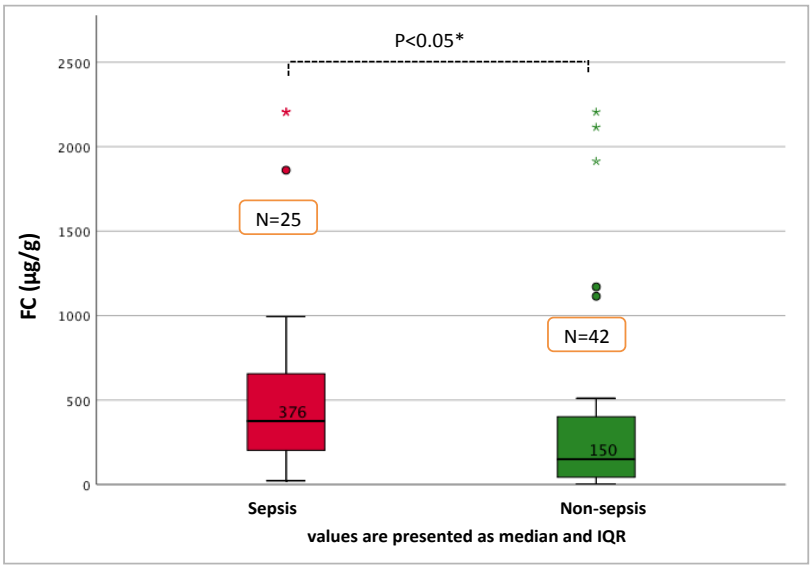

Figure 7.9 Difference in FC level between sepsis and non-sepsis patients

Figure 7.9 shows that septic patients had significantly higher FC levels compared to the non-septic group\

Table 7-13: Regression model to assess the relationship between FC and sepsis

Model	Model statistics χ2	Nagelkerke R2	Classification accuracy	B	Exp (B)	Significance
Sepsis [a]	χ2 (6) = 19.6, p-value = 0.003	0.413	81%			
FC [b]				1.226	3.407	0.045*
Highest TNFα [c]				2.5	12.18	0.079
Age [c]				0.037	1.037	0.947
PIM2 score [c]				0.149	1.161	0.851
Highest IL-6 [c]				1.037	2.819	0.105
Days of antibiotic exposure [c]				-0.057	0.944	0.701

a. **Dependent variable**
b. **Predictors**
c. **Insignificant variables**

7.3.2.6 Association between FC and clinical outcomes in critically ill children

The effect of intestinal inflammation assessed by FC (measured in the first sample) on clinical outcomes was also investigated. There was no evidence of statistical association between FC and length of ventilation; however, a weak negative association was recorded between FC and the duration of a PICU stay (Table 7-14). In children treated with inotrope (n=32), FC was negatively correlated with the duration of inotrope treatment (r=-0.437, p=0.006). In the regression model, FC accounted for 19% of the variation in the duration of inotrope treatment (Table 7-14).

Table 7-14: Association between FC and clinical outcomes

Model	R^2	Adjusted R^2	R	P-value
VFH at 30 days [a] (N=67)				
FC [c]			-0.024	0.423
Age [c]			-0.235	0.028
PIM2 score [c]			0.070	0.286
PICU-free days at 30 days [a] (N=67)	**0.95**	**0.54**		
FC [b]			-0.243	0.045*
Age [c]			-0.132	0.291
PIM2 score [c]			0.097	0.438
Hours free of Inotrope at 30 days [a] (N=32)	**0.191**	**0.164**		
FC [b]			-0.437	0.006*
Age [c]			-0.234	0.099
PIM2 score [c]			0.176	0.168

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.3.3 Relationship between serum citrulline and FC in critically ill children

In a subset of 20 children, the association between serum citrulline and FC was investigated where concomitant blood and faecal samples were available. The serum levels of citrulline and cytokines were paired to levels of FC measured in the nearest faecal sample obtained. The faecal samples were collected within a median of 3 days of the blood sampling. In this small cohort no evidence of statistical association was recorded between FC and citrulline (r=0.170, p=0.500). The regression analysis indicated that TNFα and IL-6 were the only variables statistically related to citrulline (Table 7-15).

Table 7-15: Association between FC and serum citrulline

Model	R^2	Adjusted R^2	R	P-value
Citrulline [a]	**0.545**	**0.492**		
IL-6 [b]			-0.592	0.008*
TNFα [b]			-0.548	0.015*
FC [c]			-0.170	0.500
Age [c]			0.170	0.510
PIM2 score [c]			-0.095	0.706

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

7.4 Discussion

This study showed that critically ill children had decreased levels of citrulline, and increased FC concentration, compared to age-matched healthy controls. In addition, decreased citrulline concentrations and increased FC levels were associated with elevation in pro-inflammatory cytokines levels and longer of PICU stay. In a small cohort of 20 critically ill children, where both FC and citrulline were measured, no evidence of statistical correlation was recorded between the two biomarkers.

The findings of this study regarding reduced levels of serum citrulline among the critically ill group are consistent with previously published research in critically ill adult population. Piton et al. (2010) indicated that, in critically ill adults, low plasma citrulline at 24 hours of ICU admission was an independent factor of mortality, and could be a marker of acute intestinal failure. A more recent study by Crenn et al. (2014) showed that levels of plasma citrulline were decreased at the onset of septic shock; Crenn et al. suggested that this was related to early acute intestinal dysfunction. Citrulline has been previously described as a marker of gut permeability in other disease states (Crenn et al., 2008; 2009; Hull et al., 2011). Evidence suggests that citrulline can be used as a predictive marker of enterocyte loss and villous atrophy in patients with HIV (Crenn et al., 2009). Reduced citrulline levels were also recorded in children with intestinal failure who developed catheter-related bloodstream infections (Hull et al., 2011). In addition, citrulline concentration was inversely correlated with highest endotoxin level in post-cardiac arrest patients (Grimaldi et al., 2013). These findings suggest a potential role of citrulline as a marker of impaired intestinal barrier integrity. It is important to point out that citrulline levels should be interpreted carefully, as they might be affected by factors other than compromised gut integrity. The deterioration of renal function, for instance, can potentially increase levels of circulating citrulline, since it is metabolised into arginine in the proximal convoluted tubule (Van de Poll et al., 2004; Crenn et al., 2008). However, in the current study, none of the study participants had any renal problems.

In this study, a gradual increase in serum citrulline was recorded in the time course analysis. Increasing serum citrulline could be a sign of recovery. However, the citrulline levels were low in 8 patient samples obtained between days 8 and 10. Six out of these 8 patients had MOF, and their median (IQR) PIM2 score was 5 (1.7-7.2), indicating that sicker patients who were in PICU for prolonged period had lower serum citrulline levels. However, these patients also had low admission citrulline, and it remained low through the period of their PICU stay.

This study also recorded an increase in FC concentration among critically ill children compared to age-matched healthy controls. FC has been assessed before in paediatrics as marker of intestinal inflammation in necrotising enterocolitis and inflammatory bowel disease, and also to distinguish bacterial gastroenteritis from viral gastroenteritis (Josefsson et al., 2007; Kostakis et al., 2013; Duman et al., 2015). Therefore, increased levels of FC in critically ill children could be used as a proxy of intestinal inflammation and a marker of gut homeostasis. The interaction between the host and intestinal microbiome has been shown to be highly relevant to pathophysiology and outcomes of severe and critical illness (Dethlefsen et al., 2007). Epithelial inflammation during the course of critical illness may impact the integrity of the intestinal epithelial barrier (Lupp et al., 2007; Kohler et al., 2005; Morowitz et al., 2011). Damage to the intestinal mucosal lining in severe disease may lead to the translocation of bacteria or their fragments into the bloodstream, and may contribute to systemic inflammation, sepsis and multi-organ failure (Clark & Coopersmith, 2007; Deitch, 2012). This could explain the recorded increase in FC levels among the septic children in this study.

In this study, decreased levels of circulatory citrulline was associated with elevation in all measured inflammatory mediators. In addition, increased FC levels were associated with increase in IL-6 and TNF-α levels. Septic children appeared to have statistically higher levels of FC compared to the non-septic group. Furthermore, the regression analysis showed that FC was independently associated with the incidence of sepsis. There is much speculation of a cross-link between the disruption of gut homeostasis and the progression of systemic infection and inflammation. Given the extensive vascular and

lymphatic links between the intestines and other organs, it is possible that toxic mediators released from the intestine are transported through the lymphatic system or intestinal lumen, causing systemic damage (Mittal & Coopersmith, 2014; Latorre et al., 2015). The shift of intestinal microbiota to pathogenic species has been recorded before in systemic inflammatory response syndrome and septic neonates and infants; these findings may imply that systemic infections start in the gut (De Jong et al., 2016; Madan et al., 2012; Pereira et al., 2013; Latorre et al., 2015). Increased FC levels in septic children may suggest that FC is influenced by bacterial colonisation of the gut. Intestinal barrier dysfunction is thought to contribute to the pathophysiology of sepsis (Yoseph et al., 2016); this study, however, did not record an association between the level of serum citrulline at admission and sepsis diagnosis among the patient group. In addition, there was no statistical difference in serum citrulline between the septic and non-septic groups. These findings are contradictory to a previously published pilot study of critically ill adults, where a reduction in serum citrulline in septic, critically ill adults was observed (Crenn et al., 2014). However, their study took a univariate analytical approach and did not adequately account for confounding influences, such as disease severity and antibiotic therapy.

The current study also recorded a variation in citrulline and FC levels in septic patients based on the primary site of infection. Children who had sepsis as a result of blood infection appeared to have the lowest citrulline levels and highest FC concentration, compared to septic children with primary respiratory and CNS infections. In addition, children with CNS infections had the highest citrulline and lowest FC levels. The association between gut integrity biomarkers and the presence of bloodstream infection has been investigated in children and adults. Hull et al. (2011) have shown that children who developed catheter-related bloodstream infections had reduced circulatory citrulline levels. In addition, in successfully resuscitated patients, markers of intestinal injury such as urinary FABP and circulatory citrulline were associated with incidence of endotoxemia (Grimaldi et al., 2013). Plasma citrulline concentration was inversely correlated with highest endotoxin level, while urinary FABP was positively correlated with the levels of endotoxins (Grimaldi et al., 2013). However, no studies have

investigated the association between gut health biomarkers and sepsis diagnosis, and this is an area that requires further investigation in a larger cohort of children.

Nutritional intake did not appear to influence the marker of intestinal inflammation. However, in a small cohort, a statistical association was recorded between the cumulative amount of enteral fat received and citrulline levels. Previous studies have investigated the effect of feeding on FC levels in infants, and found that there is no difference in FC levels based on the type or volume of EN received (Moussa et al., 2016; Campeotto et al., 2007; Yang et al., 2008). Conversely, Li et al. (2014) found that FC was higher in breast-fed infants than formula-fed infants. The association between feeding and gut permeability has been established before. It has been indicated that breast-fed infants have statistically lower permeability than infants receiving minimal or no human milk (Taylor et al., 2009). In addition, in a study of critically ill adults, the late initiation of EN was associated with an increase in intestinal permeability (Kompan et al., 1999). In both studies, intestinal permeability was assessed by enteral lactulose and mannitol administration and urinary measurement. Although it has been suggested that citrulline concentration may reflect the small intestinal absorptive function in various conditions, the relationship between macronutrient absorption and citrulline concentration has not been evaluated in critically ill children (Papadia et al., 2007; Van Vliet et al., 2009; Fragkos & Forbes, 2018). Rhoads et al. (2005) have shown that serum citrulline correlates linearly with the percentage of enteral calories received in children with short bowel syndrome. They suggested that higher serum citrulline is associated with better tolerance of enteral feeds (Rhoads et al., 2005). The findings of the current study might similarly indicate that improvement in gut permeability, as assessed by citrulline levels, leads to better tolerance of enteral feeds.

In the current study, negative correlation was recorded between FC and hours free of inotrope ($r=-0.437$, $p=0.006$). Many studies speculated that disruption of gut homeostasis could drive multiple organ dysfunction syndrome (Clark & Coopersmith, 2007; De Jong et al., 2016; Mittal & Coopersmith, 2014). Gut dyshomeostasis could be contributory or indeed driving cardiac dysfunction to a large extent. Typpo et al. (2015)

have recorded abnormalities in the intestinal barrier biomarker (FABP 2) in children with congenital heart diseases. In turn, impaired cardiac function may aggravate gut hypoperfusion and visceral ischemia, resulting in initiation of an immune-inflammatory event (De Jong et al., 2016). In this study, higher inotrope score was associated with increased levels of FC and reduced citrulline concentration. Inotropic drugs, particularly alpha vasoconstrictor, may reduce perfusion to the mesenteric vessel, which induce mucosal inflammation (Van Haren et al., 2003; Sharma et al., 2003). Continuous infusion of low-dose vasopressin resulted in gastrointestinal hypoperfusion in patients with septic shock (Van Haren et al., 2003). However, the contribution of the gut–heart axis in the pathophysiology of organ dysfunction in critically ill children needs to be explored in further studies.

Generally FC is affected by the age of the subject; previous studies indicated that infants normally have higher FC compared to older children (Campeotto et al., 2004). In this study, among the healthy children group, FC was negatively correlated with age and weight, indicating that younger and smaller children had higher FC levels. However, no such correlation was recorded in critically ill children. These findings suggest that during critical illness there are factors stronger than age influencing FC levels.

7.5 Limitations

The data in this chapter were derived from two separate studies. Citrulline and FC were both measured only in 20 children, but the two biomarkers were not matched in time, and no evidence of statistical correlation was recorded between them. Although the sample size in the current study was sufficient to demonstrate statistical differences between critically ill and healthy children with regard to citrulline and FC concentrations, it may have been underpowered to detect a relationship between citrulline and FC in this small cohort. Therefore, a further in-depth investigation of the association between FC and citrulline needs to be explored in a larger cohort, where both biomarkers are measured at the same time. FC has been widely used as a valid marker of intestinal inflammation. In the absence of synchronised evaluation of intestinal injury, the validity of FC as a marker of intestinal inflammation in critical illness might be questioned. This is

particularly important to differentiate whether FC is raised due to the generalised state of inflammation, or as a result of intestinal injury. Finally, the limited size of the population included in the integrated analysis (N=20) precludes reliable analysis of clinical outcomes related to intestinal injury and mucosal inflammation. A larger prospective observational study is required to explore these concepts further.

7.6 Conclusion

In this study, nutritional intake did not appear to influence markers of intestinal injury or inflammation. However, it was suggested that the enhancement of gut homeostasis may lead to better tolerance of enteral feeds. Increased FC and reduced serum citrulline were associated with increased pro-inflammatory cytokine IL-6 and TNF-α, which indicates that there is a cross-link between gut injury and systemic inflammatory response. This study has suggested that gut injury may amplify the systemic inflammatory response and contribute to MOF, and potentially death. Future work should focus on examining the possible relationship between intestinal failure and organ dysfunction such as cardiac dysfunction.

8 Chapter Eight: Clinical and Nutritional Factors Affecting the Composition of Intestinal Microbiome in Critically Ill Children

8.1 Introduction

The previous chapters have shown that the ingested macronutrients influenced the inflammatory responses in critically ill children. In addition, the intensity of inflammatory responses was closely related to the degree of intestinal inflammation and homeostasis.

The human intestines host a large and complex population of resident microbes – the microbiome, also referred to as microbiota. These bacteria and their metabolic products have a complex synergistic relationship with the host (Clark & Coopersmith, 2007). Changes to the microbiome in response to environmental influences can affect overall health, and there are many examples of how the composition and diversity of the microbiome is associated with predisposition to a range of diseases, including obesity, heart disease, renal injury and asthma (Parekh et al., 2014; Kelly et al., 2016; Zhang et al., 2018; Noval Rivas et al., 2016).

The physiological effects of critical illness on the intestines include ischaemia, hypoxia and hypotension. These, along with the iatrogenic effects of the lack of enteral feeds, and the use of antibiotics and other drugs such as vasopressors, have a damaging effect on the intestinal microbiome and result in the depletion of commensal species (Krezalek et al., 2016; Ferrer et al., 2016). Loss of commensal microbes and overgrowth of potentially pathogenic and inflammatory bacteria may contribute to metabolic dysregulation, hospital-acquired infections, sepsis, and multi-organ dysfunction syndrome (Psichas et al., 2015; Tolhurst et al., 2012; Clark & Coopersmith, 2007; Pathan et al., 2011; Deitch, 2012). It is most likely that the restoration of intestinal homeostasis could improve recovery from critical illness and reduce morbidity, particularly in children

with severe disease (De Jonge et al., 2003; De Smet et al., 2009; Daneman et al., 2013; Collins & Gibson, 1999; De Souza et al., 2015).

In health, nutrition is an important factor promoting colonisation, maturation and stability of the intestinal microbiota (Zhang & Yang, 2016). The gut microbiota utilises ingested dietary components (carbohydrates, mainly resistance starch, proteins and lipids) and host-derived components including shed epithelial cells and mucus (Ramakrishna, 2013). Dietary components may promote the growth of opportunistic microbes, while other nutrients could endorse beneficial microbes (Brown et al., 2012). Dietary fibre is the primary energy source for most commensal species and, therefore, can directly impact their growth (Graf et al., 2015). In addition, fibre is the main substrate for microbial production of SCFAs, a major bacterial metabolite known to influence the host's health (Den Besten et al., 2013). The modulation of intestinal microbiota by fibres has received interest for its potential impact on overall health. The effect of fibre supplementation on gut microbiota has been widely investigated, with data showing that the main genus influenced include *Bifidobacteria* and *Lactobacilli*, as well as the *Faecalibacterium prausnitzii* and *Roseburia* species (Bouhnik et al., 1996; Vulevic et al., 2008*;* Ramirez-Farias et al., 2009; Benus et al., 2010; Sawicki et al., 2017).

It is clinically accepted that antibiotics are the main adverse exposure on gut microbiota during critical illness. Data from adult studies have shown massive depletion of beneficial *Clostridium* clusters (XIVa, IV) and increased pathogens associated with the receipt of antibiotics (Iapichino et al., 2008; Livanos et al., 2018). However, lack of nutrient availability in the colon due to PN or EN poor in non-digestible carbohydrates might potentiate the effect of antimicrobial drugs and intensify the intestinal dysbiosis (Schneider et al., 2000). A state of energy deficit might also have a profound effect on the gut microbiota and environment (Krajmalnik-Brown et al., 2012).

The primary aim of this chapter is to explore the changes in the intestinal microbiota of critically ill children compared to their age-matched healthy controls. The secondary aim is to investigate the effect of host factors on the microbial ecology of critically ill children; these include age, diagnosis, antibiotics and feeds.

8.2 Materials and Methods

The detailed methodology of samples handling, DNA extraction, bacterial 16S rRNA gene amplification and sequencing were described in Chapter Two, pages 56–58.

For the purposes of this study the patient group was stratified by age into three groups (based on the stages of dietary progression):

- 0-6 months, mainly milk fed,
- 6-12 months, weaning diet,
- >12 months, mainly solids diet.

Where comparisons were made between healthy and critically ill states, the analysis was restricted to samples obtained from an age-matched cohort of critically ill and healthy children.

Samples collected:

Faeces:

The first available faecal sample from the child's nappy was collected in a sterile container. A 250 mg stool sample was used to perform the DNA extraction and sequencing in order to identify intestinal microbial species.

Serum:

Serial measurements of serum samples were taken between days 1–7 post-admission for the analysis of inflammatory cytokines. The maximum values were used to indicate the intensity of the inflammatory response in critically ill children.

Nutritional information:

To investigate the relationship between nutritional intake and the gut microbiome, the amount of energy and macronutrients delivered were calculated to the 24-hour period prior to each sample collection. The cumulative intake of energy and macronutrients from admission was also calculated to assess the long-term effect of feeding.

For the purposes of this study, the cut-off of underfeeding was defined as less than 65% of the calculated requirements, since the ASPEN guidelines target the delivery of at least two-thirds (approximately 65%) of the estimated requirements (Mehta et al., 2017). Overfeeding was considered when the intake exceeded 100% of the calculated needs.

8.2.1 Sample size calculation

Sample size calculation was performed using the statistical power analysis program G*Power 3 (Faul et al., 2007). The sample size was chosen to test the hypothesis that critical illness is associated with change in microbiome composition. This assumption was based on work by Rogers et al. (2016) involving the study of critically ill children, where a significant reduction was detected in the intestinal microbial diversity of critically ill children compared to controls. The mean Shannon index of Alpha diversity in critically ill children was 3, compared to 6.5 in controls. It was estimated that 39 children per group would be needed to detect the same difference between critically ill and healthy children, assuming a 5% significance level (2-sided) and 85% power.

8.2.2 Statistical analysis

Following the DNA extraction and amplification of bacterial DNA, the sequencing reads were binned into OTUs. OTUs is an operational definition used to classify groups of closely related 16s rDNA marker bacterial gene sequence. In other ward it is a way of clustering related sequences based on similarity to one another. The generation of OTUs was done on collaboration with Dr. Joseph Wanger as mentioned previously in the method chapter. I then used the generated OTU tables to perform the statistical analysis of the gut microbial profiles of critically ill and healthy children.

The normalised read counts for OTUs and functional categories were used for all statistical tests by the METAGENassist (Arndt et al., 2012) and Microbiome Analyst (Dhariwal et al., 2017) web-based programs. PCA is un-supervised method of analysis it takes the multivariate data and creates a latent variables to show how similar the

obtained data are. The PCA plots were generated first to visualise the taxonomic differences between the groups. To maximise separation in order to view important microbial characteristics of groups, PLS-DA models were used. PLS-DA models were assessed based on goodness of fit (R^2Y) and goodness of prediction (Q^2Y) metrics. In other ward Q^2Y value indicate how well the model predicts if child is critically ill/underfed or not. The models were validated with permutation multivariate analysis of variance (PERMANOVA). The PERMANOVA test was used to assess whether the observed differences (in multivariate space) were statistically significant. The PERMANOVA is the most commonly applied distance-based method to test the association of microbial composition with covariates of interest (Tang et al. 2016). The test was adjusted for participant groups (patients and controls), age groups, number of antibiotics, diagnosis and feeding status (Anderson & Walsh, 2013).

T-test was used to detect whether species statistically varied between the compared groups, and a false Discovery Rate (FDR) value of <0.05 was considered statistically significant. The Alpha diversity (the diversity within sample) determined what species and how many different types of species were present within one the sample. Alpha diversity was assessed in both patient and control samples, using the Shannon index, as it was shown to be the most reliable index to characterise microbial species diversity (He et al., 2013). T-test and ANOVA were used to compare diversity between the groups. Regression analysis was performed to assess the relationships between clinical outcomes and the proportional abundance of microbial phyla.

8.3 Results

A total of 71 critically ill children (41 males) and 44 healthy children (19 males) were enrolled in this study. For most children, a single faecal sample was collected. Nineteen children had a second sample collected during their PICU stay. The number of samples collected from each age group is shown in Table 8-1. Faecal samples were collected between day 1 and day 9 post-PICU admission. The demographics of patients and controls are shown in Table 8-2, along with the distribution of categories of primary diagnosis among patients. Amongst the patient cohort, the hospital mortality rate was 2.8% (2 out of 71 children).

Table 8-1: The number of samples collected from critically ill and healthy children

	Critically ill children		**Healthy children**	
	N of samples	**Median number of samples per patient**	**N of samples**	**Median number of samples per patient**
All children	90	1 (1-1)	44	1 (1-1)
0-6 month	29	1 (1-2)	0	0
6-12 months	11	1 (1-2)	0	0
> 12 months	50	1 (1-1)	44	1 (1-1)

Data presented as median (IQR)

Table 8-2: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometrics	**Critically ill children**			**Healthy children**
	0-6 months (N=21)	**6-12 months (N= 8)**	**>12 months (N=42)**	**> 12 months (N=44)**
Age (years)	0.1 (0.1 – 0.2)	0.7 (0.6 – 0.9)	4.1 (2.2– 8.3)*	4.8 (3.3 – 6.2)*
Weight (kg)	3.4 (3 – 4.7)	8.8 (4.2 – 10)	15.5 (12 – 24.5)	19.6 (16 – 23)
Height (cm)	51 (48 – 54.75)	73.5 (66 – 78)	101 (88.5 – 114)	110 (98 – 117)
Weight for age Z-score	-0.1.8 (-2.58 – 0.84)	-0.63 (-5.4 – -0.29)	-0.01 (-1.1 – 0.79)	0.32 (-0.06 – 1.05)
Height for age Z-score	-0.17 (-2.76– 0.83)	-0.03 (-1.2 – 1.8)	-0.07 (-2.43 – 0.76)	0.67 (-0.13 – 1.1)
Weight height Z-score	-1.2 (-1.9 – 0.31)	-0.77 (-2.8 – -0.70)	0.15 (-1.23 – 1.39)	0.65(-0.22 – 2.03)
Disease severity				
PIM2 score	1.2 (0.75 – 6.1)	3.6 (3.1 – 8.9)	4.83 (2.8 – 13.7)	
Inotrope score	0 (0 – 5)	0.0 (0.0 – 0.0)	0.0 (0.0 – 29.5)	
Maximum lactate	1.9 (1.47 – 2.6)	1.2 (0.8 – 1.3)	2 (0.975 – 3.3)	
Maximum CRP	55.5 (38.7 – 186.5)	66 (29.5 – 106)	151 (53.7 – 264)	
VFH at 30 days	621 (582 – 6536)	546 (480 – 600)	628 (532 – 657)	
Inotrope free hours at 30 days	720 (703 – 720)	720 (540 – 720)	719 (681 – 720)	
PICU -free days at 30 days	23 (17 – 25)	19 (19 – 23)	24 (17 – 25)	
Primary admission diagnosis	**N (%)**			
MOF	22 (31%)			
Respiratory Failure	35 (49%)			
CNS Disorders	11 (16%)			
Surgical Disorders	3 (4%)			

Data presented as median (IQR)

*** Age-matched population, p>0.05**

- VFH at 30 days: ventilation-free hours at 30 days
- PICU-free days at 30 days: days free of Paediatric intensive care at 30 days

8.3.1 Exploring the changes in the intestinal bacterial profiles of critically ill children compared to healthy controls

8.3.1.1 The global intestinal microbial profiles of the patients and control samples

The first step of the analysis was to compare the global gut microbiome profiles of an age-matched cohort of healthy and critically ill children. The PCA plot showed clear separation of samples obtained from critically ill and healthy children, reflecting systematic differences in the microbial profile between the two groups, [PERMANOVA] F-value: 7.8227, p-value < 0.001. Model statistics for the supervised analysis (PLS-DA) were $R^2Y = 0.78$ and $Q^2Y = 0.67$. The samples collected from healthy children were more tightly clustered than those obtained from the critically ill group (Figure 8.1).

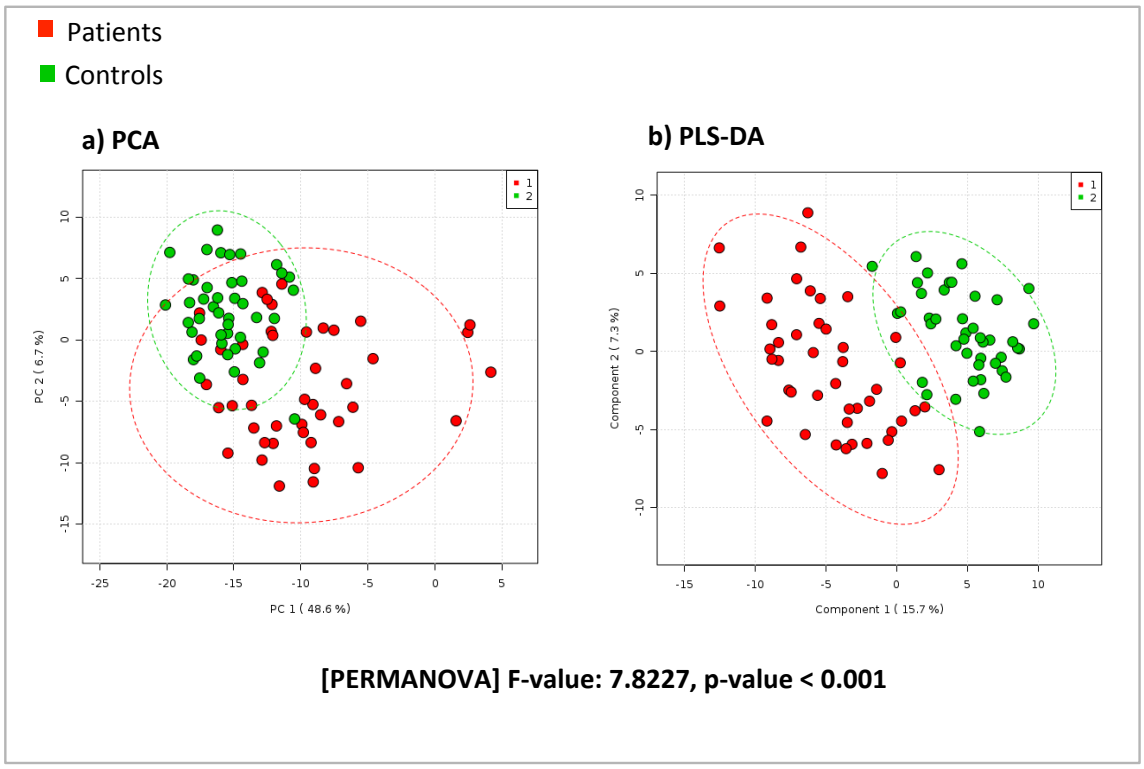

Figure 8.1 PCA and PLSDA plots to compare faecal microbial profiles in age-matched critically ill children and healthy controls

Figure 8.1 shows clear separation of samples obtained from critically ill and healthy children, reflecting systematic differences in the intestinal microbial profile between the two groups.

8.3.1.2 Assessment of the diversity of intestinal ecology in patient and control samples

The results showed that critically ill children had statistically reduced microbial Alpha diversity from a median (IQR) value of 2.3 (2 – 2.5) in healthy children to 1.7 (1.2 – 2.2) in the critically ill group (p <0.00001) (Figure 8.2).

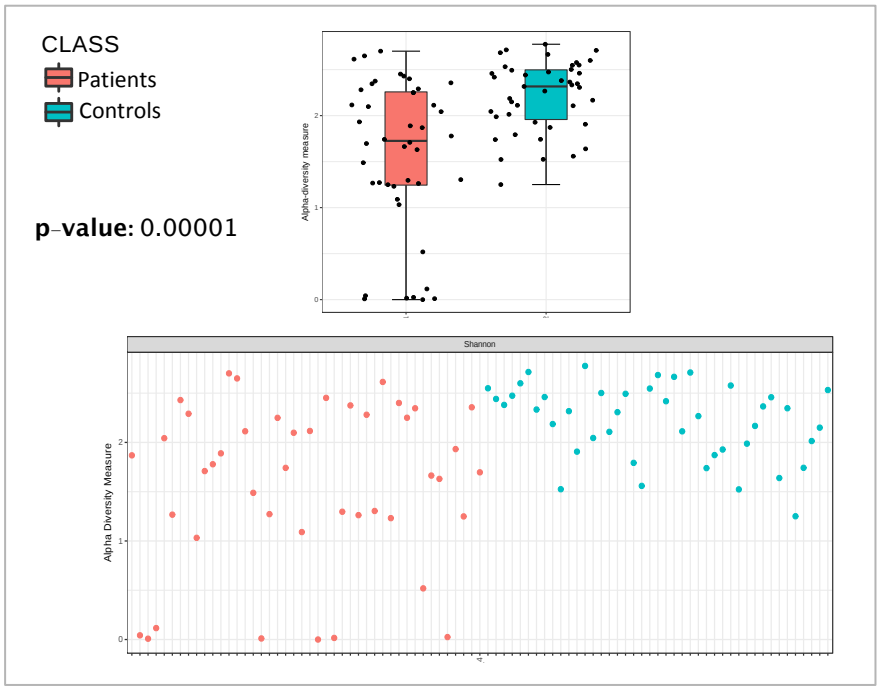

Figure 8.2 Variation in Alpha diversity between critically ill children and healthy controls

Figure 8.2 shows that healthy children had statistically higher microbial diversity compared to the age-matched critically ill children group.

8.3.1.3 Comparing the microbial ecology of patient and control samples

8.3.1.3.1 Phylum level

This section details how I compared the gut microbiome at the phylum level in critically ill children and healthy children, matching for age. The cluster analysis for the age-matched patient and control sample cohort demonstrated separation of samples by patient-control group (Figure 8.3). The absolute abundance of each phylum was

transferred to proportional abundance across the sample. *Bacteroidetes, Firmicute*s, *Actinobacteria* and *Proteobacteria* were the dominant phyla in the faecal samples of the two groups. An expansion in *proteobacteria* and reduction in *Firmicute*s abundance were recorded in critically ill children. The results also indicated that critically ill children had statistically higher relative abundance of *Proteobacteria, Actinobacteria* and lower relative abundance of *Firmicute*s compared to age-matched healthy children ($p < 0.001$) (Figure 8.4).

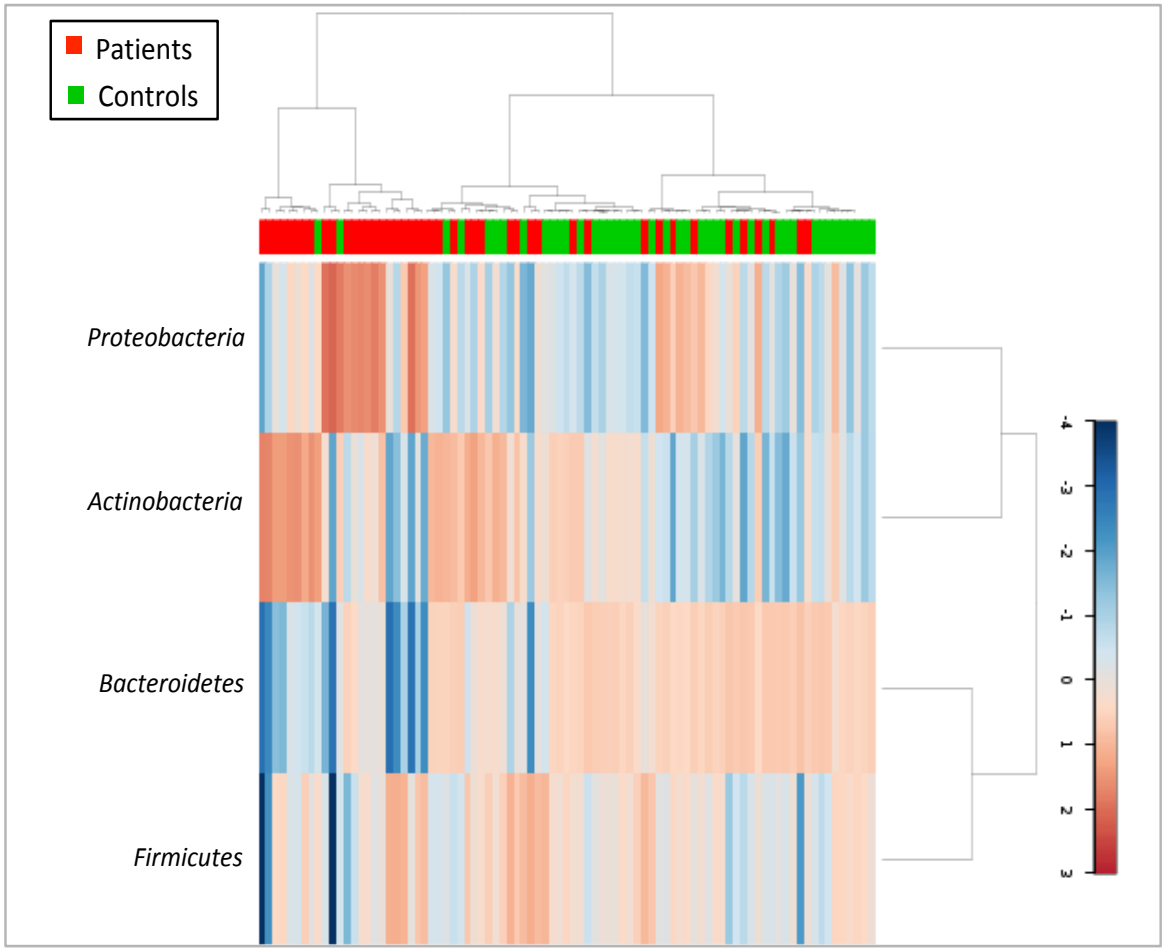

Figure 8.3 Hierarchical clustering with heatmap presentation of the gut microbiome at Phyla level using the Bray Curtis matrix and Ward method

Figure 8.3 shows a separation of samples obtained from the patient and control groups. The figure also shows that *proteobacteria* and *actinobacteria* phyla dominated in the patient group.

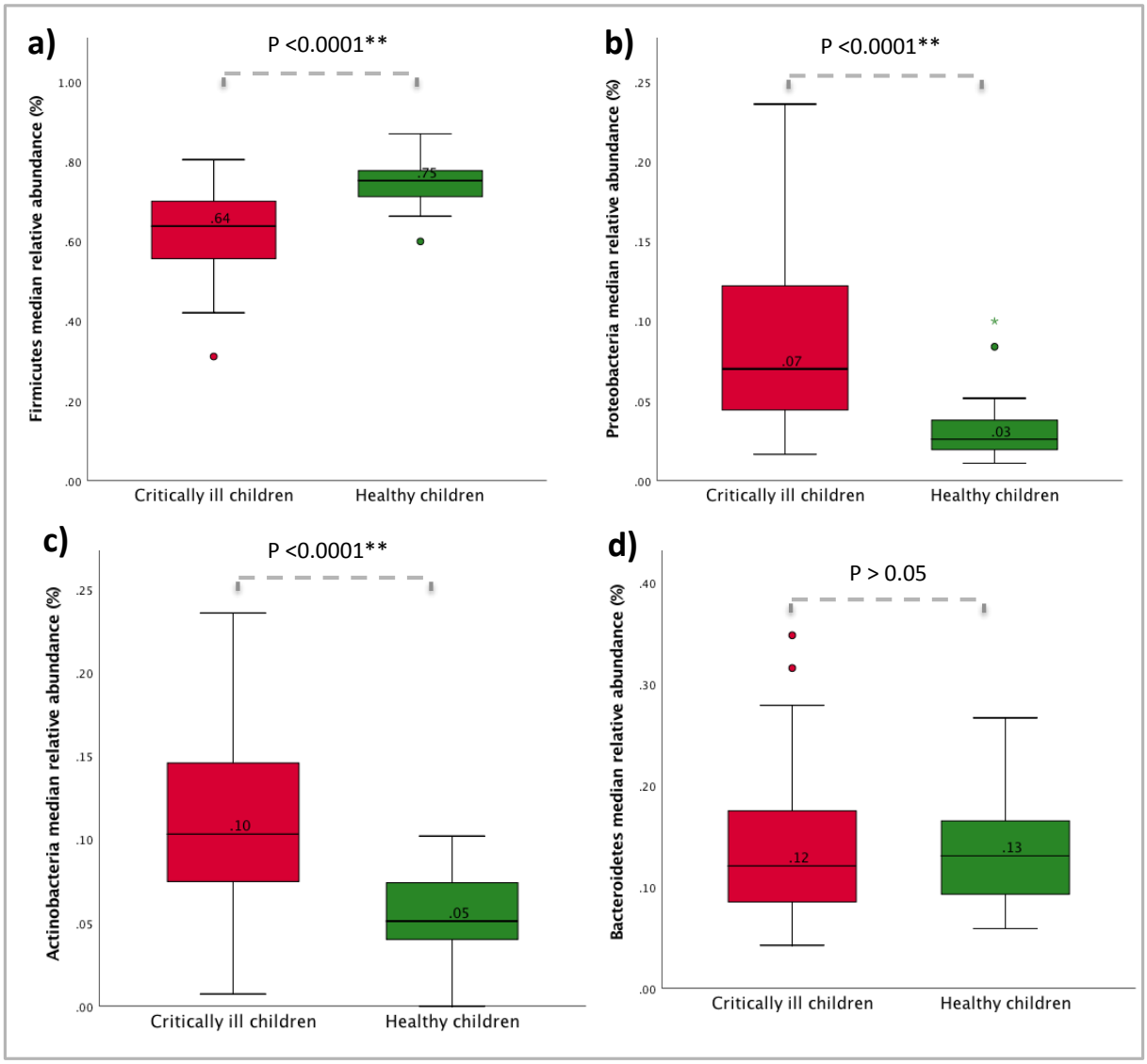

Figure 8.4 Comparison of main bacterial phyla in critically ill children and age-matched healthy controls

Figure 8.4 shows that critically ill children had statistically higher relative abundance of *Proteobacteria* and *Actinobacteria*, and lower relative abundance of *Firmicute*s compared to age-matched healthy children (p <0.001).

8.3.1.3.2 Species level

The cluster analysis for the age-matched patient and control sample cohort demonstrated a separation of samples by patient-control group at the species level (Figure 8.5). A further in-depth analysis showed that there was a major shift in the abundances of bacterial species between the two sample groups. T-test analysis showed that the patient group had statistically lower abundance of key known commensal species and an increased abundance of potential pathogens (Table 8-3) (Figure 8.6).

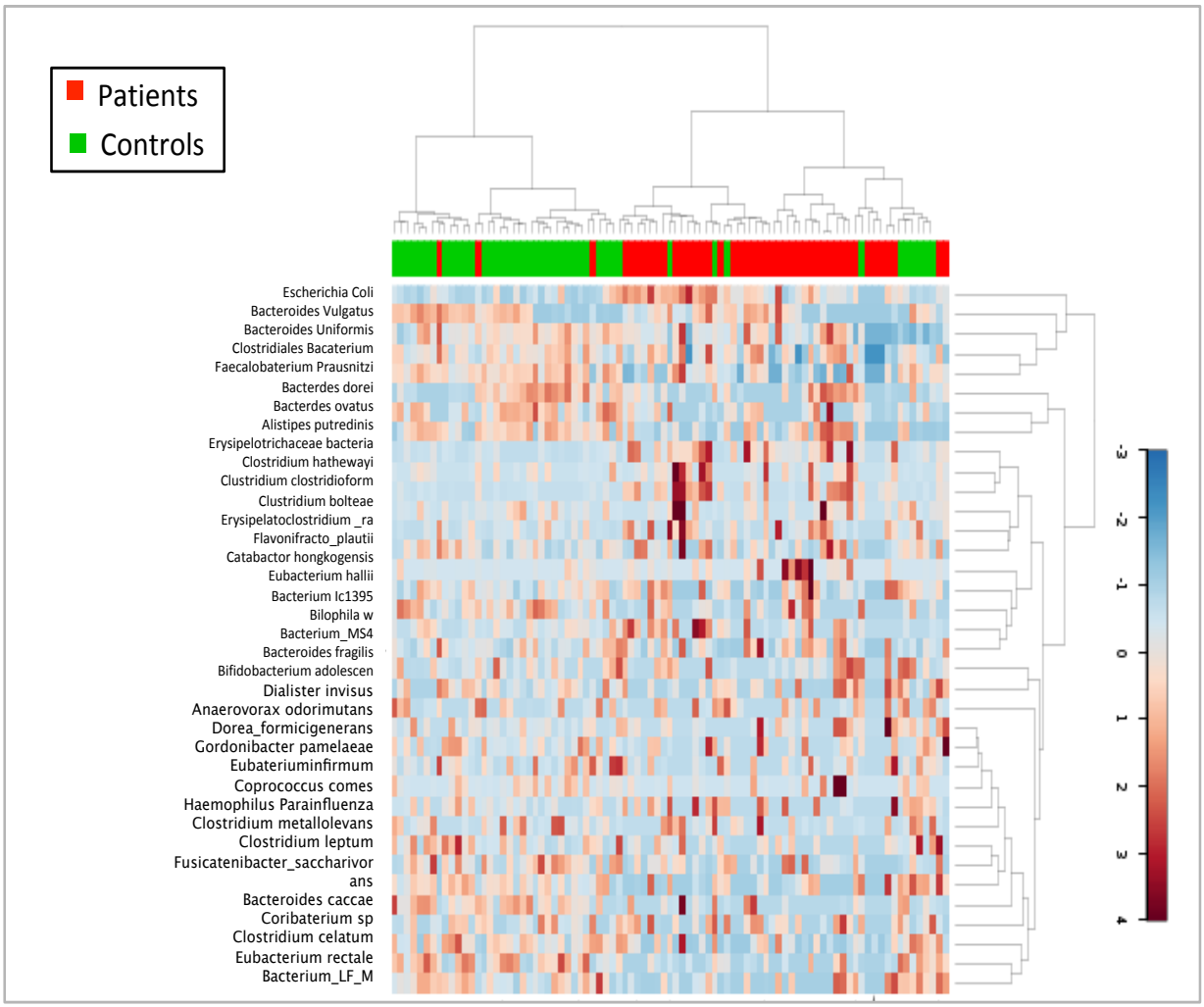

Figure 8.5 Hierarchical clustering and heatmap presentation of the gut microbiome at the species level using Bray Curtis matrix and Ward method

Figure 8.5 shows a separation of samples obtained from the patient and control groups. The figure also shows that *Escherichia coli* dominated in the patient group, while the *Eubacterium rectale* and *Faecalibacterium prausnitzi* species dominated in the control group.

Table 8-3: Identified microbial species distinguished between critically ill children and their age-matched controls

Microbial species	Mean proportional abundance (%)		FDR p-value
	Patients	Controls	
Eubacterium rectale	0.08	1.95	1.35E-06
Faecalibacterium prausnitzii	2.23	8.53	1.38E-05
Clostridium leptum	0.09	0.36	0.023177
Bacteroides caccae	0.27	0.75	0.003558
Bacteroides ovatus	0.93	2.82	0.00049311
Fusicatenibacter saccharivorans	0.08	0.51	0.00019671
Haemophilus parainfluenzae	0.09	0.35	0.040656
Clostridium celatum	0.33	1.63	0.0011364
Escherichia coli	10.47	2.10	3.34E-06
Clostridium clostridioforme	1.84	0.04	3.02E-05
Clostridium hathewayi	1.86	0.03	0.00029067
Erysipelotrichaceae bacterium	0.76	0.05	0.0060267
Catabacter hongkongensis	1.49	0.03	0.010139

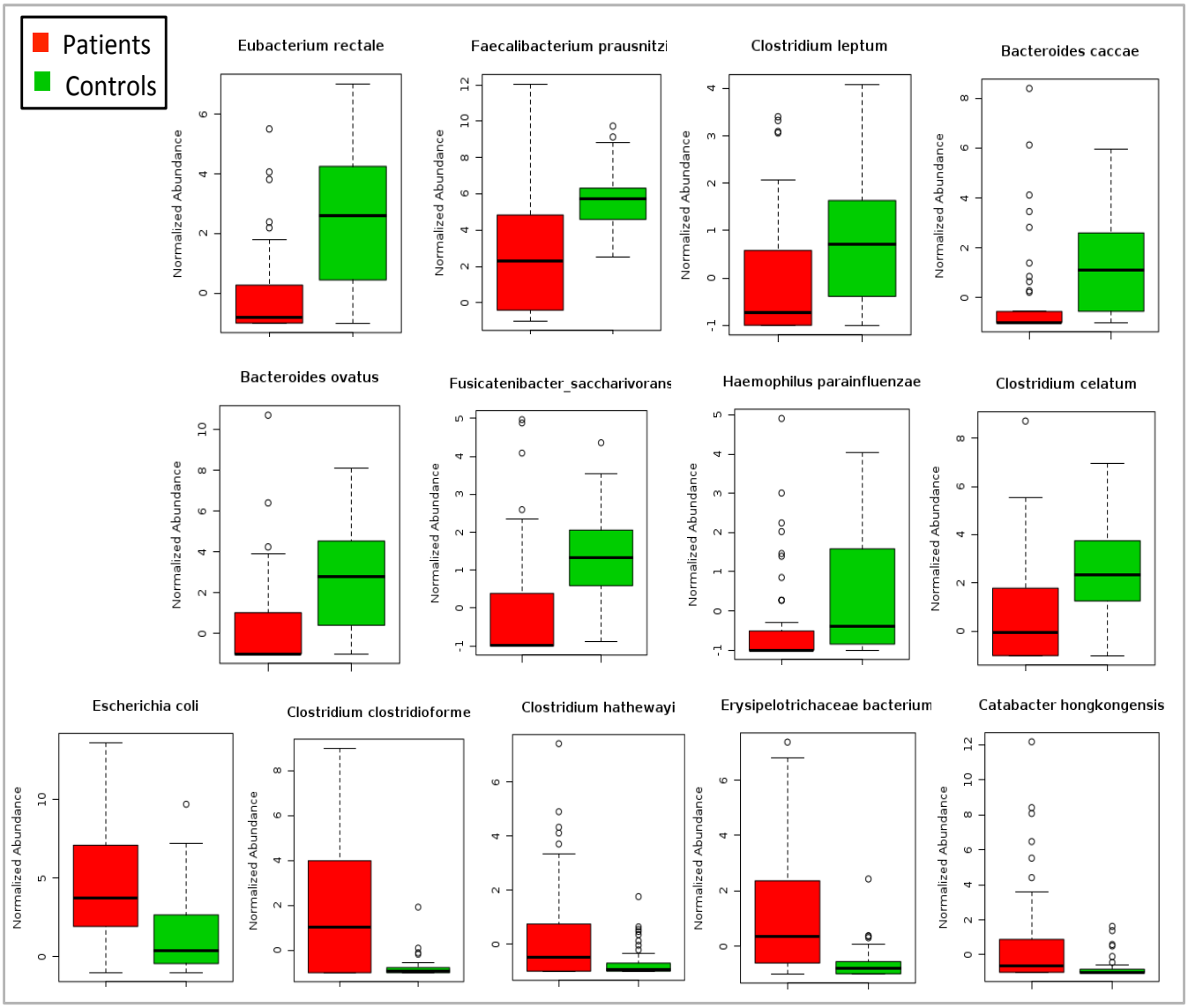

Figure 8.6 Key identified species differed between critically ill children and age-matched controls

Figure 8.6 indicates that critically ill children had statistically lower abundance of major commensal species such as *Eubacterium rectale* and *Faecalibacterium prausnitzi*, while they had higher abundance of potentially pathogenic bacteria, including *Escherichia coli*, compared to healthy children.

8.3.2 Investigating the effect of host factors on the microbial ecology of critically ill children

For the investigation of the effect of host factors, all samples collected from the patient group were included in the analysis.

8.3.2.1 Age

PCA and PLS-DA plots were used to examine the distribution of microbial profiles by age group (Figure 8.7). The plots showed the extent of separation between the different age groups. PERMANOVA analysis confirmed a statistically significant separation by age group F-value: 2.0222, p-value < 0.006. Model statistics for the supervised analysis (PLS-DA) were $R^2Y = 0.40$ and $Q^2Y = 0.20$.

The hierarchical cluster analysis demonstrated separation of samples by age group. As expected, the 6-12 months group was clustered between the younger and older groups. The hierarchical cluster analysis also showed that *Enterococcus faecalis* and *Escherichia coli* dominated in the younger group (Figure 8.8). Furthermore, the patient's age strongly influenced the diversity of the faecal microbial profile in this cohort. Younger children had statistically reduced microbial diversity compared to the older ones ($p < 0.001$) (Figure 8.9).

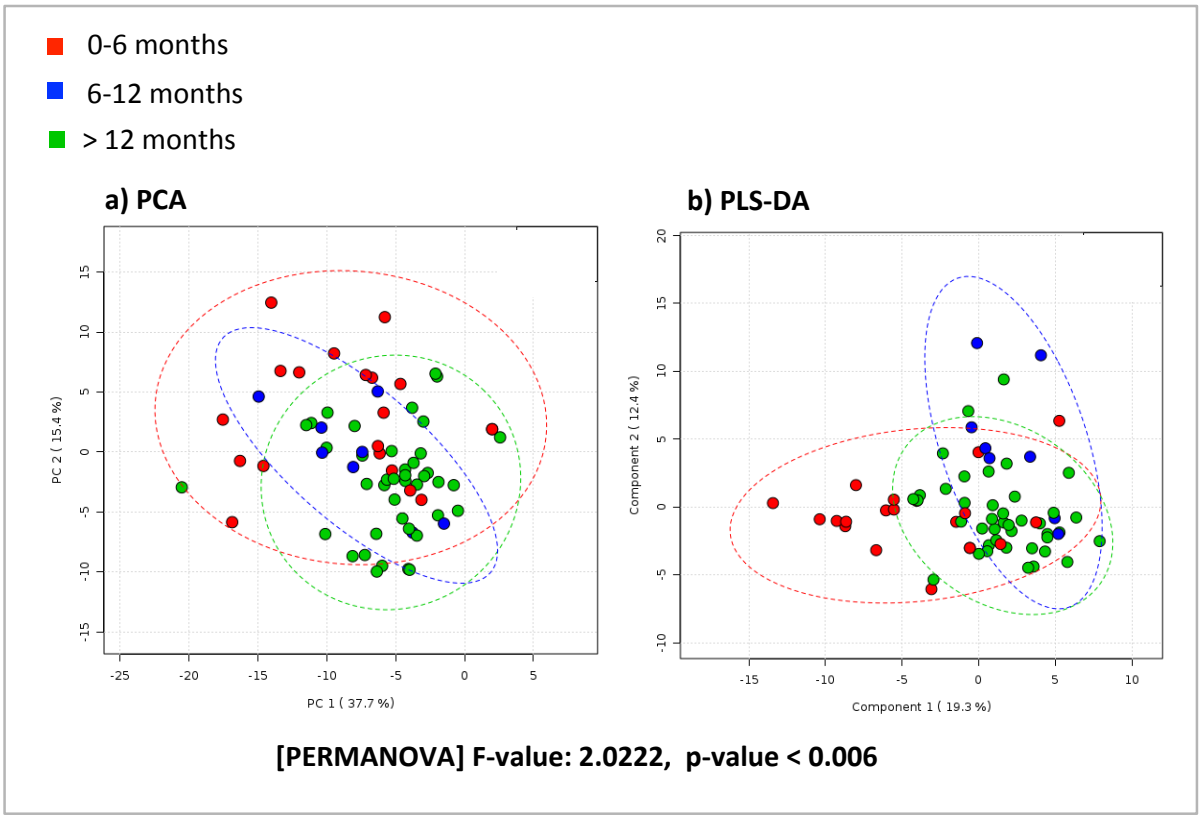

Figure 8.7 PCA and PLS-DA plots to compare faecal microbial profiles in different age groups of critically ill children

Figure 8.7 shows the extent of separation between different age groups of critically ill children, particularly in children below 6 months of age, who appear to have a distinctive microbial profile.

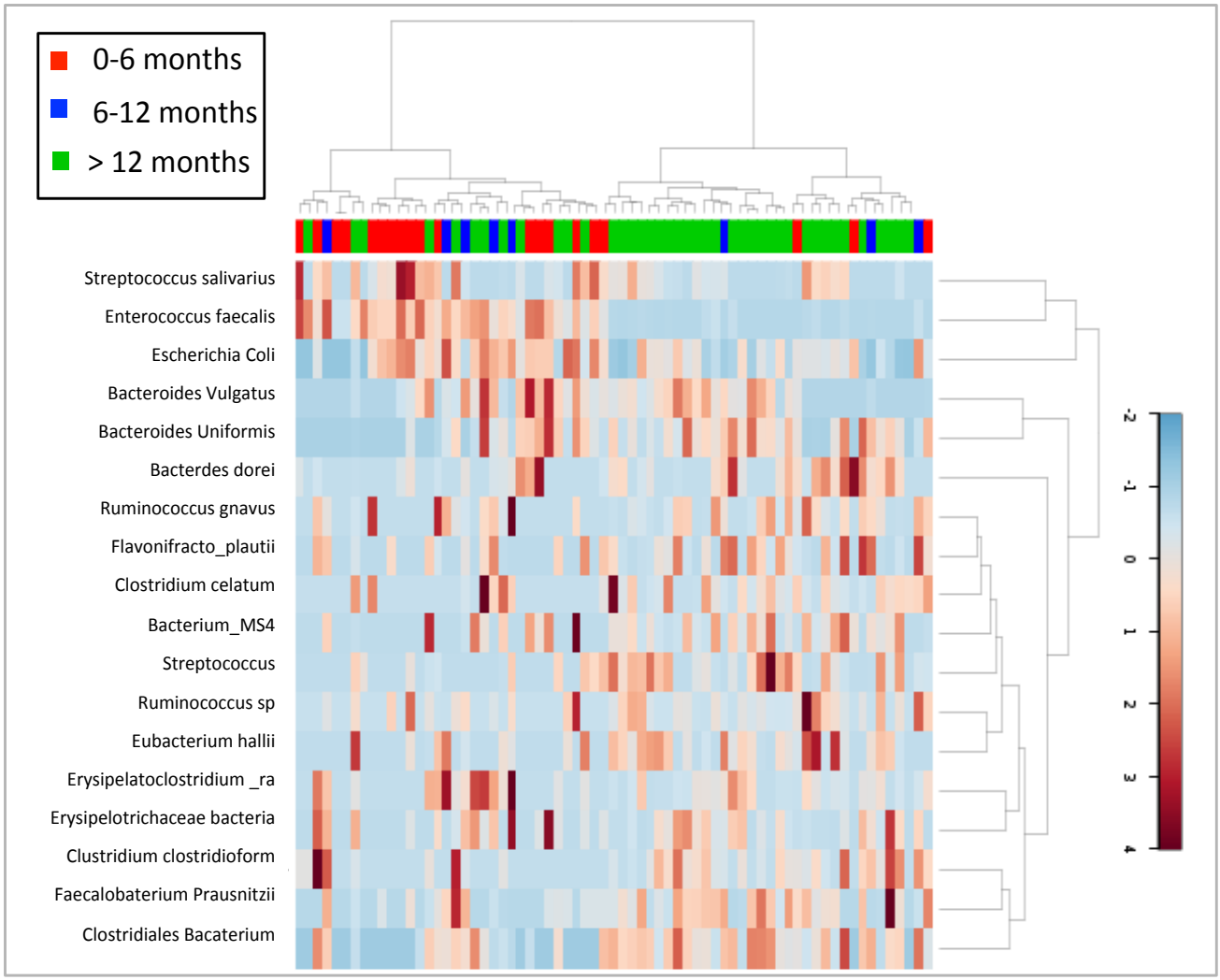

Figure 8.8 Hierarchical clustering and heatmap presentation of the gut microbiome at the species level based on age using Bray Curtis matrix and Ward method

Figure 8.8 shows a separation of samples obtained from patients > 1 year and those obtained from children < 1 year of age. However, there was an overlap between children aged 0-6 months and those aged 6-12 months. The figure also shows that *Enterococcus faecalis* and *Escherichia coli* dominated in the younger group.

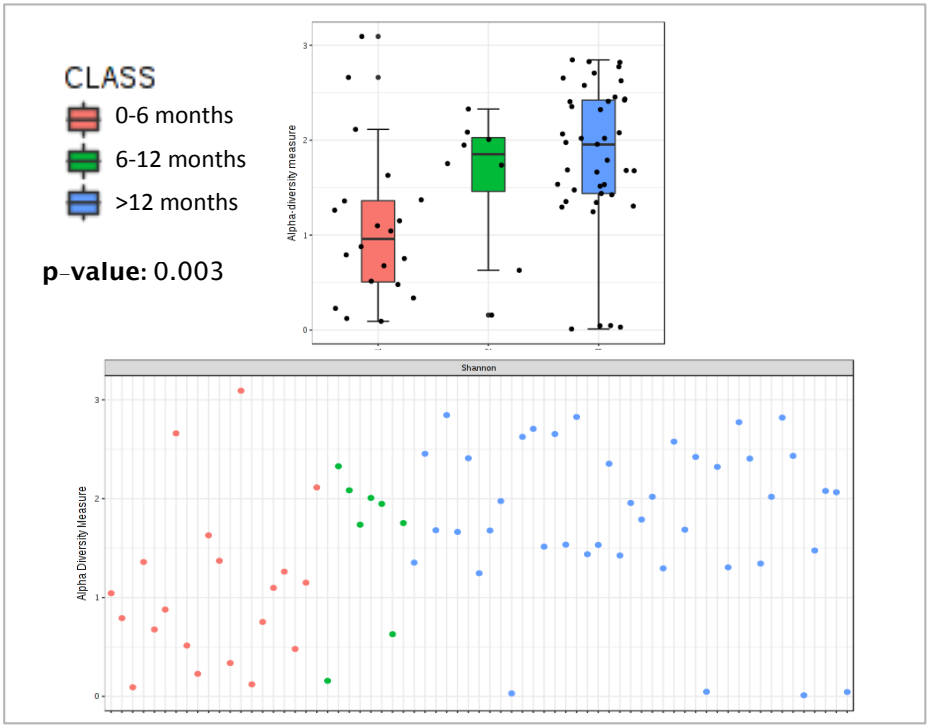

Figure 8.9 Variation in Alpha diversity among different age groups of critically ill children

Figure 8.9 shows that the microbial Alpha diversity varied among the different age groups of critically ill children; younger children had the lowest microbial diversity.

8.3.2.2 Primary diagnosis

PCA and PLS-DA plots were used to examine the distribution of microbial profiles by admission diagnosis (Figure 8.10). The faecal microbial profile did not show clustering by admission diagnosis when all groups were compared; hence they have similar bacterial profiles. The results of the [PERMANOVA] tests were not statistically significant, F-value: 1.0576, p-value < 0.333. Cross-validation of the supervised analysis (PLS-DA) was not viable, as the minimum number of samples required per group is four. The faecal microbial profile also did not show clustering by admission diagnosis when examining specific diagnostic groups compared to the remaining patients, and the results of the [PERMANOVA] tests were not statistically significant (Figure 8.10). Alpha diversity also did not vary between patients based on their admission diagnosis when all groups were compared, p-value: 0.28823. In addition, no statistical difference was recorded in Alpha diversity when specific diagnostic groups were compared to the remaining patients (MOF, p-value: 0.2396), (Respiratory, p-value: 0.076557), (CNS, p-value: 0.20226), (Surgery, p-value: 0.87481).

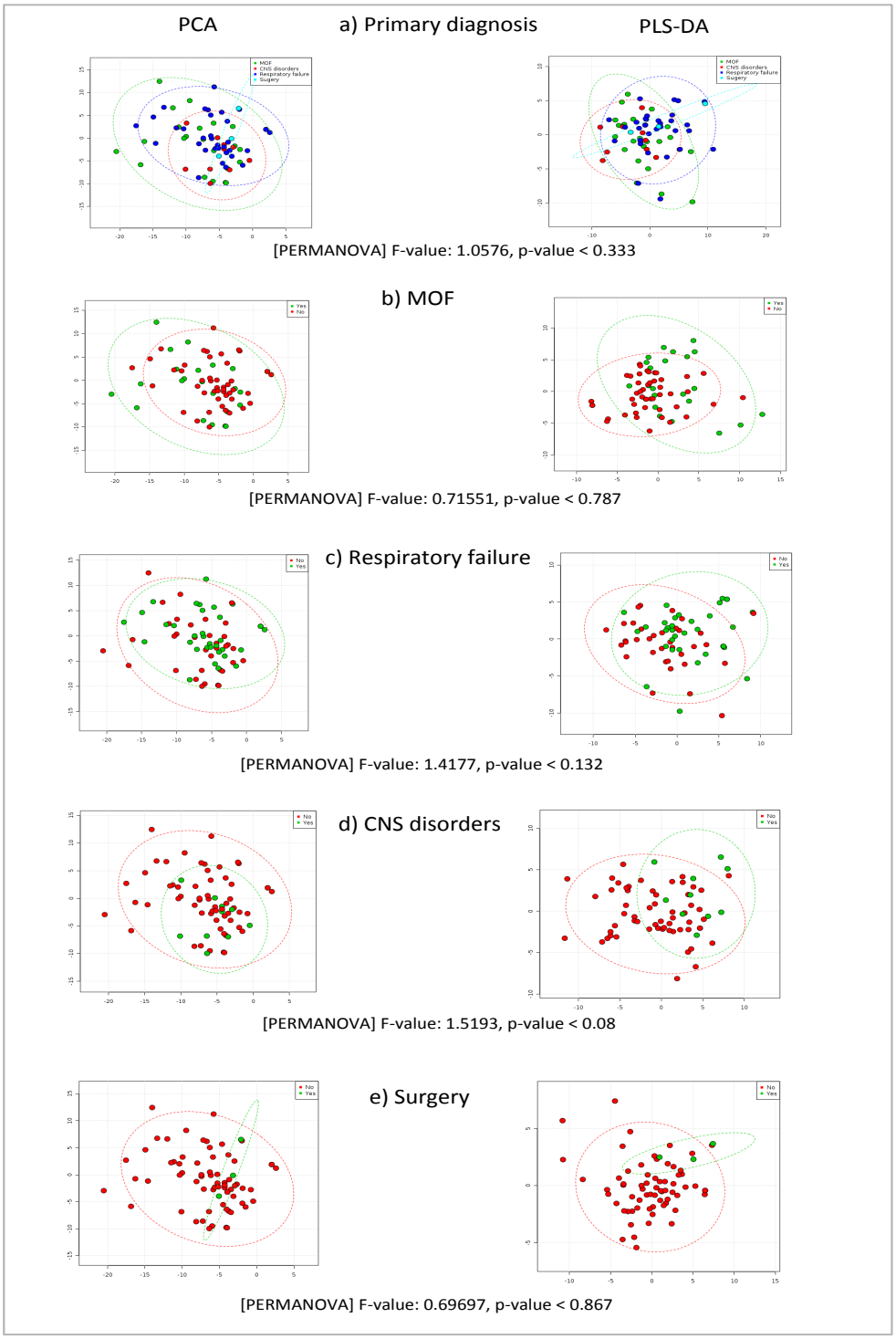

Figure 8.10 PCA and PLS-DA plots for primary admission diagnosis

Figure 8.10 does not show clustering of the patient's samples by admission diagnosis, hence they have similar bacterial profiles.

8.3.2.3 Antibiotic treatment

All patients received at least one broad-spectrum antibiotic therapy, and 59% (N=42) of them received more than one type of antibiotics (Table 8-4). The median (IQR) number of antibiotic classes received per patient was 2 (1-3). The most frequently used antibiotic classes among the current cohort were Cephalosporin (38%), Penicillin (22%), Macrolide (16%) and Aminoglycoside (12%). As the neonatal antibiotic prescription may differ due to birth-acquired infections, children were stratified by age to investigate whether antibiotic exposure is influenced by age (Figure 8.11). The results showed that the frequency of using these antibiotics did not vary statistically between the different age groups.

PCA and PLS-DA plots were used to examine the distribution of microbial profiles by the number of antibiotic classes received and days of antibiotic exposure (Figures 8.12-8.13). The faecal microbial profiles of this cohort did not show clustering based on the number of antimicrobial classes received, [PERMANOVA] F-value: 1.4058, p-value < 0.24, nor by the days of antibiotic exposure [PERMANOVA] F-value: 1.1168, p-value < 0.246. No significant features were identified in the supervised analysis (PLS-DA). In addition, the diversity of the faecal microbial profile was not influenced by the number of antibiotics received, p-value: 0.1582, or by the duration of antibiotic exposure, p-value: 0.48602.

Table 8-4: Types of antibiotic treatment provided to the patient group

Antibiotic	Class	N of patients	(%)
Ceftriaxone	Cephalosporin	36	28.3
Cefotaxime	Cephalosporin	11	8.7
Gentamycin	Aminoglycoside	13	10.2
Tobramycin	Aminoglycoside	1	0.8
Flucloxacillin	Penicillin	4	3.1
Piperacillin	Penicillin	3	2.4
CoAmoxiclav	Penicillin	8	6.3
Amoxicillin	Penicillin	7	5.5
Benzylpenicillin	Penicillin	6	4.7
Tazobactam	Penicillin	5	3.9
Clarithromycin	Macrolide	10	7.9
Erythromycin	Macrolide	4	3.1
Azithromycin	Macrolide	3	2.4
Ciprofloxacin	Fluoroquinolone	2	1.6
Vancomycin	Glycopeptide	3	2.4
Clindamycin	Lincosamide	8	6.3
Metronidazole	Nitroimidazole	2	1.6
Trimethoprim	Sulfonamides	1	0.8

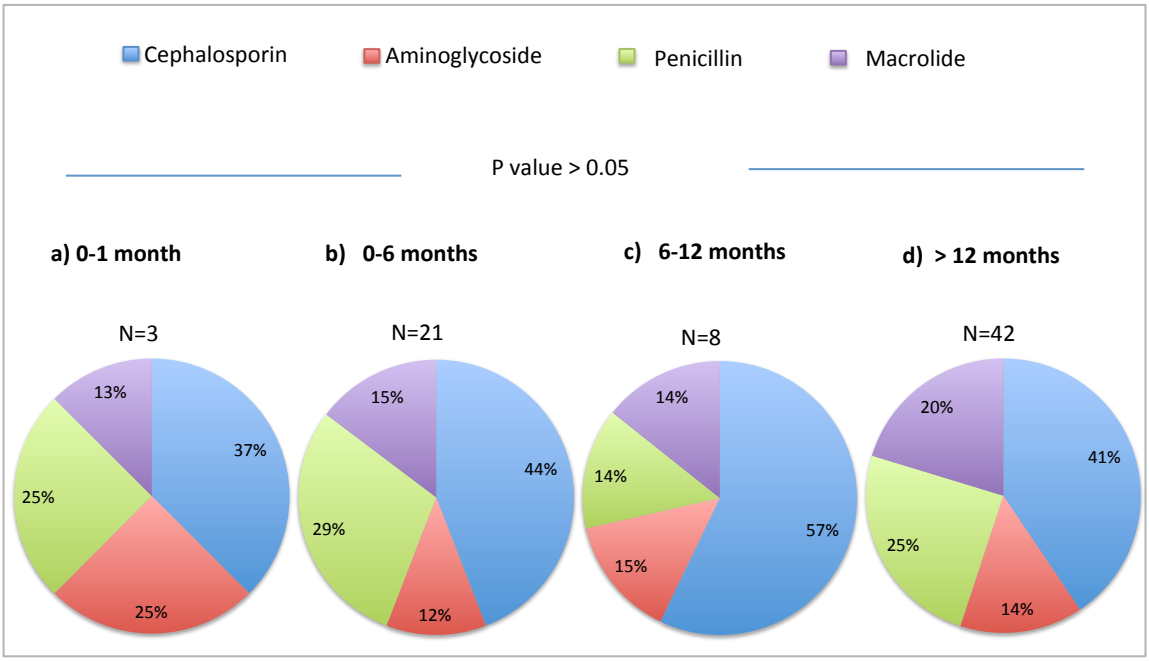

Figure 8.11 The frequency of using the major antibiotic classes in different age groups

Figure 8.11 indicates that the frequency of using Cephalosporin, Penicillin, Macrolide and Aminoglycoside did not vary statistically between different age groups.

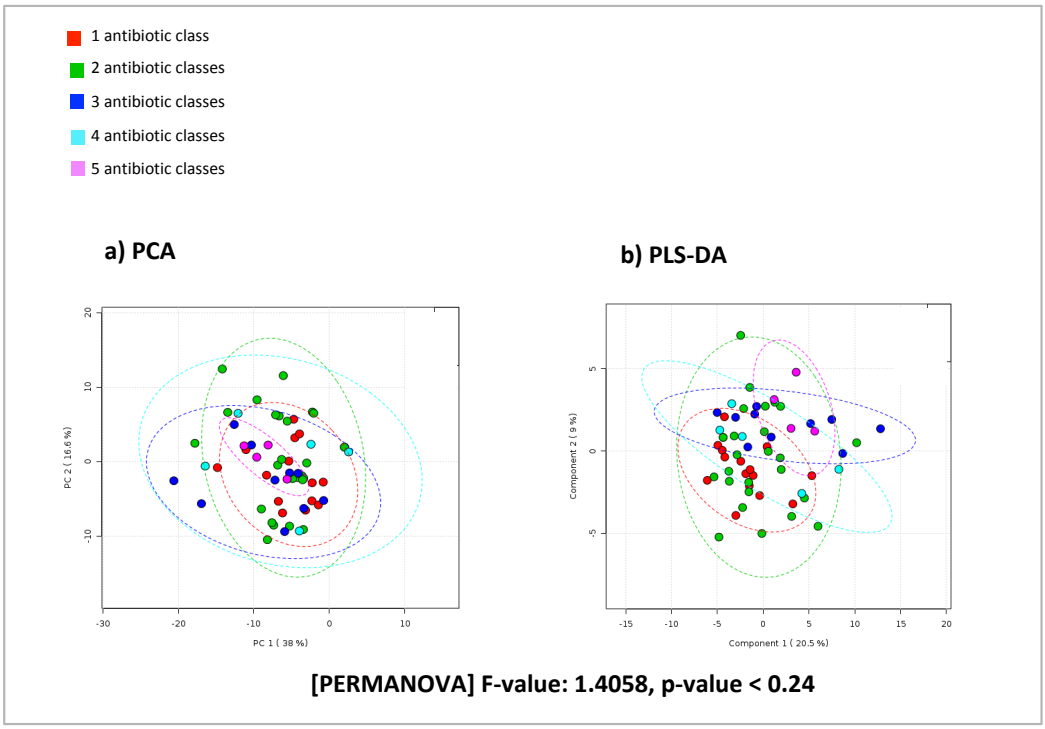

Figure 8.12 PCA and PLS-DA plots to compare faecal microbial profiles of critically ill children based on the number of antibiotic classes received at the time of sampling

Figure 8.12 shows an overlap of patient samples based on the number of antibiotics received.

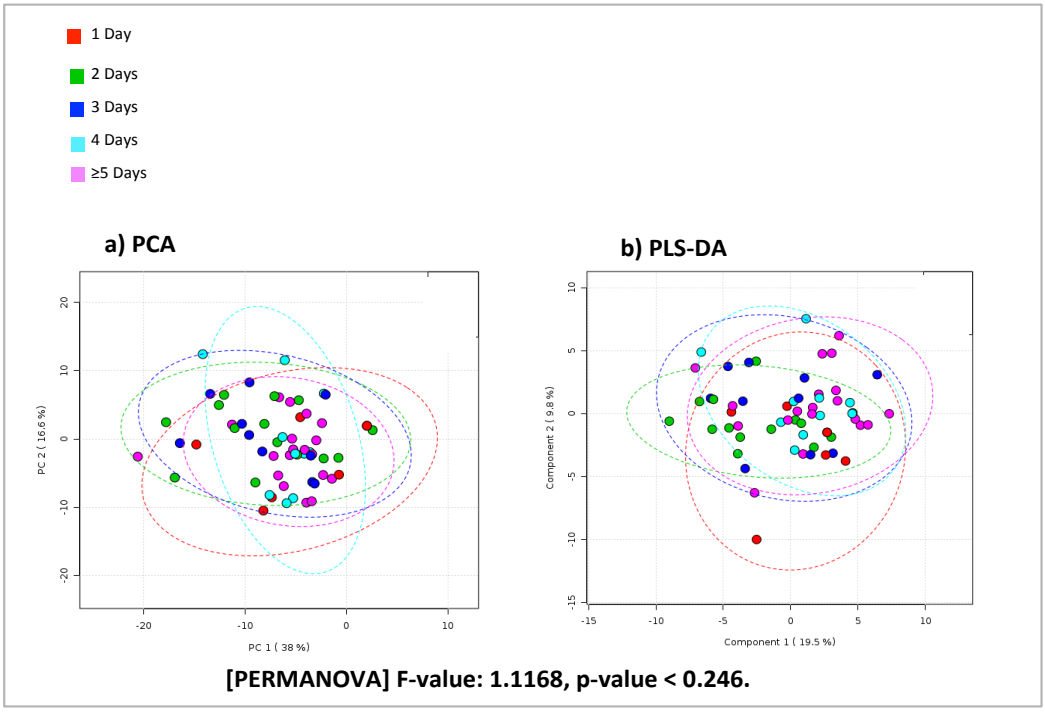

Figure 8.13 PCA and PLS-DA plots to compare faecal microbial profiles of critically ill children based on days of antibiotic exposure

Figure 8.13 shows an overlap of patient samples based on days of antibiotic exposure.

8.3.2.4 Nutrition and feeding practice in PICU

The intestinal microbial profiles were compared based on the time of commencing EN, dietary fibre received and feeding status. Dietary information was available for 43 children. The samples in each comparison group were age-matched.

1. **Time of commencing EN:**

EN was started at a median time of 9 hours post-PICU admission; however, 10 children did not receive any EN in the first 24 hours. The PCA and PLS-DA plots showed an overlap between samples in each group, [PERMANOVA] F-value: 0.75327, p-value < 0.791 (Figure 8.14). No significant feature was identified in the supervised analysis (PLS-DA). The Shannon index of Alpha diversity also did not vary significantly between patients based on the time of commencing EN, p-value: 0.78206.

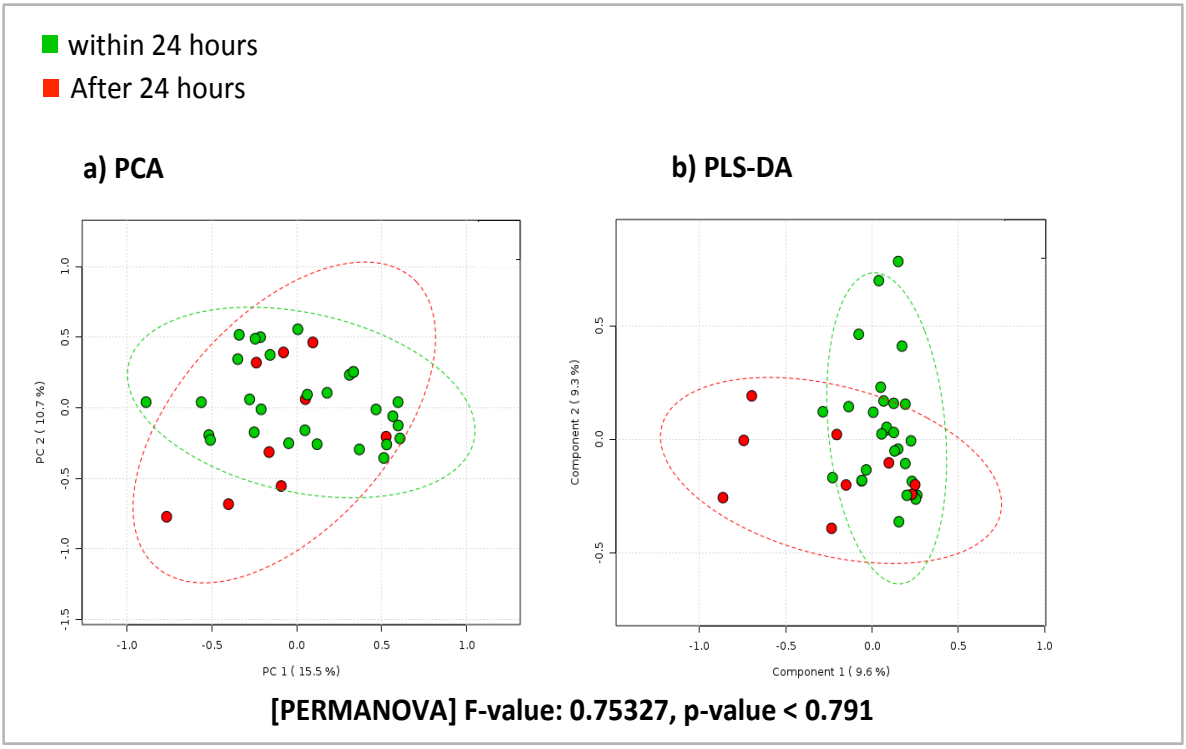

Figure 8.14 Comparison of the global intestinal microbial profiles based on the time of commencing EN

Figure 8.14 shows an overlap between samples based on time of EN.

2. Fibre-supplemented feeds:

The type of formulae provided was protocolised in the unit based on age. Most children < 1 year received fibre-supplemented formulae, and those > 1 year received standard paediatric formulae (Table 8-5).

Table 8-5: Number of children receiving fibre-supplemented formulae in each age group

Age group	N of children receiving fibre feeds	N of children receiving standard feeds
< 1 year	12	4
> 1 year	6	21

In both age groups, PCA and PLS-DA plots were used to examine the distribution of microbial profiles by the type of feed received. The plots showed an overlap between the samples based on the type of formulae received. The result of the [PERMANOVA] test was not statistically significant, and no significant feature was identified in the supervised analysis (PLS-DA) (Figure 8.15). In addition, Alpha diversity did not vary statistically between patients based on the type of EN formulae received in children < 1 year (p-value: 0.1033) nor in children > 1 year (p-value: 0.4841).

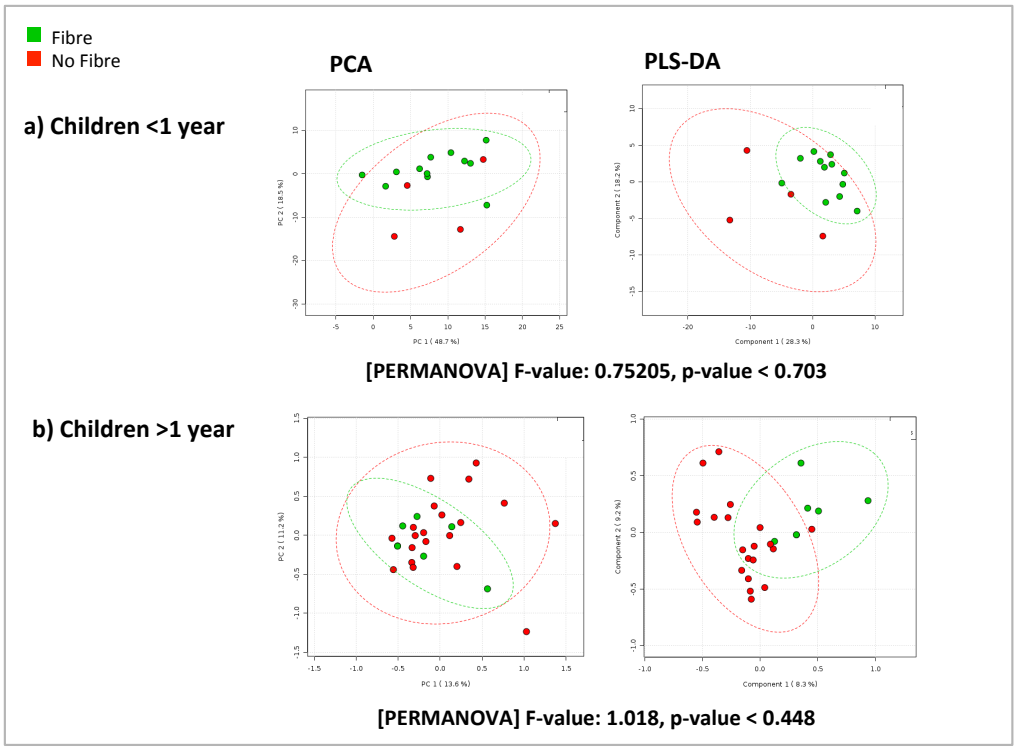

Figure 8.15 Comparison of global intestinal microbial profiles based on the type of formulae received

Figure 8.15 shows an overlap between samples based on the type of EN formulae received in both age groups.

3. Feeding status:

Both the immediate and cumulative effect of energy and macronutrient intake during the course of a PICU stay were investigated in this cohort of critically ill children. During the 24 hours prior to sample collection, under- and overfeeding were recorded among the patients. However, the cumulative amount of energy and macronutrients did not exceed requirements in any of the children. The results indicated that energy underfeeding during the 24 hours prior to sample collection was the only factor influencing the microbial composition in this cohort, [PERMANOVA] F-value: 1.7007, p-value < 0.041. Model statistics for the supervised analysis (PLS-DA) were $R^2Y = 0.57$ and $Q^2Y = 0.21$. However, neither the amount of macronutrients (carbohydrate, protein, fat) delivered in the 24 hours prior to sample collection nor the cumulative amounts affected the global microbial profiles in critically ill children [PERMANOVA] p-value > 0.05. In addition, no statistical difference in Alpha diversity measure was recorded between groups based on feeding status, p >0.05 (Figures 8.16- 8.19).

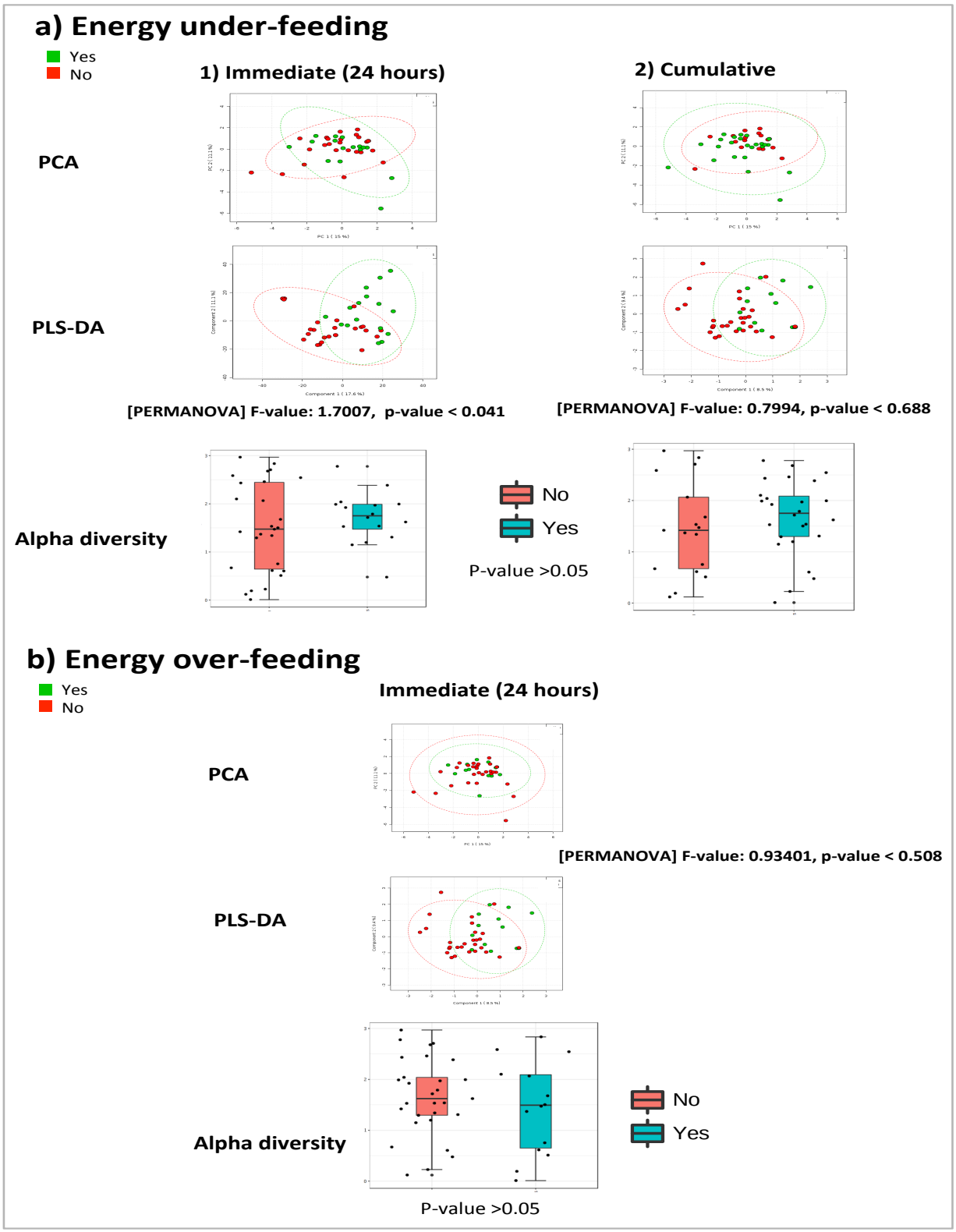

Figure 8.16 Comparison of the global intestinal microbial profiles and diversity based on the amount of enteral energy received

The above figure shows that energy underfeeding during the 24 hours prior to sample collection was the only factor influencing the microbial composition in this cohort. The figure also indicates that Alpha diversity was not affected by energy feeding state.

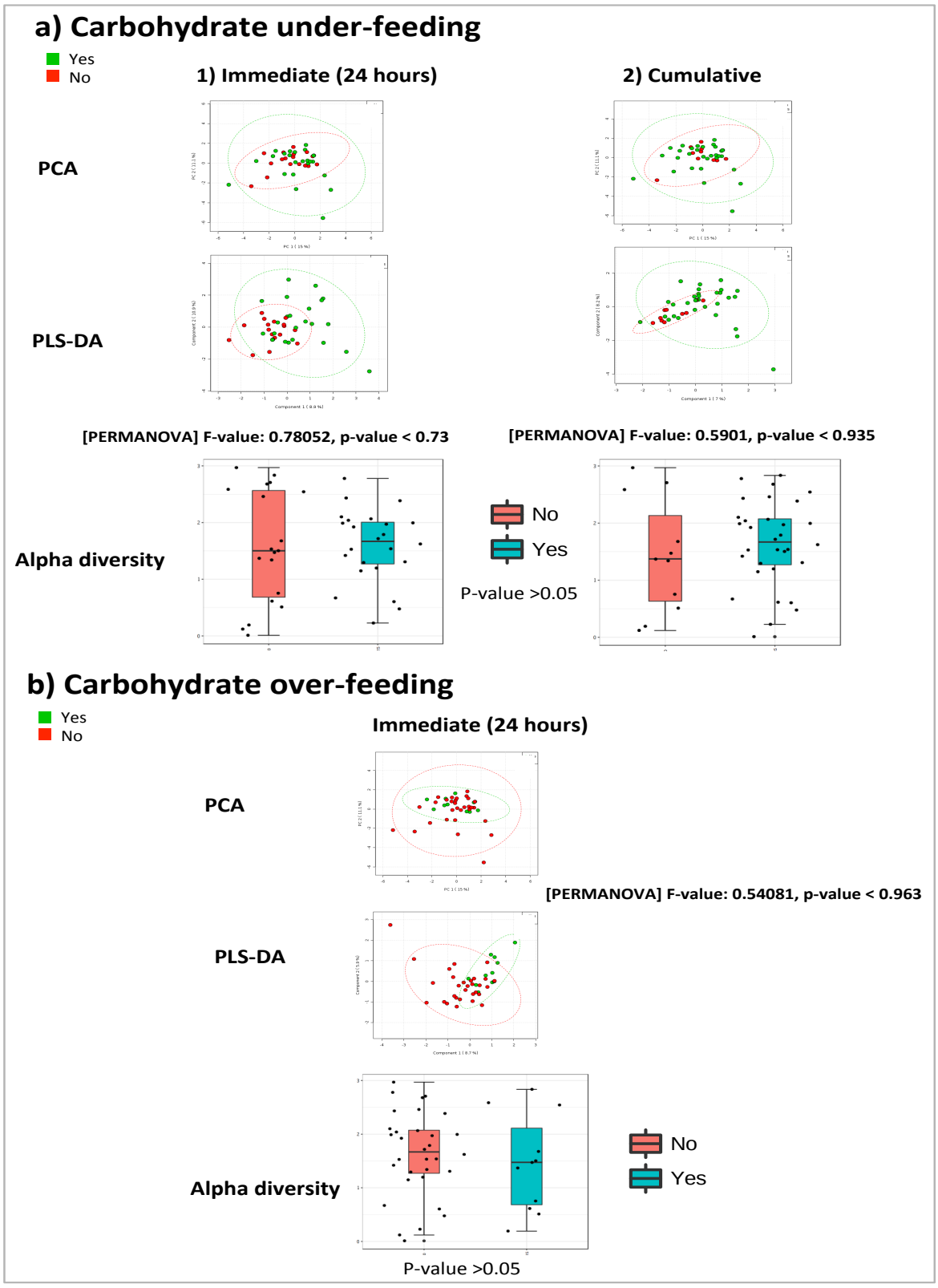

Figure 8.17 Comparison of global intestinal microbial profiles and diversity based on the amount of enteral carbohydrate received

PCA plots show an overlap between the samples based on carbohydrate feeding status. The figure also indicates that Alpha diversity was not affected by carbohydrate feeding state.

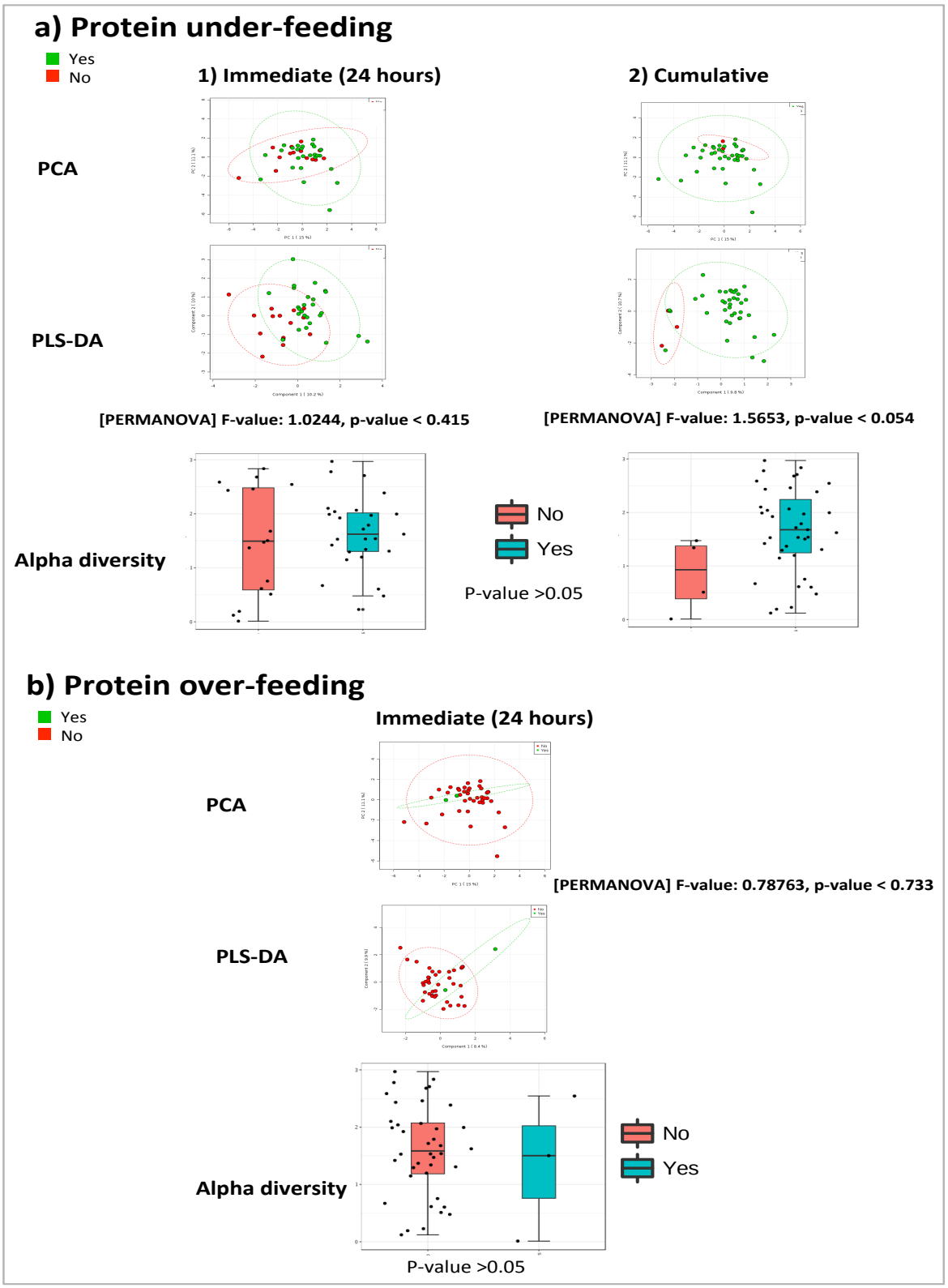

Figure 8.18 Comparison of global intestinal microbial profiles and diversity based on the amount of enteral protein received

PCA plots show an overlap between the samples based on protein feeding status. The figure also indicates that Alpha diversity was not affected by protein feeding state.

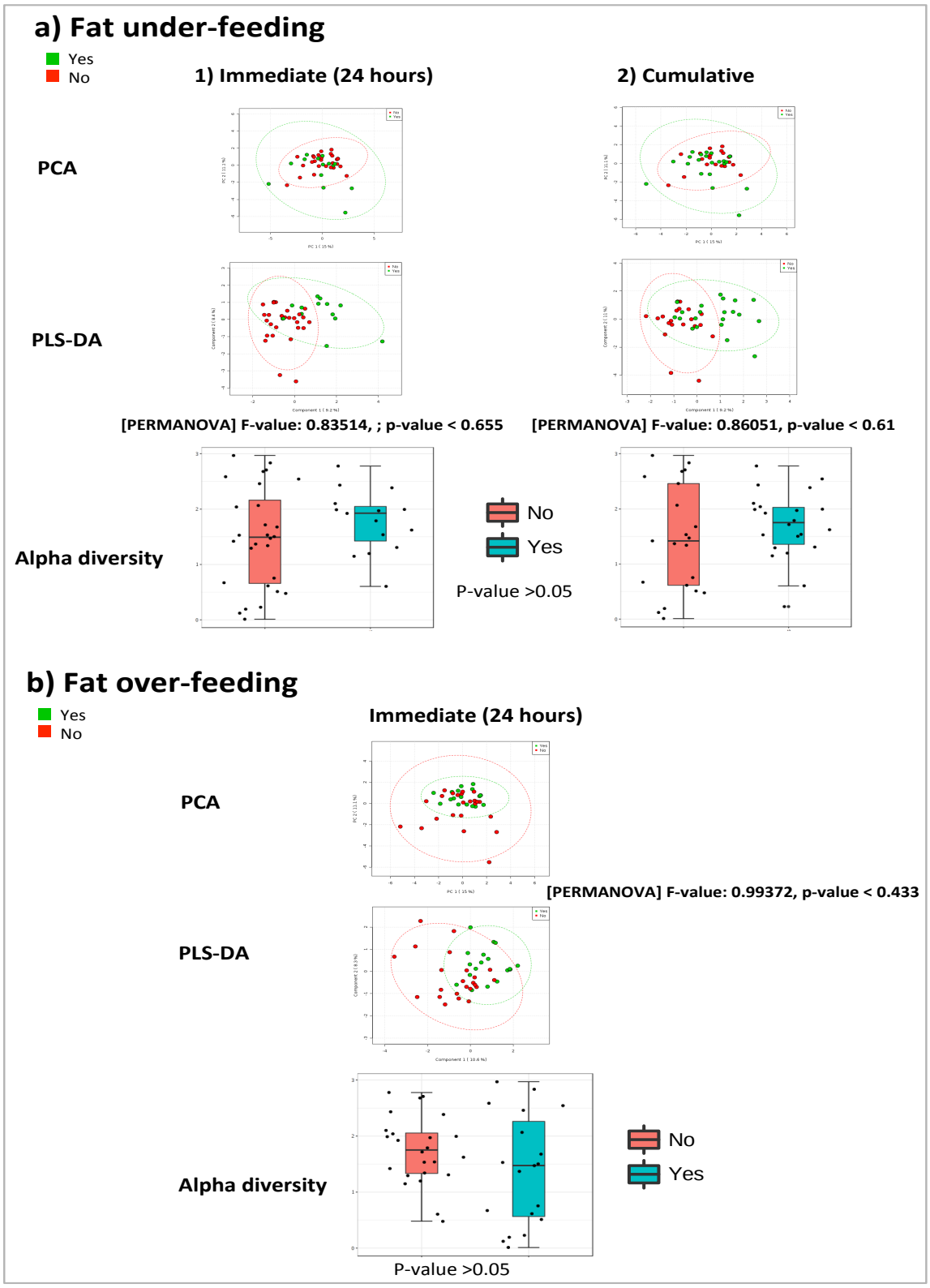

Figure 8.19 Comparison of global intestinal microbial profiles and diversity based on the amount of enteral fat received

PCA plots show an overlap between the samples based on fat feeding status. The figure also indicates that Alpha diversity was not affected by fat feeding state.

4. Metabolic pattern

In a subgroup of 10 children, the energy expenditure was performed by IC. Although the number is very small, both PCA and PLS-DA score plots showed a cluster of patients who exhibited a hypo-metabolic pattern, [PERMANOVA] F-value: 1.5402, p-value < 0.047 (Figure 8.20). Cross-validation of the supervised analysis (PLS-DA) was not viable, as the minimum number of samples required per group is 4. As the number of samples was very small, this might affect the validity of the analysis.

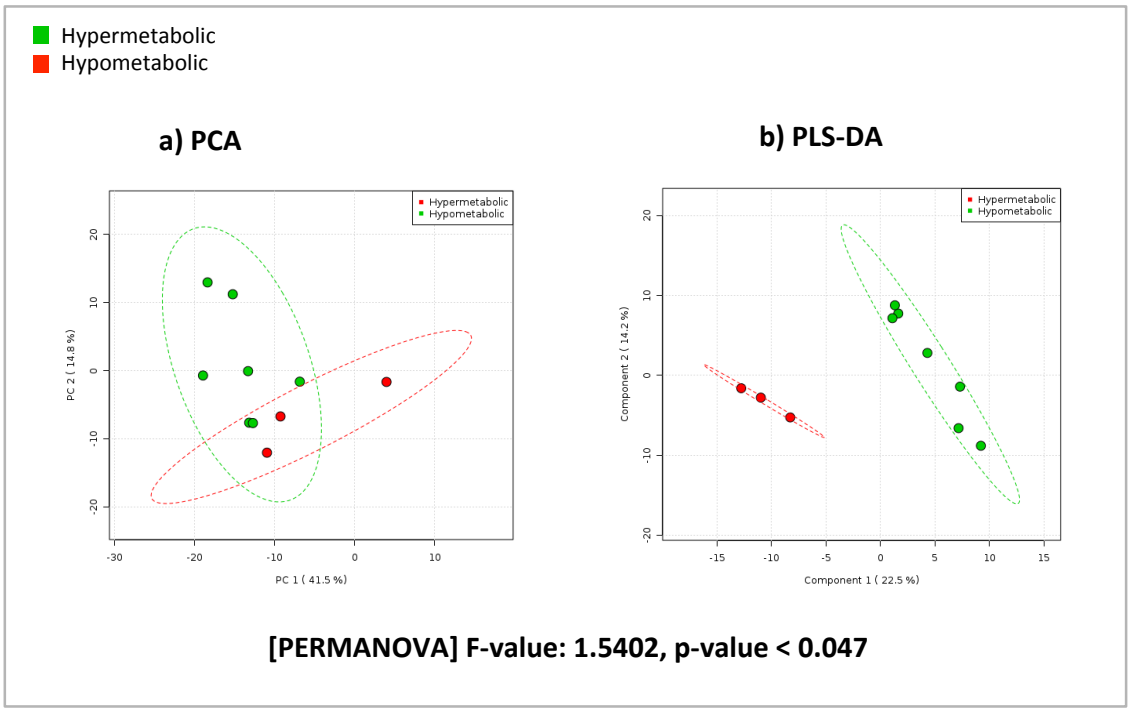

Figure 8.20 Comparison of the global intestinal microbial profiles based on the metabolic pattern

The plots show a good separation of samples obtained from children exhibiting hyper-metabolic patterns and those who did not.

8.3.3 The effect of intestinal dysbiosis during critical illness on disease severity and clinical outcomes

8.3.3.1 Systemic inflammation

Microbial data from early samples obtained between days 1 and 4 were included in this analysis. In the current study, positive correlation was recorded between the proportional abundance of *Proteobacteria* (%) with peak TNF-α (r=0.522, p=0.001), and IL1-β (r=0.523, p=0.001) (Figure 8.21). No evidence of statistical correlation was recorded between the Shannon index of Alpha diversity and inflammatory cytokines. The regression model indicated that the association between *Proteobacteria* (%) and the pro-inflammatory mediators was influenced by age and disease severity (Table 8-6).

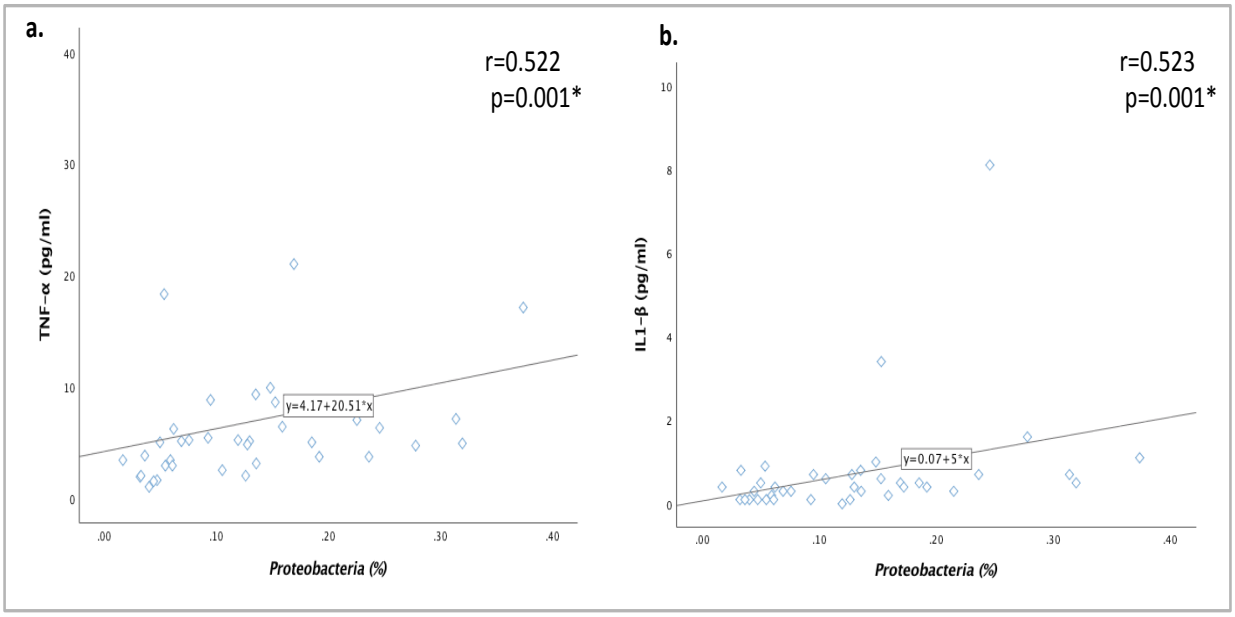

Figure 8.21 Association between and the proportional abundance of *Proteobacteria*

Figure 8.21 shows a positive correlation between the relative abundance of *Proteobacteria* and pro-inflammatory cytokines TNF-α and IL1-β.

Table 8-6: Regression model to determine the association between *Proteobacteria (%)* and pro-inflammatory mediators

Model	R	R^2	Adjusted R^2	R	P-value
TNF-α [a]	**0.669**	**0.448**	**0.416**		
Age [b]				-0.585	0.006*
PIM2 score [b]				0.076	0.327*
Proteobacteria [c]				0.406	0.006
IL1-β [a]	**0.548**	**0.301**	**0.280**		
Age [b]				-0.548	0.00*
PIM2 score [b]				-1.51	0.327*
Proteobacteria [c]				0.500	0.001

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variable**

8.3.3.2 Clinical outcomes

The association between the intestinal microbial profiles and clinical outcomes was examined using hours free of mechanical ventilation and days free of PICU at 30 days as the outcome measures. The results did not show any statistical correlation between the clinical outcome variables and *Proteobacteria* (%) or the Shannon Alpha diversity index. In the regression model all variables were excluded (Table 8-7).

Table 8-7: Regression model to determine the association between clinical outcome variables *Proteobacteria (%)* and the Shannon Index

Model	R	R^2	Adjusted R^2	R	P-value
VFH at 30 days [a]					
Age [c]				-0.046	0.368
PIM2 score [c]				0.116	0.197
Proteobacteria (%) [c]				-0.148	0.137
Shannon index [c]				0.021	0.438
PICU-free days at 30 days [a]					
Age [c]				-0.084	0.269
PIM2 score [c]				-0.034	0.4
Proteobacteria (%) [c]				-0.13	0.169
Shannon index [c]				0.111	0.207

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variable**

8.4 Discussion

This study recorded a profound loss of diversity in the faecal microbiome of critically ill children. This was associated with the loss of key commensal species and increased levels of opportunistic pathogens such as *Proteobacteria, Streptococcus* and *Enterococcus.* These findings are in agreement with previous work where changes in the microbiome composition of critically ill children and adults were reported. This shift is generally characterised by the increased abundance of opportunistic pathogens and reduced beneficial commensals (Shimizu et al., 2011; Rogers et al., 2016; Mcdonald et al., 2016; Ojima et al., 2016).

In a healthy gut, the abundance of *Proteobacteria* is usually low; the expansion of *Proteobacteria* phyla is closely correlated with disease state (Shin et al., 2015). The current study showed that the proportional abundance of *Proteobacteria* was higher among critically ill children than age-matched healthy children. Furthermore, the patient group had statistically higher abundance of bacterial species linked with diseases and infections such as *Escherichia coli, Clostridium, Erysipelotrichaceae bacterium and Catabacter hongkongensis. Escherichia coli* is responsible for up to 30% of nosocomial infections (Nagarjuna et al., 2015; Lodha et al., 2001). It is also the most common pathogen causing septicaemia and diarrhoea in critically ill children (Puentes & Dunstan, 2018). The *Erysipelotrichaceae bacterium* was found to be enriched in the patient group; this bacterial species has been implicated in inflammation-related disorders of the gastrointestinal tract (Kaakoush, 2015), and it has been shown to be increased in patients with colorectal cancer (Chen et al., 2012). *Catabacter hongkongensis* was reported in many cases of human infection and was associated with evidence of clinical sepsis, gastrointestinal diseases and prolonged hospitalisation (Elsendoorn et al., 2011; Torri et al., 2016; Lau et al., 2012).

This study also recorded a striking loss of key SCFAs forming species in critically ill children. These include *Eubacterium rectale* and *Faecalibacterium prausnitzii* (the main butyrate producers of Clostridium clusters XIVa and IV, respectively), which are usually

indicators of healthy and diverse gut microbiome composition (Antharam et al., 2013). *Faecalibacterium prausnitzii* is also recognised as an anti-inflammatory commensal bacteria (Sokol et al., 2008).

There is evidence that trauma and sepsis cause immediate changes to the microbiome and intestinal barrier. It is not quite clear whether this effect is solely due to the disease state or other environmental factors such as nutrition, or microbiome-guided therapies such as antibiotics are also involved. To the knowledge of the author, no previous studies have investigated how host factors, during critical illness, affect gut-residing microorganisms.

Among the critically ill children group, age was a determinant factor influenced the microbial profiles. Stokholm et al. (2018) indicated that the microbial profile during the first year of life is distinguished from later stages, as the composition of the human gut microbiome is still developing. In this study, the microbial profiles of critically ill children did not appear to be affected by the category of admission diagnosis. Surprisingly, the number of antibiotic agents received and the duration of antibiotic exposure did not show clustering of the microbial profiles of critically ill children. Since samples were collected after day 2 of PICU admission, where antibiotic treatment was already initiated for all children, it was less likely to see massive differences in the microbial species across the patient groups based on antibiotic therapy. However, as this population often receives extensive antibiotic treatment (Malacarne et al., 2004), this may, in addition to the functional loss of commensal species, affect the prevalence of antimicrobial-resistant genes within the intestinal microbiome. This may have a clinical impact on patients themselves or on the ward ecology (Palmer et al., 2007; Preidis & Versalovic, 2009; Theriot et al., 2014).

It has been estimated that the total antibiotic consumption in ICUs is nearly ten times greater than in general hospital wards (Malacarne et al., 2004). The effect of antibiotic exposure on gut microbiota has been widely investigated, with data showing that the main phyla influenced by antibiotic treatment are *Actinobacteria, Bacteroidetes,*

Firmicutes and *Proteobacteria* (Theriot et al., 2014; 2016; Panda et al., 2014). Broad-spectrum antibiotics in particular have shown to affect the abundance of nearly 30% of intestinal bacterial species (Dethlefsen et al., 2008). In a study of infants, a massive reduction in total bacterial densities was observed after antibiotic treatment, accompanied by delayed colonisation by beneficial species such as *Bifidobacteria* and *Lactobacilli*, and induced colonisation by antibiotic-resistant strains (Schumann et al., 2005). Data from adult studies have shown massive depletion of beneficial Clostridium clusters (XIVa, IV) and increased pathogens associated with the receipt of antibiotics (Iapichino et al., 2008; Livanos et al., 2018). Using antibiotic therapy can subsequently result in important functional alterations in the gut microbiome and increased susceptibility to infections (Palmer et al., 2007; Preidis & Versalovic, 2009). In fact, in the current study the variation seen in the microbiome of critically ill children compared to healthy children group may actually be related antibiotics exposure, since the control group did not receive any antimicrobial treatment for at least 2 week prior the recruitment. Suggesting that antibiotics are most likely the key driver of the shift observed in the microbiome of critically ill children as compared to the healthy state. The influence of antimicrobial agents on the gut microbiome could be short-term, or may last for a longer period (Theriot et al., 2014). An example of the long-lasting effect of antibiotic use is reduced colonisation resistance against some pathogens such as *Clostridium difficile* (Theriot et al., 2014). The effect of antibiotic exposure on the intestinal microbiome during critical illness needs to be examined in more detail in future studies, where a control group of critically ill children who require minimal or no antibiotic therapy should be recruited.

In the current study, energy underfeeding during the 24 hours prior to sample collection appeared to influence the microbial composition of critically ill children. The effect of energy balance on gut microbiota has not been previously investigated in the critically ill population. However, data from obesity studies has shown a noticeable shift in major intestinal bacterial phyla in response to the degree of under- and overfeeding (Cani & Delzenne, 2009; Jumpertz et al., 2011). Preclinical data in animal models have shown starvation-induced reduction in commensal bacteria including *Firmicutes Lactobacilli*

(Crawford et al., 2009; Costello et al., 2010). As critically ill children are often underfed, a pattern of alteration in the gut micobiome would be observed during periods of energy deprivation (Morowitz et al., 2011; Krajmalnik-Brown et al., 2012).

Outside of energy underfeeding, the effect of other nutritional factors on microbial composition was not evident in the current study. This could be related to the fact that most samples were obtained early after admission, where the ability to modulate the intestinal environment by feed is limited. Furthermore, during the acute phase of illness, critically ill children are heavily treated with broad-spectrum antibiotics, which are thought to be the main factor driving intestinal dysbiosis in this population (Iapichino et al., 2008; Livanos et al., 2018). Limitation in the sample size could also be a factor that compromised the reliability of the analysis in the current study.

The role of dietary fibres in stimulating the growth of beneficial commensal species is well documented, yet only 6 children > 1 year received fibre-supplemented feeds. EN practice in PICU adopting routine use of standard paediatric formulas lacking dietary fibre may potentiate the loss of beneficial species such as *Bifidobacteria*, *Lactobacilli, Faecalibacterium prausnitzii* and *Roseburia* (Bouhnik et al., 1996; Vulevic et al., 2008; Ramirez-Farias et al., 2009; Benus et al., 2010; Sawicki et al., 2017).

In a small number of children, this study showed that microbiome composition varied between children who exhibited hyper-metabolic response and those who did not. These findings are in line with previously published work on 10 healthy men, where a correlation between changes in microbiota composition with host substrate oxidation rates and REE was recorded (Kelder et al., 2014). Several mechanisms have been proposed to link the composition of the gut microbial community and host energy homeostasis. Stappenbeck et al. (2002) suggested that the normal function of gut microbiota increases the capacity of capillaries in the small intestinal villus epithelium, and accordingly helps in promoting the intestinal absorption of monosaccharide. In addition, intestinal microbial fermentation of non-digestible carbohydrates is associated with the secretion of enteroendocrine peptides, such as GLP-1 produced by L-cells (Tolhurst et al., 2012). It has also been suggested that endotoxins such as

lipopolyschariде are associated with gut injury, stimulating the secretion of GLP-1 (Lebrun et al., 2017; Nguyen et al., 2014). These mechanisms may result in increased production of GLP-1, which appears to increase REE through stimulation of insulin secretion or inducing brown adipose tissues thermogenesis (Beiroa et al., 2014; Shalev et al., 1997). In addition, the depletion of some microbial species has been shown to promote the browning of white adipose tissue, which might also affect REE (Suárez-Zamorano et al., 2015). The association between microbiota and REE signalling axis needs to be explored in a larger cohort in order to draw a valid statistical conclusion.

In the current study, the expansion of *proteobacteria* phylum was correlated with raised serum TNF-α and IL1-β in patient samples. The activation of pro-inflammatory cytokines may influence the gut microbiome and promote the growth of pathogenic species (Dickson 2016; Alverdy et al. 2000). It is also possible that systemic inflammation and tissue injury may actually be downstream consequences of disordered bacterial communities in the gut (Dickson 2016, Hakansson & Molin, 2011; Maslowski & Mackay, 2011; Lobo et al., 2016). Hypoxia in the colon facilitates the loss of obligate anaerobes and allows for expansion of facultative anaerobes (Romick-Rosendale et al. 2018). As a result of bacterial translocation, the intestinal cells secrete innate immune effector molecules such as TNF-α when exposed to bacteria or bacterial antigens (Schuijt et al., 2012). Local inflammation in the gut may further aggravate systemic inflammation and adverse outcomes among this patient group (Schuijt et al. 2012). It hard to postulate if disordered microbiome is a cause or effect systemic inflammation. An anima model of faecal microbiota transplantation from septic to healthy animals could improve our understanding about the potential role of disordered microbiome on the progression of systemic inflammation. This must be explored in future studies.

Whilst many of the triggers of intestinal dyshomeostasis may be unchangeable in critical illness, it is logical that the intestinal environment should be supported actively in affected children. Given the extensive vascular and lymphatic links between the intestines and other organs, it is possible that targeted manipulation of the intestinal microbiome by enteral feeding could be of systemic clinical benefit and improve clinical

outcomes in critically ill children. Although this has not been explored fully in this population, optimising the balance of gut ecology with pre- or probiotics could be a promising strategy to improve energy balance and clinical outcomes in critically ill children. Prebiotics such as Lactulose fructooligosaccharides and galactooligosaccharides were used in infant formulas and demonstrated a significant modulation of the gut bacteria (Collins & Gibson, 1999; Vandenplas et al., 2014; Fanaro et al., 2005). Inulin-type fructose prebiotics have been tested in a study of obese women, and showed selective changes in the gut microbiota composition (Dewulf et al., 2013). In addition, probiotics containing *Lactobacillus* and *Bifidobacteria* species have shown beneficial effects on controlling the balance of the intestinal microbiome (Collins & Gibson, 1999). Generally, using probiotics in clinical settings has been proven to be safe; however, they must be used with caution in certain patient groups (Srinivasan et al., 2006).

8.5 Limitations

This study was limited by the relatively small number of children included in the analysis of the nutritional factors. The study was originally powered to detect statistical variation in the gut microbiome between critically ill children and healthy controls; the incorporation of the nutrition study followed the completion of the metagenomic study. Dietary information was collected retrospectively from patients' electronic records. The nutritional data was not obtainable for children recruited early in the study, due to the transition of the hospital recording system from paper files to an electronic system. Accordingly, it was difficult to retrieve the data documented in the files after it was sent to the hospital archive. Due to limitation in the sample size, it was difficult to identify statistical relationships from the data, as statistical tests normally require a larger sample size to ensure a representative distribution of the population.

8.6 Conclusion

This study demonstrated that iatrogenic factors and adverse pathophysiology appear to decimate the healthy microbiome in critically ill children, resulting in the loss of commensal bacteria. The nature of the microbiome in critically ill children is shaped by

many factors. Growing evidence suggests that the gut interacts with and influences other organ systems; therefore, supporting gut health should be integrated into management protocols for critically ill children. Since the insult of critical illness is inevitable, nutritional interventions to restore normal gut-host relationships may be a potential therapeutic target. It is possible that personalised nutritional interventions to encourage the restoration of commensal species, along with improved rationalisation of antibiotic therapy, could support longer-term recovery of the intestinal mucosa and the resident microbiome. This needs to be examined in future studies.

9 Chapter Nine: Metabolic Effects of Intestinal Dysbiosis in Critically Ill Children

9.1 Introduction

The interaction between gut microbiota, nutrition, and host metabolism has undoubtedly drawn attention over the past few years (Morowitz et al. 2011). The gut microbiota uses ingested dietary components (carbohydrates – mainly resistance starch, proteins, and lipids) to generate energy for cellular processes and for growth (Ramakrishna 2013). This process is known as bacterial fermentation. The fermentation process is crucially important for maintaining host health (Cheng et al. 2013). During the process of utilising these substrates, the microbiota produces several metabolites that influence host health and metabolism (Krajmalnik-Brown et al. 2012; Ramakrishna 2013).The shift in gut bacterial population during critical illness may affect the colonic degradation capacity (Iapichino et al. 2008).

There are many bacterial metabolites derived from the fermentation of dietary components known to impact the health of the host (Table 9-1). These include SCFAs, lactate, ferulic acid and other organic compounds such as phenol, *para*-cresol and indole (Russell et al. 2013; Belenguer et al. 2011; Westfall & Lomis 2016; Russell et al. 2013). SCFAs arising from anaerobic bacterial metabolism of polysaccharides in the colon have beneficial effects on the host (Blaut & Clavel 2007; Vinolo et al. 2011; Byrne et al. 2015). SCFAs have been shown to be depleted in adults with critical illness, due to reduction in SCFAs-forming species (Shimizu et al. 2006; Osuka et al. 2012; Hayakawa et al. 2011; Vermeiren et al. 2012; Yamada et al. 2015). Based on the results from the previous chapter, the paediatric faecal microbiomes appear to lose much of their commensal population such as *Faecalibacterium prausnitzi and Eubacterium rectale* species in critical illness.

Table 9-1: Common bacterial metabolites linked to human health

Product of bacterial fermentation	Intestinal bacteria	Reference
SCFAs Butyrate Propionate Acetate	*Roseburia* *Eubacterium rectale* *Eubacterium hallii* *Faecalibacterium prausnitzii*	(Russell et al. 2013) (Benus et al. 2010) (Duncan et al. 2007)
Lactate	*Bifidobacteria* *Lactobacillales* *Klebsiella pneumoniae mutants*	(Belenguer et al. 2011) (X. Feng et al. 2017)
Ferulic acid	*Lactobacillales* *Lactobacillus fermentum*	(Westfall & Lomis 2016)
Phenol *Para*-cresol Indole	*B. thetaiotaomicron* *Bacteroides eggerthii* *Bacteroides ovatus* *Bacteroides fragilis* *Parabacteroides distasonis* *Clostridium bartlettii* *Eubacterium hallii*	(Wendy R. Russell et al. 2013)

In addition to producing metabolites that contribute to host health, gut microbiota also serve as a regulator of bile acid (BA) metabolism (Zhang et al. 2016). The human BA pool is made of the primary, secondary and tertiary BAs. The primary BAs (CA and CDCA) are synthesised in the liver, while secondary BAs are produced in the gut via modification of primary BAs by dehydroxylation, epimerisation and oxidation processes. The tertiary BAs are formed in both the liver and by gut microbiota via modification of secondary BAs through sulfation, glucuronidation, glucosidation and N-acetylglucosaminidation (Marschall et al. 1992; Ridlon et al. 2006; Wahlström et al. 2016). Intestinal microbiota are known to regulate BAs synthesis, deconjugation and metabolism (Zhang et al. 2016). Secondary BAs produced by intestinal microbiota bind to and activate a number of host nuclear receptors expressed in heart, adipose, liver and kidney tissues (Zhang et al. 2016; Ridlon et al. 2014).

The aim of this chapter is to assess the functional capacity of gut microbiota by comparing the levels of SCFAs and BA pools between critically ill children and their age-matched healthy controls. SCFAs and BAs were chosen because they are easily measured in faecal water, which provides direct information about the interaction between the host and gut.

9.2 Materials and Methods

The detailed methodology of sample handling, faecal water extraction, SCFAs and BAs measurements were described in Chapter Two page 55-56. Where a comparison was made between healthy and critically ill states, the analysis was restricted to samples obtained from an age-matched cohort of critically ill and healthy children.

Samples collected:

Faeces:

Faecal samples were collected from the child's nappy (around 300 mg) into a sterile container. Around 100 mg of stool sample was required to process the faecal water extraction needed for the quantification of SCFAs and BAs. For the purpose of this study samples were categorised into two groups early samples collected between days 1 and 5, the late samples were obtained between days 6 and 10.

Serum

Blood samples were drawn from indwelling vascular catheters at 3 time points. The maximum values were used to indicate the intensity of the inflammatory response in critically ill children.

9.2.1 Sample size calculation:

Sample size calculation was performed using the statistical power analysis program G*Power 3 (Faul et al. 2007). The level of faecal butyrate (one of the key SCFAs involved in intestinal health) was selected as the primary outcome in this study. The sample size was chosen to detect a clinically statistical difference in faecal butyrate between patients and controls. There are no data on the paediatric population. Data from studies on critically ill adults demonstrates a rapid, sustained decrease in faecal butyrate compared to healthy adults. This assumption was based on work by Yamada et al. (2015) with critically ill adults where a difference of 12 micromol/g faeces of butyrate was recorded between critically ill and healthy controls. It was estimated that 25 children per

group would be needed to detect the same difference between critically ill and healthy children, assuming a 5% significant level (2-sided) and 90% power.

9.2.2 Statistical analysis:

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as median with (25-75) interquartile ranges. The Mann-Witney U test was used to compare independent variables with non-normal distribution such as differences in SCFAs and the demographic characteristics between critically ill and healthy children. The BAs data was normalised and analysed using Metabo-Analyst, a web-based programme (Xia et al. 2012). BAs profiles were investigated using a combination of univariate and multivariate analysis. The PCA-plot was generated first to visualise the BA patterns between critically ill and healthy children. The PERMANOVA test was used to assess whether the observed differences (in multivariate space) were statistically significant. A t-test was then carried out to identify how BAs varied between critically ill and healthy children; a false Discovery Rate (FDR) of <0.05 was considered statistically significant. The regression analysis was conducted using IBM SPSS v25 USA. Stepwise linear regression analysis was applied to assess the relationship between SCFAs/ BAs with the severity of disease, inflammatory cytokines, clinical outcomes and the nutritional intake. Log transformation was performed on SCFAs and BAs prior to regression analysis.

9.3 Results

A total of 45 critically ill children (21 males) and 40 healthy children (18 males) were enrolled in this study. Fifty-four samples were obtained from the patient group and 40 from healthy controls. The demographics of patients and controls are shown in Table 9-2. Amongst the patient cohort, hospital mortality was 3 out of 45 children (6.6%).

Table 9-2: Anthropometric and clinical characteristics of children enrolled in the study

Anthropometry	All patients and controls			Age- matched patients and controls		
	Patients (N=45)	**Control (N=40)**	**P- value**	**Patients (N=27)**	**Control (N=40)**	**P- value**
Age (Years)	1.3 (0.3 – 3.9)	4.8 (3.3 – 6.2)	0.00**	3 (1.5 – 6.9)	4.3 (3.3 – 6.2)	0.086
Weight (Kg)	11 (4.8 – 15.9)	19.6 (16 – 23)	0.00**	14.6 (12 – 24)	19.6 (16 – 23)	0.029*
Height (cm)	75 (49.5 – 96)	110 (98 – 117)	0.00**	90.0 (79 – 104)	110 (98 – 117)	0.001**
Weight age Z- score	-0.5 (-1.8 – 0.42)	0.32 (-0.06 – 1.05)	0.00**	-0.11 (-0.7 – 0.75)	0.32 (-0.06 – 1.05)	0.035
Height for age Z- score	0.00 (-1.7 – 0.52)	0.67 (-0.13 – 1.1)	0.02*	-0.17 (-1.4 – 0.19)	0.67 (-0.13 – 1.1)	0.014*
Disease severity variables						
PMOD score	5 (4 – 6)			5 (4 – 6)		
PIM2 score	3.6 (1.2 – 6.4)			4 (1.9 – 7.5)		
Highest Lactate	1.6 (1.0 – 2.2)			1.6 (0.9– 2.4)		
Highest CRP	94 (34 – 222)			137 (34 – 245)		
Inotrope score	0.0 (0 – 15)			0.0 (0 – 16)		
VFH at 30 days	636 (584 – 667)			650 (571 – 681)		
PICU- free days at 30 days	22 (18 – 24)			21 (17 – 25)		
Inotrope- free hours at 30 days	720 (676 – 720)			720 (657 – 720)		
Primary admission diagnosis	**N (%)**					
MOF	16 (36%)					
Respiratory Failure	19 (42%)					
CNS Disorders	7 (16%)					
Surgical Disorders	2 (4%)					
Gastrointestinal Disorders	1 (2%)					

Data are presented as median (IQR)

** P-value is significant at 0.01 level * P-value is significant at 0.05 level

- VFH at 30 days: ventilation-free hours at 30 days
- PICU-free days at 30 days: days free of paediatric intensive care at 30 days

Faecal samples obtained from critically children were collected between days 1 and 10 post-PICU admission. A total of 30 samples were obtained between days 1 and 5 and 15 samples were obtained between days 6 and 10. The numbers of samples collected on each day are presented in Table 9-3.

Table 9-3: Number of samples collected post-PICU admission

Day	≤2 days	Day 3-5	Day 6-9	≥10 days
Number of samples	8	22	13	2

9.3.1 Measurements of faecal SCFAs

To allow for a valid comparison between critically ill and healthy children, only data from the age-matched population were included in this analysis. The results indicated that the levels of the 3 measured key SCFAs are statistically lower among the patient group compared to age-matched healthy children p≤0.05 (Figure 9.1).

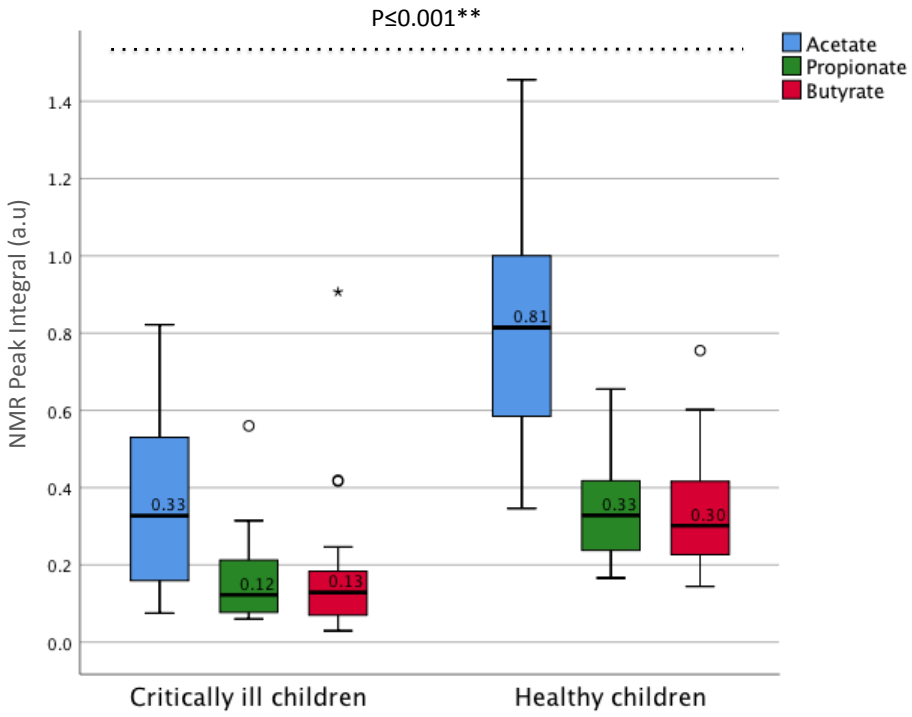

Figure 9.1 Differences in faecal SCFAs' peak integral values between critically ill children and their age-matched healthy controls

Figure 9.1 shows that critically ill children had statistically lower levels of acetate, propionate and butyrate compared to healthy children.

9.3.2 Measurements of faecal BAs

To allow for a valid comparison between critically ill and healthy children, only data from the age-matched population were included in this analysis.

In total, 18 BAs were detected in faecal water (Table 9-4). The PCA plots showed a clear separation of samples obtained from critically ill and healthy children, reflecting systematic differences in the BA profiles between the two groups. PERMANOVA analysis confirmed a statistically significant separation between the groups' F-values: 18.0222, p-value < 0.001. The samples collected from healthy children were more tightly clustered than those obtained from the critically ill group (Figure 9.2). Sixteen BAs compounds were recognised as statistically different between critically ill and healthy children (FDR ≤ 0.05), (Figure 9.3).

Table 9-4: List of 18 Bile acids detected in the faecal water of critically and healthy children

BA marker	Abbreviation
Cholic acid	CA
Chenodeoxycholic Acid	CDCA
Lithocholic acid	LCA
Deoxycholic Acid	DCA
Taurohyocholic Acid	THCA
Taurocholic Acid	TCA
Glycochenodeoxycholic Acid	GCDCA
Isolithocholic Acid	ILCA
23-nor-5b-Cholanic Acid-3a, 12a-diol	23 nor 5ß- CA-3α, 12a-diol
5beta-Cholanic Acid-3beta, 12a-diol	5ß- CA-3ß, 12a-diol
3-Ketocholanic Acid	3KCA
3a-Hydroxy-12 Ketolithocholic Acid	3a-H-12 KLCA
3,12-Diketocholanic Acid	3,12-DKCA
3a-Hydroxy-6,7-DiketoCholanic Acid	3aH-6,7-DCA
3 Dehydrocholic Acid	3DHCA
12 Dehydrocholic Acid	12 DHCA
Glycoursodeoxycholic Acid	GUDCA
Ursodeoxycholic acid	UDCA

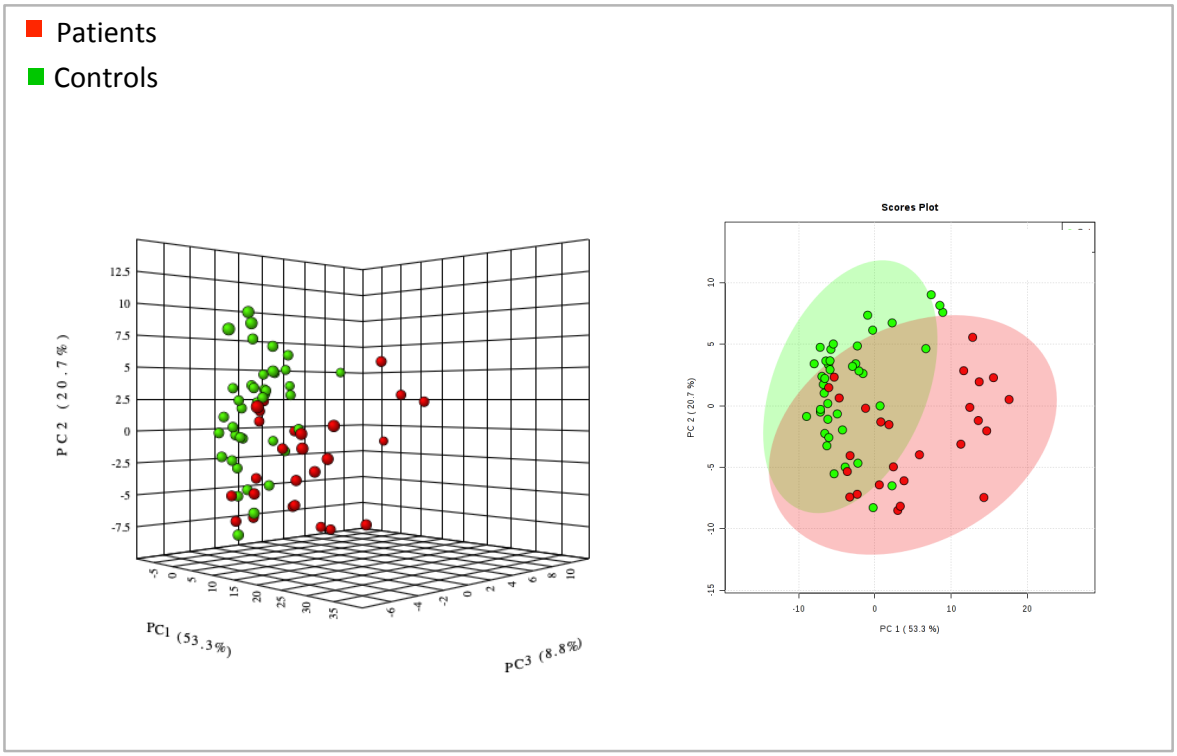

Figure 9.2 PCA score plots obtained from critically ill and age-matched healthy children's faecal water showing bile acid profiles

Data was projected into 2D and 3D for a better visualisation of data. The PCA plots showed clear separation of samples obtained from critically ill and healthy children, reflecting systematic differences in the BA profiles between the two groups.

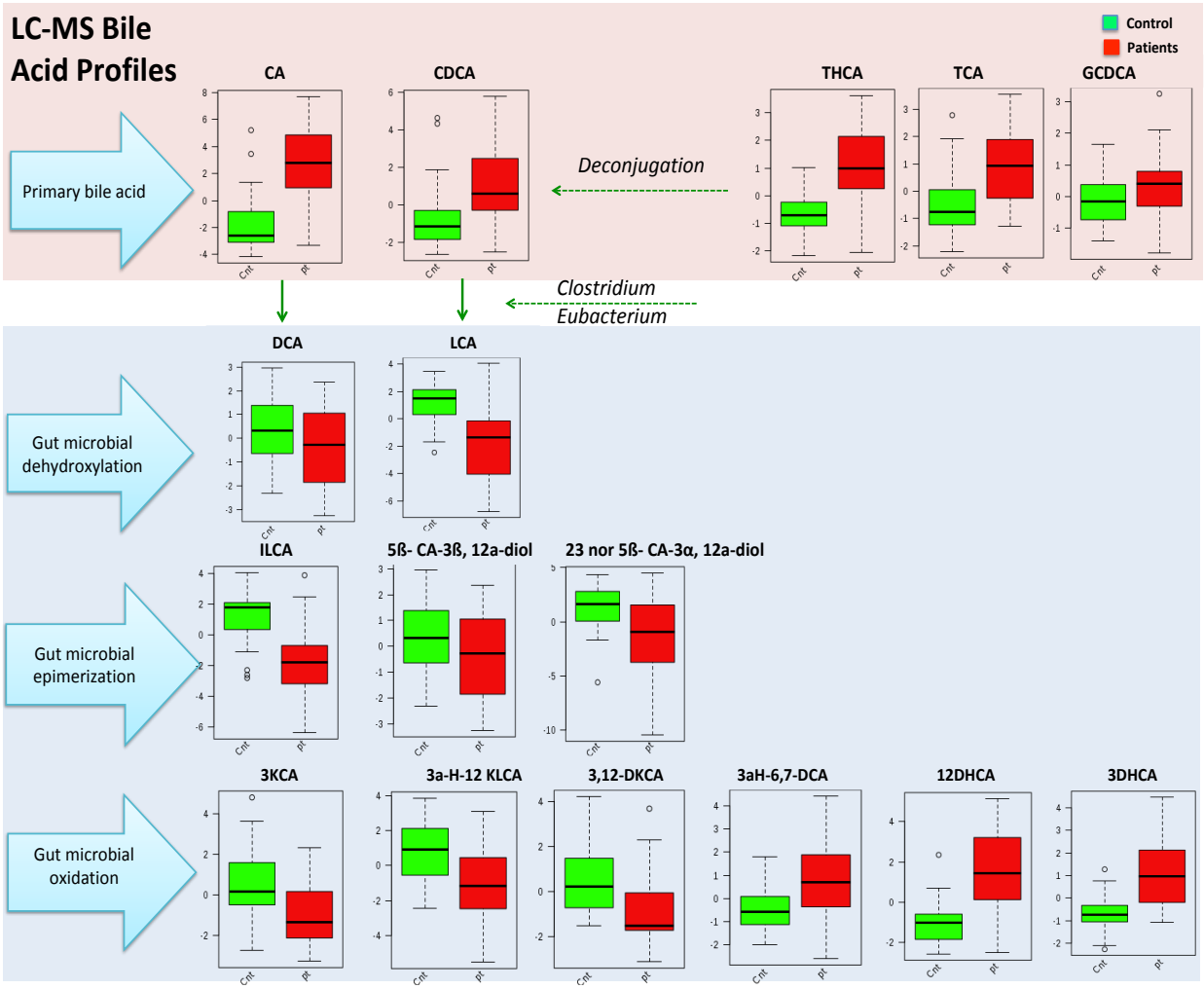

Figure 9.3 Bile acids distinguished between critically ill children and healthy children's groups

Figure 9.3 shows BAs that statistically differed between critically ill and healthy children, FDR ≤0.05. Critically ill children had higher levels of primary BAs and conjugated bile compared to their age-matched healthy controls. Healthy children group had statistically higher levels of secondary BAs, except for 3aH-6,7-DCA, 12 DHCA and 3DHCA, where they were higher among the critically ill children cohort.

9.3.3 Integrated analysis of fecal microbial and metabolic changes in critically ill children compared to healthy controls

The microbiome and metabolic data were integrated to examine the association between changes in microbial composition and the metabolic environment. A PCA of the normalised metabolic and microbial data from critically and healthy children was performed. The PCA differentiates between the bacterial species and metabolites associated with healthy and critically ill profiles (Figure 9.4). The critically ill profile was associated with a reduction in SCFAs-producing species (*Faecalibacterium prausnitzi* and *Eubacterium rectale*) and decreased levels of intraluminal SCFAs. Increased levels of primary BAs CA and CDCA were also a common feature of the critically ill state. There was also a major shift in *Clostridium and Eubacterium* (the main genera responsible for metabolising primary BA into secondary bile) towards the healthy children's profile. Secondary BAs LCA and DCA are also clustered towards the healthy children's profile.

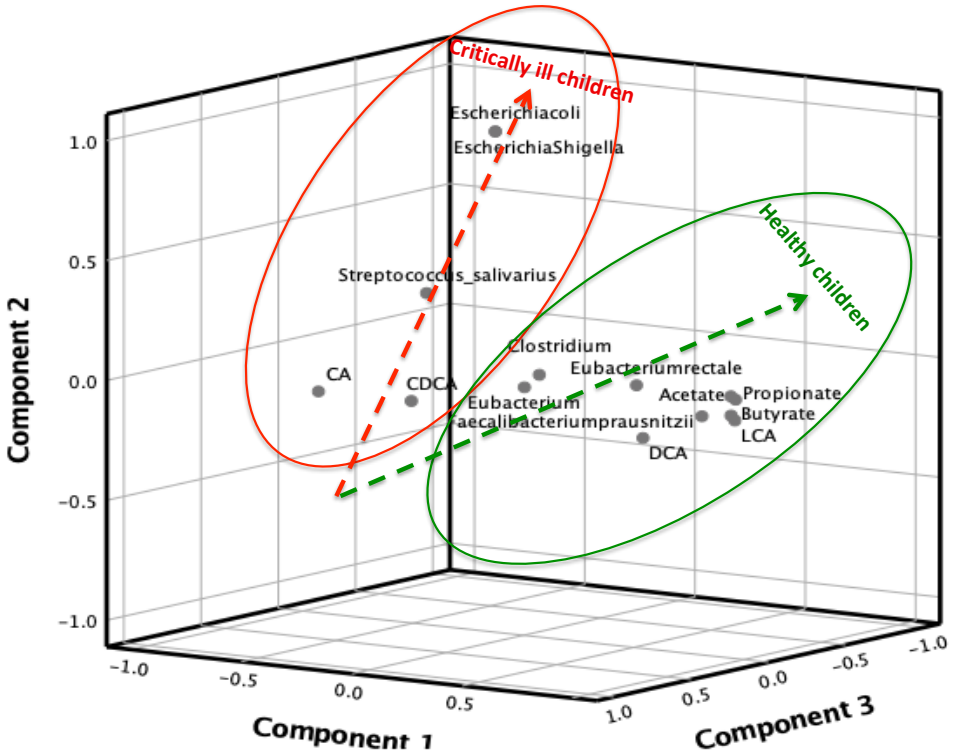

Figure 9.4 PCA analysis integrating faecal microbial and metabolic changes in critically ill children compared to healthy controls

The PCA analysis created 3 components to integrate the faecal and microbial changes in critically ill state compared to healthy state. The bacterial species and metabolites associated with healthy and critically ill profiles indicated by the arrows.

9.3.4 The effect of antibiotic treatment and disease severity on the functional capacity of gut microbiome

Regression analysis was performed to further investigate the relationship between antibiotic exposure, key commensals and SCFAs/BAs production. All critically ill children were treated with at least one broad-spectrum antibiotic agent from day of admission into PICU. The median (IQR) period of antibiotic exposure was 4 (3-7) days. The duration of antibiotic treatment did not appear to affect the levels of SCFAs or secondary BA production in this cohort (Table 9-5).

The regression analysis suggested that increased proportional abundance of *Faecalibacterium prausnitzi* was statistically associated with an increase in SCFAs peak integrals. Increased proportional abundance of *Eubacterium* was statistically associated with increased production of secondary BAs DCA and LCA (Table 9-5).

The results also indicated that children with higher PIM2 scores had reduced levels of butyrate and propionate (Table 9-5). Although the univariate analysis showed a weak positive correlation between PIM2 score primary BAs [CA (r=0.347, p-value=0.03), and CDCA (r=0.355, p-value=0.029)], in the regression analysis none of the variables were statistically related to the levels of primary BAs.

Table 9-5: Association between faecal SCFAs, secondary bile acids and the proportional abundance of key commensals

Model	R	R^2	Adjusted R^2	R	P-value
Butyrate [a] (N=30)	**0.626**	**0.391**	**0.345**		
Faecalibacterium prausnitzi (%) [b]				**0.529**	**0.004***
PIM2 score [b]				**-0.468**	**0.012***
Days of antibiotics exposure [c]				0.265	0.185
Eubacterium (%) [c]				0.161	0.424
Clostridium (%) [c]				0.004	0.982
Age [c]				0.083	0.697
Propionate [a] (N=30)	**0.613**	**0.379**	**0.328**		
Faecalibacterium prausnitzi (%) [b]				**0.511**	**0.005***
PIM2 score [b]				**-0.464**	**0.013***
Eubacterium (%) [c]				0.208	0.297
Days of antibiotics exposure [c]				0.178	0.949
Clostridium (%) [c]				-0.07	0.908
Age [c]				-0.021	0.916
Acetate [a] (N=30)	**0.416**	**0.173**	**0.142**		
Faecalibacterium prausnitzi (%) [b]				**0.416**	**0.025***
PIM2 score [c]				0.235	0.228
Clostridium(%) [c]				-0.050	0.911
Eubacterium (%) [c]				0.178	0.949
Age [c]				-0.085	0.668
Days of antibiotics exposure [c]				-0.067	0.736
DCA [a] (N=45)	**0.565**	**0.319**	**0.267**		
Age [b]				**0.505**	**0.006***
Eubacterium (%) [b]				**0.375**	**0.049***
Faecalibacterium prausnitzi (%) [c]				0.145	0.472
PIM2 score [c]				0.163	0.416
Days of antibiotics exposure [c]				0.148	0.461
Clostridium (%) [c]				-0.111	0.560
LCA [a] (N=45)	**0.774**	**0.599**	**0.568**		
Age [b]				**0.705**	**0.00***
Eubacterium (%) [b]				**0.622**	**0.00***
Faecalibacterium prausnitzi (%) [c]				-0.005	0.981
PIM2 score [c]				-0.01	0.955
Days of antibiotics exposure [c]				0.161	0.422
Clostridium (%) [c]				0.175	0.384

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

9.3.5 The association between faecal SCFAs/bile acids and inflammation

Inflammatory cytokines were measured in 40 children where serum samples were obtainable and FC was measured in 18 children. A correlation analysis was performed first to establish the association between SCFAs/BAs and the inflammatory markers (Table 9-6). The correlation table showed a weak negative correlation between Peak-TNF α with propionate (r=-0.389, p-value=0.041) and acetate (r=-0.354, p-value=0.025). No evidence of statistical correlation was recorded between the levels of BAs and the inflammatory markers.

Table 9-6: Correlation analysis of faecal SCFAs/bile acids and inflammatory markers

		Peak IL-1	Peak IL-6	Peak IL-10	Peak-TNF α	FC
Butyrate	R	-0.273	-0.275	-0.271	-0.31	0.066
	P-value	0.088	0.086	0.091	0.052	0.794
Propionate	R	-0.196	-0.184	-0.182	-0.389*	-0.012
	P-value	0.226	0.256	0.261	0.013	0.961
Acetate	R	-0.287	-0.19	-0.258	-0.354*	-0.159
	P-value	0.072	0.24	0.108	0.025	0.529
CDCA	R	0.234	0.016	0.26	0.041	-0.046
	P-value	0.147	0.924	0.106	0.803	0.855
CA	R	0.225	0.123	0.243	0.06	-0.004
	P-value	0.163	0.45	0.131	0.714	0.987
LCA	R	0.158	0.266	0.071	-0.219	0.158
	P-value	0.33	0.097	0.664	0.174	0.531
DCA	R	0.164	0.174	0.123	-0.103	0.055
	P-value	0.313	0.284	0.451	0.528	0.829

The regression analysis was then performed to assess the association between SCFAs and TNFα. The results suggested that an increase in TNFα was statistically associated with a reduction in the levels of acetate, independently from disease severity and antibiotic exposure. This model accounted for 15% of the variation in acetate levels (Table 9-7).

Table 9-7: Association between inflammatory cytokines and the levels of faecal SCFAs

Model	R	R^2	Adjusted R^2	R	P-value
Butyrate [a] (N=40)	**0.552**	**0.346**	**0.328**		
Age [b]				**0.588**	**0.00***
PIM2 score [c]				0.085	0.611
Peak TNF-α [c]				-0.173	0.298
Days of antibiotics exposure [c]				0.141	0.399
Propionate [a] (N=40)	**0.540**	**0.291**	**0.272**		
Age [b]				**0.540**	**0.00***
PIM2 score [c]				0.159	0.341
Peak TNF-α [c]				-0.211	0.211
Days of antibiotics exposure [c]				0.147	0.378
Acetate [a] (N=40)	**0.411**	**0.169**	**0.147**		
Peak TNF-α [b]				**-0.411**	**0.009***
Age [c]				0.160	0.309
PIM2 score [c]				0.059	0.724
Days of antibiotics exposure [c]				0.130	0.436

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

9.3.6 Bacterial metabolites as predictors of clinical outcomes in critically ill children

In this section, SCFAs and BAs were tested as predictors of clinical outcomes in critically ill children. VFH at 30 days, PICU-free days at 30 days and hours free of inotrope at 30 days were used as the dependent variables in the regression models. This analysis included only the SCFAs and BAs measured in the early samples obtained between day 1 and 5; samples obtained post-day 5 were excluded.

The regression models suggested that early butyrate, propionate and acetate statistically predicted the duration of mechanical ventilation, length of PICU stay and the duration of inotrope treatment (see Tables 9-8, 9-9, 9-10). Unlike SCFAs, BAs were not statistically related to any of the chosen clinical outcome measures independently of disease severity and antibiotic exposure.

Table 9-8: SCFAs measured in early samples as predictors of the duration of mechanical ventilation

Model	R	R^2	Adjusted R^2	R	P-value
VFH at 30 days [a] (N=30)	**0.397**	**0.158**	**0.127**		
Butyrate [b]				**0.397**	**0.030***
Age [c]				-0.05	0.774
PIM2 score [c]				0.04	0.818
Days of antibiotics exposure [c]				0.26	0.894
VFH at 30 days [a] (N=30)					
Propionate [c]				0.359	0.026
Age [c]				0.106	0.288
PIM2 score [c]				0.115	0.272
Days of antibiotics exposure [c]				0.083	0.332
VFH at 30 days [a] (N=30)					
Acetate [c]				0.304	0.051
Age [c]				0.106	0.288
PIM2 score [c]				0.115	0.272
Days of antibiotics exposure [c]				0.083	0.332

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

Table 9-9: SCFAs measured in early samples as predictors of the length of PICU stay

Model	R	R^2	Adjusted R^2	R	P-value
PICU free days [a] (N=30)	**0.459**	**0.210**	**0.182**		
Butyrate [b]				**0.459**	**0.011***
Age [c]				0.154	0.059
PIM2 score [c]				0.174	0.013
Days of antibiotics exposure [c]				-0.071	0.716
PICU free days [a] (N=30)	**0.434**	**0.188**	**0.159**		
Propionate [b]				**0.434**	**0.017***
Age [c]				0.150	0.438
PIM2 score [c]				0.144	0.455
Days of antibiotics exposure [c]				-0.057	0.759
PICU free days [a] (N=30)	**0.435**	**0.189**	**0.155**		
Acetate [c]				0.263	0.080
Age [c]				0.303	0.052
PIM2 score [c]				0.238	0.102
Days of antibiotics exposure [c]				0.006	0.487

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

Table 9-10: SCFAs measured in early samples as predictors of the duration of inotrope treatment

Model	R	R^2	Adjusted R^2	R	P-value
Inotrope free hours at 30 days [a] (N=30)	**0.584**	**0.341**	**0.290**		
Butyrate [b]				**0.425**	**0.004***
PIM2 score [b]				**-0.305**	**0.018***
Age [c]				0.010	0.959
Days of antibiotics exposure [c]				0.136	0.497
Inotrope free hours at 30 days [a] (N=30)	**0.591**	**0.349**	**0.299**		
Propionate [b]				**0.531**	**0.004***
PIM2 score [b]				**-0.473**	**0.010***
Age [c]				0.10	0.960
Days of antibiotics exposure [c]				0.161	0.422
Inotrope free hours at 30 days [a] (N=30)	**0.622**	**0.387**	**0.364**		
Acetate [b]				**0.622**	**0.00***
Age [c]				-0.353	0.065
PIM2 score [c]				-0.165	0.400
Days of antibiotics exposure [c]				0.078	0.693

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

9.4 Discussion

The results of this chapter indicate that critically ill children had a reduced concentration of faecal SCFAs and increased levels of the primary BAs compared to age-matched healthy children.

SCFAs arise from the anaerobic bacterial metabolism of polysaccharides in the colon and they have many beneficial effects for the host. The reduction in SCFAs levels among critically ill children group may be related to the shift in gut bacterial population, resulting in a reduction of the degradation capacity of undigested carbohydrates into SCFAs (Iapichino et al. 2008). In the current study, increased proportional abundance of *Faecalibacterium prausnitzi* was statistically associated with an increase in faecal butyrate, propionate and acetate. These findings are similar to previously published work in healthy subjects, where a strong correlation between *Faecalibacterium prausnitzi* and the production of butyrate was recorded (Benus et al. 2010*;* Duncan et al. 2007). SCFAs have been shown to be depleted in critically ill adult patients, often due to the effect of antibiotic therapy on SCFAs-forming species (Shimizu et al. 2006; Osuka et al. 2012; Hayakawa et al. 2011; Yamada et al. 2015; Vermeiren et al. 2012).

BA pool size represents the function of the microbial metabolism of BAs in the intestines (Ridlon et al. 2014). In the current study, critically ill children had a statistically higher concentration of primary and conjugated BAs compared to age-matched healthy children. Increased levels of CA, CDCA and other bile conjugates were also reported in previously published studies in critically ill patients where BA was measured in serum (Vanwijngaerden et al. 2011; Horvatits et al. 2017). CDCA are known to be toxic to the gut bacteria as they are relatively hydrophobic, therefore under normal conditions, microbial epimerisation of the 7α-hydroxy group is known to decrease the toxicity of CDCA (Ridlon et al. 2016). The transformation of primary BA through the 7α-dehydroxylation enzymatic pathway is restricted to a group of commensal bacteria belonging to the *Eubacterium* and *Clostridium* cluster XIV species (Studer et al. 2016), which has shown to be diminished in critically ill children (see Chapter Eight). In the current study, an increased proportional abundance of *Eubacterium* was statistically

associated with increases in faecal DCA and LCA. These finding may imply that reduced concentrations of secondary BAs recorded in critically ill children group is actually related to reduced the abundance or the capacity of commensal species such as *Eubacterium* to modifying primary BAs to secondary and tertiary bile (Wahlströ et al. 2016). The secondary BAs are also known to have a protective effect against the intestinal colonisation of pathogens such as *Clostridium difficile* (Studer et al. 2016; Begley et al. 2005). However, it is also important to note that increased BAs dehydroxylation by intestinal clostridia may result in increased production of hydrophobic BAs, which could be problematic (Wahlströ et al. 2016).

The integrated analysis of metabolic and microbial data showed that a critically ill profile was associated with a reduction in SCFAs-producing bacteria and decreased levels of intraluminal SCFAs. Increased levels of primary BAs CA and CDCA were also a common feature of the critically ill state. Previous studies recorded a strong correlation between *Faecalibacterium prausnitzii*, *Roseburia* and the production of butyrate in healthy volunteers (Benus et al. 2010; Duncan et al. 2007). Hence, reduced levels of SCFAs in a critically ill cohort could be related to reduced functional capacity of the microbiota to ferment dietary compounds, the substrate for SCFAs production (Morowitz et al. 2011). Similarly, an increase in the concentration of primary BAs also reflects the diminished capacity of the intestinal bacteria to metabolise primary BAs into secondary and tertiary compounds (Marschall et al. 1992; Ridlon et al. 2006; Wahlströ et al. 2016).

The results also showed that among critically ill children, those who were sicker with higher PIM2 score had reduced levels of SCFAs. In addition, a reduction in SCFAs was associated with longer duration of mechanical ventilation and length of PICU stay. TNF-α was also negatively associated with acetate levels. An important driver of multi-organ failure in critical illness is the dysregulation of innate immune pathways and loss of balance between pro-inflammatory and anti-inflammatory mechanisms (Cox 2012). Decline in SCFAs generation by commensal bacteria may contribute to local and systemic inflammatory and metabolic dysregulation. SCFAs appear to play a role in modulating inflammatory and immune responses, since they modify the migration of

leukocytes to the site of inflammation, as well as modifying the release and production of chemokine (Vinolo et al. 2011). The activation of GPR43 by SCFAs induces chemotaxis and regulates the degranulation of neutrophils (Kim et al. 2014). SCFAs, in particular butyrate, have been shown to reduce inflammatory cytokine production and inflammation in the intestine through mechanisms including nuclear factor kappa B signalling (Ramakrishna 2013; Tedelind et al. 2007). In addition, they exert an inhibitory effect on both TNFα-mediated activation of the nuclear factor kappa B pathway and lipopolysaccharide-induced TNFα release (Tedelind et al. 2007). The findings of the current study suggest that SCFAs, particularly butyrate, could be used as a prognostic biomarker predicting poor clinical outcomes in critically ill children. Future work should focus on the longitudinal monitoring of key biomolecules such as SCFAs. The reappearance of faecal SCFAs later in the PICU stay could be a sign of recovery.

In the current study, a weak correlation was recorded between both CA and CDCA and disease severity assessed by PIM2 score. However, in the multivariate analysis this correlation was confounded by other factors. The association between increased levels of primary BAs and disease severity has been established in critically ill adults. Horvatits et al. (2017) reported a positive correlation between conjugated BAs and disease severity assessed by simplified acute physiology score (SAPS2) and serum lactate in critically ill patients. The current study did not find a correlation between inflammatory markers and BAs. BA has been shown to play an important role in the regulation of the inflammatory process. It has previously been shown that serum BAs are able to directly trigger inflammatory processes via cytokine expression (Allen et al. 2011). Furthermore, BAs may also exert anti-inflammatory and immunosuppressive effects mediated via FXR and TGR5 receptors (Jia et al. 2018). Previously published in vitro studies indicated that BAs such as deoxycholic acid and CDCA acid may be implicated in intestinal injury, by promoting apoptosis of colonocytes, enhancing mucosal permeability and promoting intestinal inflammation (Barrasa et al. 2011; Münch et al. 2007; Raimondi et al. 2008). The results of this study showed that BAs were not statistically associated with any of the chosen clinical outcome measures independently of disease severity. However, in a previous study in critically ill adults, increased levels of serum BAs were associated with

increased 28-day mortality and it was proposed as an early risk stratification in critically ill patients (Horvatits et al. 2017).

9.5 Conclusion

This study provides evidence that SCFAs and BA homeostasis are significantly altered in critically ill children. Since intestinal microbiota are responsible for generating SCFAs and converting primary BAs into secondary BAs, it could be argued that abnormalities seen in SCFAs and BAs profiles are actually related to the shift in gut bacteria population observed in critical illness. Reduction in SCFAs appears to be associated with a pro-inflammatory state and disease severity. Monitoring early metabolic changes in critically ill children may identify key biomolecules that might be able to independently modulate the inflammatory and metabolic response and potentially affect clinical outcomes among this population. These findings could have potential clinical implications relating to rapid diagnostic biomarkers and the development of nutritional interventions aimed at restoring intestinal bacterial function.

10 Chapter Ten: General Discussion and Conclusion

10.1 Key findings

The main aim of this thesis was to evaluate the adequacy of enteral nutrition delivery in critically ill children in relation to the gut homeostasis and disease state. First, energy and macronutrient requirements for critically ill children needed to be established in order to assess the efficacy of the current feeding protocol. The balance between requirement and delivery of energy and macronutrients was then evaluated in a cohort of critically ill children. Finally, an integrated approach of metataxonomic and metabolomic analysis was undertaken to examine how critical illness and other iatrogenic factors including nutrition affect the composition, diversity and functionality of the intestinal microbiome in critically ill children.

Overall this project has recorded a cross link between feed, gut homeostasis in terms of mucosal integrity, and microbial composition with systemic inflammation and host metabolism (Figure 10.1). It has been shown that within the feed delivered, fat delivery was often above requirements compared to protein and carbohydrate. In addition, both protein deficit and higher delivery of fat were associated with elevation in the levels of pro-inflammatory cytokines. In particular under-delivery of protein was associated with an increase in serum levels of IL-6 and TNF-α, while higher delivery of fat was associated with elevated levels of TNF-α. The results also showed that abnormalities in gut health biomarkers were associated with elevation in inflammatory cytokines. Finally, this study also recorded a profound loss of diversity in the faecal microbiome of critically ill children. This was associated with the loss of key commensal species and increased levels of opportunistic pathogens such as *Proteobacteria, Streptococcus* and *Enterococcus*. Consequently, resulted in reduced the functionality of the gut microbiome manifested by reduced production of SCFAs and abnormalities in BAs metabolism. This project showed that SCFAs could be potential prognostic biomarkers predicting poor clinical outcomes in critically ill children. The current study showed for the first time that

energy underfeeding during the 24 hours prior the sample collection appeared to influence the microbial composition of critically ill children.

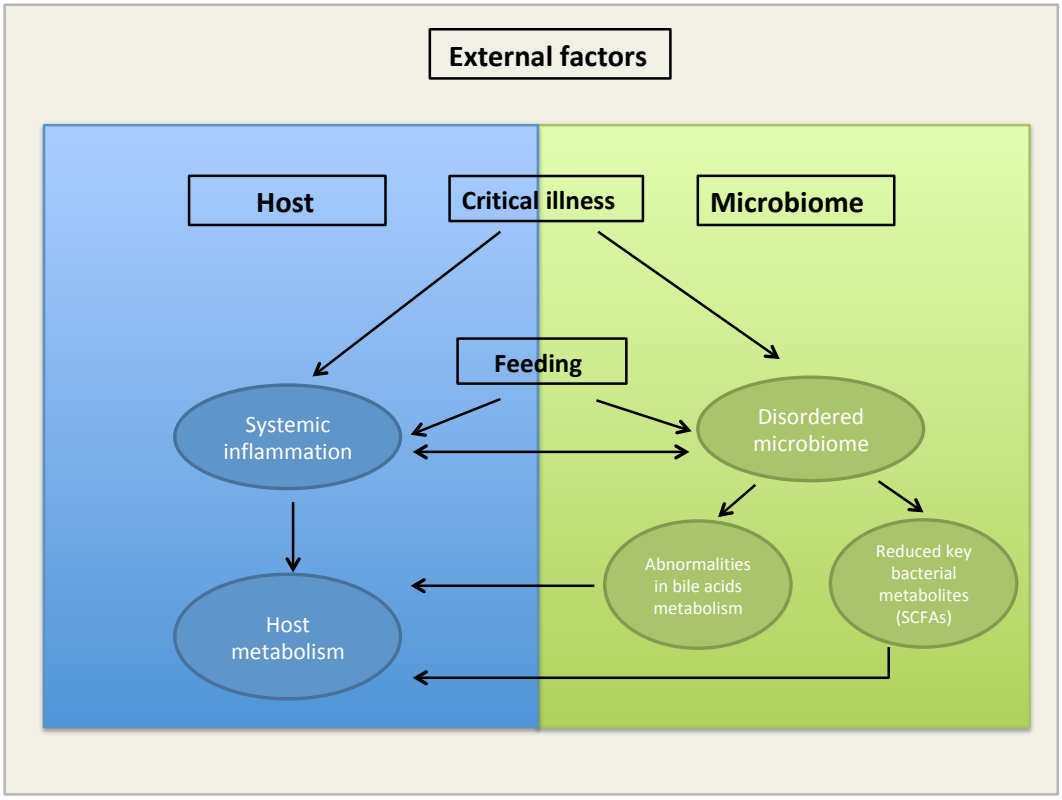

Figure 10.1 Summary of the interaction between gut microbiome, systemic inflammation and host metabolism during critical illness based on the findings from this project

It is crucial to understand the pathophysiological basis of the acute insult in order to develop new novel therapies that improve clinical outcomes of critically ill children. The findings of this thesis point to the role of gut in the pathophysiology of inflammation in critical illness. Therefore, the microbiome should be considered as an organ that can fail in critically ill patients and can impact other organs. Based on the findings from this thesis and previous work, it become obvious that critically ill children may actually be stuck in a vicious cycle of systemic inflammation and dysbiosis of gut microbiome. As the findings of this thesis suggest that EN could impact the inflammatory state and may potentially modulate the intestinal microbiome and the residing microbiota. Nutritional intervention could be a potential therapy to alleviate the disease state and improve

clinical outcome in this population by breaking this cycle and restore homeostatic state of the intestinal microbiome.

Although, nutrition is considered an integral part of the treatment plan of critically ill children, what is dismissed is whether the nutrients provided are adequate and how they are processed and utilised by the host and the microbiota. As the results suggest that aiming to achieve energy goal by using the standardised formulas may result in higher delivery of fat and compromise protein intake. It is possible that the distribution of macronutrients in these standardised feeds do not match the specific requirements of critically ill children. Therefore, there is an urgent need for new enteral formulas that closely meet the macronutrient needs of critically ill children while providing the optimum substrate necessary for the maturation and growth of commensal bacteria.

The goal of nutrition therapy in critical illness should not only be limited to improve nitrogen balance. It is about providing a good quality feeds with balance macronutrient content to feed up the entire body including the microbiota and host cells. Nutritional interventions may help to maintain commensal population and restore normal gut host relationships. Yet no clinical trials in critically ill children have tried targeted gut modulation by nutritional intervention. Future work should aim at personalising the care of critically ill children, and suggest ways to manipulate the body's response to severe illness in order to restore the homeostatic state.

10.2 Strengths and weaknesses

A particular strength of this PhD thesis is that it improved our understanding about the role of nutrition as a contributing factor affecting the disease state in critically ill children. In addition, it provided a guide for future studies aiming at targeted gut modulation by nutritional intervention to manipulate the body's response to severe illness. The findings from this thesis also provided a plausible clinical connection between inflammation host metabolism and gut microbiome. This project also

highlighted the needs for establishing macronutrient requirements that specifically meet the needs of critically ill children.

There are no previous studies investigated the effect of macronutrient delivery on disease features such as inflammation (systemic and intestinal). This project was the first to show that macronutrient delivery may influence the systemic inflammatory state in critically ill population where the inflammatory response is already stimulated by injury. These findings are of clinical significance and add to the knowledge of the field of critical care nutrition and emphasize the need to develop new feed that particularly meet the macronutrient requirements of critically ill children.

All microbiome studies in critical settings have arrived at a similar conclusion that commensal species are replaced by pathogens. None of which investigated the effect of environmental influences (e.g. Nutrition, antibiotic treatment) on the microbiome of critically ill patients. This project showed that patient's factors such as age and feeding status are important factors influencing the intestinal microbiome of critically ill children. These factors may provide a tool to identify patients at risk of extreme intestinal dybiosis (infants as they low microbial diversity or underfed children) and implement ways to support the gut environment in this group.

This work also showed that reduced SCFAs production was associated with increase in pro-inflammatory cytokines and disease severity. These findings could have potential clinical implications relating to rapid diagnostic biomarkers and the development of nutritional interventions aimed at restoring intestinal bacterial function.

Unfortunately, this project also suffered from several weaknesses. Firstly, the heterogeneity of the cohort in terms of age/disease type/severity, interventions and nutritional status are a limiting factor in this project. However, this was taken into account when conducting the statistical analysis. For instance, all regression models were adjusted for age and disease severity. In addition, where necessary, patients were stratified by age groups or diagnosis. This resulted in reduced sample size for some aspects of the work. Therefore it was difficult to find significant relationships from some

data, as statistical tests normally require a larger sample size to ensure a representative distribution of the population.

Lack of published guidelines regarding enteral fat and carbohydrate requirements for critically ill children was also a limiting factor in this study. Therefore, the recommendation for healthy children was used as a guide to establish carbohydrate and fat goal for the purpose of this project. This provided an undisputed reference range universally accepted in general nutrition but it is still considered a weakness of this study.

It is clinically accepted that antibiotics are the main adverse exposure on gut microbiota during critical illness. However, the findings of this this thesis did not record an association between the type and duration of antibiotic treatment and intestinal dysbiosis. This could be because all faecal samples obtained for the microbiome study were collected after day 2 of PICU admission where broad-spectrum antibiotic treatment was already initiated for all children. This limited the extent of observing differences in the microbial species across the patient groups based on antibiotic therapy. The effect of antibiotic exposure on the intestinal microbiome during critical illness needs to be examined in more details in future studies with reference to the type and dosing of antimicrobial agents as well as the time of exposure. However, since antibiotics are given routinely, often for life-saving purposes, they could be considered as an inevitable component in the combination of adverse exposures for the intestinal, and other microbiomes in the critically ill child.

10.3 Future work and perspective

This PhD project provided an insight about the role of host-gut interaction on host metabolism, disease severity and clinical outcomes in critically ill children. The next generation of microbiome studies in PICU settings should focus on distinguishing microbiome changes in children who do well and those who do not. In addition future work should try to develop strategies to modulate the gut environment and manipulate

the body's response to severe illness in order to restore the homeostatic state. This may include the use of nutritional interventions (such as probiotics, prebiotics and short chain fatty acids or a combination of these agents) to promote the growth of commensal species and restore the normal balance of the gut microbiota.

The findings of the current study suggest that SCFAs particularly butyrate could be used as a prognostic biomarker predicting poor clinical outcomes in critically ill children. Future work should aim at identifying key intermediates of host-gut microbiota interactions to develop a stratification and monitoring tool to guide implementation and use of gut protective treatments in critically children. The longitudinal monitoring of key biomolecules such as SCFAs could be of clinical significance; the reappearance of these intermediates later in the PICU stay could be a sign of recovery from illness.

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Appendix: Published and submitted articles

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Original article

Association between enteral macronutrient delivery and inflammatory response in critically ill children

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ABSTRACT

Background and aims: An important goal of nutrition support in paediatric critical illness is minimising catabolism. While focussing on providing full energy requirements, macronutrient balance is often neglected. Studies suggest that there is interplay between nutrition and inflammation. We aimed to assess the amount of enteral macronutrients delivered compared to estimated requirements, and the association between delivered macronutrients and systemic inflammation in critically ill children.
Method: We prospectively evaluated energy and macronutrient intake in critically ill children who required at least 72 h of mechanical ventilation. Data on enteral energy and macronutrient intake was collected and expressed as a percentage of the estimated requirements. Circulating levels of inflammatory cytokines were measured by ELISA and association assessed with delivery of macronutrients from the previous 24 h.
Results: A total of 87 children (0–16 years) were included in this study. By day 3 the median (IQR) intake of energy, fat, carbohydrate (CHO) and protein were 75% (50–103), 85% (43–120), 63% (42–102) and 45% (23–65) respectively. We have also shown that delivery of enteral fat and protein was associated with elevation in the levels of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6).
Conclusion: The inflammatory response in critically ill children is influenced by the amount of enteral fat and protein delivered. Our data suggests that within the feed delivered, fat is often higher than protein and CHO. It is crucial to take into account the proportion of macronutrients required and not only aim to achieve the energy goal.

1. Introduction

An important goal of nutrition support in paediatric critical illness is minimising catabolism and supporting basal physiological function [1]. There is often a clinical focus on delivering the energy and protein requirements of a patient, but the balance of macronutrients is often neglected. Ready-to-feed energy-dense (ED) formulae (1 kcal/ml below 1 year of age and 1.5 kcal/ml > 1 year of age) are often used to achieve energy requirements in critically ill children for a number of reasons. A higher prevalence of chronic illness and co-morbidity means that critically ill children are more likely to be malnourished on admission to the Paediatric Intensive Care Unit (PICU) than healthy children [2,3]. It is important to prevent further deterioration of their nutritional status. Furthermore, these feeds are used to compensate for restrictions in fluid intake and the often frequent interruptions to enteral feeding (EN) [4,5]. However, it is also important to point out the potential risk of overfeeding among this population particularly the malnourished children, who exhibit a hypo-metabolic pattern [6].

The effect of under and over-feeding has been previously investigated, and both have been shown to be detrimental in critically ill children [7–9,37,38]. Although more is known about energy balance and protein requirements [10], the ideal balance of macronutrients, carbohydrates, protein and fats has not yet been

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established. Current recommendations for macronutrients requirements in this population are based on the understanding of their metabolism and handling during the course of their illness [7,10]. Critical illness is characterized by impaired glucose metabolism and increased protein catabolism [10–13]. Likewise the rate of lipid turnover is generally accelerated, suggesting that critically ill children utilise fat preferentially as a substrate for their energy metabolism [7,14,15].

There is a growing body of evidence suggesting that there is interplay between nutrition and inflammation. Increased levels of inflammatory cytokines have been reported before in undernourished children with cirrhosis [16]. Studies also suggested that a diet high in fat might induce systemic low-level inflammation in adult population [17–19]. In this study, we aimed to assess the amount of macronutrient delivered compared to estimated requirements and investigate the association between delivered macronutrients and systemic inflammation in a cohort of critically ill children.

2. Material and methods

We prospectively evaluated energy and macronutrients intake in ventilated children admitted to PICU at Addenbrooke's Hospital in Cambridge, between November 2014 and May 2017, as a part of study looking at gut microbiome in critically ill children. Ethical approval was authorized by City and Hampstead LREC (Reference: 13/LO/0974).

Inclusion criteria:

- Age 1 week to 16 years.
- Mechanically ventilated for >72 h.
- Enterally fed.

Exclusion criteria:

- Preterm gestation (birth < 37 weeks).
- Known pre-existing immune paresis, oncological diagnosis and HIV.
- Children who were on full/supplemental parenteral nutrition (PN) or ketogenic diet.

The actual nutritional intake was recorded daily using patient clinical records until discharge from PICU. The type of enteral feed, total volume delivered, energy and macronutrient delivered was calculated for each patient from the nutrition data card of each formulae (in the Supplementary Appendix). Calculation of breast milk composition was based on data from previous published study on breast milk composition [20].

Enteral formulae were classified into 3 types, energy dense formula (1 kcal/ml for infant <1 year and 1.5 kcal/ml feed for older children), standard formulae (0.67 kcal/ml for infant < 1 year and 1 kcal/ml feed for older children) and breast milk. Energy requirements were estimated using the Schofield-equation [21] as suggested by The American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines in absence of indirect calorimetry [10,22,23]. Protein requirements were calculated according to ASPEN guidelines for critically ill children (0–2 years, 2–3 g/kg/day; 2–13 years, 1.5–2 g/kg/day; and >13 years, 1.5 g/kg/day) [7,10]. Due to lack of published guidelines regarding enteral CHO and fat requirements for critically ill children, the age appropriate UK reference nutrient intake (RNI) for healthy children was used as a guide for establishing CHO requirements as 50% of the total energy requirements [24]. Fat requirements of 40% for children <1 year and 35% for those >1 year were used, corresponding to European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommendations (25, 26). Energy and macronutrient intake were transformed from crude values (kcal or grams/day) into percentage of the reference requirements based on age and weight.

All anthropometry measurements were performed by PICU nurses on admission, the information required for this study including gender, age, weight, height and diagnosis were collected for each patient from the hospital electronic system. Weight/height for age z-scores were calculated based on WHO growth charts using the WHO Anthro and Anthro Plus software (version 3.2.2, January 2011) [27]. Moderate under-nutrition was considered if weight/height z-score was between −2 and -3 standard deviation (SD), and severe under-nutrition if below −3 SD [28].In addition to patient demographics, the Paediatrics Index of Mortality 2 (PIM2) scores [29] and inotrope scores [30] were calculated. Clinical outcomes such as hours free of mechanical ventilation (ventilator free hours, VFH) at 30 days and days free of intensive care at 30 days were also recorded.

Measurements of circulating levels of key inflammatory mediators including pro-inflammatory TNF-α, IL-6, interleukin-1 beta (IL-1β) and anti-inflammatory response pathways interleukin-10 (IL-10) were undertaken between Day 2 and 8. Serum levels of these cytokines were measured by ELISA (MSD, Rockville, Maryland, USA). Cytokine levels were measured at an early (acute) phase, during nutrition deprivation (day 2–3) and a later sample taken between 4 and 8 days after admission. The lower limit of detection for TNF-α was 0.01–0.13 pg/ml, IL-10 0.01–0.15 pg/ml, IL-6 0.01–0.11 pg/ml and for IL-1β was 0.01–0.27 pg/ml. Values for the amount of energy and macronutrients delivered were calculated to the 24-h period prior to each sample collection.

Statistical analysis

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro–Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as a median with interquartile ranges (IQR). Wilcoxon signed rank test, was used to compare the difference in energy and macronutrient delivery between day 1 and day 3 of admission. Whilst the Mann- Witney U was applied to assess differences in disease severity variables between children. The Spearman correlation coefficient was used to establish the correlation between variables, which was followed by stepwise linear regression analysis to assess factors that impact on cumulative energy intake, inflammatory cytokines (TNF-α, IL-10, IL-6 and IL-1β) and clinical outcomes. A p-value of $\leq$0.05 was considered statistically significant and log transformation was performed on cytokines prior to regression analysis.

3. Results

A total of 87 critically ill children [51 (58%) males] were enrolled to the study. The summary of the recruitment and consenting procedure is shown in (Fig. 1). Anthropometric and clinical characteristics of the children are shown in Table 1. The admission diagnosis for all study participants is presented (Fig. 2). Inpatient mortality was 2/87 patients (2.3%).

3.1. Feeding in PICU

EN started at a median time of 8 h (IQR 5–14) following PICU admission. Fifty-four children (62%) started their enteral feeding within the first 12 h of admission. EN was suspended for a median duration of 19 (IQR 11–28) hours within the first 3 days of admission. The most common reason for holding EN among our cohort was the presence of perceived large gastric aspirates.

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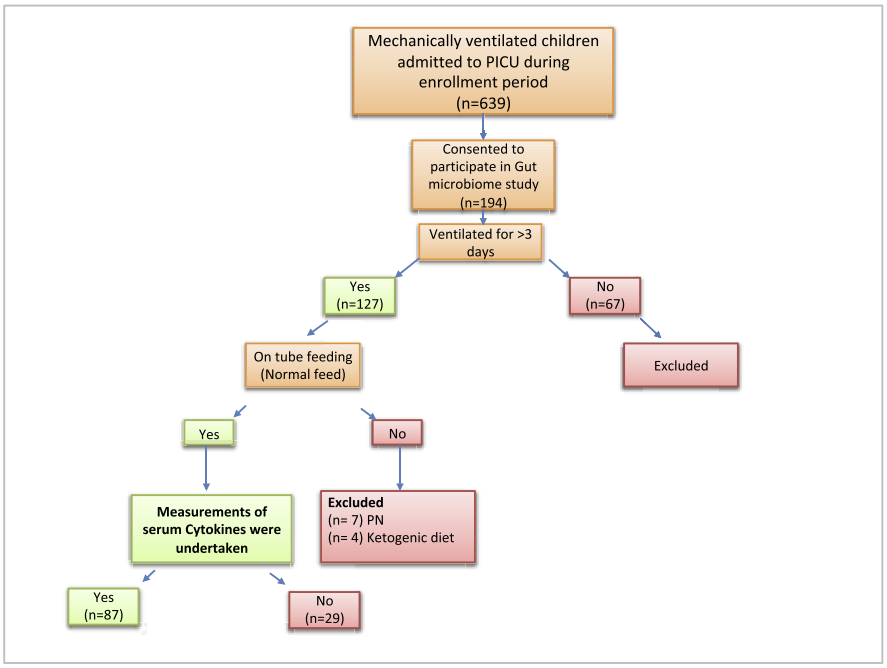

Fig. 1. Summary of recruitment procedure. The above figure shows the stages of patient recruitment and consent.

Table 1
Anthropometric and clinical characteristics of children enrolled to the study.

	All children (n = 87)	0–10 kg (n = 33)	10–20 kg (n = 31)	>20 kg (n = 23)
Anthropometrics				
Age (years)	2 (0.6–4.9)	0.4 (0.1–1)	2.6 (1.8–3.5)	11 (8–13.4)
Weight (kg)	14 (8–21)	7.3 (3.6–8.6)	14.6 (12.3–16.0)	35 (25–45)
Height (cm)	95 (74–112)	68.5 (52–75)	97 (86–101)	132 (114–149)
Weight for age Z-score	−0.1 (−1.4–0.72)	−1.0 (−2.2–−0.05)	0.47 (−0.11–1.4)	0.35 (−0.87–1.1)
% of children below <-2 z scores	15%	30%	3%	9%
Height for age Z-score	−0.15 (−1.9–1.2)	−0.5 (−7–0.8)	0.45 (−0.21–2.04)	−0.54 (−2.2–1.04)
% of children below <-2 z scores	17%	21%	9%	21%
Weight height Z-score	0.05 (−1.1–0.81)	−0.8 (−1.8–−0.02)	0.36 (−1.1–1.07)	0.69 (0.01–2.1)
% of children below <-2 z scores	8%	12%	9%	0%
Disease severity				
PIM2 score	3.2 (0.9–6.2)	4 (1.1–7.3)	2.4 (0.93–5.7)	2.9 (0.88–4.4)
Inotrope score	0 (0–22)	2 (0.0–20)	0.0 (0.0–22)	0.0 (0.0–28)
Maximum lactate	1.9 (1.3–3.3)	2.3 (1.5–3.9)	1.7 (1.2–2.5)	2.5 (1.3–3.3)
Maximum CRP	85 (29–193)	55 (18–142)	105 (25–223)	139 (34–285)
VFH at 30 days	622 (542–651)	601 (601–651)	636 (559–654)	609 (487–645)
PICU free days at 30 days	23 (19–24)	23 (18–24)	23 (20–24)	22 (18–24)

Data presented as median (IQR).
VFH at 30 days: ventilation free hours at 30 days.
PICU free days at 30 days: days free of Paediatric intensive care at 30 days.

No statistical difference in PIM2 score and inotrope score was recorded between children based on the time of commencing EN. Seventy patients (80%) of the cohort received energy dense formulae, 6 (7%) expressed breast milk and 11 (13%) were on standard formulae (Table 2).

We calculated the daily energy and macronutrient intake for the first 3 days of PICU admission (Fig. 3). The enteral intake of energy and each macronutrient on day 1 and day 2 were lower than the calculated requirements. By day 3 the median (IQR) intake of energy, fat, CHO and protein were 75% (50–103), 85% (43–120), 63% (42–102) and 45% (23–65) respectively. Fat intake on day 3 exceeded 100% of the requirements in 35 (40%) children. The delivery of energy and macronutrient was significantly improved by day 3 compared to day 1.

We went on to examine how the daily intake varied among different weight groups. Our results showed that the children with lower weight (0–10 kg) received higher doses of energy and macronutrient. The average intake of energy and macronutrient from admission up to day 3 are presented in Table 3.

Linear regression analysis was performed using the cumulative energy intake at 3 days as outcome to determine the factors affecting the delivery of energy. The regression model suggested that age and weight for age z-score statistically impacted the cumulative amount of energy delivered, this regression model

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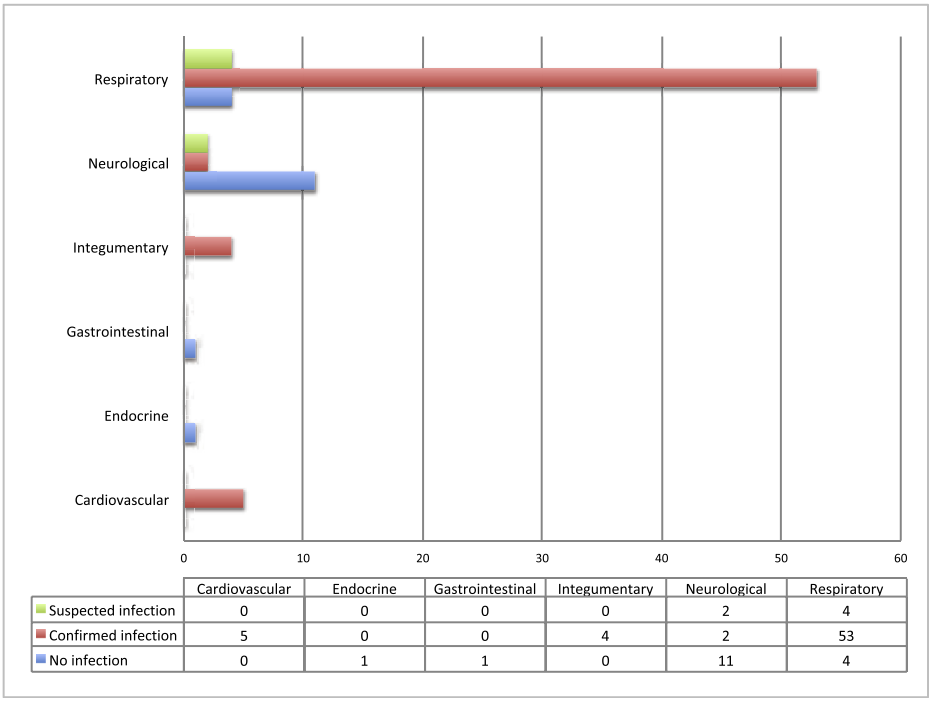

	Cardiovascular	Endocrine	Gastrointestinal	Integumentary	Neurological	Respiratory
Suspected infection	0	0	0	0	2	4
Confirmed infection	5	0	0	4	2	53
No infection	0	1	1	0	11	4

Fig. 2. Distribution of patients based on their admission diagnostic categories. Each cluster of bars represent specific diagnostic category, individual bars represent the number of patients admitted with infection, suspected infection and no infection.

Table 2
Number of patients receiving standard and ED formulae.

	All children (n = 87)	0–10 kg (n = 33)	10–20 kg (n = 31)	>20 kg (n = 23)
Breast milk	6	6	0	0
Standard infant formula	4	4	0	0
Standard paediatric formula	7	0	2	5
Energy dense infant formula	20	17	3	0
Energy dense paediatric formula	50	6	26	18

Table 2 indicates the number of children received, standard and ED formulae among each weight group.

accounted for 16% of the variation in the cumulative energy intake (Table 4).

3.2. Feeding and inflammatory response

Serum inflammatory markers were measured in a total of 125 samples collected from the 87 children enrolled to the study. The number of samples collected from each age group are presented in Table 5. All patients included in the study had an early sample, and those for whom vascular access was available had a second ('late') sample. Prior to performing regression analysis, correlation analysis was used to explore the association between macronutrient intake and the inflammatory mediators.

3.2.1. Early cytokines (samples obtained on day 2–3)

There was no correlation between macronutrient intake and early cytokine levels except for IL-6, which showed a weak negative correlation with energy ($r = -0.22$, $p = 0.04$) and protein ($r = -0.26$, $p = 0.01$).

Stepwise linear regression analysis was performed, with IL-6 as an outcome variable to determine whether feeding contributed to a change in IL-6 independently from the severity of disease and age. The results indicated that both PIM2 score and % of delivered protein were statistically related to IL6 ($p < 0.01$). This regression model accounted for 20% of the variation in IL-6 levels (Table 6).

In children <1 year of age low protein intake was associated with increase in IL-6 levels, $r = -0.554$, $p = 0.024$. Whilst in older children, no evidence of statistical association was recorded between the percentage of protein received and IL-6, $r = -0.221$, $p = 0.077$ (Table 6).

3.2.2. Late cytokines (samples obtained between day 4–8)

With the later samples, both TNF-α and IL-10 were positively correlated with the delivery of energy, fat and CHO whilst no evidence of statistically significant correlation was observed with protein intake.

Stepwise linear regression analysis was performed, with TNF-α as the outcome to determine whether energy and macronutrients intake contributed to increase in TNF-α independently from the age and severity of disease. Our results indicated that higher delivery of fat and less delivery of protein statistically contributed to increase in TNF-α, $p < 0.01$, this regression model accounted for 31% of the variation in TNF-α levels (Table 7).

We further investigated the effect of feeding on TNF-α, between different age groups. Our regression models suggested that the percentage of enteral fat delivered was the only variable

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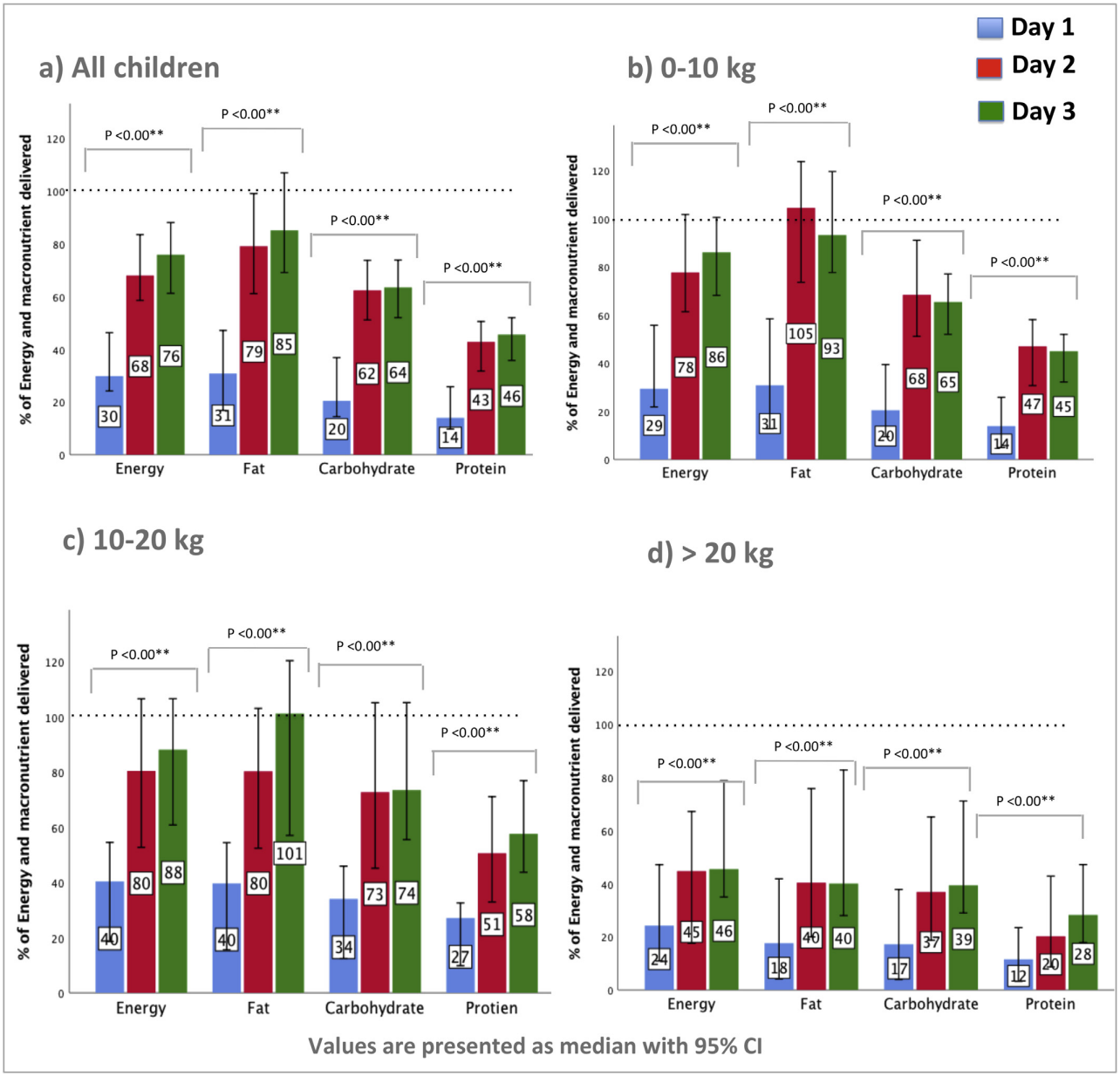

Fig. 3. Percentage of enteral energy and macronutrient delivered expressed as percentage of the calculated requirements. a) Shows the daily enteral intake (day 1–3) of energy and macronutrient in 87 critically ill children. b), c) and d) Shows the daily enteral intake (day 1–3) of energy and macronutrient in different weight groups. The above figure clearly indicated that smaller children (0–10 kg) received higher doses of energy and macronutrient. The enteral intake of both energy and macronutrient has statistically improved on day 3 compared to day 1 ($p < 0.001^{**}$).

Table 3
Average enteral intake of energy and macronutrient from admission up to day 3.

Average intake up to day 3	0–10 kg (n = 33)		10–20 kg (n = 31)		>20 kg (n = 23)	
	Crude value kcal or g/kg/day	%of reference intake	Crude value kcal or g/kg/day	%of reference intake	Crude value kcal or g/kg/day	%of reference intake
Energy	35 (27–48)	64 (52–86)	37 (25–44)	75 (48–83)	13 (6.3–25.9)	41 (21–68)
Fat	1.8 (1.2–2.3)	81 (58–100)	1.6 (0.8–1.9)	76 (42–94)	0.48 (0.18–1.07)	33 (17–72)
Carbohydrate	4.3 (3.7–5.6)	53 (39–71)	4.9 (3.5–5.6)	67 (40–81)	1.8 (1.2–3.3)	33 (15–65)
Protein	0.9 (0.6–1.1)	36 (22–45)	0.99 (0.56–1.1)	43 (31–64)	0.3 (0.17–0.69)	21 (9–43)

Data presented as median (IQR).
The above table shows the average doses of enteral energy and macronutrient presented as crude values and as a percentage of reference requirements.

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Table 4
Regression model to determine the factors affecting the cumulative energy intake.

Model	R	R^2	Adjusted R^2	R	P value
Cumulative Energy Intake[a]	0.421	0.177	0.157		
Age [b]				−0.351	0.00*
Weight for age z −score [b]				−0.326	0.001*
PIM2 score [c]				−0.160	0.07

* A p-value of <0.05.
[a] Dependent variable.
[b] Predictors: (constant).
[c] Excluded variables.

statistically related to the increase in TNF-α (r = 0.440, p = 0.032) in children > 1 year of age. This was not replicated in the younger age group (r = 0.387, p = 0.077) (Table 7).

We also investigated the effect of energy and macronutrient intake in a stepwise linear regression analysis where IL-10 was the outcome variable. This model suggested that both energy intake and PIM2 score had a statistically significant effect on IL-10. This regression model accounted for 46% of the variation in IL-10 (Table 8). In children <1 year both the percentage of energy intake and PIM2 score were statistically related to IL-10, whilst in older children PIM2 score was the only variable statistically related to increase in IL-10 (Table 8).

3.3. Effect of enteral feeding on clinical outcomes

We investigated the independent association of clinical outcomes and the average intake of energy and macronutrient. Two stepwise regression models were performed; in these regression models PICU free days and VFH were the outcome variables. There was no evidence of statistical association between energy or macronutrient intake and clinical outcomes in this cohort (Table 9).

Table 6
Factors affecting IL-6 during early admission period.

Model	R	R^2	Adjusted R^2	R	P value
IL-6[a] (all children)	**0.457**	**0.209**	**0.189**		
PIM2 score[b]				0.346	0.001*
%of enteral protein received[b]				−0.295	0.007*
% of enteral Energy received[c]				−0.053	0.638
% of enteral CHO received[c]				0.104	0.351
% of enteral Fat received[c]				0.077	0.490
Age[c]				0.213	0.055
IL-6[a] (children < 1 year)	**0.544**	**0.296**	**0.249**		
%of enteral protein received[b]				−0.544	0.024*
% of enteral Energy received[c]				−0.203	0.450
% of enteral CHO received[c]				0.459	0.074
% of enteral Fat received[c]				0.229	0.394
PIM2 score [c]				−0.074	0.0784
Age [c]				0.034	0.90
IL-6[a] (children > 1 year)	**0.505**	**0.255**	**0.232**		
PIM2 score[b]				0.429	0.00*
Age[b]				0.300	0.014*
%of enteral protein received[c]				−0.221	0.077
% of enteral Energy received[c]				−0.203	0.105
% of enteral CHO received[c]				−0.156	0.216
% of enteral Fat received[c]				−0.124	0.326

* A p-value of <0.05.
[a] Dependent variable.
[b] Predictors: (constant).
[c] Excluded variables.

4. Discussion

Protein energy malnutrition and abnormalities in glucose metabolism have been strongly linked to prolonged ventilation and hospital stay, multiple organ dysfunction and mortality [11,31,32]. Therefore it is crucial to take into account the proportion of macronutrients required and not only aim to achieve the energy goal. **In this study,** there was no association between energy and macronutrient intake and clinical outcomes. However, we recorded weak association between the amount of energy and macronutrient delivered with the inflammatory markers.

Table 5
The number of samples collected from each age group.

All children				
Early samples (Day 2–3)	**Number of samples**	**Median number of samples per patient**		
	87	1 (1–1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.8 (2.3–7.5)	6.5 (1.8–19)	2.5 (0.9–7.8)	0.2 (0.2–0.3)
Late samples (Day 4–8)	**Number of samples**	**Median number of samples per patient**		
	39	1 (1–2)		
	TNFα (pg/ml)	**1L-6(pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.7 (2.5–6.8)	3.5 (1.5–8.6)	1.6 (0.9–2.4)	0.2 (0.1–0.2)
<1 year				
Early samples (Day 2–3)	**Number of samples**	**Median number of samples per patient**		
	20	1 (1–1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	5.8 (4.5–9.6)	88 (2.8–14)	4.3 (1.1–28)	0.25 (0.2–0.5)
Late samples (Day 4–8)	**Number of samples**	**Median number of samples per patient**		
	15	1 (1–2)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	6.8 (4–7)	3. (1.5–5.9)	1.8 (0.9–3.1)	0.2 (0.1–0.4)
>1year				
Early samples (Day 2–3)	**Number of samples**	**Median number of samples per patient**		
	67	1 (1–1)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.2 (1.8–6.1)	5.9 (1.7–20.2)	2.2 (0.9–7)	0.2 (0.2 0.21)
Late samples (Day 4–8)	**Number of samples**	**Median number of samples per patient**		
	24	1 (1–2)		
	TNFα (pg/ml)	**1L-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL1-β (pg/ml)**
	3.1 (2.2–4.3)	3.6 (0.8–10.9)	1.1 (0.7–2.3)	0.2 (0.1–0.2)

Data presented as median (IQR).

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Table 7
Factors affecting TNF-α.

Model	R	R^2	Adjusted R^2	R	P value
TNF-α[a] (all children)	**0.561**	**0.315**	**0.277**		
% of enteral Fat received [b]				0.534	0.001*
% of enteral protein received [b]				−0.347	0.033*
% of enteral Energy received [c]				−0.004	0.983
% of enteral CHO received [c]				0.008	0.963
PIM2 score [c]				0.187	0.269
Age [c]				−0.191	0.257
TNF-α[a] (children < 1 year)					
% of enteral Fat received [c]				0.387	0.077
% of enteral protein received [c]				0.0.119	0.377
% of enteral Energy received [c]				0.212	0.224
% of enteral CHO received [c]				0.229	0.206
PIM2 score [c]				0.037	0.448
Age [c]				−0.202	0.337
TNF-α[a] (children > 1 year)	**0.440**	**0.193**	**0.157**		
% of enteral Fat received [b]				0.440	0.032*
% of enteral protein received [c]				0.094	0.669
% of enteral Energy received [c]				−0.094	0.669
% of enteral CHO received [c]				−0.009	0.969
PIM2 score [c]				0.322	0.134
Age [c]				−0.010	0.963

* A p-value of <0.05.
[a] Dependent variable.
[b] Predictors: (constant).
[c] Excluded variables.

The recent ASPEN guidelines targets delivery of at least two thirds of the prescribed daily energy requirement by the end of the first week in the PICU to avoid undesired clinical outcomes associated with cumulative nutrition deprivation [10]. The majority of our children achieved this energy goal by day 3, which was likely to occur as EN was generally commenced within a few hours of admission (median of 8 h) in accordance with our unit enteral feeding protocol. However, over the first 3 days protein intake on average achieved only 35% of requirements and reached 46% by day 3. In addition, our results showed that children >20 kg were at the most risk of not achieving their requirements, with only achieving an average of 21% of the requirements over 3 days. Insufficient delivery of protein is common, particularly in the early stages of admission. Our findings are similar to previously published data where under-delivery of protein was also reported [31,33–35]. On the contrary, on day 3 the median fat intake was 85% and exceeded the calculated requirements in almost 40% of children among two weight groups (0–10 kg) and (10–20 kg). This discrepancy of achieving energy requirements, under delivery of protein and exceeding fat requirements is related to two aspects: the use of standardised, ready to feed energy dense formulae, in our unit and the aim requirements for macronutrients in critically ill children. The only guidelines available for enteral nutritional support in this population is for energy and protein [7,10]. However, none have been set for fat and CHO. The ESPGHAN guidelines for healthy children were used as a guide to estimate fat requirements in our critically ill population, as we believe that younger children particularly breast-fed infants might have higher fat requirements. RNI for healthy children was used as a guide for establishing CHO requirements. In our study almost 80% of patients were fed energy dense formulae to help meet their energy needs. These formulae have a higher energy density and provide on average 50% of energy from CHO, 10% from protein and 40% from fat. The macronutrient composition of all medical formulae in European is guided by the European Commission of Food for Special Medical Purposes (FSMP) guidelines [36]. Many factors need to be taken into account with the development of these enteral feeds, including macronutrient composition, osmolality, volume of feed for nutritional adequacy, safety ranges for macro and micro nutrients for children with a wide range of diagnoses. As energy requirements are lower and protein requirements are higher in critically ill children [7,10,22,23], the distribution of macronutrients in standardised feeds do not match the specific requirements of critically ill children.

We assessed factors that had an impact on cumulative energy intake and found that age and weight for age z-score were the strongest predictor of the cumulative energy intake among our children. Our findings regarding the age are similar to Mehta et al. (2011) but contradictory to the work done by Hulst et al. (2004), where they found that younger children accumulated the highest energy deficits. This controversy could be explained by the fact that younger children had the highest percentage of malnutrition (30%) as assessed by weight for age Z-score. Due to the well-documented impact of early malnutrition [39,40], it is likely that the nutrition support team focussed more on providing full nutritional requirements particularly for smaller (lower weight for age Z scores) children to avoid further deterioration of their nutritional status during PICU stay.

Table 8
Factors affecting IL-10.

Model	R	R^2	Adjusted R^2	R	P value
IL-10[a] (all children)	**0.679**	**0.461**	**0.415**		
% of enteral Energy received [b]				0.575	0.00*
PIM2 score [b]				0.437	0.004*
%of enteral protein received [b]				0.163	0.018*
% of enteral CHO received [c]				0.082	0.636
% of enteral Fat received [c]				−0.082	0.636
Age [c]				−0.188	0.271
IL-10[a] (children < 1 year)	**0.758**	**0.575**	**0.504**		
% of enteral Energy received [b]				0.698	0.006*
PIM2 score [b]				0.540	0.046*
% of enteral protein received [c]				−0.357	0.231
% of enteral CHO received [c]				0.257	0.397
% of enteral Fat received [c]				−0.185	0.546
Age [c]				−0.143	0.642
IL-10[a] (children > 1 year)	**0.510**	**0.260**	**0.227**		
PIM2 score [b]				0.510	0.011*
% of enteral Fat received [c]				0.183	0.403
% of enteral energy received [c]				0.207	0.344
%of enteral protein received [c]				0.146	0.507
% of enteral CHO received [c]				0.177	0.420
Age [c]				−0.198	0.365

* A p-value of <0.05.
[a] Dependent variable.
[b] Predictors: (constant).
[c] Excluded variables.

Table 9
Regression model to determine the effect of feeding on clinical outcomes.

Model	R	R^2	Adjusted R^2	R	P value
VFH[a]					
PIM2 score[b]				0.132	0.114
Age[b]				−0.091	0.319
Average energy received (3 days)[b]				−0.160	0.072
Average CHO received (3 days)[b]				−0.186	0.044
Average protein received (3 days)[b]				−0.089	0.208
Average fat received (3 days)[b]				−0.184	0.046
PICU free hours at 30 days					
PIM2 score[b]				−0.198	0.034
Age[b]				0.45	0.203
Average energy received (3 days)[b]				0.028	0.397
Average CHO received (3 days)[b]				−0.002	0.493
Average protein received (3 days)[b]				−0.090	0.206
Average fat received (3 days)[b]				−0.009	0.466

VFH at 30 days: ventilation free hours at 30 days.
PICU free days at 30 days: days free of Paediatric intensive care at 30 days.
[a] Dependent variable.
[b] Excluded variables.

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In this study we also assessed the association between macronutrient intake and inflammatory mediators. We found that both protein deficit and higher delivery of fat were associated with elevation in the levels of inflammatory cytokines; in particular under-delivery of protein was associated with an increase in serum IL-6 and TNF-α. The association between dietary protein and inflammatory markers has not previously investigated in critically ill population. However, increased levels of inflammatory markers have been recorded in malnutrition studies where presumably energy and protein intake is compromised [40,41]. Low protein intake has been shown to be associated with increased serum IL-6 in a murine model, in addition, IL-6 and TNF-α have been proposed as a possible biomarker of nutritional deficits [16,42].

Despite the current knowledge about the contribution of dietary fatty acids in modulating the production of lipid mediators and signalling molecules in cells that are involve in immune regulation and inflammation [42], to our knowledge no previous data has been published linking fat intake with elevated levels of circulatory cytokines in critical illness. The current study recorded an association between enteral fat delivery and elevation in TNF-α, particularly in children >1 year of age. Although the younger group received higher doses of enteral fat and their median TNF-α was higher, but surprisingly we did not record such a statistical association. This could be attributed to variation in fat requirements, particularly in young children who have a higher fat requirement [25,26]. There are limited data describing the changes in lipid metabolism in relation to inflammation during critical illness. In septic patients the changes in plasma fatty acids profiles appear to be related to the intensity of the inflammatory response, besides interleukins have shown to be inversely related to low-LDL/low-HDL [43,44]. In septic children increased expression of Neutrophil CD64 was associated with low HDL and LDL levels [45]. None of the above mentioned studies recorded the EN intake or investigated the dietary factors. Studies linking inflammation to dietary factors do exist in other populations including obesity, heart disease and inflammatory bowel disease. In patients with heart failure TNF-α levels were elevated with higher intake of saturated fat [46]. In an obese rat model, use of a high fat diet induced expression of TNF-α, IL1β and IL-6 in skeletal muscle, visceral fat and blood [47–50]. Studies also suggest that the high fat western diet might induce systemic low-level inflammation as a result of changes in gut microbiota [17,18]. In a study conducted on 15 overweight men, a low-fat diet resulted in statistically significant reduction in TNF-α [19]. However, it is important to point out that the type of fat delivered might also impact the inflammatory response. In an experimental induced colitis rat model, medium-chain triglycerides (MCT) rich diet reduced IL-6, IL-8 levels, indicating that MCT rich formulae exert an anti-inflammatory effect in colitis [51].

Although the exact mechanism that links dietary fat to inflammation is not fully understood, but several mechanisms have been proposed. It is generally accepted that pro-inflammatory fatty acids may act directly and activate receptors that signals inflammatory response [52]. It has also been suggested infiltration of macrophages associated with adiposity accounts for the increased adipose expression of TNF-α [47]. However, one of the recently proposed mechanisms that links dietary fat to inflammation, is related to it's effect on promoting the translocation of microbial products from the gut into the bloodstream [53]. Given the well-documented evidence of intestinal dysbiosis during critical illness makes this explanation plausible and of clinical justification.

In the current study association between energy intake and IL-10 could be related to stimulation of insulin secretion. IL-10 signalling has been proposed as a potential mechanism to increase energy expenditure and improve insulin sensitivity [54,55].

This study suggests that the energy and macronutrient delivery in the later phase are associated with alterations in the inflammatory response when data was analysed in a single cohort. However, we recorded in consistency in the findings with further stratification to different age groups, which could be related to reduction in the sample size. This could also be attributed to variation in macronutrient requirements between infants and older children. It is worth mentioning that the changes in clinical condition could be contributory or indeed driving this association to a large extent and this needs to be explored in further studies.

In the absence of guidelines regarding enteral macronutrient requirements, it is not surprising that there have been no trials of enteral macronutrient targets in critically ill children. In a landmark study of parenteral nutrition in this patient group (the Paediatric Early versus Late Parenteral Nutrition In Critical Illness (PEPaNIC) trial [56], the dose of amino acids delivered was associated with an increased rate of infection and a longer ventilation time. The authors postulate this to be due to repression of autophagy by amino acids in critical illness. Another potential mechanism is the early induction of Resistin leading to repression innate immunity and changes in amino acids kinetics, including increase levels of phenylalanine and serine and reduce glutamine concentrations [57–59]. The PEPaNIC trial indicated that parenteral delivery of glucose and lipids were associated with fewer infections and earlier PICU discharge respectively. We did not observe such an association between enteral nutritional intake and the duration of ventilation or the length of PICU stay. It is important to note that the route of delivering nutrition was different from our study; also a distinctive statistical plan was carried out. Unlike the PEPaNIC study, we monitored the intake for 3 days which may be insufficient to detect a statistical effect of nutritional intake on clinical outcomes. Importantly, our cohort had significantly lower protein intake compared to requirements than patients enrolled into the PEPaNIC trial (36% vs 80%) [56]. although we only measured intake to 3 days compared to 7 in the PEPANIC trial, making a comparison between data from the two studies challenging.

There is a lack of published guidelines regarding enteral fat and CHO requirements for critically ill children. Although the PePANIC study contributes significantly to our knowledge for PN nutrient delivery in critically ill children, the requirements used for PN are not applicable for enteral nutrition. There are key differences in the metabolisms of enteral versus parenteral carbohydrates, lipids and protein are related to intestinal absorption of nutritional substrates, insulin and inflammatory stimulation and visceral protein synthesis [59]. We therefore used guidelines from ESPGHAN for fat and RNI for CHO for healthy children, whilst utilising published guidance for recommended protein and energy intake in paediatric critical illness [7,10,22,23].

Our study has several limitations including the relatively small sample size and heterogeneity of the patient cohort. Ideally we would divide the children into more than two age groups and investigate the relationship between macronutrient and cytokines more deeply. As this study suggests that fat intake may drive a pro-inflammatory response, it seems prudent to investigate the relationship between omega3:omega6 in this study. In our smaller patient cohort, where formula delivery was protocolised and most children received a similar type of formula, this type of analysis was not feasible. Within the linear regression model, there were some variables that had a relatively low r-value that limit how well the changes in inflammatory cytokines can be attributed to them. However the results of our analysis appear to be in agreement with reported associations between macronutrient intake and inflammation in other disease state [19,41,42,44,50–53].It is clear that further work is needed to establish appropriate enteral CHO and fat requirements in critically ill children, and also to examine the effect

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of specific fat sources such MCT and omega-3 on inflammatory response and other clinical outcomes. Imminent studies must also include data from children discharged after a PICU admission in order to consider long-term effects of enteral macronutrient delivery compared to requirements.

Conclusion

The inflammatory response in critically ill children is complex and mediated by many factors. Our study suggests that the enteral fat and protein intake is associated with changes in the inflammatory response. This wider significance of our findings need to be examined in a larger study based on clinical endpoints. Our data suggests that within the feed delivered, fat intake is often above requirements compared to protein and CHO delivery. This imbalance of nutrient provision may result from using the standard paediatric feeds that may not necessarily match the macronutrient requirements of critically ill children. Therefore it is crucial to take into account the proportion of macronutrients required and not only aim to achieve the energy goal. These findings are based on generalised guidelines for macronutrient requirements in healthy children and future work should examine whether critically ill children have specific macronutrient needs.

Statement of authorship

SZ conceived the study, participated in its design, collected, analysed and interpreted the data, collected and processed serum samples, performed the statistical analysis, obtained funding and draughted the manuscript. DW and JR recruited patients, acquired clinical data, collected serum samples and revised the manuscript. RB supervised statistical analysis and interpretation and revised the manuscript. FV participated in data interpretation and revised the manuscript. RM participated in conception and design the study, supervised statistical analysis and interpretation revised the manuscript. NP conceived the study, participated in its design and coordination, obtained funding, supervised statistical analysis, draughted and revised the manuscript.

Ethics approval and consent to participate

Ethical approval was authorized by City and Hampstead LREC (Reference: 13/LO/0974). Informed consent for participation and publication was obtained from parents of the children.

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Conflicts of interest

No conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2018.10.001.

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Relationship between inflammation and metabolic regulation of energy expenditure in critically ill children.

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Abstract

Background and aims: Critical illness is associated with derangement in the metabolic and inflammatory response. The persistent state of inflammation may derive a range of metabolic abnormalities in critically ill children. Previous work highlighted the cross-link between feeding, inflammation and gut homeostasis. The aim of this study was to assess the relationship between inflammatory activation and metabolic regulation of energy expenditure by assessing cytokine release, levels of gut-derived hormone [Glucagon like peptide-1 (GLP-1)] and energy expenditure in a cohort of critically ill children.

Method: Two serum samples were collected from each child between day 1-5 of PICU admission, for the analysis of inflammatory cytokines (TNF-α, IL-10, IL-6 and IL-1β) and GLP-1. Indirect Calorimetry (IC) measurements were performed concurrently. The metabolic index was determined using the ratio of Measured Energy Expenditure (MEE) / Predicted Basal Metabolic Rate (PBMR) based on the Schofield equation.

Results: A total of 42 children (0-14 years) were included in this study. The regression analysis showed a statistical association between TNF-α and the GLP-1 ($r=0.453$, $p=0.003$). Where IC measurements were performed (N=21) GLP-1 showed a statistical association with the metabolic index ($r=0.632$, $p=0.002$). No evidence of statistical assocaition was recorded between TNF-α and the metabolic index.

Conclusion: Circulating GLP-1 appears to be increased in response to inflammatory stimuli by TNF-α in critically ill children. Our findings suggest that GLP-1 could provide a novel link between the inflammatory response and the gut with strong relevance for metabolic dysregulation in critically ill children.

Key wards: Critical illness, Inflammation, Metabolism, Energy expenditure, GLP-1

1. Introduction

Critical illness is associated with derangement in the metabolic and inflammatory response (1). The persistent state of inflammation may derive a range of metabolic abnormalities in critically ill children. Inflammatory cytokines have the capacity to regulate energy metabolism through suppression of appetite and by inducing lipolysis in fat tissue and protein breakdown in muscles (2). It is still not clear if the inflammatory cytokines affect stress mediators such as energy expenditure directly or through intermediaries that influence the metabolic pattern and other nutritional markers.

GLP-1 is a gut hormone that plays an important role in the regulation of metabolism. GLP-1 induces a state of negative energy balance through controlling the appetite, gastric motility and to a lesser extent energy expenditure. (3), (4), (Parekh *et al.* 2014). The levels of GLP1 appears to be increased in critically ill adults (5), (6) which, may be related to the inflammatory stimuli by pro-inflammatory cytokines (7).

Critically ill children generally have reduced energy expenditure (8), however the hypermetabolic pattern has also been reported (9). Therefore it is critical to identify factors, which contribute to increase resting energy expenditure (REE) in order to provide sufficient nutritional requirements.

Previously published studies have highlighted the cross-link between feeding, inflammation and gut homeostasis. The aim of this study was to assess the relationship between inflammatory activation and metabolic regulation of energy expenditure by assessing cytokine release, levels of gut-derived hormones and energy expenditure in a cohort of critically ill children.

2. Methods

This prospective pilot study was conducted in critically ill children at Addenbrookes Hospital, Cambridge, UK. Written informed consent was obtained from patients' guardians before inclusion in this study.

2.1 Biological samples

Two serum samples were collected from each child between day 1-5 for the analysis of inflammatory mediators and GLP-1. Cytokines were measured using Electrochemical luminescence immunoassay from MSD, Rockville, Maryland, and USA. The lower limit of detection for TNF-α was 0.01-0.13 pg/ml, IL-10 0.01-0.15 pg/ml, IL-6 0.01-0.11 pg/ml and for IL-1β was 0.01-0.27 pg/ml. GLP-1 was collected in a special tube with added inhibitor cocktail (DPPIV inhibitor, serine protease inhibitor) and measured using a singleplex multi array assay system (MSD, Rockville, Maryland, USA). The lower limit of detection was 0.98 pg/ml.

2.2 Patient's clinical and nutritional data

Patient's data were obtained from the hospital electronic system (EPIC, Madison, Wisconsin, United States). Information including gender, age, weight, and Height was collected for each child. Weight/height for age z- scores were calculated based on WHO growth charts using the WHO Anthro and Anthro Plus software (version 3.2.2, January 2011) (10). Patients' primary diagnosis necessitating PICU admission was recorded and then categorized into multi-organ system failure (MOF) or single organ failure categories (11). In addition to patient demographics, the Paediatrics Index of Mortality 2 (PIM2) scores (12), Paediatric multiple organ dysfunction score (PMODS) (13) and inotrope scores (14) were calculated. Clinical outcomes such as hours free of mechanical ventilation (ventilator free hours, VFH) at 30 days and days free of intensive care at 30 days were also recorded.

The actual energy intake was recorded for each child from the hospital electronic system. The type of enteral feed, total volume delivered, energy and macronutrient delivered was calculated for each patient based on the nutrition data card of each formula (in the Supplementary Appendix). Calculation of breast milk composition was based on data from previous published study on breast milk composition (15).

2.3 Measuring Energy expenditure

Resting Energy Expenditure (REE) measurements were performed for mechanically ventilated children Weighing more than 10 kg. For those who had chest drain, peritoneal dialysis, Fraction of inspired oxygen (FiO_2)>60% or endotracheal tube leak >10% , the measurements of REE was not feasible.

The metabolic index was determined using the ratio of MEE / PBMR based on Schofield equation. To assess the association between REE, inflammatory cytokines and GLP-1, a serum sample was obtained concurrently with IC measurement.

Standard measurement protocol for indirect calorimetry (IC)

REE was measured using Ultima CCM™® indirect calorimeter. The measurement was undertaken when the child was in resting state. No nursing care or physical therapy had been provided for at least 1 hour before the test to ensure the volume of oxygen (VO_2) and volume of Carbone dioxide (VCO_2) was as stable as possible. IC was calibrated to reference gases prior to each participant according to the Manufacturers Manual. The IC was then attached to mechanical ventilator for at least 10 minutes before starting the test to ensure patient and ventilator stability prior to measuring REE. The test lasted for 40 minutes in resting state, where intra variation between VO_2 and VCO_2 was less than 10% (16). During the test if any medical intervention had to be provided the test was stopped and the measurement was repeated to ensure the continuity of a resting state.
IC measured VO_2 consumption and VCO_2 production and calculated REE automatically according to weir equation: REE = [3.9 (VO_2) + 1.1 (VCO_2)] 1.44 (17).

2.4 Statistical analysis:

The statistical analysis was conducted using IBM SPSS v25 USA. The Shapiro-Wilk Test was used to assess the normality of the data distribution. Quantitative variables with non-normal distribution were expressed as a median with interquartile ranges (IQR). Spearman correlation coefficient was used to establish the correlation between variables, which was followed by a stepwise linear regression analysis to assess factors affecting GLP-1 and REE. A p-value of ≤ 0.05 was considered statistically significant and log transformation was performed on cytokines prior to regression analysis.

3. Results

Inflammatory cytokines and GLP-1 were measured in 42 children enrolled in this (24 males). Of those, 21 children fulfilled the criteria for energy expenditure study and REE was measured within 5 days of Paediatric Intensive Care Unit (PICU) admission (Table 1). Anthropometric and clinical characteristics of the children are shown in (Table 2). None of the study participants died within the study period.

Table 1: Number of IC measurement taken on each day of PICU admission

Day	Day1	Day2	Day3	Day 4	Day5
Number of children	2	5	9	2	3

Table 2: Anthropometric and clinical characteristics of children enrolled to the study

Patients characteristics (N = 42)	**Median**
Age (years)	3.7 (1 – 8.3)
Weight (kg)	16.8 (9.8 – 25.2)
Height (cm)	103 (79 – 123)
Weight age Z score	-0.1 (-1.4 – 0.8)
Height age Z score	-0.08 (-1.9 – 1.1)
Weight for height Z score	0.3 (-0.9 – 0.8)
TNF-α (pg/ml)	3.7 (2.7 – 5.4)
IL-1β (pg/ml)	0.2 (0.1 – 0.3)
IL-6 (pg/ml)	9.8 (2.2 – 25.7)
IL-10 (pg/ml)	1.8 (1 – 6.5)
GLP1 (pg/ml)	11.3 (8.1 – 19)
Maximum glucose (mmol/l)	6.5 (5.2 – 7.9)
Disease severity variables	**Median**
PMOD score	6 (5 – 7)
PIM2 score	3.1 (0.9 – 6.1)
Inotrope score	3 (0 – 27)
VFH of mechanical ventilation at 30 days	619 (535 – 645)
Hours free of inotrope treatment at 30 days	714 (675 – 720)
PICU free days at 30 days	23 (18 – 24)
Primary admission diagnosis	**N**
MOF	14 (33%)
Respiratory Failure	17 (41%)
CNS Disorders	9 (21%)
Surgical Disorders	2 (5%)

Data are presented as median (IQR).

- VFH at 30 days: ventilation free hours at 30 days
- PICU free days at 30 days: days free of paediatric intensive care at 30 days

3.1 GLP-1 and the inflammatory response in critically ill children

Prior to performing the regression analysis, a correlation analysis was performed to establish the association between the inflammatory mediators and GLP1. The results showed a strong positive correlation between GLP-1 with TNF-α ($r=0.453$, $p=0.003$) and IL-10 ($r=0.433$, $p=0.004$). A weak correlation was recorded between GLP-1 and IL-1β ($r=0.314$, $p=0.043$).

To further investigate this association a stepwise regression analysis was performed to determine if TNF-α, IL-10 and IL-1β independently influenced plasma GLP-1 concentrations. The regression analysis indicated that TNF-α and IL-6 statistically influenced GLP-1 concentrations ($p < 0.01$) (Table 3). Although the univariate analysis showed a correlation between GLP-1 and IL-10 but this correlation was confounded by other factors in the regression analysis (Table 3).

Table 3: Factors affecting GLP-1 level in critically ill children

Model (N=42)	R	R^2	Adjusted R^2	R	P value
GLP-1 [a]	**0.433**	**0.187**	**0.166**		
TNF-α [b]				0.433	0.003*
Maximum glucose [c]				-0.021	0.45
Age [c]				0.078	0.315
PIM2 score [c]				0.077	0.319
Total energy received (%) [c]				0.218	0.088
EN at the time of sampling (Yes/No)				0.182	0.131
GLP-1 [a]	**0.493**	**0.243**	**0.202**		
IL-6 [b]				0.348	0.014*
Total energy received (%)[b]				0.218	0.019*
Maximum glucose [c]				-0.021	0.45
Age [c]				0.078	0.315
PIM2 score [c]				0.077	0.319
EN at the time of sampling (Yes/No)				0.182	0.131
GLP-1 [a]	**0.376**	**0.141**	**0.119**		
IL-1β [b]				0.376	0.008*
Maximum glucose [c]				-0.021	0.45
Age [c]				0.078	0.315
PIM2 score [c]				0.077	0.319
Total energy received (%) [c]				0.218	0.088
EN at the time of sampling (Yes/No)				0.182	0.131
GLP-1 [a]					
IL-10 [c]				0.309	0.026
Maximum glucose				-0.021	0.45
Age [c]				0.078	0.315
PIM2 score [c]				0.077	0.319
Total energy received (%) [c]				0.218	0.088
EN at the time of sampling (Yes/No)				0.182	0.131

a. **Dependent variable**

b. **Predictors: (constant)**

c. **Excluded variables**

3.2 GLP-1 and the metabolic response

Where energy expenditure measurements were performed (N=21) GLP-1 showed a strong positive correlation with the metabolic index (r=0.632, p=0.002), (Figure 1). However, no evidence of statistical correlation was recorded between inflammatory cytokines and the metabolic index. Regression analysis was then performed to determine whether GLP1 and pro-inflammatory markers contributed to a change in EE independently from the severity of disease and age. The results indicated that only GLP1 was statistically related to the pattern of EE ($p < 0.01$). This regression model accounted for 39% of the variation in EE (Table 4).

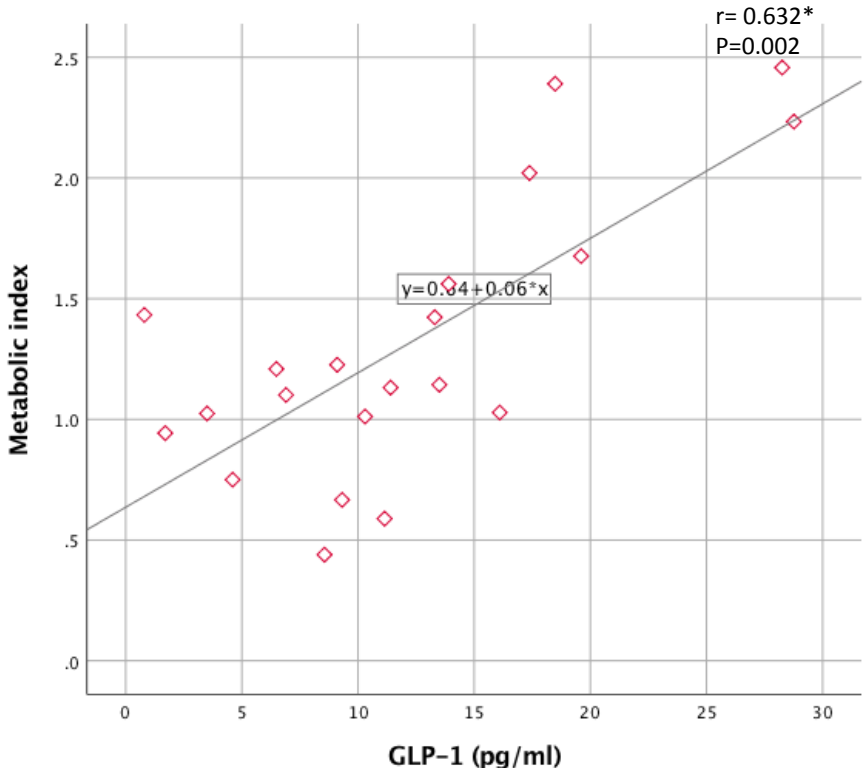

Figure 1. Association between REE and serum GLP1 concentration

Figure 1 shows a positive correlation between GLP-1 and the metabolic index.

Table 4: Association between energy expenditure

Model (N=21)	R	R^2	Adjusted R^2	R	P value
Metabolic index [a]	**0.649**	**0.421**	**0.388**		
GLP1 [b]				0.649	0.001*
TNF-α [c]				0.031	0.448
Age [c]				-0.311	0.091
PIM2 score [c]				-0.23	0.165
Total energy received (%) [c]				0.263	0.132
Metabolic index [a]	**0.649**	**0.421**	**0.388**		
GLP1 [b]				0.649	0.001*
IL-6 [c]				-0.063	0.396
Age [c]				-0.23	0.165
PIM2 score [c]				-0.311	0.091
Total energy received (%) [c]				0.261	0.133
Metabolic index [a]	**0.433**	**0.187**	**0.166**		
GLP1 [b]				0.649	0.001*
IL-1β [c]				-0.117	0.312
Age [c]				-0.23	0.165
PIM2 score [c]				-0.311	0.091
Total energy received (%) [c]				0.261	0.133

a. **Dependent variable**
b. **Predictors: (constant)**
c. **Excluded variables**

3.3 The GLP-1 as a predictor of clinical outcomes and disease severity in critically ill children

To establish the association between GLP-1 and disease severity a correlation analysis was performed between GLP-1 and a range of disease severity and clinical outcome variables. The results showed a positive correlation between GLP-1 and inotrope score (r= 0.485, p=0.001), and a negative correlation was recorded with hours free of inotrope treatment (r= -0.312, p=0.047). However, the regression analysis showed that GLP-1 did not predict the duration of inotrope treatment and mechanical ventilation or length of PICU stay.

4. Discussion

The findings of this study suggest that the inflammatory response to stress may induce metabolic abnormalities, and hence affect energy expenditure. Although no direct associated was recorded between TNF-α and REE, we hypothesized that GLP1-1 mediates a crosstalk between the inflammatory stimuli and REE.

The association between inflammation and REE has been established in both critically ill adults and children (18), (19). Numerous studies also have shown a connection between inflammatory markers and REE in other disease states such as obesity (2), (20), kidney disease (21) and cancer (22). In critically ill adults energy expenditure was increased in septic patients with systemic inflammatory response syndrome (SIRS) (Moriyama et al. 1999). In contrast, Briassoulis et al. (2010) reported reduced energy expenditure and increased inflammatory markers in children with sepsis and SIRS. In patients with chronic kidney disease inflammation assessed by C-Reactive Protein (CRP) was associated with increased REE (21). While in cancer patients, an increase in TNF-α was associated with hypermetabolism (22). Inflammatory cytokines such as IL6, IL-1β and TNFα may directly regulate REE by acting through cell membrane receptors in the brain or peripheral tissues to induce energy expenditure (2), (23). These pathways involve induction of GLP-1 and Leptin expression by the inflammatory stimuli (2), (20).

The inflammatory mediators are thought to contribute to the induction of GLP-1 secretion through stimulation of intestinal L cells (24). Mechanistically, the activation of STAT-3 transcription factor by cytokines appears to enhance the expression of GLP1 (24). In critically ill adults plasma GLP-1 levels were strongly influenced by inflammation (6), (7). Furthermore, GLP-1 secretion has shown to be increased in diabetic patients in response to inflammatory stimuli (25). In a study by Kahles et al. (2014) they also indicated that septic patients had statistically higher levels of GLP-1 compared to none-septic patients, implying an association between GLP1 with inflammatory response and immune system activation. GLP-1 secretion also appeared to be increased in response to endotoxin stimulation (26). In critically adults the levels of GLP1 was positively correlated with the severity of disease (7), (6).

New emerging researches recorded increased levels of GLP1 in critically ill children and adults (6), (5), (7) which could be implicated in the abnormalities seen in REE among this

population. Higher fasting plasma GLP-1 levels were associated with higher rates of REE in adults with glucose intolerance (27). In addition, an increase in energy expenditure was recorded after peripheral infusion of GLP1 in human studies (28). In a murine model the administration of GLP1 was associated with an upsurge in oxygen consumption and REE (29), (30). Contradictory to the finding of the current study, Poggiogalle et al. (2018) indicated that fasting plasma GLP-1 was not associated with changes in REE in adults

The available data on how GLP-1 affects energy expenditure is still not conclusive. However, the effect of GLP1 on insulin stimulation was suggested as a factor accounting for the increase in REE (28). In addition, GLP-1 appears to promote browning of white adipose tissues and stimulates brown adipose tissues thermogenesis through a process involving uncoupling of the mitochondrial respiration by uncoupling protein-1 to generate heat (32). (Figure 3)

It is also important to note that there might be other factors contributing to the increase of GLP-1 concentration in our cohort. These factors might be related to the nature of medications given and the pathological state. For example, increased GLP1 levels could also be related to decreased renal clearance (6). It has also been suggested that endotoxins associated with gut injury might stimulate the secretion of GLP-1 (33). Since GLP-1 secretion is stimulated by nutrient intake (34), it is possible that the feeding status of the patients contribute to the variation in circulating GLP1, although in the current study no such association was recorded between GLP1 and the amount of energy delivered.

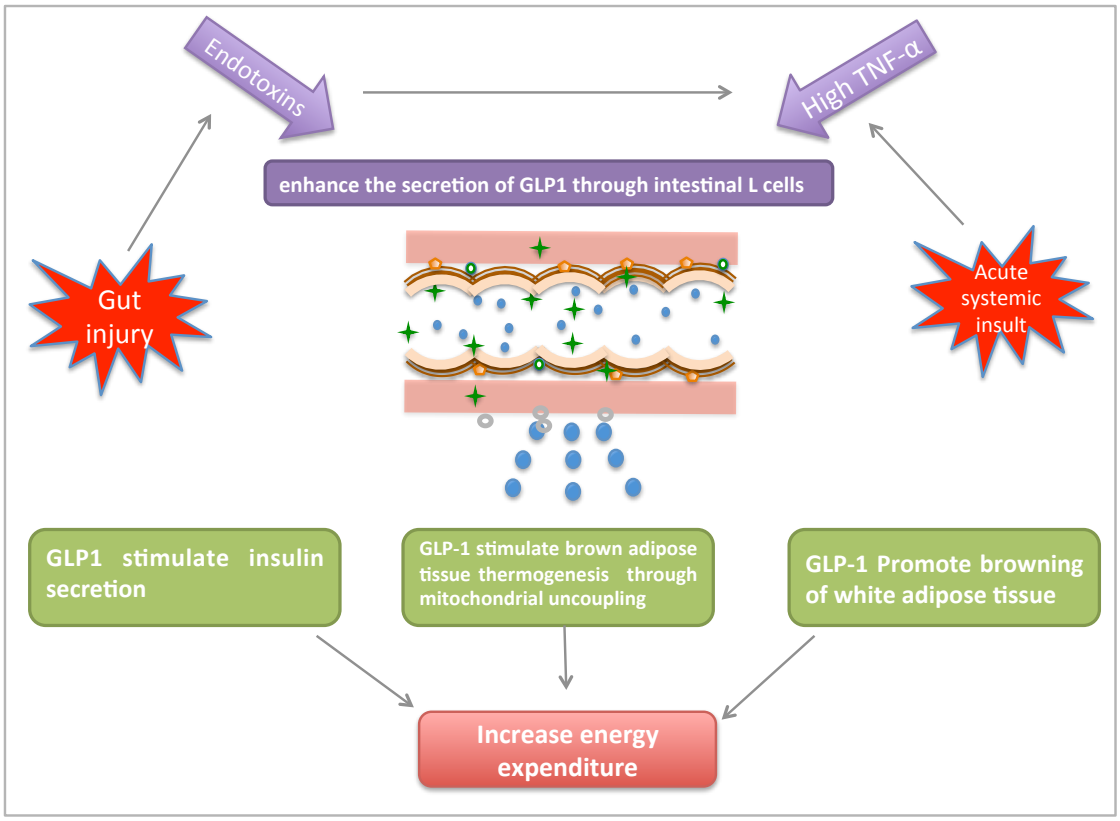

Figure 2. Potential mechanisms for the association between inflammation and REE mediated by GLP-1

Figure 2 shows the potential mechanistic association between pro-inflammatory cytokines, GLP1 and REE. TNF-α/IL-6 levels are increased in response to initial systemic or intestinal insult. Inflammatory cytokines or and endotoxins acutely enhance the secretion of GLP1 through intestinal L cell. GLP1 increase REE though 3 mechanisms:

1. Stimulation of insulin secretion.
2. Promote browning of white adipose tissues.
3. Stimulation of brown adipose tissues thermogenesis.

Brown adipose tissues regulate REE through the adaptive thermogenesis process that involves uncoupling of the mitochondrial respiration by uncoupling protein 1 to generate heat. (33), (32).

5. Conclusion

Circulating GLP-1 appears to be increased in response to inflammatory stimuli by TNF-α in critically ill children. These findings suggest that GLP-1 could be a potential marker of the severity of the immune system activation. This study also revealed that elevation in GLP-1 is associated with increase in REE in this population. In summary, GLP-1 could provide a novel link between the inflammatory response and the gut with strong relevance for metabolic dysregulation in critically ill children.

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Authors' contribution:

SZ conceived the study, participated in its design, collected, analysed and interpreted the data, collected and processed serum samples, performed the IC measurements, performed the statistical analysis, obtained funding and drafted the manuscript. DW and JR recruited patients, acquired clinical data, collected serum samples and revised the manuscript. RB supervised statistical analysis and interpretation and revised the manuscript. RM participated in conception and design the study, supervised statistical analysis and interpretation revised the manuscript. NP conceived the study, participated in its design and coordination, obtained funding, supervised statistical analysis, drafted and revised the manuscript.

Ethics approval and consent to participate:

Ethical approval was authorized by City and Hampstead LREC (Reference: 13/LO/0974). Informed consent for participation and publication was obtained from parents of the children.

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Conflict of Interest:

No conflict of interest to declare

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Figure and Table Legends:

Table 1: Number of IC measurement taken on each day of PICU admission

Table 2: Anthropometric and clinical characteristics of children enrolled to the study

Data are presented as median (IQR).

- VFH at 30 days: ventilation free hours at 30 days
- PICU free days at 30 days: days free of Paediatric intensive care at 30 days

Table 3: Factors affecting GLP-1 level in critically ill children

d. Dependent variable
e. Predictors: (constant)
f. Excluded variables

Table 4: Association between energy expenditure

a. Dependent variable
b. Predictors: (constant)
c. Excluded variables

Figure 1. Association between REE and serum GLP1 concentration

Figure 1 shows a positive correlation between GLP-1 and the metabolic

Figure 2. Potential mechanisms for the association between inflammation and REE mediated by GLP-1

Figure 2 shows the potential mechanistic association between pro-inflammatory cytokines, GLP1 and REE. TNF-α/IL-6 levels are increased in response to initial systemic or intestinal insult. Inflammatory cytokines or and endotoxins acutely enhance the secretion of GLP1 through intestinal L cell. GLP1 increase REE though 3 mechanisms:

1. Stimulation of insulin secretion.
2. Promote browning of white adipose tissues.
3. Stimulation of brown adipose tissues thermogenesis.

Brown adipose tissues regulate REE through the adaptive thermogenesis process that involves uncoupling of the mitochondrial respiration by uncoupling protein 1 to generate heat.